Lecture Supplements

Plant Bacteriology

Bacterial Disease Management-Part 3

Compiled by N. Hassanzadeh

Version 4.25

January 1, 2025

Website Address:
http://www.phytobacteriology.com

Contact address

- Department of Plant Protection, Faculty of Agricultural Sciences and Food Industries, Science & Research Branch, Islamic Azad University, Tehran-Iran.
- P.O. Box: 14155/775, Postal Code: 1477893855
- Branch website: www.srbiau.ac.ir
- e-mail addresses:
- hasanzadehr@srbiau.ac.ir
- hasanzadehr@yahoo.com

Table of Contents

Table of Contents

Table of Contents

Deleterious Bacteria

- **INA bacteria and Indirect damage to plants:**
- This can occur when bacteria promote the formation of ice crystals on soft fruit, like strawberries.
- **Bacteria and Plant Diseases:**
- The 'tumors' caused by Agrobacteria typically grow on sunflowers, tomatoes, or roses.

1. Protection from INA bacterial damage to plants

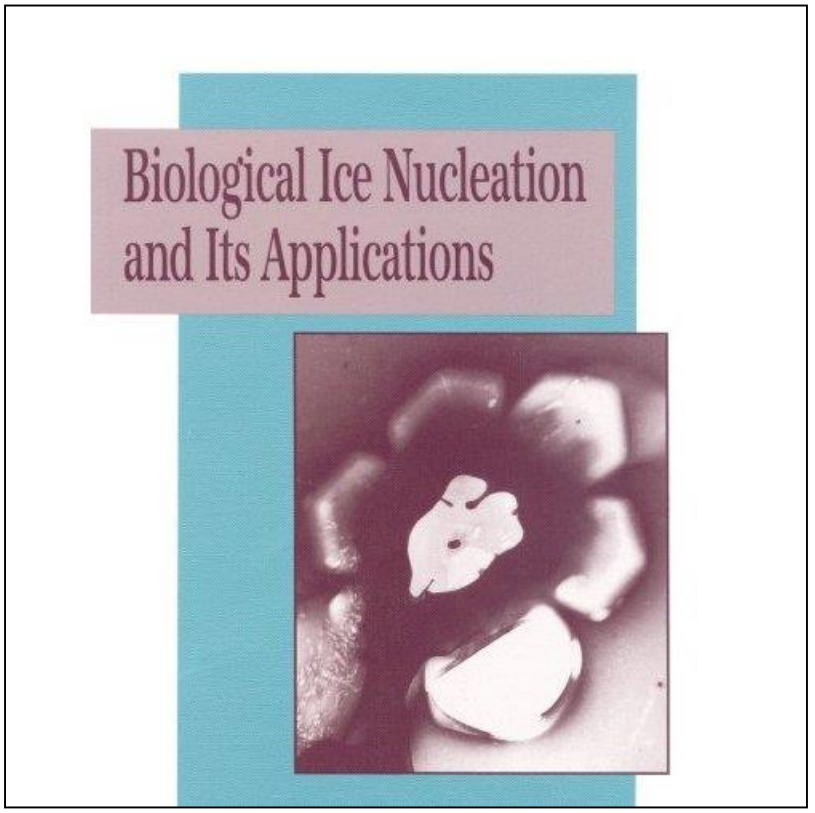
Ice Nucleation-Active Bacteria

Biological Ice Nucleation and Its Applications
Bacteria

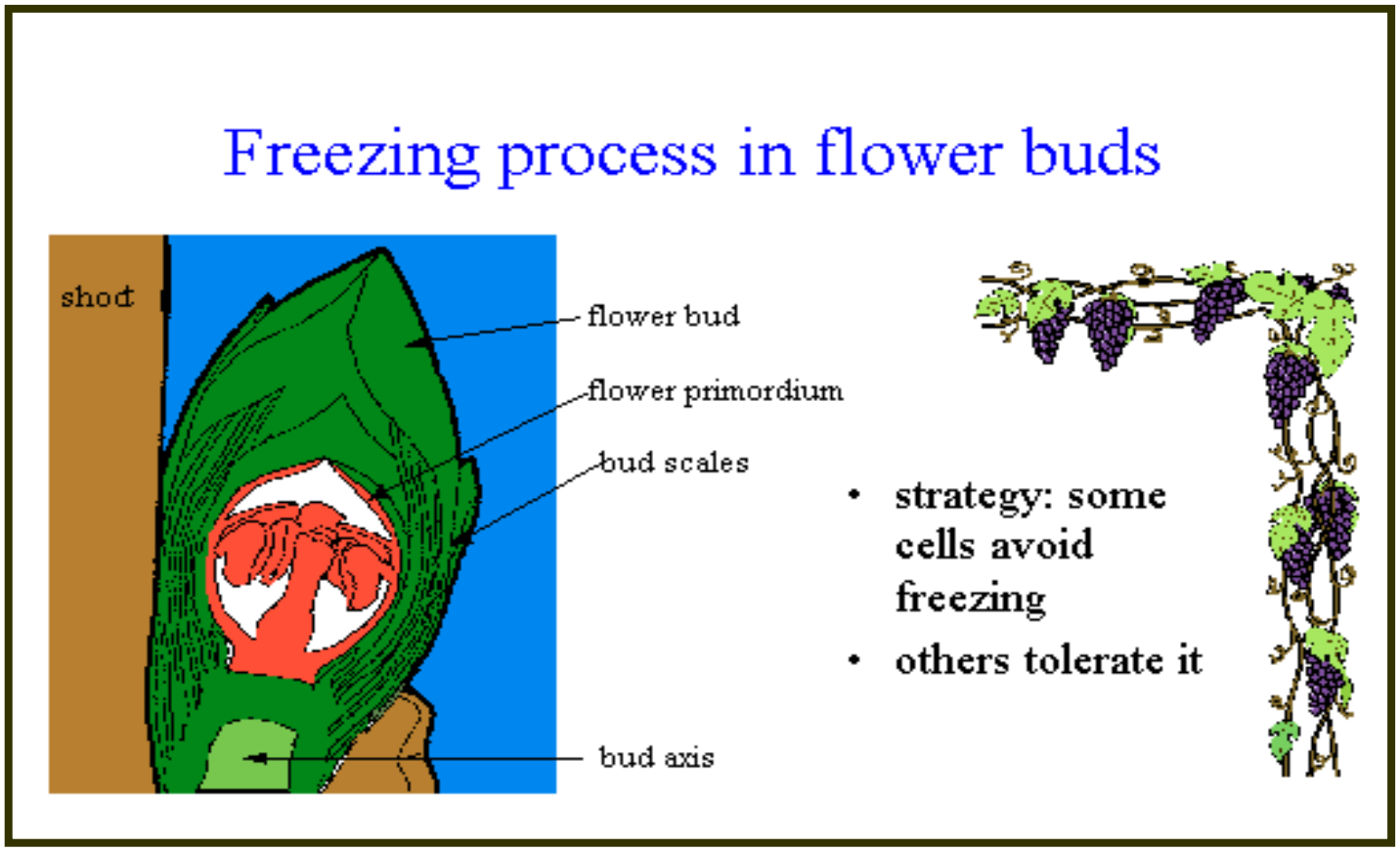
- **Biological Ice Nucleation and Its Applications**
- Richard E. Lee, Gareth J. Warren, L. V. Gusta.
- Publisher: Amer. Phytopathological Society; 1 edition (May 15, 1995).
- 370 pp.

Product Description

- A select group of bacteria plays a key role in the phenomenon of ice nucleation; their actions having an impact on the frost sensitivity of plants, the winter survival of certain insects, and even on weather systems.
- This is the first book to integrate the ice nucleation research of plant physiologists, crop scientists, microbiologists, biochemists, bacteriologists, entomologists, and food scientists worldwide.



Freezing process in flower buds



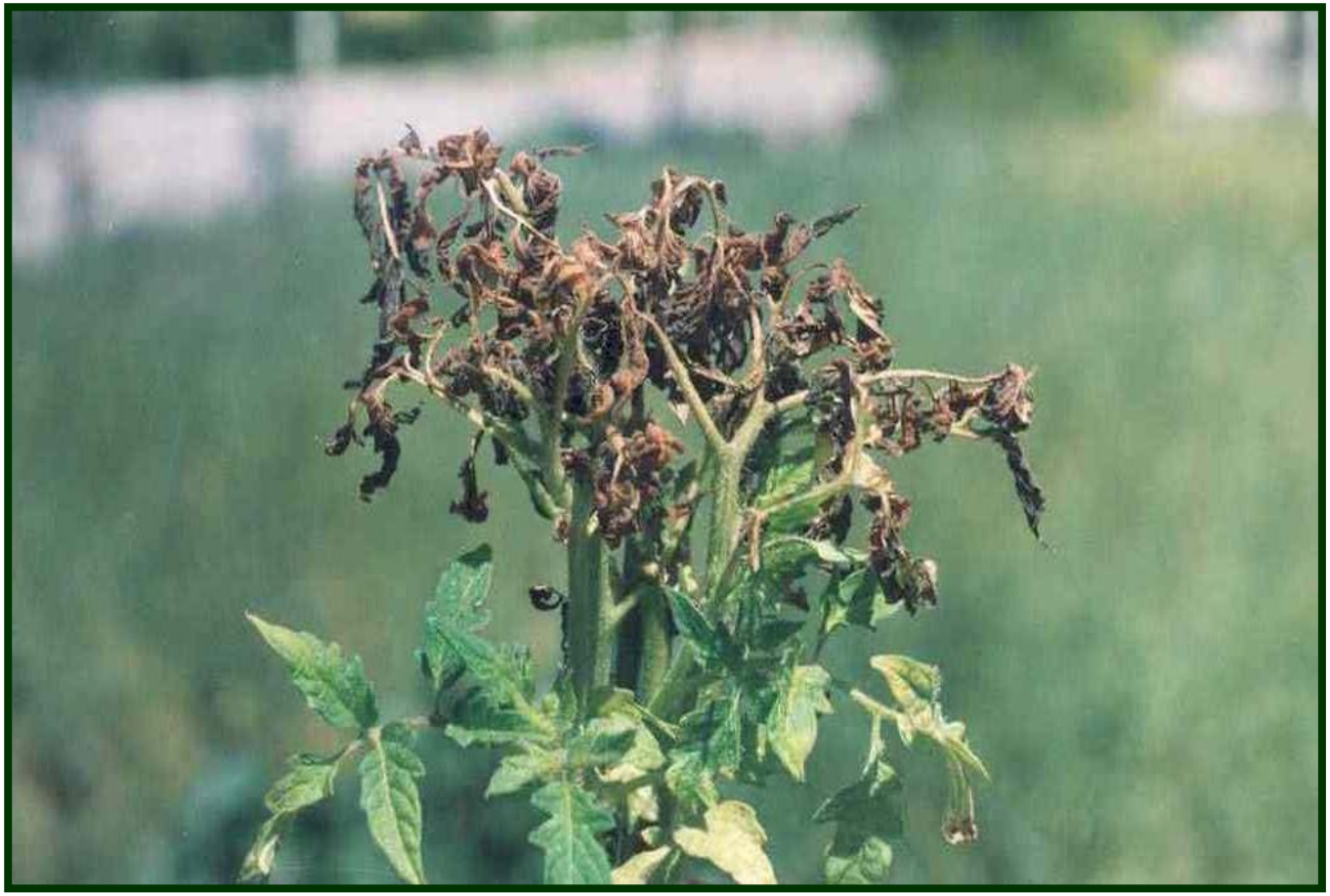
Icy Lingonberry

Wikipedia,2014

Some bacteria can induce ice formation at sub-freezing temperatures (ice nucleation active [INA] bacteria

Examples of frost injury in Iran

Tomato plant



Examples of frost injury in Iran

Walnut trees

Examples of frost injury in Iran

Examples of frost injury in Iran

Pistachio plant

Ice nucleation-active bacteria

Ice nucleating bacteria on plants

1. *Pseudomonas syringae* pv. *syringae*
2. *Pseudomonas syringae* pv. *coronafaciens*
3. *Pseudomonas syringae* pv. *pisi*
4. *Pseudomonas syringae* pv. *lachrymans*
5. *Pseudomonas savastanoi* pv. *glycinea*
6. *Pseudomonas viridiflava*
7. *Pseudomonas fluorescens*
8. *Pantoea ananatis*
9. *Pantoea agglomerans*
10. *Xanthomonas translucens*

Ice$^+$genes

Chromosomal or plasmid genes?

HGT

- In spite of many studies being done, the location of ice nucleation gene whether in chromosomal or plasmid DNA has not been identified.
- The ice-nucleation gene for *P. viridiflava* KUIN-2 is in the plasmid DNA.
- The ice nucleation gene are all highly conserved between species and genera, leading to the hypothesis that the Ina$^+$ gene only evolved once and has since been horizontally transmitted among diverse species and genera (Edwards *et al.*,1994).

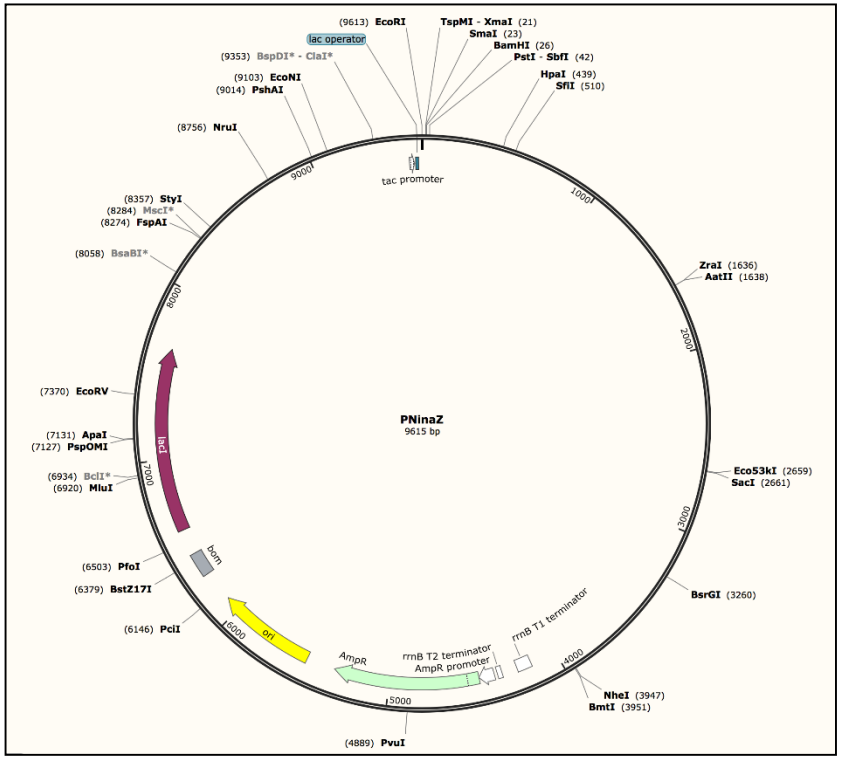
McCorkle,2009

Ice^{+}genes

Chromosomal or plasmid genes?

Plasmid map of the plasmid containing inaZ gene

- The plasmid with the inaZ gene in *P. syringae*.
- After putting the samples in the cooling bath again, one with INP froze after a few minutes while the control remained liquid.



Ksobecka,2015

Ice nucleation-active bacteria
Inducing precipitation

- Humans have been trying to induce precipitation since the 1940s to this day using much the same mechanism – except the ice nucleating agent in this case is the chemical Silver Iodide, which when sprayed into clouds freezes cloud droplets which then fall to the ground.
- *Pseudomonas* are a better ice nucleator, and are commonly used in production of artificial snow or ice rinks, but not – as far as I know – sprayed into clouds.

Ksobecka,2015

Ice nucleation-active bacteria
INA$^+$ bacteria in precipitation

- A growing body of circumstantial evidence suggests that ice nucleation active (Ice$^+$) bacteria contribute to the initiation of precipitation by heterologous freezing of super-cooled water in clouds.
- Two Ice$^+$ strains, however, were identified as *Lysinibacillus*, a Gram-positive genus not previously known to include Ice$^+$ bacteria.
- These culturable Ice$^+$ bacteria in precipitation support a role for Ice$^+$ bacteria in the initiation of precipitation.

Lysinibacillus is a Gram-positive, rod-shaped, and round-spore-forming bacterial genus of the family Bacillaceae.

Failor *et al.*,2017

Ice nucleation-active bacteria
Pseudomonas syringae

- In regions that have very cold climates, frost can be a hindrance to a successful crop.
- It has been estimated that frost alone accounts for crop losses of about approximately $1 billion in the United States.
- *Pseudomonas syringae*, a commonly occurring bacterium, is present on leaves and other plant surfaces.
- It has a peculiar ice-nucleating character which allows the formation of frost which, in turn, damages the plant.

Sugavanam,2010

Ice nucleation

Distilled water vs sterile D.H_2O

- The term ice nucleation describes the initiation of the phase transition of water from a liquid to a solid state.
- When a water sample of moderate size is cooled, it will normally not freeze at 0°C. If the water is pure, it can be cooled to temperatures near to -40°C before it freezes.
- Liquid water at temperatures lower than 0°C is termed supercooled water, and this supercooled state is metastable.

Bisht,2011

Ice nucleation

Distilled water vs sterile D.H_2O

Heterogeneous vs homogenous ice nucleation

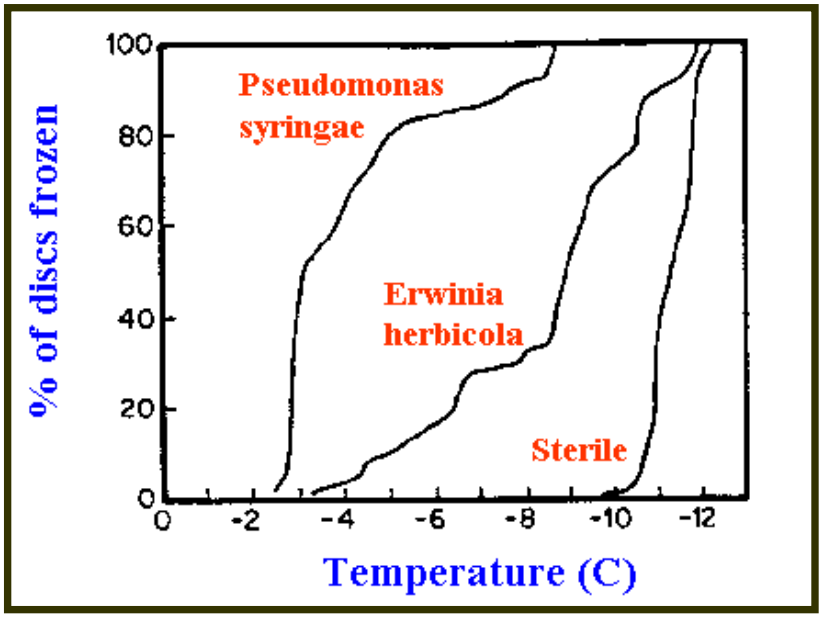
- To enable ice formation to take place, water molecules must cluster in an ice-like pattern and this cluster must reach a critical size.

1. If the initial aggregation of water molecules takes place on a foreign structure, the process is termed heterogeneous ice nucleation.
2. If the water molecules aggregate without the help of another structure, the nucleation is termed homogenous.

Bisht,2011

Two efficient ice nucleators

P. syringae and *P. agglomerans* compared with sterile D.H_2O



Ice nucleation phenomenon

Catalysis of ice crystal formation in supercooled water has been demonstrated for some plant pathogenic bacteria e.g. *Pseudomonas syringae*

These epiphytic bacteria initiate ice formation at temperatures higher than normally required.

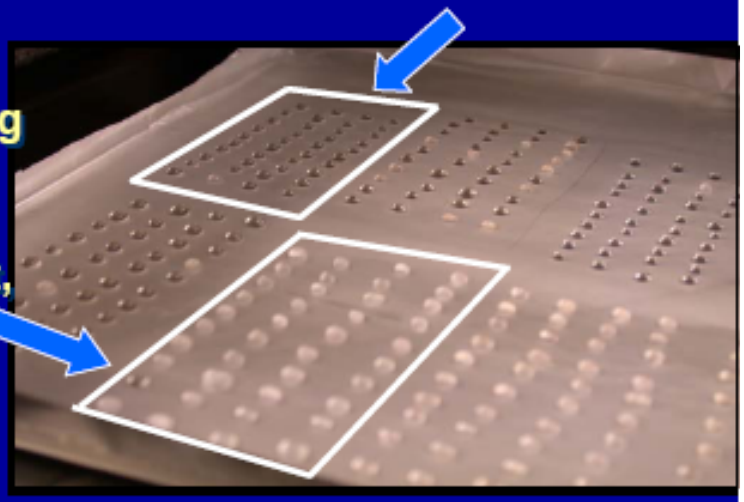
A gene in the bacterium codes for protein in cell wall that confers ice nucleating ability.

Ice damage creates wounds

Droplet freezing assay:

In this sample, ice-nucleating bacteria were not present. The droplets supercool but do not freeze.

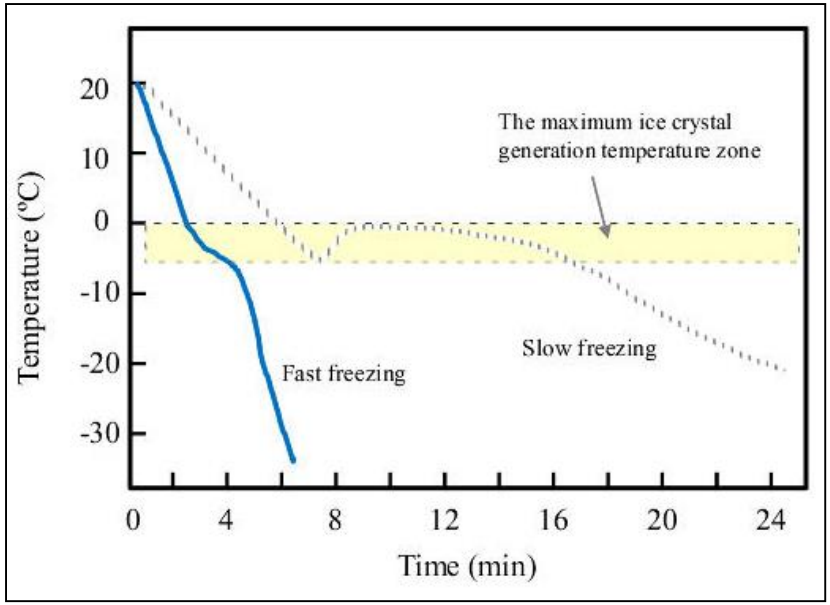
In this sample, ice-nucleating bacteria were abundant, and ice was formed



The maximum ice crystal generation temperature zone
Ranging from 0°C to -7°C

- The maximum ice crystal generation temperature region is from 0°C to -7°C.
- This temperature region is important for ice crystal structure formation.

1. When the time to pass through this temperature region is short, a detailed ice crystal is formed, and
2. When the time is long, a large and rough ice crystal is formed.



Kawarhara *et al.*,2013

Characteristics of ice nucleation-active bacteria

P. syringae and a handful of other bacterial species

- The ability of bacteria to nucleate supercooled water to form ice is uniquely limited to *P. syringae* and a handful of other bacterial species, many of which dwell in the phyllosphere.
- Of course, ice-nucleating bacteria were first found associated with leaves, and this is where people have looked.
- Such bacteria usually make up a small proportion (0.1-10%) of the bacteria found on leaf surfaces.

Hirano *et al.*,2000

Characteristics of ice nucleation-active bacteria

P. syringae and a handful of other bacterial species

- Not all strains within these species are ice nucleation active.
- Indeed, ice nucleation activity has been used as one (among several) trait to distinguish strains among some of the *P. syringae* pathovars.

1. Strains within pv. *syringae* as agents of frost injury to plants frequently exhibit the ice phenotype.
2. While none of the strains tested thus far within pvs. *tomato* or *morsprunorum* are ice nucleation active.

- Among strains that are active in ice nucleation, not every cell is active at a given time and temperature.

No nuclei no frost

Frost-sensitive plants vs. pure water

- Frost-sensitive plants are injured when temperatures drop below 0°C because ice forms within their tissues.
- Small volumes of pure water can be supercooled to -10°C or below without ice formation, provided no catalyst centers or nuclei are present to influence ice formation.

Supercooling

- Supercooling in the temperature range of 0 to roughly 5°C is primarily limited by the presence of INA bacteria.
- Below 5°C, other heterogeneous ice nuclei, including those produced by plants themselves, probably also limit supercooling.
- Thus, INA bacteria are responsible for ice formation, and hence injury to plants, mainly in the range from 0 to 5°C.
- The larger the bacterial population, the greater function of the population sizes of INA bacteria in the phyllosphere.

Ice nucleation bacteria or fungi

Ina protein found on the outer bacterial cell wall

- Ice nucleation-active (INA^+) bacteria capable of synthesizing a secretory protein, the ice nucleation protein (INP), which confers on cells the ability to nucleate ice crystals at subfreezing temperatures (as high as -2°C to -3°C), thereby causing frost injury to plants.
- "ice-plus" protein (Ina protein, "Ice nucleation-active" protein) found on the outer bacterial cell wall acts as the nucleating centers for ice crystals.

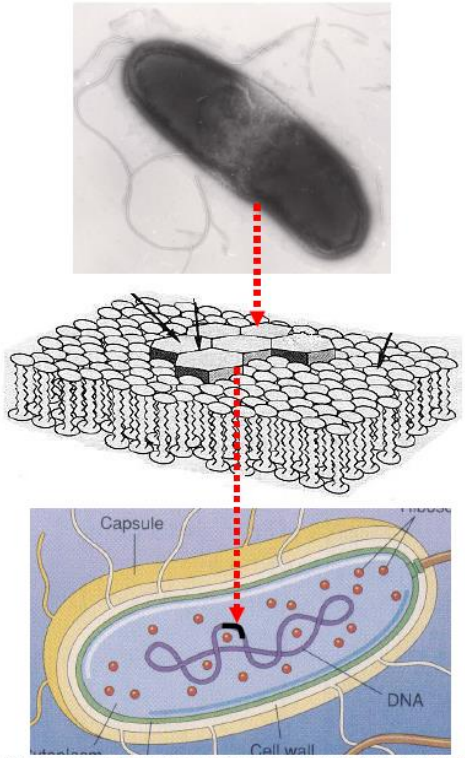
The wild-type *P. syringae* bacterium is known as ice-plus bacteria. It contains a surface protein in its outer cell wall that helps with frost formation, hence the name ice-plus.

In the case of the mutant *P. syringae*, the frost-facilitating surface protein is missing, so these types of bacteria cannot facilitate frost formation and are therefore known as ice-minus.

Ice nucleation bacteria

Ina protein found on the outer bacterial cell wall

Detection targets



http://morayeel.louisiana.edu/SeaweedsLab/Gavio/bacterial%20cell%20copy

- Whole, viable cells
  (culture on nutrient medi
- INA protein
  (serology)
- Gene for the INA protein
  (polymerase chain reacti
  PCR)

Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

Ice nucleation genes

Specific ice genes

The bacterial phenotype is due to a protein product of a single gene

- The genes corresponding to the INA^+ phenotype:
  1. *inaZ* for *P. syringae*
  2. *inaW* for *P. fluorescens*
  3. *iceE* for *E. herbicola*
  4. *inaU for Pantoea ananatis* pv. *uredovora*
- Three ice-nucleation genes i.e. *inaK*, *inaV* and *inaZ* which were characterized from *P. syringae* strains, exhibit high similarities in sequences and in primary organization.
- *inaQ*, ice nucleation protein gene from *P. syringae* MB03.

Ice-nucleation-active bacteria contain a single gene coding for a protein that seems to be responsible for catalyzing ice.

Li *et al.*,2012

Characterization of ice nucleation proteins

- So far, six different genes that transcribe INPs have been sequenced from six different bacterial strains.
- The genes that have been isolated thus far are known as:

1. *inaW* from *Pseudomonas fluorescens* (Warren *et al.*, 1986),
2. *inaZ* from *Pseudomonas syringae* (Green and Warren, 1985),
3. *inaA* and *inaU* from *Erwinia* (*Pantoea*) *ananatis* (Abe *et al.*, 1989, Michigami *et al.*,1994),
4. *iceE* from *Erwinia herbicola* (*Pantoea agglomerans*) (Warren and Corotto, 1989), and
5. *inaX* from *Xanthomonas campestris* (Zhao and Orser, 1990).

Orser et al. (1983) demonstrated that the INA phenotype is coded by a single gene localized in a 3.5- to 4.0-kb DNA region.

McCorkle,2009

Ice nucleation bacteria

Ice genes

Horizontal gene transfer

- A phylogenetic analysis of 16S ribosomal RNA gene sequences from a total of 14 ina^+ and ina^- bacterial strains indicated that the ina^+ bacteria are not monophyletic but instead phylogenetically interspersed among ina^- bacteria.
- The relationships of ina^+ bacteria inferred from ina sequence did not coincide with those inferred from the 16S data.
- These results suggest the possibility of horizontal transfer in the evolution of bacterial ina genes.
- Recent evidence supports a role for the horizontal transfer of ice-binding protein genes from bacteria to sea-ice diatoms.

Edward *et al.*,1994; Raymond and Kim, 2012

Ice nucleation protein (INP)

Three domains proteins

Proteins that help ice crystals to form. The crystals pierce holes in plants

- An ice-nucleating protein is made up monomers of about 150 KDa.
- One monomer is formed by:

1. A N-terminal domain (around 180 amino acids), probably involved in the phenomenon of maturation of the nucleation site,
2. A central repetitive region (around 1000 amino acids), which seems essential in the ice-producing activity, and
3. A C-terminal domain (around 50 amino acids) presumably involved in the aggregation of monomers (Green *et al.*,1988).

Lagriffoul *et al.*,2010

Ice nucleation protein (INP)

The central repeating domain act as a template for ice crystal formation

- All INPs (1200 aa to 1500 aa) encoded by these three genes comprise of three distinct structural domains:

1. The N-terminal domain;
2. C-terminal domain;
3. The central repeating domain.

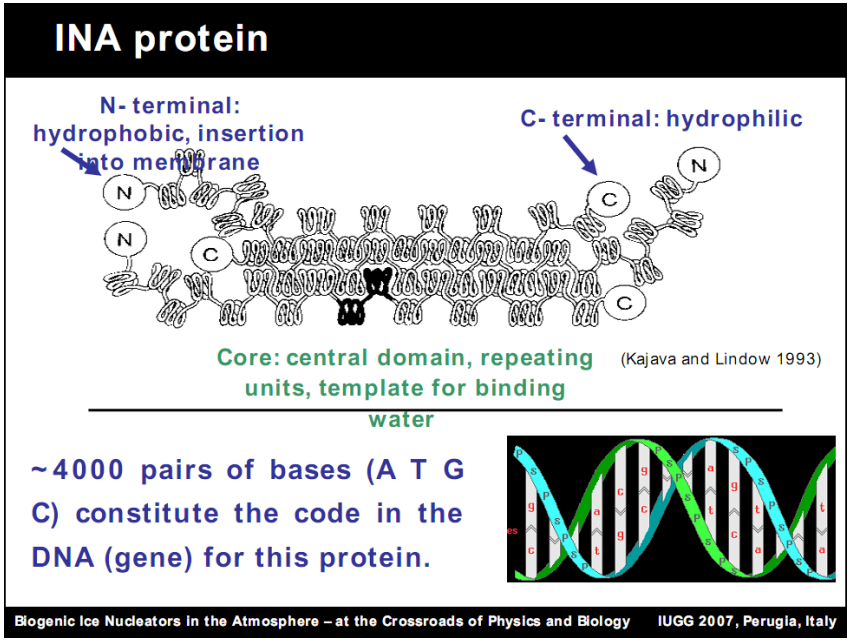
- The central repeating domain (CRD) (approximately 81%), which constitutes contiguous repeats given by 16-residue (or 48-residue) periodicities with a consensus octapeptide (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr).
- This domain presumably acts as a template for ice crystal formation.

Li *et al.*,2012

Ice nucleation protein (INP)

The central repeating domain act as a template for ice crystal formation

- INPs comprises of continuous repeat of a consensus octapeptide (Ala-Gly-Thr-Gly-Ser-Thr-Leu-Thr) and function as templates for the formation of small ice crystal seeds termed "ice nuclei".



Guilbaud *et al.*,2007; Bisht,2011

Ice nucleation proteins(INP)

The repeat structure

- A family of large and unusual proteins (ice nucleation proteins, mw 118 kDa, or larger) are a key component of bacterial ice nuclei.
- Predicted amino acid sequences of two such proteins from *P. syringae* and *P. fluorescens*, respectively, have revealed:
- An internal repeating consensus octapeptide Ala-Gly-Tyr-Gly-Ser-Thr-Gly-Thr, which makes up 70% of the protein, contains two threonine residues and one serine residue.
- All three bacterial ice genes have the same type of unique structure.

Ice nucleation proteins

Three domains proteins

The repeat structure

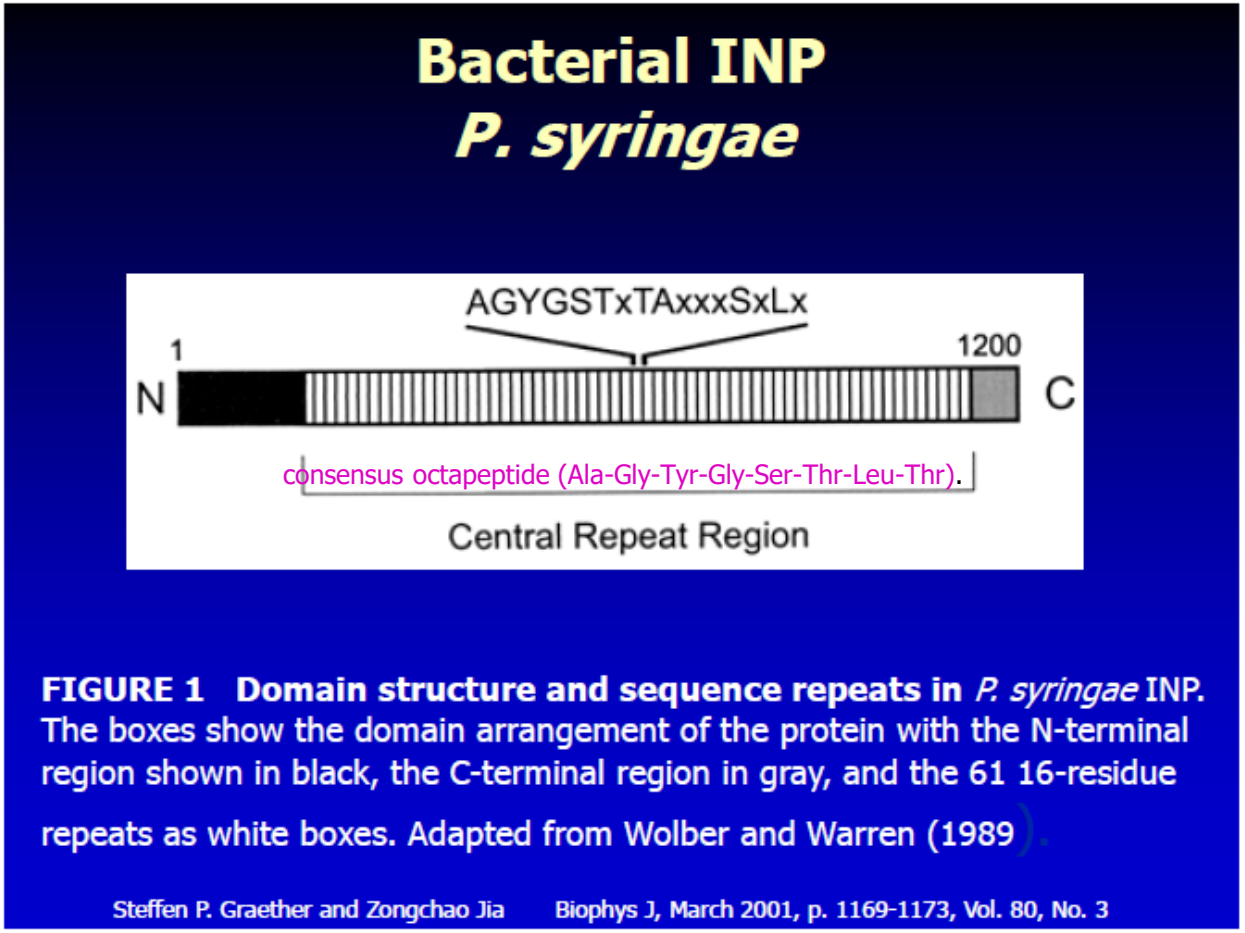


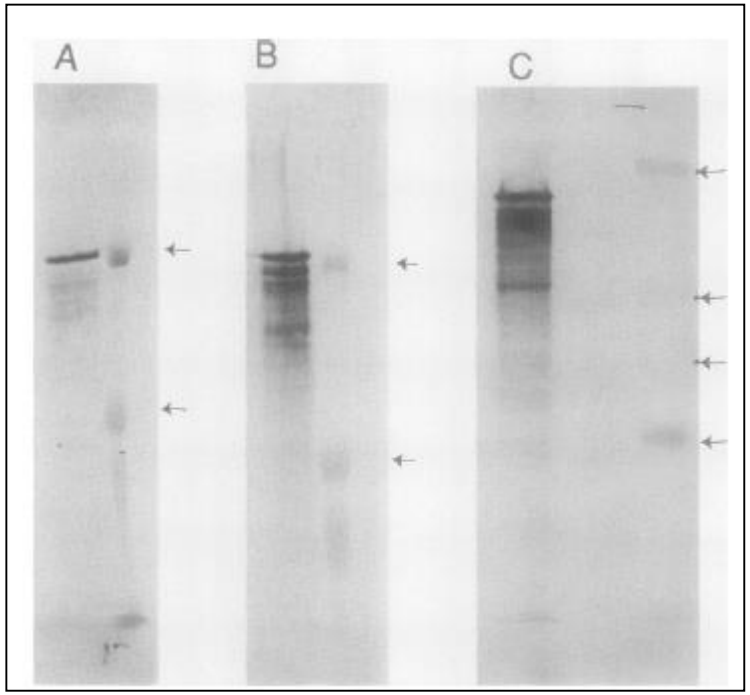
FIGURE 1 Domain structure and sequence repeats in *P. syringae* INP. The boxes show the domain arrangement of the protein with the N-terminal region shown in black, the C-terminal region in gray, and the 61 16-residue repeats as white boxes. Adapted from Wolber and Warren (1989).

Steffen P. Graether and Zongchao Jia Biophys J, March 2001, p. 1169-1173, Vol. 80, No. 3

Bacteria Fall,2008

PAGE analysis on 8.5% polyacrylamide-SDS gels of whole-cell extracts of Ina$^+$ bacteria
Ice nucleation proteins, mw 118 kDa, or larger

- PAGE analysis on 8.5% polyacrylamide-SDS gels of whole-cell extracts of Ina$^+$ bacteria showing immoblotting against the inaZ protein:
- (A) *P. syringae* C9, (B) *E. coli* C9la, (C) *P. syringae* S203.
- The material applied to each gel came from 5 x 10^9 cells.
- The arrows indicate the following standards run simultaneously (from the top) 200, 92.5, 69, and 43 kDa.
- For gels A and B, only the 200- and 92.5-kDa standards have arrows.



Turner *et al.*,1991

Ice nucleation proteins
Protein prediction

- p*I* and molecular mass were predicted with the ExPASy p*I* tool (http://ca.expasy.org/tools/pi_tool.html).
- The presence of a signal peptide was predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Raymond *et al.*,2008

Ice nucleation proteins(INPs)

Three classes of ice-nucleating structures

- Turner et at. (1990) have classified ice nucleating protein into three chemically distinct class depending on A, B, C structure.
- Properties of ice-nucleating cells indicating that there are three chemically distinct classes:

1. Class A structures,
2. Class B structures, and
3. Class C structures as well as
4. Intermediate or mixed structures on the surfaces of ice nucleation-active cells.

- These suggest that the structures of the classes are chemically heterogeneous.

Ice nucleation proteins

Three classes of ice-nucleating structures

- In general and with some differences between strains:
- Class A structures, which are the most active, nucleate at - 4.5°C or warmer.
  - e.g. *P. syringae* and *P. agglomerans*.
- Class B structures nucleate between - 5 and - 8°C
  - e.g. *P. s.* pv. *glycinea*.
- Class C structures are the least active and nucleate at - 8°C or colder.
  - e.g. *P. s.* pv. *glycinea* and *P. s.* pv. *phaseolicola*.

Ice nucleation proteins

Three classes of ice-nucleating structures

- The ice nuclei activity has been classified by the range of temperature in which they initiate freezing:
- type 1 ice nuclei are active between -2°C to -5°C,
- type 2 are active between -5°C to -7°C, and
- type 3 are active between -7°C to -10°C.
- Very potent ice nucleators, active at high subfreezing temperature, are produced by bacteria such as *Erwinia herbicola*.
- Other bacterial genera viz., *Pseudomonas*, *Pantoea* (*Erwinia*) and *Xanthomonas* can nucleate the crystallization of ice from super-cooled water.

Bisht,2011

Ice nucleation proteins(INPs)

Three classes of ice-nucleating structures

- The class C structure was composed of aggregates of ice-nucleating protein (INP),
- The class B structure was a glycoprotein with sugar residue including glucose, mannose etc., attached to the protein core, and
- The class A structure was a lipoglycoprotein that was covalently anchored to the cell surface via a mannose-PI (phosphotidylinositol) that is similar to the anchoring of many proteins to cell membranes I eukaryotic cells.

Bisht,2011

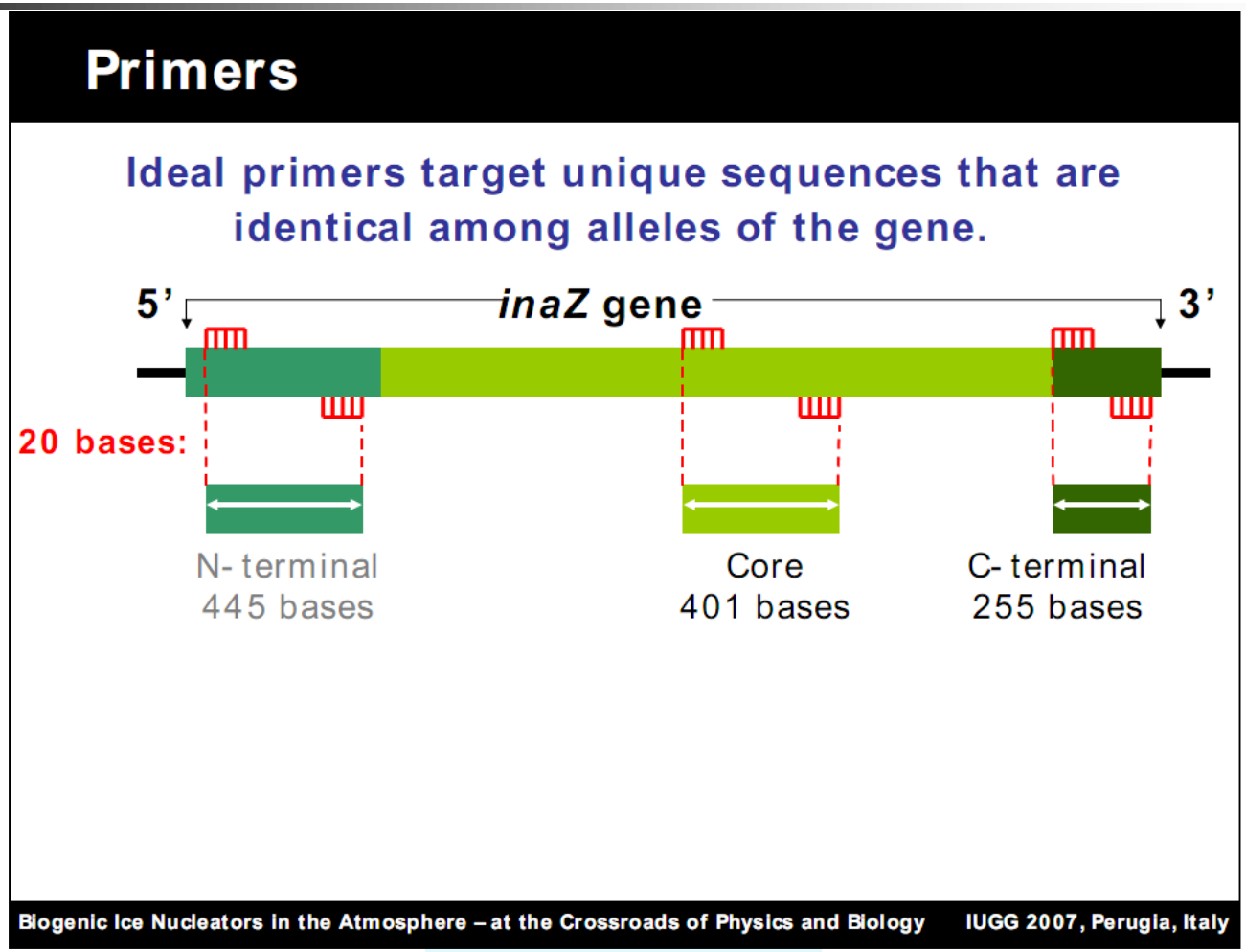
Ice nucleation genes

Transform the bacterium *E. coli* with the recombinant plasmid

- Plasmids containing known amounts and structures of DNA from INA^+ bacteria have been introduced into host *Escherichia coli*, converting the *E. coli* phenotypically from Ina^- (no ice-nucleating activity) to Ina^+.

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity



Guilbaud *et al.*,2007

Ice nucleation protein (INP)

Three domains proteins

Proteins that help ice crystals to form. The crystals pierce holes in plants

- INP is a monomeric protein composed of more than 1,200 amino acid residues with a deduced molecular mass of 118 kDa.
- One monomer is formed by:

1. A N-terminal domain (around 180 amino acids), probably involved in the phenomenon of maturation of the nucleation site,
2. A central repetitive region (around 1000 amino acids), which seems essential in the ice-producing activity, and
3. A C-terminal domain (around 50 amino acids) presumably involved in the aggregation of monomers (Green *et al.*,1988).

Lagriffoul *et al.*,2010;..

Ice nucleation protein (INP)

Three domains proteins

- Both the C and N-termini of INP are free and exposed on the cell surface, so foreign proteins fused to the C- or the N-terminus of INP can be localized to the cell surface.
- INP has the ability to maintain its ice nucleation activity after fusion to a foreign protein, which allows the detection of the recombinant proteins on the cell surface by ice nucleation activity assay.

Prasanth *et al.*,2015

Ice nucleation protein (INP)

Three domains proteins

Deletion mutagenesis of the ice nucleation gene from *Pseudomonas syringae*

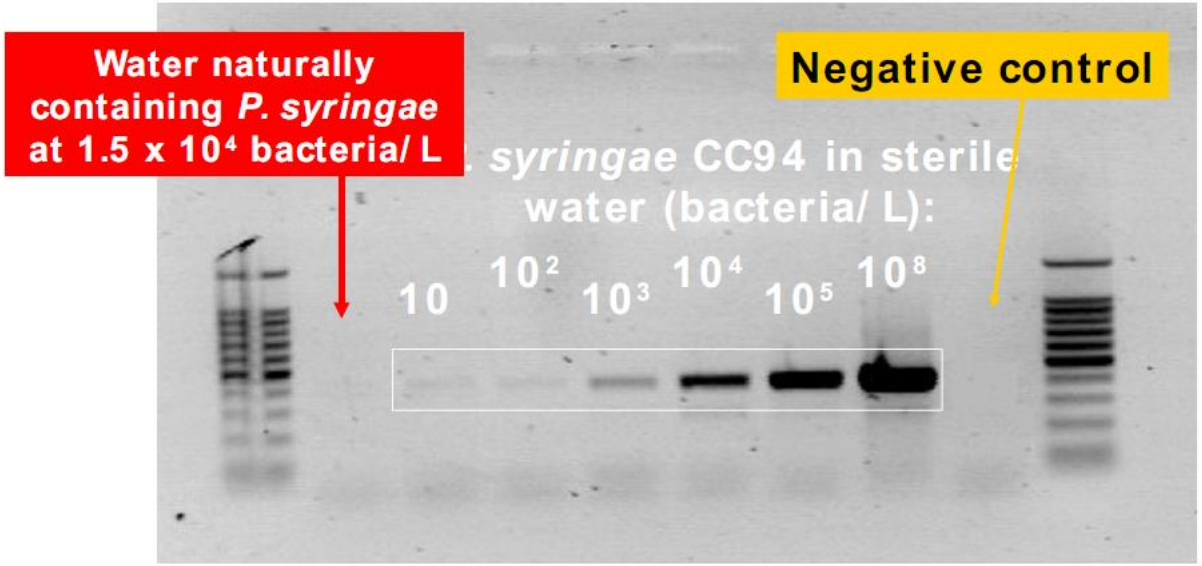
- Deletions which disrupted the periodicity of 16 codons, in a repetitive region of inaZ, caused the frequencies of ice nuclei in the bacterial population to be significantly depressed.
- Deletions removing part or all of one of the non-repetitive regions (that encoding the amino-terminal domain of the InaZ protein) did not abolish nucleation activity, but caused it to be limited to cooler threshold temperatures.
- In contrast, the non-repetitive carboxy-terminal do main of the InaZ protein was shown to be essential for ice nucleation at all temperatures.

Green *et al.*,1988

PCR Primers for *Pseudomonas* spp.

Detecting ice-nucleating bacteria in environmental samples using PCR of the gene conferring ice nucleation activity

Applications



P. syringae seeded into river water at 10^7 and 10^6 bacteria/ L was detected (lower concentrations not yet tested).

Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

Control of INA$^+$ bacteria

Frost control

By ice-minus strains

- By isolating, culturing, mass producing, and applying non-ice nucleation-active bacteria (ice-minus strains) antagonistic to ice nucleation-active bacteria on the plant surfaces.
- This treatment protects frost-sensitive plants from injury at temperatures at which untreated plants may be severely injured.

Both ice-plus and ice-minus strains can be found in nature. However, genetic engineering has allowed the gene to be altered in an ice-plus strain so that the protein is not produced. Ice-minus bacteria to be used for spraying crops are made on a large scale using recombinant DNA technology.

Control of INA$^+$bacteria

Frost control

By producing genetically modified bacteria

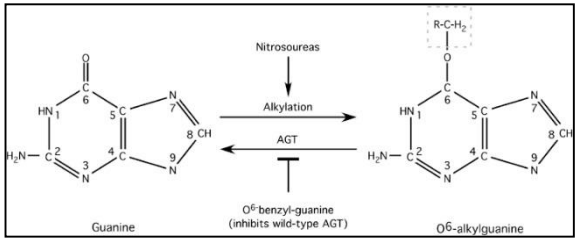
- **Ice-minus Bacteria (*P. syringae*) - Frost-fighting Superman?**
- Molecular studies indicate that ice-nucleation-active bacteria contain a single gene coding for a protein that seems to be responsible for catalysing ice.
- The genetically modified bacteria were to used to protect crops.
- The ice-minus variant of *P. syringae* is a mutant, lacking the gene responsible for ice-nucleating surface protein production.

Sugavanam,2010; Arnold,2009

Control of Ice bacteria

Chemically induced Ice$^-$ strains by ethyl methanesulfonate

- Induced mutations on the molecular level can be caused by chemicals such as methylating agents e.g. liquid methagen ethyl methanesulfonate (EMS), a colorless liquid, it is classified as an alkylating agent. EMS as an alkalizing mutagen.
- EMS ($C_3H_8O_3S$) is often used in genetics as a mutagen.
- It produces random mutations in genetic material by nucleotide substitution; specifically by guanine alkylation.
- Mutations induced by EMS can then be studied in genetic screens or other assays.



Alkylation is the transfer of an alkyl group from one molecule to another.

Control of Ice bacteria

Deletion mutagenesis of the ice nucleation gene(*InaZ*) from *Pseudomonas syringae*

- The effects of mutations on the ice nucleation phenotype of ice nucleation gene *inaZ*, from *Pseudomonas syringae* S203 were determined in a heterologous host, *Escherichia coli* K12.
- Deletions which disrupted the periodicity of 16 codons, in a repetitive region of inaZ, caused:

1. the frequencies of ice nuclei in the bacterial population to be significantly depressed;
2. the nuclei with thresholds at warmer temperatures were most affected.

McCorkle,2009

Control of Ice bacteria

Deletion mutagenesis of the ice nucleation gene(*InaZ*) from *Pseudomonas syringae*

- In contrast, when the periodicity was left intact, deletions and duplications in the same region had only slight effects on nucleation activity.
- Deletions removing part or all of one of the non-repetitive regions (that encoding the amino-terminal domain of the InaZ protein) did not abolish nucleation activity, but caused it to be limited to cooler threshold temperatures.
- In contrast, the non-repetitive carboxy-terminal do main of the InaZ protein was shown to be essential for ice nucleation at all temperatures.

McCorkle,2009

Control of Ice bacteria

Deletion mutagenesis of the ice nucleation gene from *Pseudomonas syringae*

- Mutation's which disrupted periodicity ("dysperiodic" mutations) in the repetitive region appeared to reduce the "quality" of ice nuclei (i.e., reduce threshold temperatures) rather than reduce their quantity.
- Mutations of inaZ were constructed in plasmid pRLG12 and pRLG27.
- The Ina phenotypes of plasmids were determined in *Escherichia coli*.
- In pRLG12, the inaZ gene is downstream from its native *ina* promoter, whereas in pRLG27, the *inaZ* gene is downstream from the *lac* promoter, and the *ina* promoter is absent.

Green *et al.*,1988

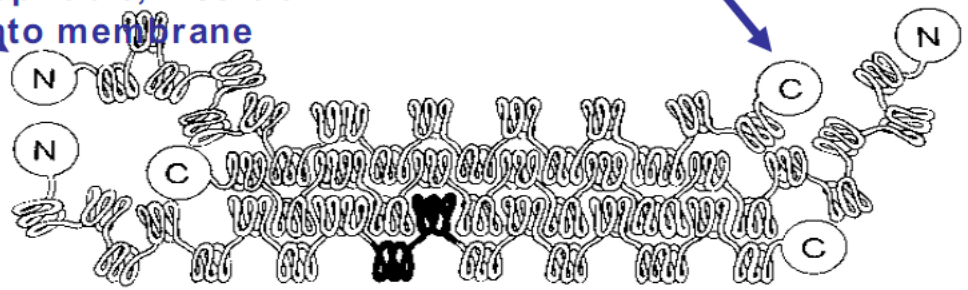
Ice nucleation protein (INP)

The central repeating domain act as a template for ice crystal formation

INA protein

N- terminal: hydrophobic, insertion into membrane

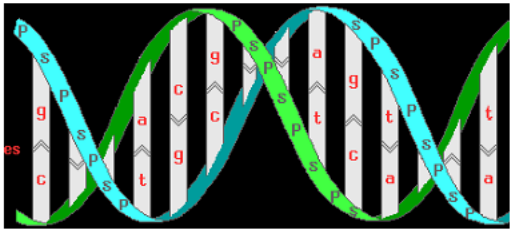
C- terminal: hydrophilic



Core: central domain, repeating units, template for binding water

(Kajava and Lindow 1993)

~4000 pairs of bases (A T G C) constitute the code in the DNA (gene) for this protein.



Biogenic Ice Nucleators in the Atmosphere – at the Crossroads of Physics and Biology IUGG 2007, Perugia, Italy

Guilbaud *et al.*,2007

Control of Ice bacteria

Genetically-engineered Ice$^-$ strains by recombinant DNA

- To systematically create the ice-minus strain of *P. syringae*, its ice-forming gene must be isolated, amplified, deactivated and reintroduced into *P. syringae* bacterium.
- The following steps are often used to isolate and generate ice-minus strains of *P. syringae*:
  - Digest *P. syringae*'s DNA with restriction enzymes.
  - Insert the individual DNA pieces into a plasmid.
  - Pieces will insert randomly, allowing for different variations of recombinant DNA to be produced.
  - Transform the bacterium *E.coli* with the recombinant plasmid.
  - The plasmid will be taken in by the bacteria, rendering it part of the organism's DNA.

Control of Ice bacteria

Genetically-engineered Ice$^-$ strains by recombinant DNA

- Identify the ice-gene from the numerous newly developed *E. coli* recombinants. Recombinant *E. coli* with the ice-gene will possess the ice-nucleating phenotype, these will be "ice-plus".
- With the ice nucleating recombinant identified, amplify the ice gene with techniques such as polymerase chain reaction (PCR).
- Create mutant clones of the ice gene through the introduction of mutagenic agents such as UV radiation to inactivate the ice gene, creating the "ice-minus" gene.

Wikipedia,2014

Control of Ice bacteria

Genetically-engineered Ice$^-$ strains by recombinant DNA

- Repeat previous steps (insert gene into plasmid, transform *E. coli*, identify recombinants) with the newly created mutant clones to identify the bacteria with the ice-minus gene. They will possess the desired ice-minus phenotype.
- Insert the ice-minus gene into normal, ice-plus *P. syringae* bacterium.
- Allow recombination to take place, rendering both ice-minus and ice-plus strains of *P. syringae*.

Wikipedia,2014

Control of Ice bacteria

Genetically-engineered bacteria

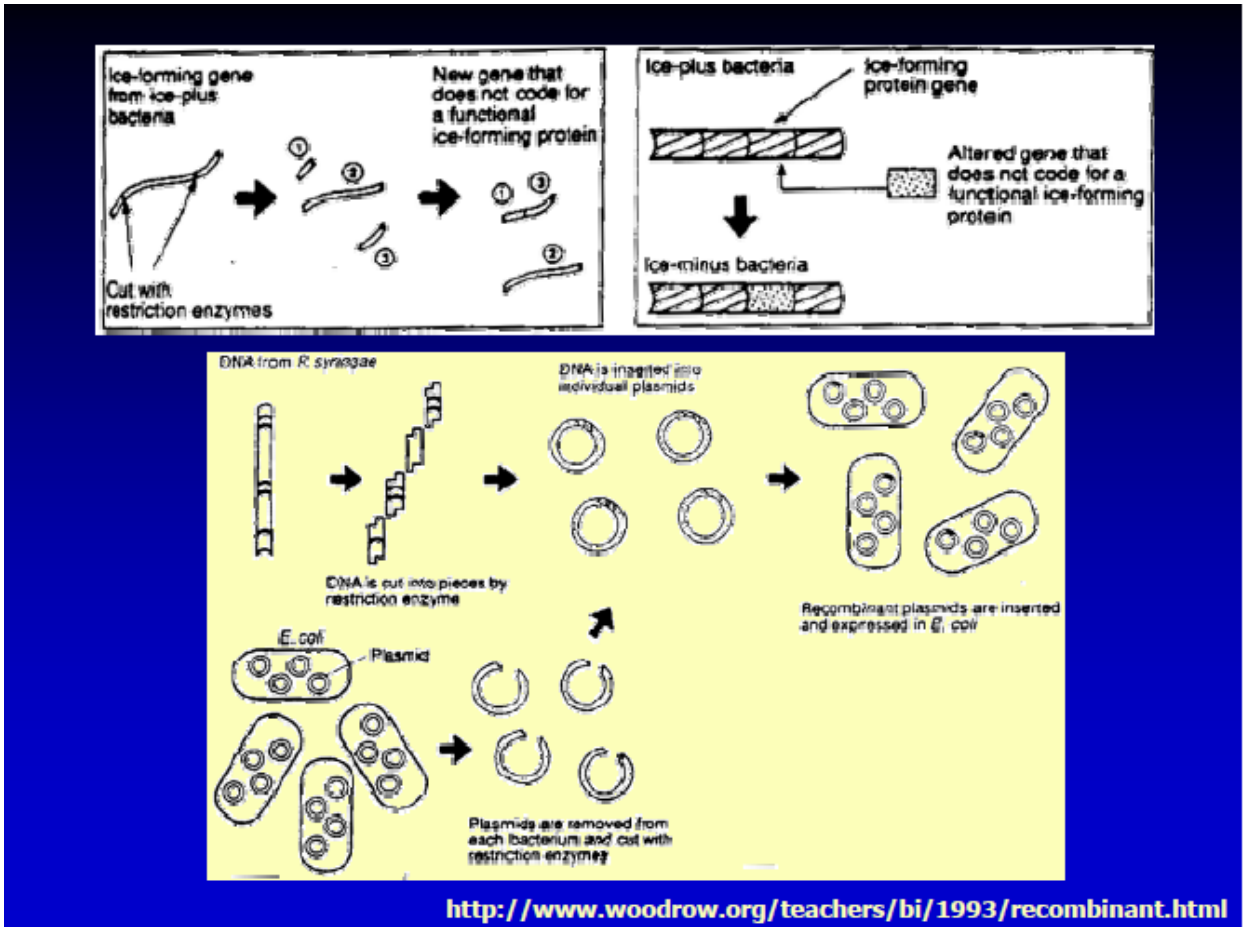
Tn-5 mutagensis

- Site-directed deletion of the ice nucleation gene from competent nonpathogenic strains of *P. syringae* and *P. fluorescens* (ice-minus mutants) have been generated as biological control agents of frost injury to plants, representing the first genetically-engineered bacteria to be released outside the laboratory under Environmental Protection Agency (EPA) permit in the USA.

Stephen Lindow, on carrying out further research, discovered the ice-minus strain of bacterium in 1977. Later he discovered a way of recreating these ice-minus bacteria using restriction enzymes and recombinant DNA technology.

Biocontrol of Ice bacteria

Genetically-engineered Ice^{-} strains by recombinant DNA



BacteriaFall,2008

Control of Ice bacteria

Genetically-engineered bacteria

Recombinant DNA to Protect Crops

'Ice minus' bacteria

Steven Lindow, Professor of Plant Pathology, UC-Berkeley

produced ice-minus *P. syringae*

Control of Ice bacteria

Genetically-engineered bacteria

BacteriaFall,2008

Control of Ice bacteria
Genetically-engineered bacteria

- Berkeley plant scientists under direction of Steven Lindow spraying a field of potatoes with ice-minus, a genetically engineered version of *Pseudomonas syringae* that prevents frost, in 1987.

Ksobecka,2015

Efficacy of the Ice mutants

Reduction but not elimination

Frostban: a commercial product

- *Pseudomonas syringae* - ice-minus bacteria- recombinant strains can't make proteins which act as nuclei for ice crystal formation.
- Recombinant DNA techonlogy has allowed for the synthetic removal or alteration of specific genes, enabling the creation of the ice-minus strain.
- The recombinant form was developed as a commercial product known as Frostban.
- Field-testing of Frostban was the first release of a genetically modified organism into the environment. The testing was very controversial and drove the formation of US biotechnology policy. Frostban was never marketed.

Wikipedia,2014

Efficacy of the Ice mutants

Reduction but not elimination

- Ice mutant strains were effective in preventing or minimizing colonization of plants by INA bacteria but not in eliminating established populations of the target microbes.
- Establishment of relatively large population sizes of the antagonists on leaf surfaces was required for effective exclusion of INA bacteria from leaf surfaces.

Efficacy of the Ice mutants

Reduction but not elimination

- The efficacy of the Ice mutants was similar to that found with some naturally occurring non-INA strains.
- The reduced population sizes of Ice^+ bacteria treated with the Ice strains correlated with reduced amounts of frost injury (ca. 70 to 80%) to potato plants during a natural radiative frost event.
- Radiation frosts occur because of heat losses in the form of radiant energy.

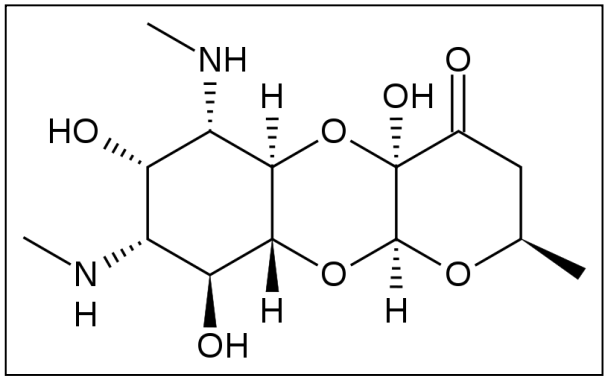
Chemical control of Ice bacteria

Frost control

By spectinomycin

- Some chemicals are effective in reducing INA as we found in laboratory conditions but how they work in nature is yet to be clarified.
- While spectinomycin has been reported to cause rapid reduction in ice nucleation activity at higher temperatures, its effect may also be dependent on the environmental conditions during or after such treatment.

Spectinomycin is produced in nature by many organisms including cyanobacteria and various plant species. It is also produced by the bacterium *Streptomyces spectabilis*.



Nejad,2005

Control of winter frost

Application of plant anti-stress polymer against different climate stresses including frost

- Use a liquid polymer.
- The non-toxic liquid sold at nurseries and hardware stores gives an extra five degrees of protection.
- One application coats leaves with an invisible polymer film, that can protect a plant for as long as three months.

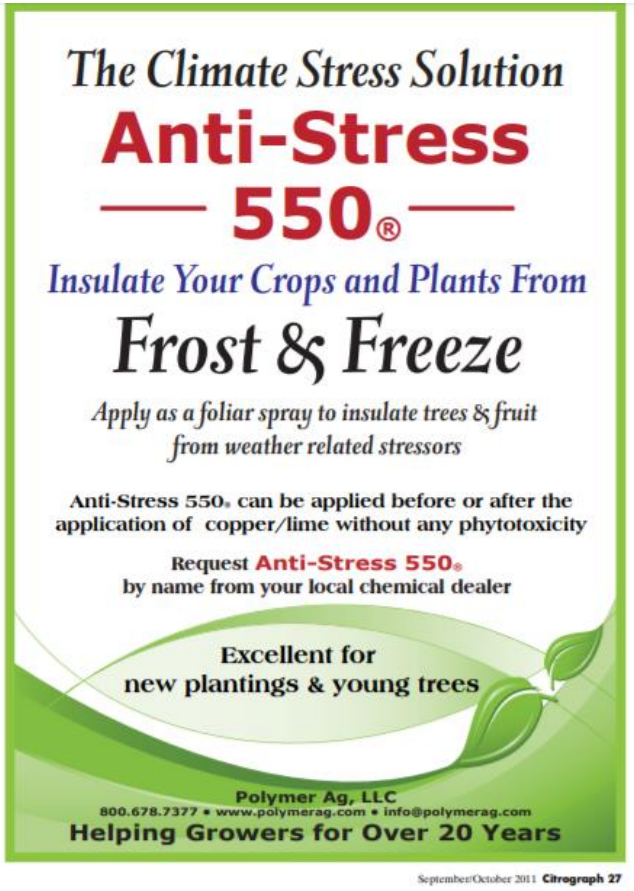
Klapp,2013; Smith,2017

Control of winter frost

Application of plant anti-stress polymer against different climate stresses including frost

Anti-Stress 550 and CM-96-018

- Frost damage to potatoes, grapevine and citrus plants was assessed following treatment with either an acrylic polymer (Antistress™) or with a hydrophobic particle film (CM-96-018).
- In large freezing tests, the application of the hydrophobic particle film (CM-96-018) consistently led to less damage compared to acrylic polymer (Antistress™).



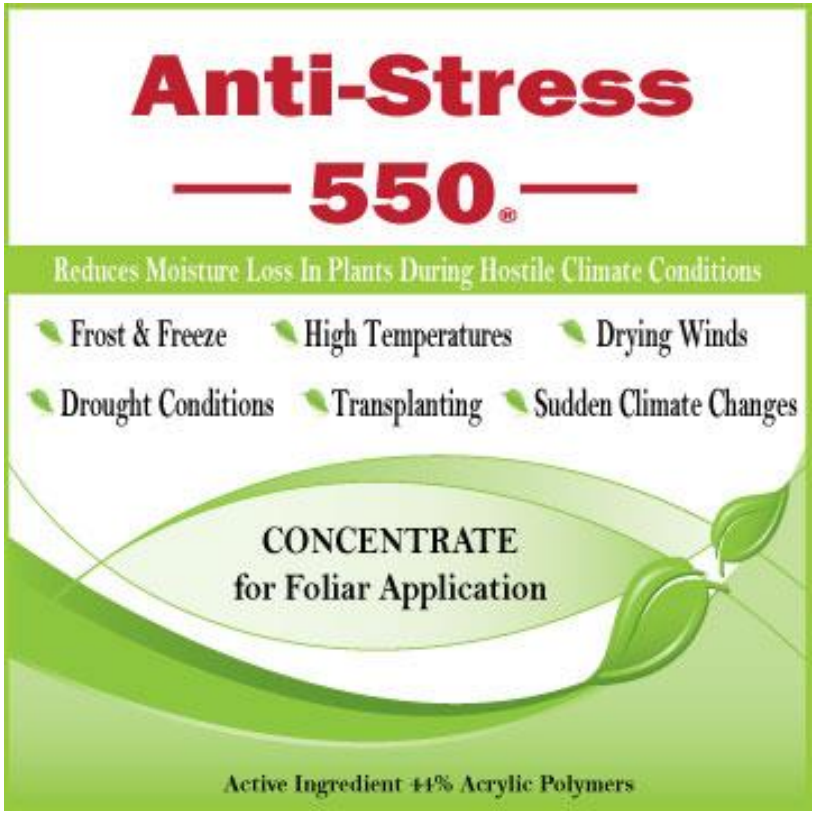
Fuller *et al.*,2006

Control of winter frost

Application of plant anti-stress polymer against different climate stresses including frost

Anti-Stress 550

- Plant Anti-Stress 550, 2.5 gal.
- SKU: SSS1172
- Categories: Anti Transpirant, Clearance, Tree Care
- Tag: planting
- Price: $190.00



Terra Tech

Control of frost and heat

Application of Envy, a bio-shield polymer against summer heat and winter frost

Envy

- Bio-shield polymer for protection against summer heat and winter frost.
- Envy is a frost protector and anti transpirant, which can substantially

1. reduce moisture losses by up to 50%,
2. eliminate sun & windburn damage, as well as
3. increase frost tolerance by an additional 4 Degrees.

AgroBest

Control of frost
DEMETER, a seaweed-based product

- AgroBest has developed this seaweed-based product as it offers a degree of frost protection and nutrition to a wide range of crops.
- With its seaweed base and the additive of potassium, crops will be protected short term, with possible yield benefits long term.
- The potassium provides protection to the plant within 6 hours of application, and the seaweed will continue protection after 36 hours to keep plants protected for a period of 10-12 days.
- Demeter will provide approximately 2-3°C extra frost tolerance, whilst also providing nutritional benefits essential to plant quality.

Control of Ice bacteria
Application of polymers

POLYMERS FOR PROTECTING MATERIALS FROM DAMAGE

Patent Number:	WO2004030455
Publication date:	2004-04-15
Inventor(s):	GUILLET JAMES (CA); HAYNES MICHAEL N (US); ZYCHICK JOEL (US)
Applicant(s):	AGROSHIELD LLC (US); GUILLET JAMES (CA); HOFHEIMER ADAM (US); ZYCHICK JOEL (US)
Requested Patent:	WO2004030455
Application Number:	WO2003US31385 20031002
Priority Number(s):	US20020415461P 20021003
IPC Classification:	A01N1/00; A01N3/00; A01N25/04; A01N25/28
EC Classification:	
EC Classification:	
Equivalents:	AU2003282921
Cited Documents:	US6180562

Control of Ice bacteria

Antifreeze proteins (AFPs) vs. nucleation proteins (INPs)

- Ice-nucleating bacteria (INBs):

1. Produce ice nucleation proteins (1200 aa to 1500 aa);
2. Promote the growth of ice in freezing-sensitive plant tissues at temperatures as high as -2°C;
3. Modifying freezing processes to obtain nutrients (release nutrients from host tissues that fuel bacterial proliferation).

- In contrast, some bacteria from permanently or seasonally frozen habitats secrete antifreeze proteins (AFPs) to inhibit the growth of external ice and promote survival.

Muryoi *et al.*,2004

Antifreeze proteins

AFPs(antifreeze non-glycoproteins) vs. AFGPs (antifreeze glycoproteins)

- Biological antifreezes consist of:

1. Antifreeze proteins (AFPs), and
2. Antifreeze glycoproteins (AFGPs).

- Glycoproteins are proteins contain carbohydrates.
- antifreeze glycoproteins (AFGPs) were the first AFPs discovered in fish.
- AFGPs circulates in the blood of Arctic fish enable them to avoid freezing in their perpetually icy environment.

Wikipedia,2010;..

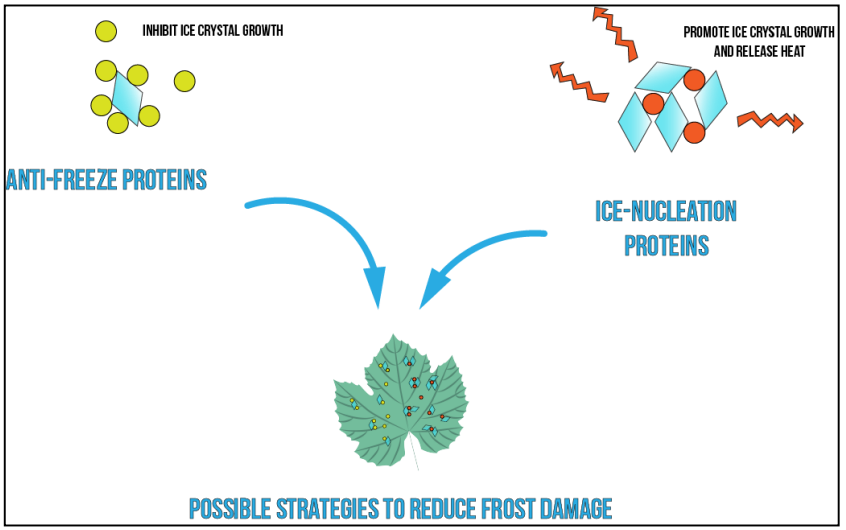
Antifreeze proteins (AFPs)
Function

- This antifreeze activity makes the organisms less sensitive to cold temperatures.
- Antifreeze protein (AFP) promotes survival of bacteria at subzero temperatures.
- Antifreeze proteins adsorb onto the surface of ice and inhibit its recrystallization.
- Inhibitors of heterogeneous ice nucleation, which can favour supercooling, have been found in various organisms.
- Note: Larger ice crystals are more likely to injure biological organisms.

Muryoi *et al.*,2004;..

Antifreeze proteins (AFPs)
Function

- Ice-Binding Proteins (IBPs) have the ability to bind ice crystals and operate different actions on them. Among this particular protein family, Antifreeze Proteins (AFPs) encircle crystals to inhibit their growth.



Inhibiting recrystallisation, and lowering the water freezing temperature.

Antifreeze proteins (AFPs)
Function

- Cropaid Natural Plant Antifreeze contains *Thiobacillus* subspecies and minerals used by these bacteria.
- It is freeze resistant and lowers the freezing point of the plant.
- It also stimulates plants to produce antifreeze proteins and antifreeze amino acids so the plants get stronger against cold and frost temporarily (15-20 days).

Cropaid

Inhibiting recrystallisation, and lowering the water freezing temperature.

Antifreeze proteins
AFPs(antifreeze non-glycoproteins)

- AFPs have been discovered in:
- various fish,
- insects,
- bacteria,
- fungi, and
- overwintering plants including ferns, gymnosperms, monocotyledonous, dicotyledonous, angiosperms, etc.

Wikipedia,2010;..

AFP diversity

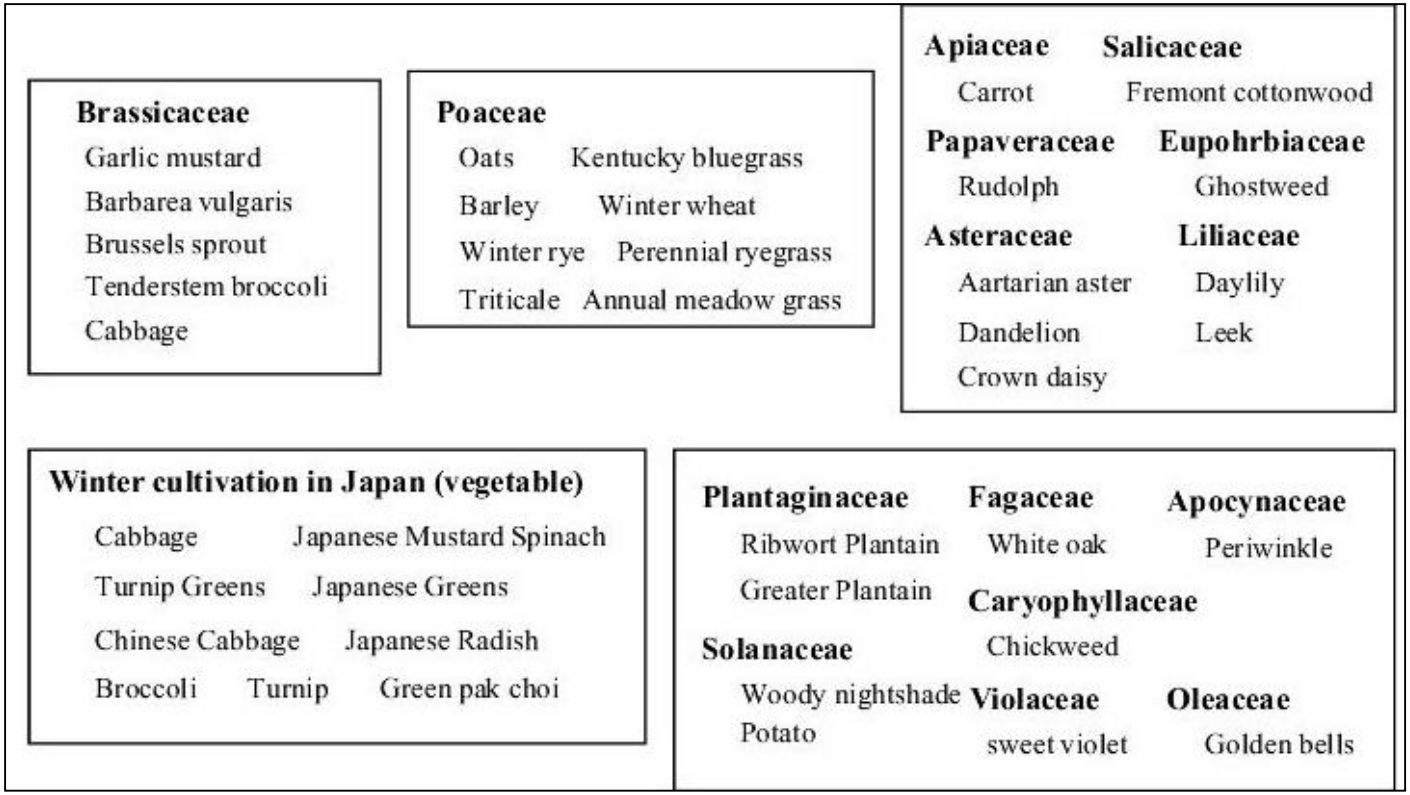
In different organisms rather bacteria

Number	Protein	Species
1	Fish type I	sculpins; righteye flounders
2	Fish type II AFP	Atlantic herring; sea raven, rainbow smelt
3	Fish type III AFP	eel pouts
4	Fish type IV AFP	longhorn sculpin
5	AFGP	cods and Antarctic notothenioids
6	Chitinase AFP	winter rye
7	Glucanase AFP	winter rye
8	Thaumatin AFP	winter rye
9	Budworm AFP	spruce budworm
10	Beetle AFP	mealworm beetle; *Dendroides canadensis*

Ewart *et al.*,1999

AFP diversity

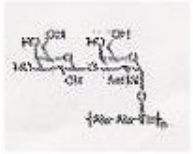
List of some plants having antifreeze activity



Kawarhara *et al.*,2013

AFP diversity

Structure and properties of AFP and AFGP from various fishes

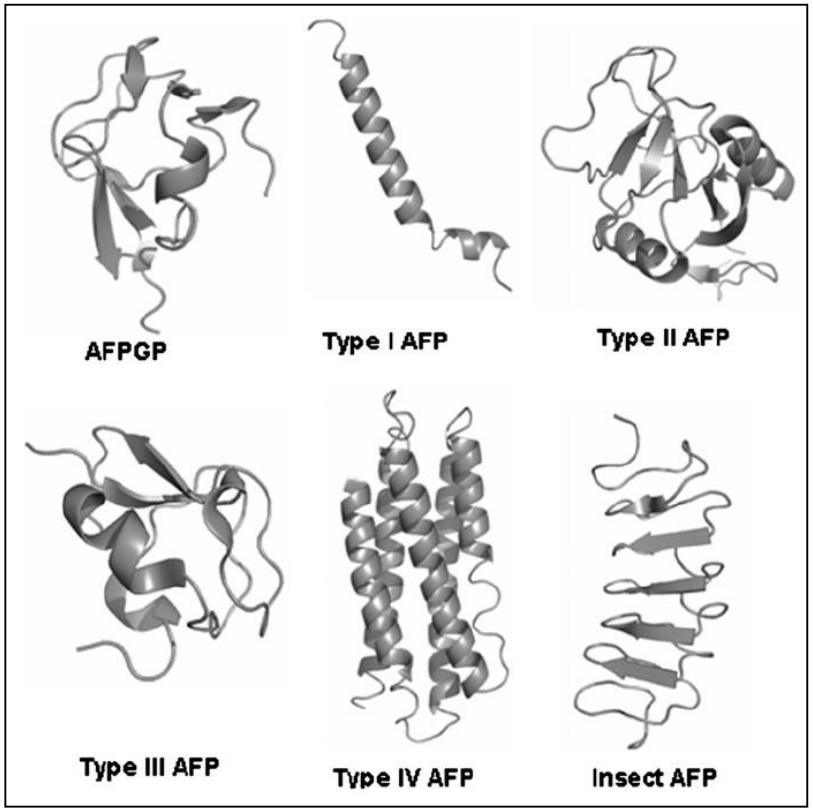
Characteristics	AFGP	Type Ⅰ AFP	Type Ⅱ AFP	TypeⅢAFP	TypeⅣAFP
Mass (Da)	2600-33000	3300-4500	11000-24000	6500	12000
Key Properties	AAT repeat Disaccharide	Alanine-rich α-helix	Disulfide bonded	β-sandwich	Alanine rich herical bundle
Representative Structure					
Natural Sources	Antarctic-nototheniods	Right-eyed, flouders	Sea raven, Smelt, herring	Ocean pout, Wolfish, eel pout	Longhorn, sculpin
					

Kawarhara *et al.*,2013

Antifreeze proteins (AFPs)

Types of antifreeze proteins

- In fish, AFPs are classified into five known types namely AFGPs, AFPI, AFPII, AFPIII and AFPIV.
- AFGPs are made up of 4 to more than 50 tandem repeats of Ala-Ala-Thr with a disaccharide attached to each Thr OH.



Kandaswamy *et al.*,2011

Antifreeze microorganisms
Yeasts

- Some cold-adapted microorganisms increase their survival at sub-zero temperatures by producing proteins that bind to and inhibit the growth of ice crystals.
- These included:
- Two yeast species:

1. *Cryptococcus* sp. and
2. *Rhodotorula* sp.

Raymond *et al.*,2008

Antifreeze bacteria

Antifreeze proteins of bacteria (AFPs)

INP about 150 KDa vs. AFP between 22-164 KDa

- To our knowledge only five bacteria have been shown to possess AFP activity:

1. A PGPR *Pseudomonas putida*, isolated from plants in the high Arctic,
2. *Micrococcus cryophilus*, isolated from chilled sausages,
3. *Rhodococcus erythropolis*, was isolated from the mid-gut of beetle larvae from an unknown location.
4. *Marinomonas protea*,
5. A *Moraxella* species, isolated from Antarctic soils.

- Only two bacterial AFPs have been characterized to date:
- A 164-kDa lipoglycoprotein from *P. putida* GR12-2, and
- A 52-kDa lipoprotein from a *Moraxella* sp.

6. *Acinetobacter calcoaceticus* KlNI-1 was also produced anti-nucleating protein with molecular mass of 55 kDa (Kawahara,2002).

Gilbert *et al.*,2004

Antifreeze bacteria
Antifreeze proteins (AFPs)

7. *P. syringae* pv. *syringae* B728a

- Recently it was shown that *P. syringae* pv. *syringae* B728a also has an unlinked gene encoding an antifreeze protein (Feit *et al.*,2005).
- Antifreeze proteins are secreted into the medium, where they inhibit the growth of external ice by:

1. adsorbing onto the ice surface, and
2. lowering the temperature at which it can grow.

Höfte & De Vos,2006

Antifreeze bacteria
Pseudomonas putida

8. *Pseudomonas putida*

- The AFP of *Pseudomonas putida*, a common soil bacterium, has been purified, and it has an amino acid composition and other compositional properties similar to those of the bacterial ice nucleating proteins.
- This protein differs from other known antifreezes in having both:

1. ice nucleating, and
2. Antifreeze activities.
3. Sequencing and structural studies on this protein will be of great interest.

Ewart *et al.*,1999

Antifreeze bacteria
Pseudomonas putida

- *Pseudomonas putida*
- It was found that the secreted antifreeze protein from *P. putida* GR12-2 lacks a conserved canonical N-terminal signal peptide.
- However, they speculate secretion may occur using either:

1. a hemolysin-like secretion, or
2. type V autotransportation system.

Hemolysins or haemolysins are lipids and proteins that cause lysis of red blood cells by destroying their cell membrane.

Lorv *et al.*,2014

Antifreeze bacteria
Acinetobacter and *Bacillus*

9. *Acinetobacter* and *Bacillus*

- Some known anti-nucleating bacteria belong to the genus *Acinetobacter* or *Bacillus*.
- Additional bacterial genera with ice recrystallization inhibition activity, but the source of the activity remains to be confirmed as antifreeze proteins; these include
- *Buttiauxella*,
- *Chryseobacterium*, and
- *Idiomarina*.

Phosphatidylinositol was purified from *Bacillus cereus*, and an activity inhibitory to ice nucleation(Warren 1987).

Lorv *et al.*,2014

Antifreeze bacteria
Ice recrystallization inhibition activity

10. Other bacteria observed to have ice recrystallization inhibition activity include:

- *Sphingomonas* sp.,
- *Halomonas* sp.,
- *Pseudoalteromonas* sp.,
- *Stenotrophomonas maltophilia*,
- *Psychobacter* sp.,
- *Enterobacter agglomerans*,
- *Pseudomonas fluorescens*, *Rahnella* sp.,
- *Duganella zoogloeoides*,
- *Erwinia billingiae*, and
- *Sphingobacterium kitahiroshimense*.

Lorv *et al.*,2014

Thermal hysteresis
TH activity

- AFPs create a difference between the melting point and freezing point known as thermal hysteresis(TH).
- AFP activity is commonly defined in terms of thermal hysteresis(TH), which is the difference observed for the solution freezing and melting temperatures.

The freezing point of pure water is 0°C, but that melting point can be depressed by the adding of a solvent such as a salt.

Kawarhara *et al.*,2013

Thermal hysteresis
TH activity

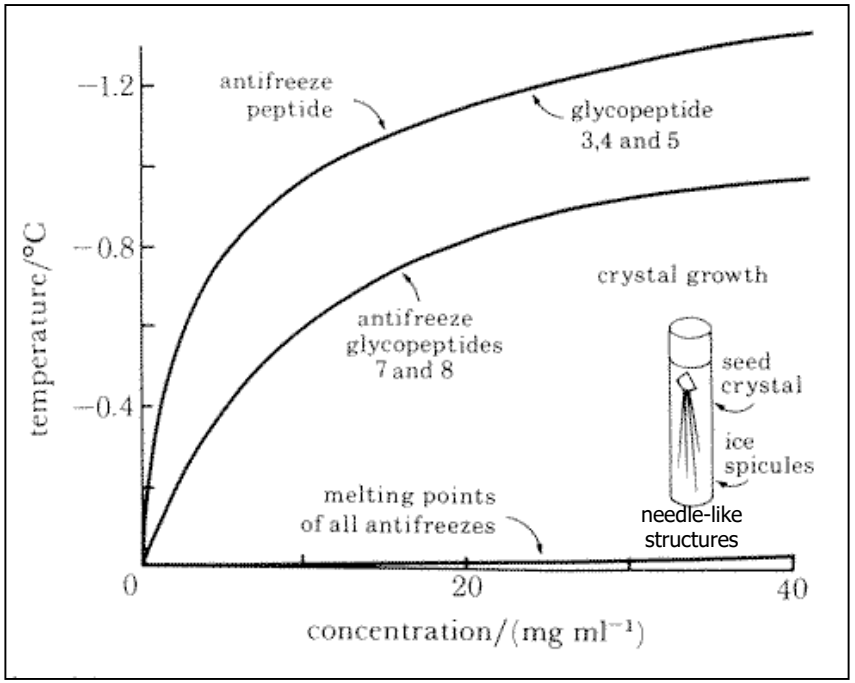
- Organisms differ in their values of thermal hysteresis.
- The maximum level of thermal hysteresis shown by fish AFP is approximately -1.5°C.
- However, insect antifreeze proteins are 10-30 times more active than fish proteins.
- In contrast, aquatic organisms are exposed only to -1 to -2°C below freezing.
- During the extreme winter months, the spruce budworm resists freezing at temperatures approaching -30°C.
- The Alaskan beetle *Upis ceramboides* can survive in a temperature of -60°C by using antifreeze agents that are not proteins.

Wikipedia,2015

The thermal hysteresis

The difference between the freezing point and the melting point

- All of the AFP and antifreeze glycoprotein (AFGP) could act only to lower the freezing point of water without altering the melting point.
- This activity was defined by the difference between both temperatures (Kawahara,2002).
- This value is called thermal hysteresis.
- The thermal hysteresis, or the difference between the freezing point (crystal growth point) and the melting point is shown in this graph for several of the antifreeze compounds.

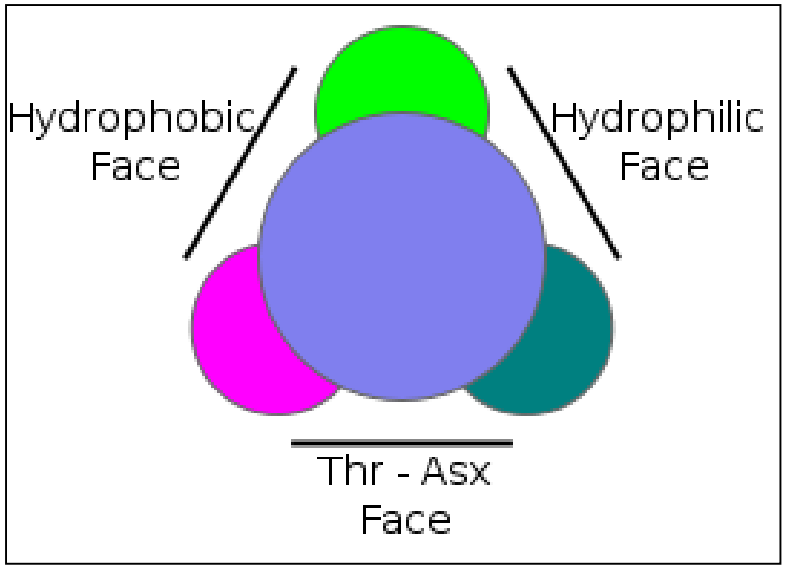


Muldrew,1999

Antifreeze proteins (AFPs)
Diversity in their tertiary structures

- Despite their common function, AFPs display remarkable diversity in their tertiary structures.
- The five types of AFPs differ in their primary sequence of amino acids.

1. Type I -IV AFPs were found in fishes.
2. Type V AFPs are found in different insect families.

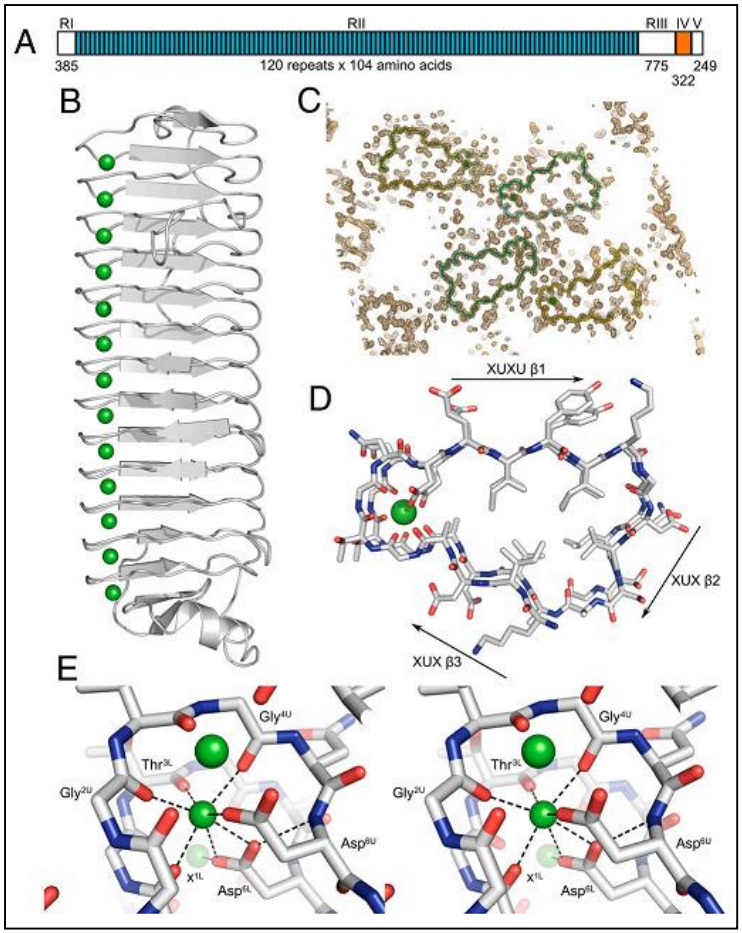


The three faces of Type I AFP
3D structure of Type I AFP the best documented AFP.

Wikipedia,2013

Antifreeze proteins (AFPs)
Ca-dependent AFP (MpAFP)

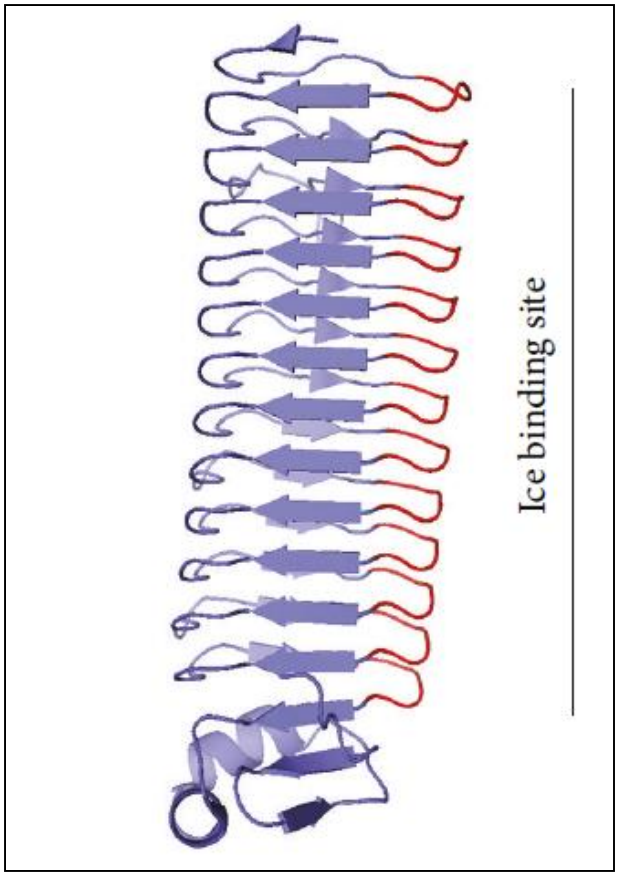
- The Antarctic bacterium *Marinomonas primoryensis* produces an exceptionally large (ca. 1.5 MDa) Ca-dependent AFP (MpAFP).
- The protein contains two highly repetitive segments that divide it into five distinct regions.
- The antifreeze activity of Mp AFP resides in region IV (Mp AFP_RIV), a 322-aa segment of the protein that contains 13 tandem 19-aa repeats.



Garnham *et al.*,2011

Antifreeze proteins (AFPs)
Diversity in their tertiary structures

- Protein structure of *Mp*AFP retrieved from RCSB Protein Data Bank (PDB).
- Ice binding site of the antifreeze protein lies along the aligned calcium binding turns, X-Gly-Thr-Gly-Asn-Asp.
- These calcium binding turns are highlighted in red.
- The figure was created using the program PYMOL (http://www.pymol.org)..



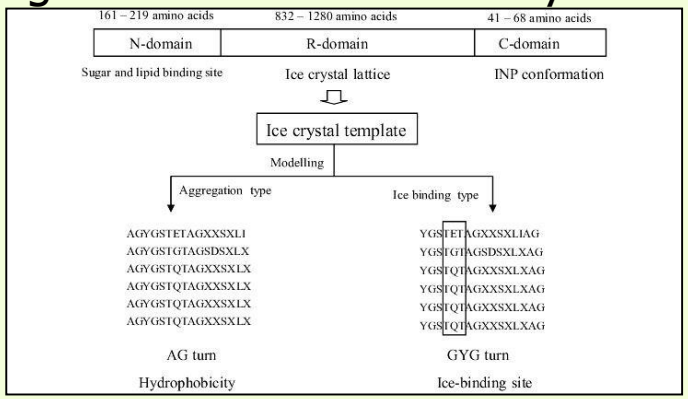
Lorv *et al.*,2014

Antifreeze proteins (AFPs)

The structure of ice-nucleating protein and both models of different properties

- Based on the structure and component of ice nucleation materials, we could predict that each domain has the following important role for the nucleation:
- N-domain through association with lipid and saccharide thereby increasing hydrophobicity;
- R-domain through structuralization of ice lattice-resembling protein; and
- C-domain through stabilization of tertiary structure of the complex.

Lattice(order)



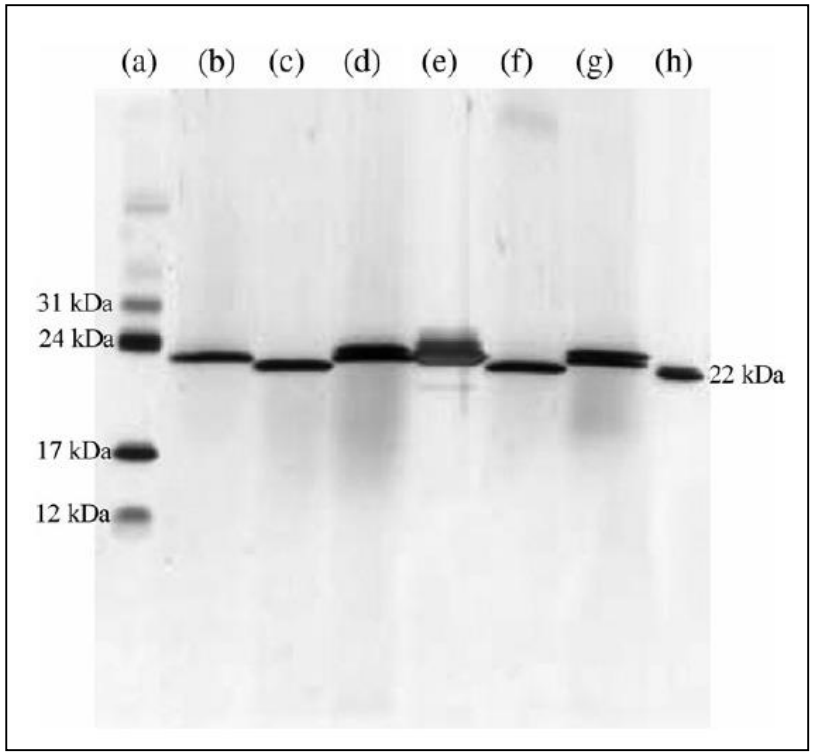
Kawarhara *et al.*,2013

Antifreeze bacteria

SDS-PAGE analysis of bacterial AFP purification

- SDS-PAGE analysis of bacterial AFP purification.
- Six of bacterial AFPs were purified on SDS-PAGE followed by silver staining.

a) Polypeptide marker (Bio-Rad),
b) Isolate code cry-c(see table),
c) Isolate code cry-g,
d) Isolate code cry-k,
e) Isolate code cry-l,
f) Isolate code cry-n,
g) Isolate code cry-21,
h) *Typhula ishikariensis* AFP (TisAFP) with a molecular weight of 22 kDa (Hoshino *et al.*,2003a, b) as a standard.



Singh *et al.*,2013

Biocontrol of Ice bacteria

Interactions of antifreeze proteins (AFPs) and ice nucleation proteins (INPs)

- Sometime some AFPs have the ability to interact with ice nucleators (INAs), which may result in either:

1. the inhibition, or
2. the enhancement of ice nucleation activity.

- **E.g.**
- *P. syringae* pv. *syringae* B728a an economically important species of plant pathogenic bacteria with ice nucleating gene (Psyr1608) also has an unlinked gene encoding for an anti-freeze protein (Psyr0931).

Kandaswamy *et al.*,2011;..

Biocontrol of Ice bacteria

Interactions of antifreeze proteins (AFPs) and ice nucleation proteins (INPs)

- These inhibitors are known to exist in the xylem parenchyma cells of Katsura trees (*Cercidiphyllum japonicum*).
- Other ice crystal-controlling materials, which can play a crucial role in the second step of ice formation, are antifreeze proteins, antifreeze glycoproteins and antifreeze glycolipids.
- The function of AFP is to inhibit ice formation and ice crystal growth by suppressing the binding of water molecules to the ice crystal surface.

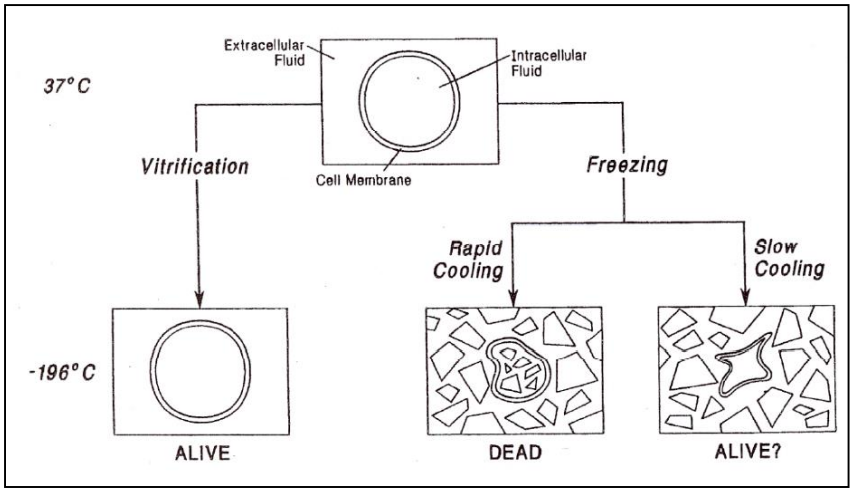
Kawarhara *et al.*,2013

The freezing process in plants

Physiochemical process during cryopreservation of the cells

1. Freezing can occur exclusively in the extracellular space (right),
2. it can occur in both the extracellular and the intracellular space (middle), or
3. it may occur not at all (left).

- Adapted from Coger and Toner(1995) in Fahy (1995).



Water that becomes solid without freezing is said to be vitrified. Vitrification(transformation of a substance into a glass) can occur usually through very rapid cooling or the introduction of agents that suppress the formation of ice crystals.

Nejad,2005;..

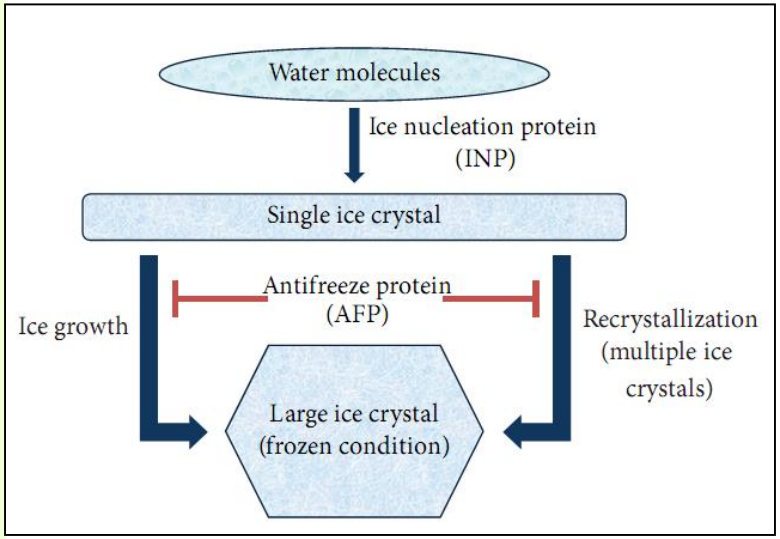
The interactions of ice crystal controlling proteins on the two stages of ice crystal growth

1. Ice nucleation proteins induce growth of single ice crystal nuclei, while
2. Antifreeze proteins inhibit further ice crystal growth.

- Blue lines represent ice growth direction at low subzero temperatures;
- Red lines indicate inhibitory action.
- Ice crystal formation occurs in two stages:

1. ice nucleation, and
2. ice growth.

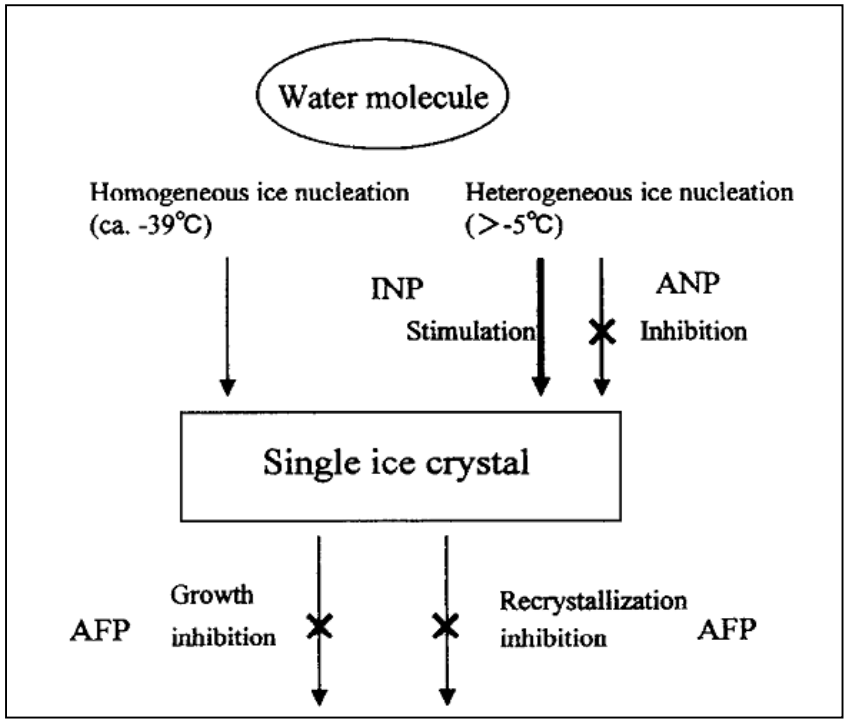
- Each class of ice crystal controlling protein targets one of these two stages.



Lorv *et al.*,2014

The scheme of action modes on various ice crystal-controlling proteins(AFPS)

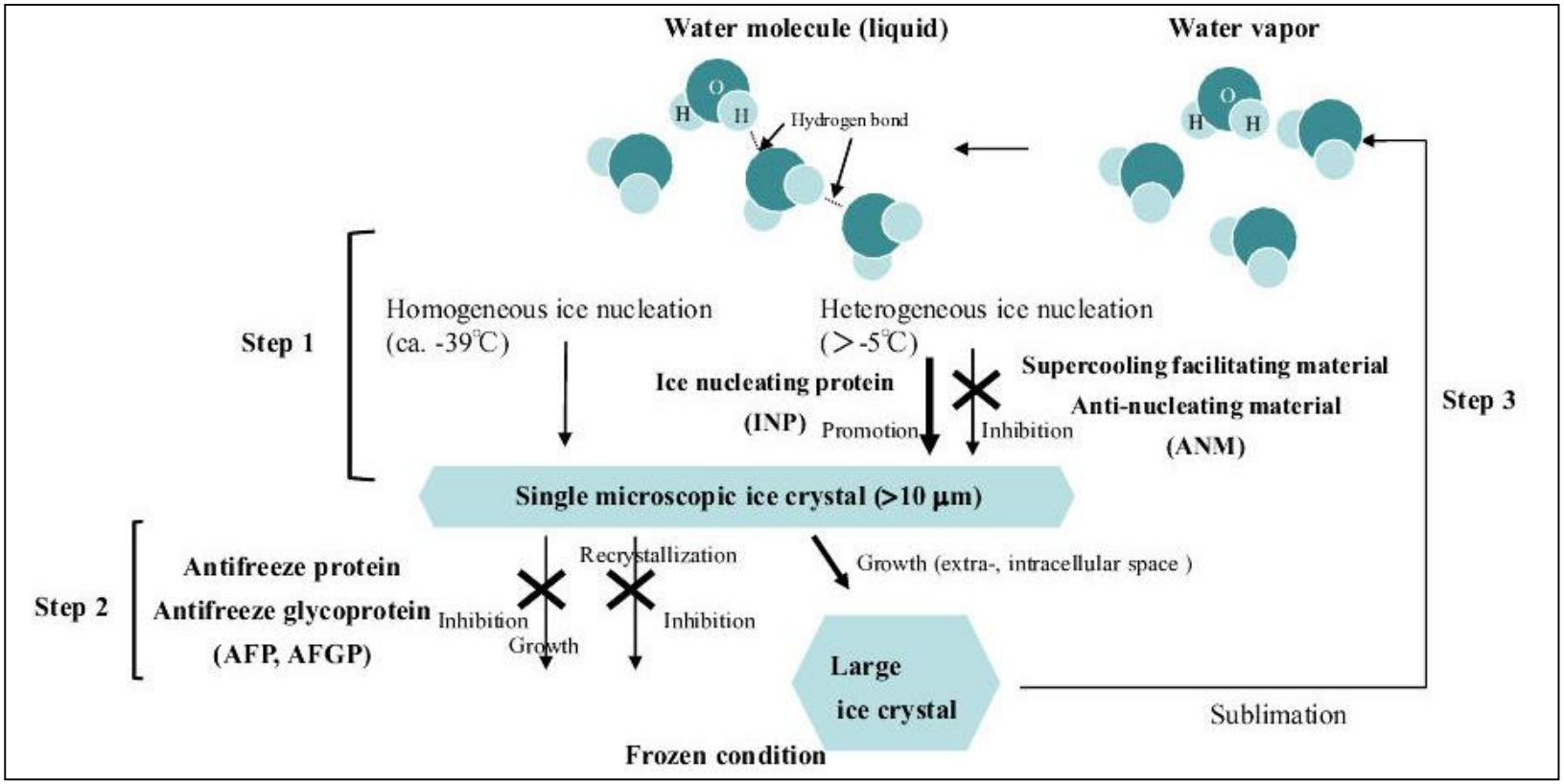
- Homogeneous water(pure water) was reported to freeze at -40°C.
- A heterogeneous water solution can contain additional molecules, such as dust particles and ice active bacteria, that act as seeds for ice nucleation.
- In these situations, a solution can freeze at high subzero temperatures, up to -2°C.



Kawahara,2002; Lorv *et al.*,2014

Antifreeze proteins (AFPs)

The representative functions on various ice crystal-controlling materials

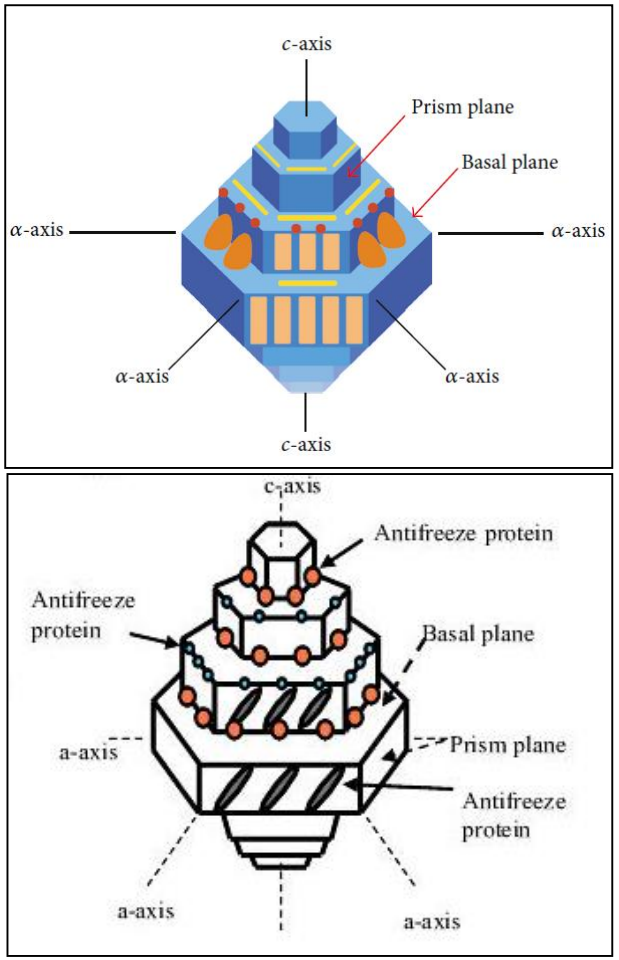


Kawarhara *et al.*,2013

Antifreeze proteins (AFPs)

AFP binding to hexagonal ice crystal

- Potential interactions of antifreeze proteins on ice crystal surfaces.
- Antifreeze proteins (non-blue objects) bind to two major planes: prism and basal plane.
- Location of binding is dependent on the protein characteristics.
- Antifreeze proteins bound to the prism plane inhibit ice growth along the α-axes, but when bound to the basal plane *c*-axis ice growth is inhibited.

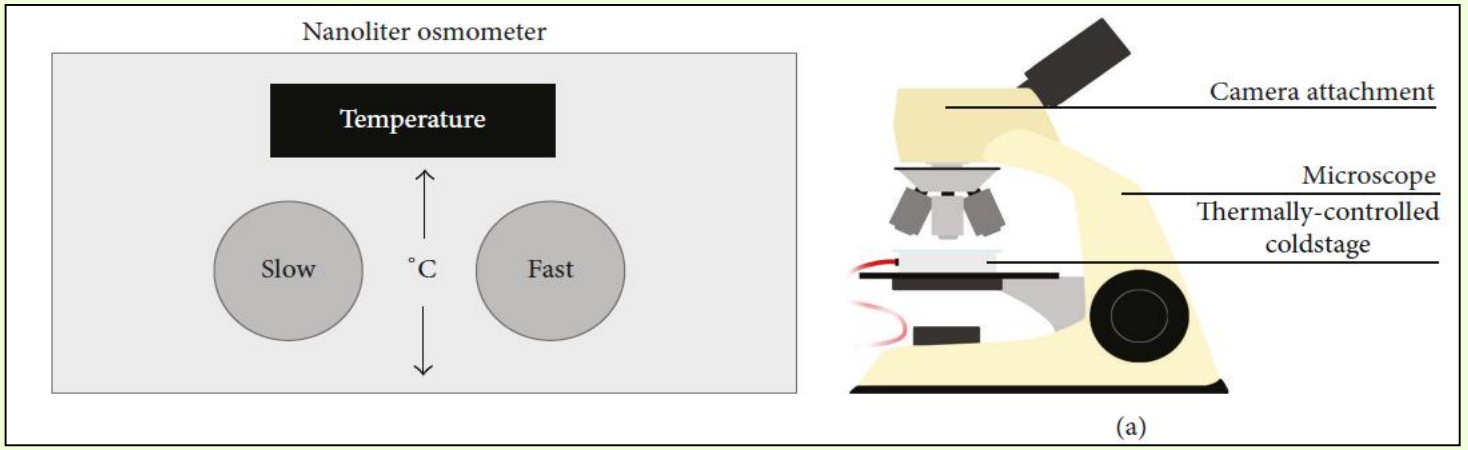


Kawarhara *et al.*,2013; Lorv *et al.*,2014

Antifreeze proteins (AFPs)

Instruments used to determine thermal hysteresis

- The operator uses the nanoliter osmometer to carefully control the temperature of the cold plate:
- knobs are used to sensitively increase and decrease temperatures.
- A solution droplet placed in immersion oil is observed for ice crystals.
- Following flash freezing, the solution is melted until a single ice crystal remains to visually determine the melting and freezing temperature.



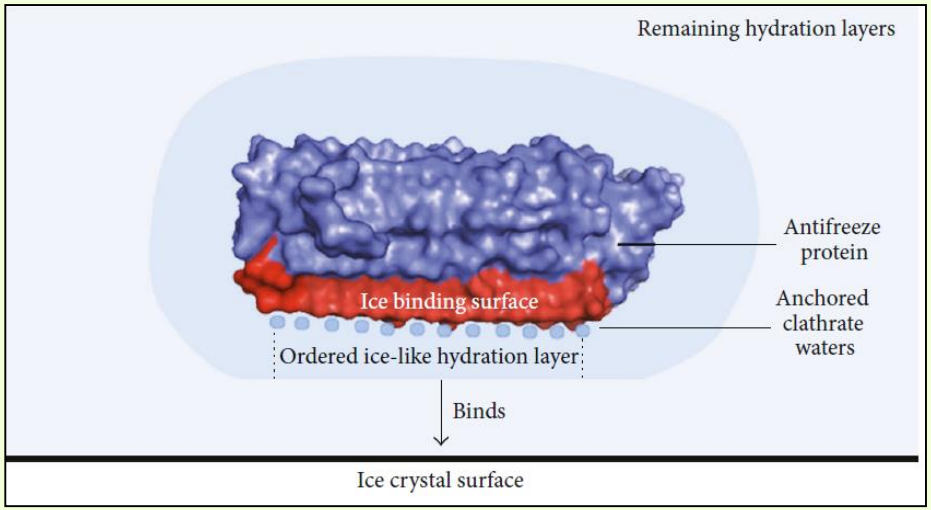
B. Marshall, C. L. DiPrinzio et al.,2007. "Fluorescence microscopy evidence for quasi-permanent attachment of antifreeze proteins to ice surfaces," *Biophysical Journal* 92(10), 3663-3673.

Lorv *et al.*,2014

Antifreeze proteins (AFPs)

Schematic diagram of ice binding using an ice-like hydration layer(*Mp*AFP retrieved from RCSB Protein Data Bank)

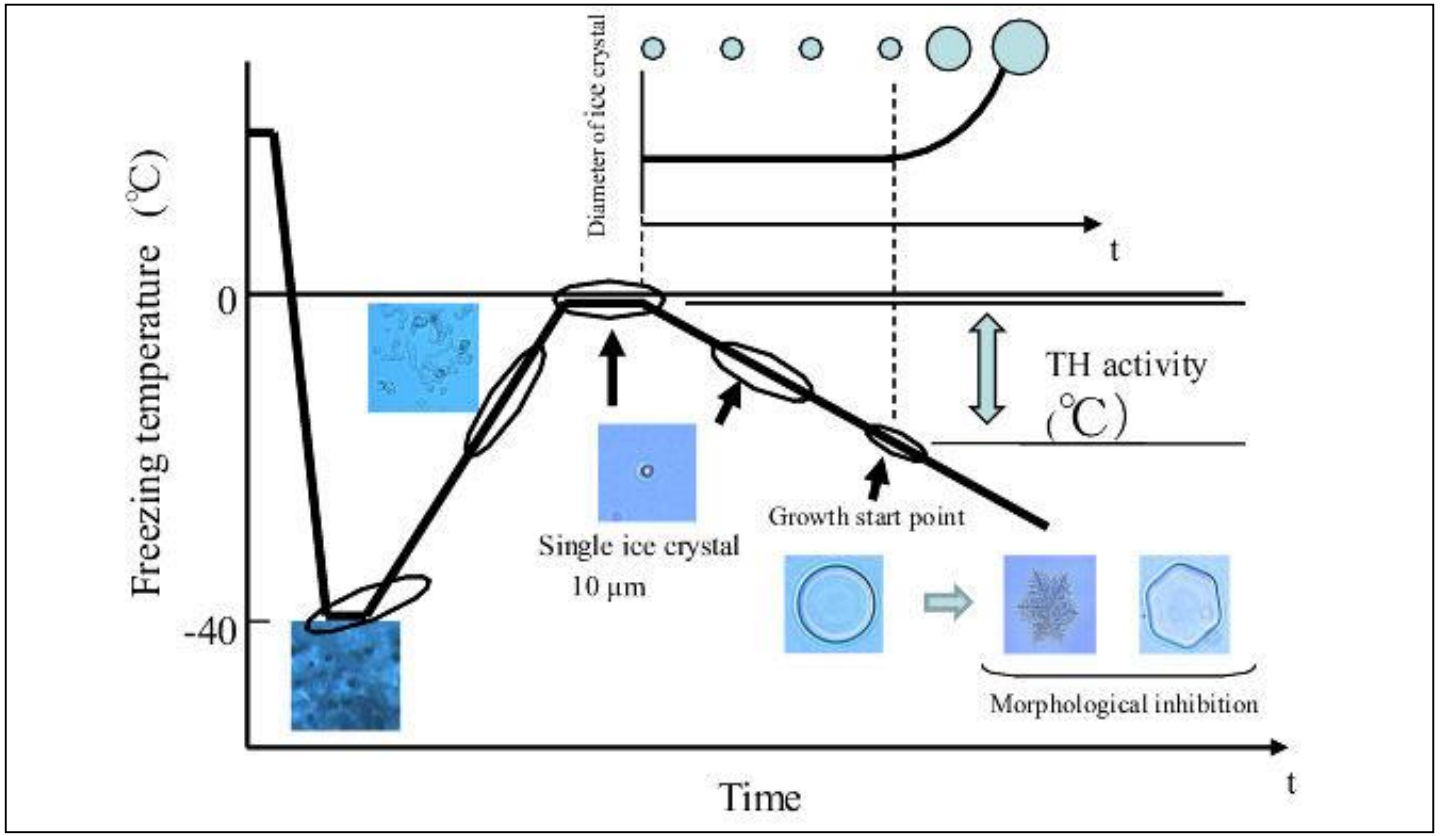
- Anchored clathrate waters (crystalline solids which look like ice) in the troughs of the ice binding surface orders the hydration layer to become ice-like.
- This hydration layer become an ice template allowing binding to ice crystal surfaces.
- The ice binding surface of the antifreeze protein is highlighted in red using pyMOL.



Lorv *et al.*,2014

Antifreeze proteins (AFPs)

Scheme of freezing curve and ice morphology of the TH activity using microscope with temperature-controlled sample stage



Kawarhara *et al.*,2013

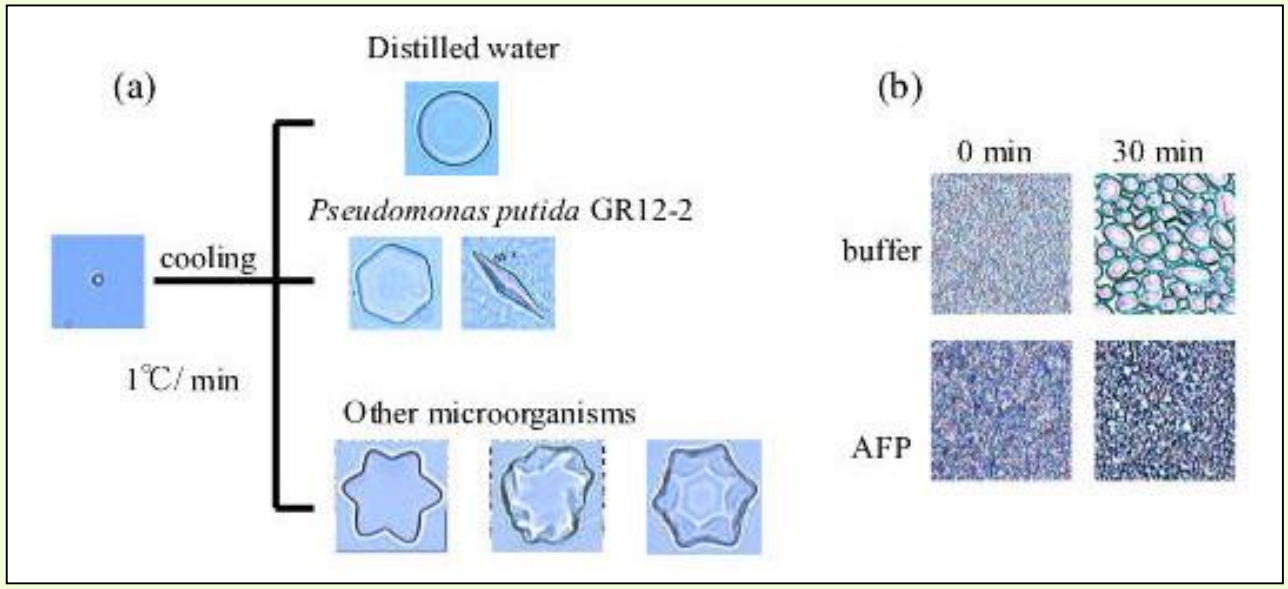
Antifreeze proteins (AFPs)

Ice crystal regulation by some antifreeze inhibition

(a) Ice crystal morphology

(b) Ice crystal recrystallization inhibition

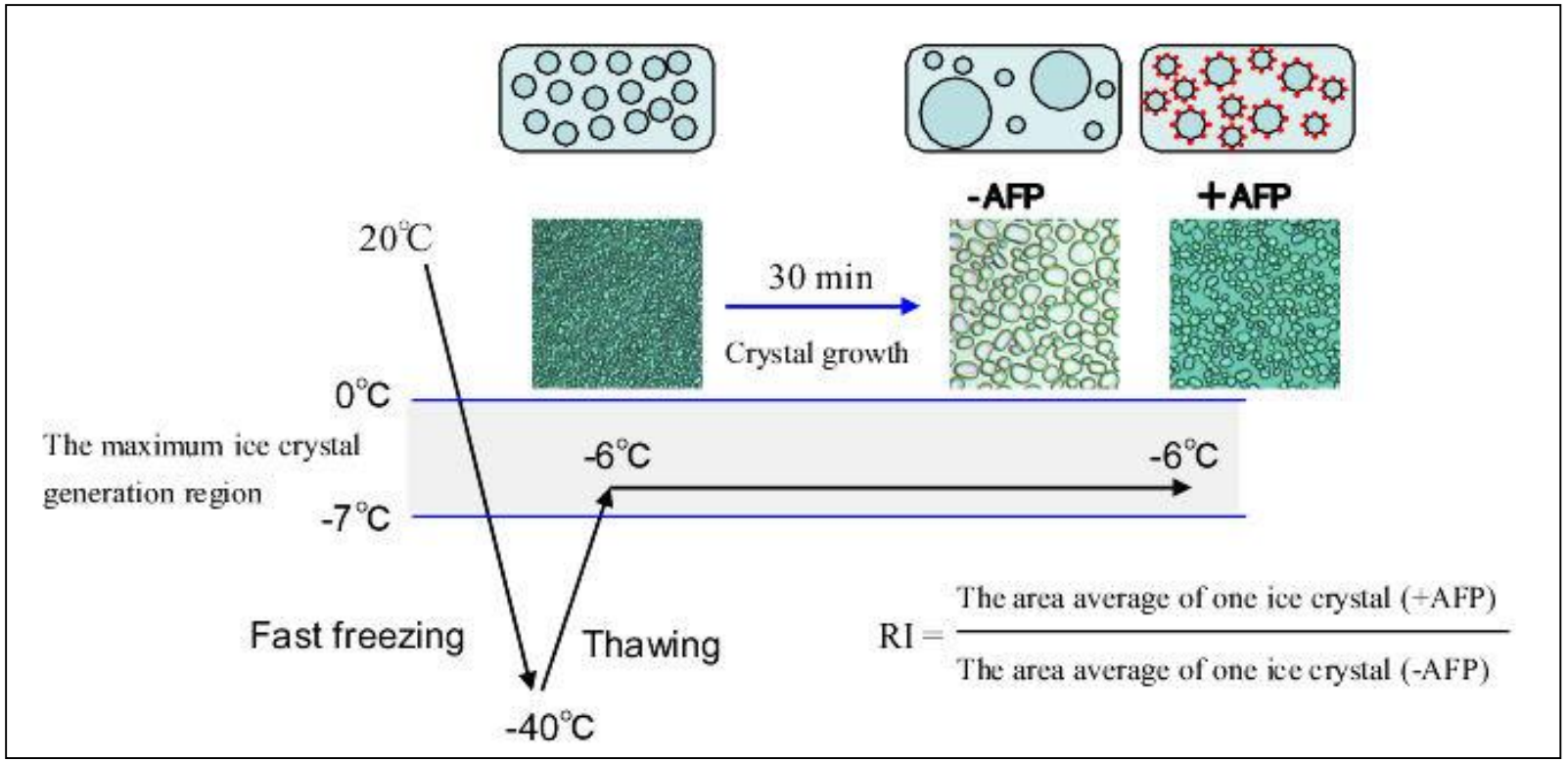
- AFPs appeared to inhibit the normal growth direction of ice by preferentially adsorbing to the prism faces of ice crystals, thereby forming needle-shape crystals (a).



Kawarhara *et al.*,2013

Antifreeze proteins (AFPs)

Scheme of freezing curve and ice morphology of sucrose sandwich assay using microscope with temperature-controlled sample stage



Kawarhara *et al.*,2013

Antifreeze bacteria

Isolation and culturing of bacteria

- One gram of sediment from Cryoconite holes (water filled cylindrical melt-holes on glacial ice surface) was suspended in 9 mL sterile saline (0.9% NaCl solution) and diluted serially (decimal dilutions 10^1, 10^2, 10^3).
- Enumeration of culturable bacteria was made using the spread plate method (0.1 mL) on nutrient agar (NA), 1/10 NA, marine broth (MB), 1/10 MB, Antarctic bacterial medium (ABM), and 1/10 ABM and incubated at two different temperatures, 4 and 15 °C, for 14-30 days.
- Isolates for the study were picked both based on unique morphotypes and also randomly from each plates.
- The pure isolates obtained were stored in glycerol stock at-70°C and the important isolates deposited at RIKEN-BRC JCM, Japan.

Singh *et al.*,2013

Antifreeze bacteria
Screening for AFPs activity

- Fourteen bacterial strains were used for primary screening of AFP activity (Table).
- One loopful of pure culture was inoculated into 50 mL autoclaved nutrient broth and incubated at 1°C and nonagitated for 1-2 months (56 days).
- Five microlitre culture broth was taken and observed under Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK) for AFP activity.
- By the shape of the ice crystals, the positive and negative activities of the strains were noted.

Singh *et al.*,2013

Taxonomic characterization of antifreeze bacteria

PCR, DNA sequencing, sequence alignment

- Total DNA was extracted from cultures grown on nutrient agar plate at 4 and 15°C using InstaGene DNA Extraction Kit (Bio-Rad).
- The 16S rRNA gene was amplified from the extracted DNA by PCR method using KOD-plus DNA polymerase using the following primers:
- 9F (GAGTTTGATCCTGGCTCAG) and
- 1541R (AAGGAGGTGATCCAGCC).
- Sequences were obtained with ABI prism 3100 Sequencer (Applied Biosystems) using ABI standard protocol.

Singh *et al.*,2013

Taxonomic characterization of antifreeze bacteria

PCR, DNA sequencing, sequence alignment

- Sequence alignment of the 16S rRNA gene region of each isolate was performed using CLUSTALW option of the software Molecular Evolutionary Genetics Analysis (MEGA) software v4.0. (Tamura *et al.*, 2007).
- The sequences were deposited at GenBank and were subjected to a NCBI BLAST search.
- In a second alignment, the 16S rRNA gene region sequences were aligned using CLUSTALW together with the homologous sequences (retrieved from GenBank) of closely related species of bacteria.

Singh *et al.*,2013

Taxonomic characterization of antifreeze bacteria

PCR, DNA sequencing, sequence alignment

- Fourteen Arctic (is a polar region located at the northernmost part of the Earth) bacterial strains belonging to five genera, *Cryobacterium*, *Leifsonia*, *Polaromonas*, *Pseudomonas*, and *Subtercola* isolated from sediments found in cryoconite holes of Arctic glaciers, were subjected to screening for antifreeze proteins

Isolate code	Sequence deposition no.	Species identification/16S rRNA gene sequences closest similarity (%)	AFPs activity
Cry-l (MLB-33)	JX205200	*Cryobacterium psychrophilum* (AM410676) by 95.7%	+
Cry-c (MLB-29)	JX205196	*Cryobacterium psychrotolerans* (JN637331) 98.2%	+
Cry-21(MLB-37)	JX205204	*Cryobacterium psychrotolerans* (JN637331) by 99.6%	+
Cry-9 (NA-9)	JF790966	*Cryobacterium* sp. (FJ464984) by 98.0%	–
Cry-5 (MB-5)	JF790972	*Cryobacterium* sp.DR9 (FJ464984) by 98.3%	–
Cry-k	AB872307	*Cryobacterium* sp. AsdMX-L1 (JX123060) by 99.0%	+
Cry-6 (MLB-40)	JX205207	*Leifsonia antarctica* (AM931710) by 98.4%	–
Cry-b (NA-b)	JF790973	*Leifsonia* sp. (GU213322) by 99.3%	–
Cry-15 (MLB-44)	JX205211	*Polaromonas naphthalenivorans* (AY166684) by 99.0%	–
Cry-16 (MLB-45)	JX205214	*Polaromonas naphthalenivorans* (AY166684) by 97.8%	–
Cry-g (MB-g)	JF790967	*Pseudomonas ficuserectae* (AB021378) by 96.9%	+
Cry-n (MLB-46)	JX205212	*Pseudomonas ficuserectae* (AB021378) by 97.3%	+
Cry-2 (MLB42)	JX205209	*Pseudomonas ficuserectae* (AB021378) by 96.9%	+
Cry-8 (MLB-47)	JX205212	*Subtercola frigoramans* (AM410673) by 97.8%	+

AFP, antifreeze protein.

Singh *et al.*,2013

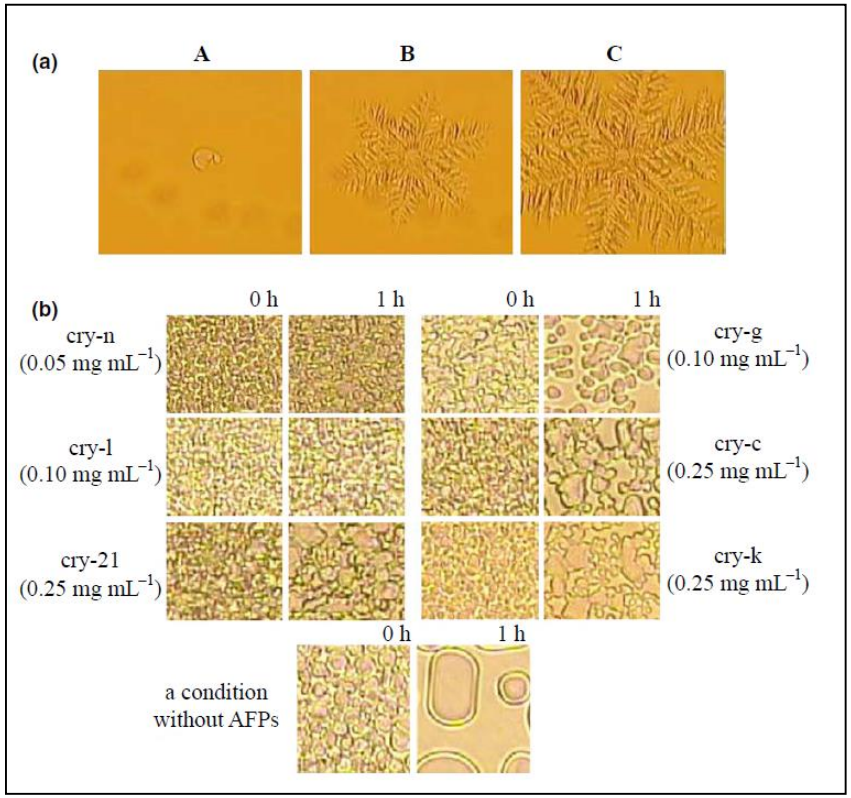
Antifreeze bacteria

Ice growth patterns in the presence of bacterial AFPs

a) Ice growth patterns in the presence of bacterial AFPs. These snapshots were taken during ice growth at temperatures above freezing point (frame A), around freezing point (frame B), below freezing point (frame C).

b) Images of ice recrystallization observed in bacterial AFP solutions.

- Left panels show start points (i.e. 0-h incubation), and right panels show end points (i.e. after 1-h incubation).
- The lower figures indicate the measurements performed without AFPs as a negative control.



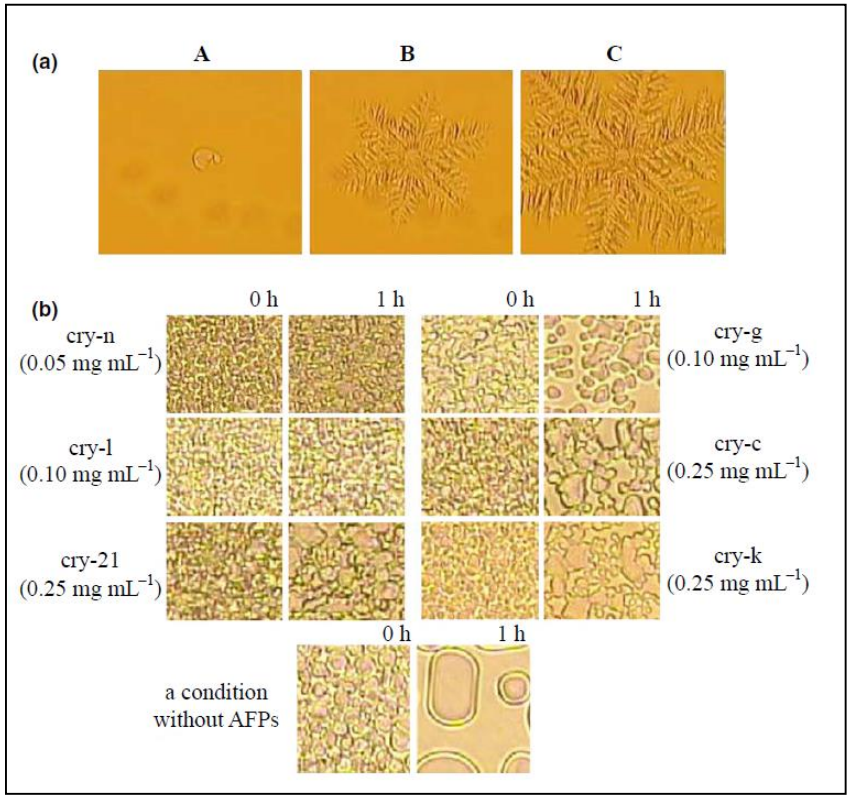
Singh *et al.*,2013

Antifreeze bacteria

Ice recrystallization inhibition activity

IRI assay

- The bacterial AFPs tested all had a different degree of ice recrystallization inhibition (IRI) activity (4b).
- As mentioned above, the highest TH activity was obtained with both cry-g and cry-n AFPs.
- The IRI assay showed that cry-g AFP failed to inhibit ice recrystallization even at a conc. of 0.1 mg mL^{-1}, while cry-n AFP inhibited ice recrystallization at a concentration of 0.05 mg mL^{-1}.
- Therefore, it appears that the strength of IRI activity of AFPs does not relate to the strength of TH activity.



Singh *et al.*,2013

Antifreeze bacteria
Purification of AFPs

- Bacterial strains showing AFP activity were selected for purification.
- They were cultured in nutrient broth (Eiken Chemical, Japan) at 1°C under static conditions. After a month, the culture media were centrifuged to remove bacterial cells. The supernatants were dialyzed against 25 mM glycine-HCl buffer (pH 3.0).
- The dialysates were applied to an Econo-pac High-S column (Bio-Rad) and eluted with the same buffer containing 200 mM NaCl.
- One strain did not bind to Q-resin at pH 6, and the fractions showing AFP activity were dialyzed against 25 mM MES-NaOH buffer (pH 6.0) and then loaded onto an Econo-pac High-Q column (Bio-Rad) and eluted with 25 mM MES–NaOH buffer (pH 6.0) containing 150 mM NaCl.
- Flow-through fractions showing AFP activities were dialyzed against 25 mM sodium acetate buffer (pH 4.0) and then loaded onto an Econo-pac High-Q column (Bio-Rad).
- The active fraction was eluted with the same buffer containing 150 mM NaCl.

Singh *et al.*,2013

2. Protection from plant Pathogenic Bacteria

Biosafety and Biosecurity

Crop Biosecurity
Agricultural bioterrorism

- Although the precise causes for the emergence of these diseases are not known, numerous explanations for these phenomena have been postulated by a variety of sources:

1. Some explanations include natural causes such as:
2. Climatological changes,
3. Man-made alterations in certain ecosystems,
4. Release of new germplasm,
5. Introduction of contaminated seeds,
6. An increase in international air travel and trade, and so on.

- However remote, the possibility that some diseases could have occurred as a result of deliberate introduction cannot be dismissed.

Schaad *et al.*,1999

Global production of eight major crops and estimated losses by crop and region (1988-90)

Crop	Actual Crop Production (billions of US$)	US$ (billions) Losses due to			
		Pathogens	Insects	Weeds	Total
Rice	106.4	33.0	45.4	34.2	112.5
Wheat	64.6	14.0	10.5	14.0	38.5
Barley	13.7	1.9	1.7	2.0	5.7
Maize	44.0	7.8	10.4	9.3	27.4
Potatoes	35.1	9.8	9.6	5.3	24.8
Soybeans	24.2	3.2	3.7	4.7	11.6
Cotton	25.7	4.3	6.3	4.9	15.5
Coffee	11.4	2.8	2.8	2.0	7.6
Region					
Africa	13.3	4.1	4.4	4.3	12.8
N. America	50.5	7.1	7.5	8.4	22.9
Latin America	30.7	7.1	7.6	7.0	21.7
Asia	162.9	43.8	57.6	43.8	145.2
Europe	42.6	5.8	6.1	4.9	16.8
Former Soviet Union	31.9	8.2	7.0	6.7	22.1
Oceania	3.3	0.8	0.6	0.5	1.9

Rosenzweig *et al.*,2001

Crop biogeography and origins

- Of the major food crops:
- rice, wheat, maize and potato, only rice is grown predominantly where it originated in SE(Southeast) Asia.
- wheat originated in the Middle East
- maize originated in the Americas
- potato originated in the Andes
- Can be considered beneficial plant invasions.

Jeger, 2010

Crop Biosecurity
Agricultural bioterrorism

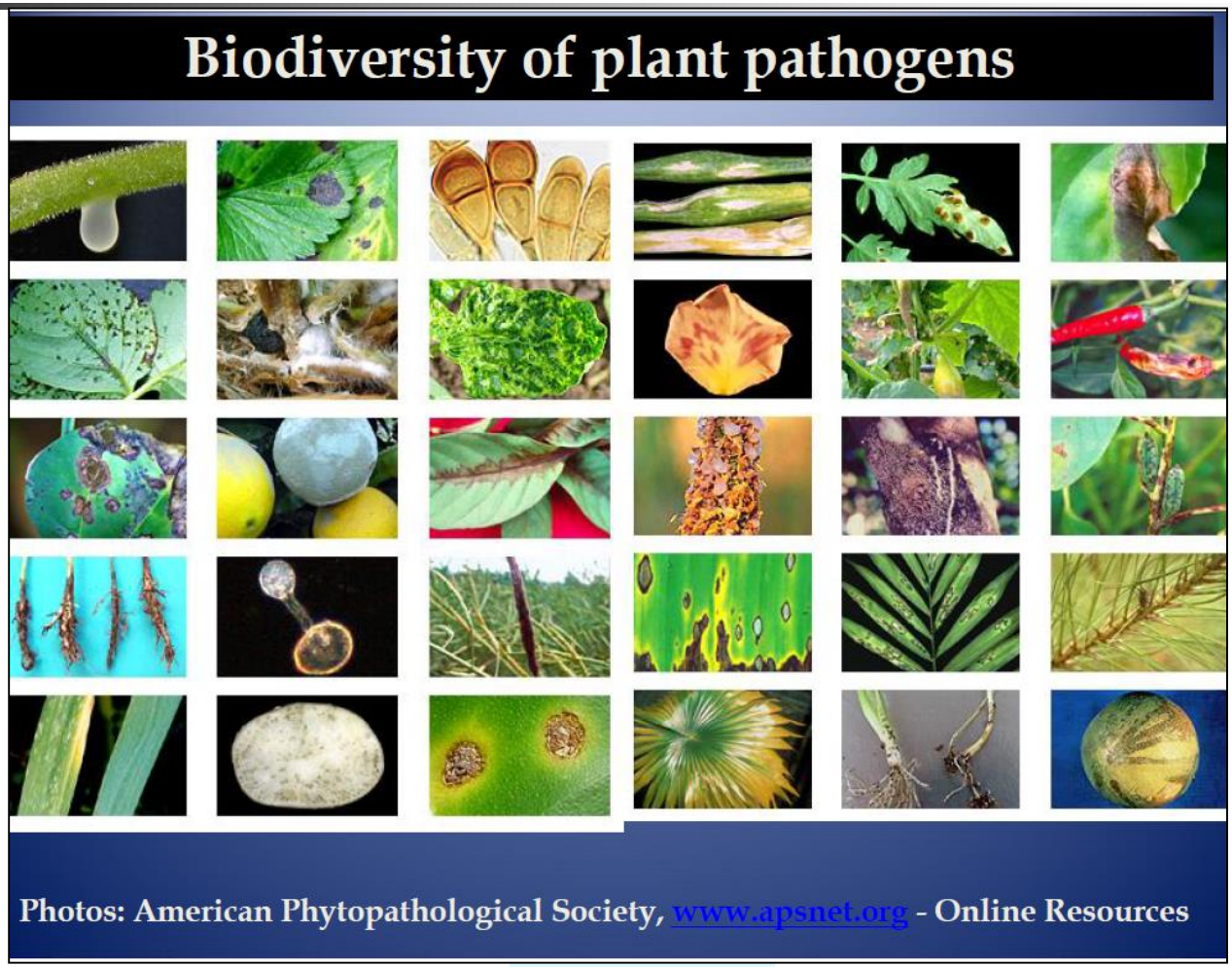
- Introduced crops may carry plant pathogens with them or acquire pathogens as a consequence of new encounters in the new environment.
- Invasive plant pathogens occur over:

1. different time scales;
2. Evolutionary time;
3. Centuries/decades;
4. Within a cropping season.

Jeger, 2010

Crop Biosecurity

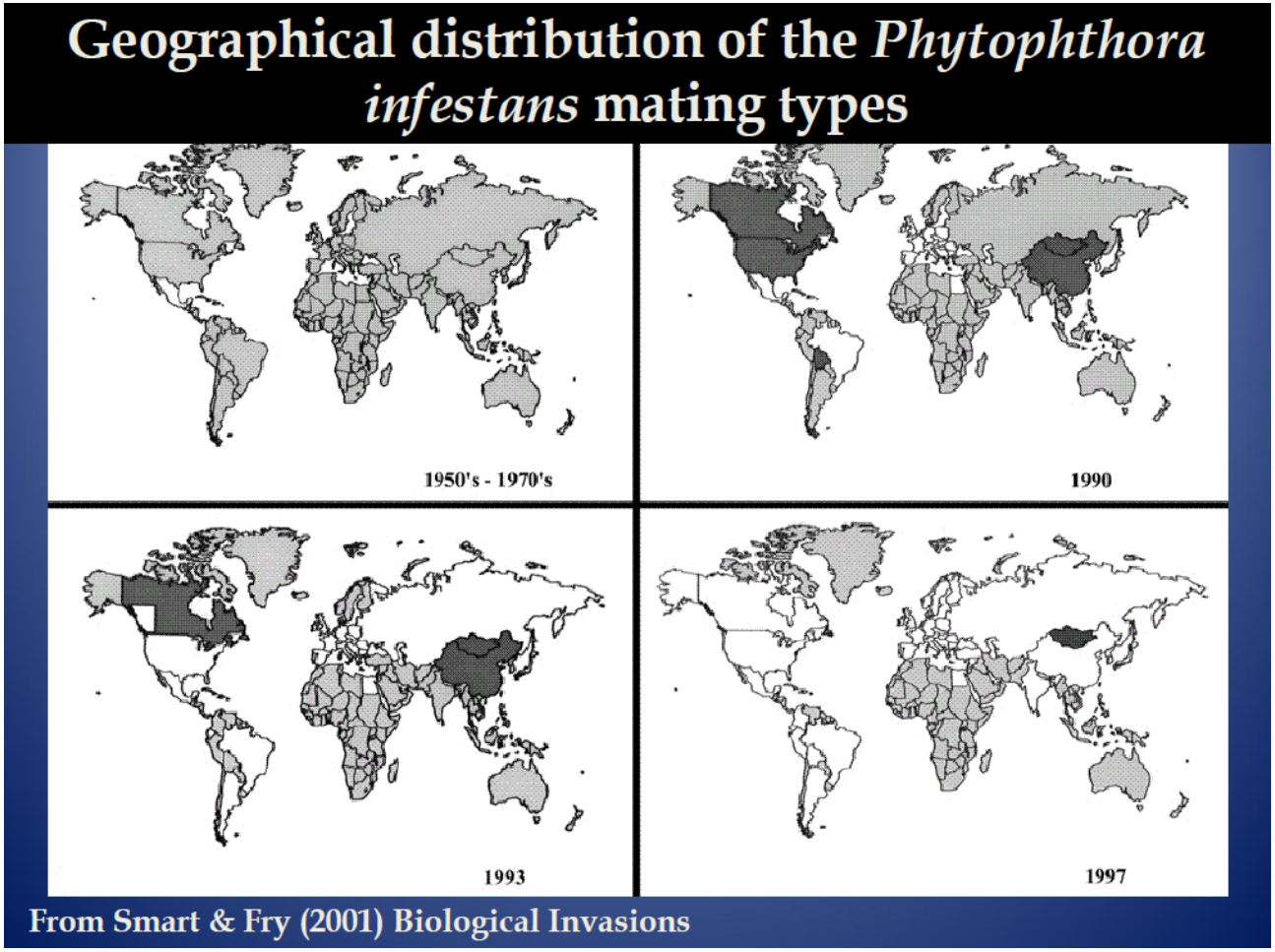
Biodiversity of plant pathogens



Jeger, 2010

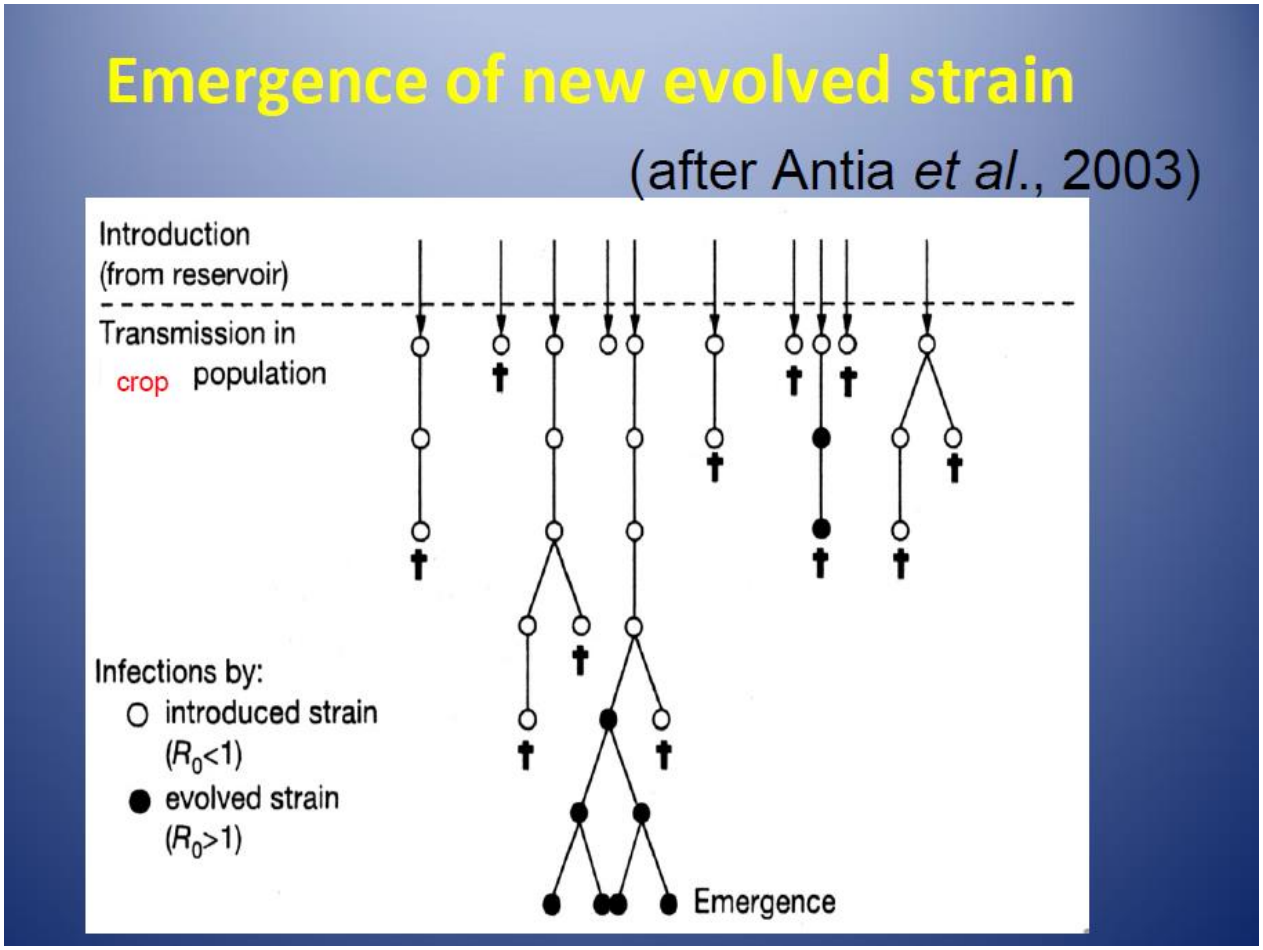
Crop Biosecurity

Geographical distribution of the *Phytophthora infestans* mating types



Jeger, 2010

Crop Biosecurity
Emergence of new evolved strain



Jeger, 2010

Definition
Biosafety and Biosecurity

- 'Biosafety' and 'Biosecurity' are different terms.
- **Biosafety:** The set of measures taken to ensure the safe handling of biohazardous materials, such as pathogens, biological contaminants, and genetically modified organisms, especially to prevent their accidental spread beyond a laboratory or research facility.
- **Biosecurity:** refers to measures that are taken to stop the spread or introduction of harmful organisms to human, animal and plant life.

Corona,2009

Definition
Bioterrorism and Agroterrorism

- **Terrorism:**
- Threat and intimidation of governments and societies by surppresive mass destruction of life (humans, animals, plants and/or cultural heritage).
- **Bioterrorism:** Threat or use of biological agents [to cause harm] by individuals or groups motivated by political, religious, ecological or other ideological objectives (Carus, 2001).
- **Agroterrorism:** Threat or use of biological agents to cause harm to agriculture and the economy and eventually cause starvation.

Corona,2009

Definition
Bioterrorism and Agroterrorism

- **Agroterrorism:** is a subset of bioterrorism, and it is defined as the deliberate introduction of an animal or plant disease/pest with the goal of generating fear, causing economic losses, and/or undermining stability.
- Agroterrorism in its widest meaning, including biological warfare, bioterrorism, biocrime and sabotage, is defined as the deliberate and malevolent use of pathogens by an individual, organization or State in order to damage the health of plants (crops, trees or agricultural commodities) or animals, or even affect the use able to be made of them in terms of production.

European Union,2015

Basic concepts in Ag Biosecurity and Forensics

Issues with Biosecurity concepts

Numerous terms and few meanings

'agro', 'bio', 'terror' and 'security' wording roots are creating unwanted analogies, expectations and misunderstanding among governments, scientists and general public.

- **Biosecurity**
  - **Agricultural biosecurity**
- **Biosafety**
- **Biodiversity/Invasive species**
- **Forensics**
  - **Microbial forensics/bioforensics**
    - **Terrorism**
  - **Bioterrorism**
  - **Agroterrorism**

Corona,2009

Crop biosecurity/Ag-biosecurity

Microbes in court

The emerging field of microbial forensics

- What is microbial forensics?
- You have probably heard of commonly used forensic methods such as the analysis of striations on bullets to identify the gun used to commit a crime.
- But what if a microbe is the weapon of choice, as can occur if a bioterrorist comes to town?
- Microbes as weapons is not a new topic.
- There have been reported cases, for example of HIV-infected people intentionally infecting others.

Forensic relating to courts of law. Forensic science is the scientific method of gathering and examining information about the past which is then used in a court of law.

Salyers,2003

Agricultural bioterrorism

History of plant pathogens as bioweapons

- Biological warfare against agricultural targets is not a new idea.
- Over many decades, various state-sponsored research programs have been established with the goal of using weaponized microorganisms as part of the country's military arsenal.
- The United States conducted research involving a number of pathogenic microbes, including the biological agents causing

1. anthrax,
2. foot and mouth disease, and
3. rice blast.

Fletcher *et al.*,2006

Agricultural bioterrorism

History of plant pathogens as bioweapons

- Germany had programs during both the First and Second World Wars, whereas the former Soviet Union conducted programs from the Second World War through the Cold War, as did Iraq, beginning with the Iran-Iraq War.
- Other countries are thought to have had biological weapon programs aimed at agriculture and food production.
- Evidence found in caves in Afghanistan suggested interest by Islamic militants in the weaponization of the fungus that causes wheat rust.
- Other countries that have explored microbes as potential weapons include Canada, France, Japan, and the United Kingdom.

Fletcher *et al.*,2006

Plant pathogens important for the BWC

Agricultural Bioterrorism

- AD HOC GROUP OF THE STATES PARTIES TO THE CONVENTION ON THE PROHIBITION OF THE DEVELOPMENT, PRODUCTION AND BWC/AD HOC GROUP/WP.124 STOCKPILING OF BACTERIOLOGICAL (BIOLOGICAL) AND TOXIN WEAPONS AND ON THEIR DESTRUCTION

- Sixth session
- Geneva, 3-21 March 1997

Crop Biosecurity

Defining biological agents and Toxin

- Biosecurity is the protection of a country's economy, environment and people from unwanted exotic pests and diseases.
- It includes trying to prevent new pests and diseases from arriving, and eradicating or controlling those already present.

Crop Biosecurity
Defining biological agents and Toxin

- We define biological agent as "any microorganism, virus, infectious substance, or biological product that may be engineered as a result of biotechnology, or any naturally occurring or bioengineered component of any such microorganism, virus, infectious substance, or biological product, capable of causing:

1. Death, disease, or other biological malfunction in a human, an animal, a plant, or another living organism;
2. Deterioration of food, water, equipment, supplies, or material of any kind, or
3. Deleterious alteration of the environment.

Crop biosecurity
Agricultural bioterrorism

- There are human and animal pathogens that could be devastating if deliberately spread.
- However, when plant pathogens are compared with human and animal pathogens, it is clear that the latter present a far greater threat as potential biological weapons.

Young *et al.*,2008

Crop biosecurity
Agricultural bioterrorism

- Over the past several years there has been an increase in new and emerging animal and plant diseases.
- Examples in the plant realm include:

1. Bacterial canker of citrus (*Xanthomonas citri*),
2. Watermelon fruit blotch (*Acidovorax avenae* subsp. *citrulli*),
3. Sorghum ergot (*Claviceps africana*),
4. Karnal bunt of wheat (*Tilletia indica*),
5. Scab of wheat (*Fusarium graminearum*).
6. Fiji disease is caused by a Phytoreo virus which is very destructive only on sugarcane.

Schaad *et al.*,1999;..

Deleterious bacteria

Etiological and epidemiological considerations

- The most severe crop devastation is associated with systemic, fastidious xylem- or phloem-limited bacteria, such as *Ca*. Liberibacter spp. and *X. fastidiosa*, whose epidemiology is determined, at least in part, by their insect vectors.
- However, their long latency periods and multi-year epidemic development allows time to anticipate economic impacts from crop losses, making them poor weapons.

Young *et al.*,2008

Crop biosecurity

Agricultural bioterrorism

Putative plant-pathogenic bacterial biological weapons

1. '*Candidatus* Liberibacter africanus', '*Ca*. L. americanus', and '*Ca*. L. asiaticus'.
2. *Clavibacter michiganensis* subsp. *sepedonicus*
3. *Erwinia amylovora*
4. *Ralstonia solanacearum* races 2 and 3
5. *Xanthomonas albilineans*
6. *Xanthomonas axonopodis* pv. *citri*
7. *Xanthomonas oryzae* pv. *oryzae*
8. *Xanthomonas oryzae* pv. *oryzicola*
9. *Xylella fastidiosa*

Young *et al.*,2008

Crop biosecurity
Agricultural bioterrorism

	
Bacterial canker of tomato caused by *Clavibacter michiganensis* pv *michiganensis*.	Bacterial wilt of potato, caused by *Ralstonia solanacearum*.
	
Halo blight of beans, caused by *Pseudomonas syringae* pv. *phaseolicola*.	Black rot of cabbage (photograph) and other crucifer crops, caused by *Xanthomonas campestris* pv. *campestris*.

Agricultural bioterrorism

Examples of the arrival in the United States of bacterial plant pathogens and their consequences

Disease	Pathogen	Situation and consequences
Asiatic citrus canker	*Xanthomonas axonopodis* pv. *citri*	Florida's $1.4 billion citrus industry is severely impacted.
Bacterial wilt	*Ralstonia solanacearum* race 3, biovar 2	introduced into the United States via infected geraniums shipped from Africa and Central America to nurseries.
Pierce's disease of grapevine	*Xylella fastidiosa*	Threatens California's $2.8 billion wine, table, and raisin grape.

Fletcher *et al.*,2006

The important criteria in Biological weapons production

1. Agents known to have been developed, produced or used as weapons.
2. Agents which have severe socio-economic and/or significant adverse human health impacts, due to their effect on staple crops, to be evaluated against a combination of the following criteria:

a) Ease of dissemination (wind, insects, water, etc.);
b) Short incubation period and/or difficult to diagnose/identify at an early stage;
c) Ease of production;
d) Stability in the environment;
e) Lack of availability of cost-effective protection/treatment;
f) Low infective dose;
g) High infectivity;
h) Short life cycle.

BWC/AD HOC GROUP/WP.124 3 March 1997

Possible Bioterrorist Pathogen Rating Criteria and Points

- A better approach is to develop criteria and a numerical rating system or "Effective Pathogen Index" (EPI) to assess risk and probability of harm.
- A perfect organism would have a EPI of 10 (for example, add the total points and divide by 10).
- Similar criteria for a BW agent as part of a state supported BW program could be developed.

!	Produces toxin	15
!	Easy to obtain, handle, and deliver	10
!	Easy to grow in large amounts	10
!	Highly infectious under many conditions	10
!	Results in the establishment of a quarantine	10
!	No chemical control or host resistance available	10
!	No method for rapid or reliable detection	10
!	Infects systemically by natural means	10
!	Spreads quickly by natural means	5
!	Causes severe crop losses	5
!	Survives long periods and is persistent	5

Schaad *et al.*,1999

Evaluation table of plant pathogens- The important criteria in Biological weapons production.

+ Conforms to criterium

- Does not conform to criterium

BWC/AD HOC GROUP/WP.124 3 March 1997

		Criteria										
	PLANT PATHOGEN	1	2	2a	2b	2c	2d	2e	2f	2g	2h	BW potential
1	Citrus greening disease bacteria	-	-	-	+	-	-	+	-	-	+	Doubtful
2	*Colletotrichum coffeanum* var *virulans*	-	-	+	-	+	+	-	±	-	NA	Good
3	*Chochliobolus miyabeanus*	-	±	+	-	+	+	-	±	-	NA	Doubtful
4	*Dothistroma pini (Scirrhia pini)*	-	+	+	+	-	+	+	+	+	NA	Good
5	*Erwinia amylovora*	-	+	+	±	+	-	+	+	+	NA	Good
6	*Microcyclus ulei*	-	-	+	±	+	-	-	-	-	NA	Doubtful
7	*Phytophthora infestans*	-	+	+	+	+	+	-	+	+	NA	Doubtful
8	*Pseudomonas solanacearum*	-	+	+	+	+	+	+	+	+	NA	Good
9	*Puccinia erianthi*	-	-	+	-	-	-	+	-	+	NA	doubtful;
10	*Puccinia graminis*	+	+	+	-	-	+	+	+	+	NA	Medium
11	*Puccinia striiformiis (Puccinia glumarum)*	-	+	+	-	-	-	-	-	+	NA	Limited
12	*Pyricularia oryzae*	+	+	+	-	+	+	-	+	+	NA	Good
13	Sugar cane Fiji disease virus	-	+	-	-	-	-	-	-	-	+	Limited
14	*Tilletia indica*	-	-	+	+	-	+	-	+	+	NA	Weak
15	*Ustilago Maydis*	-	+	+	+	+	+	+	+	+	NA	Good
16	*Xanthomonas albilineans*	-	+	+	+	+	+	+	-	+	NA	Good
17	*Xanthomonas campestris* pv *citri*	-	-	+	+	+	-	±	+	+	NA	Limited
18	*Xanthomonas campestris* pv *oryzae*	-	+	+	-	+	+	+	+	+	NA	Good

Crop biosecurity

Who is responsible for biosecurity?

- The central government is responsible for:

1. border management,
2. national-scale events,
3. agency co-ordination, and
4. the legislative framework.

Better Border Biosecurity,2007

Crop biosecurity
American Phytopathological Society

- The members of American Phytopathological Society (APS) include a broad representation of the national and international scientific leadership and expertise for protecting agriculture against crop diseases, regardless of nature of the threat agent and whether it is accidentally or intentionally introduced.
- Two fundamentally different strategies for assuring crop biosecurity:

1. Prevention, and
2. Preparedness.

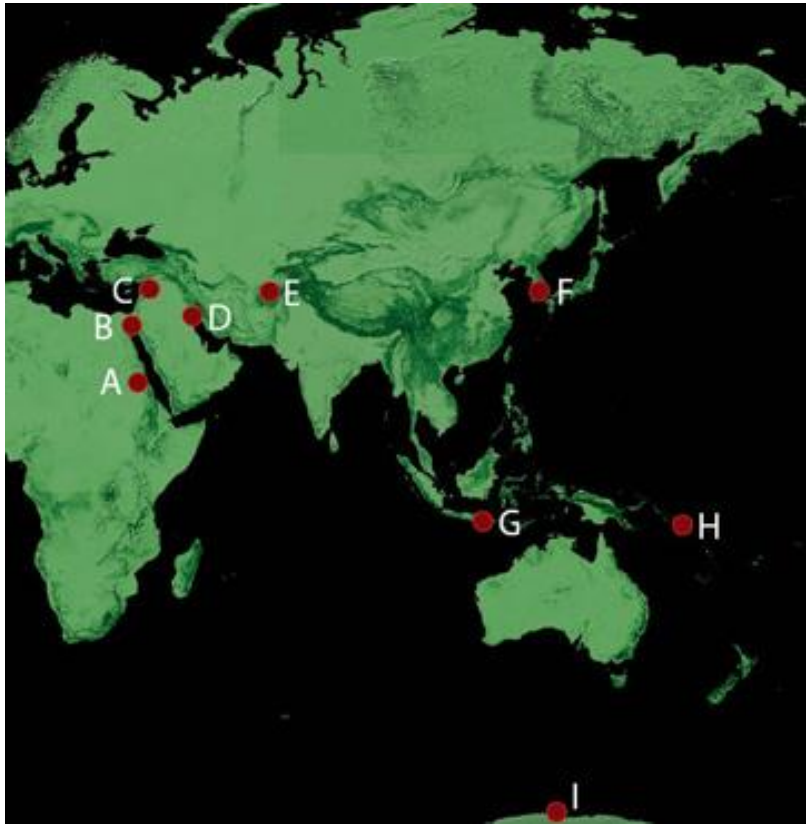
Crop biosecurity
Prevention and preparedness

- Prevention is currently focused on increased security, secrecy(secrete), and border protection.
- Preparedness focuses on early detection, rapid diagnosis, and rapid recovery.
- Both are important, but too much focus on security and secrecy will impair the necessary free and open conduct of science and information exchange fundamental to both prevention and preparedness.

Crop biosecurity

Ag-biosecurity issues regarding military and defense systems

- Currently New Zealand Defense Force personnel are deployed on 14 operations.
- Peace keeping UN missions and defense exercises in 10 countries around the world, including:
  - A - Sudan
  - B - Sinai
  - C - Middle East
  - D - Iraq
  - E - Afghanistan
  - F - Korea
  - G - Timor-Leste



Corona,2009

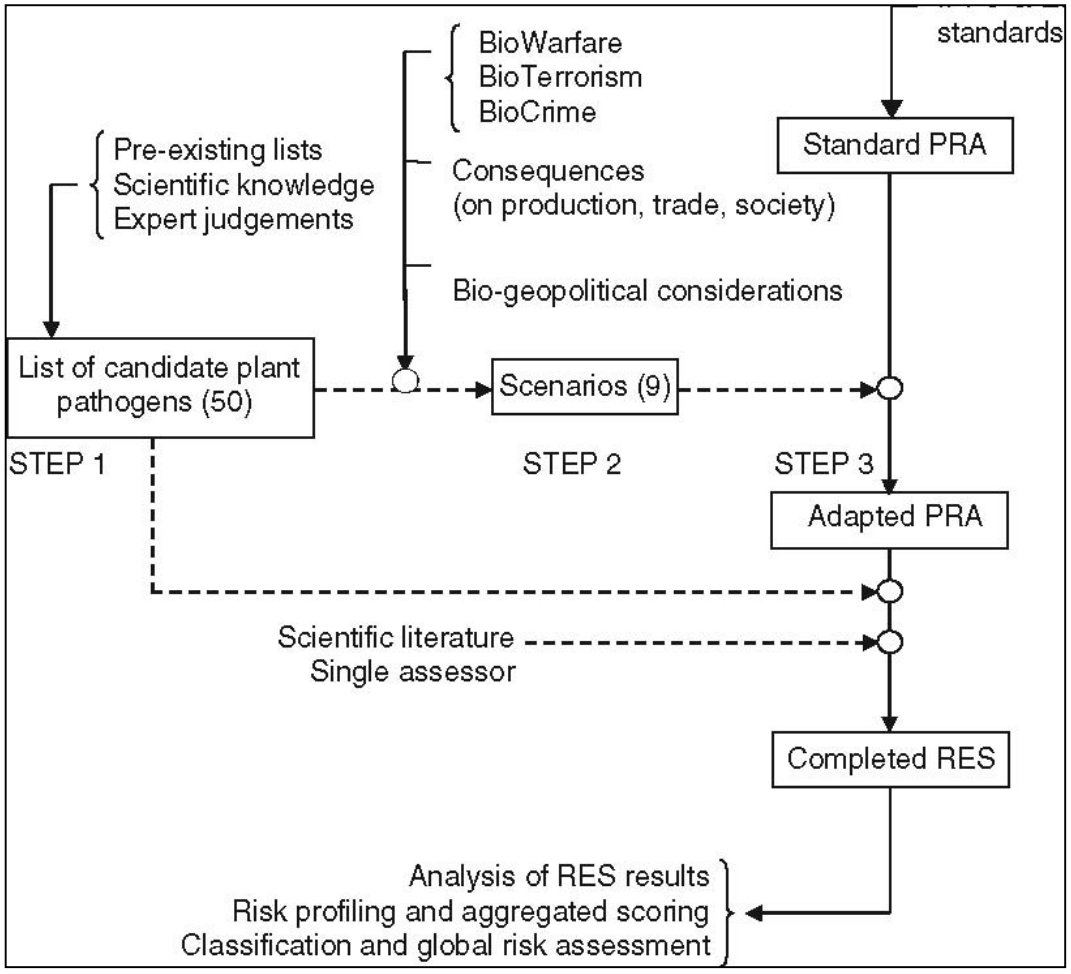
Crop biosecurity

American Phytopathological Society ad hoc committee on Crop Bioterrorism,2002

Julie Beale	jbeale@ca.uky.edu
Bill Brown	wbrown@lamar.colostate.edu
Molly Cline	molly.n.cline@monsanto.com
R. Jim Cook	rjcook@wsu.edu
Anne Desjardins	desjarae@mail.ncaur.usda.gov
Jacque Fletcher	jaf2394@okstate.edu
Jan Leach	jeleach@plantpath.ksu.edu
André Lévesque	levesqueca@em.agr.ca
Larry Madden	madden.1@osu.edu
Norm Schaad	schaad@ncifcrf.gov

Crop Biosecurity

Methodology used by Latxague et al. (2007) for assessing the risk posed by agroterrorism in Europe



Jeger,2010

PLANTFOODSEC

Plant and Food Biosecurity

- PLANTFOODSEC started on February 1st, 2011 for five years duration.
- PLANTFOODSEC aims to build a virtual Centre of Competence in order to increase the quality and impact of plant and food biosecurity training and research in Europe.
- During the first (February 2011 – July 2012) and the second (August 2012 – January 2014) reporting period the work was performed as follows:

European Union,2015

PLANTFOODSEC

Plant and Food Biosecurity

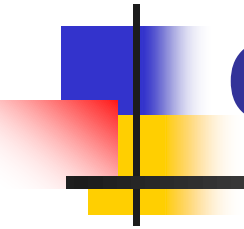
- (WP1) Plant disease epidemiology applied to crop biosecurity;
- (WP2) Food biosecurity;
- (WP3) Analysis of risks;
- (WP4) Diagnostic and detection systems;
- (WP5) Responder systems on eradication and containment;
- (WP6) Training on Plant and Food Biosecurit;y
- (WP7) Dissemination;
- WP8 Management.

European Union,2015

Disease Management Strategies

Chemical control

Biological control



Chemical Control

Pesticides

The Global Pesticides Market

$130 Billion Pesticides Market Outlook, 2023 - Herbicides Will Gain $27 Billion of Global Annual Sales by 2023

1. The global pesticides market size reached a value of nearly $84.5 billion in 2019, having grown at a compound annual growth rate (CAGR) of 4.2% since 2015, and
2. expected to grow at a CAGR of 11.5% to nearly $130.7 billion by 2023.

ResearchAndMarkets,2020

The Global Pesticides Market

Herbicides Will Gain $27 Billion of Global Annual Sales by 2023

- The pesticides market is segmented by type into:

1. fungicides,
2. herbicides, and
3. insecticides.

- The herbicides market was the largest segment of the pesticide market, accounting for $43.8 billion or 51.9% of the total in 2019 and this market is expected to be the fastest-growing segment going forward at a CAGR of 12.8%.

ResearchAndMarkets,2020

Formula and Calculation

CAGR Calculator - Compound Annual Growth Rate Calculator Online

Formula

$$\mathrm{CAGR} = \left(\frac{V_{\text{final}}}{V_{\text{begin}}}\right)^{1/t} - 1$$

CAGR = compound annual growth rate

V_{begin} = beginning value

V_{final} = final value

t = time in years

ResearchAndMarkets,2020; Wikipedia,2021

The Global Pesticides Market

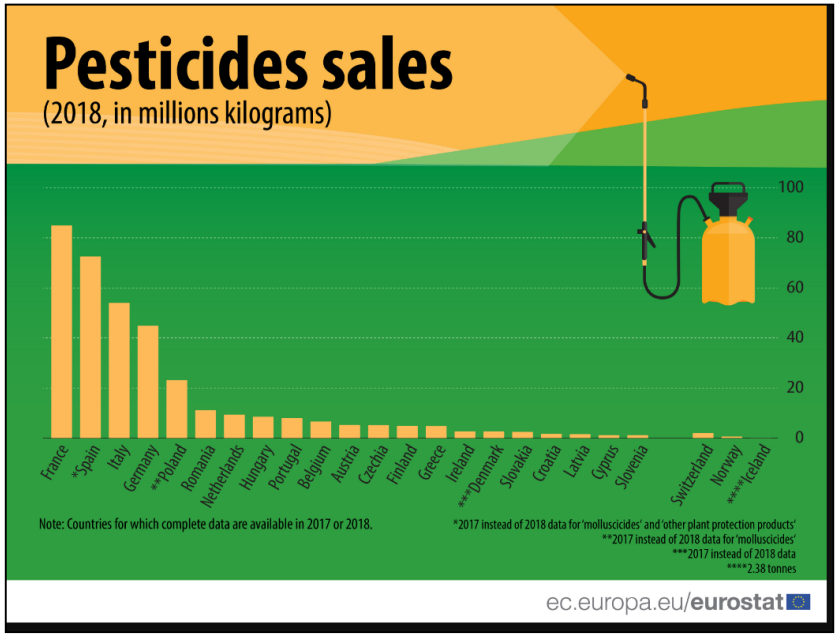
The pesticide market size in different regions

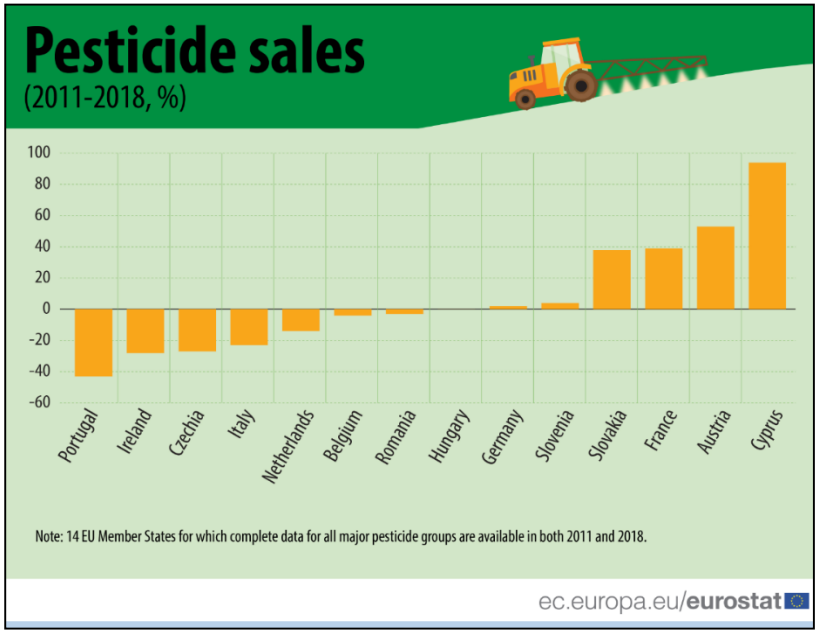
1. The Asia Pacific was the largest region in the pesticide market, accounting for 26.8% of the global market in 2019.
2. It was followed by South America, North America and then the other regions.
3. Going forward, the fastest growing regions in the pesticide market will be Africa and South America, where growth will be at CAGRs(compound annual growth rates) of 18.2% and 17.9%, respectively.
4. These will be followed by the Middle East and Eastern Europe where the markets are expected to grow at CAGRs of 15.8% and 10.3%, respectively.

ResearchAndMarkets,2020

The Global Pesticides Market

Sales of pesticides in the EU





Products eurostat-news,2020

The Global Pesticides Market

Number and volume of agriculture pesticide imported to Iran during 2012-2014

- Number and average annual volume of technical material (AI) of pesticides imported to Iran during 2012-2014.

Use category	No. of technical material (AI)	Volume (tonnes)
Herbicide	42	4918
Fungicide	22	1419
Insecticide & acaricide	35	4470
Rodenticide	3	0.007
Nematicide	1	325
Molluscicide	1	24
Total	104	10831

- Number and volume of agriculture pesticide formulated products imported to Iran during 2012-2014.

Use category	Product	No. of products	Average annual volume (tonnes of AI[a])
Herbicide	Single AI	36	916
	Mixture AI	14	181
Fungicide	Single AI	25	1139
	Mixture AI	13	60
Insecticide & acaricide	Single AI	45	785
	Mixture AI	3	2,8
Rodenticide	Single AI	5	16
Other[b]	Single AI	6	23
Sub-total	Single AI	116	2889
	Mixture AI	30	244
Grand total			3133

[a] *AI* active ingredient
[b] Includes 2014 importation of 30.4 tonnes (annual average 10.1 tonnes) methyl bromide for use in quarantine of imported plant products into Iran

Zaim *et al.*,2017

The Global Pesticides Market

Companies Mentioned

- Adama Agricultural Solutions
- Ag-Chem Africa
- Arysta Life Science
- Astranova Tarim Ticaret Ve San. A.S.
- BASF SE
- Bayer AG
- Botanical Resources Australia
- Chema Industries
- ChemChina
- Coromandel International
- Dhanuka Agritec
- Dow DuPont Inc.
- E.I.D. Parry
- FMC Corporation
- Hockley International
- Hubei Sanoda
- Interfarm (UK) Ltd
- Jiangsu Huifeng Agrochemical Co.
- Jiangsu Huifeng Agrochemical Co.
- Hubei Sanoda
- Interfarm (UK) Ltd
- Jiangsu Huifeng Agrochemical Co.
- Jubaili Agrotec ltd.
- Kumiai chemical
- Menta Co. Ltd.
- Monsanto
- Nanjing Red Sun Co.
- PI Industries
- Platform Specialty Products Corporation
- Shandong Weifang Rainbow Chemical Co.
- Sharda Crop
- Sichuan Leshan Fuhua Tongda Agro-Chemical Technology Co.
- Sumitomo Chemicals
- Syngenta
- UPL

ResearchAndMarkets,2020

Chemical control

Advantages and disadvantages

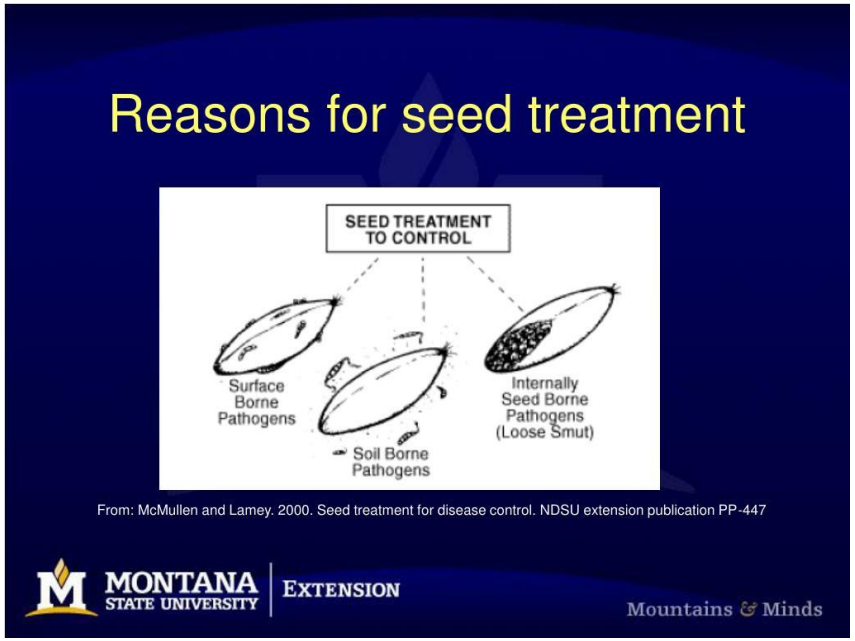
- Chemical control is the use of synthetic chemical pesticides to eliminate pests or reduce their effects.
- Chemical control has proven effective in some cases in freeing basic material from bacterial plant pathogens.
- Limitations of chemical control methods are:

1. Phytotoxicity and difficulties with penetration into internal tissues of plants, where the bacteria may remain protected.
2. Most fungicidal seed treatments do not control bacterial pathogens.

Chemical control
Benefits of seed treatment

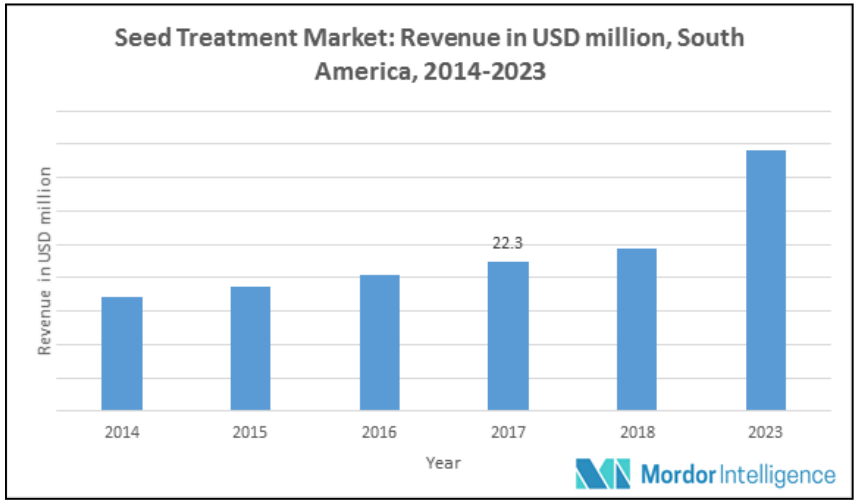
- Five reasons to use seed treatments:

1. Prevents spread of plant diseases.
2. Protects seed from seed rot and seedling blights.
3. Improves germination.
4. Provides protection from storage insects.
5. Controls soil insects.



South America wheat seed treatment market

- The South America wheat seed treatment market was estimated at USD 22.3 million in 2017 and is expected to reach USD 39.2 million by the year 2023, registering a robust(firm) CAGR of 10% during the forecast period (2018-2023).



Contact bactericides

1. Copper-based bactericides
2. Other contact bactericides including antibiotics.

Fungicides

Types of Fungicides

1. Protective (preventative): application prevents the establishment of an infection.
2. Curative: application interrupts the development of an established infection before visible symptoms. e.g. copper sulfate pentahydrate (Phyton 35).
3. Eradicant: application interrupts further development of an established infection having visible symptoms.
4. Residual: remains on surface of the leaf and provides protection. E.g. Evocide is a new genre of liquid surface disinfectant with broad spectrum antibacterial efficacy on application.
5. Systemic: movement of fungicide inside the plant (locally or throughout the plant).

Fungicides

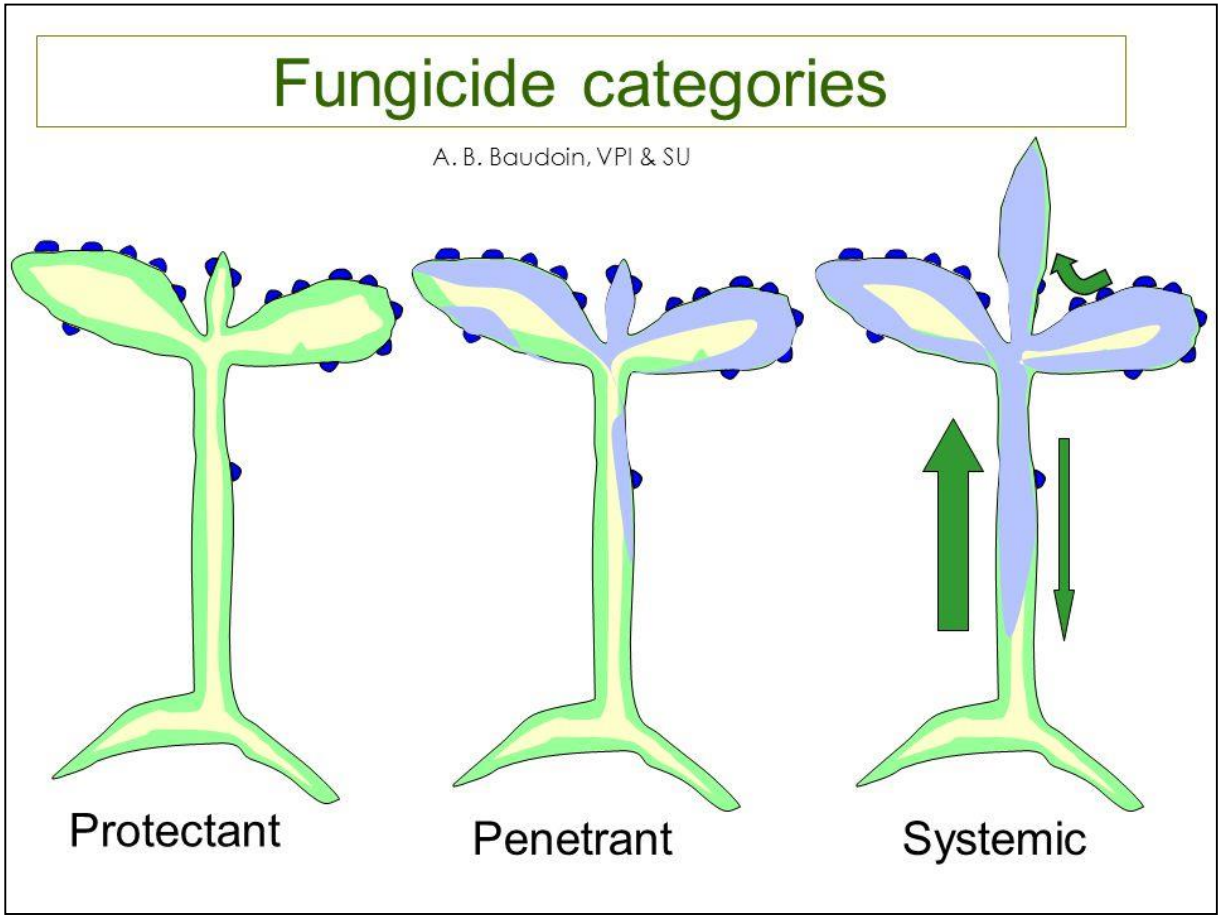
Types of fungicides

- Fungicides can be classified as protectants, eradicants and systemic fungicides:

1. Protectant: Fungicide which is effective only if applied prior to fungal infection is called protectant. Example – Copper (single-site inhibitor), Mancozeb & Zineb (Multi-site inhibitor).
2. Eradicant: Is the one, which removes fungi from an infection court. An eradicant can penetrate the host tissues to a limited extent & eliminate an established infection. Example – Lime Sulphur.
3. Systemic Fungicides: Systemic fungicides are the compounds, which are transported over a considerable distance in plant system after-penetration.

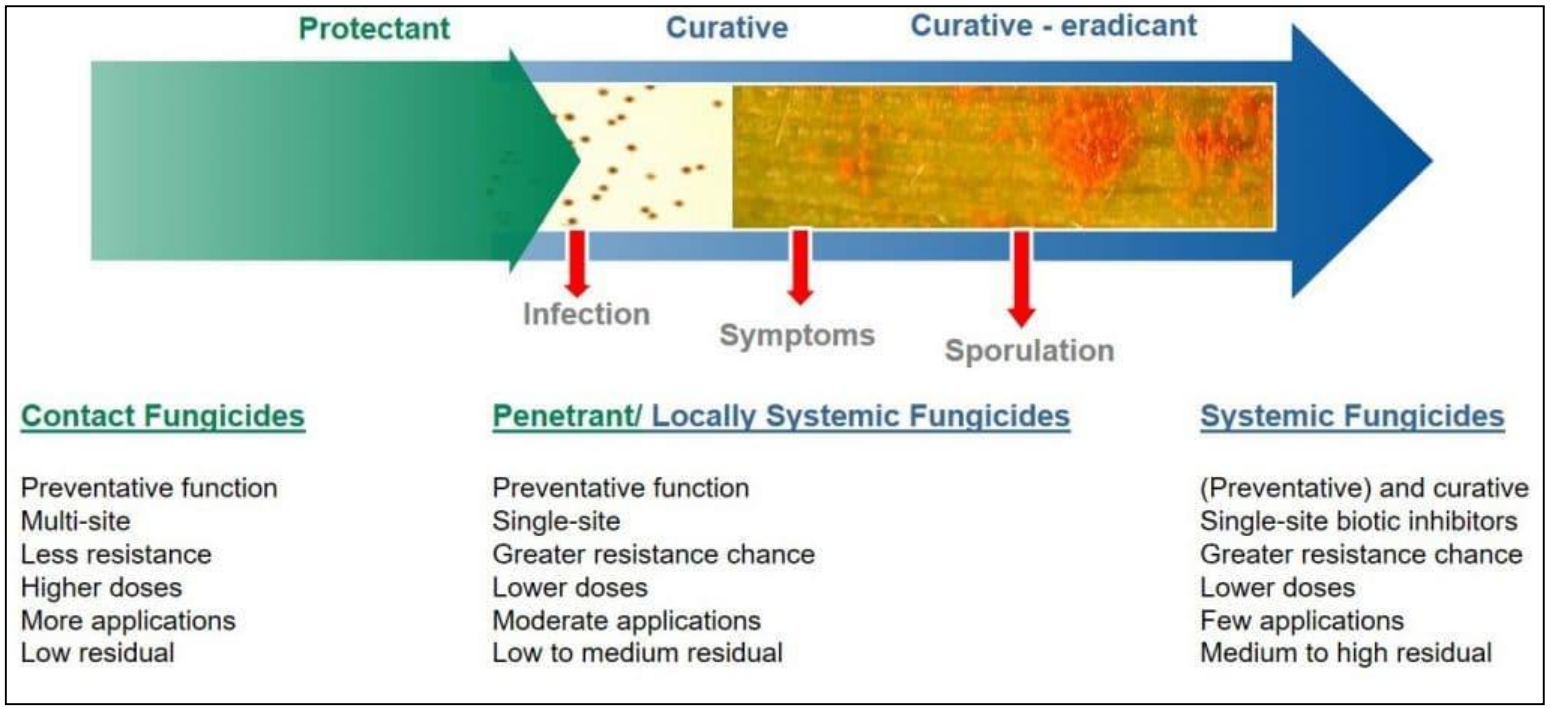
Fungicides

Types of fungicides



Types of Fungicides

Application timing for contact (protectant) versus systemic (curative) fungicides



Contact Fungicides	Penetrant/ Locally Systemic Fungicides	Systemic Fungicides
Preventative function	Preventative function	(Preventative) and curative
Multi-site	Single-site	Single-site biotic inhibitors
Less resistance	Greater resistance chance	Greater resistance chance
Higher doses	Lower doses	Lower doses
More applications	Moderate applications	Few applications
Low residual	Low to medium residual	Medium to high residual

Protectant Fungicides
1. Copper (inorganic or mineral)

- Copper-based bactericides introduced in the 1880s are still used extensively to combat many bacterial plant diseases.
- These are broad spectrum compounds; useful as fungicides and bactericides; protective (preventative) fungicides.
- Free Cu - Copper sulfate: Bordeaux mixture (copper sulfate and hydrated lime).
- Fixed Cu - Copper hydroxide, copper oxide, copper oxychloride, copper octanoate.
- Copper is bound to organic and inorganic molecules in fixed-type coppers, less toxic to plants.
- Copper, are limited in their usefulness due to inferior efficacy and phytotoxicity, especially russeting.

Phytotoxic to plants in elemental form (use uncommon)

Copper spray limitations

Usually multiple sites of activity

Limitations:

Spring weather conditions are very favourable
rapid bacterial multiplication and infections
Copper must be sprayed frequently (removal by rainfall)
low penetration of copper into buds, flowers,..

Copper tolerant or resistant strains

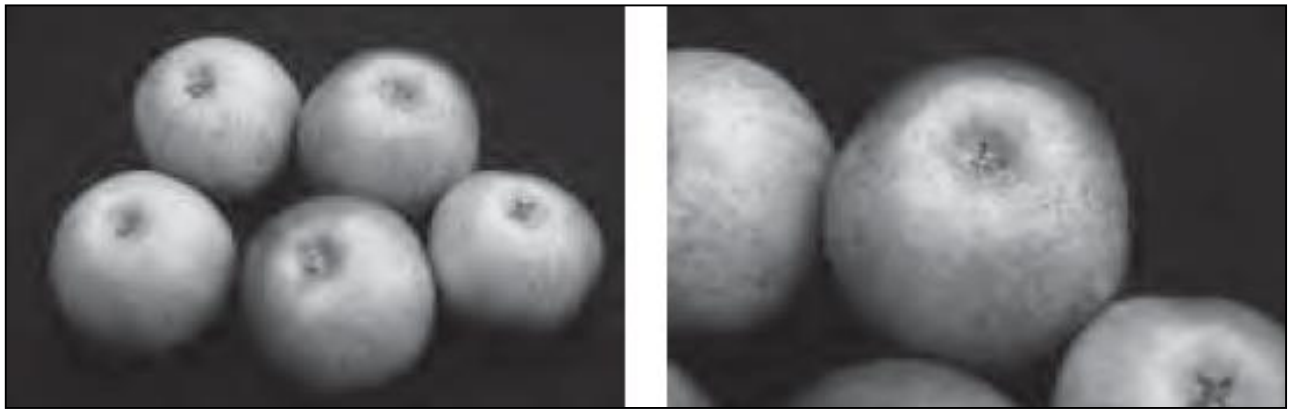
Moderate efficacy in disease control

Copper accumulation in soil (reduction in total amount of copper)

Moragrega et al.,

Copper and russetting

- Black speckling and russet resulted from copper applications in cover sprays on Twenty Ounce apples.
- These are destined for the processor because they are not acceptable for fresh fruit.



1 g = 0.0022 lbs or pounds.

Breth *et al.*,2001

Protectant fungicides

2. Organics

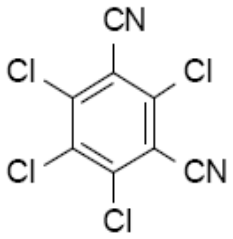
Increase copper formulations efficacy

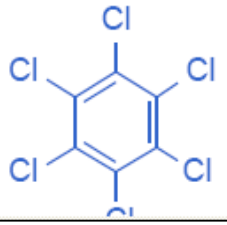
- Organics are protective (preventative) fungicides.
- Broad spectrum control, multi-site activity.
- Represent 60-70% of fungicides used.
- For Example:
- Dithiocarbamate fungicides including:
- Mancozeb,
- Maneb(Manganese-based fungicide),and
- Zineb (zinc-based fungicide).

Protectant fungicides
Organics (Cont'd)

- Dithiocarbamates (*e.g.* maneb, mancozeb) and chlorothalonil still in widespread use

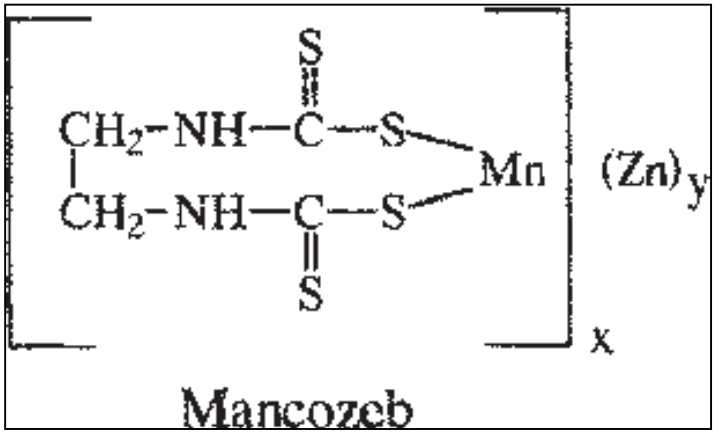
Maneb $[\text{-SCSNHCH}_2\text{CH}_2\text{NHCS}_2\text{Mn-}]_x$ 1955

Chlorothalonil  protective 1964

c.f. HCH (lindane) 

Mancozeb
Structure

- Chemical name:
- Zinc ion coordination product with manganese ethylene-1,2-bisdithiocarbamate polymer.
- Empirical formula:
- $(C_4H_6N_2S_4Mn)_a \cdot (C_4H_4N_2S_4Zn)_y$
- Structural formula:



Mancozeb

Mancozeb
Mode of action

- Mancozeb was introduced in 1961 as a material a superior to maneb or mixture of maneb and zineb in the protection of certain agricultural crops from plant pathogenic fungi.
- Activity: contact
- Mode of action: Multi-site inhibitor.

New products with antibacterial activity
Increase copper formulations efficacy

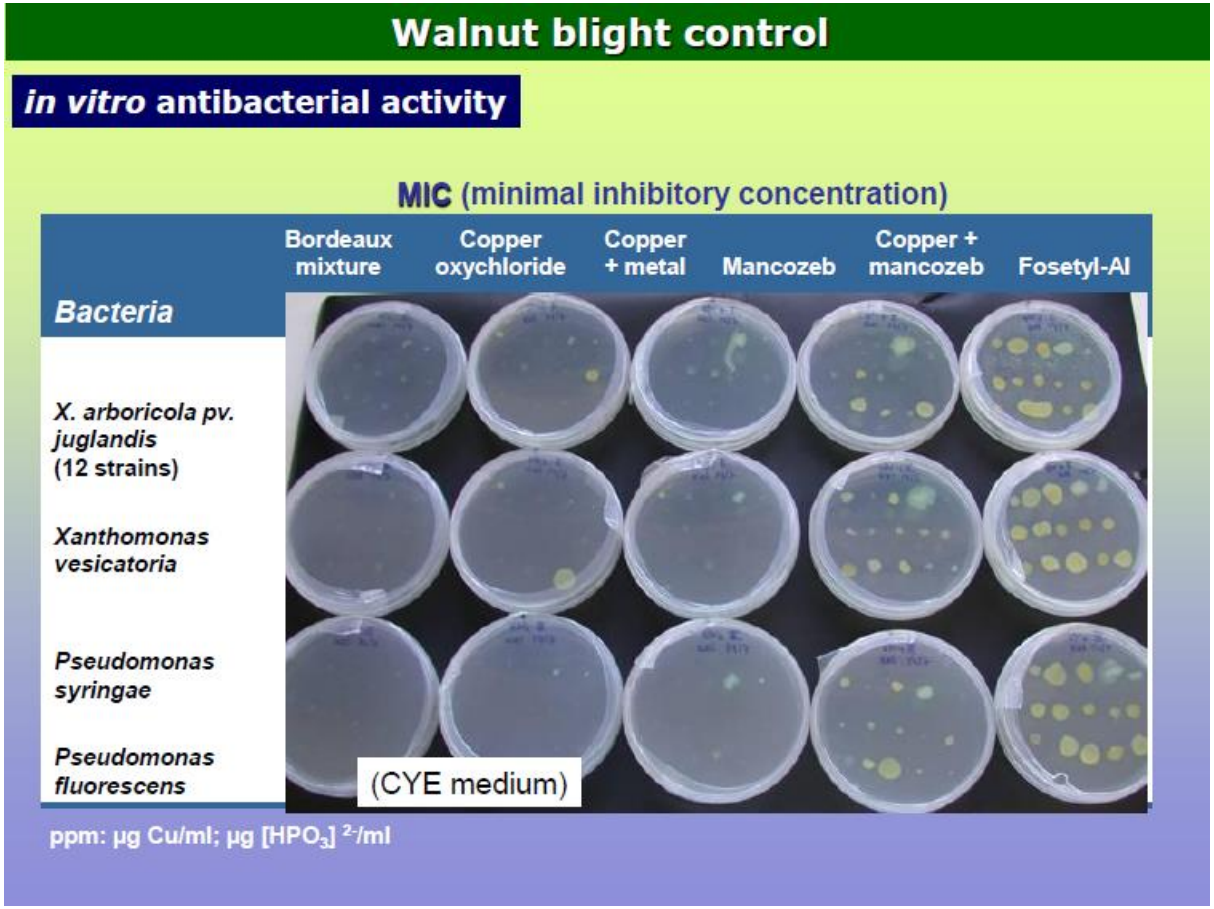
Copper + mancozeb mixtures
seem to be more effective than copper alone
(Buchner et al., 2001)

Addition of metallic ions (salts: Fe, Mg, Ca, Mn, Zn)
enhances the copper toxicity to *X.a.j*
(Lee et al, 1983)
(Buchner et al., 2001)

Reduction of populations of copper resistant strains

Moragrega *et al.*,

New products with antibacterial activity
Increase copper formulations efficacy



Moragrega *et al.*,

New products with antibacterial activity
Increase copper formulations efficacy

Walnut blight control

***in vitro* antibacterial activity**

MIC (minimal inhibitory concentration)

Bacteria	Bordeaux mixture (ppm Cu)	Copper oxychloride (ppm Cu)	Copper + metal (ppm Cu)	Mancozeb (ppm ai)	Copper + mancozeb (ppm Cu)	Fosetyl-Al (ppm $[HPO_3]^{2-}$)
X. arboricola pv. juglandis (12 strains)	12 (9) 48 (2) >96 (1)	12 (9) 48 (2) >96 (1)	12 – 24	> 100	12	316 – 632
Xanthomonas vesicatoria	12	12	6	> 100	12	316
Pseudomonas syringae	12	12	12	> 100	12	632
Pseudomonas fluorescens	24	24	24	> 100	12	632

ppm: µg Cu/ml; µg $[HPO_3]^{2-}$/ml

Moragrega *et al.*,

New copper products with antibacterial activity

The new copper standards

- CUPROFIX MZ DISPERSS consists of 30% mancozeb plus 12% copper (from the Bordeaux Mixture), with all the benefits of the DISPERSS formulation.
- This new bactericide/fungicide pre-mix combination of copper and mancozeb, provides enhanced bacterial control versus copper alone.
- Research has shown that EBDC fungicides, such as mancozeb, increase the activity of copper for control of bacteria, especially where copper resistant strains occur.
- This combination will also provide enhanced control of fungal diseases controlled by both copper and mancozeb, and the mancozeb will provide control of diseases not affected by copper.

EBDC fungicides: ethylene bisdithiocarbamate

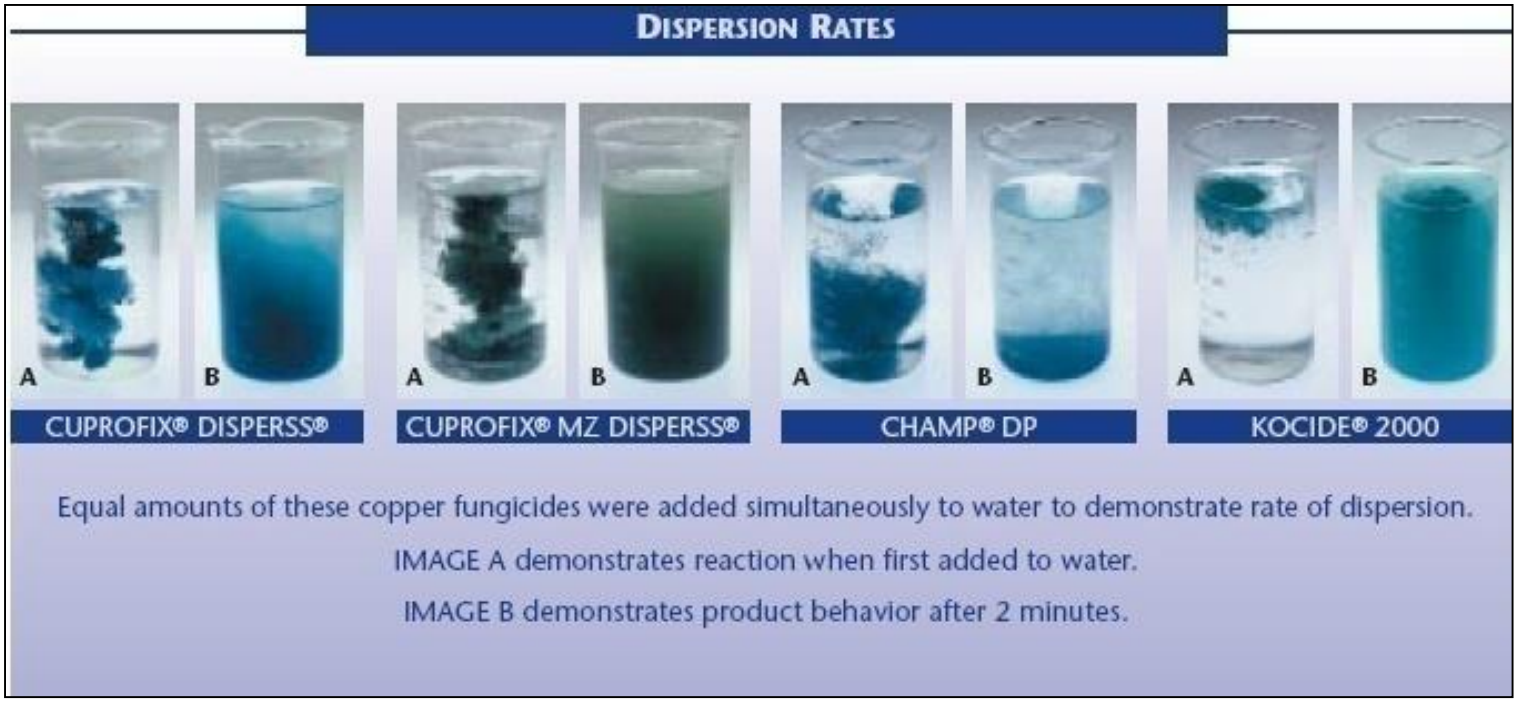
Cuprofix Mz Disperss

The new copper standards

- Cuprofix Mz Disperss is a antimicrobials from United Phosphorus Limited.
- It starts with very fine copper hydroxosulfate particles (Bordeaux Mixture).
- Each particle is coated with surfactants, wetting and anti-foaming agents, creating a superior dry flowable granule formulation.
- The result is an essentially neutral pH, very stable, highly dispersible formulation providing maximum disease protection when applied to a multitude of crops.

Active Ingredient	Percent (%)
Basic copper sulfate	22.1
Mancozeb	30.4

Dispersion rates of different commercial copper compounds



Systematic fungicides

- Examples:

1. Thiabendazole: It is commercially available as Tecto and Mertect.
2. Probenazole: Its commercial name is oryzemate. It is also specific against rice blast.
3. Benomyl: Is effective against many fungi.

Systematic fungicides
Mode of actions

1. Disruption of membrane function (DMIs- triazoles, etc.
2. Disruption of nuclear processes- oomycete fungicide e.g. metalaxyl.
3. Respiration inhibitors: large group
4. Inhibition of tubulin biosynthesis e.g. benomyl

- Miscellaneous other mechanisms: Antibiotic type agents e.g. kasugamicin.

Systematic fungicides

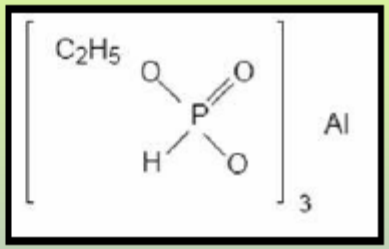
Fosetyl-Al (Alliett)

P. syringae, E. amylovora, Xanthomons sp.

PHOSPHONATE DERIVATIVES

Fosetyl-Al

(aluminum tris-O-ethyl phosphonate)



Systemic fungicide against fungi (Oomicetes)

Efficacy in control of some bacterial diseases

***Pseudomonas syringae* pv. *syringae* (pear)**
Fireblight of rosaceous (*Erwinia amylovora*)
Ornamentals (*Xanthomonas sp.*)

(Chase, AR 1993; Moragrega et al, 1997; Ruz et al, 1999)

No *in vitro* antibacterial activity

Translocation inside the plant

induction of plant defences

Moragrega *et al.*,

Antibiotics

With a broad-spectrum effects

Bacterial disease management

- Type of antimicrobial substance active against bacteria.

1. The term antibiotic strictly refers to substances that are of biological origin; whereas,
2. The term chemotherapeutic agent refers to a synthetic chemical.

- Many of newer "antibiotics" are actually:

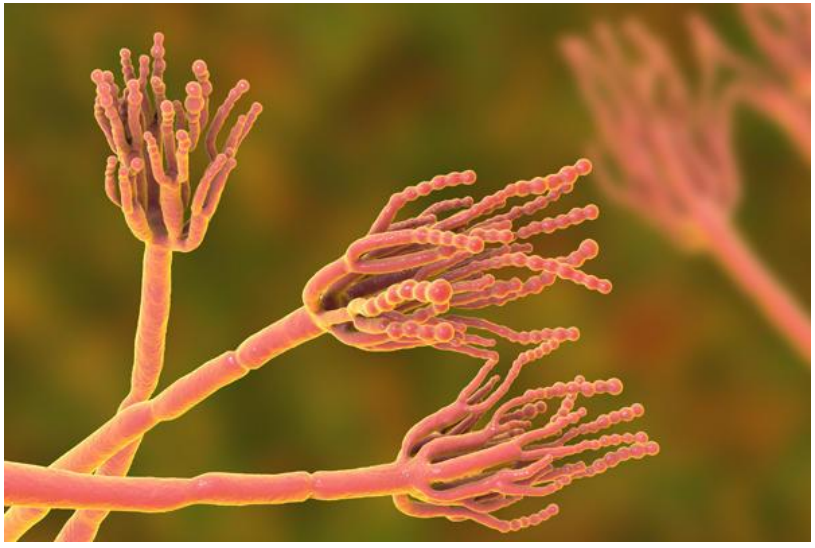
1. chemically modified biological products, or
2. even chemically synthesized biological products.

- However, the term antibiotic is often used to refer to all types of antimicrobial agents.

Source of antibiotics

Bacteria and fungi

- Antibiotics are chemicals that kill or inhibit the growth of bacteria and are used to treat bacterial infections. They are produced in nature by soil bacteria(esp. *Streptomyces* spp.) and fungi.



Fungi Penicillium which cause food spoilage and are used for production of the first antibiotic penicillin. 3D illustration showing spores conidia and conidiophore.

Actinomycetes

- Diverse but usually gram-positive "bacteria"
- Include pathogens and spp. that are sources for antibiotics
- … includes *Streptomyces*

Potato scab (*Streptomyces* sp.)

Systematic fungicides
Antibiotics

- Antibiotics are agents that are selectively toxic for bacteria either:

1. Killing (bactericidal), or
2. Inhibiting their growth (inhibitors of protein synthesis, mostly bacteriostatic).

- Antibiotics have been used since the 1950s to control certain bacterial diseases of:
- high-value fruit,
- vegetable, and
- ornamental plants.
- Today, the antibiotics most commonly used on plants are oxytetracycline and streptomycin.

McManus and Stockwell,2000;..

Antibiotic

Used in plant disease control

- The Centers for Disease Control and Prevention (CDC) estimate that 50 million pounds of antibiotics are produced annually in the United States.

1. About half of the antibiotics are used by humans in prescriptions from doctors, and
2. 40% are used for animal diseases and production.
3. In the USA, antibiotics applied to plants account for less than 0.5% of total antibiotic use.

McManus *et al.*,2002; Sundin,2018

Production of antibiotics

- About 100 antibiotics manufactured in large quantities since first production of penicillin in 1940s.
- Improve yields by using mutated strains and improving fermentation procedures.
- e.g. strain that once produced 60 mg of penicillin per litre of culture now makes 20 g/l.
- Many antibiotics now are semi-synthetic i.e. made partly by microbes and modified by chemists.

Antibiotics

Used in plant disease control

- In the United States, streptomycin is registered for use on twelve fruit, vegetable, and ornamental plant species.
- Oxytetracycline is registered for use on four fruit crops.
- Both antibiotics are applied primarily for the control of bacterial diseases, although:

1. streptomycin is also used to a limited extent to control diseases caused by water molds, and
2. oxytetracyline has been used to control certain diseases caused by phytoplasmas that infect plants.

Antibiotic
Used in plant disease control

Antibiotics examined for plant disease control (1940s)

- Penicillin
- Streptomycin
- Aureomycin
- Chloramphenicol
- Oxytetracycline

Streptomycin

- Utilized in plant disease management since the early 1950's
- 100 ppm solution
  - Targets:
  - Fire blight of apple and pear
  - Bacterial blight of celery
  - Shoot tip dieback of nursery trees
  - Bacterial spot of tomato and pepper

Antibiotics
Used in plant disease control

Antibiotic use for plant disease control in the U.S.

- Streptomycin – use started in the 1950s
  - Mostly for fire blight of apple and pear
  - Some usage on tomato, discontinued
- Oxytetracycline – use started in the 1980s
  - in response to streptomycin resistance in the fire blight pathogen
- Kasugamycin – registered in 2015
  - Fire blight
  - Also developed in response to streptomycin resistance
  - No uses outside of plant agriculture

Streptomycin

- Utilized in plant disease management since the early 1950's
- 100 ppm solution
  - Targets:
  - Fire blight of apple and pear
  - Bacterial blight of celery
  - Shoot tip dieback of nursery trees
  - Bacterial spot of tomato and pepper

Sundin,2018

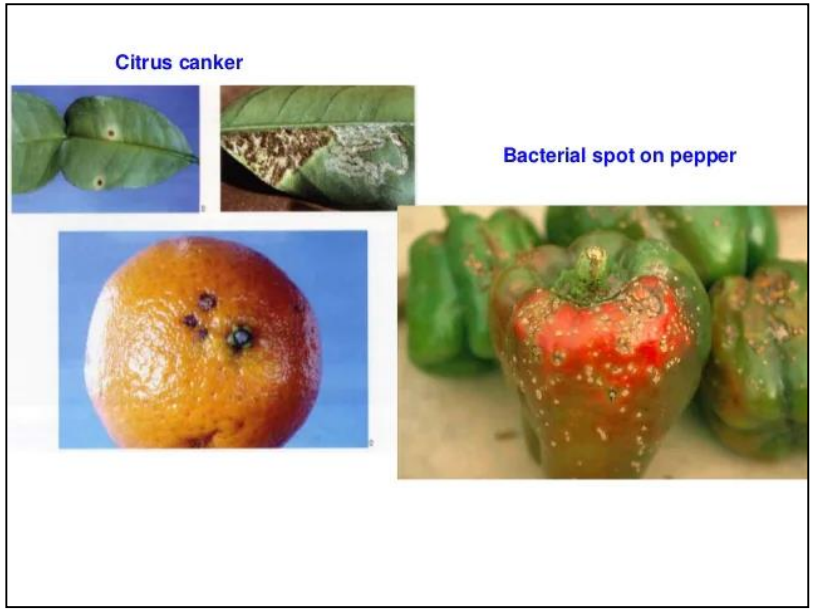
Antibiotics

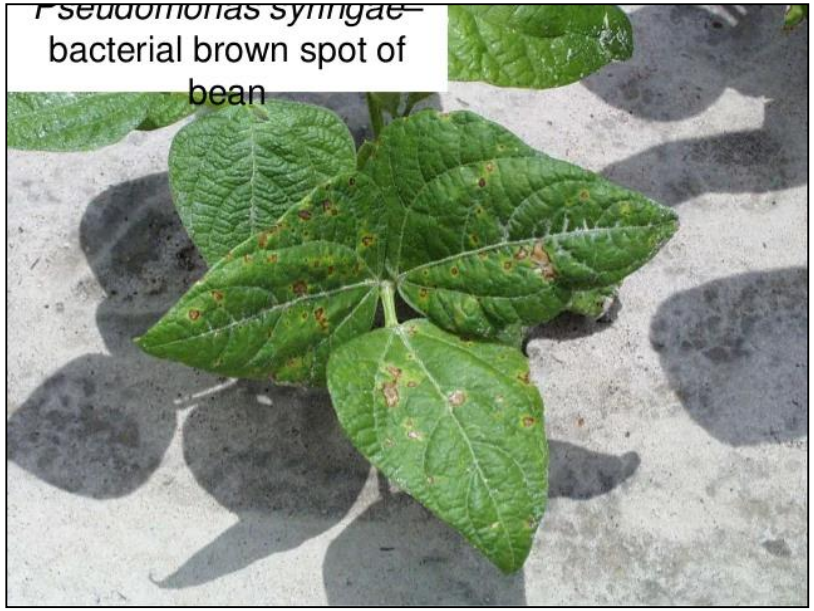
Used in plant disease control

1. **Aureofungin**
   - It is a broad spectrum antibiotic produced by *Streptoverticillium cinnamomeus* var. *terricola*. It is effective only when it is mixed with copper sulphate. It controls wilt of coconut, citrus gummosis, chillies powdery mildew & grapes downy mildew.
2. **Streptomycin**
   - It is produced by *Streptomyces griseus*. It controls bacterial diseases viz. citrus canker, black arm of cotton and rice bacterial blight.
3. **Tetracycline**
   - It is produced by *Streptomyces* sp. and effective in controlling mycoplasma diseases like sandal spike, brinjal little leaf and gingelly phyllody.

Antibiotics

Used in plant disease control





Sundin,2012

Antibiotics

Used in plant disease control

Bacterial Diseases are Exceedingly Difficult to Control

- Lack of host resistance is a critical issue
- Most popular varieties are typically the m

'Gala' 'Golden Delicious

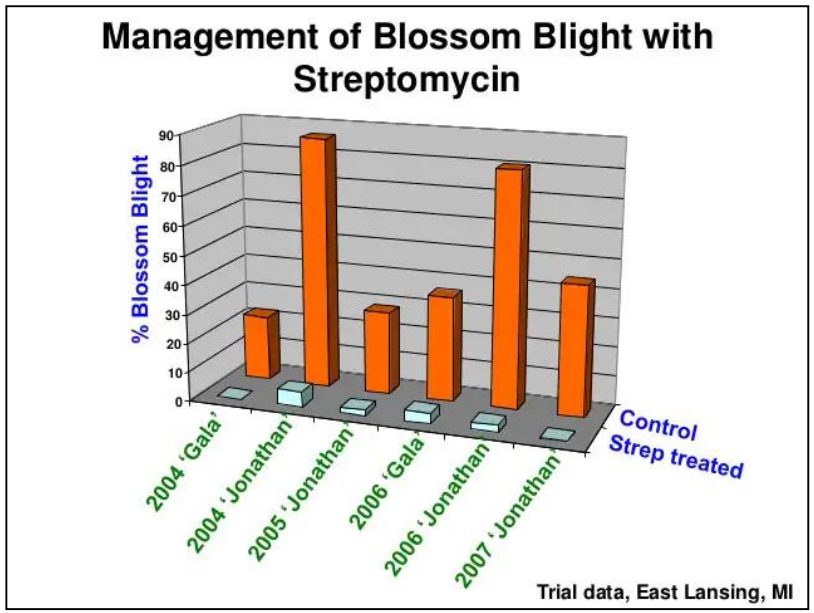
Fire Blight Disease

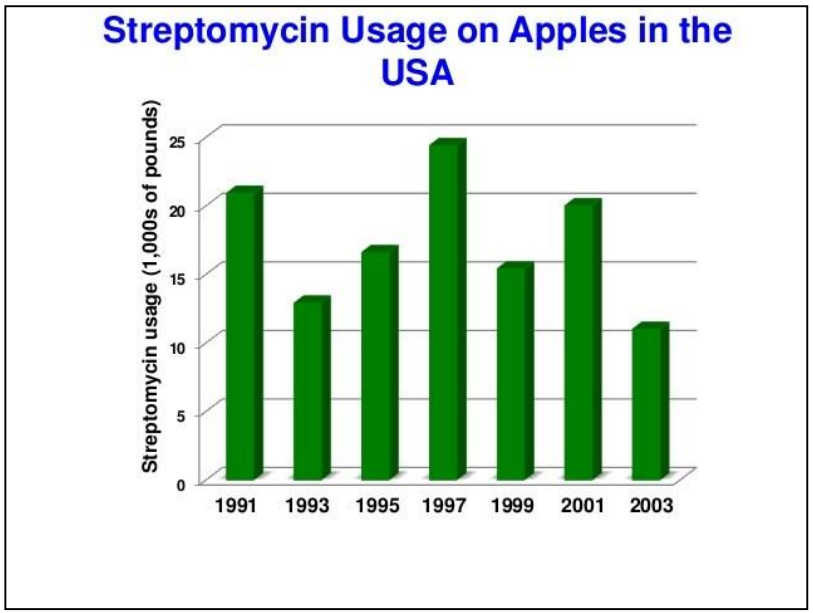
- Reduces fruit yields
- Kills branches
- Kills roots (tree death)

Sundin,2012

Antibiotics

Used in plant disease control





Sundin,2012

Bacterial pathogens and crops targeted by streptomycin

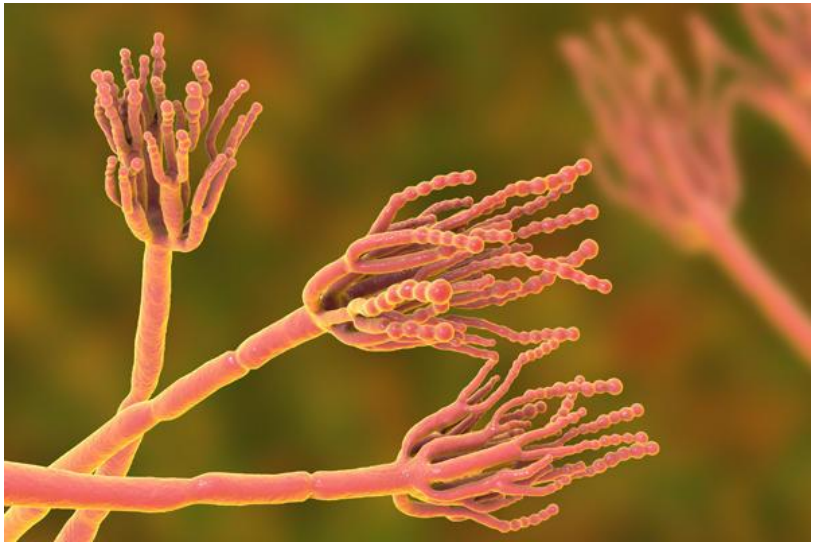
Pathogen	Disease	Crop
Erwinia amylovora	fire blight	Apple, pear and nashi
Pseudomonas syringae pv. *syringae*	bacterial blast	Stone fruit
Xanthomonas arboricola pv. *pruni*	bacterial spot	Stone fruit
Pseudomonas syringae pv. *tomato*	tomato speck	Tomato
Pseudomonas syringae pv. *syringae*	Leaf spot	Tomato
Xanthomonas vesicatoria	bacterial spot	Tomato
Clavibacter michiganensis pv. *michiganensis*	bacterial canker and wilt	Tomato

Antibiotics registered for use on plants in the U.S.

Antibiotic	Formulation	Trade name (s)	Primary uses
Streptomycin	22.4% streptomycin sulfate; equivalent to 17% streptomycin	Agri-mycin 17	Apple, pear, ornamental plants, tomato, pepper, potato
Oxytetracycline	31.5% oxytetracycline calcium complex; equivalent to 17% oxytetracycline	Mycoshield; Agricultural Terramycin	Peach and nectarine, pear, apple

Source of antibiotics
Bacteria and fungi

- Antibiotics are chemicals that kill or inhibit the growth of bacteria and are used to treat bacterial infections. They are produced in nature by soil bacteria(esp. *Streptomyces* spp.) and fungi.



Fungi Penicillium which cause food spoilage and are used for production of the first antibiotic penicillin. 3D illustration showing spores conidia and conidiophore.

Actinomycetes

- Diverse but usually gram-positive "bacteria"
- Include pathogens and spp. that are sources for antibiotics
- … includes *Streptomyces*

Potato scab (*Streptomyces* sp.)

Antibiotics
Used as a spray

- Application of honeybee-safe antibiotic spray in a pear orchard in bloom to protect trees from fire blight caused by the bacterium *Erwinia amylovora*.

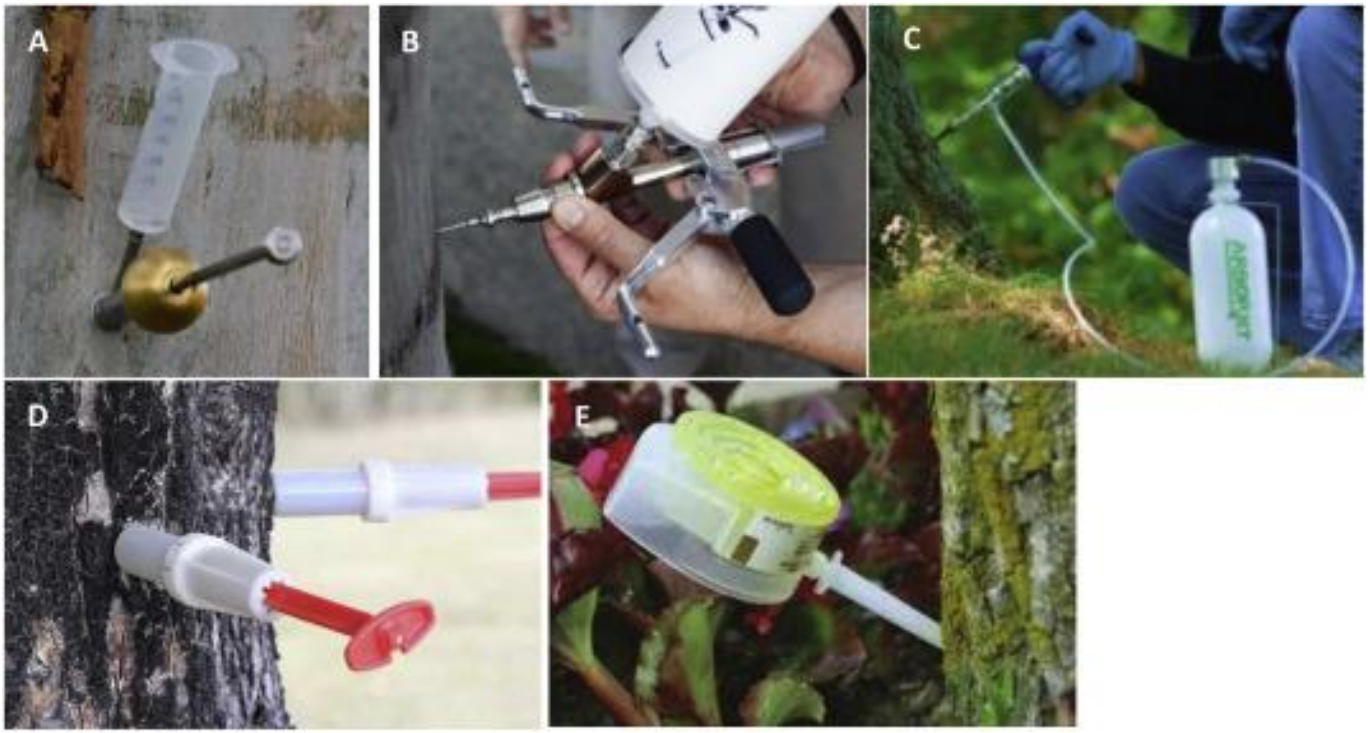
Cabi.org;..

Antibiotics
Used as injection

- When injected into trees infected with mollicutes or fastidious bacteria, tetracyclines stop the development of the disease and induce the remission of symptoms, i.e., the symptoms disappear and the trees resume growth as long as some tetracycline is present in the trees.
- Usually one injection at the end of the growing season is sufficient for normal growth of the tree during the following season.

Antibiotics used as injection

Trunk injection of plant protection products to protect trees from pests and diseases



Berger and Laurent,2019

Antibiotics used as injection

Trunk injection of plant protection products to protect trees from pests and diseases

- **How Chemjet works:**
- The Chemjet® Tree Injector is a spring-loaded syringe-like device, with a 20mm tapered nozzle. The chemical is drawn through the nozzle into the chamber of the injector in measured 10, 15 or 20ml quantities. The injector handle is pulled back to energize the spring pressure can be locked open at 20 ml by twisting the handle in a clockwise direction.
- Holes are drilled into the tree using a 11/64 bit to a depth of about one inch, and the Chemjet® nozzle is pressed into the hole by hand (no hammering) to make a tight seal.
- Note: you do not have to twist while inserting! While holding the Chemjet® in place, the red handle is pulled back slightly then turned counterclockwise to allow the spring pressure to inject chemical into the tree. The injector is then left unattended to inject at a steady low pressure of about 20 PSI which does not damage the inner bark layers, and allows discharge of chemical into the xylem at a rate that is accommodated by the natural capacity of the tree to accept more liquid.

Berger and Laurent,2019

Antibiotics used as injection

Trunk injection of plant protection products to protect trees from pests and diseases

While holding the Chemjet® in place, the red handle is pulled back slightly then turned counterclockwise to allow the spring pressure to inject chemical into the tree. The injector is then left unattended to inject at a steady low pressure of about 20 PSI.

Berger and Laurent,2019

Antibiotics
Used as a soil drench

- Streptomycin has also been used as:

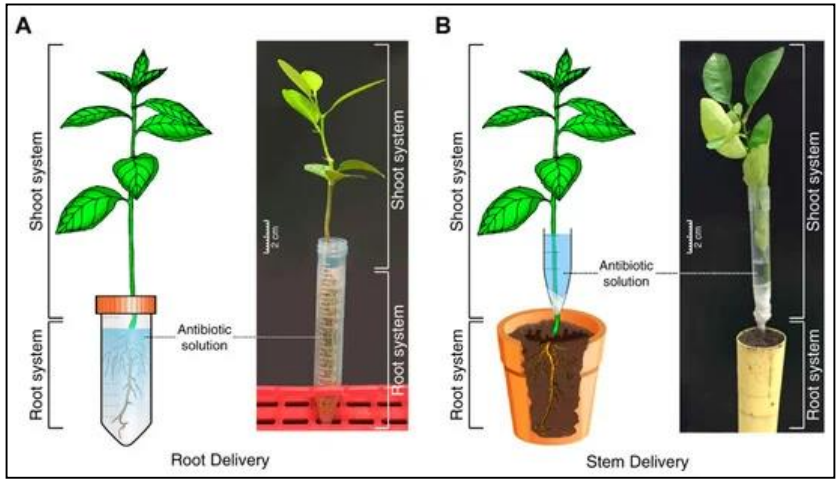
1. A soil drench(to wet thoroughly, to soak), e.g., in the control of geranium foot rot caused by *Xanthomonas* sp.,
2. As a dip for potato tuber pieces used for seed against various bacterial rots of tubers, and
3. As a seed disinfectant against bacterial pathogens of beans, cotton, crucifers, and cereals.

Antibiotics

Used as root drench and stem delivery

Oxytetracycline and streptomycin against the *Candidatus* Liberibacter asiaticus (CLas)

- Antibiotics used for plant disease control are generally absorbed and translocated systemically by the plant to a limited extent.
- The presence of oxytetracycline and streptomycin in the phloem of treated plants suggested that these antibiotics could be effective against CLas.



Delivery of streptomycin and oxytetracycline into 3-month-old citrus seedlings. Root drench (A) and stem delivery (B).

Al-Rimawi *et al.*,2019

Antibiotics

Oxytetracycline and streptomycin against the *Candidatus* Liberibacter asiaticus (CLas)

Use information for antibiotics for HLB management in FL

Product Details	Firewall SO WP™ (streptomycin)	Fireline 17 WP™* (oxytetracycline)	Mycoshield® * (oxytetracycline)
Preharvest Interval (days)	40	40	21
Maximum Number of Applications per Calendar Year	3	3	8
Minimum Retreatment Interval (days)	21	21	21

New usage in Florida likely will significantly increase the amount of antibiotics used in plant agriculture in the U.S.

367,500 acres of oranges in Florida (NASS, 2015)

Antibiotics are applied with penetrating surfactants in an attempt to increase uptake by flush leaves

90% of oxytetracycline applied to plants is degraded by sunlight within 3 days
(Christiano et al. 2010; Plant Dis. 94:1213-1218)

Sundin,2018

Antibiotics

Antibiotic use on crops in low and middle-income countries based on recommendations made by agricultural advisors

- Bacterial pathogens are present throughout the world and on all crops.
- In many low-income economies(LMICs), including those in Africa, antibiotics are freely available through unregulated supply chains and over-the-counter sales.
- In other regions of the world antibiotics are not freely available. So the use of antibiotics is limited in this region.
- In China, the use of antibiotics in crop production is higher than that recorded within our data (Zhang *et al.*,2017).
- The use of antibiotics on crops in China is at least partially fuelled by government subsidies aimed at promoting their use.

Taylor & Reeder,2020

Antibiotics
Mode of actions

- Targeting one of five organelles/biosynthetic pathways:
- Ribosomes (inhibition of protein synthesis, translation);

1. Cell wall (inhibition of cell wall synthesis);
2. Cytoplasmic membrane (alteration of cell membranes);
3. DNA (inhibition of nucleic acid synthesis);
4. Cell metabolism.

Antibiotics

Mechanisms of action of antibiotics

Action	Alteration of bacterial envelope	Inhibition of protein synthesis	Inhibition of nucleic acid synthesis	Inhibition of metabolic pathway
Antibiotic family	ß-lactam	MLS	Quinolone	Sulfamide
	Glycopeptide	Phenicol,	Rifamycine, Ansamycine	Folic acid
	Polymyxin, daptomycin	Oxazolidinone		Nitro-imidazole
	Cathelicidin antimicrobial peptide (CAMP)	Aminoglycoside		
		Cycline (tetracycline)		

Scnehir,2009

Antibiotics

Mode of actions

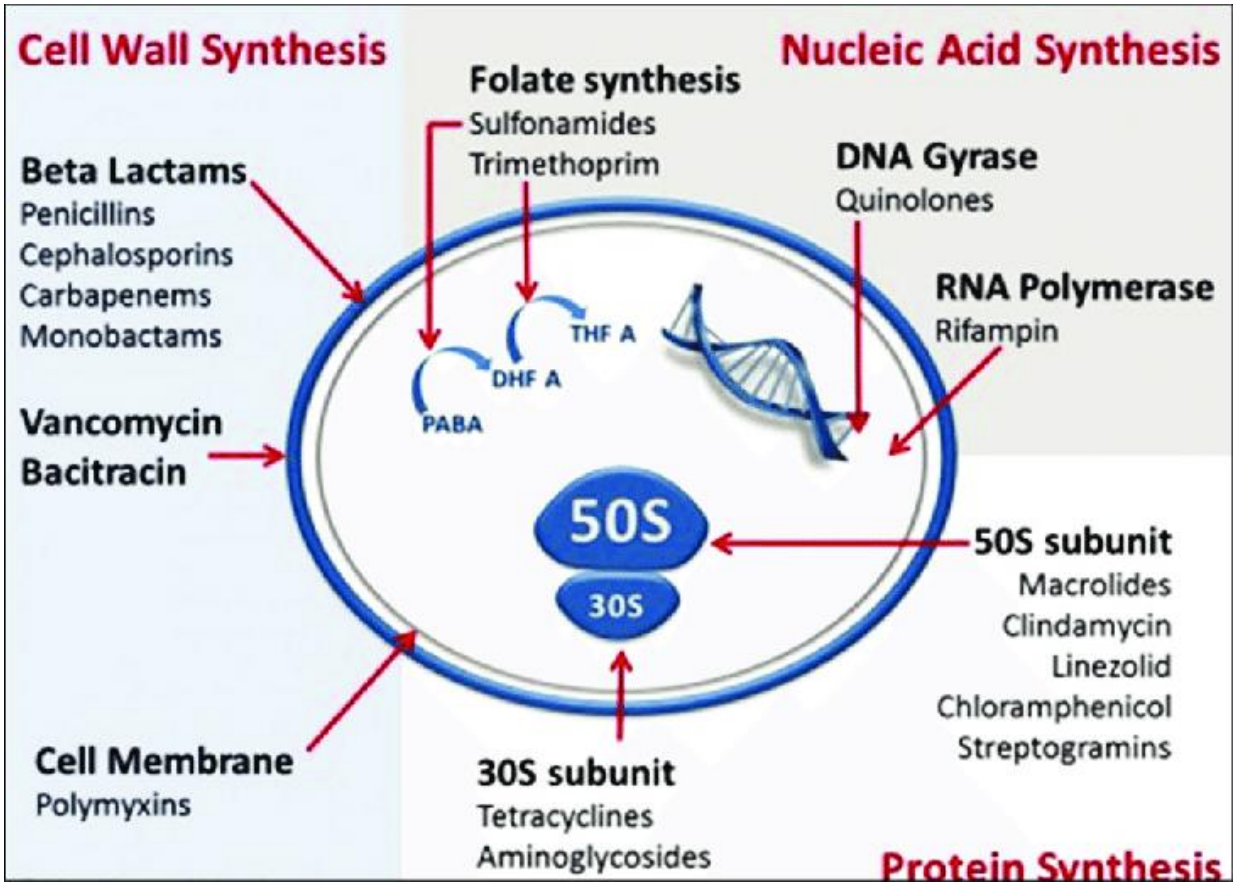
Classes of antimicrobial agents(antibiotics)

Classification: Mode of action

- Cell wall synthesis inhibitors
  - Beta-lactams (penicillins, cephalosporins, aztreonam, imipenem)
  - Poly-peptides (bacitracin, vancomycin)
- Protein synthesis inhibitors
  - Aminoglycosides
  - Tetracyclins
  - Macrolides
  - Chloramphenicol
  - Clindamycin
- Inhibitors of essential metabolites (folate)
  - Sulfonamides
  - Trimethoprim
- Injury to plasma membrane
  - -polymyxin B
  - - mystatin
  - - amphotericin B
  - - miconazole
- Inhibition of nucleic acid replication and transcription
  - - Quinolones
  - - Rifampin

Maiyoh,2015

Antibiotics
Mode of actions



Kapoor *et al.*,2017

Antibiotics

Antibiotics targets ribosomes and inhibits protein synthesis

- A better classification therefore utilizes their site of action at the ribosome, that is whether they bind primarily to the 30S or 50S ribosomal subunits.

Those which act on the 30 S ribosomal subunit
The aminoglycoside antibiotics
The tetracyclines
Those which act on the 50 S ribosomal subunit
Puromycin
Chloramphenicol
Erythromycin
Lincomycin
Clindamycin
Fusidic acid

Edwards,1980

Antibiotics

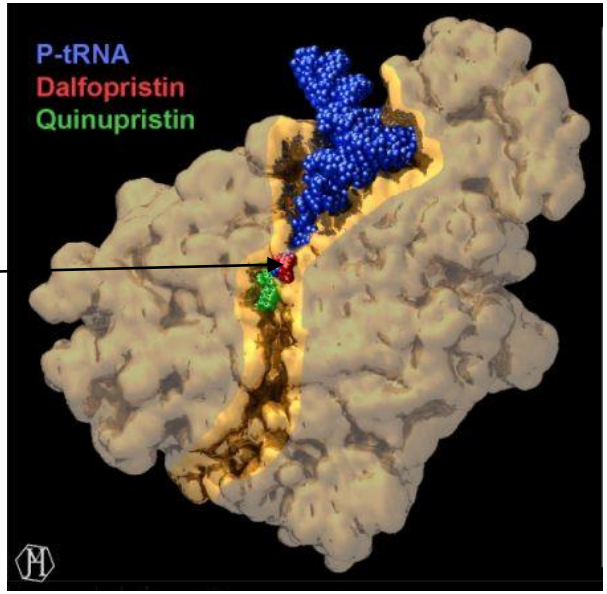
Antibiotics targets ribosomes and inhibits protein synthesis

- Antibiotics including: Minocycline, Minocycline hydrochloride, Demeclocycline, Demeclocycline hydrochloride, Tigecycline, Tetracycline, Oxytetracycline, Doxycycline, Doxycycline hyclate, Spectinomycin, Hygromycin, Paromomycin, Streptomycin, Kanamycin, Gentamicin, Tobramycin, Amakacin, Netilmicin, Neomycin, bind to the 30S ribosomal subunit of 16S rRNA and inhibit protein synthesis.
- Erythromycin binds to the 50S unit and stops mRNA movement in the ribosome.

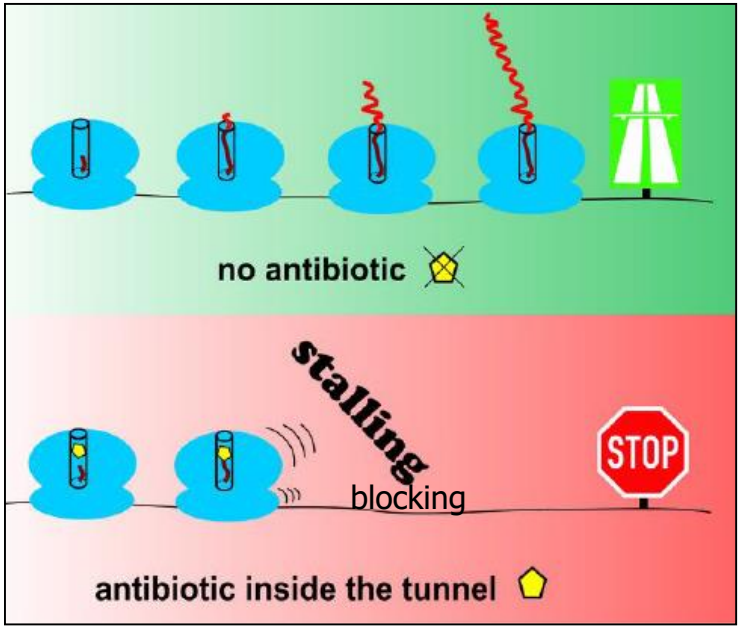
The function of the 30S subunit is primarily determined by the 16S RNA of which it is primarily comprised, while the 50S subunit's function is primarily determined by the bacterial 23S RNA.

Antibiotics

Antibiotics affecting protein synthesis



Antibiotic

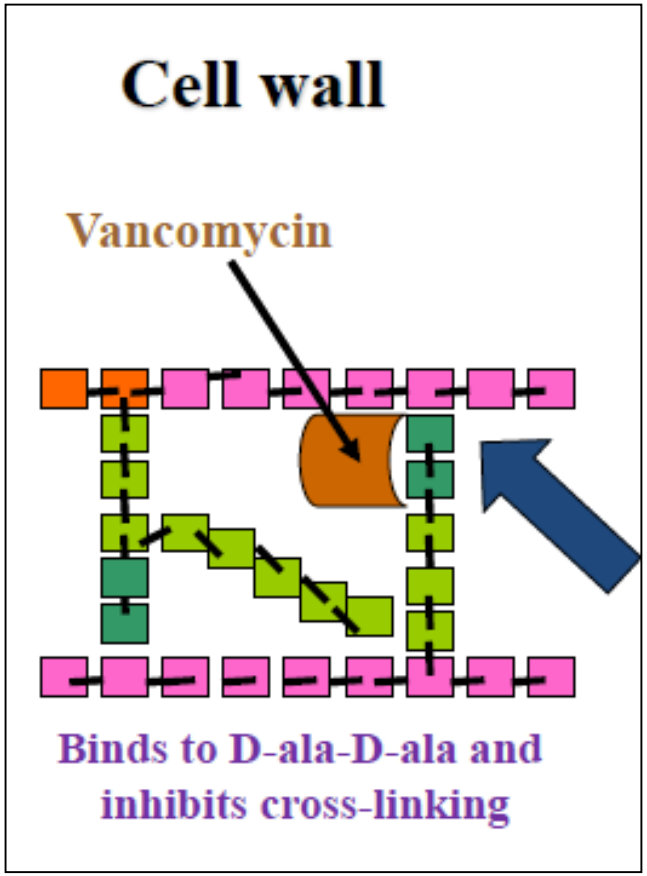


Koch *et al.*,2017,..

Vancomycin

Antibiotics affecting the cell envelope

- Vancomycin is classified as an aminoglycoside antibiotic.
- This class of antibacterials is effective against many gram-positive bacteria.
- Vancomycin binds to D-ala-D-ala thus sterically inhibits transpeptidation (cross-linking).
- Replacement of one of the D-ala in the peptide side chain of peptidoglycan leads to resistance.



Fox,2008;..

Antibiotic resistance

In plant pathogenic bacteria

Antibiotics
Bacterial resistant to antibiotics

- Spread of antibiotic resistance to animal and human pathogens.
- There is great concern over the use of antibiotics in agriculture due to the potential for resistance to spread to medically important bacteria.
- The regulations pertaining to antibiotic use on plants differs widely between countries and regions.

1. The European Union and Brazil do not approve any antibiotics as active ingredients in pesticides (Donley,2019), whereas
2. Some countries permit their use for certain crops or in emergency situations,
3. others have no legislation on this topic at all.

- Many countries of South East Asia (SEA) and Western Pacific (WP), consider the use of antibiotics in crop production as an important means of controlling pathogens whilst at the same time protecting the environment.

Taylor & Reeder,2020

Antibiotics

How is resistance spread?

Inherent, or acquired

- Antibiotic resistance can either be:

1. Inherent, or
2. acquired.

- Some bacteria are naturally resistant to some antibiotics due to their physiological characteristics.
- This is inherent resistance.
- Acquired resistance occurs when a bacterium that was originally sensitive to an antibiotic develops resistance.

Antibiotics

Bacterial resistant to antibiotics

- *Pseudomonas*,
- Enterobacteriaceae, and
- The full family of anaerobes are among the vast number of "bad news" bacterial organisms that have become resistant to antibiotics.

Antibiotics

Reports of antibiotic resistance in plant-pathogenic bacteria

Antibiotic	Organism	Location	Genetic mechanism	Reference
Kasugamycin	*Acidovorax avenae* ssp. *avenae*	Japan	*aac(2′)-IIa*	138
	Burkholderia glumae	Japan	*aac(2′)-IIa*	138
Oxolinic acid	*Erwinia amylovora*	Israel	Probable chromosomal mutation	53
	Burkholderia glumae	Israel	Probable chromosomal mutation	53
		Japan	Probable chromosomal mutation	41
Streptomcyin	*E. amylovora*	California, USA	Chromosomal mutation	97
		California, USA	*rpsL* mutation	14
		Michigan, USA	*rpsL* mutation	14
		Oregon, USA	*rpsL* mutation	14
		Washington, USA	*rpsL* mutation	14
		New Zealand	*rpsL* mutation	14
		California, USA	*strAB* on plasmid RSF1010	80
		California, USA	Tn*5393*a	32
		Michigan, USA	Tn*5393* on pEa34	13
		Michigan, USA	Tn*5393* on pEa29	63
		New York, USA	Tn*5393* on pEa29	119
	Pseudomonas syringae			
	P. syringae	Oregon, USA	*strAB*[a]	93
	P. syringae pv. *actinidiae*	Japan	Tn*5393*a	39
		Japan	*rpsL* mutation	
	P. syringae pv. *papulans*	New York, USA	*strAB*[b]	75
		Michigan, USA	*strAB*[b]	52
	P. syringae pv. *syringae*	Oklahoma, USA	Tn*5393*a	111
	X. axonopodis pv. vesicatoria	Argentina	Tn*5393*b	113
	X. citri subsp. *citri*	Korea	*strB*[c]	48
	X. oryzae pv. *oryzae*	China	*aadA1*	134

[a]Presence of the *strAB* genes was determined by hybridization, but structural genes of Tn*5393* were not screened for.
[b]The probe SMP3 was utilized to detect streptomycin resistance; this probe contains portions of the *strA* and *tnpR* genes from Tn*5393*a.
[c]Presence of the *strB* gene was determined by PCR but *strA* or structural genes of Tn*5393* were not screened for.

Sundin and Wang,2018

Antibiotic resistance

Bacterial resistance to streptomycin

- Resistance to streptomycin has been also reported in:
- *P. cichorii*,
- *P. syringae* pv. *lachrymans*,
- *P. syringae* pv. *papulans*, and
- *P. syringae* pv. *syringae*,
- *Erwinia amylovora*, and
- *Xanthomonas campestris*.

Antibiotics

Streptomycin resistance in plant pathogenic bacteria

Pathogen	Plant (s) affected	Location(s)
Erwinia amylovora	apple, pear	California, Idaho, Isreal, Michigan, Missouri, New Zealand, Oregon, Washington
Pseudomonas cichorii	celery	Florida
Pseudomonas syringae	apple, pear, ornamental and landscape trees	Michigan, New York, Oklahoma, Oregon
Xanthomonas campestris	tomato, pepper	Argentina, Brazil, California, Florida, Georgia, Ohio, Pennsylvania, Taiwan, Tonga

Antibiotic resistance
Bacterial resistance to streptomycin

- Oxytetracycline is a broad-spectrum antibiotic active against Gram-positive and Gram-negative bacteria.
- Resistance of plant pathogens to oxytetracycline is rare.
- Besides, oxytetracycline is generally less effective compared to streptomycin because it does not kill existing bacteria.
- Oxytetracycline is very sensitive to degradation by sunlight and can be easily washed off the leaf surfaces during rain events.

Archer *et al.*,2020

Antibiotic resistance

Bacterial resistant to tetracycline

- Tetracycline resistance genes can be found on Tetracycline-resistant (Tc) plasmids (Chopra and Roberts, 2001).
- There have been up to 29 tetracycline resistance genes (*tet*) that have been identified and at least 3 oxytetracycline resistance genes (*orp*) that have been characterized in both Gram-positive and Gram-negative organisms.
- *P. aeruginosa* is known to express four "*tet*" genes: *tet*(A), *tet*(C), *tet*(E) and *tet*(G).
- These genes code for multi-drug resistant efflux pumps which function to expel the antibiotic from the bacterial cell.

See also kind and functions of efflux pumps in bacterial pathogenesis file.

Nagy,2010

Antibiotic resistance

Bacterial resistant to penicillin

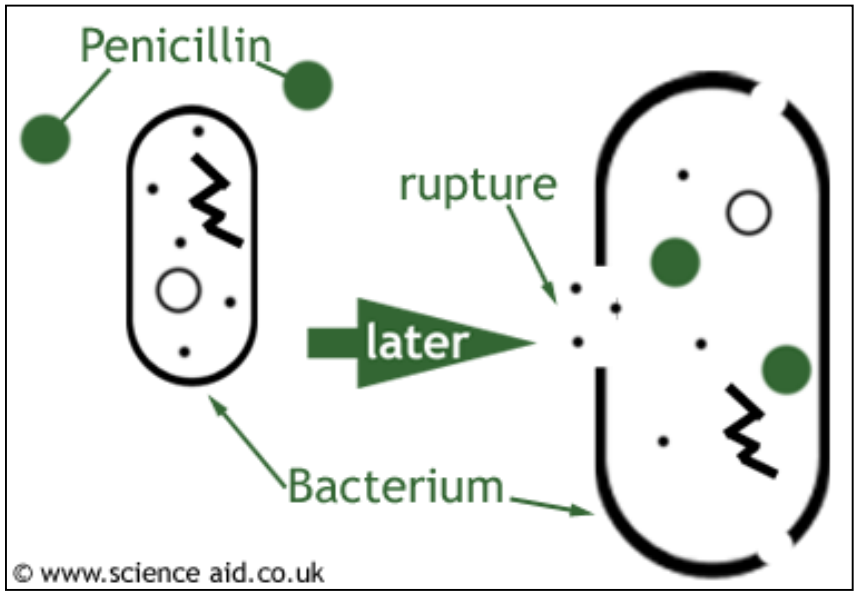
- Many older penicillins display little activity against Gram negative bacteria, since they do not penetrate the outer membrane.
- Cephalosporins and other newer penicillins are active against Gram negative bacteria, since they can penetrate the outer membrane.
- In the case of Gram negative bacteria, penicillins pass across the outer membrane using porins.
- Resistance may develop from mutation leading to modified porins.
- Penicillins can be destroyed by β-lactamase (penicillinase) produced by resistant G+ve bacterial strains.

Alvin Fox,2008

Penicillin

Penicillins inhibit bacterial cell wall synthesis, leading to bacterial lysis

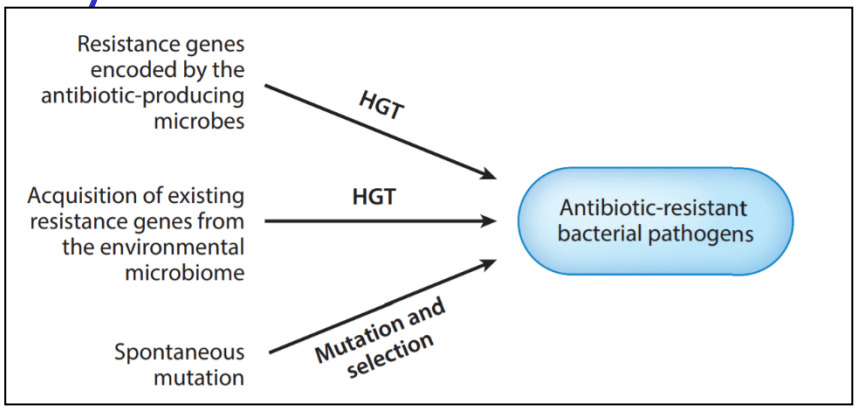
- Penicillin kills bacteria by inhibiting the proteins which cross-link peptidoglycans in the cell wall.
- When a bacterium divides in the presence of penicillin, it cannot fill in the "holes" left in its cell wall.



Antibiotic

Transfer of streptomycin resistance efficiently to different bacteria

- Common fluorescent epiphytic bacteria, which were not associated with disease, were found to transfer streptomycin resistance efficiently to the pathogen, *P. syringae* pv. *papulans*, in the laboratory, suggesting that these bacteria may provide a reservoir for streptomycin.



Schematic illustration of the origin of antibiotic-resistant bacterial pathogens. Abbreviation: HGT, horizontal gene transfer.

Sundin and Wang,2018;..

Antibiotic resistance

Emergence and spread of antibacterial-resistant bacteria

- The emergence and spread of antibacterial-resistant bacteria has continued to grow due to both:

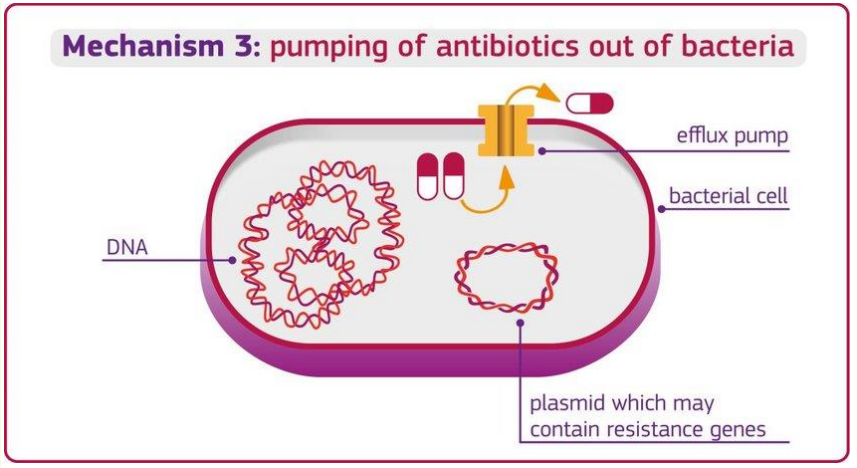
1. the over-use, and
2. misuse of antibiotics.

- Treating a patient with antibiotics causes the microbes to adapt or die; this is known as 'selective pressure'.
- If a strain of a bacterial species acquires resistance to an antibiotic, it will survive the treatment. As the bacterial cell with acquired resistance multiplies, this resistance is passed on to its offspring.
- In ideal conditions some bacterial cells can divide every 20 minutes; therefore after only 8 hours in excess of 16 million bacterial cells carrying resistance to that antibiotic could exist.

Antibiotic resistance

Emergence and spread of resistant bacteria

- For example resistance genes can be transferred:
  1. from one plasmid to another plasmid, or chromosome,
  2. resistance can occur due to a random spontaneous chromosomal mutation.
- **A mutation that arises naturally and not as a result of exposure to mutagens.**



Sanseverino *et al.*,2018;Microbiology Society,2021

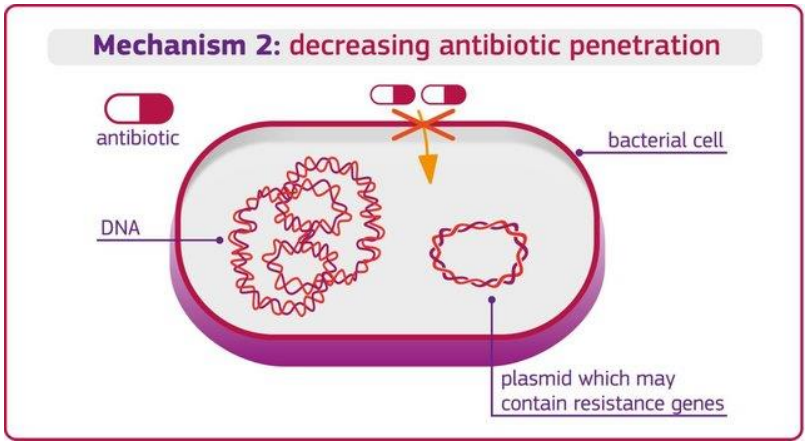
Spontaneous mutation
Stability of mutants

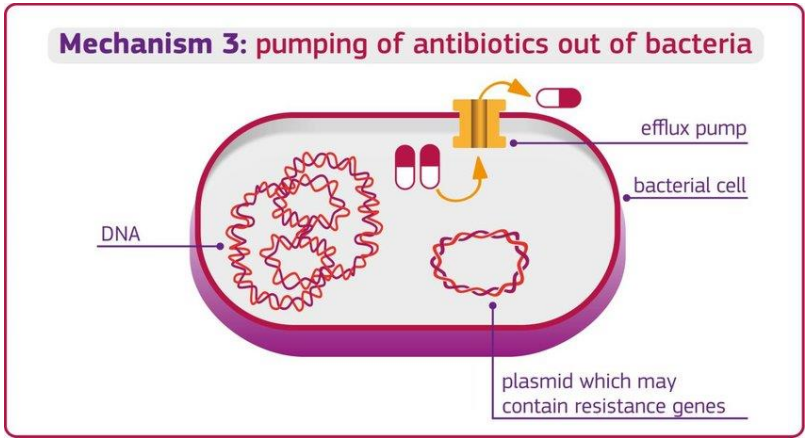
- Streptomycin resistant strain of *Acidovorax citrulli* 30290^{str} was a spontaneous mutant (Weller and Saettler, 1978) obtained by plating strain *Acidovorax citrulli* 30290 onto an ethanol bromcresol purple/brilliant blue R (EBB) agar (Schaad and Sechler, 1999) gradient plate containing 0 to 100 µg/ml streptomycin.
- The stability of strain 30290^{str} was confirmed by growing the bacterium for at least 30 generations in the absence of streptomycin.

Zaho *et al.*,2009

Mechanisms of antibiotic resistance

Decreasing antibiotic penetration or pumping of antibiotic out of bacterial cells





Bacteria can acquire or develop resistance to antibiotics by reducing the antibiotic intracellular concentration, or pumping of antibiotic out of bacterial cells.

Sanseverino *et al.*,2018

Antibiotic resistance

Emergence and spread of resistant bacteria to nontarget bacteria

- Resistance to streptomycin, which has been used since the late 1950s to control disease in fruit orchards, was detected in the pathogen *Ps. syringae* pv. *papulans* and a number of other Gram-negative bacteria present in apple (*Malus* sp.) orchards in the USA (Norelli *et al.*,1991).
- Common fluorescent epiphytic bacteria, which were not associated with disease, were found to transfer streptomycin resistance efficiently to the pathogen, *P. syringae* pv. *papulans*, in the laboratory, suggesting that these bacteria may provide a reservoir for streptomycin.

Antibiotic resistance

Resistance to streptomycin through plasmid or transposon

- Streptomycin resistance in *E. amylovora*, *P. syringae* and *X. campestris* pv. *vesicatoria* have shown that resistance is conferred by the *strAB* genes, which encode aminoglycoside phosphotransferase enzymes that modify streptomycin to a non-toxic form.
- Resistance to streptomycin in *Pseudomonas* bacteria is plasmid/transposon determined.
- The *strAB* genes are located on the transposon Tn5393.

Sundin,2016

Antibiotic resistance

Plasmid-borne resistance to bactericide genes

In Gram-negative phytopathogenic bacteria

IS	Size (bp)	IR (bp)	Family*	Pathogen	Comment
51	1311	26	IS3	*Ps. savastanoi* pv. *glycinea*	One copy downstream of the CMA cluster in p4180A
				Ps. savastanoi pv. *savastanoi*	Multiple copies; inactivates *iaaM*
52	1209	9/10	IS5	*Ps. savastanoi* pv. *savastanoi*	Few copies; inactivates *iaaM*
53	2568	27	IS*21*	*Ps. savastanoi* pv. *savastanoi*	In IS*51*
100	1053	20/28	IS*21*	*Ps. savastanoi* pv. *phaseolicola*	Flanking avirulence/virulence genes in the pathogenicity island of plasmid pAV511
476	1225	26	IS3	*X. campestris* pv. *vesicatoria*	Present in Cu^r strains
801	1517	0	IS*91*	*Ps. savastanoi* pv. *phaseolicola*	Race-specific
870	1074–1085	0	IS*630*	*Ps. savastanoi* pv. *glycinea*	Three copies of IS*801*-like sequences flank the coronatin cluster in p4180A
911	1250	25/36	IS3	*Pa. agglomerans*	Associated with Tn*10*; Tc^r
1133	1231	27	IS3	*Er. amylovora*	Associated with Tn*5393*; Sm^r
1327	812	17	IS6	*Er. herbicola* pv. *gypsophilae*	Related to IS*26* in *Proteus vulgaris*, only on pPATH
1416	1322	29	IS3	*B. glumae*	Four copies in *B. glumae*
1417	1335	17	IS3	*B. glumae*	Widespread but restricted to *B glumae* and related species
1418	865	15	IS5	*B. glumae*	Widespread but restricted to *B glumae* and related species
1419	1215	36	Novel	*B. glumae*	Widespread but restricted to *B glumae* and related species
6100	880	14	IS6	*X. campestris* pv. *vesicatoria*	Associated with Tn*5393*; Sm^r

* Based on data of Mahillon & Chandler (1998).

Vivian *et al.*,2001

Antibiotic resistance

Plasmid-borne resistance to bactericide genes

In Gram-negative phytopathogenic bacteria

Gene	Pathogen	Plasmid	Compound*	Reference
copA–D	*Ps. syringae* pv. *tomato*	pPT23D	Cu	Mellano & Cooksey (1988)
ND	*Ps. syringae* pv. *syringae*	pPSR12	Cu, As, Co	Kidambi *et al.* (1995)
strA strB	*Ps. syringae* pv. *syringae*	pPSR14	Cu, STR	Sundin *et al.* (1994)
strA strB	*X. campestris* pv. *vesicatoria*	ND (68 kb)	STR	Minsavage *et al.* (1990a)
tetB	Orchard epiphytes†	ND	TET	Schnabel & Jones (1999)
tmp	*Ps. savastanoi* pv. *glycinea*	pPg2	TMP	Leary & Trollinger (1985)

ND, Not designated.

* As, arsenate; Co, cobalt; Cu, copper; STR, streptomycin; TET, tetracycline; TMP, trimethoprim.

† Identified only as *Pa. agglomerans* (= *Er. herbicola*) with TetB or *Pseudomonas* spp. with TetA, TetC or TetG.

Vivian *et al.*,2001

Antibiotic resistance

Multiple mechanisms of resistance

Resistance to antibiotics and copper compounds

- Multiple mechanisms of resistance can occur in a single isolate, leading to higher levels of resistance.
- The nine strains of *Ps. syringae* pv. *syringae* isolated from pear, each harbouring:

1. six of the plasmids carried only Cu^r,
2. two carried Cu^r plus Sm^r, and
3. one carried only Sm^r.

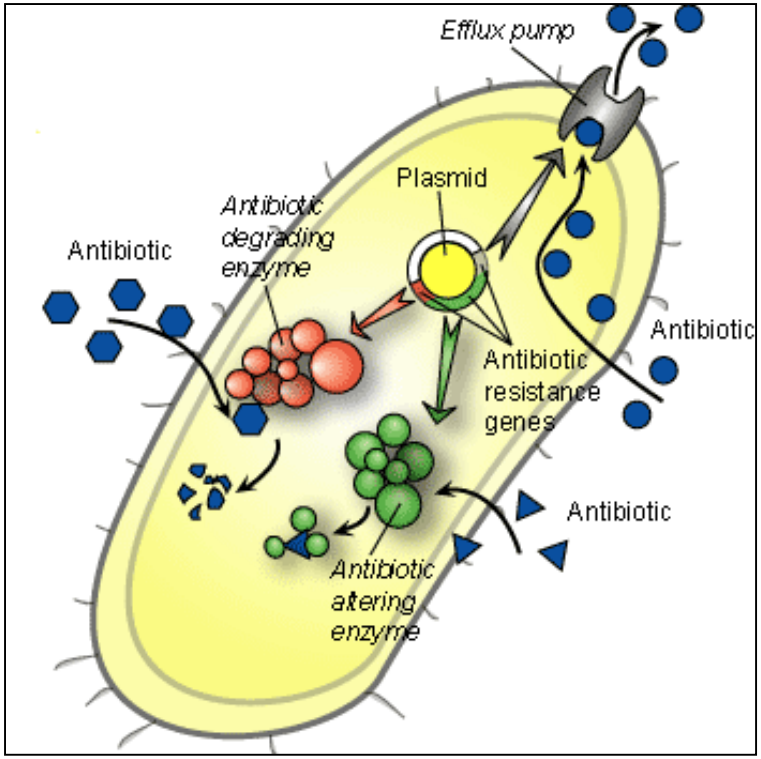
- The spread of resistance to copper and streptomycin may owe more to the agency of transposable elements rather than to plasmid transfer *per se.*

Antibiotic resistance

Mechanisms of antibiotic resistance

- Bacteria are able to exchange genes in nature by three processes:

1. conjugation,
2. transduction, and
3. transformation.

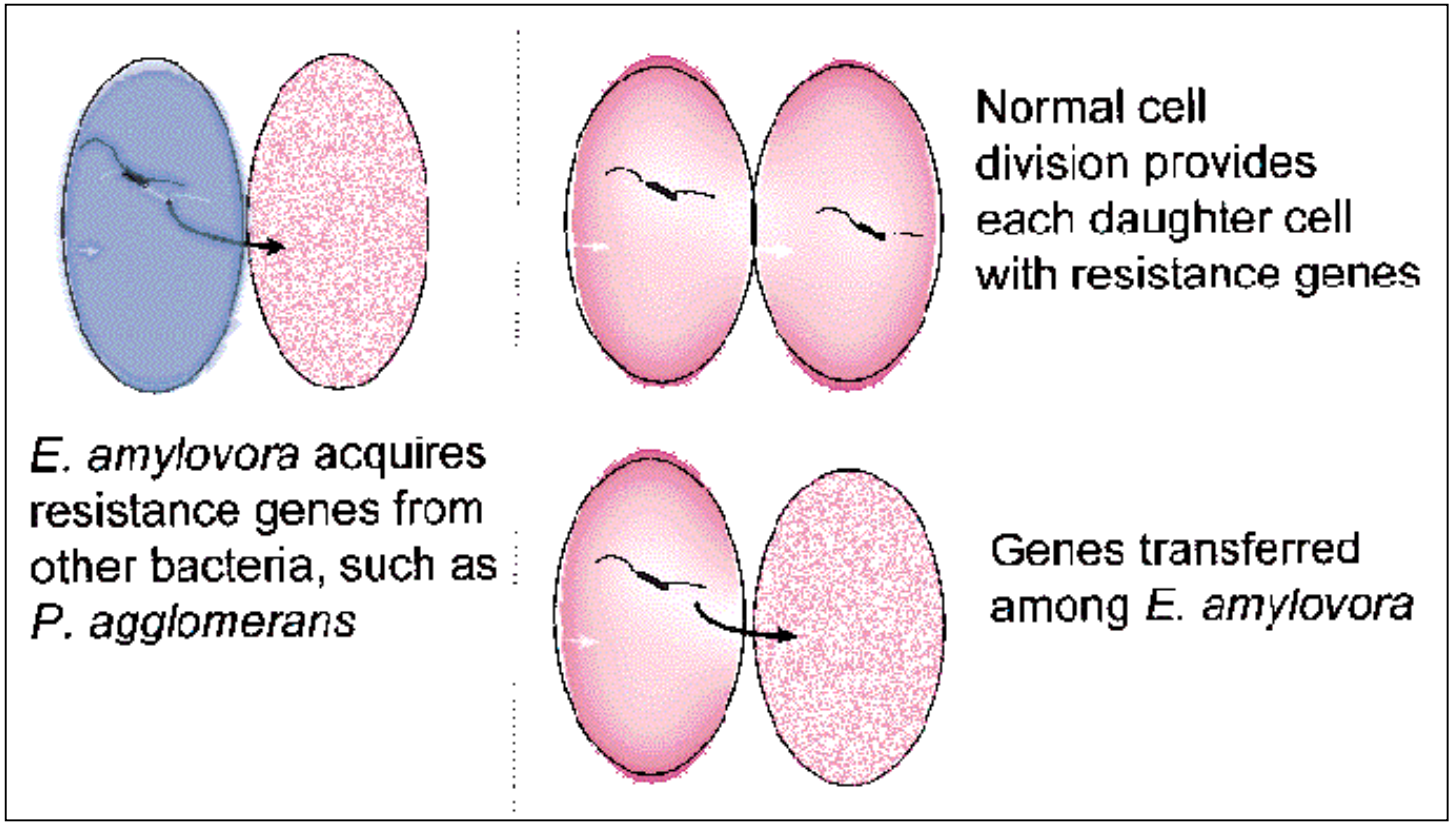


Transfer of streptomycin-resistance genes between the two species
Conjugation

- Nonpathogenic bacteria are being exploited for biological control of fire blight.
- *Pantoea agglomerans* (formerly *Erwinia herbicola*) occupies some of the same niches on apple as *E. amylovora*.
- This may provide an opportunity for transfer of streptomycin-resistance genes between the two species.
- The presence of an identical plasmid carrying *strA* and *strB* in *E. amylovora* and *P. agglomerans*, and conjugal transfer of this plasmid between these species *in vitro*, suggests that *P. agglomerans* might be a reservoir for, or an intermediary in the transfer of, resistance genes to *E. amylovora*.

Antibiotic resistance

Conjugative transfer of resistance



Patty McManus

Antibiotic resistance

Mechanisms of antibiotic resistance

Plasmid and chromosomal-borne resistance

1. Early studies on the genetics of copper and antibiotic resistance in plant pathogenic bacteria indicated that resistance evolved most often through acquisition of plasmid-borne resistance genes.
2. However, in *E. amylovora*, mutation of a chromosomal gene is the most common mechanism for streptomycin resistance.

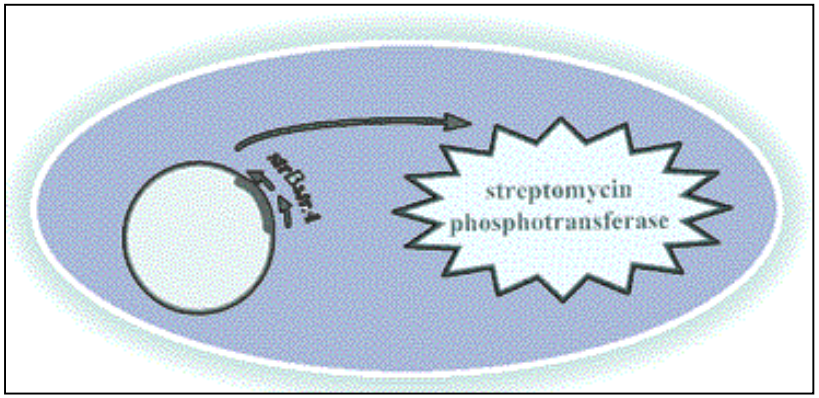
- In *E. amylovora*, the first instances of streptomycin resistance were conferred by a chromosomal mutation altering the ribosomal protein target of the antibiotic (Moller *et al.*,1981).

McManus *et al.*,2002; Sundin,2016

Molecular analyses of streptomycin-resistant *E. amylovora*

- The streptomycin-resistance genes *strA* and *strB* have been found in at least 16 different genera of clinical and environmental bacteria, often associated with:

1. transposons,
2. conjugative plasmids, or
3. both.



Patty McManus

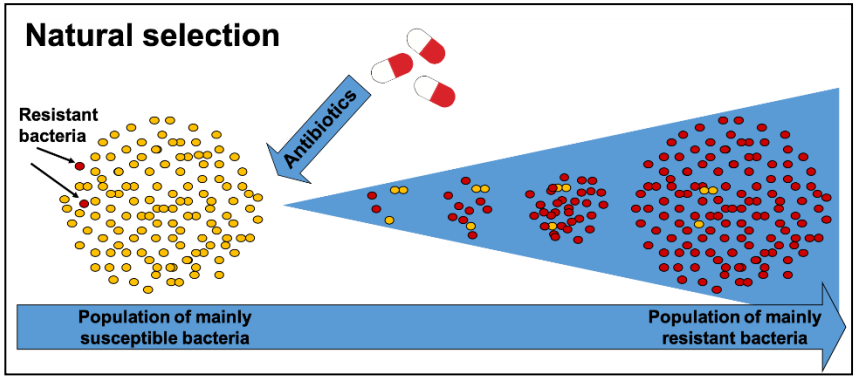
Distribution of fire blight resistance to streptomycin in the World

The stars indicate regions with Streptomycin-resistant *Erwinia amylovora*.

Patty McManus

Antibiotic resistance
Calculation of mutation rates

- If we were to treat the bacterial population with that specific antibiotic, only the resistant bacteria will be able to multiply; the antibiotic selects for them.
- These bacteria can now increase in numbers and the end result is a population of mainly resistant bacteria.



Antibiotic resistance
Calculation of mutation rates

- The mutation rate is the number of mutations per cell division. Because the cell population is so large, the number of cell divisions is approximately equal to the number of cells in the population (N).

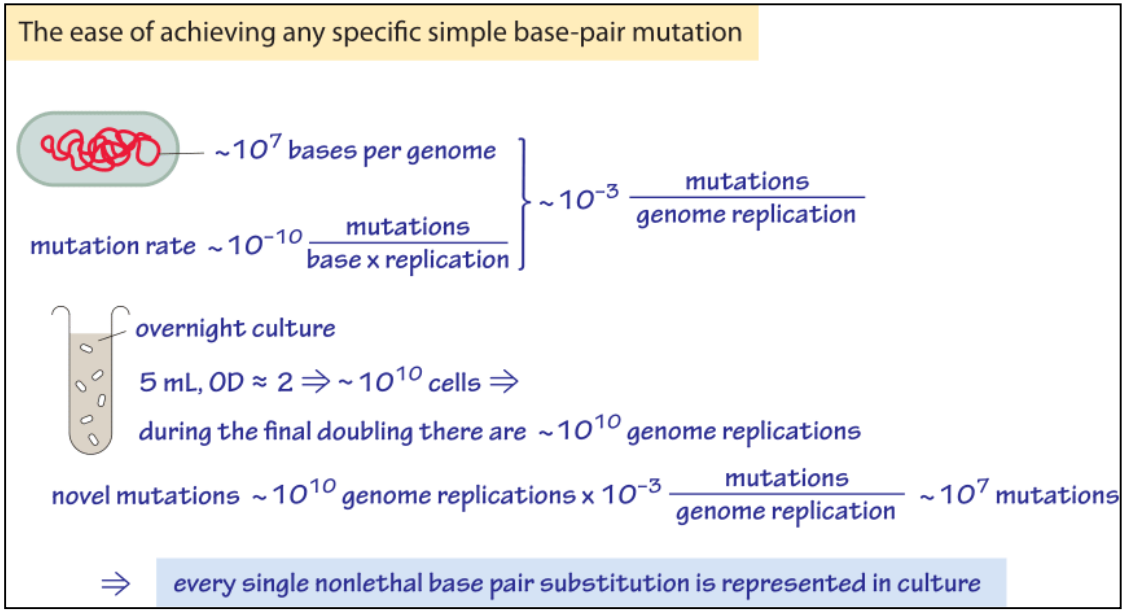
$$a = \frac{h}{N}$$

- In the example shown above, if h was determined from a fluctuation test with 10^7 cells per tube, then the mutation rate would be:

$$a = \frac{h}{N} = \frac{0.6 \text{ mutations / tube}}{10^7 \text{ cell divisions / tube}} = 6 \times 10^{-8} \text{ mutations / cell divisions}$$

Antibiotic resistance
Calculation of mutation rates

- Back of the envelope calculation of the mutations in an overnight culture of bacteria. One finds that every possible base pair change in explored.



Buckhaults,2007

Antibiotic resistance

Methods to determine antibiotic resistance

Antibiotic susceptibility testing

- The resistance to antibiotics was determined with the commercial antibiograms e.g. ATB UR 14030 and ATB ANA 14260 (BioMerieux).
- Antibiograms ATB ANA 14260 were incubated under aerobic conditions.
- Visual observations were made after 24 and 48 hours.

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC), and The minimum bactericidal concentration (MBC)

- What is the difference between MIC and MBC?
- MIC is defined as the lowest concentration of antimicrobial or drug that will inhibit the visible growth of bacteria after overnight incubation(Levison, 2004), while
- MBC is the lowest concentration of antibacterial agent required to kill a particular bacterium(Wiegand *et al.*,2008).

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC), and The minimum bactericidal concentration (MBC)

- The basic quantitative measures of the *in vitro* activity of antibiotics are:

1. The minimum inhibitory concentration (MIC) and
2. The minimum bactericidal concentration (MBC).

- The MIC is the lowest concentration of the antibiotic that results in inhibition of visible growth (i.e. colonies on a plate or turbidity in broth culture) under standard conditions.
- The MBC is the lowest concentration of the antibiotic that kills 99.9% of the original inoculum in a given time.

Buckhaults,2007

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC), and The minimum bactericidal concentration (MBC)

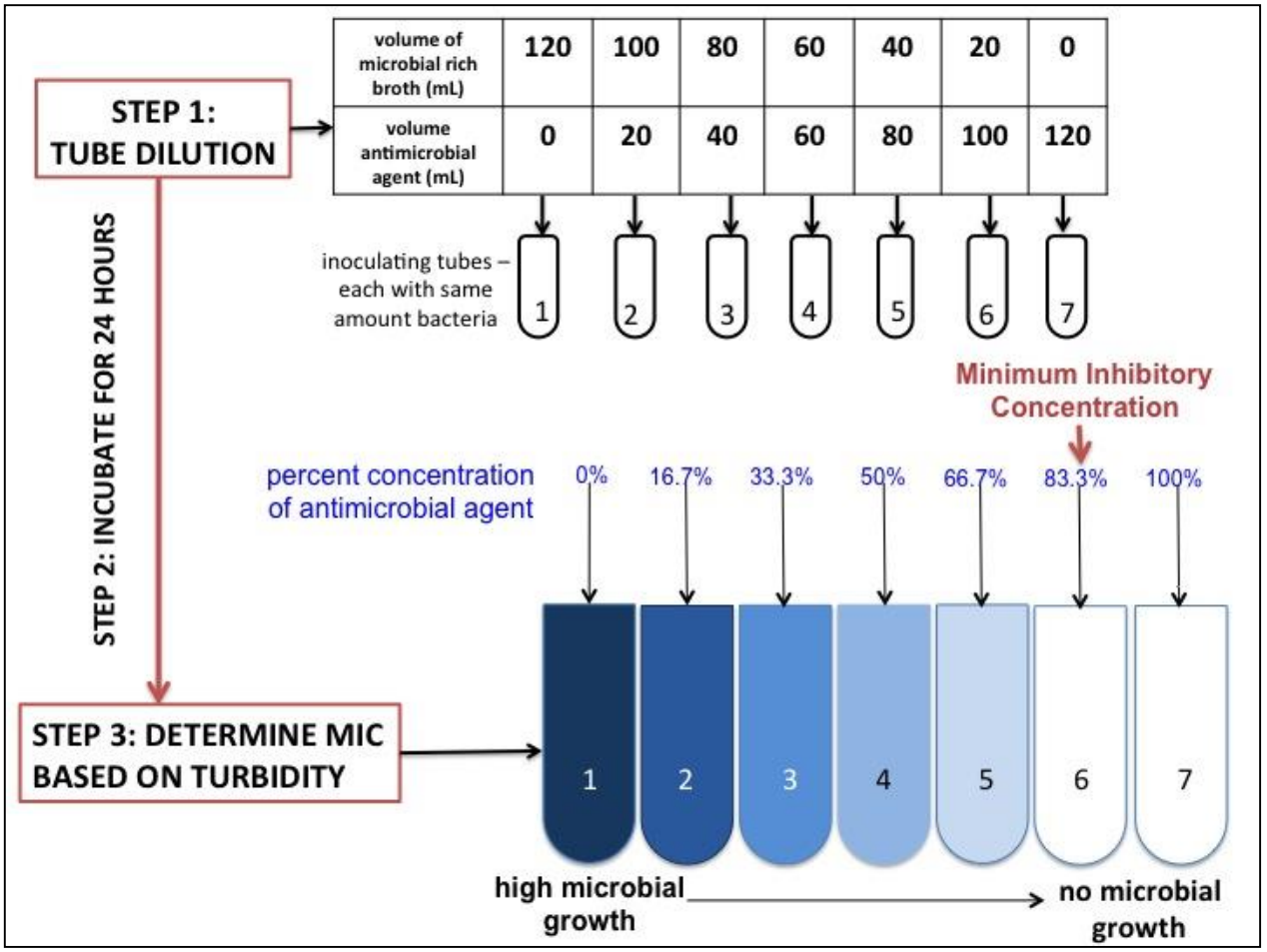
- The MIC and MBC were determined by the following methods:

1. The tube dilution test
2. Broth microdilution test
3. Disk diffusion test

Antibiotic susceptibility testing

MIC and MBC Assays

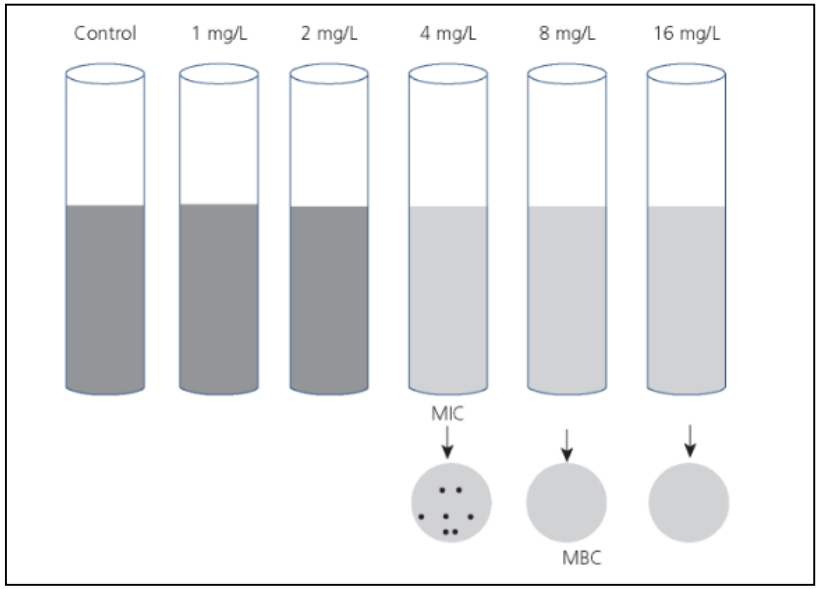
The tube dilution test



Wikipedia,2017

Antibiotic susceptibility testing
MIC and MBC Assays
The tube dilution test

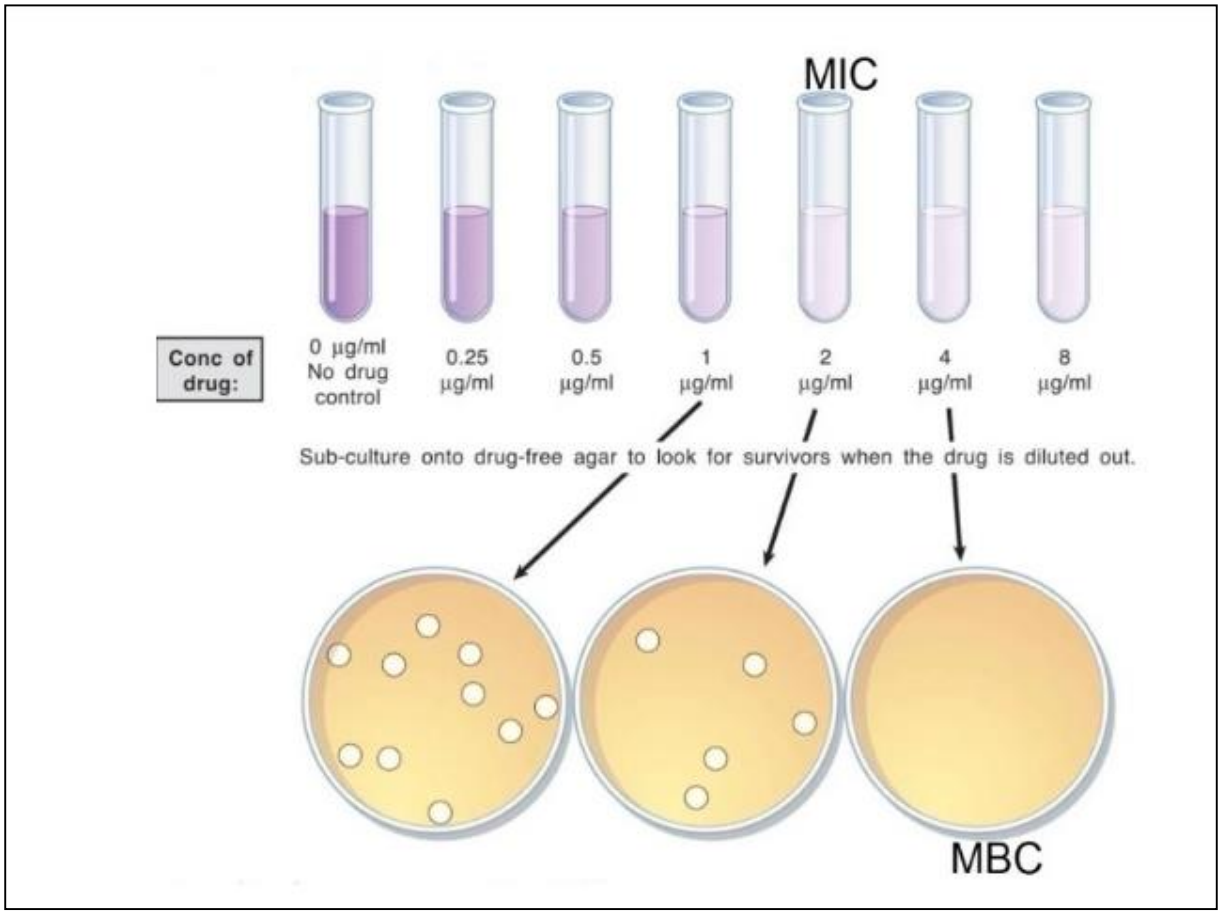
- Most effective plant extracts and showed bacteriostatic and bactericidal activities against *S. aureus* with MIC's ranged from 3.0 to 4.0 mg/ml and MBC of 8 mg/ml.
- *P. aeruginosa* was less sensitive and thus, its MBC reached to 12.5 mg/ml.



Antibiotic susceptibility testing

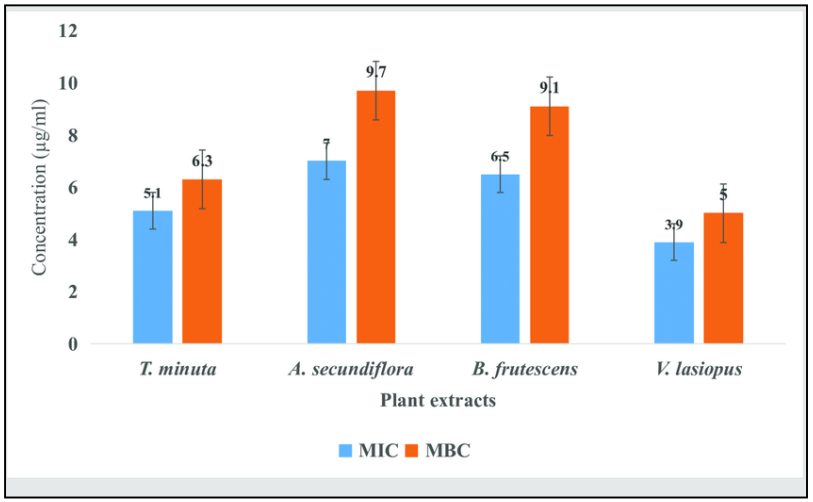
MIC and MBC Assays

The tube dilution test



Antibiotic susceptibility testing
MIC and MBC Assays
The tube dilution test

- Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of plant extracts against *Enterococcus faecalis*.
- Key: Error bars-represent standard error of mean (SEM).



Vernonia lasiopus was more active at low concentrations (MIC 3.9µg/ml; MBC 5.0µg/ml) while *Aloe secundiflora* was less active (MIC 7.0µg/ml; MBC 9.7µg/ml). Ciprofloxacin (5µg/ml) was used as a positive control producing an average zone of inhibition of 21.67±2.66mm while Methanol (33.50±2.56mm) and 4% Dimethyl sulphoxide (0.00±0.00mm) were used as negative control.

Opinde,2018

Antibiotic susceptibility testing
MIC and MBC Assays
Broth microdilution test

- To set up an MIC/MBC assay:

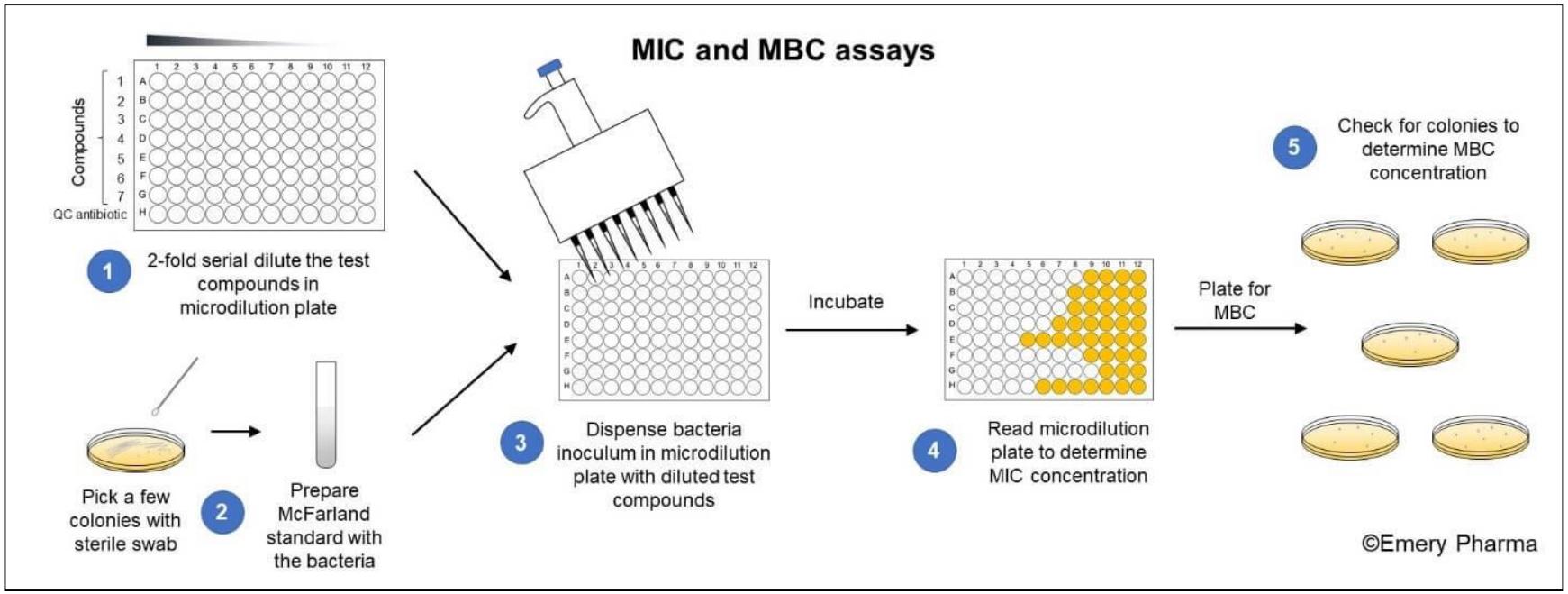
1. first prepare 2-fold serial dilutions of the test compounds (up to 7) and one quality control (QC) antibiotic in a microdilution plate.
2. Create the inoculum by taking a few colonies from an agar plate with a sterile swab, preparing a McFarland standard, and diluting the McFarland standard into media.
3. Dispense the inoculum into the microdilution plate with the serial diluted test compounds and incubate the microdilution plate.
4. Read the microdilution plate to determine the MIC value.
5. Plate a portion of each well on an appropriate agar media, incubate the agar, and check for colonies to determine the MBC.

Emery Pharma,2022

Antibiotic susceptibility testing

MIC and MBC Assays

To set up an MIC/MBC assay



Emery Pharma,2022

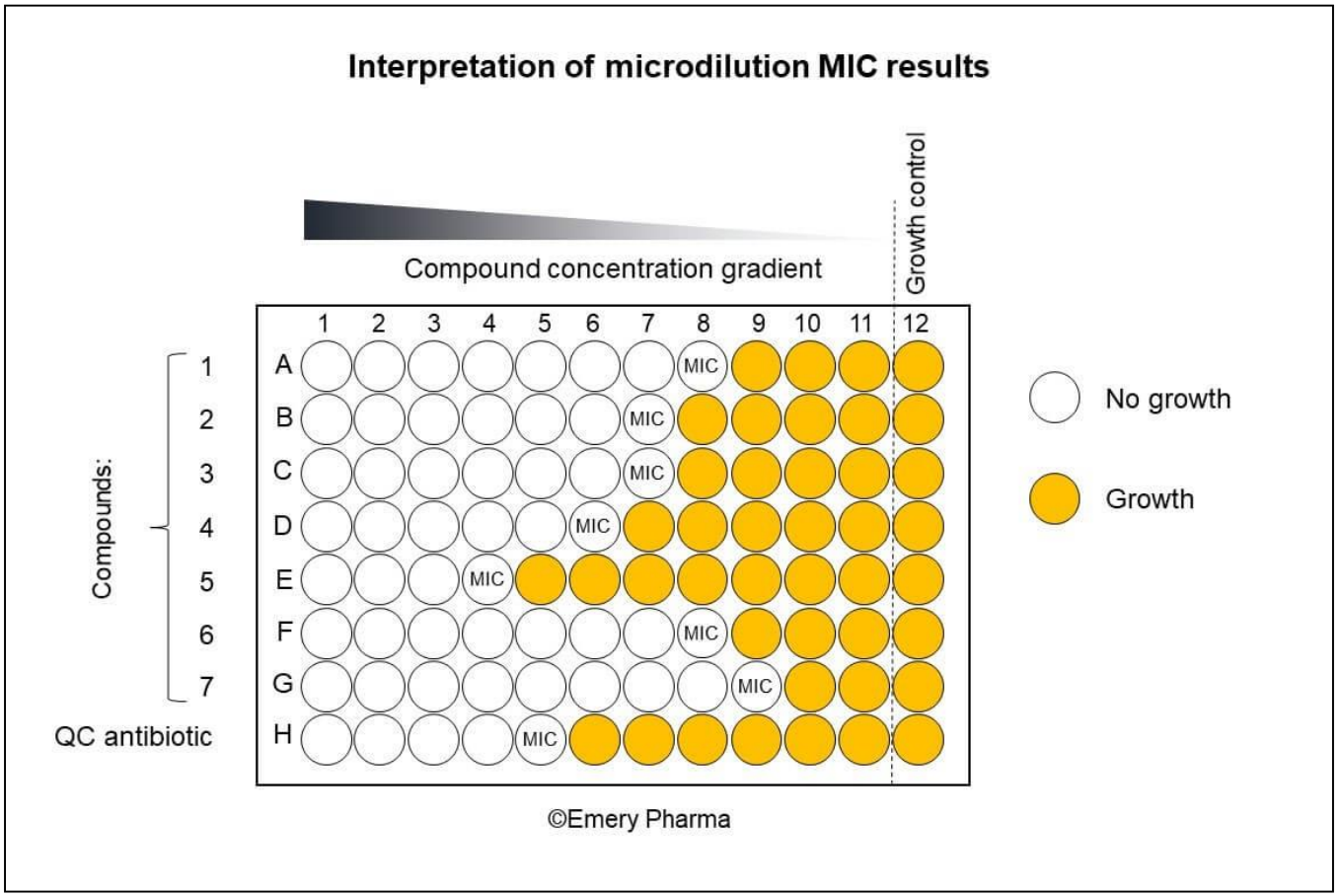
Antibiotic susceptibility testing
MIC and MBC Assays
Broth microdilution test

- Interpretation of Microdilution Results:
- Depicted here is a typical MIC assay conducted according to CLSI microdilution guidelines.
- Up to 7 compounds and one quality control (QC) antibiotic are serially diluted from column 1 to column 11 of a 96-well microplate to form a concentration gradient.
- Column 12 serves as a positive growth control.
- In the illustration, "no growth" is represented by white circles and "growth" is represented by yellow circles.
- The MIC value is the lowest concentration of a compound/antibiotic at which no growth is observed.

Antibiotic susceptibility testing

MIC and MBC Assays

Interpretation of Microdilution Results



Emery Pharma,2022

Antibiotic susceptibility testing

MIC and MBC Assays

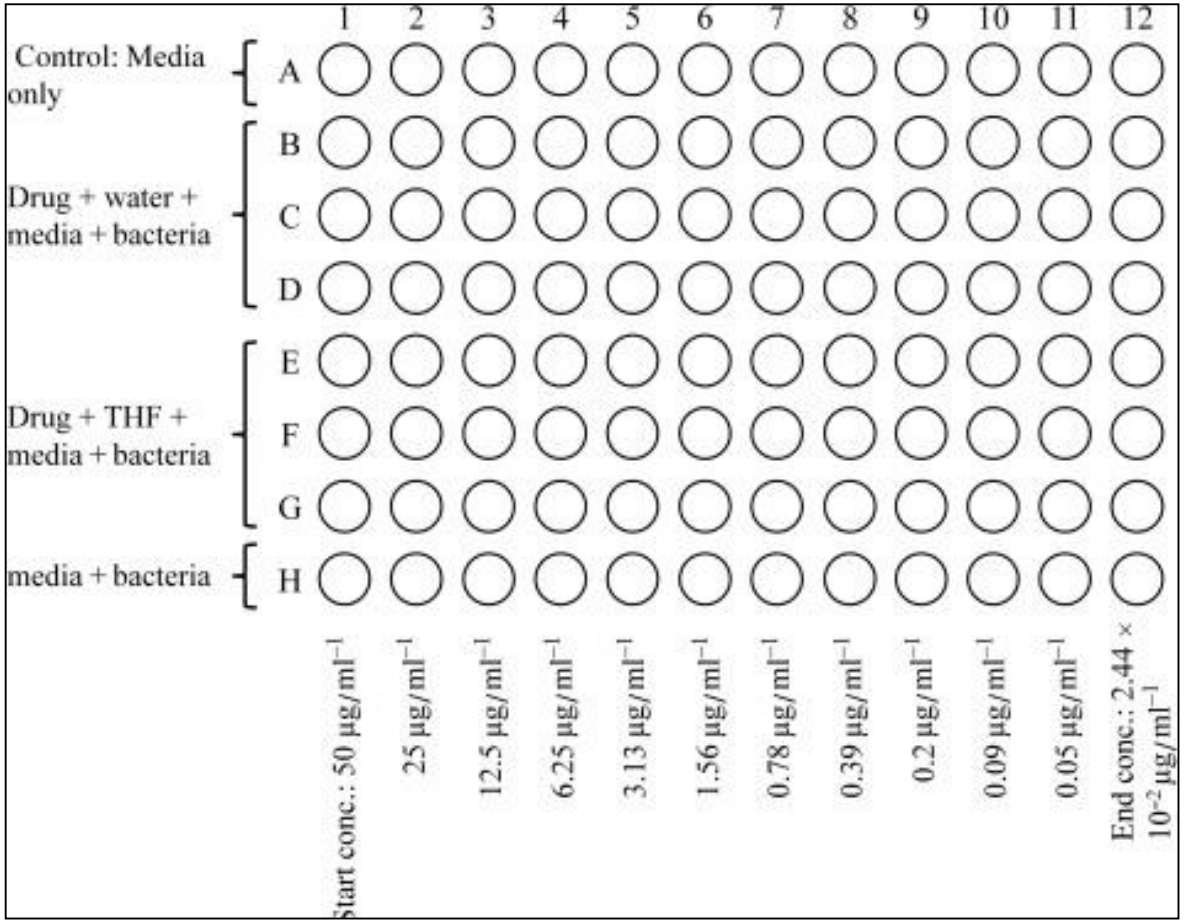
More information about dilution broth microdilution test

- Two-fold dilutions of antibiotics (e.g., 1 µg/mL, 2 µg/mL, 4 µg/mL etc.) are prepared and added to individual wells in disposable plastic microdilution trays containing a liquid bacterial growth medium.
- The wells are then inoculated with a bacterial suspension of a standardized cell density.
- Following incubation for 16 to 20 hours, the trays are examined for evidence of bacterial growth in the form of turbidity.
- The lowest concentration of antimicrobial which prevents visible growth represents the MIC, or minimum inhibitory concentration.
- These trays usually contain 96 wells, which allows 12 antibiotics to be tested in a range of eight two-fold dilutions in a single tray.

Antibiotic susceptibility testing

MIC and MBC Assays

More information about dilution broth microdilution test



Hassounah *et al.*,2016

Antibiotic susceptibility testing
MIC and MBC Assays
Disk diffusion test

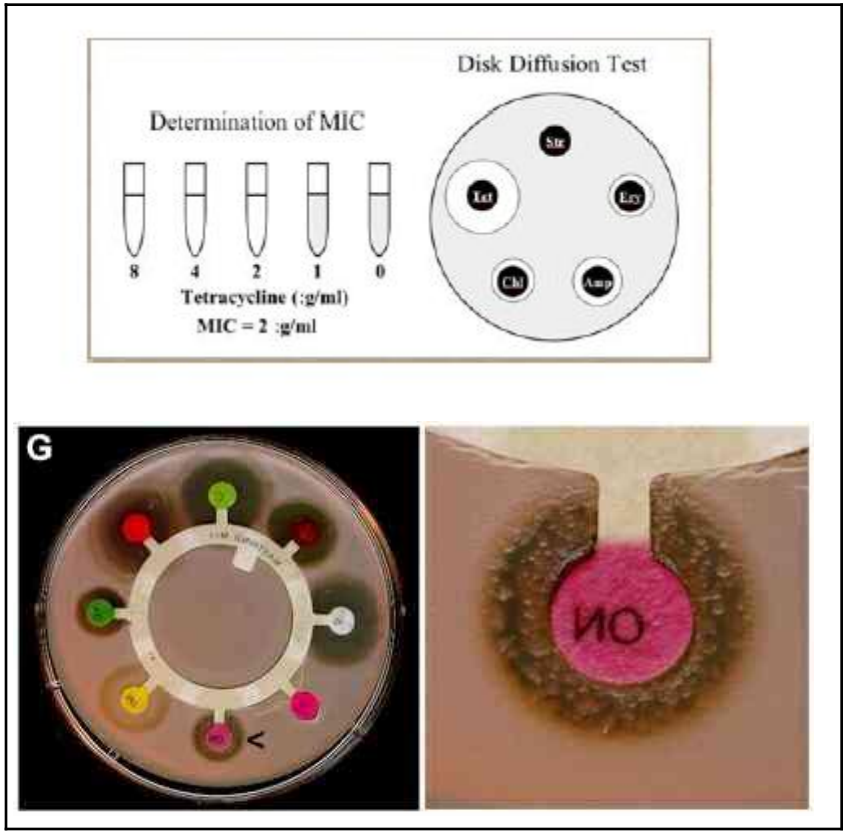
- The disk diffusion test is also known as the Kirby-Bauer procedure.
- A standardized inoculum is applied onto the entire surface of an agar medium in a large Petri plate. Uniform paper discs, each impregnated with a different antibiotic, or the same antibiotic in varying concentrations, are placed on the surface of the agar. The plates are then incubated for 16 to 18 hours.
- The antibiotic agent diffuses from the paper disk into the agar, thereby preventing the growth of the organism in a zone around the disc.
- The width of the zone is measured in millimeters and gives an indication of the sensitivity of the organism to the agent or agents being tested (Frobisher *et al*.,1974).

Antibiotic susceptibility testing

MIC and MBC Assays

Disk diffusion test

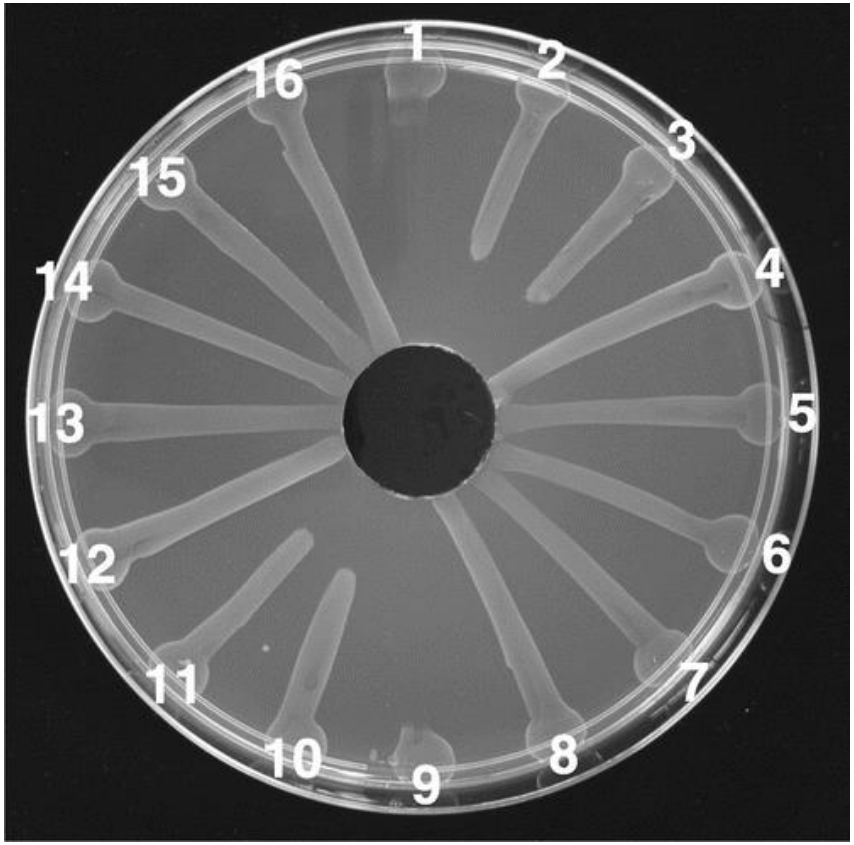
- Tests for sensitivity and resistance to antibiotics.
- The size of the zones of inhibition of microbial growth surrounding the antibiotic disks on the plate are an indication of microbial susceptibility to the antibiotic.



Disk diffusion test

Differences in streptomycin sensitivity of sensitive, moderately resistant-low (MR-L), moderately resistant-high (MR-H), and highly resistant (HR) strains of *Erwinia amylovora*.

- A concentration gradient from approximately 0.5 µg/ml (edge of plate) to 50 µg/ml (center of plate) streptomycin was established by applying a stock concentration of 10,000 µg/ml using a spiral plater.
- Eight strains are evaluated on a single plate with each strain duplicated in opposing streaks.
- Strains are as follows:
- 1 and 9, 529 (sensitive from California);
- 2 and 10, 538 (MR-L from California);
- 3 and 11, 547 (MR-L from California);
- 4 and 12, NW 1-1 (MR-H from Michigan); 5 and 13, NW 18-6 (MR-H from Michigan),
- 6 and 14, 552 (HR from California); and
- 7 and 15, 9-1 (HR from California), and S5 (HR from Michigan).



Förster *et al.*,2015

Median lethal dose
LD_{50} value

- LD stands for "Lethal Dose".
- LD_{50} is the amount of a material (e.g. fungicides), given all at once, which causes the death of 50% (one half) of a group of tested population.
- The LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a material.

Median lethal dose
LC_{50} value

- LC stands for "Lethal Concentration".
- LC values usually refer to the concentration of a chemical in air but in environmental studies it can also mean the concentration of a chemical in water.
- The concentrations of the chemical in air that kills 50% of the test animals during the observation period(traditionally 4 hours) is the LC_{50} value.
- Other durations of exposure (versus the traditional 4 hours) may apply depending on specific laws.

Minimum inhibitory concentrations MIC/ED50

- The most widely used measure of resistance is the median effective dose (ED50), that which kills half the sample being tested.
- Another is the minimum inhibitory concentration (MIC), the lowest dose sufficient to kill the pathogen.
- The MIC might appear at first sight to be the easier to estimate, as it is simply an empirical observation of the lowest dose that kills all individuals.
- However, the estimated MIC is positively correlated with the inoculation density.
- The MIC observed at the higher dose is therefore likely to be higher than that at the lower dose.

Effective doses (EDs)
ED50/ED95 values

- The aim of applying a fungicide to a crop is to achieve near-complete disease control.
- It is therefore often useful to estimate effective doses (EDs) higher than the ED50 such as the ED95, which kills 95% of the target pathogen.

Effective doses (EDs)
EC50-EC90

- It is preferable, where possible, to express potency in terms of EC50 but ED50 is appropriate for *in vivo* measurements and for those *in vitro* experiments where the absolute concentration is uncertain.
- **EC50-EC90:**
- The concentrations at which 50 or 90 per cent of a population reacts.
- The concentration at which the reaction of an organism is 50 per cent or 90 per cent less than the control value.

Monitoring methods

Use of EC values for monitoring studies

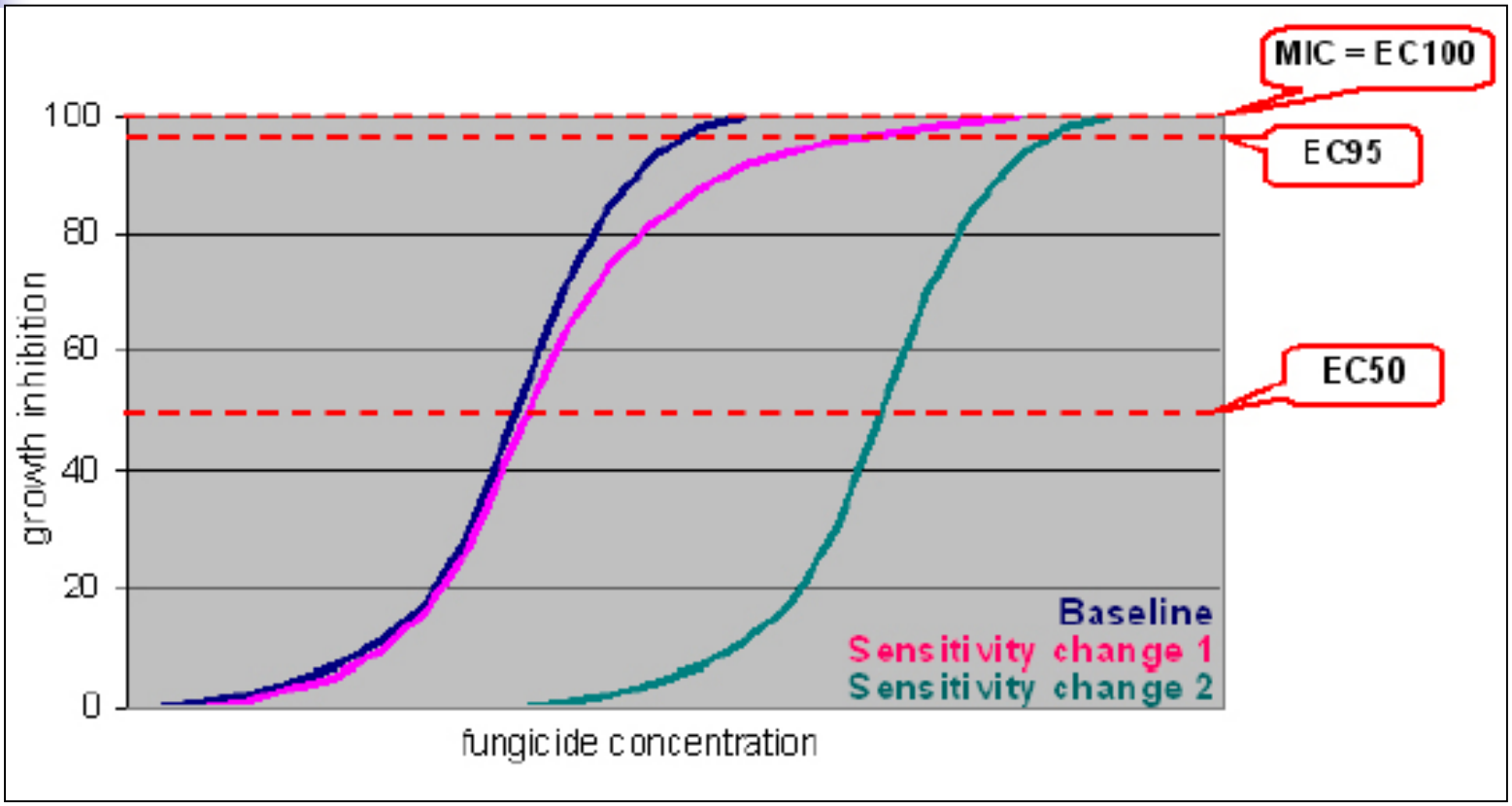
MIC= EC100

- MIC= EC100, i.e. the concentration at which 100% of a fully sensitive population is inhibited.
- Modes of action with shifting type of resistance (Annex graph: Types of resistance) need to be tested minimum at 2, but preferably at 4 to 5 different concentrations.
- In these cases the concentration range should include the EC50 and EC95 values.

Banana working group, Fungicide resistance action committee,2006

Use of EC values for monitoring studies

Sensitivity distribution curves and different EC values



Use of different EC values for the detection of different types of resistance

Observed sensitivity change	Monitoring parameter		
	EC50	EC95	MIC = EC100
Sensitivity change 1 "Shifting" e.g. triazoles	×	✓	✓
Sensitivity change 2 "Disruptive resistance" e.g. QoIs, BCMs	✓	✓	✓

✓ Monitoring parameter suitable for detection of certain sensitivity change
× Monitoring parameter not suitable for detection of certain sensitivity change

Summary for the use of different EC values according to the observed or expected changes in population sensitivity:

EC 50	Stable, less data variability	Shifting type of resistance
EC 95	Sensitive, small changes detectable; Risk: false positive possible	
MIC or EC100	Small changes difficult to detect	Disruptive type of resistance

Antibiotic resistance

How to prevent antibiotic resistance

- Antibiotic resistance occurs when bacteria develop defenses against the antibiotics designed to kill them.
- This renders the drugs useless against the new resistant strains, allowing resistance to grow and spread to other germs, creating drug-resistant infections that can be difficult to treat.

Centers for Disease Control and Prevention,2020

Antibiotic resistance

How to prevent antibiotic resistance

1. Learn the right ways to use antibiotics

- Antibiotic resistance occurs when bacteria develop defenses against the antibiotics designed to kill them.
- This renders the drugs useless against the new resistant strains, allowing resistance to grow and spread to other germs, creating drug-resistant infections that can be difficult to treat.

Centers for Disease Control and Prevention,2020

Antibiotic resistance

How to prevent antibiotic resistance

1. Learn the right ways to use antibiotics

- Not all infections need antibiotics.
- Otherwise, misuse and overuse of these drugs, however, have contributed to a phenomenon known as antibiotic resistance.

Centers for Disease Control and Prevention,2020

Antibiotic resistance

2. Combination antibiotic therapy

- Antibiotic should be mixed and rotated with different modes of action.
- The combined use of streptomycin and oxytetracycline on plants accounts for about only 0.1% of all antibiotics produced annually in the United States.
- Nearly all of this is applied to fruit trees (apples and pears) for management of fire blight (*Erwinia amylovora*).
- The combination of streptomycin and copper can increase efficacy and may reduce some of the risk in selecting for antibiotic resistant bacteria.

Antibiotic resistance

3. Use nanoantibiotics

- The metal and metal oxide-based nanoparticles and antibiotics, due to less toxicity and enhanced antibacterial, antiviral and anticancer efficacy, are regarded as promising therapeutic candidates for future applications in biomedical sciences.
- Their size provides them with unique properties such as an increased surface area to volume ratio, which makes them efficient drug carriers and enhance their solubility, compatibility as well as ease of delivery.
- Due to these diverse mechanisms of action, nano-antibiotics are likely to be effective against antibiotic resistant bacteria.

Kumar *et al.*,2021

Antibiotic resistance

4. Insect antimicrobial peptides used against antibiotic-resistant isolates

- Pyrrhocoricin is a antibacterial peptide originally isolated from the European sap-sucking bug *Pyrrhocoris apterus*, is non-toxic to eukaryotic cells and healthy mice, has good *in vitro* activity against model bacterial strains.

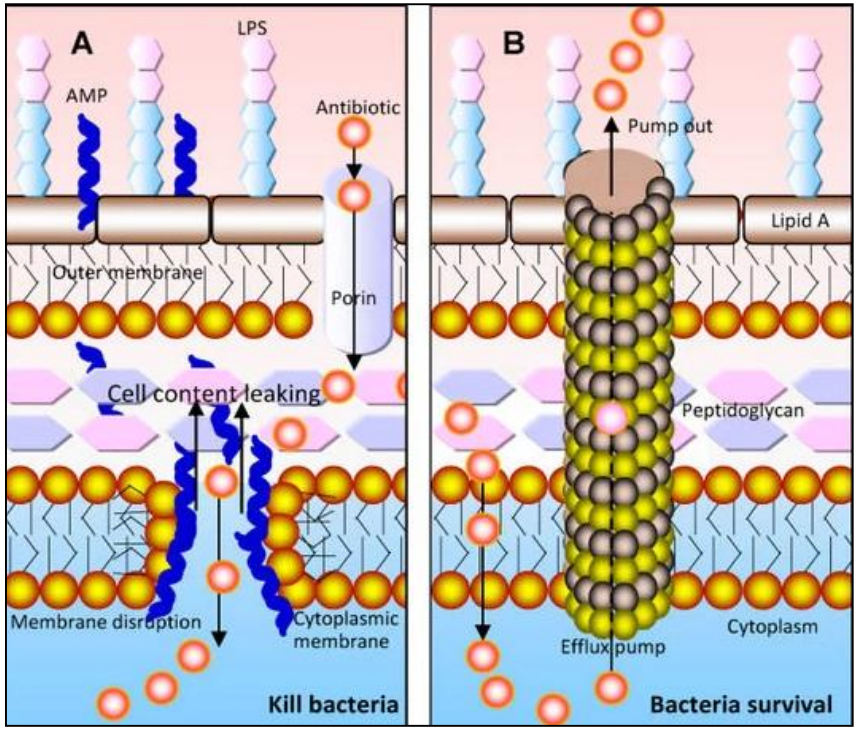
Pyrrhocoricin:	H-Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn-NH_2
Chex-pyrr-DapAc:	H-Chex-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Dap(Ac)-NH_2
unprotected dimer:	H-Chex-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Dap | H-Chex-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Dap(Ac)-NH_2
N-MeArg dimer:	(H-Chex-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab-NH_2
Pip-dimer:	(H-Pip-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab-NH_2
Pyrr-tetramer:	(H-Chex-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn)$_4$-Lys$_3$βAla-OH

Antibiotic resistance

5. Combined effects of antimicrobial peptides (AMPs) and antibiotics on bacteria

A. AMPs can disrupt the bacterial membrane to cause the leakage of the cell content into the extracellular medium and kill the bacteria. The AMPs can facilitate more antibiotics to enter the cytoplasm of bacteria and finally interact with their target.

B. In bacterial cells, antibiotics are pumped out of the cells by the multidrug efflux pumps, which is how bacteria exert their resistance properties.



Wu *et al.*,2018

Copper resistance

In plant pathogenic bacteria

Copper resistance

Copper resistance in plant pathogenic bacteria

- Copper bactericides:

1. Not highly effective, issues with copper resistance;
2. Can be phytotoxic to plant;
3. Long-term copper buildup in soils.

- The extensive use of copper and antibiotic sprays over multiple years and/or the use of high numbers of applications within individual seasons is correlated with the selection of resistance in pathogen populations.

Sundin,2016

Copper resistance

Copper resistance in plant pathogenic bacteria

- Copper-based fungicides such as Bordeaux mixtures are used extensively to control bacterial pathogens on fruit trees such as *P. syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum* on stone fruit trees.
- The use of copper, however, has several disadvantages:

1. Phytotoxicity can occur and resistance to copper develops rapidly in bacteria.
2. Copper accumulation in the soil.

Höfte & De Vos,2006

Copper resistance

Copper resistance in plant pathogenic bacteria

- Copper-based fungicides such as Bordeaux mixtures are used extensively to control bacterial pathogens on fruit trees such as *P. syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum* on stone fruit trees.
- The use of copper, however, has several disadvantages:

1. Phytotoxicity can occur and resistance to copper develops rapidly in bacteria.
2. Copper accumulation in the soil.

Höfte & De Vos,2006

Copper resistance

Bacterial resistant to copper compounds

- Bordeaux mixture, primarily a fungicide based on copper plus lime has been used to control plant disease in crops for over 100 years.
- Plasmid-borne resistance to copper has been found in several phytopathogenic bacteria, including:

1. *X. campestris* pv. *vesicatoria* pathogenic on pepper (*Capsicum annuum*),
2. in *Ps. syringae* pv. *syringae* pathogenic to ornamental fruit trees [mainly pear, and
3. *P. syringae* pv. *tomato*, a pathogen of crucifers and tomato.
4. *Xanthomonas juglandis* on walnut.
5. Xanthomonas citri, causal of citrus canker disease, and
6. *Xanthomonas alfalfae* subsp. *citrumelonis*, citrus bacterial spot and is more aggressive on trifoliate citrus and its hybrids.

Copper resistance

Copper resistance in plant pathogenic bacteria

- Both plasmid-borne resistance and chromosomal resistance were observed.
- Hwang *et al.*,2005 have recently shown that most *P. syringae* strains are copper resistant.
- Copper resistance genes, including the:

1. *copABCD* operon, and a
2. *copRS* two-component regulatory system are present in the genome of *P. syringae* pv. *syringae* B728a (Feil *et al.*,2005).

- These proteins appear to be 92-96% identical to plasmid-encoded CopABCDRS proteins found in other strains of *P. syringae*.

Höfte & De Vos,2006

Copper resistance

Bacterial resistant to copper compounds

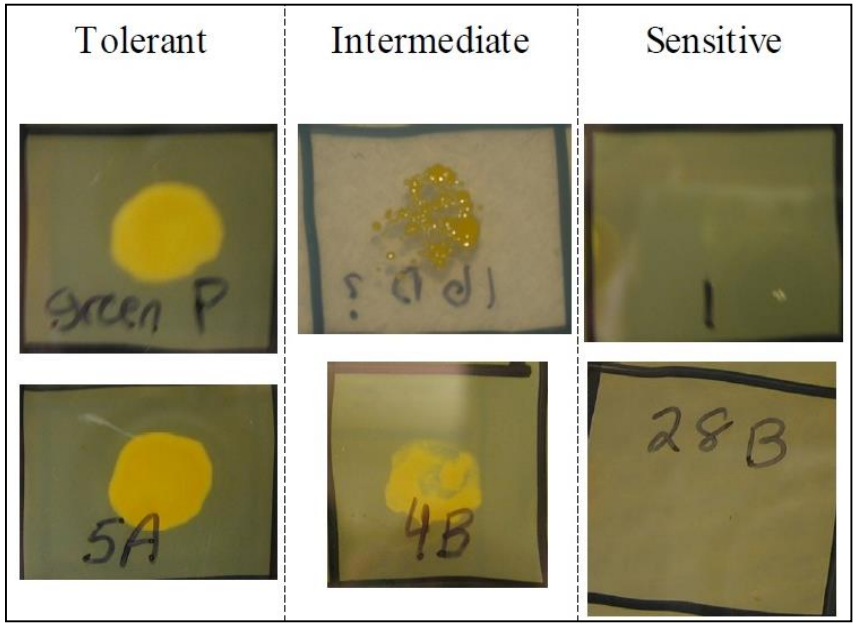
- The genes specifying resistance to copper appear to be widely conserved among the two genera and are generally located on large plasmids, with the exception of a walnut (*Juglans regia*) pathogen, *X. a.* pv. *juglandis*, in which they are chromosomal.

Copper resistance

Copper tolerance groupings of *Xanthomonas axonopodis* pv. *vesicatoria* strains using a copper amended agar plate assay

- Images demonstrate tolerance groups (tolerant, intermediate, and sensitive).

1. The tolerant group was defined by full, confluent growth after 2 days.
2. Intermediate strains were defined by having uneven, variable growth after 2 days.
3. The sensitive group comprised strains that had no observable growth after 2 days.



Garton *et al.*,2009

Dual resistance

Resistance to copper and antibiotics

- Copper resistance is often linked to streptomycin resistance and dual resistance to these bactericides was detected on conjugative plasmids in *P. syringae* pv. *syringae*, ranging in size from 68 to 220 kb.
- One such determinant is the Sm^r transposon, Tn5393, first identified in *E. amylovora* and subsequently identified as the basis of streptomycin resistance seen in *P. syringae* and *X. campestris*.
- Insertion sequences, IS1133 in *E. amylovora* and IS6100 in *X. campestris*, increase the expression of the resistance genes, leading to higher resistance among the bacteria.

Biological Control

Abbreviated synonym "biocontrol"

BACs

Biological Control Agents

Biological Control
Definition

- Biological control is defined broadly as the "use of natural or modified organisms, genes, or gene products" to reduce the effects of pests and diseases.

Biological Control
Prospects

- Biological control is considered as a promising alternative to pesticide and plant resistance to manage plant diseases.
- The introduction of biological control agents (BCAs) alters:

1. the interaction among plants, pathogens, and environments, leading to
2. biological and physical cascades that influence pathogen fitness, plant health, and ecological function.

He *et al.*,2021

Biological Control
Prospects

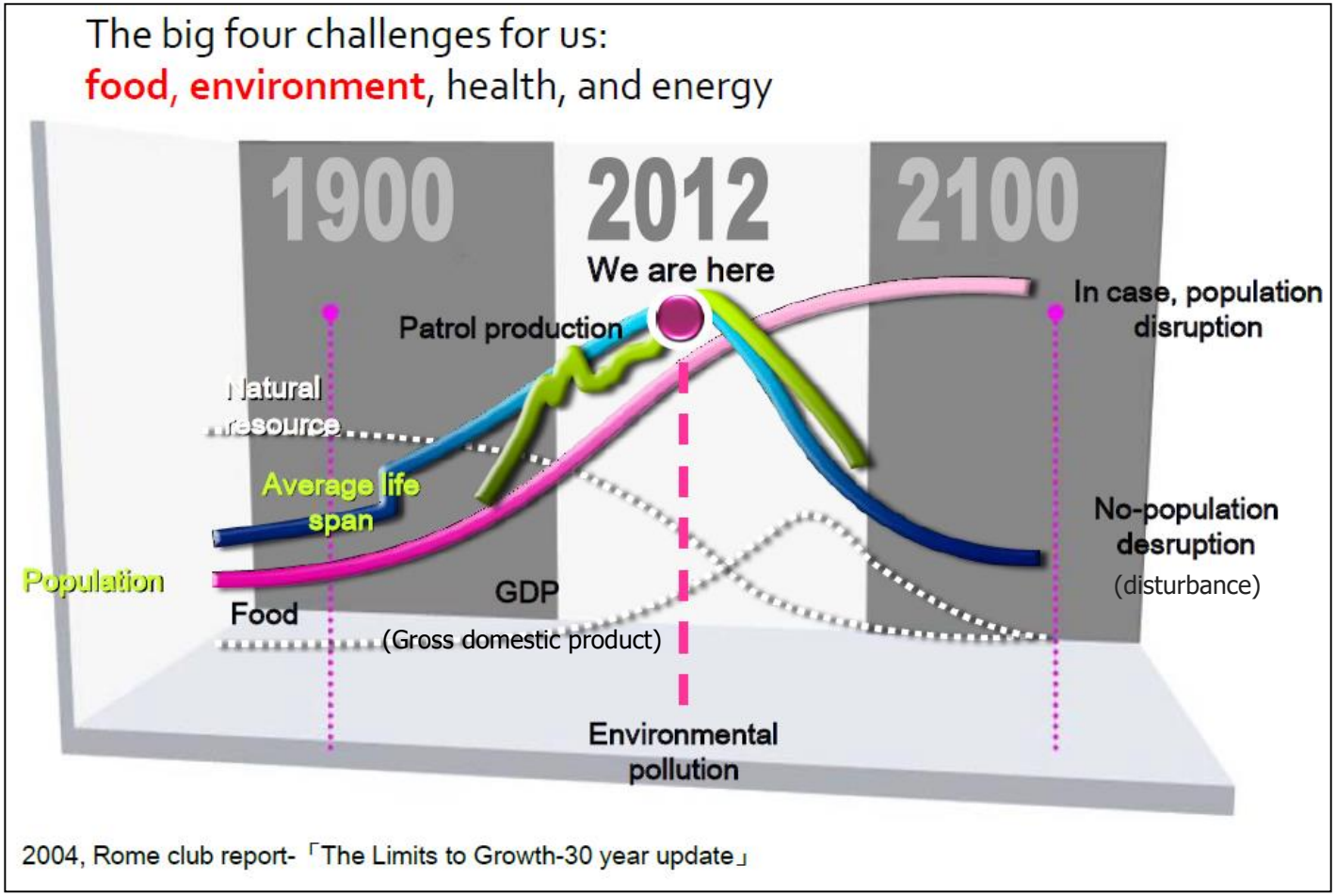
- Attempts should make to develop "green" BCAs used dynamically and synthetically with other disease control approaches in an integrated disease management scheme, and evolutionary biologists should play an increasing role in formulating the strategies.
- Governments and the public should also play a role in the development and implementation of biological control strategies supporting positive externality.

He *et al.*,2021

Biological control

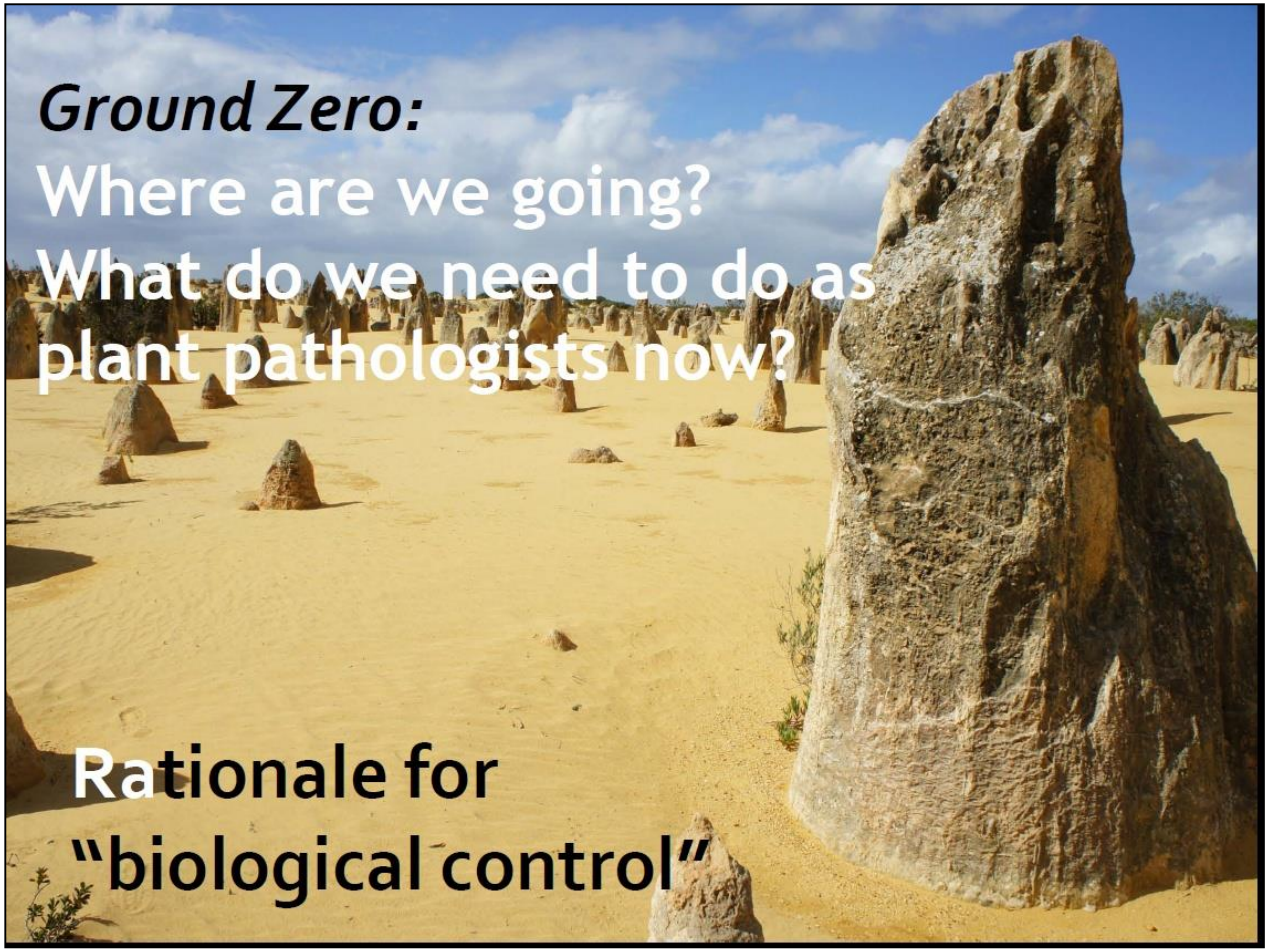
The global challenge

Food, environment, health and energy



Biological control of plant pathogens

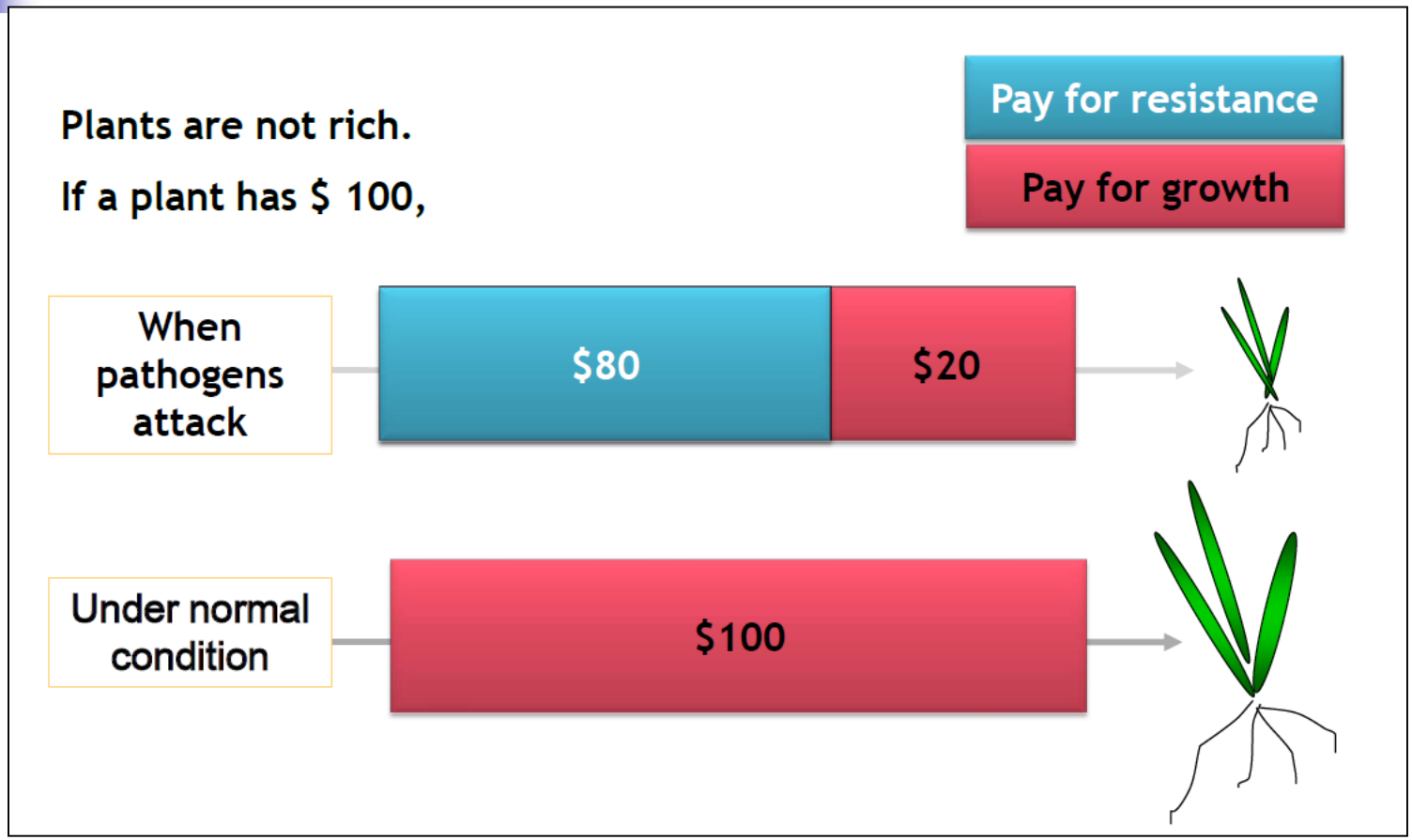
Where are we going? What do we need to do as plant pathologists now?



Choong-Min Ryu, Geun-Cheol Song

Biological control of plant pathogens

The concept of "allocation fitness cost"



Microbial biological control agents against plant diseases

Direct or indirect modes of action

- Microbial biological control agents (MBCAs) are applied to crops for biological control of plant pathogens where they act via a range of modes of action.
- Indirect interaction with the pathogen:

1. induce resistance or prime enhanced resistance against infections by a pathogen;
2. competition for nutrients and space.

- With direct interaction with the pathogen:

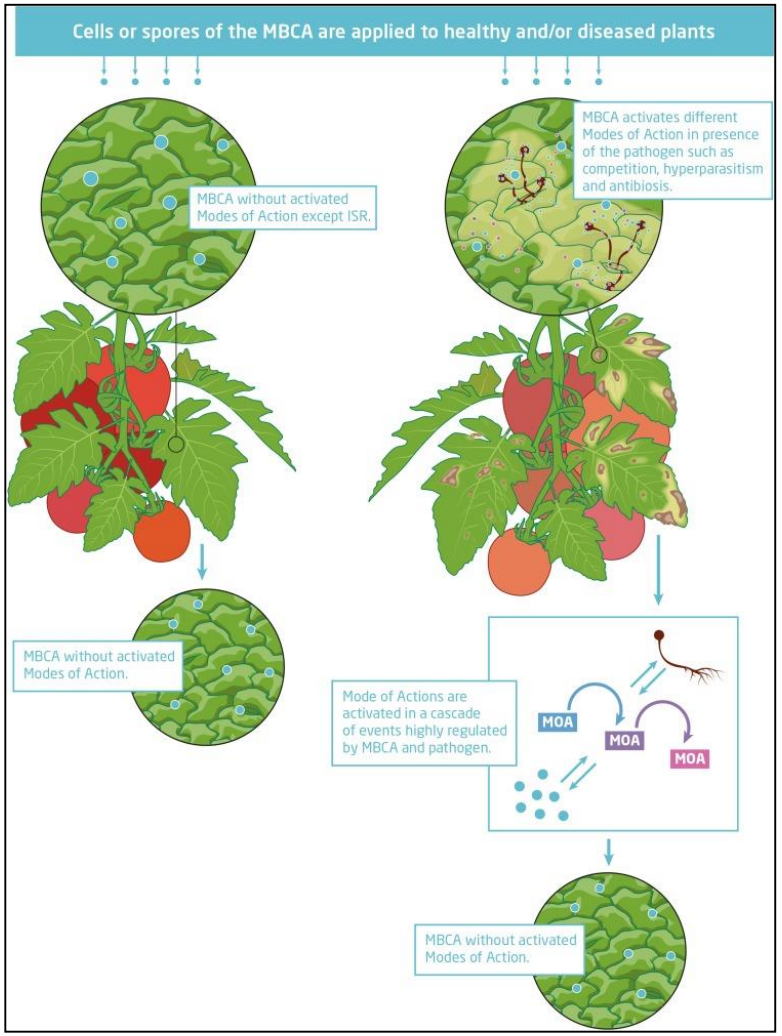
1. by hyperparasitism or antibiosis.
2. Production of antimicrobial secondary metabolites.

Köhl *et al.*,2019

Microbial biological control agents against plant diseases

Direct or indirect modes of action

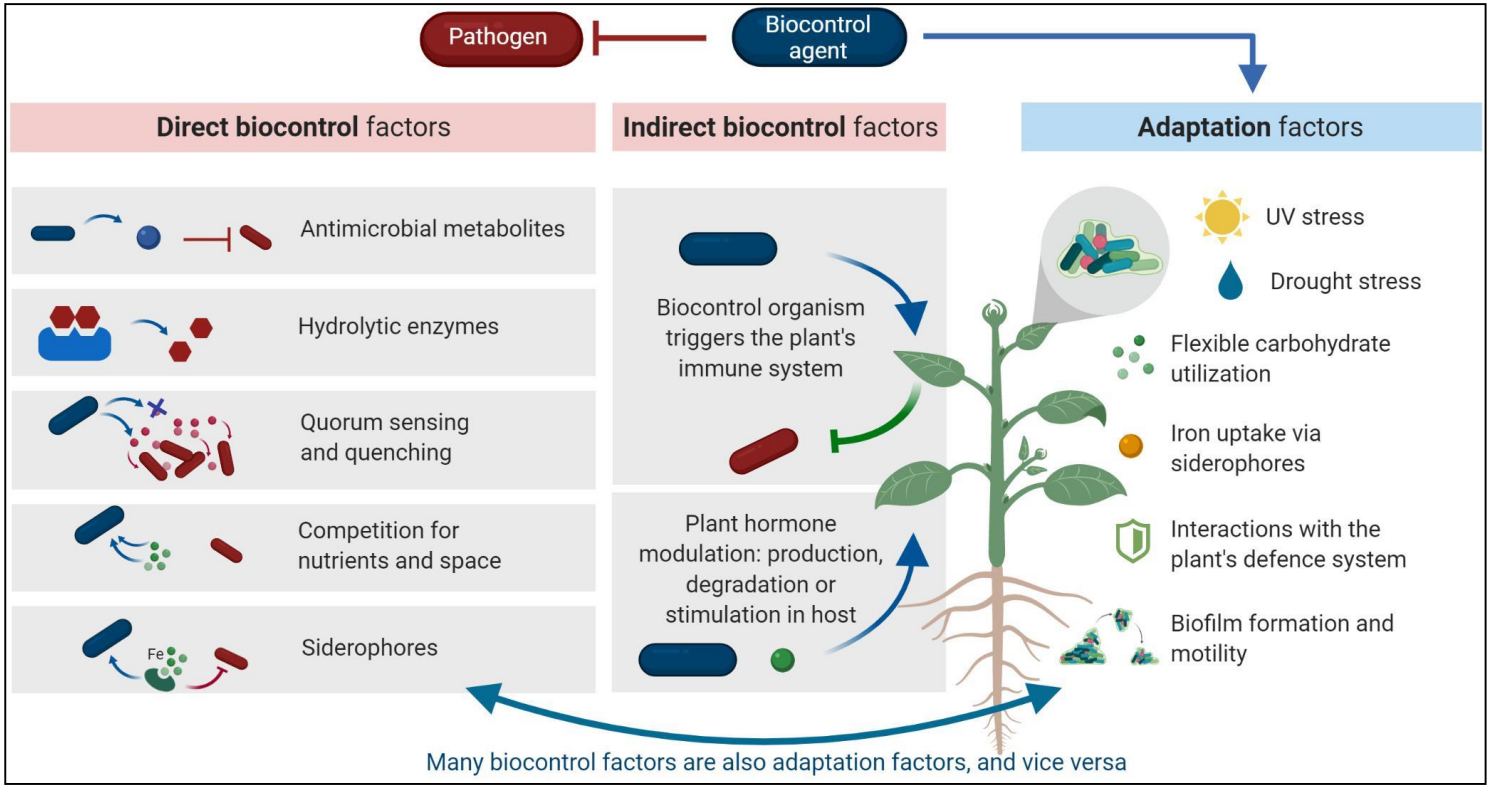
- Microbial biological control agent (MBCA) temporally interacting *in situ* (in the original place) with the targeted pathogen activating different modes of action in cascades of events.



Köhl *et al.*,2019

Microbial biological control agents against plant diseases

Direct or indirect modes of action



Legein *et al.*,2020

Microbial biological control agents against plant diseases

Direct or indirect modes of action

1. The biocontrol agent colonized the plant root surface and produced antimicrobial compounds such as surfactin.
2. In the plant rhizosphere, antibiosis and nutrient competition interaction suppressed the growth of pathogens.
3. Due to the production of antimicrobial compounds and in the simultaneous presence of pathogens, the induced systemic resistance (ISR) is enhanced.

- Thus, this mediated the defense response of the plant towards pathogens and consequently improved plant growth and the defense mechanism against pathogens.

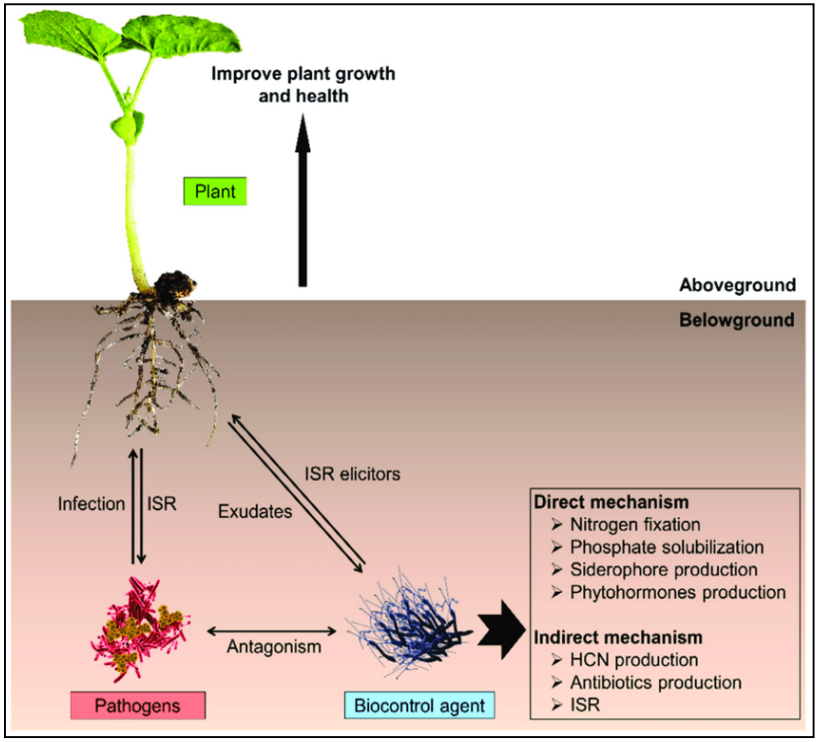
Ngalimat *et al.*,2021

Microbial biological control agents against plant diseases

Direct or indirect modes of action

- Microbial biological control agent (MBCA) temporally interacting in situ with the targeted pathogen activating different modes of action in cascades of events:

1. inducing resistance;
2. antibiosis and nutrient competition;
3. antimicrobial compounds.



Ngalimat *et al.*,2021

Biological control

Modes of action in relation to development and use of microbial biological control agents

Mode of action	Method for screening	Pathogen specificity	Risk of resistance[1]	Dependency on environmental conditions	Dependency on plant physiology	Use by distributors and end user
Induced resistance	Complex bioassay on plants	Specific to broad	Low	Low	High	Knowledge transfer needed
Competition	Simplified bioassays	Broad	Low	High	Low	Knowledge transfer needed
Hyperparasitism	Simplified bioassays	Pathogen specific interactions	Low	High	Low	Knowledge transfer needed
Antimicrobial metabolites produced *in situ*	Simplified bioassays	Specific to broad	Low	Moderate	Low	Knowledge transfer needed
Antimicrobial metabolites in product	*In vitro* assays	Broad	Moderate	Low	Low	Similar to use of fungicides
Helper strains[2]	Complex bioassays	Depends on MBCA	Low	Reduced	Reduced	Knowledge transfer needed
Assembled consortia combining different modes of action	*In silico* design followed by complex bioassays	Broad	Low	Low	Low	Knowledge transfer needed
Modulation of indigenous microbiota	Complex site-specific bioassays	Broad	Low	Medium	Low	Site-specific knowledge needed

[1] *Also depending on the specific evolutionary potential of targeted pathogen.* [2] *Applied in combination with MBCAs.*

Köhl *et al.*,2019

Biological control

Modes of action in relation to risk assessment and registration of microbial biological control agents

Mode of action	Risk of acute toxicity	Risks of metabo-lites	Environmental risks	Risks by environ-mental fate	Risks of phyto-toxicity	Analytical method	Recommended modification of current regulations
Induced resistance	Very low	Very low	Very low	Very low	Low	Strain-specific	Simplification because of low intrinsic risks
Competition	Very low	Very low	Very low	Very low	Very low	Strain-specific	Simplification because of low intrinsic risks
Hyperparasitism	Very low	Very low	Very low	Very low	Very low	Strain-specific	Simplification because of low intrinsic risks
Antimicrobial metabolites produced *in situ*	Low	Low	Low	Low	Low	Strain-specific	Simplification because of low intrinsic risks
Antimicrobial metabolites in product	Risk assess-ment relevant	Risk assess-ment relevant	Risk assess-ment relevant	Risk assess-ment relevant	Risk assess-ment relevant	Metabolite-specific	Use current regulations for PPPs
Helper strains[1]	Low	Low	Low	Low	Low	Strain-specific	No registration required
Assembled consortia combining different modes of action	Low	Low	Low	Low	Low	Multiple strain-specific	New concept needed for overall risk assessments instead of risk assessment per active ingredient
Modulation of indigenous microbiota	Low	Low	Low	Low	Low	Microbiome characterization	No registration required

[1] *Applied in combination with MBCAs.*

Köhl *et al.*,2019

Why use biological control?

- Chemical pesticides:
- Implicated in ecological, environmental, and human health problems
- Require yearly treatments
- Broad spectrum
  -Toxic to both beneficial and pathogenic species.
- Biological control agents:
- Non-toxic to human
- Not a water contaminant concern
- Once colonized may last for years
- Host specific
  - Only effect one or few species.

Why use biological control?

- Biological control agents are:

1. Expensive
2. Labor intensive
3. Host specific.

- Chemical pesticides are:

1. cost-effective
2. easy to apply
3. Broad spectrum.

Mechanisms of biological control of plant pathogens

- Nutrient competition – competition between microorganisms for:

1. carbon,
2. nitrogen,
3. O_2,
4. iron, and
5. other nutrients.

Mechanisms of biological control of plant pathogens

- Destructive mycoparasitism – the parasitism of one fungus by another:

1. Direct contact
2. Cell wall degrading enzymes
3. Some produce antibiotics

- Example:
- *Trichoderma harzianum*, used as seed treatment against pathogenic fungus.

Requirements of successful biocontrol

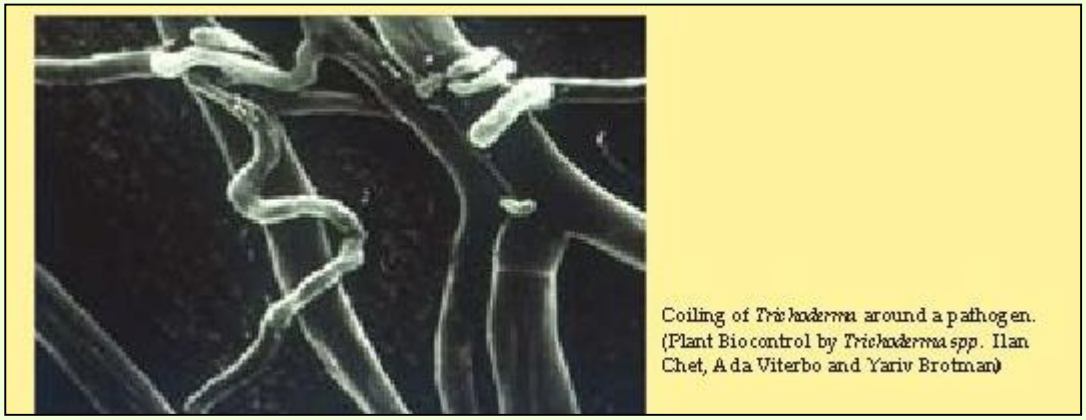
- Highly effective biocontrol strain must be obtained or produced:

1. Be able to compete and persist
2. Be able to colonize and proliferate
3. Be non-pathogenic to host plant and environment.

Requirements of successful biocontrol

- Inexpensive production and formulation of agent must be developed:

1. Must ensure agents will grow and achieve their purpose Coiling of *Trichoderma* around a pathogen.



Coiling of *Trichoderma* around a pathogen. (Plant Biocontrol by *Trichoderma spp.* Ilan Chet, Ada Viterbo and Yariv Brotman)

Requirements of successful biocontrol

- Delivery and application must permit full expression of the agent:

1. Production must result in biomass with excellent shelf live
2. To be successful as agricultural agent must be:

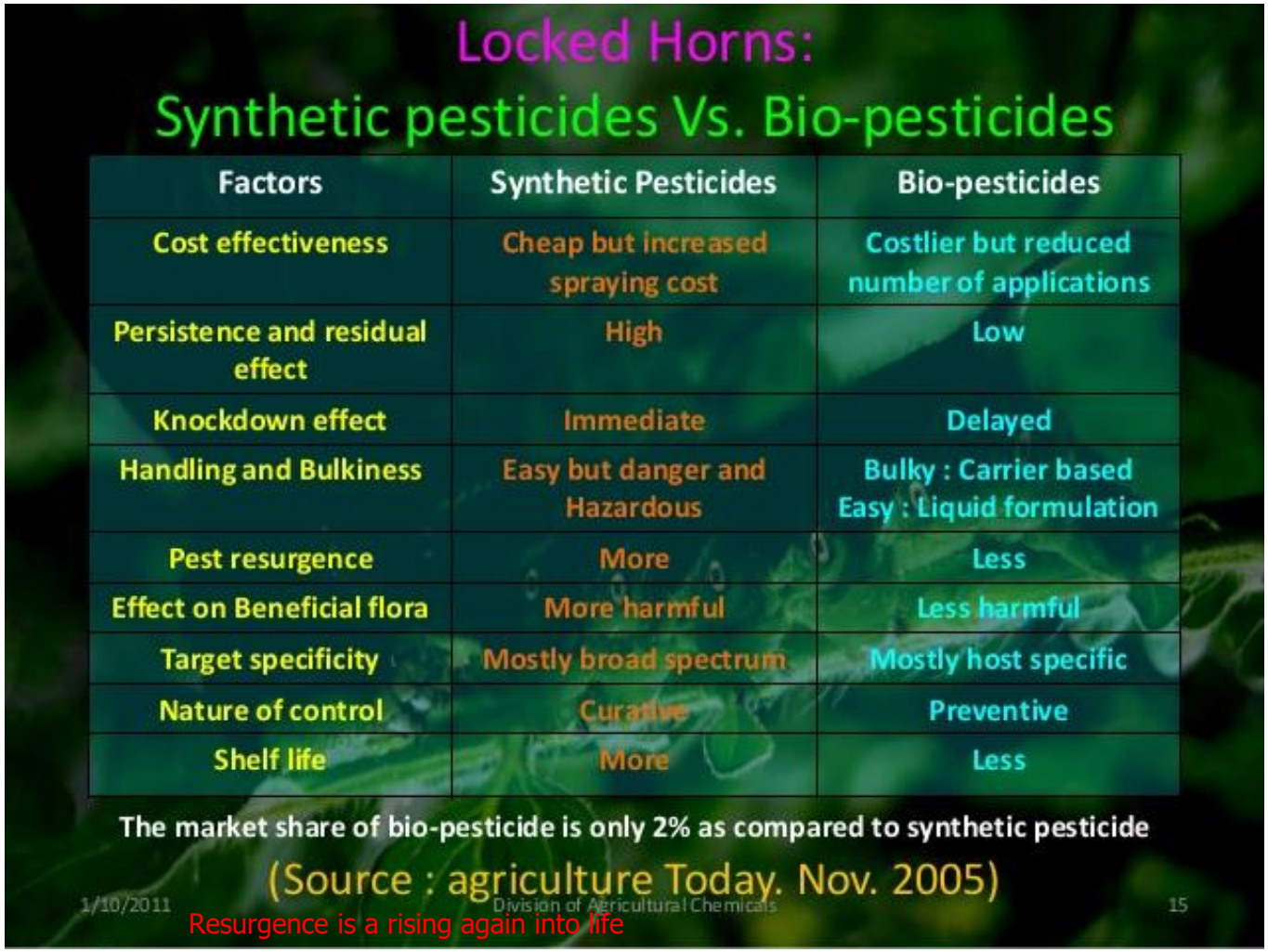
i. Inexpensive
ii. Able to produce in large quantities
iii. Maintain viability.

Shelf life is the length of time that a commodity may be stored without becoming unfit for use, consumption, or sale.

Biological control

Advantages

Synthetic pesitcides vs. bio-pesticides



Biological control

The natural and economic considerations of plant disease management with biological control agents

- A diagram showing the interconnection of natural and economic properties of farmer adoption for biological control.
- Biological control of plant diseases can generate multifaced effects, including:

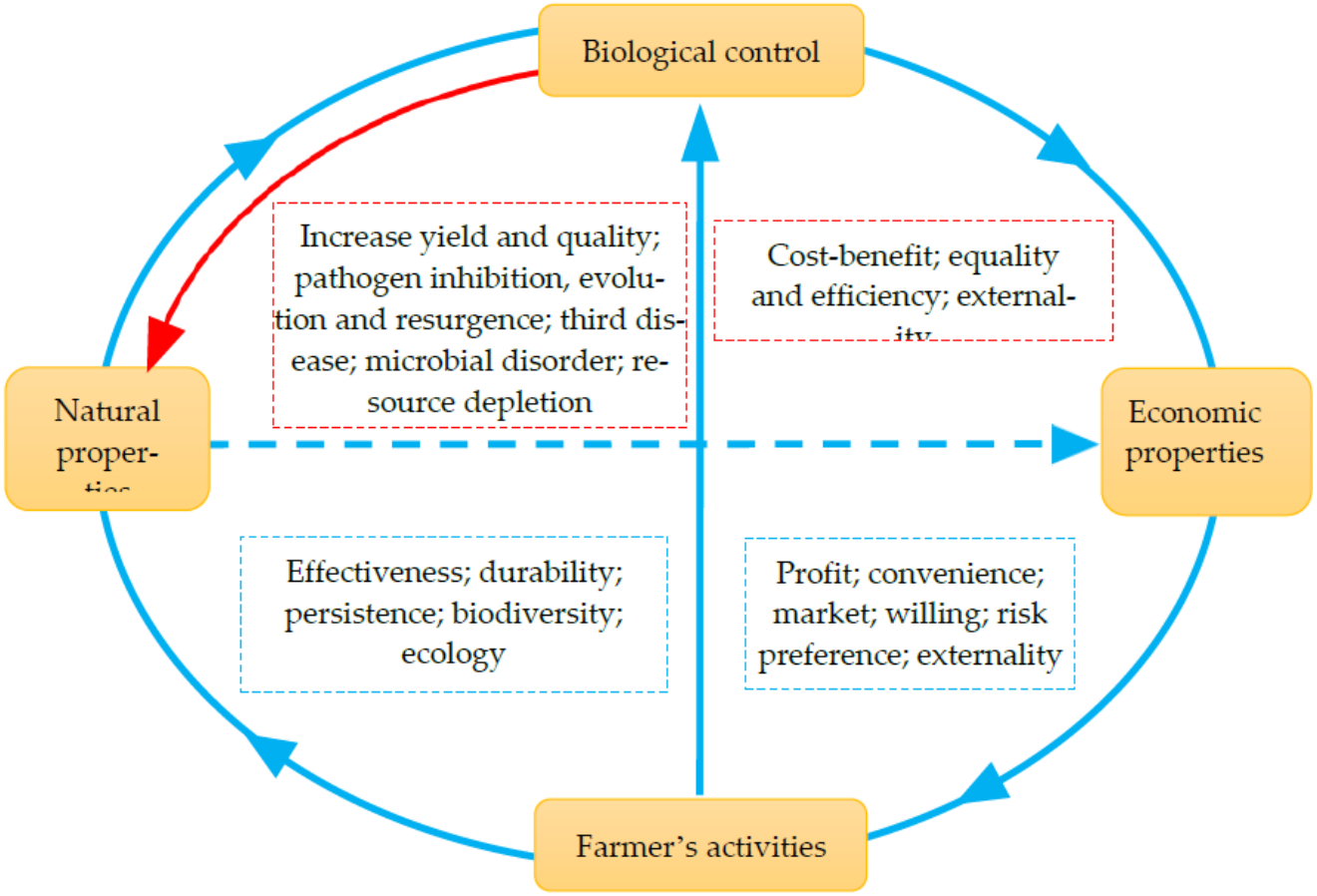
1. natural (e.g., pathogen inhibition, evolution, the third-party epidemics, nutrient supply, plant growth support, and resistance against biotic and abiotic stresses, saving yield and quality), and
2. economic (e.g., cost, efficiency, benefit, externality) properties.

- In turn, these properties, particularly economic profit, convenience, and supply–demand market of technology and products, determine the choice of farmers in using biological control and other agricultural practices through the adaptation of their willingness, risk preferences, and expectations.

He *et al.*,2021

Biological control

The natural and economic considerations of plant disease management with biological control agents



He *et al.*,2021

Biological control

The natural and economic considerations of plant disease management with biological control agents

- Among the alternatives, biological control appears to be one of the most promising approaches for eco-friendly and sustainable agriculture to protect crop plants and safeguard food.
- Nevertheless, currently, it is insufficient to draw a conclusion that the application of biological control could actually:

1. improve the efficiency,
2. profit,
3. convenience of disease management, and
4. agricultural productivity.

He *et al.*,2021

Plant disease biocontrol
Assessment of 'eco-friendly index

- 'Eco-friendly index' for assessing pros and cons (for and against) of various disease management technologies, using biocontrol, allelopathy and soil amendment as examples, and to ensure :

1. the technologies developed are not only effective and economical,
2. but also ecologically sound and socially acceptable are discussed here.

Huang and Chou,2012

Plant disease biocontrol

Assessment of 'eco-friendly index

Pythium damping-off

- The following groups were found according to the assessment of 'eco-friendly index':
- Group 1. Very high 'eco-friendly index'.
- Group 2. Moderately high 'eco-friendly index'.
- Group 3. Moderately low 'eco-friendly index'.
- Group 4. Very low 'eco-friendly index'.

Huang and Chou,2012

Plant disease biocontrol

Assessment of 'eco-friendly index

Pythium damping-off

- Group 1. Very high 'eco-friendly index'.
- The 'eco-friendly index' of *Rhizobium leguminosarum* bv. *viceae* is very high because:

1. the bacteria not only effectively controlled damping-off of pea and sugar beet,
2. but also improved soil fertility through its symbiotic relationships with legume crops.

- Group 2. Moderately high 'eco-friendly index'.
- The 'eco-friendly index' of *Pseudomonas fluorescens*, *P. putida*, *Pantoea agglomerans* and *Bacillus cereus* is moderately high.
- They are effective biocontrol agents for *Pythium* damping-off disease.
- However, *P. fluorescens*, *P. putida* and *Pantoea agglomerans* were reported as human pathogens and *B. cereus* was linked to outbreaks of foodborne illness associated with fresh fruits and vegetables.

Plant disease biocontrol

Assessment of 'eco-friendly index

Pythium damping-off

- Group 3. Moderately low 'eco-friendly index'. The 'eco-friendly index' for *Erwinia rhapontici* and *E. carotovora* is moderately low.
- Despite effective control of Pythium damping-off of crops, these bacteria cause disease on a wide range of crops.
- For example:
- *Erwinia rhapontici* causes pink seed disease of pea bean, lentil, chickpea, durum wheat and common wheat as well as crown rot of other plants.

- Group 4. Very low 'eco-friendly index'. **This group includes chemical pesticides** that are highly toxic to target and non-target organisms and are highly persistent in the environment.
- For examples:
- The 'eco-friendly index' of the organic mercury fungicide, the DDT insecticide, and the atrazine herbicide is very low because of their possibly harmful side effects on the entire ecosystem.

Huang and Chou,2012

Plant disease biocontrol

'Eco-friendly index' of rhizobacteria as biocontrol agents of Pythium damping-off of field crops

Bacterial agent	Control of Pythium diseases	Environmental impact	Eco-friendly Index*
Rhizobium leguminosarum bv. *viceae*	Yes	-improve plant health -improve soil fertility	+++
Pseudomonas fluorescens	Yes	-improve plant health -a human pathogen	++
Pseudomonas putida	Yes	-improve plant health -a human pathogen	++
Bacillus cereus	Yes	-improve plant health -a foodborne human pathogen on fresh produce	++
Pantoea agglomerans	Yes	-improve plant health -a human pathogen	++
Erwinia rhapontici	Yes	-cause pink seed disease of pea, bean, lentil, chickpea and wheat -cause crown rot of rhubarb	+
Erwinia carotovora	Yes	-cause soft rot of carrot and other crops	+

* Eco-friendly Index: -= Very Low; + = Moderately Low; ++ = Moderately High; +++ = Very High

Huang and Chou,2012

Biological control of bacterial plant diseases

- Increased global trade, together with climate change and the limitations in plant protection products, has favored the emergence and establishment of new plant diseases which, in turn, cause significant crop losses.
- Fruit production, for instance, is threatened by several bacterial plant diseases such as:

1. the bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa),
2. the bacterial spot of stone fruits caused by *Xanthomonas arboricola* pv. *pruni* (Xap), and
3. the angular leaf spot of strawberry caused by *Xanthomonas fragariae* (Xf).

Daranas *et al.*,2018

Biological Control

1. Phytopathogen biocontrol

Biological Control

2. Plant associated beneficial bacteria(PGPR)

Biological control

Gram-positive bacteria vs. Gram-negative bacteria

- Certain Gram-positive bacteria have a natural formulation advantage over their Gram-negative counterparts: the spore.
- Although the Gram-positive bacteria have not been as well represented in the biocontrol literature.
- Their spore-forming abilities and historical industrial uses bode well for biocontrol success.

Biological control

Plant associated beneficial bacteria

PGPR and PHPR

- The beneficial bacteria were classified two major groups according to their mode of action:

1. Plant growth promoting rhizobacteria (PGPR) or Beneficial plant rhizobacteria (PR) or Plant growth-promoting bacteria (PGPB, PGPR), or
2. Plant health promoting rhizobacteria (PHPR).

Lian *et al.*,2007;..

Biological control
Plant associated beneficial bacteria(PGPR)

- Bacteria are estimated to occupy between 7% and 15% of the total root surface area.
- Of these, some bacteria positively affect plants and have been designated as plant growth-promoting rhizobacteria (PGPR).
- PGPR suppress diseases by:

1. directly synthesizing pathogen-antagonizing compounds, as well as
2. by triggering plant immune responses.

Salete Mota *et al.*,2017; Jiao *et al.*,2021

Biological control
Plant associated beneficial bacteria(PGPR)

- Rhizobacteria can indirectly or directly promote positive effects on plants.

1. Indirectly, they suppress pathogens mediated by competition and the production of antimicrobial compounds and lytic enzymes.
2. Directly, they solubilize minerals and cause a wide range of changes in the rhizosphere, which promotes higher efficiency in the absorption of water and macro- and micronutrients by plants and changes in phytohormone concentrations, nitrogen fixation, and siderophore production.

Salete Mota *et al.*,2017

Biological control

Plant associated beneficial bacteria(PGPR)

- Bacteria that colonize the rhizosphere are commonly referred to as rhizobacteria (Sikora 1992; Lugtenberg and Dekkers 1999).
- Rhizobacteria can play significant roles in plant health.

1. Some rhizobacteria are significant plant pathogens, while
2. Others are beneficial mutualists.

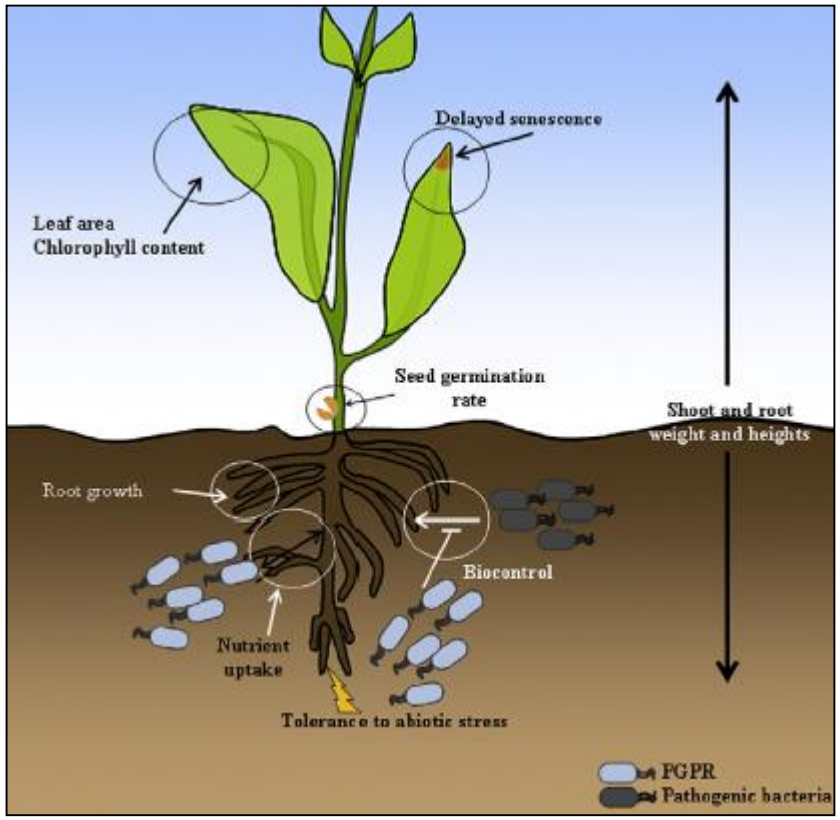
- Approximately 10% of all isolated rhizobacteria affect plant growth and/or health.

Lian *et al.*,2007;..

The agents of biological control

Schematic diagram represents the mechanism of PGPR (plant growth-promoting rhizobacteria)

- Benefits to plants from host–PGPR interactions.
- These benefits have been shown to include increase in:
- seed germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights and heights, bio-control, and delayed senescence.



Pérez-Monta *et al.*,2013

The agents of biological control

Schematic diagram represents the mechanism of PGPR (plant growth-promoting rhizobacteria)

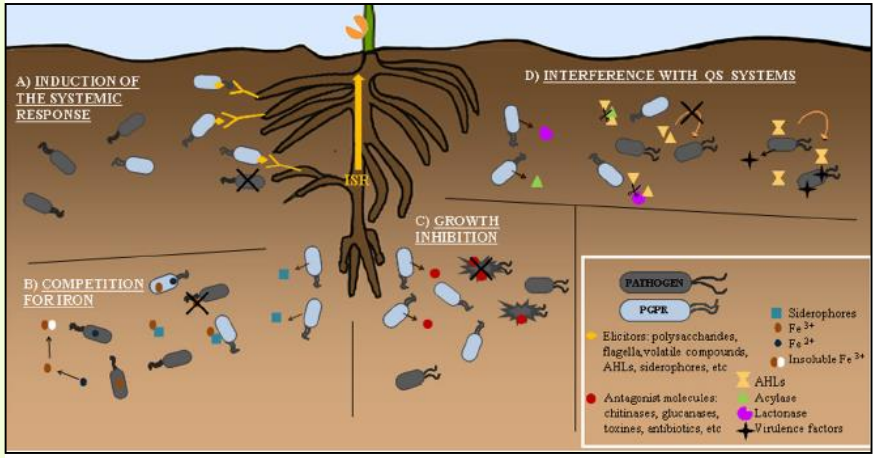
- Some forms of PGPR antagonism against plant pathogens.

A. Induction of the systemic response, ISR reducing plant disease incidence

B. Competition for iron, making it unavailable to plant pathogens.

C. Growth inhibition by producing antibiotics, toxins, biosurfactants and cell wall degrading enzymes.

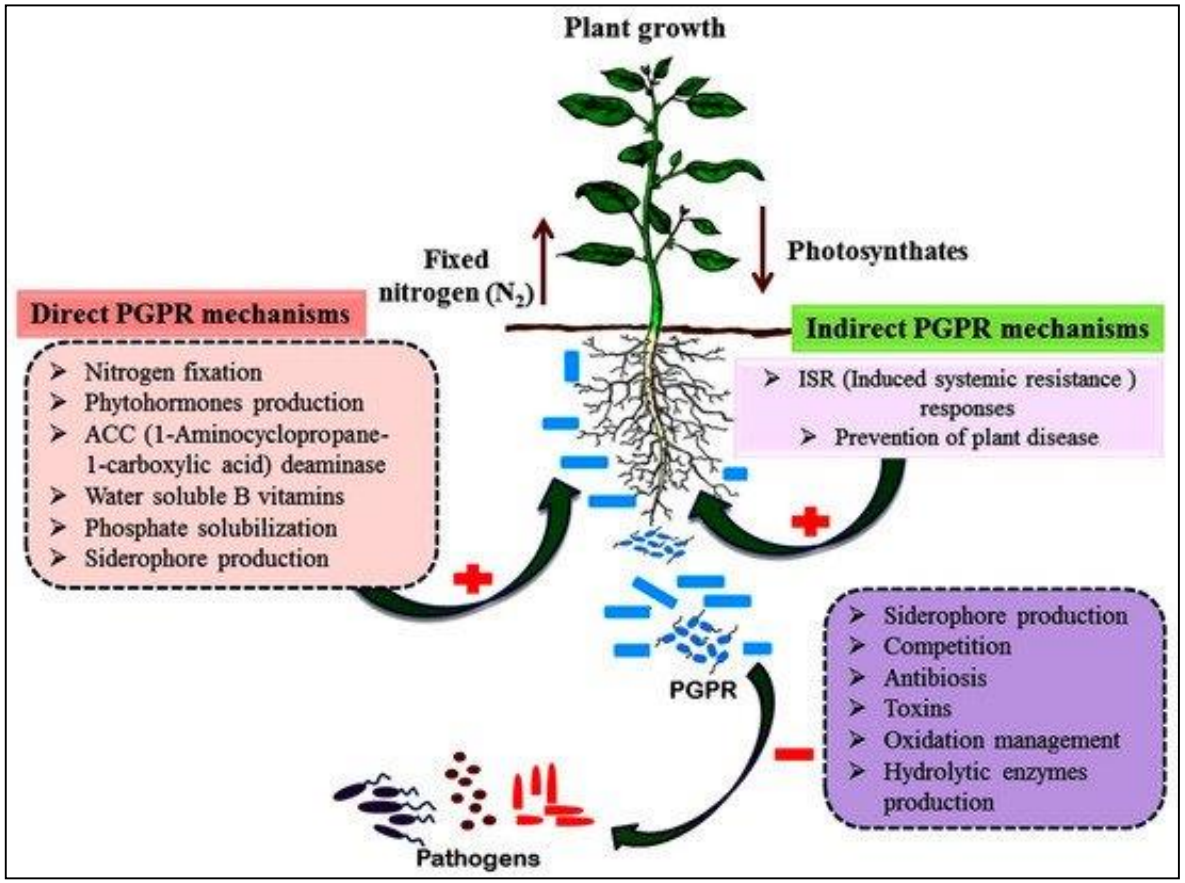
D. Interference with QS systems.



Pérez-Monta *et al.*,2013

The agents of biological control

Schematic diagram represents the mechanism of PGPR (plant growth-promoting rhizobacteria)

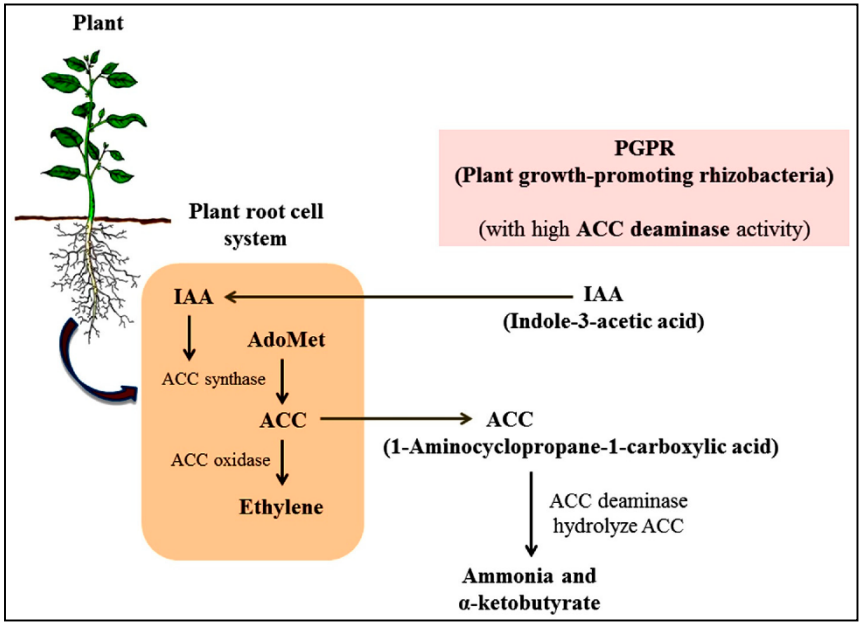


Chandran *et al.*,2021

PGPR use IAA and ACC deaminase enzyme for plant growth promotion

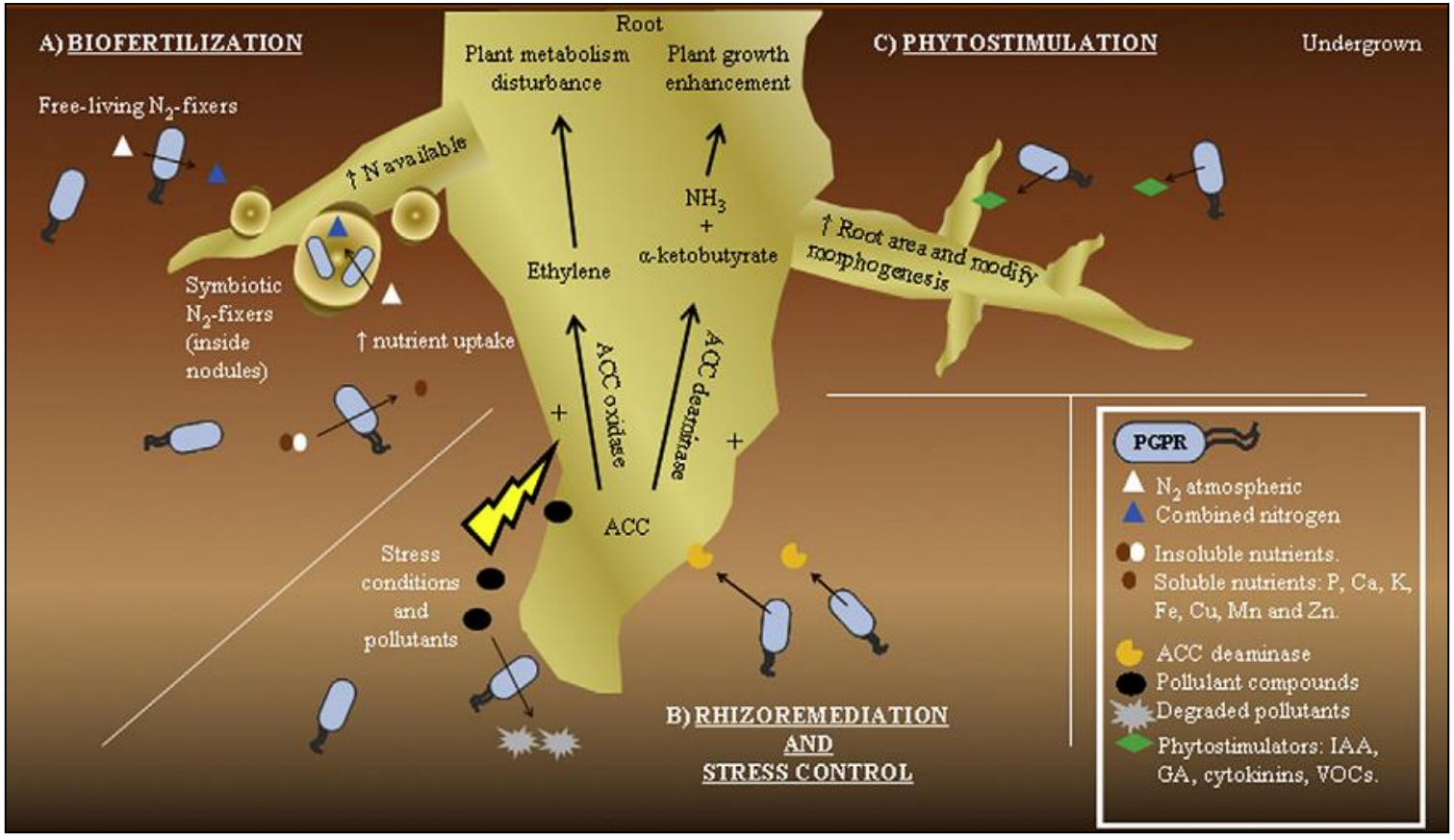
1. A number of PGPR produce IAA (indole-3-acetic acid) for plant growth promotion and development.
2. Likewise, plant growth-promoting rhizobacteria (PGPR) stimulate plant growth by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

- ACC deaminase causes a lowering of plant ethylene levels resulting in longer roots.
- The ACC deaminase in PGPR decreases ethylene level in plants by reducing ACC to ammonia and α-ketobutyrate.



Glick and Pasternak,2003; Chandran *et al.*,2021

PGPR use IAA and ACC deaminase enzyme for plant growth promotion

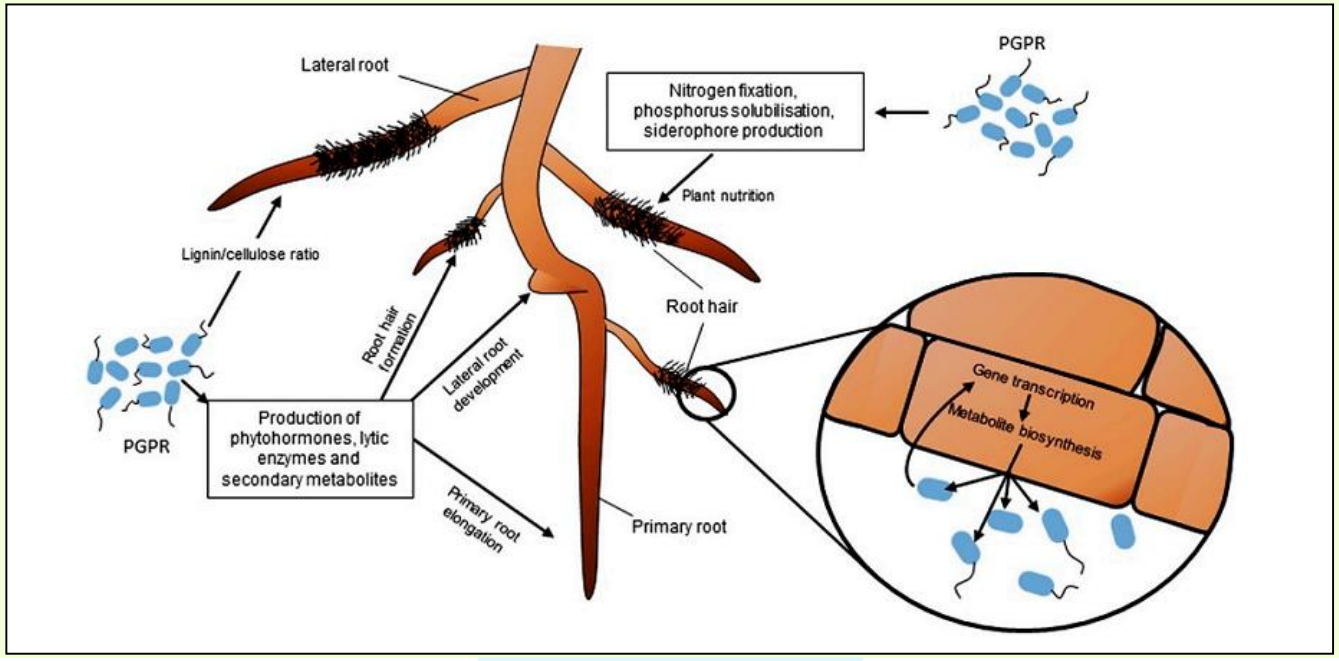


Pérez-Monta *et al.*,2013

PGPR

Nitrogen fixation, phosphorus solubilization, and siderophore production

- The possible mode of action used by plant growth promoting rhizobacteria (PGPR) towards growth promotion in plants. The flow and location of nitrogen fixation, phosphorus solubilization, and siderophore production are shown.



Vejan *et al.*,2016

Biological Control of Phytopathogens

Mechanisms and Applications

Compounds related to

1. **phytopathogen biocontrol and/or**
2. **promotion of plant growth (PGPR) or Plant health promoting rhizobacteria (PHPR).**

Mechanisms of biological control

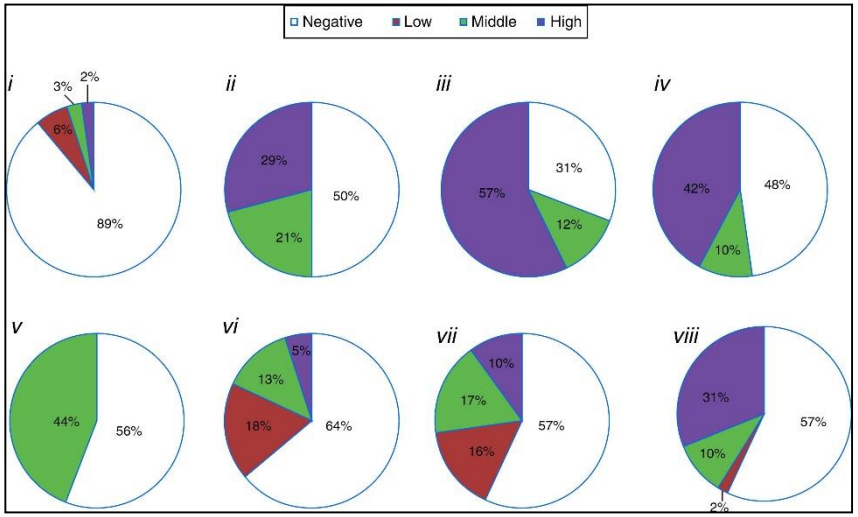
Well known inhibitory substances produced by bacteria

- Different inhibitory substances produced by bacteria have been reported.
- Several inhibitory substances have not been elucidated yet;
- In fact, we only know 1% of the bacterial diversity in a natural environment,
- leading us to assume that many other inhibitory substances remain to be explored.

Cesa-Luna *et al.*,2019

Bacterial selection for biological control of plant disease

- **Origin of the bacteria**
- The bacteria were obtained from different niches (phylloplane, rhizosphere, and soil) and grouped according to their isolation source: fig tree, Gramineae, Leguminoseae, Liliaceae, peach tree, Tagetes sp., non-rhizospheric soil, and others (tomato plant, Brassicae, and culture medium contaminants).



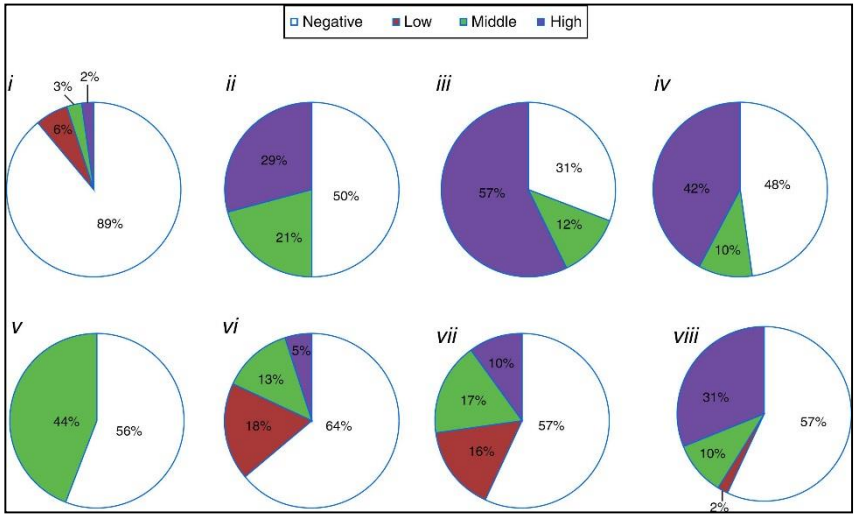
Of the 1219 bacterial isolates, 92% produced one or more of the eight compounds evaluated, but only 1% of the isolates produced all the compounds.

Salete Mota *et al.*,2017

Bacterial selection for biological control of plant disease

- Percentage of isolates (total = 1219) grouped as reaction intensity:

i. Chitinolytic (11% positive:, 89% negative);
ii. lipolytic;
iii. proteolytic on gelatin medium;
iv. proteolytic Litmus milk medium;
v. ammonia-production;
vi. amylase-production;
vii. antibiotic against *Monilinia fructicola*;
viii. phosphate-solubilization.



Of the 1219 bacterial isolates, 92% produced one or more of the eight compounds evaluated, but only 1% of the isolates produced all the compounds.

Salete Mota *et al.*,2017

Mechanisms of biological control
Methods to evaluate microbial antagonisms

- Among the well known inhibitory substances produced by bacteria are the broad-spectrum of:

1. antibiotics,
2. organic acids,
3. siderophores,
4. volatile organic compounds,
5. antifungal, and
6. bacteriocins.

Cesa-Luna *et al.*,2019

Mechanisms of biological control
Plant growth-promoting bacteria (PGPR)

- Rhizobacteria (PGPR) has been successfully used for the biocontrol of fungal, nematode, bacterial and viral diseases of plants in different parts of the world.
- Some of the biocontrol mechanisms that have been dealt with in detail and will be discussed are as follows:

1. Interactions of PGPR with pathogens;
2. Interactions of PGPR with plants;
3. Interactions of PGPR in the rhizosphere.

Siddiqui,2006

Mechanisms of biological control
Plant growth-promoting bacteria (PGPR)

- Plant growth-promoting bacteria (PGPR) are associated with the surfaces of plant roots and may increase plant yield by mechanisms that impart improved:

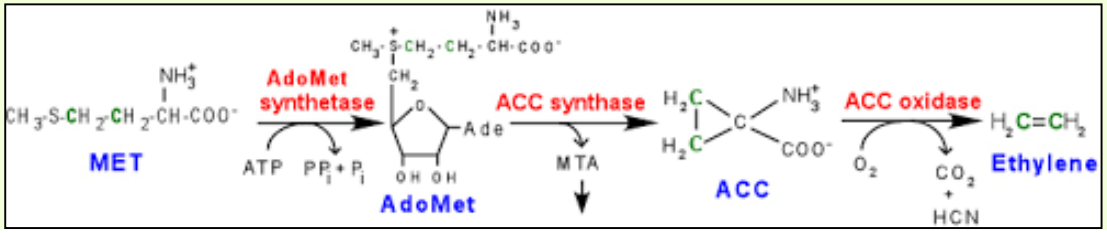
1. Mineral nutrient uptake (e.g. nitrogen fixation)
2. Disease suppression,
3. Phytohormone production,
4. Some species as bioferilizers (a product that contains living microorganisms).

Bais *et al.*,2004; Siddiqui,2006

Mechanisms of biological control
Plant growth-promoting bacteria (PGPR)

- All endophytic and rhizosphere bacteria were screened for plant growth promoting (PGP) traits including:

1. Phosphate solubilization
2. Indole-3-acetic acid (IAA) production
3. Siderophore
4. 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. ACC is a precursor of ethylene.



Ethylene is synthesized in flowering plants from methionine via a cyclic pathway, which preserves the methylthio group of methionine while using ATP. Two notable intermediates in the cycle are S-adenosyl-methionine and 1-aminocyclopropane-l-carboxylic acid; the latter compound is the immediate precursor of ethylene.

Mechanisms of biological control

Some more plant growth-promoting traits characterized *in vitro*

1. Phosphate (P) solubilization was determined on rock phosphate buffered medium.
2. Phytase production was determined on plates with phytic acid (inositol hexaphosphate sodium salt) as sole P source.
3. The presence of ACC deaminase activity in bacteria was detected on plates with DF salts minimal medium with ACC as sole nitrogen source.
4. Indole acetic acid (IAA) production was detected on nitrocellulose membrane disk.
5. Hydrolysis of chitin was studied on defined medium amended with 1.5% colloidal chitin as sole carbon source.
6. Hydrocyanic acid (HCN) production was tested on 35-mm petri dish containing Kings B agar medium amended with 4.4 g/l glycine with filter.
7. Bioremediation via degradation of pollutants.

Hameeda *et al.*,2006;..

Screening for putative biological control agents and PGPRs

Methods to evaluate microbial antagonisms

1. **Agar assays**
2. **Liquid assays**
3. **The '*in planta*' assay**

Screening for putative biological control agents and PGPRs

Antagonism among bacterial or fungal strains

1. Agar or liquid Assays

Screening for putative biological control agents and PGPRs

Agar or liquid assays

- Different methods can evaluate microbial inhibition, the most used comprise:

1. the double-layer agar, and
2. Simultaneous inhibition assays.
3. Assays in liquid media have also been frequently reported and represent a variant of simultaneous active interaction.

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

Agar or liquid assays

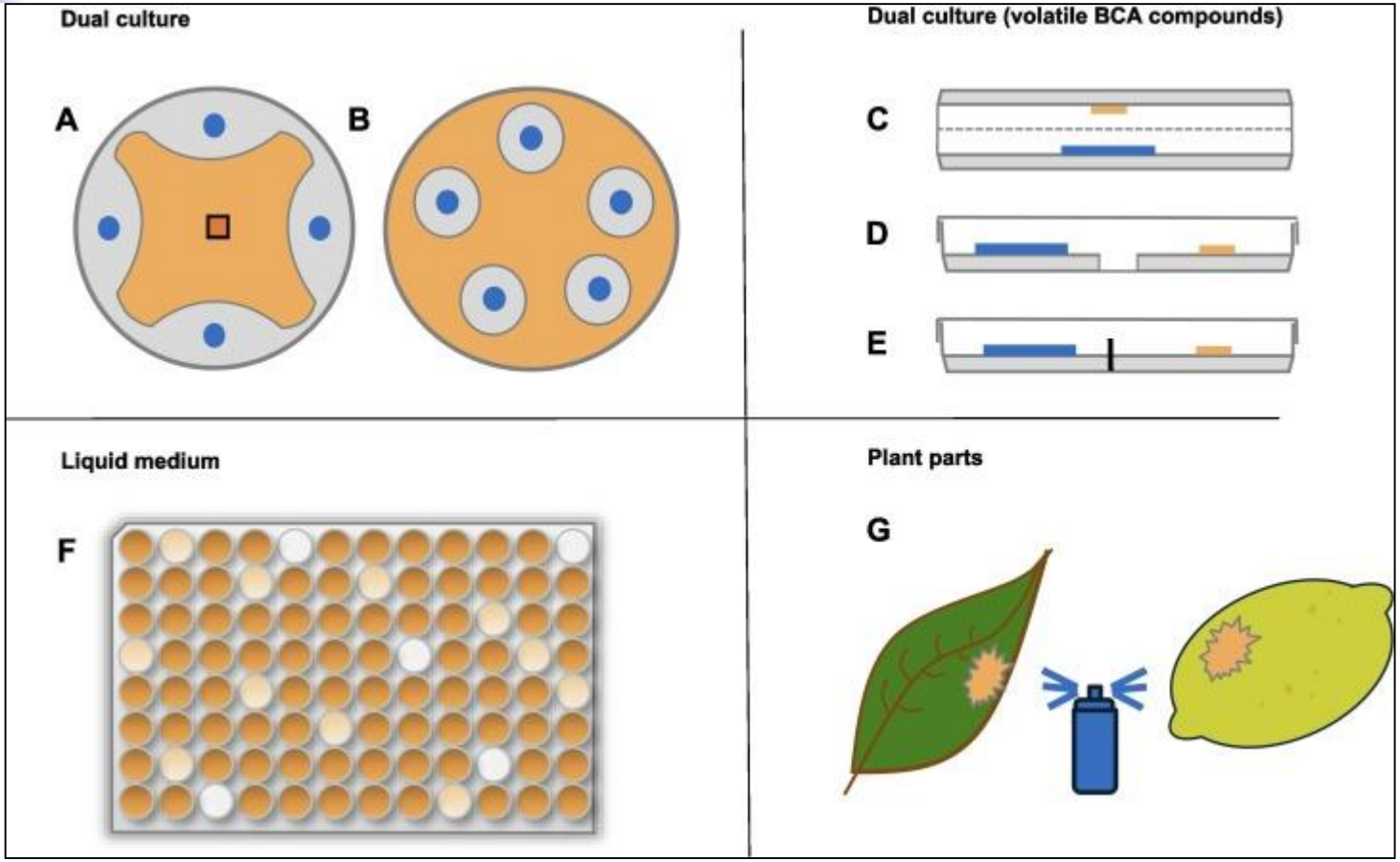
- *In vitro* tests are appropriate during the initial selection steps due to the large number of microorganisms that can be evaluated and, especially, their low cost.
- These include:

1. characterize bacteria to determine their *in vitro* potential for the production of compounds related to phytopathogen biocontrol and/or promotion of plant growth (CRBPGs);
2. select bacterial isolates with the highest number of CRBPGs, and
3. validate the selection process by studying the effect of the bacteria selected on different pathogens.

Salete Mota *et al.*,2017

Screening for putative biological control agents and PGPRs

Agar and liquid assays



Raymaekers *et al.*,2020

Screening for putative biological control agents and PGPRs

1. In the double-layer agar method

- In the double-layer agar method bacteria never interact between them; however, bacteria explored as sensitive should be able to grow in the presence of metabolites previously produced by the antagonistic strain grown on the first agar-layer (Mukherjee and Ghosh, 2014).
- The double-layer agar method consists of growing a producer strain on the surface of an agar-medium during 24-48 h.
- After incubation time, producer colonies are removed with a sterile glass slide and the remain cells are killed by exposing the glass Petri dish to the vapor of chloroform during 1.5 h.

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

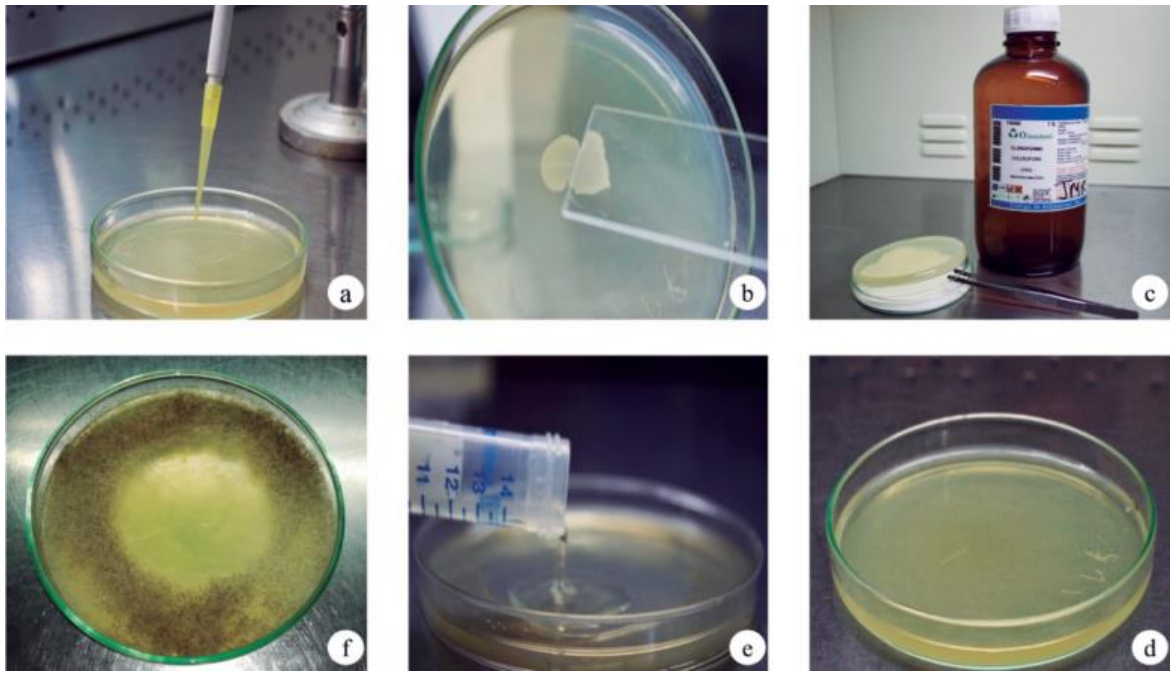
In the double-layer agar method(contd)

- Plates are left in a laminar flow cabinet until the residual chloroform is evaporated and the second layer of soft agar inoculated with the indicator strain is poured over the first layer of agar, where the producer strain had grown previously.
- Plates are incubated at the optimal temperature for each microorganism analyzed.
- Inhibition halos formed in the upper layer are considered indicative of antibacterial activity.

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

In the double-layer agar method(cont.)



Double-layer agar assay. In this process the producer strain is grown in the middle of a plate with a specific culture media (a). After incubation of 48 h bacterial colonies are removed (b) and killed under chloroform vapors (c). Once the remaining chloroform is evaporated (d), a double layer of soft agar (inoculated with an indicator strain) is poured (e). Once more the plates are incubated to look for an inhibition halo (f).

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

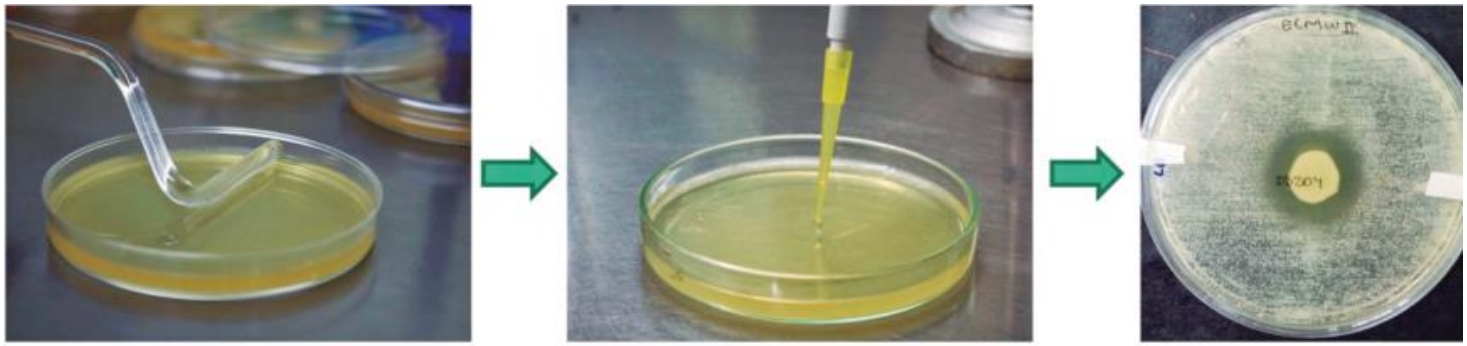
2. The simultaneous inhibition assay

- In the simultaneous inhibition assay, both bacterial species are co-interacting all the time during the assay.
- For this methodology, overnight cultures of strains explored as sensitive are placed over the surface of an agar plate by the spread-plating method (Sanders, 2012; Molina-Romero *et al.*, 2017b), and a 20 µl-drop of the producer strain is placed on the middle of the agar plate.
- After the drop dried, Petri plates are inverted and incubated at the right temperature for the microorganism analyzed.
- Surrounding halos of the producer strain are indicative of antibacterial activity.

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

The simultaneous inhibition assay



Simultaneous inhibition assay. An indicator strain is massively grown on the surface of an agar plate and a drop of the producer strain is placed in the middle of the plate. Once the drop dried, plates are incubated and the inhibition halo surrounding the producer strain is observed as shown in the last step.

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

3. Antagonisms in liquid media

- In this assay, the producer and the sensitive strains are grown in a defined liquid media, both separately and together in co-culture.
- Bacterial growth observed in the mixed culture is compared to the observed in the individual culture.
- When a producer bacterium inhibits the growth of a sensitive strain, the bacterial number of the sensitive strain decreases sharply in the mixed culture.
- In this experiment, the bacterial number is determined by counting CFU/ml using a selective medium, the media selection play an essential role in the screening of co-interacting strains (Muñoz-Rojas *et al.*,2005).

Cesa-Luna *et al.*,2019

Screening for putative biological control agents and PGPRs

4. Inhibitory substance produced by bacteria

- Some inhibitory substances produced by beneficial bacteria are:

1. Broad-spectrum antibiotics,
2. Siderophores
3. Bacteriocins, and
4. volatile compounds.

Cesa-Luna *et al.*,2019

Mechanisms of biological control
Methods to evaluate microbial antagonisms
1. Antibiotics

- The basis of antibiosis as a biocontrol mechanism of PGPB has become increasingly better understood over the past two decades (Table 2).
- A variety of antibiotics have been identified:
- **Compounds produced by pseudomonads:**
- Amphisin,
- 2,4-diacetylphloroglucinol (DAPG),
- hydrogen cyanide,
- oomycin A,
- phenazine,
- pyoluteorin,
- Pyrrolnitrin,
- tensin,
- tropolone, and
- cyclic lipopeptides.
- **Compounds produced by *Bacillus*, *Streptomyces* and *Stenotrophomonas* spp.**
- oligomycin A,
- kanosamine,
- zwittermicin A, and
- Xanthobaccin.

Mechanisms of biological control
Antibiotics produced by rhizobacteria

- Antibiotics produced by different PGPR have a broad-spectrum activity.
- The major antibiotics that play a vital role in the suppression of plant pathogens are grouped into:

1. Non-volatile antibiotics:

- Polyketides (2,4 Diacetyl phloroglucinol; Pyoluteorin; Mupirocin)
- Heterocyclic nitrogenous compounds (Phenazine derivatives)
- Phenylpyrrole (Pyrrolnitrin)
- Cyclic lipopeptides
- Lipopeptides (Iturin, Bacillomycin, Plipstatin, Surfactin)
- Aminopolyols (Zwittermycin - A).

2. Volatile antibiotics:

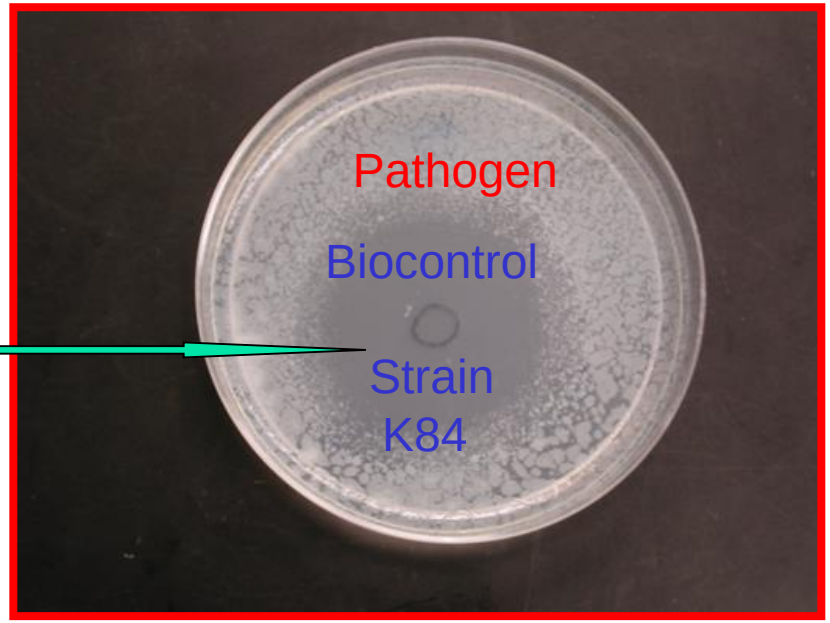
- Hydrogen cyanide
- Aldehydes, alcohols, ketones and sulfides.

Mechanisms of biological control
Antibiotics

- In all cases, the antibiotics have been shown to be particularly effective at suppressing growth of the target pathogen *in vitro* and/or *in situ*.
- To be effective, antibiotics or other related compounds must be produced in sufficient quantities near the pathogen to result in a biocontrol effect.

The mechanism of suppression influences the quality of the biocontrol interaction

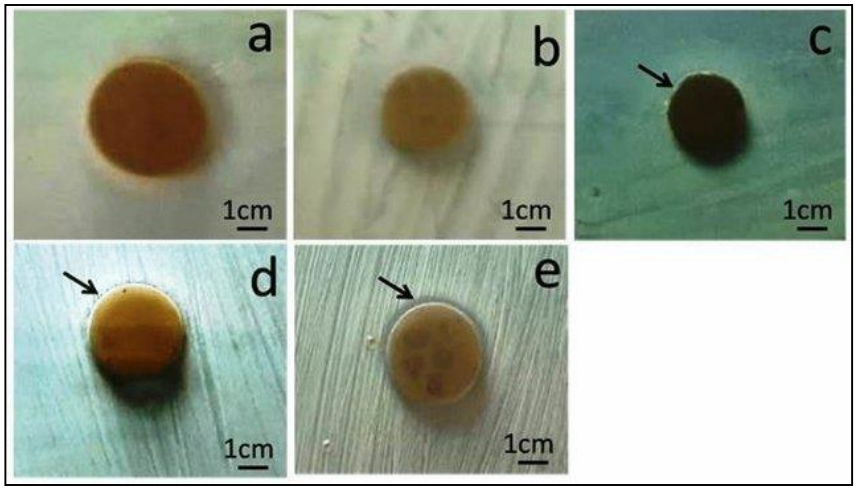
- **Mechanisms of biocontrol:**
- Competition
- Antibiosis
- Parasitism



Sonk,2007

The mechanism of suppression influences the quality of the biocontrol interaction

- Petri dish photographs after conducting agar disc-diffusion assays at 37°C for 24 h against *S. aureus* as test micro-organism for the tested nanocomposites with scale bar = 1 cm.



Abulyazied *et al.*,2021

Some of antibiotics produced by BCAs

Antibiotic	Source	Target pathogen	Disease
2, 4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium spp.*	Damping off
Agrocin 84	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*	Crown gall
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*	Aflatoxin contamination
Bacillomycin, fengycin	*Bacillus amyloliquefaciens* FZB42	*Fusarium oxysporum*	Wilt
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Damping off
Gliotoxin	*Trichoderma virens*	*Rhizoctonia solani*	Root rots
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Fire blight
Iturin A	*B. subtilis* QST713	*Botrytis cinerea* and *R. solani*	Damping off
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*	Damping off
Phenazines	*P. fluorescens* 2-79 and 30-84	*Gaeumannomyces graminis* var. *tritici*	Take-all
Pyoluteorin, pyrrolnitrin	*P. fluorescens* Pf-5	*Pythium ultimum* and *R. solani*	Damping off
Pyrrolnitrin, pseudane	*Burkholderia cepacia*	*R. solani* and *Pyricularia oryzae*	Damping off and rice blast
Zwittermicin A	*Bacillus cereus* UW85	*Phytophthora medicaginis* and *P. aphanidermatum*	Damping off

Pal & Gardener,2006

Antibiotics produced by rhizobacteria

PGPR	Antibiotics	Reference
Pseudomonas sp.	**Antifugal antibiotics**	
	Phenazines	Burkhead *et al.* (1994)
	Phenazine-1-carboxylic acid	Pierson and Pierson (1996)
	Phenazine-1-carboxamide	Chin-A-Woeng *et al.* (1998)
	Pyrrolnitrin	Thomashow and Weller (1988)
	Pyoluteorin	Howel and Stipanovic (1980)
	2,4diacetylphloroglucinol	Shanahan *et al.*(1992b)
	Rhamnolipids	
	Oomycin A	Kim *et al.*(2000)
	Cepaciamide A	Howie and Suslow (1991)
	Ecomycins	Jiao *et al.*(1996)
	DDR	Miller *et al.*(1998)
	Viscosinamide	Hokeberg *et al.*(1998) Nielsen *et al.* (1999)
	Butyrolactones	Thrane *et al.* (2000)
	N-butylbenzene sulphonamide	Gamard *et al.*(1997) Kim *et al.*(2000)
	Pyocyanin	Baron and Rowe (1981)
	Antibacterial antibiotics	
	Pseudomonic acid	Fuller *et al.*(1971)
	Azomycin	Shoji *et al.*(1989)
	Antitumour antibiotics	
	FR901463	Nakajima *et al.*(1996)
	Cepafungins	Shoji *et al.*(1990)
	Antiviral antibiotic	
	Karalicin	Lampis *et al.*(1996)
Bacillus sp.	Kanosamine	Milner *et al.*(1996)
	Zwittermycin A	Silo - Suh *et al.*(1994)
	Iturin A (Cyclopeptide)	Constantinescu (2001)
	Bacillomycin	Volpon *et al.*(1999)
	Plipastatins A and B	Volpon *et al.*(2000)

Siddiqui,2006

Antibiotics and their primers for the detection of antibiotic producers (Zhang,2004)

Table 3

Primer	Sequence	Antibiotics related
PHZ1	GGC GAC ATG GTC AAC GG	PCA
PHZ2	CGG CTG GCG GCG TAT AT	PCA
PHZX	TTT TTT CAT ATG CCT GCT TCG CTT TC	PCA
PHZY	TTT GGA TCC TTA AGT TGG AAT GCC TCC	PCA
PCA2a	TTG CCA AGC CTC GCT CCA AC	PCA
PCA3b	CCG CGT TGT TCC TCG TTC AT	PCA
Phl2a	GAG GAC GTC GAA GAC CAC CA	2,4-DAPG
Phl2b	ACC GCA GCA TCG TGT ATG AG	2,4-DAPG
BPF2	ACA TCG TGC ACC GGT TTC ATG ATG	2,4-DAPG
B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	2,4-DAPG
BPF3	ACT TGA TCA ATG ACC TGG GCC TGC	2,4-DAPG
BPR2	GAG CGC AAT GTT GAT TGA AGG TCT C	2,4-DAPG
BPR3	GGT GCG ACA TCT TTA ATG GAG TTC	2,4-DAPG

Continued table 3.

BPR4	CCG CCG GTA TGG AAG ATG AAA AAG TC	2,4-DAPG
PrnAF	GTG TTC TTC GAC TTC CTC GG	Pyrrolnitrin
PrnAR	TGC CGG TTC GCG AGC CAG A	Pyrrolnitrin
PRND1	GGG GCG GGC CGT GGT GAT GGA	Pyrrolnitrin
PRND2	YCC CGC SGC CTG YCT GGT CTG	Pyrrolnitrin
PrnCf	CCA CAA GCC CGG CCA GGA GC	Pyrrolnitrin
PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	Pyrrolnitrin
PltCreg1F	AGG CAA TCA CTA CCA TCC GTG CGC	Pyoluteorin
PltCreg2r	ATG AGG AGC AGG AGG TGT CGA GCA C	Pyoluteorin
PLTC1	AAC AGA TCG CCC CGG TAC AGA ACG	Pyoluteorin
PLTC2	AGG CCC GGA CAC TCA AGA AAC TCG	Pyoluteorin
PltBf	CGG AGC ATG GAC CCC CAG C	Pyoluteorin
PltBr	GTG CCC GAT ATT GGT CTT GAC C	Pyoluteorin
Plt1	ACT AAA CAC CCA GTC GAA GG	Pyoluteorin
Plt2	AGG TAA TCC ATG CCC AGC	Pyoluteorin
678	ATG TGC ACT TGT ATG GGC AG	Zwittermicin A
667	TAA AGC TCG TCC CTC TTC AG	Zwittermicin A

Siddiqui,2006

Rhizobacteria

Mode of action of *Pseudomonas* biocontrol agents

- Antibiotic production is a key mechanism by which some rhizosphere bacteria (Rhizobacteria/PGPR)can inhibit plant pathogens and suppress diseases.
- Two of the best-studied antibiotics responsible for the activity of *Pseudomonas* biocontrol agents are the:

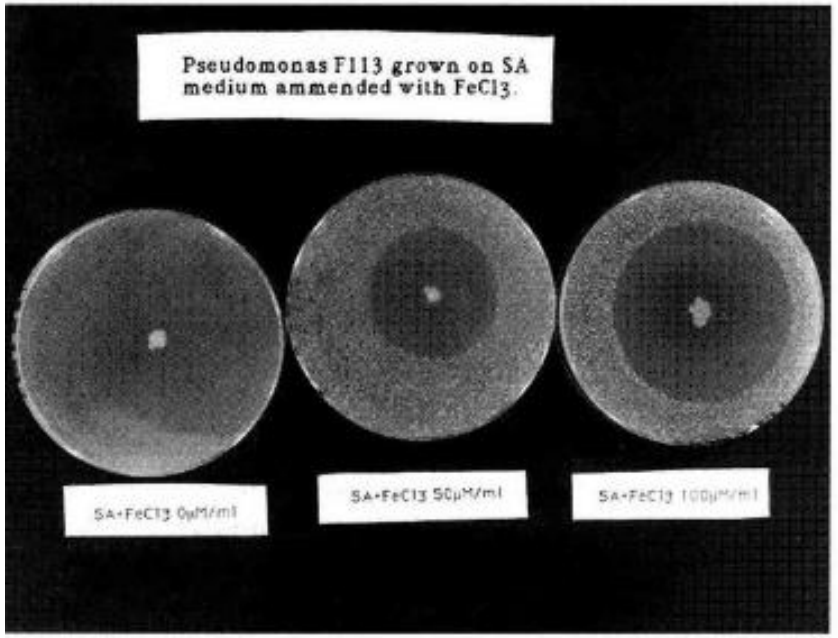
1. Phenolic compound 2,4-diacetylphloroglucinol (DAPG),
2. Phenazines.

- The activity of these two antibiotics is broad.

Modes of action of *Pseudomonas* biocontrol agent

Plate inhibition bioassay

- Effect of iron availability on the biocontrol of *Pseudomonas fluorescens* F113 against diacetylphloroglucinol (Phl) sensitive *Bacillus* strain.
- The concentration of available Fe^{+3} ions enhances Plh production in F113.
- In a plate inhibition assay, in which overnight cultures of F113 were grown on SA media amended with increasing concentrations of ferric chloride, increased Plh production resulted in increased inhibition of the *Bacillus* test culture.



Dunne *et al.*,1996

Rhizobacteria

Presence of genes encoding fengycin A and bacillomycin D biosynthesis

- To determine whether the collected tomato-associated rhizobacteria have the potential to produce different types of antimicrobial lipopeptides, PCR amplifications were performed todetect genes responsible for fengycin (A, B, D and E) and bacillomycin D biosynthesis.
- Among the 5 primer pairs tested, 2 genes encoding fengycin A and bacillomycin D biosynthesis were detected. Among the 25 isolates screened using FENA1F/FENA1R primer pairs, 18 yielded the indicative presence of *fen*A gene involved in fengycin A biosynthesis.
- Screened using BACC1F/BACC1R primer pairs, 16 isolates were found to be bacillomycin D-producing agents.

Ouhaibi-Ben Abdeljalil *et al.*,2016

Rhizobacteria

Presence of genes encoding fengycin A and bacillomycin D biosynthesis

- The 8 genes were amplified under the same PCR conditions.
- Briefly, each PCR mixture contained 2 µL of extracted DNA (10 ng/µL), 1 µL of $MgCl_2$ (50 mM), 0.6 µL of dNTP (10 mM), 0.6 µL of each primer (10 ng/µL), 3 µL Bovine Serum Albumine (10 µg/µL) (BioLabs), 0.1 µL of Taq polymerase, 3 µL of PCR buffer (10X) & 19.7 µL of SDW in a total volume of 30 µL.

Primer name	Sequence	Amplicon size (pb)	Annealing Temperature (°C)
799f	AACMGGATTAGATACCCKG	400	52
1492r	GTTACCTTGTTACGACTT	400	52
rpoB-f	GACGATCATYTWGGAAACCG	500	52
rpoB-r	GGNGTYTCRATYGGACACT	500	52
gyrB-f	AAAACAACCRATTCATGAAG	500	52
gyrB-r	TCGCTTCACTATTYCCAAGT	500	52
FENA1F	GACAGTGCTGCCTGATGAAA	757	54
FENA1R	GTCGGTGCATGAAATGTACG		
FENB1F	CAGCCGCTGTCAACAAGATA	950	54
FENB1R	ACACGACATTGCGATTGGTA		
FEND1F	TTTGGCAGCAGGAGAAGTTT	964	53
FEND1R	GCTGTCCGTTCTGCTTTTTC		
FENE1F	GCCAAAAAGAAACGAGCAAG	756	53
FENE1R	GTCGGAGCTAACGCTGAAAC		
BACC1F	GAAGGACACGGCAGAGAGTC	814	60
BACC1R	CGCTGATGACTGTTCATGCT		

Ouhaibi-Ben Abdeljalil *et al.*,2016

Rhizobacteria

Presence of genes encoding fengycin A and bacillomycin D biosynthesis

Molecular, morphological and biochemical characterization of tomato-associated rhizobacteria and assessment their ability to produce lipopeptide antibiotics and plant growth-promoting compounds. Positive reaction (+); Negative reaction (–); Fen A : Fengycin A, Bac : Bacillomycin D, IAA: Indole-3- acetic acid, P. solubilization: Phosphate solubilization. All isolates were negative for hypersensitive reaction (HR) on tobacco leaves, and they were negative for the detection of Fengycin B, D and E.

Molecular identification			Morphological characterization	Biochemical characterization				Detection of antibiotic biosynthesis genes		PGPR traits		
Isolate	Similarity (%)	Accession No.	Colony morphology	Gram	Oxidase	Catalase	Levan	Fen A	Bac D	IAA	P. Solubilization	Siderophore production (halo diameter in mm)*
Bacillus megaterium B1	99	KU168423	White, flat, opaque	-	+	+	-	-	+	+	-	48.3
B. thuriengiensis B2	99	KU158884	White, flat, opaque	+	+	+	-	-	+	+	+	31.7
Enterobacter cloacae B3	99	KT923049	White, flat, opaque	-	-	+	-	+	-	+	+	55.0
E.cloacae B4	100	KT923050	Transparent, flat, Translucide	-	-	+	+/-	+	+	+	+	50.0
B. megaterium B5	100	KT923054	Transparent, flat,Translucide	-	+/-	+	+/-	+	-	+	+	45.0
B. subtillis B6	100	KT921427	White, convex, translucide	+	-	+	-	+	+	-	+	0.0
B. amyloliquefaciens B7	99	KT921428	White, flat, opaque	+	-	+	-	+	+	+	-	83.3
B. subtillis B8	99	KU158885	White, flat, opaque	+	+	+	+/-	+	+	+	+	35.0
B. amyloliquefaciens B9	100	KU158887	Transparent, convex, opaque	+	+	-	-	+	+	+	+	36.7
B. subtillis B10	99	KT921327	Light yellow, flat, opaque	+	+	+	-	+	+	+	+	90.0
Chryseobacterium jejuense B11	99	KU158886	White, convex, translucide	+	-	-	-	-	+	+	+	43.3
Klebsiella pneumoniae B12	99	KT921328	Transparent, convex, translucide	-	-	+	-	+	-	+	+	90.0
B. amyloliquefaciens B13	99	KT951658	Transparent, convex, translucide	+	+	+/-	-	+	-	+	+	36.7
B. subtillis B14	99	KU161090	White, flat, opaque	+	+	+/-	-	+	-	-	-	73.3
B. amyloliquefaciens B15	99	KT923051	White, plate, opaque	+	+	+/-	-	-	+	-	-	28.3
E. cloacae B16	100	KT921429	Cream, flat, opaque	-	-	+	-	+	+	+	+	90.0
B. subtillis B17	100	KT923055	White, flat, opaque	+	-	+	-	+	+	+	+	0.0
B. amyloliquefaciens B18	100	KT923052	White, plate, opaque	+	+	-	-	+	+	+	+	0.0
B. subtillis B19	99	KT921430	Cream flat, opaque	+	+	+/-	-	+	-	-	-	75.0
B. subtillis B20	99	KT921431	White, plate, opaque	+	+	+	-	+	+	+	+	66.7
B. amyloliquefaciens B21	100	KT923047	Transparent, plate, translucide	+	+	+	-	+	+	+	+	0.0
B. amyloliquefaciens B22	100	KT923053	White, plate, opaque	+	+	+	-	+	+	+	+	0.0
B. thuriengiensis B23	99	KT923056	White, plate, opaque	+	+	+	-	+	+	+	+	13.3
B. megaterium B24	100	KT923048	Cream, flat, opaque	-	+	+	-	-	+	-	-	75.0
B. subtillis B25	99	KU161091	Transparent, flat, Translucide	+	+/-	+/-	-	-	+	-	-	10.0

Mechanisms of biological control

2. Enzymes

- A variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases.
- Lytic enzymes as microbial byproducts hydrolyze a wide variety of polymeric compounds, including:
- Chitin,
- Proteins,
- Cellulose,
- Hemicellulose, and
- Lignin.
- Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly.

See also hydrolysis activities of PGPR.

Mechanisms of biological control

Lytic enzymes

Chitinases

- Chitinases, the hydrolytic enzymes that specifically degrade chitin, are gaining much attention worldwide.
- Chitinases are produced by several bacteria, actinomycetes, fungi and also by higher plants.
- Microorganisms produce the chitinase primarily for assimilation of chitin as carbon and (or) nitrogen source.
- These chitinases are used in various applications such as biological control of fungal pathogens.

Mechanisms of biological control

Preparation of chitin from chicken feather and shrimp shell

Isolation of chitin degrading bacteria

- Chitin is a long-chain polymer of a N-acetylglucosamine, a derivative of glucose.
- It is the main component as carbon and (or) nitrogen source.

1. In terms of structure, chitin may be compared to the polysaccharide cellulose, and
2. In terms of function, to the protein keratin.

- For keratinolytic and chitinolytic/activity they usually use chicken feathers and shrimp shell, respectively.
- **Chitin/chitosan is the major waste product of marine and fishery industry.**
- These materials were also used for detecting chitinolytic bacteria.

Keratin is a protein that adds strength to hair, nails and the outer layer of skin.

Mechanisms of biological control
Preparation of chitin from chicken feather
Isolation of chitin degrading bacteria

- Poultry feather(CF) was cut into small fragments, washed extensively with water and immersed overnight in a neutral detergent solutions such as ethanol, methanol, sod. Hypochlorite, etc.
- The treated chicken-feathers were then washed and rinsed thoroughly with tap water to remove the detergent.
- The sun-dried CF fragments were ground in a Willey/ball/ hammer-milled and passed through a small mesh grid (20 mesh sieve) to remove coarse particles (Wawrzkiewicz *et al.*,1991).
- 1% of chicken feather powder as a sole source of carbon and nitrogen was added in an appropriate basal media and autoclaved.
- Chitinolytic/Keratinolytic activity was detected as a clear zone around the colony after incubation up to 5 days at room temperature.

Cervantes-González *et al.*,2008; Kim *et al.*,2001; Saber *et al.*,2010

Mechanisms of biological control
Preparation of chitin from shrimp shell
Isolation of chitin degrading bacteria

- The process with shrimp shells is the same as described for poultry feather(CF), except that detergent treatment was omitted and a 40 mesh sieve was used.

Cervantes-González *et al.*,2008

Mechanisms of biological control

Preparation of colloidal chitin from flakes

Isolation of chitin degrading bacteria

- Colloidal chitin was prepared from the chitin flakes (Sigma Chemicals Company, USA) by the method of Mathivanan (1995).
- The chitin flakes were ground to powder and added slowly to 10 N HCl and kept overnight at 4°C with vigorous stirring.
- The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25°C.
- The precipitate was collected by centrifugation at 10000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0).
- It was freeze dried to powder and stored at 4°C until further use.

Shanmugaiah *et al.*,2008

Mechanisms of biological control

Detection of chitinase activity on chitin agar plate

- Chitin agar plates were prepared using 0.5% acid swollen chitin or 0.5% glycol chitin per liter.
- Chitin agar plate was prepared with different dyes:
- Calcofluor white M2R,
- fluorescein isothiocyanate,
- rhodamine B,
- uthenium red, and
- Congo red, at 0.001% (w/v).

Gohel *et al.*,2005

Detection of chitinase activity on chitin agar plate

- Chitinase activity on solid plate method, containing:

A) acid swollen chitin with calcofluor white;

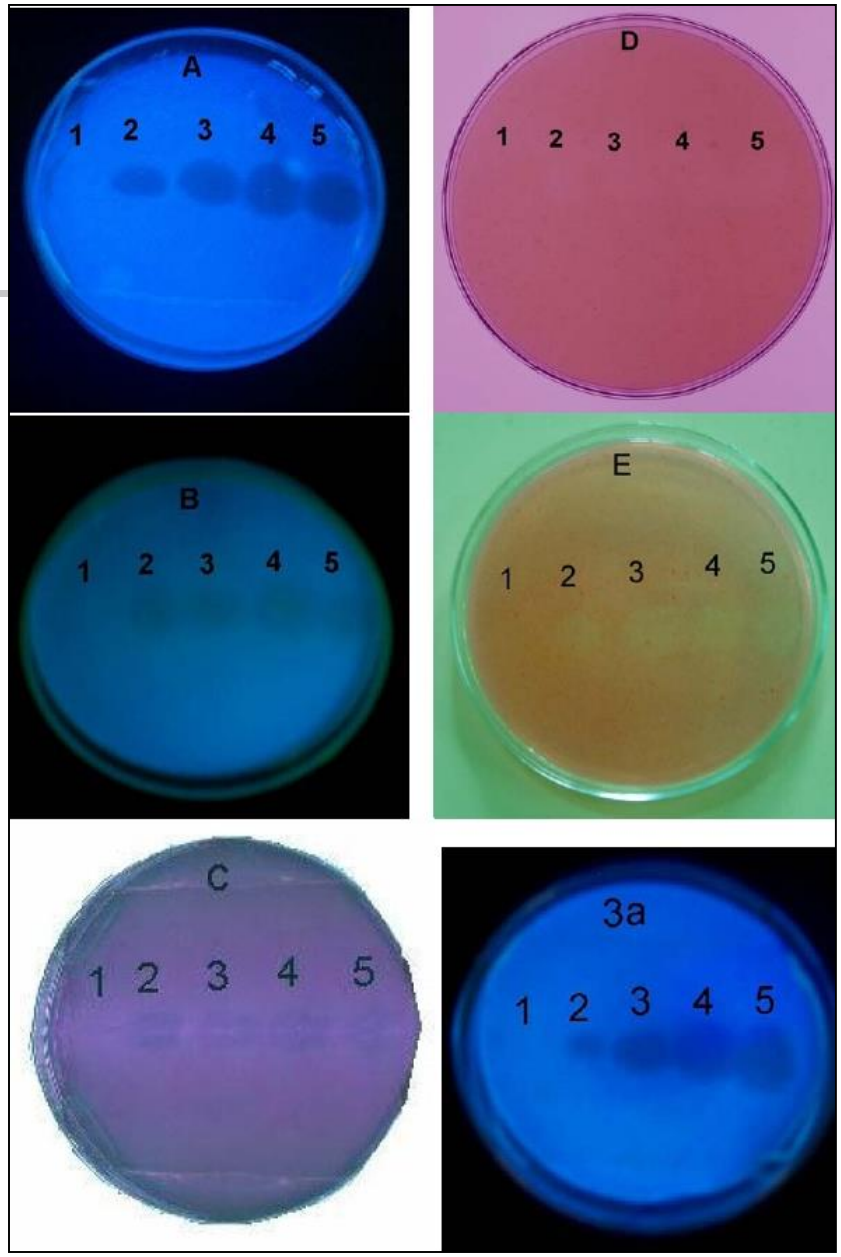
B) acid swollen chitin with fluorescein isothiocyanate;

C) acid swollen chitin with rhodamine B;

D) acid swollen chitin with ruthenium red;

E) acid swollen chitin with congo red; and

3a) glycol chitin with calcofluor white M2R.



Gohel *et al.*,2005

Mechanisms of biological control

Lytic enzymes

ß-1,3-glucanase production by bacteria

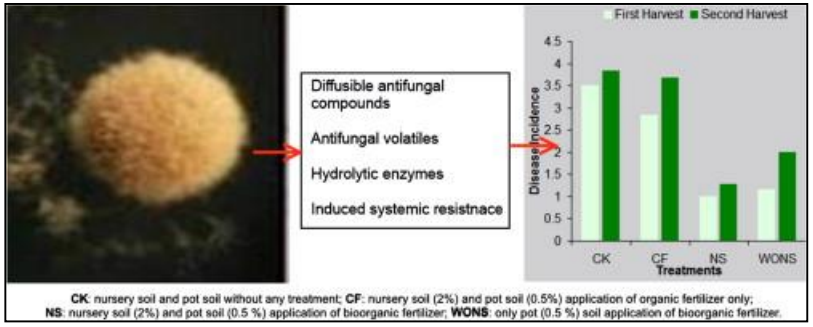
- The ß-1,3-glucanase synthesized by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 lyse fungal cell walls of *F. oxysporum* f. sp. *cucumerinum*.
- *B. cepacia* synthesizes ß-1,3-glucanase that destroys the integrity of *R. solani*, *S. rolfsii* and *Pythium ultimum* cell walls.
- ß-1,3-glucanase production was determined as described by Renwick *et al.*,1991 in the previously defined medium, except the C source was ß-1,3-glucan (5 g L^{-1}).

Mechanisms of biological control

Lytic enzymes

ß-1,3-glucanase and more enzymes production

- The strain, coded as *Streptomyces goshikiensis* YCXU, inhibited *in vitro* a broad range of phytopathogenic fungi.
- The strain could utilize different carbon sources and exhibited catalase, β-1,3-glucanase, chitinase and urease enzyme activities.
- The stain was also able to produce antifungal diffusible and volatile organic compounds.



Faheem *et al.*,2015

Mechanisms of biological control

Lytic enzymes

Lignolytic activity

- Lignin is the most structurally complex carbohydrate possessing a high molecular weight and the most recalcitrant, consisting of various biologically stable linkages.
- The lignocellulose material of plant consists of three main compounds, namely cellulose, hemicellulose and lignin.
- Lignocellulytic enzymes have significant potential application in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture.

Sasikumar *et al.*,2014

Mechanisms of biological control

Lytic enzymes

Alkali lignin preparation

- Lignocellulose is the major structural component of woody plants and non-woody plants such as grasses and represents a major source of renewable organic matter.
- The plant sources used for extraction of lignin were dried and bark grinded to powder.
- With 10 g of powdered bark (lignin sources), 5 mL of 1% sulfuric acid was added and heated in hot air oven at 80°C for 20 min and allowed to cool followed by 100 mL of 4% sodium hydroxide and boiled for 30 min.
- The dark brown colored alkali lignin was filtered and autoclaved at 15 lbs for 10 min (Bholy *et al.*, 2012).

Sasikumar *et al.*,2014

Mechanisms of biological control

Lytic enzymes

Alkali lignin preparation

- Preparation of lignin sources

a. Dried plant barks,

b. Powdered bark.

Sasikumar *et al.*,2014

Mechanisms of biological control

Lytic enzymes

Lignin rich medium

- The sample was collected in a sterilize polythene bag and transported to the laboratory and stored at -20°C.
- The lignin degrading bacteria was enriched using a medium in which lignin provided the sole carbon and energy source (MSM-L).
- MSM-L consisted of 1% alkaline lignin minimum salt medium solution which contained (g/L of deionized water) K_2HP0_4, 4.55; KH_2PO_4, 0.53; $MgSO_4$, 0.5; NH_4NO_3, 5 (Chandra *et al.*, 2008).
- The components were mixed and the resulting suspension was autoclaved. Enrichment culture were performed in 250 mL Erlenmeyer flask by placing 5 g sample in 95 mL MSM-L and culture were incubated at 120 rpm for 7 d at 30 to 45°C.

Enriched medium cultured with lignin degrading microorganisms.

Sasikumar *et al.*,2014

Mechanisms of biological control

Lytic enzymes

Lignin rich medium

- Enriched sample of 1 mL were transferred to 99 mL of sterile 0.9% NaCl.
- The solution were stirred vigorously and allowed to settle down. Using 1 mL of the liquid mixture, serial dilution technique was performed from each dilution.
- About 100 µL of serially diluted sample were spread on plate containing minimal salt medium agar containing alkaline lignin.
- The plates were incubated at 30°C for 7 d until colonies developed.
- The isolated bacteria were plated onto fresh MSM-L agar plates repeatedly to obtain pure cultures (Rahman *et al.*,2013).

Enriched medium cultured with lignin degrading microorganisms.

Sasikumar *et al.*,2014

Mechanisms of biological control

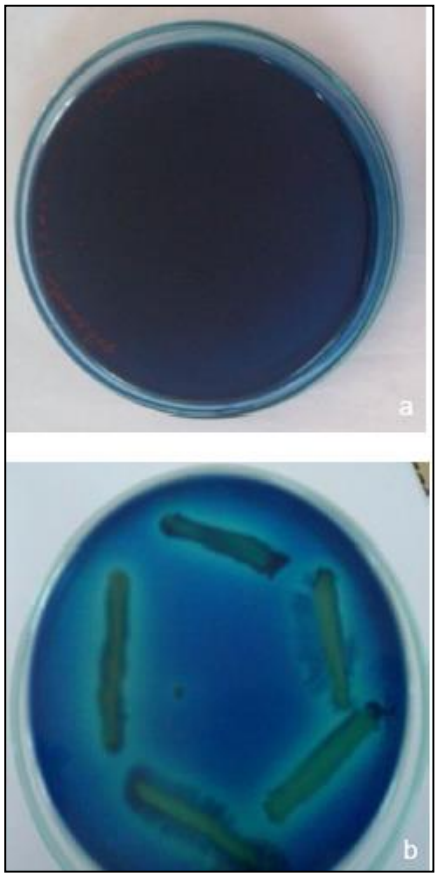
Lytic enzymes

Lignolytic activity

- The bacterial isolates were further screened using methylene blue dye as an indicator.
- The microbes possess lignolytic enzymes undergoes oxidation of indicator dye.
- The isolated bacteria were streaked on methylene blue indicator dye (0.25 g/L) containing LB agar plate.
- The plates were incubated at 30°C for 72 h.
- The agar plates were monitored daily for bacterial growth and decolorization of the methylene blue dyes (Bondounas *et al.*,2011).
- Lignolytic activity plate:

a. Control plate,

b. Decolorization of methylene blue plate.



Sasikumar *et al.*,2014

Mechanisms of biological control

Lytic enzymes

Protease activity

- Protease, a hydrolytic enzyme accounts 60% of total worldwide sale of industrial enzymes.
- Protease are commonly classified according to their optimum pH:
- Acidic protease
- Neutral protease
- Alkaline protease

Kezia *et al.*,2011

Mechanisms of biological control

Lytic enzymes

Protease activity

- The proteases including different families of enzymes:

1. Serine protease with nematocidal activity,
2. Cysteine (thiol) protease,
3. Aspartic proteases, and
4. Metallo-protease.

- Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases.
- And among bacteria, *Bacillus* species are specific producers of extracellular alkaline proteases.

Olajuyigbe and Ajele,2005

Mechanisms of biological control

Lytic enzymes

Screening for proteolytic activity

- Casein Degradation Test:
- Protease (proteolytic activity) was determined from clearing zones in skim milk agar:
- 50 ml of sterilized skim milk mixed at 55°C with 50 ml of one-fifth volume of tryptic soy agar and 4% agar) after 5 days of incubation at 30°C.
- Agar plates containing gelatin (1% w/v) were used for proteases.

See also biological control of nematodes.

Berg *et al.*,2002

Mechanisms of biological control
Screening for proteolytic activity

- Casein Degradation Test:
- Protease (proteolytic activity) was determined from clearing zones in skim milk agar:
- 50 ml of sterilized skim milk mixed at 55°C with 50 ml of one-fifth volume of tryptic soy agar and 4% agar) after 5 days of incubation at 30°C.

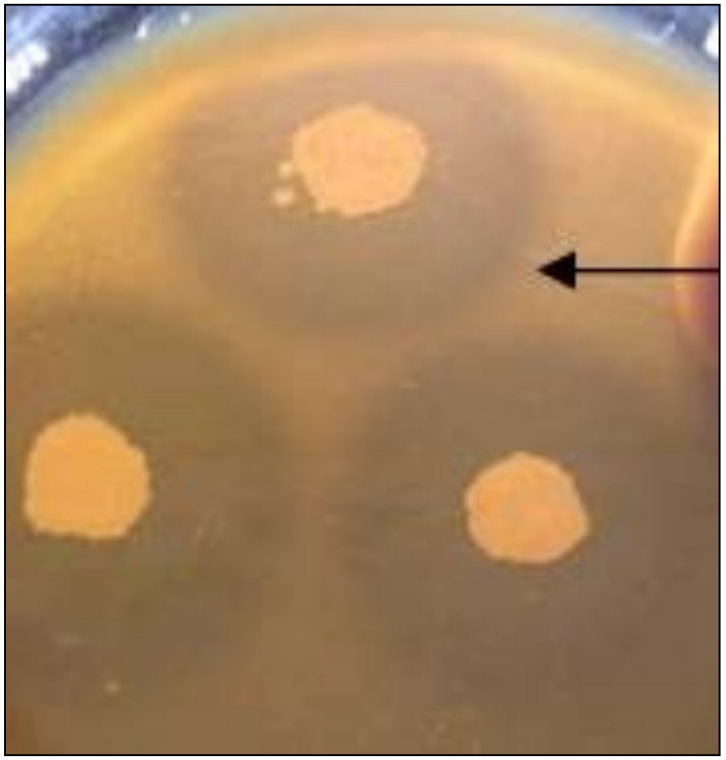
Berg *et al.*,2002;Qureshi *et al.*,2010

Mechanisms of biological control

Lytic enzymes

Screening for proteolytic activity

- Protease production was tested as described by Denizci *et al.*,2003, on Skim milk agar medium that contained 0.1% glucose, 0.2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.5% skim-milk (skim-milk was sterilized separately).
- The pH of the medium was adjusted to 10 by addition of 10% Na_2CO_3 solution after sterilization.
- Inoculated plates were incubated for 72 h at 30°C.
- Positive reaction: Production of a clear zone around the inoculated area.



Tariq *et al.*,2008

Mechanisms of biological control

Lytic enzymes

Screening for proteolytic activity

- Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rate,...
- Therefore, depending on the:

1. Zone of clearance,
2. pH, and
3. Growth temperature of organism.

- The bacterial isolates such as *Bacillus* species (*B. macerans*, *B. licheniformis* and *B. subtilis*) were selected for further experimental studies.

Olajuyigbe and Ajele,2005;..

Mechanisms of biological control

Lytic enzymes

Crude protease preparation

- The culture medium used in this work for protease production contained 0.5% glucose (w/v), 0.75% peptone (w/v), 0.5% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) KH_2PO_4, and 0.01% (w/v) $FeSO_4.7H_2O$ maintained at 37°C for 24 to 72 h in a shaking incubator (140 rpm).
- At the end of each fermentation period, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C for 15 min and the clear supernatant was used as crude enzyme preparation.

Olajuyigbe and Ajele,2005

Mechanisms of biological control

Lytic enzymes

Determination of protease activity (Protease assay)

- Protease activity was determined by spectrophotometer method in triplicate by incubating 500 ml of 0.5% azocasein in Tris-HCl buffer with 100 ml enzyme solution for 60 minutes at 37°C.
- Reaction was stopped by adding 500 ml of 15% Trichloroacetic acid (TCA) with shaking.
- This was left for 15 min and centrifuged at 4°C for 15 min at 3000 rpm.
- 1 ml of supernatant was added to 1 ml of 1 M NaOH and absorbance was read at 440 nm.
- One unit of the enzyme activity was defined as 1µg of tyrosine liberated ml^{-1} under the assay conditions.

Najafi *et al.*,2005; Olajuyigbe and Ajele,2005

Mechanisms of biological control

Lytic enzymes

Determination of protease activity (Protease assay)

- Production of protease by various strains of *P. aeruginosa*.
- PU=Protease units. The specific activity (protease units) was expressed as 1 µg of tyrosine released per minute per milliliter of enzyme solution.
- Note: part of original table was represented.

Group	Strain[a]		Proteolytic activity (PU/ml, × 10^3)
I	IFO 3455	(C)	8.0
		(S)	6.0
	No. 4	(C)	3.0
		(S)	3.6
	No. 20	(C)	4.2
		(S)	2.2
	TM 13	(C)	7.0
		(S)	6.0
	TM 14	(C)	6.6
		(S)	5.7
	TM 49	(C)	7.3
		(S)	7.4
	TM 97	(C)	0.9
		(S)	No growth
II	IFO 3080	(C)	0
		(S)	2.0
	No. 3	(C)	0
		(S)	1.5
	No. 6	(C)	0.1
		(S)	1.7
	No. 15	(C)	0
		(S)	2.3
	No. 17	(C)	0

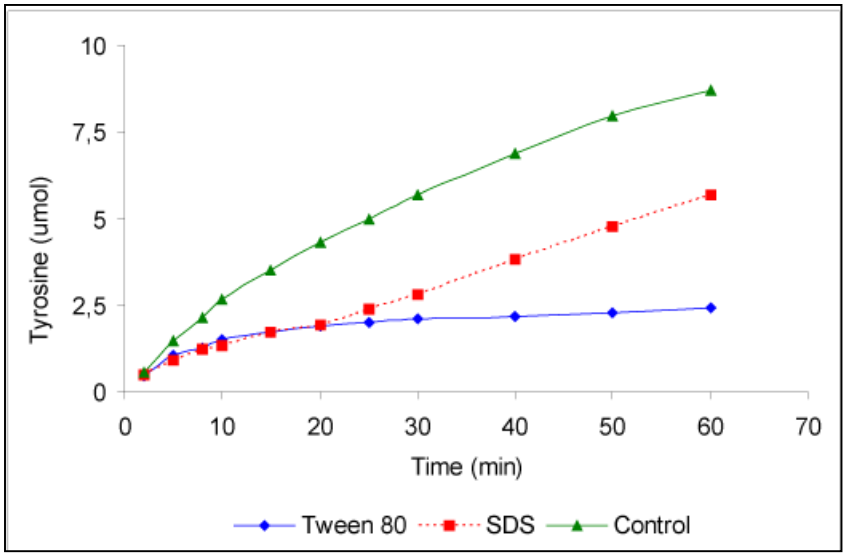
Morihara and Tsuzuki,1977

Mechanisms of biological control

Lytic enzymes

Determination of protease activity (Protease assay)

- **Effect of SDS and Tween 80 on protease.**
- The enzyme was incubated with 1% (w/v) SDS and 3% (v/v) Tween 80 along with1.5% (w/v) casein in 50 mM Tris-HCl (pH 8) at 50ºC.
- One unit of protease activity is defined as the amount of the enzyme that gives an absorbance value equivalent to 1 µg of tyrosine per min at 30 °C.



Morihara and Tsuzuki,1977; Izrael-Živkovići *et al.*,2010

Mechanisms of biological control

Determination of protease activity (Protease assay)

Alternative method

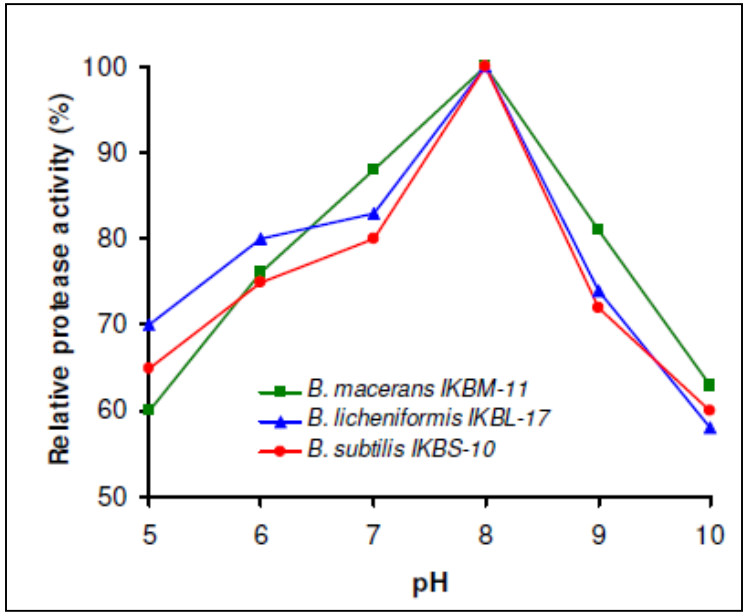
- To 0.5 ml culture broth, 0.5 ml of substrate (1% soluble casein) and 1.5 ml sodium phosphate buffer pH 7.6 were added and incubated at 35°C for one hour.
- After incubation, 2.0 ml of each sample was taken and 2.0 ml of 15% TCA was added and centrifuged for 5 min at 4000 rpm.
- To 1 ml aliquot, 4.0 ml 0.5 N NaOH and 1 ml Folin-phenol reagent (1:1) were added and then final volume was made up to 10 ml by adding 4.0 ml double distilled water.
- The absorbance was read at 625 nm.
- One unit of protease activity was defined as the amount of protease required to catalyze the liberation of 1 µg of tyrosine under the assay conditions.
- Protein content was determined by Lowry et al.(1951) method.

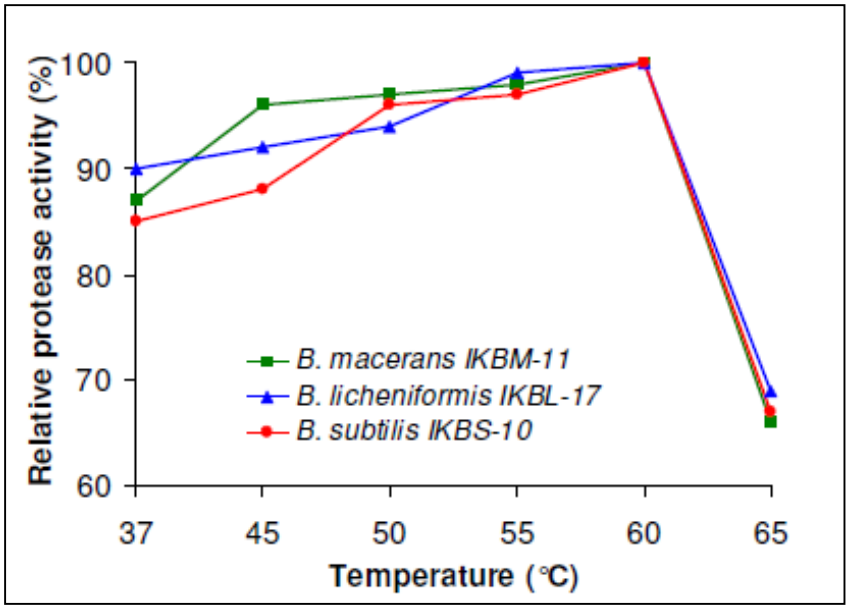
Qureshi *et al.*,2010

Mechanisms of biological control

Lytic enzymes

Determination of protease activity (Protease assay)





Effect of pH and temperature on protease production.

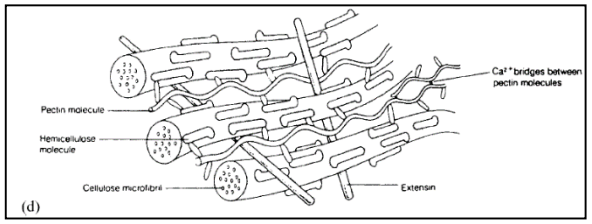
Olajuyigbe and Ajele,2005

Mechanisms of biological control

Amylase, pectinase and polygalacturanase, xylanases activities

- Amylase, pectinase and polygalacturanase, xylanases activities were detected using soluble starch (1%), pectin (0.5%), polygalacturanic acid (1%), beech xylan (0.5 % w/v), respectively.
- After incubation at 30°C for 4-5 days, the plates were flooded with specific staining solutions; congo red 0.03% for cellulase and glucanase; lugol solution for amylase; CTAB 2% for pectinase; and, ruthenium red 0.1% for polygalacturanase.
- The enzymatic activity was taken as evidence by appearance of clear zones around the colonies and their diameters were measured in millimeters.

Elshafie *et al.*,2012;..



Cellulase production

- Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase).
- Endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain.
- Exoglucanase is necessary for cleavage of the nonreducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose.

Cellulase production

Isolation and screening of cellulose-degrading bacteria

- The macerated gut of the collected organisms was inoculated in a basal salt media ($NaNO_3$ 2.5 g; KH_2PO_4 2 g; $MgSO_4$ 0.2 g; NaCl 0.2 g; $CaCl_2 \cdot 6H_2O$ 0.1 g in a liter) containing filter paper (Whatman filter paper no. 1 of area 70.541 cm^2) for the isolation of cellulolytic bacteria.
- These cultures were incubated for 7 days in a shaker incubator at 37°C at 100 rpm.
- Bacterial colonies capable of utilizing cellulose as sole source of carbon were isolated on cellulose agar media composed of KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2.0 g, agar 15 g, gelatin 2 g and distilled water lL and at pH 6.8–7.2.

Gupta *et al.*,2012

Cellulase production

Confirmation of cellulose-degrading ability of bacterial isolates

- Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the M9 medium (Miller,1974) amended with yeast extract (1.2 g L^{-1}) and cellulose (10 g L^{-1}).
- After 8 d of incubation at 28°C, isolates surrounded by clear halos were considered positive for cellulase production.

Singh *et al.*,2015 also used an alternative minimal medium salts consisting of KH_2PO_4 (1.5% w/v), $(NH_4)_2SO_4$ (0.5% w/v), $MgSO_4.7H_2O$ (0.06% w/v) and $CaCl_2.2H_2O$(w/v) +1.8% agar (w/v) for qualitative screening enzymatic activities of bacteria such as cellulase, xylanase, lipases, proteases.

Cattelan *et al.*,1999; Singh *et al.*,2015

Extracellular cellulase activity
Congo red clearing zone assay

- Nutrient agar plates were supplemented with 1% carboxy methyl cellulose (CMCA plates).
- Strains were streaked and petri plates were incubated at 37ºC for 48 hours.
- Petri plates were flooded with 0.1% Congo red reagent and left for 20 minutes.
- Then the plates were washed with 1M NaCl.
- Clearance zones called halo zones are seen against the red color of Congo red for the positive test.
- The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity.

Ponnambalam *et al.*,2011; Upadhyaya *et al.*,2012

Extracellular cellulose/xylanse activity
Congo red clearing zone assay

- Microorganisms were grown on CMC/xylan agar containing NaCl 0.5, KH_2PO_4 1.0, $MgSO_4$.$7H_2O$ 0.5, $MnSO_4.H_2O$ 0.01, NH_4NO_3 0.3, $FeSO_4.7H_2O$ 0.01, CMC/xylan 10.0, Agar 12.0 (g/L) at 7.0 pH.
- For the secretion of cellulase enzyme CMC agar plates were incubated at 30±2°C for 7-8 days.
- After incubation culture plates are flooded with 0.1% Congo red solution for 15 minutes and Congo red solution poured off and further plates washed by flooding with 1M NaCl for 10 minutes.
- A clear zone formation around the microbial colonies.

Rathore *et al.*,2014

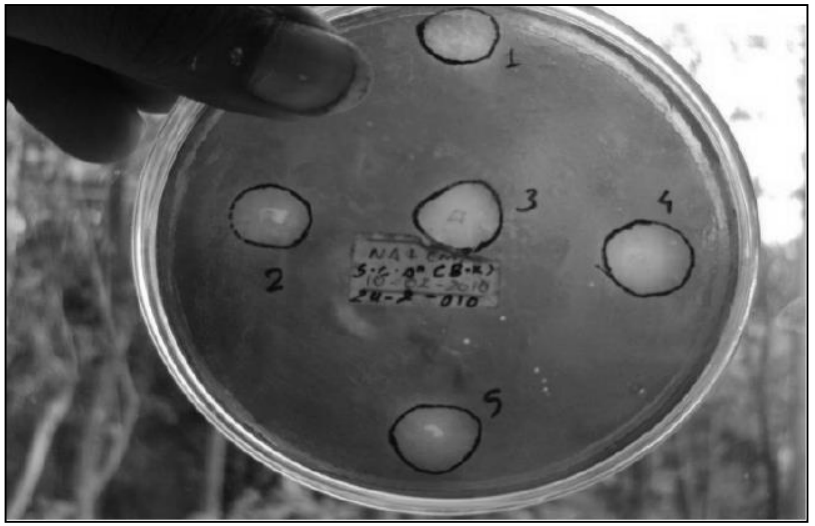
Extracellular cellulose/xylanse activity
Test tube assay

- Primary screening was made by minimal basal salt media supplementing with cellulose and xylan.
- Agar tubes were allowed to solidify in ice-bath to ensure the uniform distribution of cellulose in the tube.
- Isolated microbial cultures were inoculated on agar slant and tubes were incubated at 30±2°C.
- Vertical zone of clearance from agar surface was measured for cellulytic activity after 7 days.

Jahangeer *et al.*,2005; Rathore *et al.*,2014

Extracellular cellulase activity
Congo red clearing zone assay

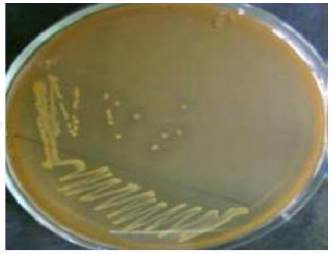
- Clearance zones (halo zones) are seen against the red color of Congo red for the positive test.



Upadhyaya *et al.*,2012

Extracellular cellulase activity

Congo red clearing zone assay



- **Cellulose Congo-Red agar media:**
- KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1L and at pH 6.8-7.2.
- The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria.
- Then the plates were washed with 1M NaCl.
- Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies.

Gupta *et al.*,2011; Mekar *et al.*,2014

Cellulase production

Pure, solidified CMC plate-based clearing assay

- A solution of 7% CMC was heated to 70°C, poured into petri dishes and was allowed to polymerize at room temperature overnight.
- Spot plating was performed with 5 µL of cellulase (1 µg/µL), amylase (1 µg/µL) and agarase (1 µg/µL), followed by an incubation at 27°C for 12-16 h.
- Hydrolysis zones were visualized by flooding of the plates with Gram's iodine (2 g potassium iodide and 1 g iodine in 300 mL water) as described above.

Johnsen and Krause,2014

Extracellular cellulase activity
Cellulase production

- The selected CDB isolates were cultured at 37°C at 150 rpm in an enzyme production media composed of KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, and gelatin 2 g, distilled water 1 L and containing Whatman filter paper No.1 (1 × 6 cm strip, 0.05 g per 20 mL) and at pH 6.8-7.2.
- Broth culture after three days of incubation period was subjected to centrifugation at 5000 rpm for 15 min at 4°C.
- Supernatant was collected and stored as crude enzyme preparation at 4°C for further enzyme assays.
- Pellet recovered after centrifugation of broth culture was subjected to gravimetric analysis in order to determine the residual cellulose of filter paper.

Gupta *et al.*,2012

Extracellular cellulase activity
Enzyme(cellulase) Assay

- Total cellulose activity was determined by measuring the amount of reducing sugar formed from filter paper.
- Endoglucanase (β 1-4 endoglucanase-EC 3.2.1.4) activity was assayed by measuring the amount of reducing sugar from amorphous cellulose.
- The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology.
- Endoglucanase activity was determined by incubating 0.5 mL of supernatant with 0.5 mL of 2% amorphous cellulose in 0.05 m sodium citrate buffer (pH 4.8) at 50 for 30 min.

Gupta *et al.*,2011

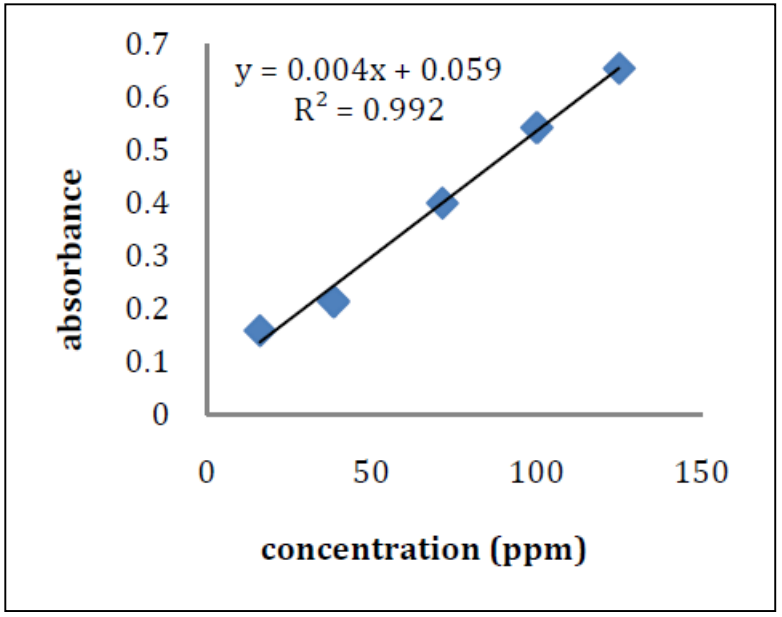
Extracellular cellulase activity
Enzyme(cellulase) Assay

- FPC (filter paper cellulase) activity was determined by incubating 0.5 mL of supernatant with 1.0 mL of 0.05 M sodium citrate buffer (pH 4.8) containing Whatman no.1 filter paper strip-1.0 × 6.0 cm (=50 mg).
- After incubation for an hour at 50°C, the reaction was terminated by adding 3 mL of 3, 5-dinitrosalicylic acid (DNS) reagent to 1 mL of reaction mixture. In these tests, reducing sugars were estimated spectrophotometrically with 3, 5-dinitrosalicylic acid using glucose as standards.
- The enzymatic activity of total FPCase and endoglucanase were defined in international units (IU).
- One unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol reducing sugars (measured as glucose) per mL per minute.

Gupta *et al.*,2011

Extracellular cellulase activity
Enzyme(cellulase) Assay

- Cellulase system consist of: 1,4-β-endoglucanase, 1,4-b-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase).
- The synergy of third enzymes do complete hydrolysis of cellulose to glucose.
- In this study, glucose used as standard and DNS reagent used to stop the enzymatic reaction, so the reaction product can be measured.
- The reaction between glucose and the DNS reagent gave maximum absorption at 450 nm.



Glucose calibration curve

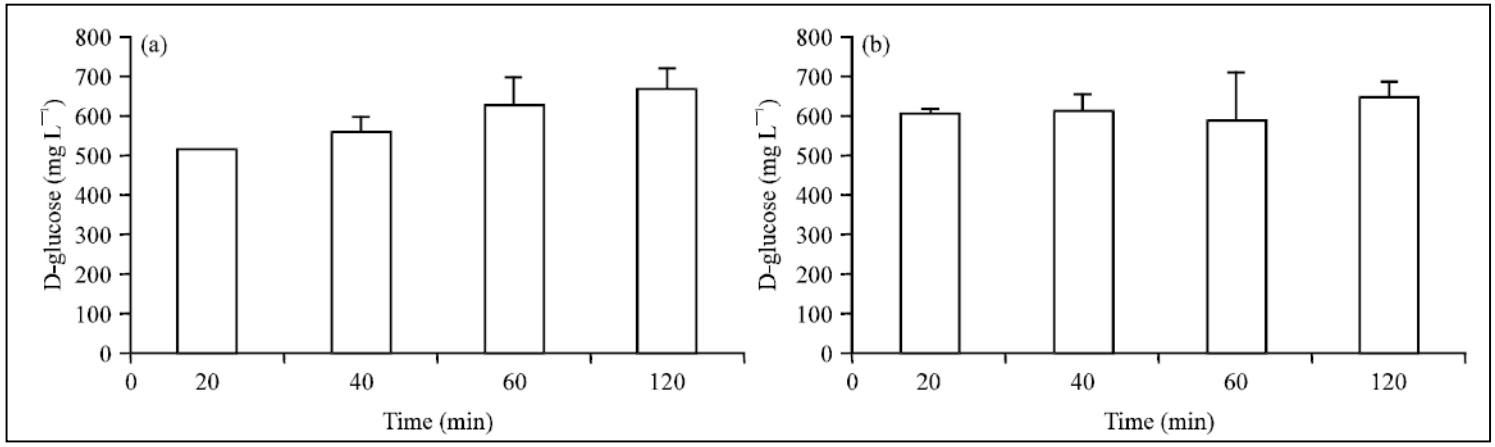
Mekar *et al.*,2014

Extracellular cellulase activity
Enzyme(cellulase) Assay

- Comparison of cellulase activity *Aspergillus niger* cellulase using carboxymethyl cellulose as a substrate at different time points.
- Detection of reducing sugars using

a) The 2-cyanoacetamide method, and

b) The dinitrosalicylic acid (DNS) method.

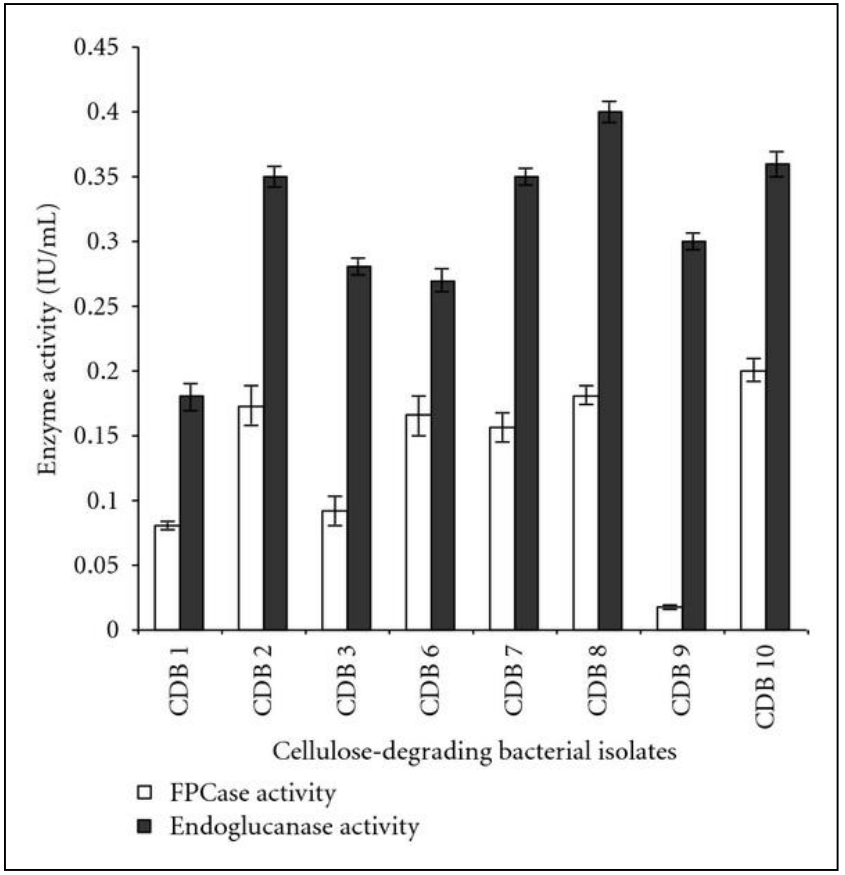


Bars represent the mean values assayed in triplicate that were adjusted for background, The error bars indicate the standard deviation.

Jurick *et al.*,2012

Extracellular cellulase activity
Enzyme(cellulase) Assay

- Extracellular cellulase activity of two enzymes: FPCase (filter paper cellulase) and endoglucanase of all cellulose-degrading bacterial (CDB) isolates.
- The activities ranged from 0.012 to 0.196 IU/mL for FPCase and 0.1622 to 0.400 IU/mL for endoglucanase assay.
- Values in figure are means of three replicates with standard deviation.



Gupta *et al.*,2011

Pectinase production

- Pectinase production was determined in the same M9 medium except the cellulose was replaced with pectin (4.8 g L^{-1}).
- After 2 d of incubation at 28°C, the plates were flooded with 2 M HCl (T. Denny, 1997, personal communication), and isolates surrounded by clear halos were considered positive for pectinase production.

Cattelan *et al.*,1999

Pectinase production
Fermentation and media

- A basic liquid medium was used for the production of pectinase having compostion (g/ml), Pectin (1.0), Ammonium dihydrogen sulphate (0.14), Potassium dihydrogen phosphate (0.2), Potassium hydrogen phosphate (0.6)/Magnesium sulphate (0.02) at pH 7.2.
- The production media was incubated for 48 h for submerged fermentation.

Tripathi *et al.*,2014

Pectinase production
Fermentation and media

- The enzyme pectinase was produced by using isolated bacterial species was carried out with a basic pectinase production media (pH 7.2) by submerged fermentation after incubation of 48 h.
- The crude enzyme was recovered.
- The specific activity of the crude enzyme was found to be 3.1 Umg^{-1}.
- The crude enzyme was purified by ammonium sulphate precipitation and followed by Dialysis and aliquates are applied to agarose ion- exchange column (1*30).
- After this step Specific activity was 4.5 Umg^{-1} and enzyme was 1.5 fold purified.
- Maximum yield observed about 80% for the process most recently this was found specific activity 0.185 for *Bacillus subtilis*.

Tripathi *et al.*,2014

Pectinase production

Enzyme extraction and purification+Ion exchange chromatography

- **Enzyme extraction and purification:**
- Culture medium was centrifuged and supernatant was used as crude enzyme source. The crude enzyme was precipitated by 60% ammonium sulfate saturation, incubated for night at 40°C and centrifuged at 5000 rpm for 20 min and palates were dissolved in T.E. buffer at pH 7.
- Dialysis was also performed against T. E. buffer overnight at 40°C.
- **Ion exchange chromatography:**
- Dialyzed buffer was directly applied to the agarose column (1×30 cm.) equilibrated with TE buffer (pH 7.0). Elution was carried out by liner gradient of NaCl (0.1M - 0.6M). About 5 ml of fraction collected and activity was observed.

Tripathi *et al.*,2014

Pectinase production

Enzyme assay

NS and DNS methods

- Pectinase activity was measured by estimation of glucose by DNS method using pectin as substrate.
- The Nelson-Somogyi (NS) and 3,5-dinitrosalicylic acid (DNS) assays for reducing sugars(Rss) are widely used in measurements of carbohydrase activities against different polysaccharides.
- The RSs released in hydrolysis were analyzed using the NS and DNS.

The reducing sugar can reduce the nitro of 3,5-dinitrosalicylic acid (DNS) to amino, thereby generating a reddish brown colour for amino compounds.

Tripathi *et al.*,2014

Pectinase production

Enzyme assay

Crude and ion exchange

- Standard graph prepared by concentration of standard glucose solution.
- One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µm glucose per min.

Purification table for Pectinase Enzyme. The yield of purified enzyme was about 80% and maximum specific activity was obtained 4.5 Umg^{-1}.

sample	Total Protein (mg)	Activity (Unit)	Specific Activity (U/mg)	Fold	Yield
Crude	0.56	0.18	3.1	1	100
Ion Exchange	0.45	0.10	4.5	1.5	80

Tripathi *et al.*,2014

Pectinase production

Pectinolytic activity assay

NS and DNS methods

- **Pectinase production in apple and orange Pomaces Media:**
- The ability of the active *Streptomyces* sp. (strain J9) to grow in apple and orange broth media was tested.
- The media was prepared as follows: 1 g of dried fruit pomaces (apple or orange) was suspended in 100 ml distilled water, then supplemented with 0.1 ml trace salt solution composed of 1 ml/L of $FeSO_4.7H_2O$, 0.1 g of $MnCL_2.4\ H_2O$ and 0.1 g of $ZnSO_4.7H_2O$.
- The pH of the media was adjusted to 7.5 prior to autoclaving.
- Flasks containing the media were inoculated with 1 ml spore suspension. Cultures were incubated at 28°C for 6 days.
- Enzyme activity was performed using the standard DNS method as mentioned before.

Saadoun *et al.*,2013

Pectinase production

Pectinolytic activity assay

NS and DNS methods

- Pectinase assay was performed following the procedure of Miller,1959 with some modification.
- Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution, plus 1.8 ml of 1.0% (w/v) citrus pectin in 50 mm sodium phosphate buffer (pH 7.0) was incubated at 37°C in a shaker water bath (GFL, Germany) for 30 min.
- The reaction was terminated by adding 3 ml of DNS reagent.
- The color was then developed by boiling the mixture for 5 min.
- Optical densities of samples were measured at 575 nm against a blank containing the reaction mixture minus the crude enzyme.
- Results were then compared to controls inoculated with an inactive pectinolytic streptomycete isolate.
- Results were interpreted in terms of enzyme activity in which one unit of enzyme activity (U) was defined as the mount of enzyme releasing one μmol reducing groups (D – galacturonic acid) per min under these assay conditions.

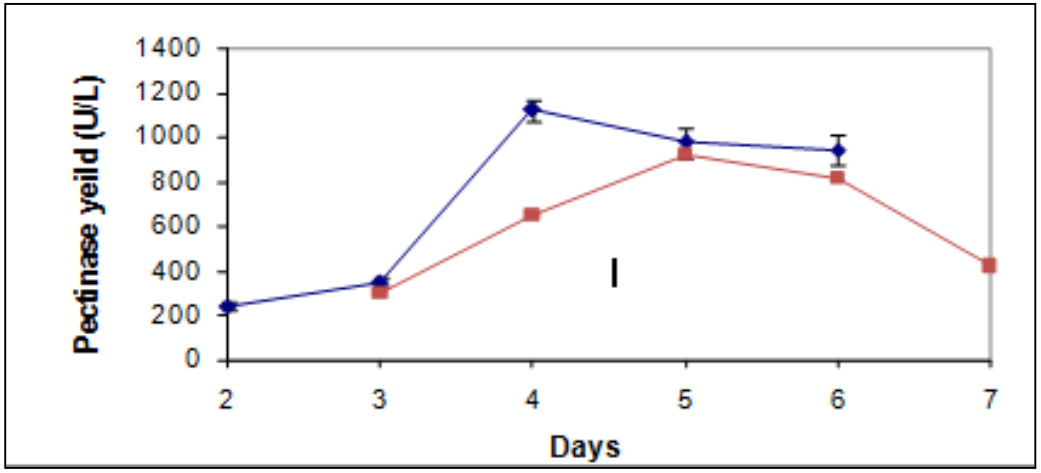
Saadoun *et al.*,2013

Pectinase production

Pectinolytic activity assay

NS and DNS methods

- Production of pectinase enzyme by the J9 *Streptomyces* isolate in apple (♦) and orange (▪) pomace medium.
- Points represent the standard errors for means at α=0.05



The standard error(SE) is very similar to standard deviation. Both are measures of spread. The higher the number, the more spread out your data is. To put it simply, the two terms are essentially equal — but there is one important difference. While the standard error uses statistics (sample data) standard deviations use parameters (population data). The SE tells you how far your sample statistics (like the sample mean) deviates from the actual population mean.

Saadoun *et al.*,2013

Lipase activity

- The lipase activity of the selected endophytic bacterial isolates was determined by supplementing the nutrient agar media with 0.01% $CaCl_2.H_2O$, followed by adding sterilized Tween 80 to the media to give a final concentration of 1%.
- The media was poured into the Petri plates, and presence of opaque halo zone around the colonies was considered as positive (Sierra, 1957).

Prasad and Dagar,2014

Tannase activity

- Tannase activity was detected employing the modified method devised by Osawa,1990 in which nutrient agar was used in place of brain heart infusion agar.
- Hydrolysis capacity (HC) index for all ten isolates was calculated by dividing the diameter of the clear zone around the colony with bacterial colony diameter.
- Hydrolysis capacity (HC) index provides a semi-quantitative measure of these bacteria's enzyme production.

Singh *et al.*,2015

Mechanisms of biological control

Hydrogen cyanide (HCN)

An volatile antibiotic

- Hydrogen cyanide (HCN) is a volatile toxic substance. It effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations.
- The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens.
- *P. fluorescens* CHA0 produces antibiotics, siderophores and HCN, but suppression of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be due primarily to HCN production (Voisard *et al.*,1989).
- Howell *et al.*,1988 reported that volatile compounds such as ammonia produced by *Enterobacter cloacae* were involved in the suppression of *Pythium ultimum*-induced damping-off of cotton.

Mechanisms of biological control
Antifungal volatile compounds assay

- For antifungal volatile compounds production assay, divided plates were used.
- Both compartments of plates were added with PDA medium.
- One compartment was inoculated with a plug of freshly grown *Fusarium oxysporum* f. sp. *niveum* and other compartment was inoculated with *Streptomyces goshikiensis* YCXU.
- The plates were sealed with Parafilm and fungal growth was measured on daily basis. Two control treatments were included: one was containing *Escherichia coli* DH5ά in place of strain YCXU and second without any bacterial inoculation.

Faheem *et al.*,2015

Mechanisms of biological control
Hydrogen cyanide (HCN) production assay

- Test for HCN production was carried out by the method of Lorck, modified by Alstrom and Burns,1989.
- The HCN vapors react with the picric acid, reduced to coloured isopurpuric acid and formed red color on a test.
- Bacteria were heavily inoculated in nutrient agar plates supplemented with 4.4 g/L glycine and incubated in an inverted position at 30°C with filter paper strips dipped in picric acid solution (0.5% picric in 2% Na_2C0_3 aqueous solution) placed inside the lids.
- Change of colour of the indicator strip from yellow to brown was considered as a positive result.

Mechanisms of biological control

Hydrogen cyanide (HCN) production assay

- The production of HCN was estimated by the method of Wei et al.,1991).
- The cultures were grown on KM plates supplemented with 4.4 g/l glycine as a precursor and the filter paper strips soaked in saturated picric acid solution were exposed to the growing *Pseudomonas* isolates.
- The plates were incubated for 7 days at 28 ± 2°C and observations were recorded as change in the colour of filter paper to brown as positive indicator for HCN production.

Kotasthane *et al.*,2017

Mechanisms of biological control

Hydrogen cyanide (HCN) production assay

Qualitative assay

- Hydrogen cyanide (HCN) production of fungal and bacterial biocontrol agents was tested qualitatively following the method of Bakker and Schipper,1987.
- The antagonistic bacteria were streaked on King's B medium amended with glycine at 4.4g/l. sterile filter paper saturated with picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3, 1000 ml of distilled water) was placed in the upper lid of the Petri plate.
- The dishes were sealed with parafilm and incubated at 28°C for 48 h.
- A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction respectively.

Reetha *et al.*,2014

Mechanisms of biological control

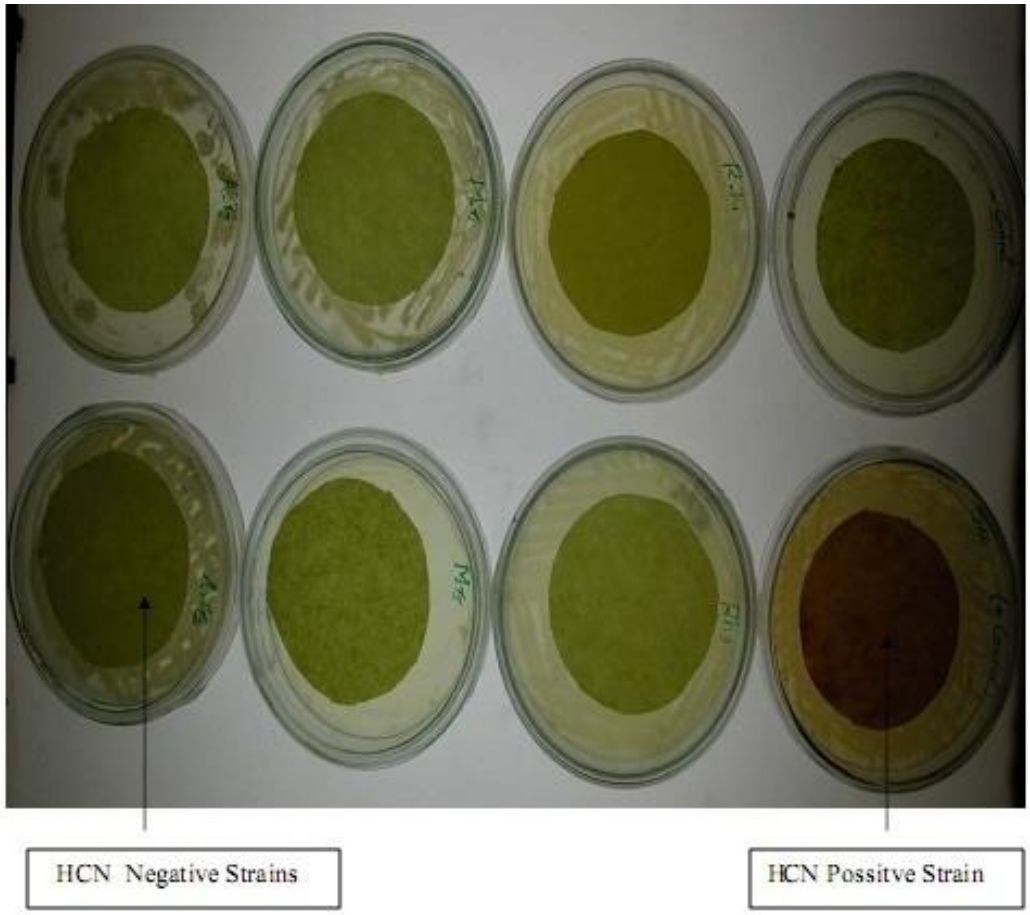
Hydrogen cyanide (HCN) production assay

- Production of HCN was observed according to method of Lork (1948).
- Freshly grown cells were spread on king's B medium containing glycine (4.5 g/L).
- A sterilized filter paper saturated with 2% solution of sodium carbonate and 1% solution of picric acid was inserted in the upper lid of a petri plate.
- The Petri plate was sealed with parafilm and incubated at 30°C for 4 days.
- A change in colour of the filter paper from yellow to reddish brown was used an index of cynogenic activity.

Tariq *et al.*,2008

Mechanisms of biological control

Hydrogen cyanide (HCN) production assay



Tariq *et al.*,2008

Mechanisms of biological control

Hydrogen cyanide (HCN) production assay

Quantitative assay

- Antagonistic bacteria were grown in King's B broth amended with glycine (4.4 g/l) and uniform strips of filter paper (10 x 0.5 cm^2) were soaked in alkaline picrate solution and kept hanging inside the conical flask.
- After incubation at 28 ± 2°C for 48 h the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of HCN evolved.
- The colour was eluted by placing the filter paper in a test tube containing 10 ml of distilled water and its absorbance was read at 625 nm (Sadasivam and Manickam, 1992).

S. No.	Isolates	HCN	
		Qualitative	Quantitative (O.D. Value)
1.	Bs10	-	0.015
2.	CBs5	-	0.004
3.	Pf1	+++	0.094
4.	CPf5	+++	0.085

HCN production - negative, + weak, ++ moderate, +++ strong

Reetha *et al.*,2014

Screening for putative biological control agents and PGPRs

2. The '*in planta*' assays

PGPR

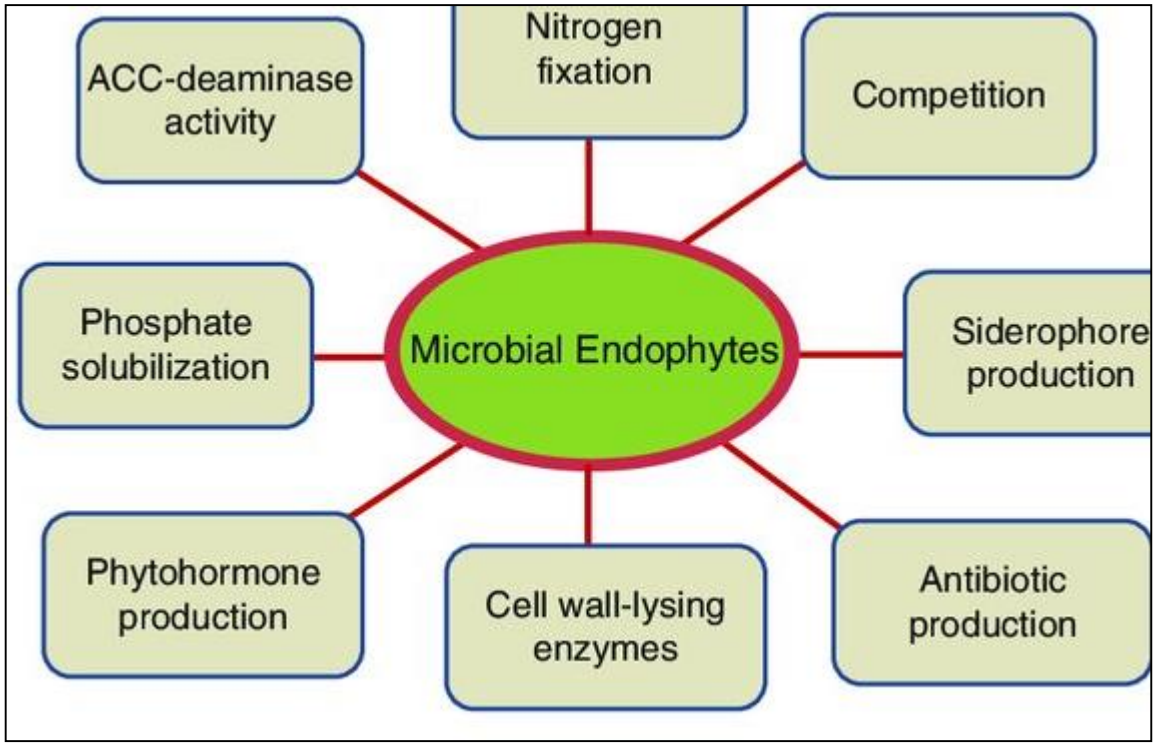
Role of PGPR in sustainable agriculture

- In the present work, two bacterial isolates *A. aneurinilyticus* (ACC02) and *Paenibacillus* sp. (ACC06) with a high amount of ACC deaminase activity were selected from the rhizosphere of *A. sativum* crop plant for further assessment for growth promoting abilities such as:
- IAA production,
- insoluble phosphate,
- zinc solubilization,
- siderophore secretion,
- HCN, and
- ammonia production.

Bücking *et al.*,2019

PGPR

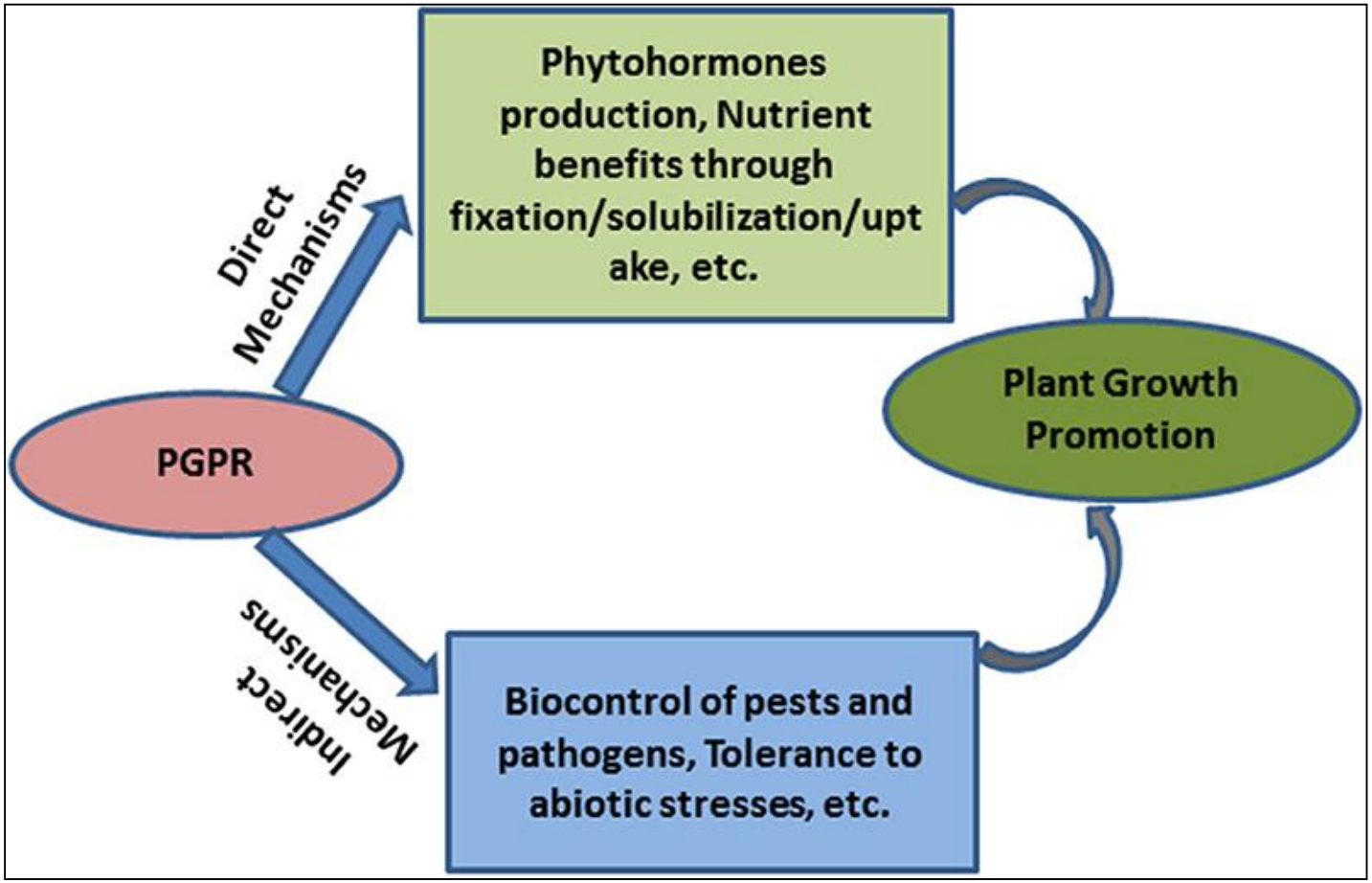
Role of microbial endophytes in sustainable agriculture



Vyas,2018

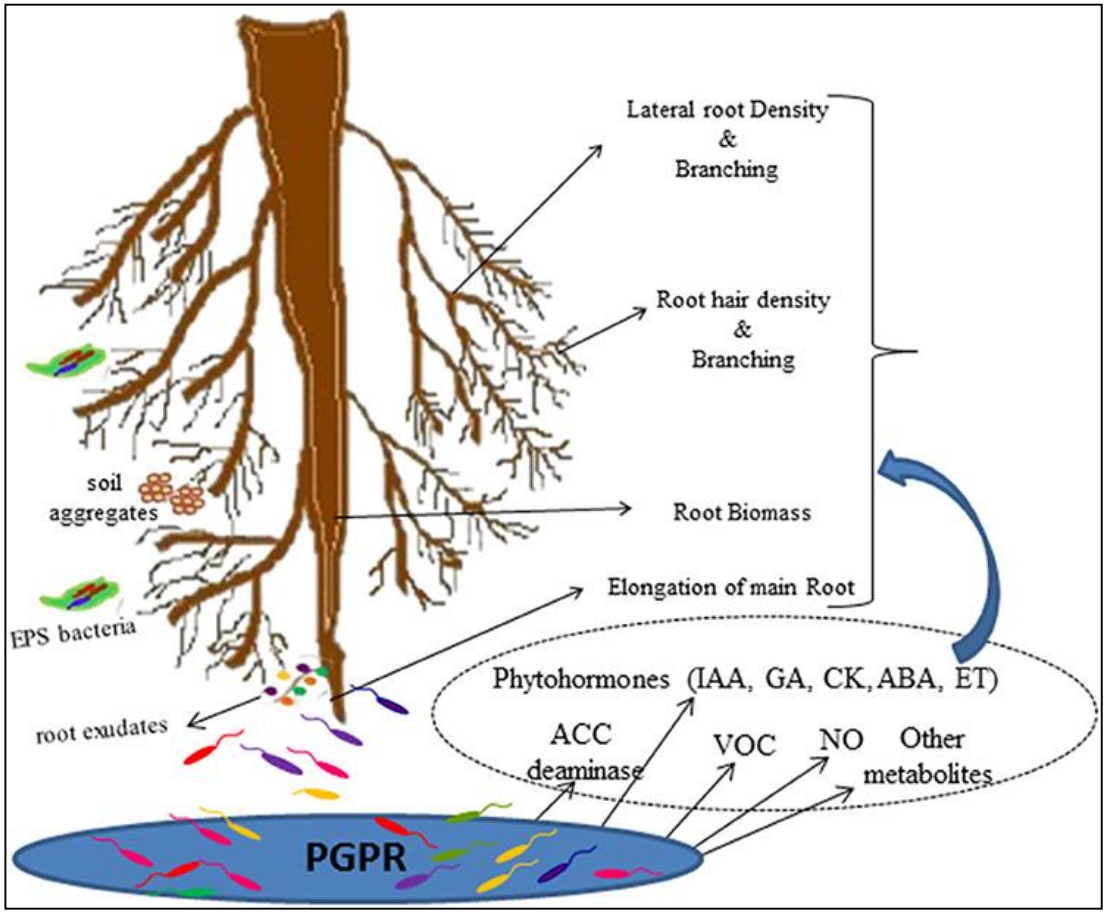
PGPR

Mechanisms of plant growth promotion by PGPR

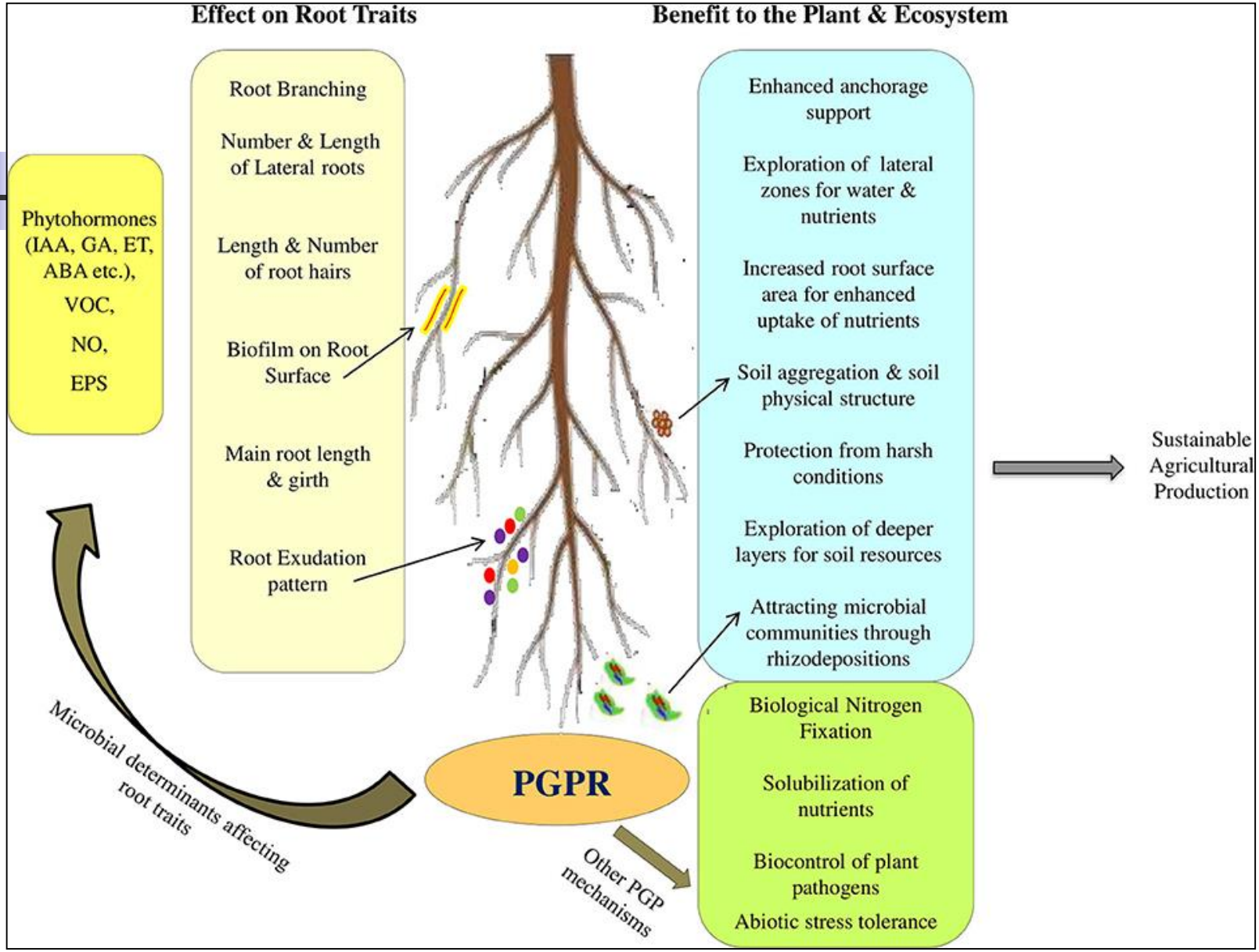


Grover *et al.*,2021

PGPR-Root interaction



Grover *et al.*,2021

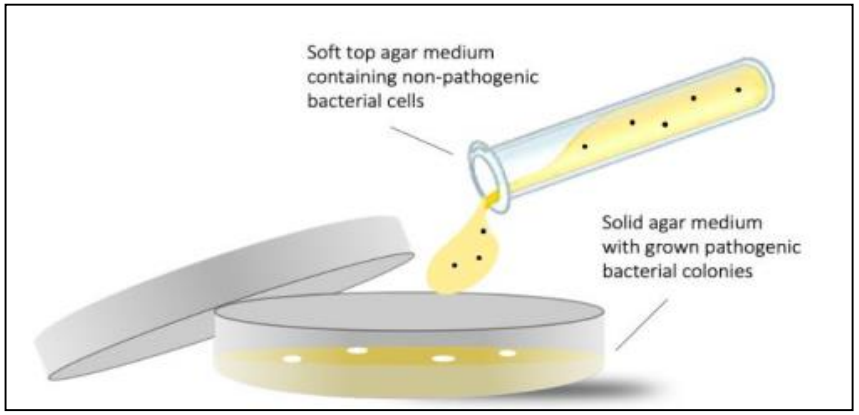


Grover *et al.*,2021

Screening for putative biological control agents and PGPRs

Overlay method for antagonism evaluation

- Bacteria against bacteria:
- Pathogenic strain was planted on solid NA media.
- Next, the top soft agar containing non-pathogenic test bacteria was immediately poured into each solid medium.
- Then incubated at 37°C for 24 h to allow all media to solidify.

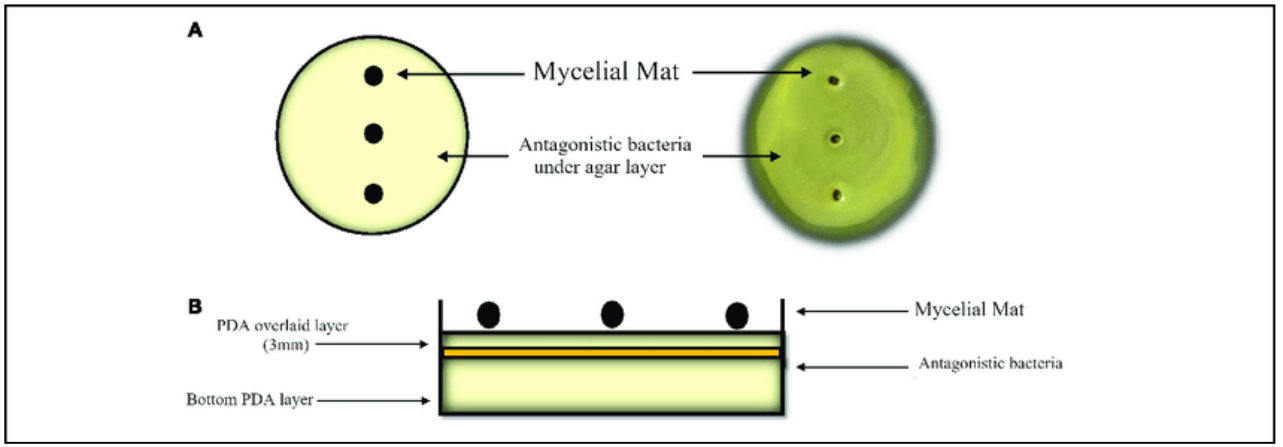


Norma Ethica *et al.*,2019

Screening for putative biological control agents and PGPRs

Antagonistic assay by the agar double layer method

A. Bacteria against fungi:
B. Top view,
C. Side view of the first agar layer (bottom) to support bacterial growth and biofilm development after 24 h. A three mm PDA layer (overlaid) is placed onto bacterial grown as top layer for mycelial mat grown.



Macedo-Raygoza *et al.*,2019

PGPR
Seed bacterization

- Pearl millet seeds were surface sterilized with 1% sodium hypochlorite for 5 min and washed five times with sterilized distilled water.
- Seeds were coated with peat based inoculum of bacteria (10^8-10^9/g peat) using 1% carboxymethylcellulose(CMC) as adhesive, dried in air and the cell count was 10^6-10^7 CFU per seed before sowing.
- Increase in germination, shoot length, leaf area, root length, and plant dry weight (biomass) after seed treatment in repeated experiments at glasshouse conditions were recorded.

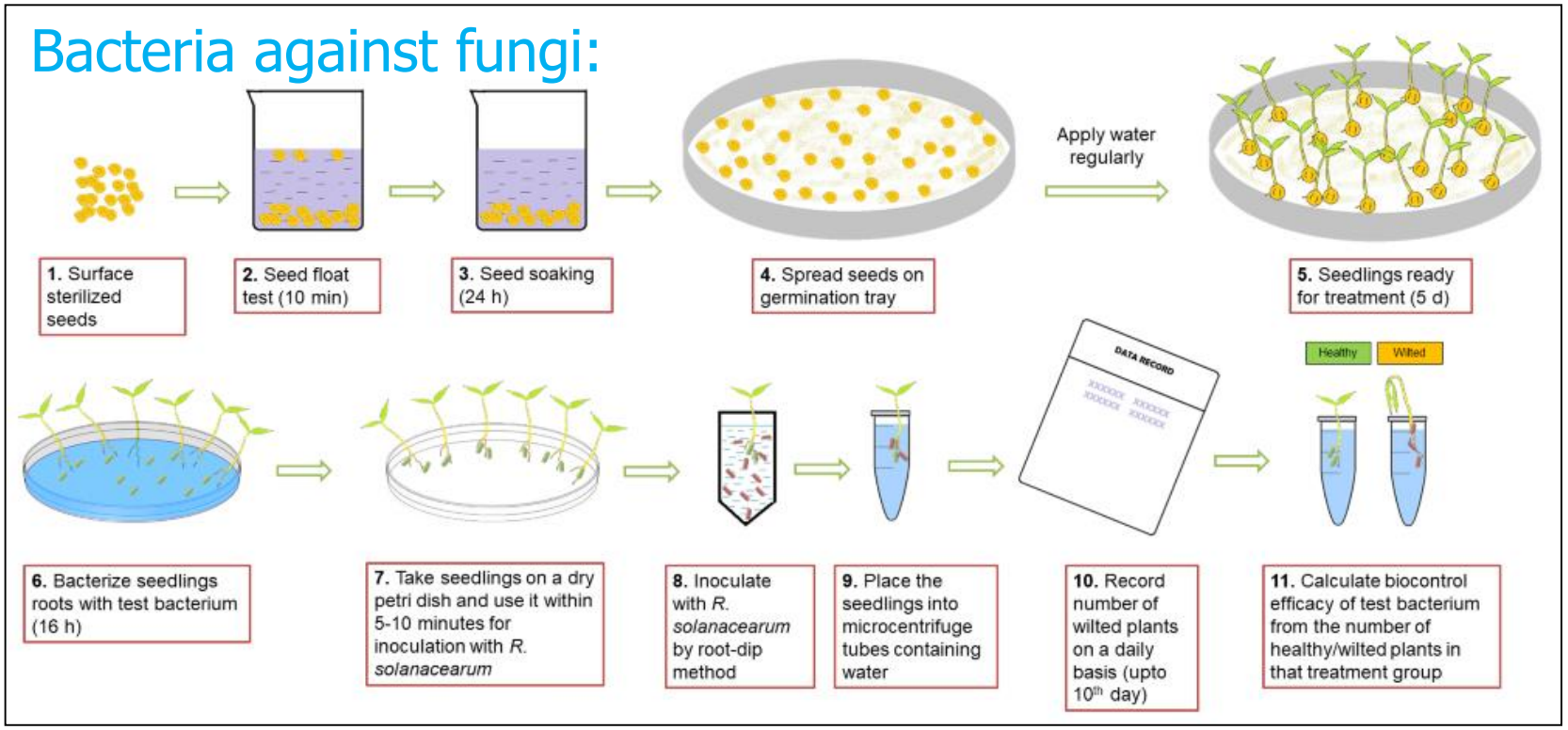
Hameeda *et al.*,2006

Biocontrol agents

Root bacterization

A stepwise demonstration of the protocol is given below

A Quick Method for Screening Biocontrol Efficacy of Bacterial Isolates Against Bacterial Wilt Pathogen *Ralstonia solanacearum* in Tomato



Agarwal *et al.*,2020

PGPR

Seed bacterization

Against fungal pathogens

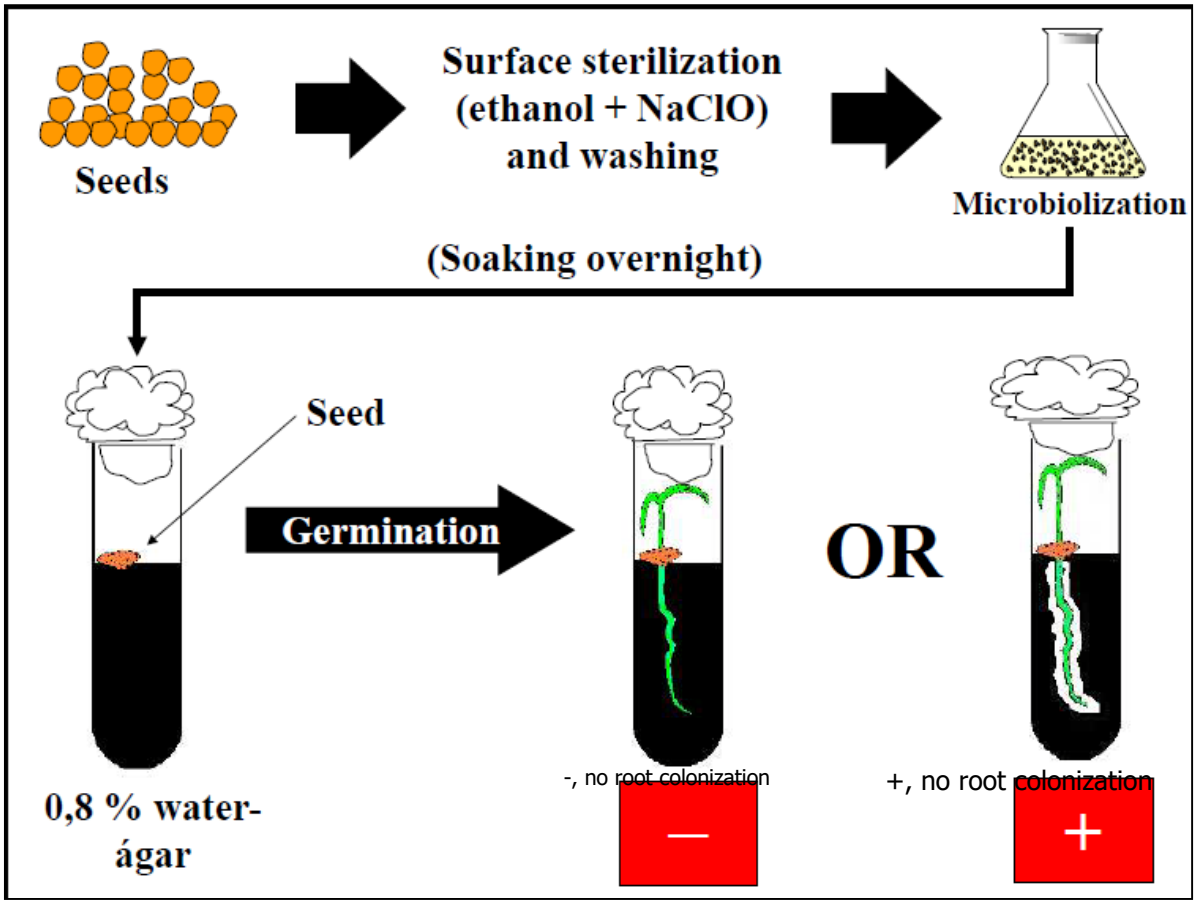
- Surface-sterilized seeds are placed on the soil surface and inoculated with approximately 50 ml of 10^7 cfu/ml of the tested PGPR suspension.
- The seeds are then covered with an additional 2.0 cm layer of infected soil mixture, and an additional 3 ml of nutrient solution are added.
- Untreated seeds planted in pathogen-inoculated in mixtures of sand and vermiculite and reference PGPR strains, are used as positive and negative controls, respectively.
- A strain is considered a promising biocontrol agent if it performs as well as one of the reference strains.

Bashan *et al.*,1993

PGPR

Seed bacterization

Root colonization

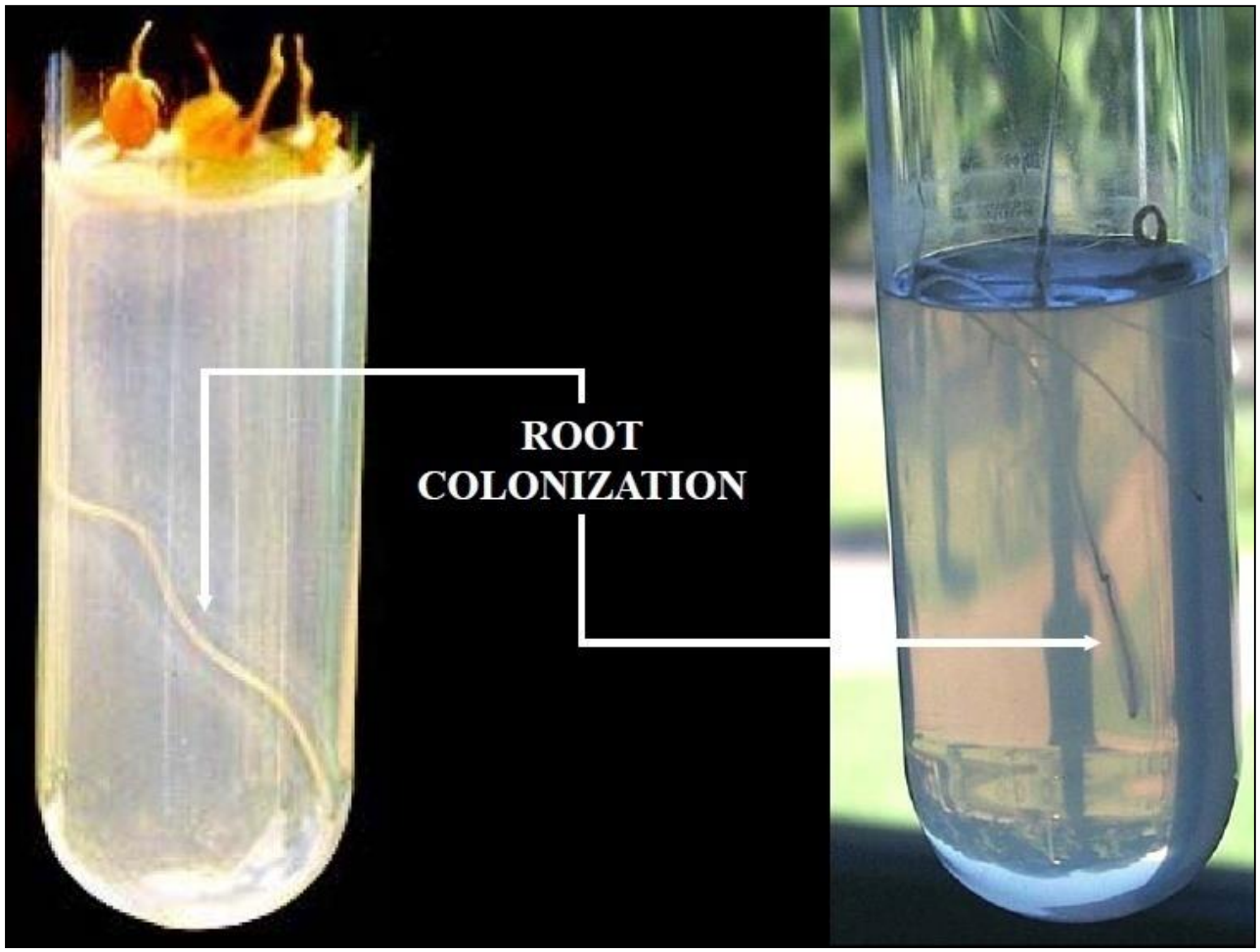


Romeiro,2010

PGPR

Seed bacterization

Root colonization



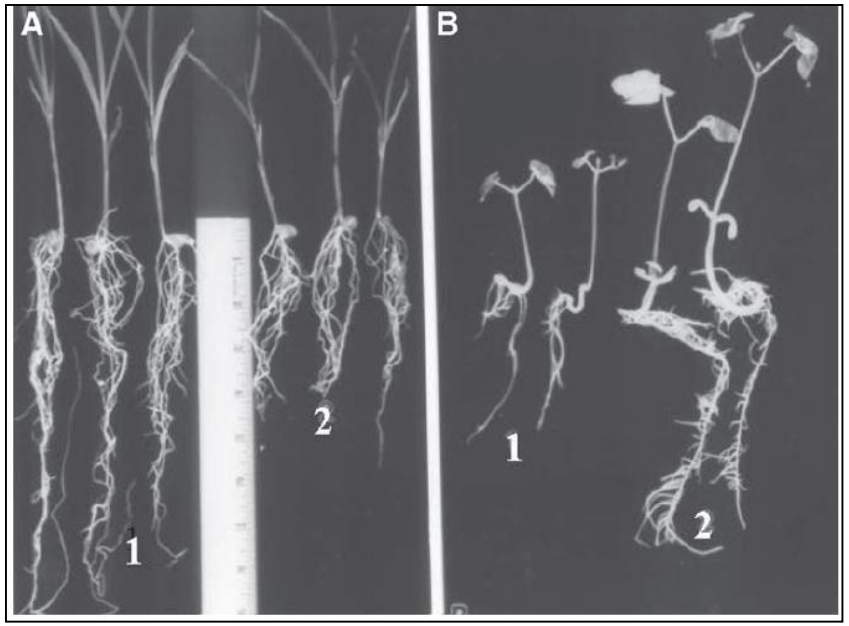
PGPR

Effects *of Bacillus mojavensis* on corn and bean seedlings growth

- Effects of *Bacillus mojavensis* RRC101 on plant growth.

A. Effects of *Bacillus mojavensis* RRC101 on 3-wk-old corn seedling root growth, with (1) and without (2) the bacterium.

B. Effects on bean seedling growth, with (1) and without (2) the bacterium.



Spencer and Spencer,2004

PGPR

Root colonization by fluorescent pseudomonads

- Disease classes of *Rhizoctonia* root rot on beans in which
- 0 = healthy, absence of symptoms;
- 1 = small black or brown lesions less than 1mm in diameter;
- 2 =lesion covering less than 75% of the stem and/or root surface;
- 3 = lesion covering more than 75% of the stem and /or root surface;
- 4 = seedling dead.

Olorunleke *et al.*,2011

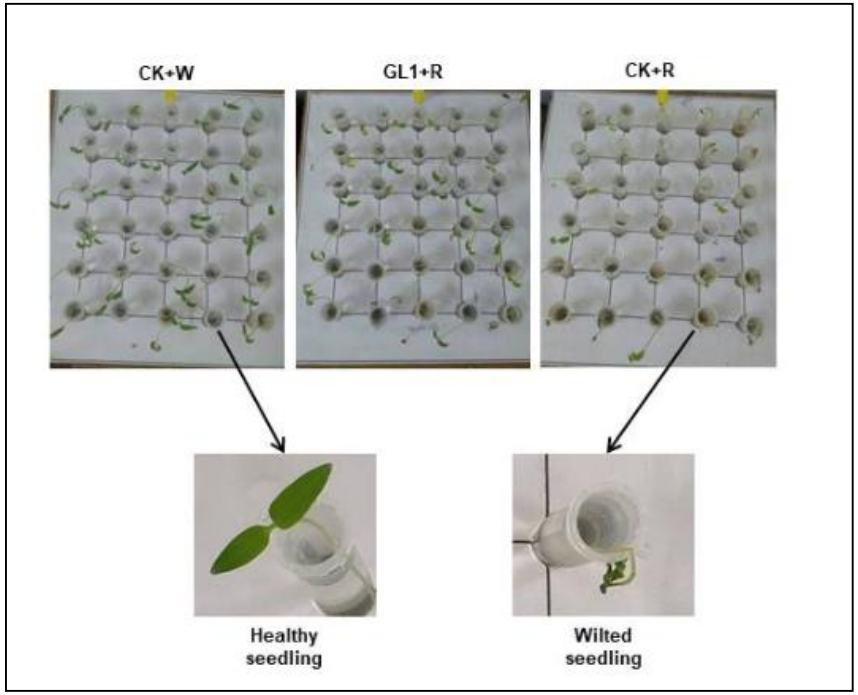
PGPR

Seed bacterization

A stepwise demonstration of the protocol is given below

Schematic diagram representing biocontrol efficacy assay for *R. solanacearum* antagonizing bacteria

- GL1+R: inoculation with antagonizing bacterium *Staphylococcus warneri* GL1 followed by *R. solanacearum*, and
- CK+R: mock(replicate) inoculation with sterile water followed by *R. solanacearum* inoculation.



Agarwal *et al.*,2020

Nitrate reducing bacteria
Two-step process

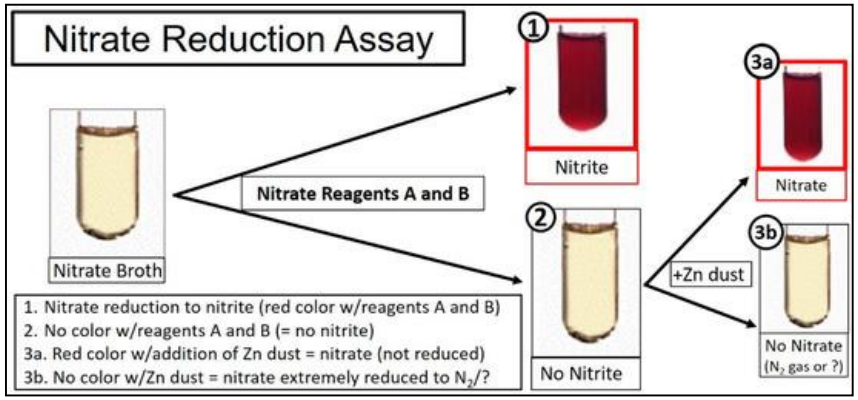
- Nitrite detection is a two-step process whereby the reduction of nitrate to nitrite is determined by the addition of nitrate reagent A (sulfanilic acid) followed by reagent B (alpha-napthylamine).

1. If a red color develops, this then confirms the presence of nitrite.
2. If there is no color change, then either nitrate remains unreduced and is determined by the addition of zinc powder which is a strong reducer that will reduce nitrate to nitrite to turn the culture red, confirming there was unreduced nitrate in the tube.
3. If there is still no color change with the addition of zinc powder, then the only explanation is that nitrate was reduced to nitrite, and then further reduced to other nitrogen compounds.

Bhusal and Muriana,2021

Nitrate reducing bacteria
Two-step process

- Nitrate reagent A: sulfanilic acid (Fisher); 1 g in 200 mL of 5 N acetic acid,
- Nitrate reagent B: alpha-napthylamine (Fisher); 2 g in 250 mL of 5 N acetic acid,
- Reagent C:
- Zinc powder (50 mg; Fisher).



Bhusal and Muriana,2021

Isolation and identification of nitrate reducing bacteria

Nitrate reduction-PCR analysis

- Colonies with red color zones were isolated from the on-agar nitrate reduction assay plates, cultured, and confirmed for nitrate reducing activity in nitrate broth.
- Further identification was obtained by PCR amplification of DNA corresponding to the 16S rRNA sequences using universal 16S rRNA primers designated
- 8-Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and
- 1541-Reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) to provide amplification of a 1533-bp stretch of 16S rRNA sequence for bacterial identification.
- Both primers have a melting temperature (T_m) of 55°C.

Bhusal and Muriana,2021

Isolation and identification of nitrate reducing bacteria

Nitrate reduction-PCR analysis

- Thermal cycling was performed:
- initial denaturation at 95°C for 4 min, followed by 40 cycles of 94°C for 1 min (denaturation), 60°C for 45 s (annealing), 72°C for 1 min (extension), followed by a final extension cycle at 72°C for 4 min, and a final hold at 4°C.
- Amplified products were cleaned up using the GenCatch™ PCR Cleanup Kit (Epoch Life Sciences, Sugarland, TX, USA) to purify amplimers after PCR from enzymes, dNTPs, and salts, and then submitted to DNA Sequencing.

Bhusal and Muriana,2021

Isolation and identification of nitrate reducing bacteria

Nitrate reduction-PCR analysis

- Thermal cycling was performed:
- initial denaturation at 95°C for 4 min, followed by 40 cycles of 94°C for 1 min (denaturation), 60°C for 45 s (annealing), 72°C for 1 min (extension), followed by a final extension cycle at 72°C for 4 min, and a final hold at 4°C.
- Amplified products were cleaned up using the GenCatch™ PCR Cleanup Kit (Epoch Life Sciences, Sugarland, TX, USA) to purify amplimers after PCR from enzymes, dNTPs, and salts, and then submitted to DNA Sequencing.

Bhusal and Muriana,2021

PGPR
Nitrogen fixation-PCR analysis

- The nitrogen-fixation ability of endophytic bacteria was tested using a tube containing a semisolid LGI medium(next slide).
- After inoculating with the corresponding bacteria, the tubes were incubated at 30°C for up to 5 days.
- The nitrogen-fixation ability was shown by pellicle growth on the surface of the media.
- Detection of diazotrophic isolates was accomplished by targeting *nifH* gene via PCR amplification using the primers of
- PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and
- PolR (5′-ATSGCCATCATYTCRCCGGA-3′).

Ester de Fretes *et al.*,2018

Plant growth promoting rhizobacteria (PGPR)

Nitrogen fixation-PCR analysis

- **Semisolid LGI medium:**
- Sucrose: 5.0 g
  K_2HPO_4: 0.2 g
  KH_2PO_4: 0.6 g
  $MgSO_4 . 7H_2O$: 0.2 g
  $CaCl_2 . 2H_2O$: 0.02 g
  $Na_2MoO_4. 2H_2O$: 0.002 g
  Bromthymol blue solution (0.5% in 0.2N KOH): 2 ml
  Fe(III) EDTA (1.64%): 4.0 ml
  Vitamin solution: 1.0 ml
  Distilled water: 1.0 L
  Adjust pH to 6.0
  For semisolid medium, add 0.5 g of agar; for solid medium, add 15 g of agar.
  Autoclave at 105°C for 30 min.
- **Vitamin solution:**
  Biotin: 10 mg
  Pyridoxol HCL: 20 mg
  Distilled water: 0.1 L

Ester de Fretes *et al.*,2018

PGPR
Nitrogen fixation-PCR analysis

- **Alternative medium:**
- The Beijerinckia (BJK) medium (Becking, 1959) was prepared as follows:
- sucrose 20.0 g,
- KH_2PO_4 0.8 g,
- K_2HPO_4 0.2 g,
- Mg $SO_4.7H_2O$ 0.5 g,
- $FeCl_3$ 0.1 g,
- molybdenum 0.005 g and
- distilled water 1.0 L.
- The medium was set to pH 6.5.

Emmyrafedziawati and Stella,2018

PGPR
Nitrogen fixation-PCR analysis

- The nitrogen-fixation ability of endophytic bacteria was tested using a tube containing a semisolid LGI medium. After inoculating with the corresponding bacteria, the tubes were incubated at 30°C for up to 5 days. The nitrogen-fixation ability was shown by pellicle growth on the surface of the media.
- Detection of diazotrophic isolates was accomplished by targeting *nifH* gene via PCR amplification using the primers of
- PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and
- PolR (5′-ATSGCCATCATYTCRCCGGA-3′).

Ester de Fretes *et al.*,2018

PGPR
Nitrogen fixation-PCR analysis

- The 25 µL PCR mixture contained 12.5 µL GoTaq reaction buffer, 0.8 µM of both sets of primers, 2 µL DNA template, and 8.5 µL ddH_2O.
- PCR conditions were as follows:
- 95°C for 5 min; 30 cycles of 95°C for 45 s; 58°C for 45 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.
- Amplification product was visualized using agarose electrophoresis with 1% (w/v) on a 1% TBE buffer and stained with SYBR (Invitrogen).

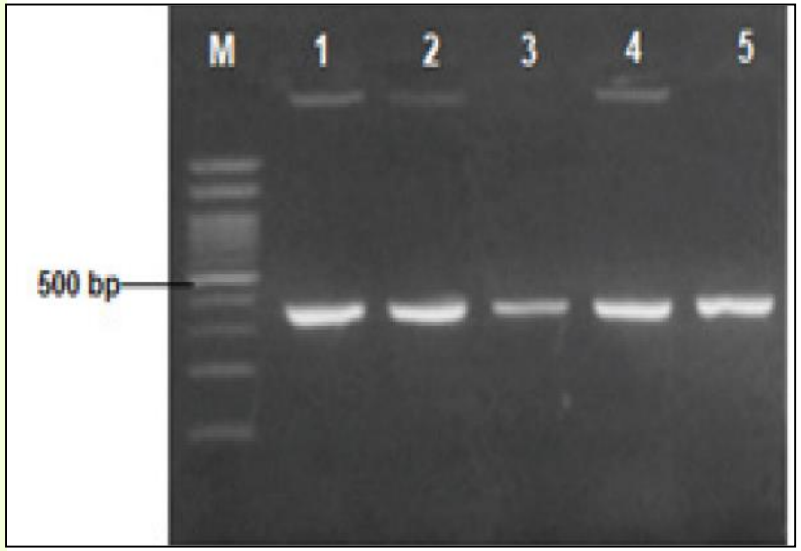
Ester de Fretes *et al.*,2018

PGPR

Nitrogen fixation-PCR analysis

- Screening of endophytic bacteria for diazotrophy using partial amplification of the nifH gene.
- Lanes: M, 100 bp DNA size marker;

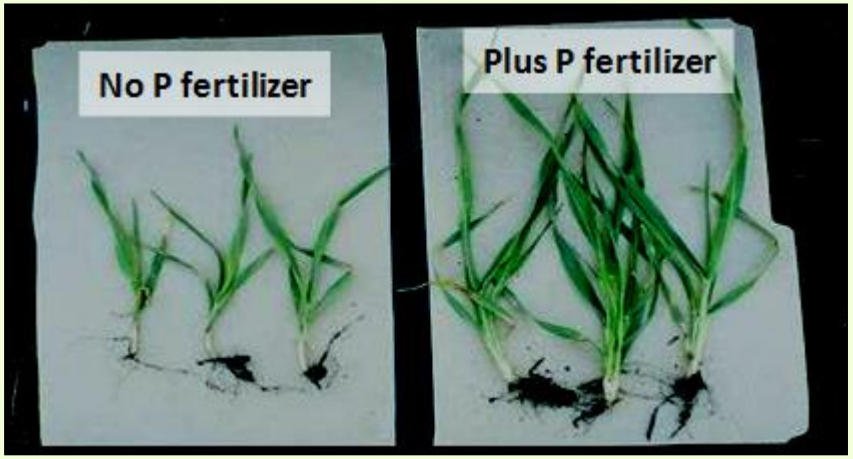
1. isolate BA8 (*Microbacterium oleivorans*);
2. isolate BA10(*Curtobacterium citreum*);
3. isolate MA11(*Beijerinckia fluminensis*);
4. isolate MA32(*Bacillus subterraneus*);
5. isolate MA33(*Staphylococcus pasteuri*).



Ester de Fretes *et al.*,2018

Phosphate (P) solubilization
Phosphate-solubilizing bacteria

- Phosphorus is one of 17 nutrients essential for plant growth. In fertilizers, it is normally found in the form of phosphoric acid (H_3PO_4).
- Phosphate is key for:
- Quick emergence
- Early vigor
- Root growth
- Maturity
- Quality
- Yield.



Novozymes,2015

Phosphate (P) solubilization
Phosphate-solubilizing bacteria

- It is well known that phosphate-solubilizing bacteria can increase the availability of P to plants in deficient soils.
- These bacteria solubilize phosphate through the production of acids, and possibly by means of other mechanisms as well (Nautiyal *et al.*,2000).
- Diverse bacteria, including *B. subtilis*, *K. terrigena*, *Pseudomonas* spp., and *Streptomyces griseus* produce phytases for degrading organic phosphate compounds.
- Phosphorous acid(H_3PO_3) is a compound normally marketed as a fungicide is able to control fungal diseases(water molds and downy mildew) belong to the Oomycota (or oomycetes) on agronomical and horticultural crops.

Siddiqui,2006; Brunings *et al.*,2012

Phosphate (P) solubilization
Phosphate-solubilizing bacteria

- Phosphate-Solubilizing Bacteria (PSB) improve plant growth, yield and phosphorus content of several crops, and may be used as bioinoculant to enhance sustainable production.
- Accordingly, several works reported that inoculation with PSB belonging to the genera *Achromobacter*, *Agrobacterium*, *Aerobacter*, *Bacillus*, *Burkholderia*, *Escherichia*, *Erwinia*, *Enterobacter*, *Flavobacterium*, *Microccocus*, *Pseudomonas*, *Paenibacillus*, *Pantoea*, *Serratia* and *Rhizobium* resulted in improved growth, yield and P uptake in several crops.

Viruel *et al.*,2014

Phosphate (P) solubilization
Phosphate-solubilizing bacteria

- The bacterium was inoculated into 200 mL liquid media supplemented with either $Ca_3(PO_4)_2$ or lecithin and cultured at 28°C for 12 days with continuous agitation (150 r/min).
- 10 ml culture was sampled aseptically every 24 hours for the determination of acidity and available phosphorus.
- The acidity was assayed simply by reading on a pH meter, and the phosphorus availability was determined with Mo-blue method(Watanabe and Olsen,1965).
- Optimum pH and temperature for P-solubilization in liquid $Ca_3(PO_4)_2$ medium were determined following the above method.

Zhu *et al.*,2011

Phosphate (P) solubilization

Phosphate-solubilizing bacteria

- The modified $Ca_3(PO_4)_2$ culture medium contained with the following ingredients (l^{-1}): glucose 10 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 30 g, KCl 0.3 g, $FeSO_4.7H_2O$ 0.03 g, $MnSO_4.4H_2O$ 0.03 g, $MgSO_4.7H_2O$ 0.3 g, $Ca_3(PO_4)_2$ 10 g, agar 20 g, H_20 1000 mL, pH 7.0-7.5.
- The lecithin culture medium was composed of (l^{-1}): glucose 10 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 30 g, KCl 0.3 g, $FeSO_4.7H_2O$ 0.03 g, $MnSO_4.4H_2O$ 0.03 g, $MgSO_4.7H_2O$ 0.3 g, lecithin 0.2 g, $CaCO_3$ 5 g, yeast extract 0.4 g, agar 20 g, H_20 1000 mL, pH 7.0-7.5.

Zhu *et al.*,2011

Phosphate (P) solubilization
Phosphate-solubilizing bacteria

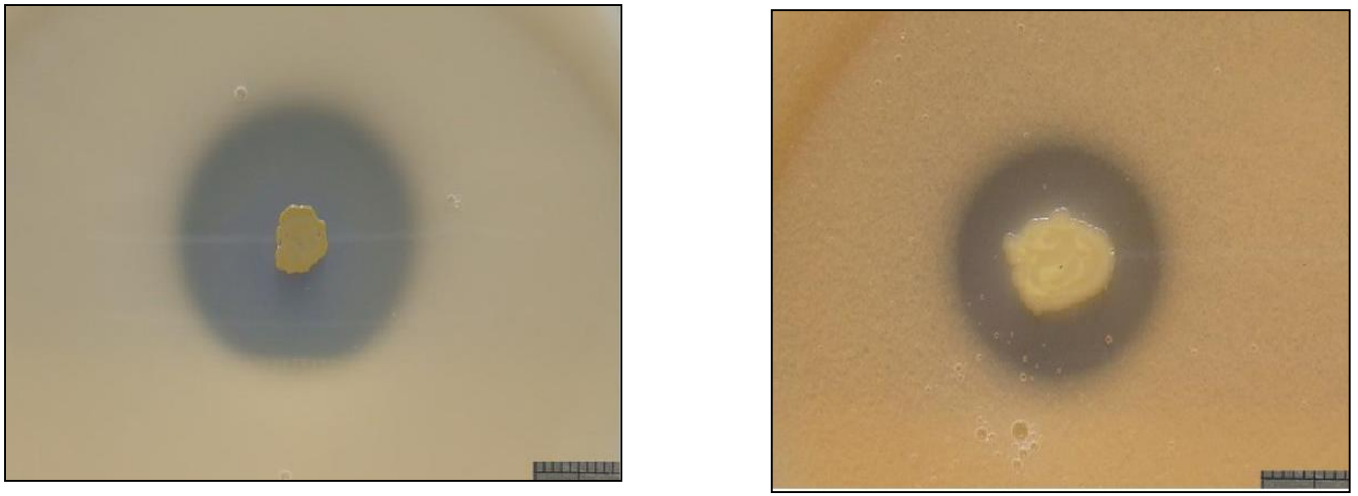
- The bacterium was isolated and screened on Pikovskaya's agar (PKA) medium consisting of constituents:
- glucose 10 g; tri-calcium phosphate(TCP) 5 g; yeast extract 0.5 g; ammonium sulphate 0.5 g; potassium chloride 0.2 g; sodium chloride 0.2 g; magnesium sulphate 0.1 g; ferrous sulphate trace; manganese sulphate trace; agar agar 15 g; distilled water 1L; the pH was adjusted to 7.0±0.2 before sterilization, by pour plate technique.
- After 48 h of incubation at 28±2°C discrete colony showing halo zones were picked up, sub-cultured in PKA slants and preserved.

Paul and Sinha, 2016

Phosphate (P) solubilization

Phosphate-solubilizing bacteria

Growth in $Ca_3(PO_4)_2$ and lecithin-containing solid media



The phosphate-solubilizing zone formed on medium containing $Ca_3(PO_4)_2$ (left) and lecithin (right). Bar: 1 cm. The isolate grows well at 28°C on lecithin and $Ca_3(PO_4)_2$ containing solid media.

- In 3 days (lecithin containing medium) or 5 days ($Ca_3(PO_4)_2$ containing medium), clear phosphate-solubilizing zone forms.
- In 10 days, the phosphate-solubilizing zone expanded to the biggest (about 2.5-3.0 cm on $Ca_3(PO_4)_2$ containing plate, and about 1.7-2.0 cm on lecithin containing medium.

Zhu *et al.*,2011

Phosphate (P) solubilization

Phosphate-solubilizing bacteria

Growth on solid agar media

- Quantitative determination of phosphate solubilizing activity on agar medium.
- The clearing zones formed by the bacteria on the re-spotted plates were quantified on the 7th day of incubation using the following equation:

$$\text{Phosphate solubilizing index} = \frac{\text{colony diameter} + \text{clearing zone}}{\text{colony diameter}} \times 100$$

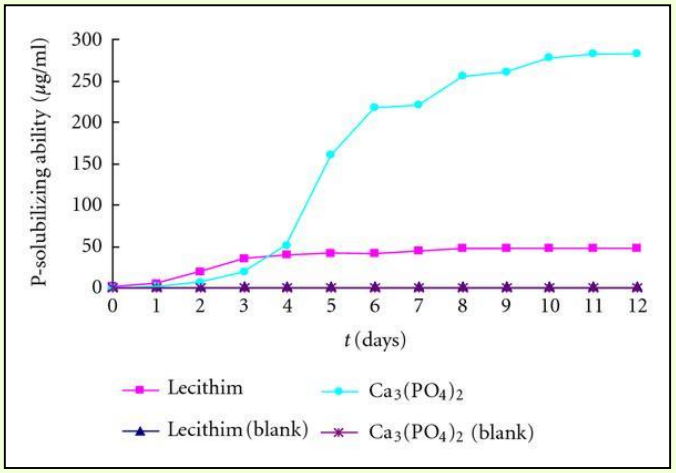
Sharon *et al.*,2016

Phosphate (P) solubilization

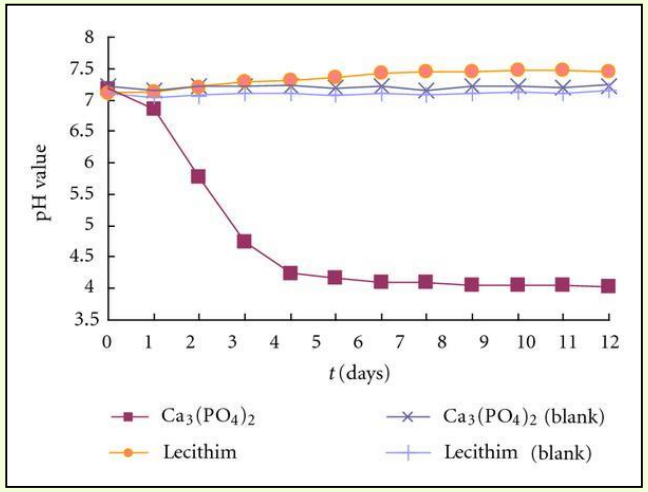
Phosphate-solubilizing bacteria

Growth in $Ca_3(PO_4)_2$ and lecithin-containing liquid media

- Phosphorus-solubilizing performance of the isolate: P-solubilizing ability.



- Phosphorus-solubilizing performance of the isolate: pH value of the medium.



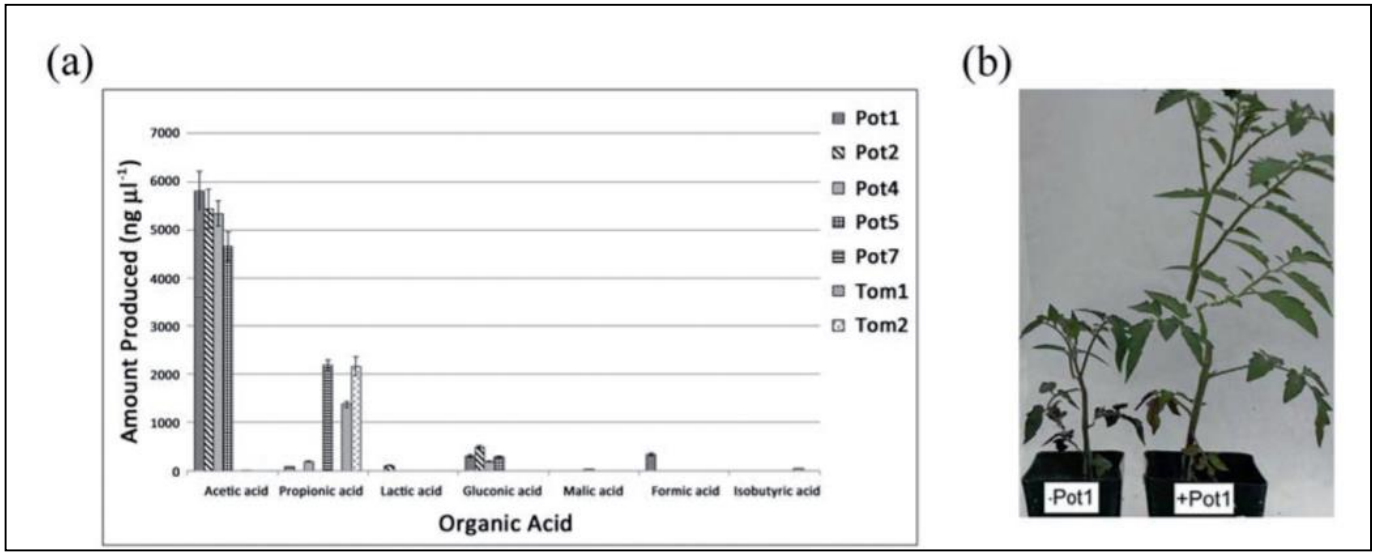
$Ca_3(PO_4)_2$ solubilization is slow in the first two days and then becomes fast, reaching the highest (283.16 μg/mL) in about 11 days. Lecithin solubilization starts to increases in 1 day, reaching the highest 47.52 μg/mL in 8 days. It was found that the growth of the isolate caused a significant increase of acidity in $Ca_3(PO_4)_2$ containing medium. In about 4 days, the acidity increased from pH 7.21 to pH 4.24. In contrast, the acidity decreased from pH 7.1 to pH 7.46 in lecithin-containing medium.

Zhu *et al.*,2011

Phosphate (P) solubilization

Phosphate-solubilizing bacteria

Growth on solid agar media



Bacterial acid production and effect on plant growth. (a) Production of organic acids by PSB isolates grown in PVK liquid medium after 24 h of incubation. Bars indicate standard error (n=3). (b) Greenhouse trial testing the application of *Pantoea* sp. Pot1 on soluble phosphorus-deprived tomato plants. Left, plant provided with only insoluble $Ca_3(PO4)_2$. Right, plant provided with both *Pantoea* sp. Pot1 and insoluble $Ca_3(PO4)_2$.

Sharon *et al.*,2016

Phytohormones
Acetic acid production

- Large numbers of experiments have shown that bacterial participation raises the phytohormone levels in plants.
- Many studies suggest the involvement of indole-3-acetic acid (IAA) in morphological and physiological changes of the inoculated plant roots.

The addition of L-tryptophan into bacterial culture medium can increase the biosynthesis of IAA up to 2.7 times. L-tryptophan is an amino acid with an indole group that can function as a physiological precursor of IAA in plants and microorganisms because it contains active compounds that can trigger microbial growth.

Siddiqui,2006

Indole acetic acid production

Quantification of IAA production

Colorimetric analysis

- This was obtained from 3 days and 20°C cultures of Enterobacterias and *Pseudomonas* sp., in King B broth/LB/NB with and without additional L-tryptophan (3 g/l). Tubes were incubated in a shaker (150 rpm, 30°C).
- The culture broth was centrifuged at 10,000 rpm for 10 min.
- One ml of the supernatant was mixed with 2 ml of Salkowski reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water and 7.5 ml of 0.5 M $FeCl_3.6H_2O$).
- The tubes containing the mixture were allowed to stand for 30 min to 60 min in the dark at room temperature for color development.
- Appearance of brownish pink color indicates the presence of IAA.
- The intensity of color was spectrophotometrically measured at 530 nm using a standard curve for calibration by using authentic IAA.

See also bacterial diagnosis-Part 2. section Agrobacterium.

Torres *et al.*,2000; Prasad and Dagar,2014; Sukweenadhi *et al.*,2018

Indole acetic acid production

Quantification of IAA production

Colorimetric analysis

- Bacteria were inoculated into 30 ml of Luria-Bertani Tryptophan solution. At room temperature, bacterial cultures were incubated and shaken at 150 rpm for 7 days. Every two days in a week, the IAA level generated during cultivation was measured. The measuring of IAA level was done in colorimetry way with a spectrophotometer at 535 nm wavelength.
- Culture fluid was centrifuged for 25 minutes at 5000 rpm.
- The obtained filtrate was mixed with the Salkowski reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water and 7.5 ml of 0.5 M $FeCl_3.6H_2O$) with a ratio of 2:1.
- The mixture was then incubated at room temperature for an hour before the absorbance was measured at a wavelength of 535 nm. IAA level produced by endophytic bacteria was determined from the linear plot of the absorbance value of a standard IAA.
- Un-inoculated media was used as a control.

Herlina *et al.*,2017

Indole acetic acid production

Quantification of IAA production

Other formula for preparation Salkowski reagent

1. Salkowski reagent is a mixture of 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$) which upon reaction with IAA yields pink color, due to IAA complex formation with and reduction of Fe^{3+}(Gang *et al.*,2019). kept the ragent in the dark.
2. Salkowski reagent: A mixture of 15 ml 0.5 M $FeCl_3$, 500 ml distilled water, and 300 ml of concentrated H_2S0_4(Meudt and Gaines, 1967).
3. Salkowski's reagent R1: 12 g liter^{-1} $FeCl_3$ in 429 ml liter^{-1} H_2SO_4 (Gutierrez *et al.*,2009).

Salkowski reaction test: 2 mg of dried extract was shaken with 1 mL of chloroform and a few drops of concentrated sulphuric acid were added along the side of the test tube. A red brown colour formed at the interface of two layers indicates the presence of steroids.

Indole acetic acid production

Preparation of Indole-3-Acetic Acid (IAA) Stock Solution (10 mg/mL)

1. Add 1.0 g of Indole-3-acetic acid (GoldBio Catalog # I-110) to a 100 mL flask or beaker.
2. Add 2.0-5.0 mL of ethyl alcohol (EtOH) or 1N NaOH to dissolve the powder. Bring volume to 100 mL with molecular biology grade water.
3. If necessary agitate the solution to dissolve the IAA.
4. Sterilize by autoclaving or filtering through a 0.2 micron filter.
5. Store aliquots of the IAA at -20°C.

Note: The final concentration of the stock solution is 10 mg/ml. However, the concentration used in the experiment may be dependent upon the media used in the experiment. The typical final experimental concentration can vary widely and can range from 0.01 mg/ml to 3.0 mg/ml.

Indole acetic acid production

How to prepare the calibration curve for IAA determination with colorimetric method?

- For calibration I prepared IAA stock solution of 1000 µg/ml.
- For proper dissolving IAA, I added few drops of 1 M NaOH. Using the stock I prepared the following concentrations: 0, 0.5, 1, 2.5, 5, 10, 20, 50 and 100 µg/ml, each 2 ml.
- Next, I added to each standard 4 ml of Salkowsky reagent.
- All samples and standards I incubated in dark for 20 minutes.
- After that I observed pink color.
- I measured absorbance of pink-samples and standards at 530 nm.
- I zeroed spectrophotometer using the standard of 0 µg IAA/ml (2ml H_2O + 4 ml Salkowsky reagent).

The pink-standards.

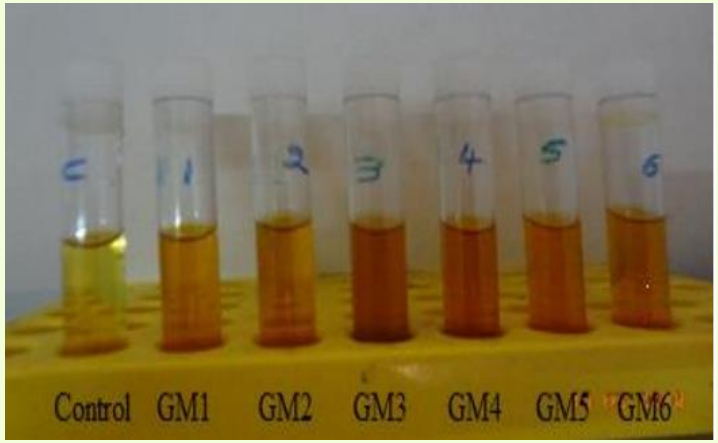
Banach,2019

Indole acetic acid production

Quantification of IAA production

Colorimetric analysis

- IAA production by GM1, GM2, GM3, GM4, GM5 and GM6.



- O.D values of standard IAA at 530nm.

Concentration of IAA(µg/ml)	Optical density at 530nm
100	0.262
50	0.133
25	0.063
12.5	0.024
6.25	0.015
3.125	0.004

A standard curve of various concentrations of pure IAA in the range of 0-250 µg mL^{-1} was prepared by plotting IAA concentration to optical density (at 530 nm).

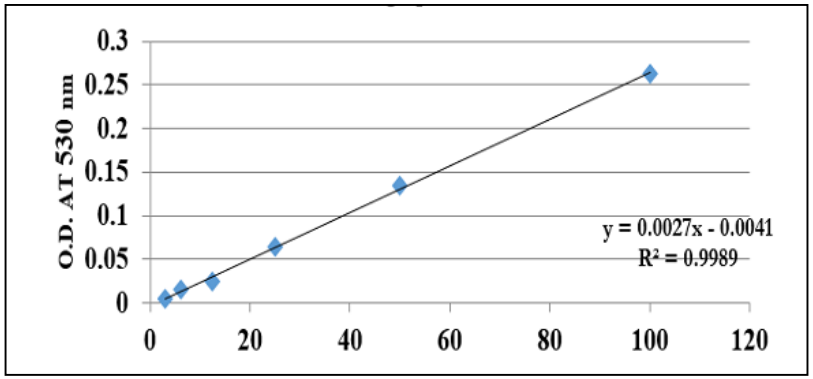
Shaik *et al.*,2016;..

Indole acetic acid production

Standard graph of IAA

Colorimetric analysis

- An IAA concentration curve can be made with 0, 0.2, 1, 2, 3, 6, 11, 20, 45, 100, 200 and 300 µg of synthetic IAA /mL and quantified using a spectrophotometer at wavelength of 530 nm (Gordon & Weber, 1951).



Standard graph of IAA (concentration of IAA on X-axis vs O.D value at 530nm on Y-axis) by plotting the concentration of IAA against O.D. a straight line was constructed the straight-line equation was represented on the graph.

Shaik *et al.*,2016;..

Indole acetic acid production

Standard graph of IAA

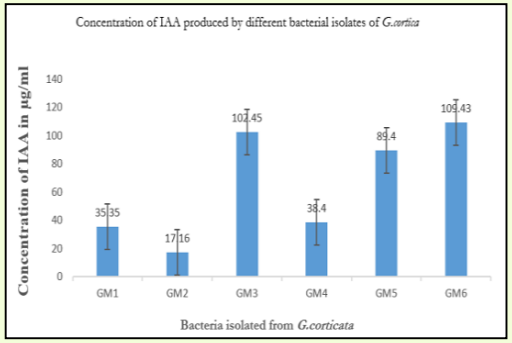
Colorimetric analysis

- Concentration of IAA produced by bacteria GM1-GM6 isolated from red alga *G. carticata*.

Bacterial isolate	Concentration of IAA (µg/ml)
GM1	35.35 ± 0.41
GM2	17.16 ± 0.18
GM3	102.45 ± 0.39
GM4	38.40 ± 0.36
GM5	89.40 ± 0.17
GM6	109.43 ± 0.08

Values are tabulated as means ± the standard error of the mean (SEM) and N=3.

- Standard error bar diagram representing amount of IAA produced by the bacteria isolated from *G. carticata*.



The above figure depicts that all the bacterial isolates are producing IAA but the Strains GM3, GM5 and GM6 are producing more amount of IAA in these GM6 is producing significant amount of IAA.

Shaik *et al.*,2016

Indole acetic acid production

Quantification of IAA production

Colorimetric analysis

Isolated strain	Host plant	Growth OD at 540 nm	Production of IAA (µg/mL)
Rhizobium undicola strain N30	*Neptunia oleracea* Lour.	1.24 ± 0.01	152.0 ± 0.52
*Rhizobium undic*ola strain N32	*Neptunia oleracea* Lour.	1.40 ± 0.02	148.0 ± 0.66
Rhizobium undicola strain N34	*Neptunia oleracea* Lour.	1.56 ± 0.01	197.0 ± 0.88
Rhizobium undicola strain N35	*Neptunia oleracea* Lour.	1.62 ± 0.03	163.0 ± 0.88
Rhizobium undicola strain N37	*Neptunia oleracea* Lour.	1.99 ± 0.02	226.0 ± 1.54

IAA production by different isolates obtained from the nodules of *N. oleracea*. Results presented are the mean of 3 individual experimental setups.
Bacteria were grown in L-tryptophan (0.1%) supplemented yeast extract medium for 24 h at 30 ± 2°C. The control set was devoid of any carbon sources.

Ghosh *et al.*,2015

Phytohormones
Inoculation and ethylene measurement
Gas chromatography

1. Inoculate each isolate and the positive control strain into respective isolation medium (NFb, LGI, LGI-P, or TB) supplemented with N source, or in another rich medium (e.g., King B), until each one reach OD of ~ 0.05 to 0.1. 2.
2. Inoculate 20 µl of each bacterial culture in the bottom of Bijou or penicillin vials, 600in triplicate.
3. Close the vials with cotton plugs and incubate them ~ 72 h at 28° to 30°C, until a veil-like pellicle is formed on the surface of the culture medium (Next figure).
4. Substitute the cotton plug for a rubber stopper and add 0.6 ml of acetylene gas (10 % of gas phase) in each vial using the 1 ml plastic syringe.
5. Incubate ~ 60 min at 28° to 30°C (for some bacterial genera the period could be longer). **Be careful do not disturb the veil-like pellicle!**
6. Remove 0.5 ml of the gaseous phase of each vial using the 10 to 500 µl gas tight syringe and inject this gas amount into the gas chromatograph, previously stabilized.
7. Read the amount of ethylene produced.

- Ethylene (100 ppm in N) is used as standard and 0.5 ml contains 2.232 nmol of ethylene at 25°C and 1 atm. To calculate the specific activity of nitrogenase is necessary to determine the culture's protein concentration.

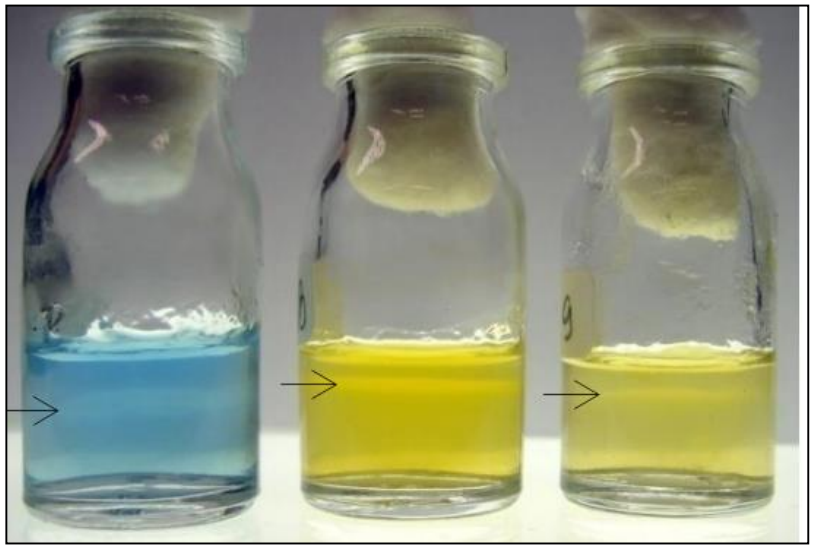
Ambrosini *et al.*,2010

Phytohormones

Inoculation and ethylene measurement

Gas chromatography

- Bijou or penicillin vials and the growth of diazotrophs cultivated in N-free semi-solid media (left to right: NFb, LGI-P, and LGI media).
- Black arrows indicate the height of pellicles which are positioned near at surface of the culture media.



Ambrosini *et al.*,2010

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- IAA accumulation in plants induces the transcription of ACC synthase genes, which increases the ACC concentration, leading to the production of ethylene.
- Plant growth-promoting bacteria that both produce ACC deaminase and synthesize IAA, produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase promote plant growth by sequestering and cleaving plant-produced ACC, and thereby lowering the level of ethylene in the plant.

Mo Kang *et al.*,2019:..

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- Ethylene (gaseous hormone) production induces different observable changes in plants, including reduction in the growth rate.
- Rhizobacteria having ACC-deaminase enzyme can facilitate plant growth to over come these deleterious effects.
- It has been proposed that PGPR may enhance plant growth by lowering the plant ethylene levels.
- ACC (1- aminocyclopropane-1-carboxylate), is a precursor of ethylene.

Siddiqui,2006; Shahzad *et al.*,2010

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- ACC deaminase and IAA producing bacteria assist plant growth and can effectively protect plants against various environmental stresses, including salinity stress.
- IAA accumulation in plants induces the transcription of ACC synthase genes, which increases the ACC concentration, leading to the production of ethylene.
- PGPR containing ACC deaminase:
- may break down some of the excess ACC, and
- lower plant ethylene levels during the advent of environmental stress.

Kang *et al.*,2019

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- 1-Aminocyclopropane-1-carboxylate (ACC) deaminase catalyses the cleavage of ACC, the immediate precursor of ethylene in plants, to α-ketobutyrate and ammonia.
- The enzyme ACC deaminase (ACCD) cleaves ACC to form α-ketobutyrate and ammonium.
- ACC is as a source of nitrogen and precursor of ethylene.
- Thus, the bacterial enzyme ACC deaminase (1-aminocyclopropane-1-carboxylate) promotes plant growth by lowering plant ethylene levels.

Jacobson *et al.*,1994;..

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

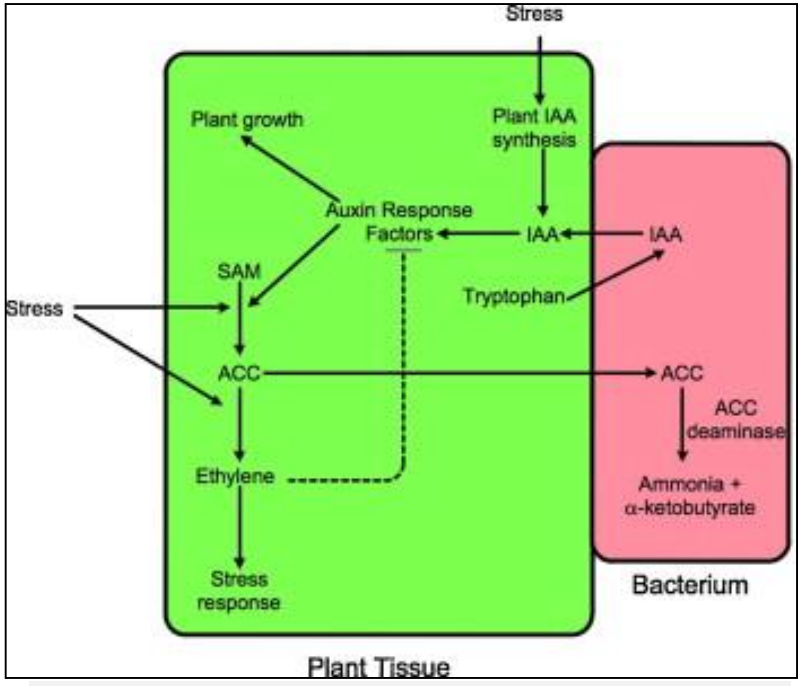
- This enzyme is present in plant growth-promoting bacteria (PGPR) and lowers the ethylene level by catalyzing the conversion of ACC to ammonia and α-ketobutyrate (α-KB).
- ACC deaminase is present in the cytoplasm of bacteria at a low level until it is induced by ACC, and the induction of enzyme activity is a relatively slow process.
- ACC deaminase activity and IAA production was slightly increased in some strains in response to an increase in NaCl concentration in the growth media.

Jacobson *et al.*,1994; Yaish *et al.*,2015;..

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- A schematic model of how plant growth-promoting bacteria that both produce ACC deaminase and synthesize IAA may facilitate plant growth.
- Immediately following an abiotic or biotic stress, the pool of ACC in the plant is low as is the level of ACC deaminase in the associated bacterium.



Abbreviations:
ACC, 1-aminocyclopropane-1-carboxylate;
IAA, indole-3-acetic acid;
SAM, *S*-adenosyl methionine.

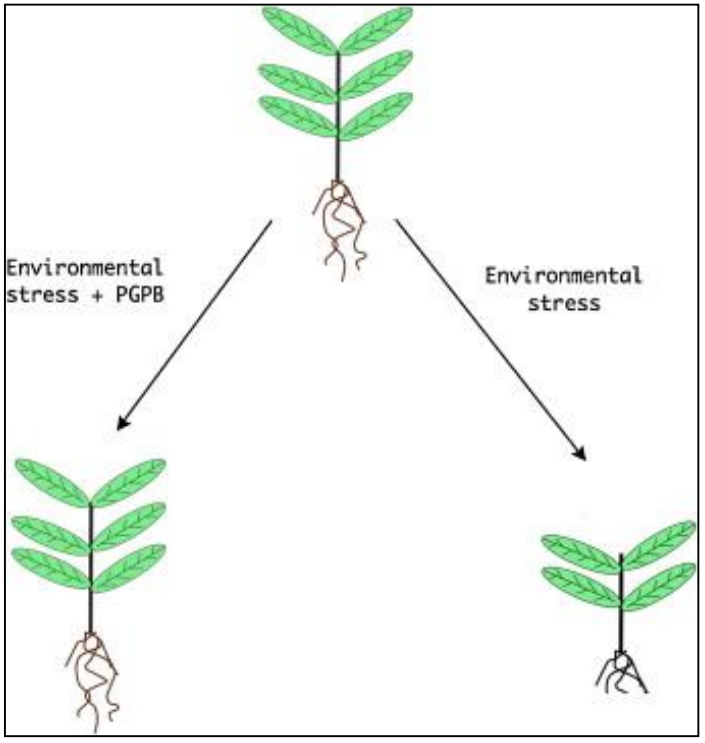
Glick,2014

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- A schematic view of the inhibition of plant growth as a consequence of:

1. stress induced ethylene synthesis, and
2. the ability of ACC deaminase-containing PGPB to decrease the stress ethylene level and thereby limit the growth inhibition.



Soil microorganisms that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase promote plant growth by sequestering and cleaving plant-produced ACC, and thereby lowering the level of ethylene in the plant.

Glick,2014

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- The objectives of the present study were:

1. to isolate efficient ACC deaminase producing PGPR from the rhizosphere of rice plants grown in coastal saline soils, and characterize them;
2. to evaluate other plant growth promoting (PGP) activities including production of indole acetic acid (IAA) by the most promising ACC deaminase producing isolates;
3. to determine the effect of potent PGPR isolates on root elongation under salinity stress, and
4. To estimate ethylene from rice seedlings treated with ACC deaminase containing PGPR or chemical ethylene inhibitors(AVG=10^{-4} M L-α-(2-aminoethoxyvinyl) glycine hydrochloride.

Bal *et al.*,2012

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- **Root elongation assay:**
- A total of 17 ACC utilizing bacteria were isolated from
- the coastal rice field soil from five different locations and screened for their ACC deaminase metabolism.
- Results indicated that all the strains metabolized ACC but with variable degrees of efficacy (next Table).
- Highest ACC deaminase activity per hour was exhibited by the isolate SB1.ACC2 (2664.08 nmol α-ketobutyrate mg^{-1} h^{-1}).
- 11 strains exhibiting high ACC utilization rate were further selected to screen their growth-promoting activity in rice under axenic conditions (root elongation assay).

Bal *et al.*,2012

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- ACC deaminase activity (nmol α-ketobutyrate mg^{-1} protein h^{-1}) of the isolates.
- Highest ACC deaminase activity per hour was exhibited by the isolate SB1.ACC2 (2664.08 nmol α-ketobutyrate mg^{-1} h^{-1}).
- Mean values sharing the same letter (s) in column do not differ significantly according to Duncan's multiple range test (P=0.05).
- *Mean of five replicate observations ± SD (Standard deviation.

S. No.	Isolates	ACC deaminase activity*
1	SB1.ACC1	894.11^{f}±41.81
2	SB1.ACC2	2664.08^{j}±63.21
3	SB1.ACC3	1468.90^{h}±31.60
4	SB2.ACC1	397.04^{d}±21.16
5	SB2.ACC2	2049.42^{i}±52.20
6	SB2.ACC3	295.72^{bc}±19.09
7	SB2.ACC4	236.05^{b}±18.58
8	SB2.ACC5	1145.43^{g}±66.44
9	SB2.ACC6	526.04^{e}±42.65
10	SB3.ACC1	440.51^{d}±27.24
11	SB3.ACC2	329.09^{c}±28.15
12	SB3.ACC3	919.42^{f}±73.05
13	SB3.ACC4	906.07^{f}±56.02
14	SB4.ACC1	61.29^{a}±6.24
15	SB4.ACC2	92.49^{a}±8.43
16	SB5.ACC1	62.02^{a}±15.50
17	SB5.ACC2	91.28^{a}±12.16

Bal *et al.*,2012

As isolates have the ability to produce both ACC deaminase and IAA they promoted root, shoot and other growth indices of rice to a greater extent.

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- Morphological and biochemical characters the isolates.
- We tested 11 high ACC deaminase producers for their growth-promoting activity without salt stress and three most promising strains:
- SB1. ACC2 (*Alcaligenes* sp.);
- SB1.ACC3 (*Bacillus* sp.), and
- SB2.ACC2(*Ochrobactrum* sp.) With IAA production(μM ml^{-1})ca.49.56 ,45.91 and 152.37, respectively.

Characters	Isolates		
	SB1-ACC2	SB1-ACC3	SB2-ACC2
Morphological			
Gram Reaction	–	+	–
Cell shape	Rod	Rod	Rod
Cell Length (μ)	2.0±0.1	2.0±0.1	4.0±0.2
Colony Color	White	White	White
Motility	+	+	+
Biochemical			
MR	–	+	+
MRVP	–	–	–
Citrate utilization	–	–	+
Nitrate reduction	–	+	+
Starch hydrolysis	–	–	–
Oxidase	+	+	+
Catalase	+	+	+
Tributyrin hydrolysis	+	+	+
Tween 80 hydrolysis	–	–	+
Urease	–	–	–
Plant growth promoting traits			
IAA production (μM ml^{-1})	49.56±2.81	45.91±1.79	152.37±2.38
HCN production	–	–	–
Ammonia production	+	+	+
Phosphate solubilization	+	+	+
Siderophore Production	+	+	+
Salt tolerance			
Growth in maximum salt concentration (M NaCl)	1.54	1.03	0.68

Bal *et al.*,2012

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

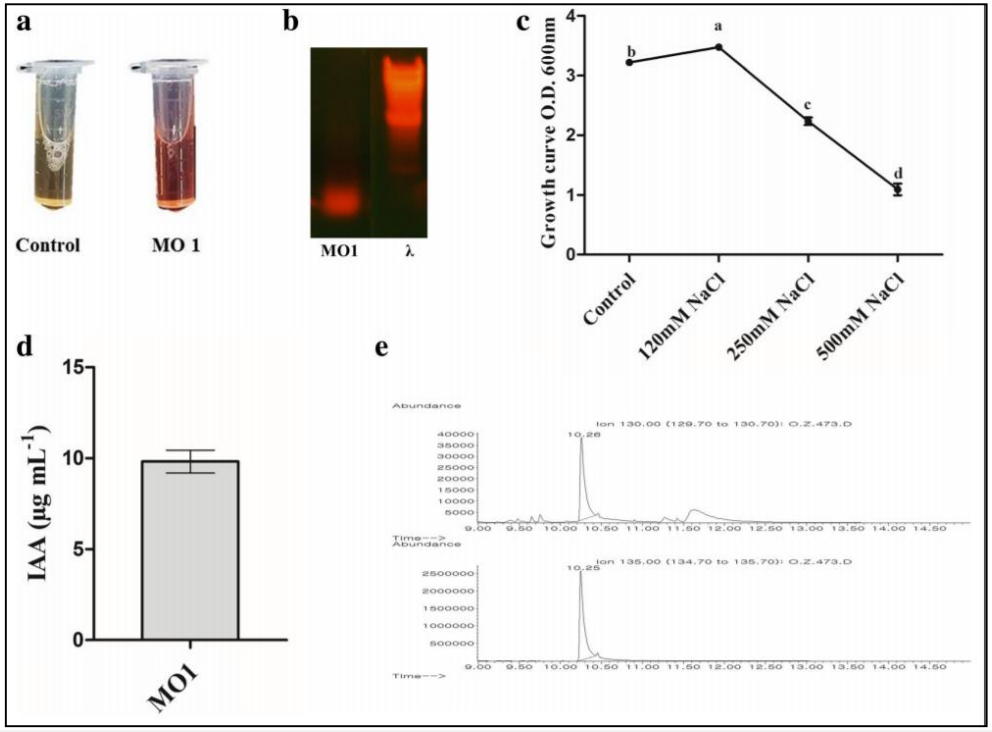
- Salt stress effect on shoot and root growth of 7 day old rice seedlings ex-posed to salt stress(150 mM) under gnotobiotic conditions.
- a, Negative Control (0.03 M $MgSO_4$);
- b, SB1. ACC2 (*Alcaligenes* sp.);
- c, Heat killed SB1. ACC2;
- d, SB1.ACC3 (*Bacillus* sp.); e, Heat killed SB1ACC3; f, SB2.ACC2(*Ochrobactrum* sp.); g, Heat killed SB2ACC2;
- h, AVG (a known inhibitor of ethylene production).

SB1.ACC2 was the most efficient strain which enhanced both length and fresh weight of root and shoot up to 73.8, 63.2, 105.8 and 59.5%, respectively.

Bal *et al.*,2012

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene



Leclercia adecarboxylata MO1, ACC deaminase and indole-3-acetic acid (IAA) producing halotolerant bacterium. (a) Salkowski test for IAA (b) The presence of ACC deaminase responsible *acdS* gene (c) Salinity stress tolerance of MO1 (d) The amount of IAA produced by strain MO1 (e) GC–MS/SIM spectrometry analysis of IAA produced by strain MO1.

Kang *et al.*,2019

Phytohormones

Salinity stress, IAA, ACC accumulation, ACC deaminase, ethylene

- PGPRs from date palm tree (*Phoenix dactylifera*).
- ACC deaminase activity (μmol mg^{-1} h^{-1}), and IAA and similar compounds (μg ml^{-1}) produced by newly isolated strains.
- Activity or product not detected in the assays is denoted by N.D.

Strain	ACC-Deaminase	IAA and simila compounds
Group 1		
PD-R1	3.9	30.2
PD-R3	5.1	N.D.
PD-R6	12.5	25.9
PD-R10	13.5	N.D.
PD-R12	13.1	21.2
PD-R13	3.4	70.8
PD-R34	N.D.	78.1
PD-L4	N.D.	45.1
PD-L5	N.D.	101.5
PD-L6	3.3	N.D.
Group 2		
PD-P1	4.2	110.2
PD-P7	4.5	N.D.
PD-P8	11.2	N.D.
PD-P11	N.D.	N.D.
PD-P12	22.5	206.4
PD-P14	8.1	178.2
PD-P26	15.1	N.D.
PD-P33	26.6	N.D.
PD-P40	7.4	N.D.
PD-P42	10.5	N.D.

Yaish and Glick,2015

Phytohormones

ACC deaminase activity indirect assay

DF culture media emended with different concentration of ACC

- Different concentration of ACC were recommended:

1. ACC (50 µM): Make a stock solution of 10 mM by dissolving 2 mg of ACC in 2 mL distilled water. Dilute the stock solution to 50 µM.
2. Initial ACC concentration of approx. 5 $mmole^{-1}$(0.5 g^{-1}) (Chang *et al.*,2011).
3. DF agar with 1.5% ACC(Cedeño-García *et al.*,2018).

- NOTE: The heat-labile ACC was filter-sterilized and the filtrate was added to the salt medium. Store the ACC solution at -20°C(Bulens *et al.*,2011).

Phytohormones

ACC deaminase activity indirect assay

Growth on DF solid culture medium

- **DF salts solid medium, for ACC deaminase activity indirect assay:**
- The morphologically distinct colonies were screened for ACC deaminase activity on the sterile minimal DF (Dworkin and Foster) salts media (DF salts per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH 7.2) amended with 3 mM ACC instead of $(NH_4)_2SO_4$ as sole nitrogen source (Dworkin and Foster, 1958; Penrose and Glick, 2003).
- The inoculated plates were incubated at 28°C for 3 days and growth was monitored on a daily basis.
- Colonies growing on the plates were taken as ACC deaminase producers and were purified by sub culturing the isolates.

Gupta and Pandey,2019

Phytohormones

ACC deaminase activity indirect assay

Growth on DF solid culture medium (contd..)

- To prepare agar plates containing DF salts solid medium (below) with ACC, spread 50 µL of 0.5 M ACC (1 mM final concentration) with a glass loop on the solidified medium (~ 25 ml).
- Only defrost the tube containing 0.5 M ACC when it will be used (since it is very labile) and then store it again at -20°C.
- After spreading ACC over the solidified medium, wait until the plate is completely dry, and then inoculate the bacteria immediately.

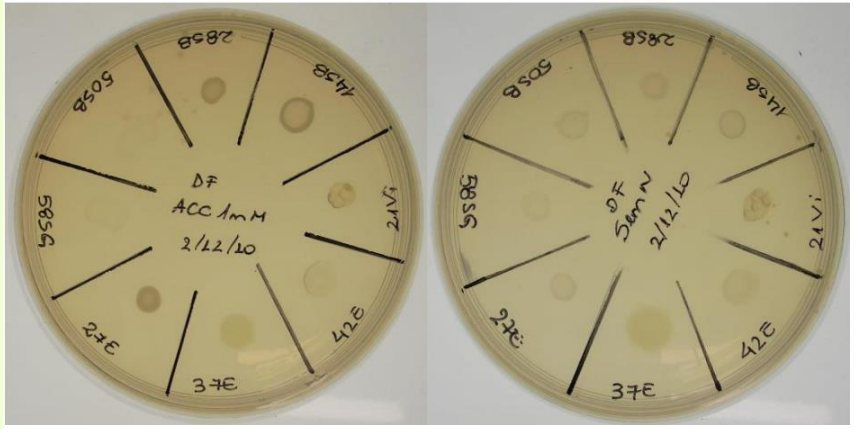
Ambrosini *et al.*,2010

Phytohormones

ACC deaminase activity indirect assay

Growth on DF solid culture medium(contd..)

- DF salts agar plates with ACC (left) and without ACC (right).
- The isolates named 27E, 14SB, and 28SB show visible differences in the growth of their colonies when ACC was present on the medium.
- The isolates named 37E, 42E, and 21Vi do not show differences regarding to the colonies growth in both agar plates.
- The isolates named 50SB and 58SG are not also able to grow in the plates containing ACC, or show a residual growth in DF salts medium without any other N source.



Isolates 27E, 14SB, and 28SB show visible differences in the growth of their colonies when ACC was present on the medium (left).

Ambrosini *et al.*,2010

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on M9 broth culture medium

- Bacterial cultures grown in Tryptic Soy Broth(TSB) and washed with sterile physiological water were used to inoculate tubes of M9 Minimal Medium containing per liter of distilled water: Na_2HPO_4. 7 H_2O, 12.8 g, KH_2PO_4, 3 g, NaCl, 0.5 g, NH_4Cl, 1 g, $MgSO_4$ (1 M), 2 ml, glucose (20%), 20 ml, $CaCl_2$ (1 M), 0.1 ml, and 3 mM ACC as sole nitrogen source.
- M9 medium without ACC served as a control.
- All inoculated tubes were incubated at 28°C.
- The absorbance was recorded after 24 h and then after 48 h at 600 nm.
- **Strains having ACC deaminase activity provided high values in the ACC tube.**

Aarab *et al.*,2015

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- The bacterial isolates were grown in 15 ml of LB broth at 30°C until they reached the stationary phase after which, cells were collected by centrifugation (at 8000 g).
- To induce ACC deaminase activity, the cells were resuspended in 7.5 ml of DF minimal salts medium supplemented with 5 mM of ACC as a sole nitrogen source and then incubated for 40 h at 30°C with shaking (120 rpm).
- ACC deaminase activity was determined by measuring the production of α-ketobutyrate and expressed as nM of α-ketobutyrate formed min^{-1} mg $protein^{-1}$ (Penrose and Glick, 2003).

Yim *et al.*,2010

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- An aliquot of 100 µL of suspended cells (10^7 CFU mL^{-1}) of the selected strains, were inoculated onto 3 mL nutrient broth and incubated for 24 h at 200 rpm at a constant temperature of 25°C.
- Then 1 mL of cell suspension was transferred to microtubes and centrifuged at 8000 rpm for 10 min at 4°C.
- The supernatant was extracted and washed twice in Dworkin-Foster (DF) minimal salts medium and centrifuged at 8000 rpm for 10 min at 4°C.
- The cell pellet was then suspended in 500 µL DF minimal media and incubated in a rotary shaker at 200 rpm and 25°C for 24 h.

Cedeño-García *et al.*,2018

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- The quantitative assessment of ACC deaminase activity was done spectrophotometrically in terms of α-ketobutyrate production at 540 nm by comparing with the standard curve of α-ketobutyrate, which ranged from 0.1 to 1.0 µmol (Honma and Shimomura, 1978).
- The protein estimation was done as per Bradford methodology (Bradford, 1976).
- One unit of ACC deaminase activity was expressed as the amount of α-ketobutyrate liberated in nmol per milligram of cellular protein per hour.

Gupta and Pandey,2019

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- Protein concentrations determination:
- The protein concentration of toluenized cells was determined by the method of Bradford (1976).
- A 26.5 µl aliquot of the toluene-labilized bacterial cell sample used for the ACC deaminase enzyme assay was diluted with 173.5 µl of 0.1 M Tris-HCl (pH 8.0), and boiled with 200 µl of 0.1 N NaOH for 10 min.
- After the cell sample was cooled to room temperature, the protein concentration was determined by measuring the absorbance at 595 nm immediately after mixing the solution with 200 µl of Bradford's reagent.
- Bovine serum albumin (BSA) was used to establish a standard curve.

Zulfikar Ali *et al.*,2013

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- Bacterial strains isolated from rhizosphere of alfalfa: compatibility with *Ensifer meliloti*, indole acetic acid (IAA) production, phosphate solubilization and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity.

Strain	Origin	Phosphate solubilization PO_2	IAA production	ACC deaminase activity
		mg L^{-1}		
GN-1	Endorhizosphere	14.70d	18.14bc	-
GN-2	Endorhizosphere	90.73ab	26.22a	-
GN-4	Endorhizosphere	15.13c	15.13c	-
GN-7	Endorhizosphere	93.77a	22.47ab	-
GN-8	Endorhizosphere	-	14.03c	+
GN-9	Endorhizosphere	93.37a	19.07bc	-
GN-15	Endorhizosphere	-	15.70bc	-
GN-18	Endorhizosphere	-	13.71c	-
GE-4	Exorhizosphere	80.50b	19.70abc	-
GE-5	Exorhizosphere	-	20.13abc	-
GE-6	Exorhizosphere	88.40ab	16.79bc	-
GE-11	Exorhizosphere	87.05ab	17.18bc	-

Different letters represent significant differences according to LSD test ($P < 0.05$).
GN: Isolated from the endorhizosphere; GE: isolated from the exorhizosphere.

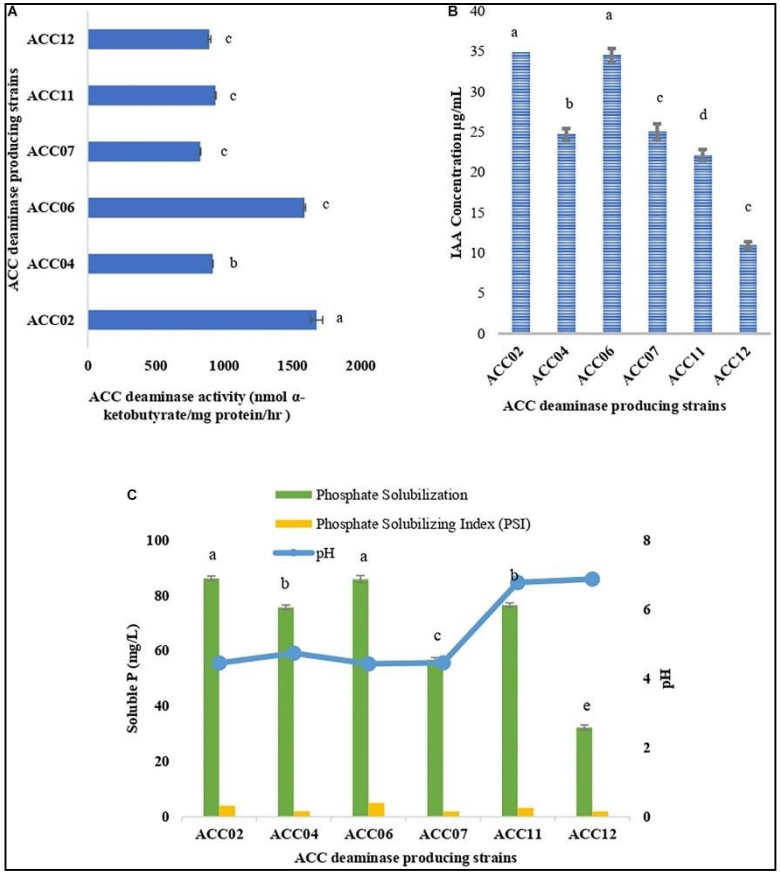
Cedeño-García *et al.*,2018

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- The quantitative assessment of ACC deaminase activity was done spectrophotometrically in terms of α-ketobutyrate production at 540 nm by comparing with the standard curve of α-ketobutyrate, which ranged from 0.1 to 1.0 µmol.



Bücking *et al.*,2019

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- Effect of ACC deaminase producers, ACC02 and ACC06, as individual strains and consortium on plant growth promotion of French bean under salinity stress (A) and normal condition (B), as compared to positive (uninoculated plants growing in normal conditions) and negative control (uninoculated plants growing saline stress conditions).

Two potential strains, ACC02 and ACC06, were found to possess other growth promoting potential like IAA production, phosphate solubilization, siderophore and ammonia production.

Bücking *et al.*,2019

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity on DF broth culture medium

- Plant growth promoting traits (including ACC deaminase acitivity) of strain AJS-15.
- Enzyme activity was expressed as µmol/mg protein/h.

Plant growth promoting traits	Activity
ACCD activity (nmoles of α-KB/mg pr. h^{-1})	191.90 ± 16
IAA production (µg ml^{-1})	0.531±0.050
Phosphate solubilization (µg ml^{-1})	8.612±2.148
Growth on N-free medium	+
Siderophore index	-
Ammonia production	+

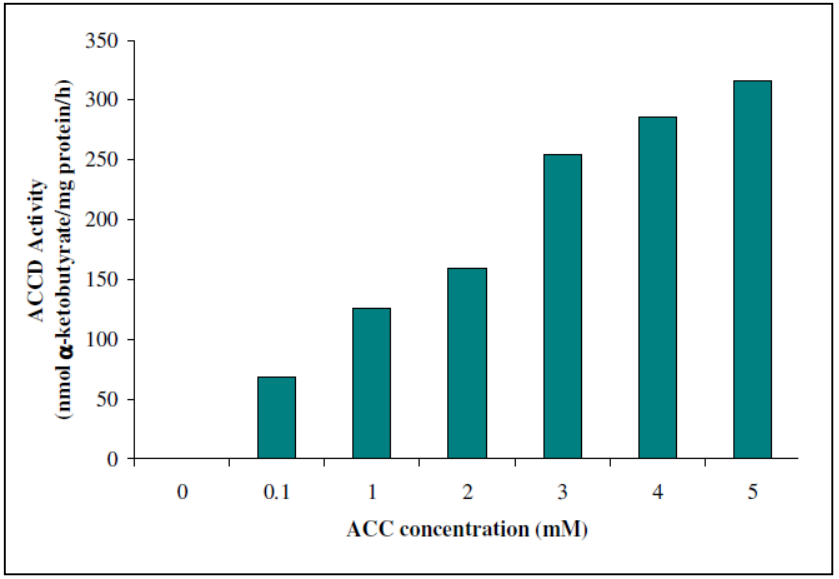
Singh and Nath Jha,2015

Phytohormones

Quantification of ACC deaminase activity (cont.)

At different concentrations of ACC

- ACCD activity in *Microbacterium* sp. strain ECI-12A in the presence of varying concentrations of ACC.
- Data shown is the average of two independent experiments performed separately in identical condition.



The amount of µmol of α-ketobutyrate produced by this reaction was determined and compared with a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µmol. For the purpose of standard curve generation a sock solution of 100 mM α-ketobutyrate (Sigma Aldrich Co., USA) was prepared in 0.1M Tris-HCl pH 8.5 and stored at 4°C. Enzyme activity was expressed as µmol/mg protein/h.

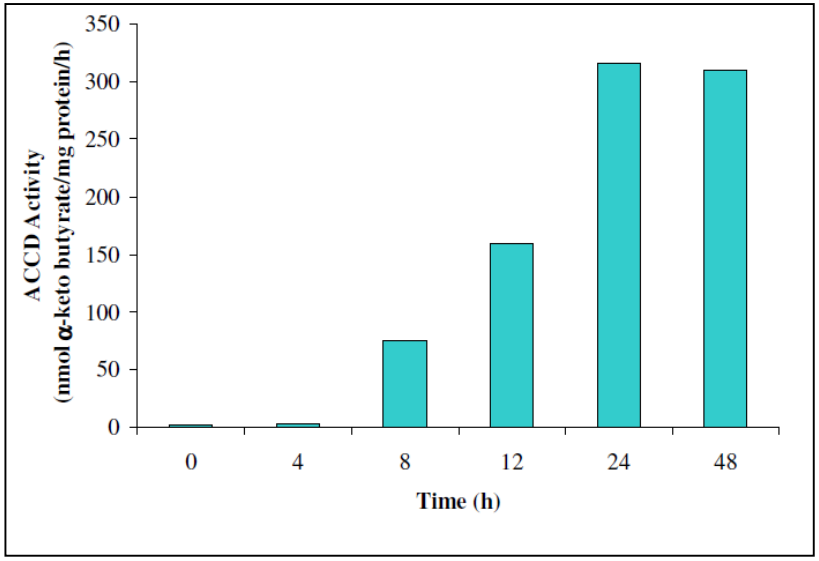
Shrivastava and Kumar, 2013

Phytohormones

Quantification of ACC deaminase activity (cont.)

At different concentrations of ACC and time intervals

- Time course induction of ACCD activity in *Microbacterium* sp. strain ECI-12A.
- Culture was grown with 5 mM ACC and activity was measured at desired time intervals.
- Data shown is the average of two independent experiments performed separately in identical condition.



The amount of µmol of α-ketobutyrate produced by this reaction was determined and compared with a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µmol. For the purpose of standard curve generation a sock solution of 100 mM α-ketobutyrate (Sigma Aldrich Co., USA) was prepared in 0.1M Tris-HCl pH 8.5 and stored at 4°C. Enzyme activity was expressed as µmol/mg protein/h.

Shrivastava and Kumar, 2013

Phytohormones

ACC deaminase activity indirect assay

Quantification of ACC deaminase activity (cont.)

- Estimation of ACCD activity in selected isolates ACCD activity is indirectly responsible for growth promotion in plants; therefore, its activity was measured.
- Out of nine isolates tested highest activity was found in *Klebsiella* sp. strain ECI-10A followed by *Pseudomonas* sp. strain AF-4B.

Bacterial strains	ACC deaminase activity (nmol α-keto butyrate/ mg protein/ h)
Klebsiella sp strain ECI-10A	539.1
Microbacterium sp strain ECI-12A	122.0
Agrobacterium sp strain AF-1D	237.3
Pseudomonas sp strain AF-4B	435.2
Klebsiella sp strain AF-4C	171.9
Serratia sp strain AF-5A	305.7
Pseudomonas sp strain PN-4D	358.4
Agrobacterium sp strain BN-2A	316.0
Klebsiella sp strain BN-4A	261.9

All the isolates showed activity in the range of 122-539.1 nmol αketobutyrate/mg protein/h. Data shown is the average of two independent experiments performed in identical conditions. The induction of ACCD activity was tested with 5mM ACC.

Shrivastava and Kumar, 2013

Phytohormones

ACC deaminase activity indirect assay

ELISA plate assay

- 5 ml of ½ Tryptic soy broth (TSB) were inoculated with rhizobacterial isolates. The cultures were incubated for 48 h at 30°C under shaking conditions (100 rpm). Cultures were diluted 10 times in sterilized 0.1 M $MgSO_4$ solution.
- In 96-well plate, 120 µL MSM (minimal salt medium containing ACC as sole nitrogen source) was added to each well.
- In first 4 lanes, 15 µL 0.1 M $MgSO_4$, and in second 4 lanes, 15 µL 0.1 M $(NH_4)_2SO_4$ were added.
- The 3 mM ACC was filter sterilized with 0.2 µm membrane filter and was stored at -20°C before the assay. This was allowed to thaw before use; 15 µL of thawed ACC were filled in the rest of the 4 lanes. For inoculation of each well, 15 µL bacterial culture were used.
- In untreated control wells, 15 µL 0.1 M $MgSO_4$ were used instead of inocula.
- Optical density (OD) was measured after 48 h at 600 nm using Biolog® identification system.
- The OD value of ACC and $(NH_4)_2SO_4$ wells were compared along with $MgSO_4$ wells to determine the ability of bacteria to utilize ACC for their growth.

Shahzad *et al.*,2010

Phytohormones

ACC deaminase activity indirect assay

ELISA plate assay(Contd..)

- Rhizobacterial isolates showing variable growth (measured as OD) on the media containing ACC as sole N source.

Rhizobacterial isolates	Grouping of isolates based on their ACC-utilization rate		
	Group-H O.D>0.75	Group-M O.D=0.75-0.50	Group-L O.D<0.50
J4			✓
J5		✓	
J6			✓
J7		✓	
J10		✓	
J14			✓
J15		✓	
J16	✓		
J17		✓	
J18	✓		
J19		✓	
J24		✓	
J26			✓
J27		✓	
J28			✓
J41			✓
J105			✓
J107	✓		
J108	✓		
J109		✓	
J112	✓		
J114			✓
J115		✓	
J117			✓
J118	✓		
J119	✓		
J120	✓		
J122		✓	
J127			✓

Shahzad *et al.*,2010

Phytohormones

Effects of ACC deaminase producing strains on French bean growth under salt stress

- **Pot Experiment Assay:**
- Three inoculated and uninoculated French bean seeds of respective treatment were then sown per plastic pots (30 cm in height and 30 cm in diameter), filled with autoclave-sterilized potting mixture of garden soil and coco peat in 1:1 ratio (3 kg soil pot^{-1}). The experimental soil was characterized as sandy loam with pH 4.5, EC 0.0354 dS m^{-1}, 66% sand, 9% slit, and 26% clay.
- The pots were placed in a growth chamber and maintained under optimum light and temperature condition, i.e., 80% relative humidity, 16:8 light: dark photoperiod and at 25°C for 30 days.
- After 10 days of seedling emergence, French bean seedlings were irrigated daily, twice a day, with either sterile distilled water or solution of EC 2.5 ds m^{-1} (25 mM NaCl; to artificially induce salinity stress) as per the treatment condition.
- The unbacterized plants subjected to salinity stress were presented as a negative control group while non-saline, unbacterized plants served as positive control group.

Bücking *et al.*,2019

Phytohormones

Effects of ACC deaminase producing strains on French bean growth under salt stress(Contd..)

- **Pot Experiment Assay:**
- Effect of ACC deaminase producers, ACC02 and ACC06, as individual strains and consortium on plant growth promotion of French bean

A. under salinity stress, and

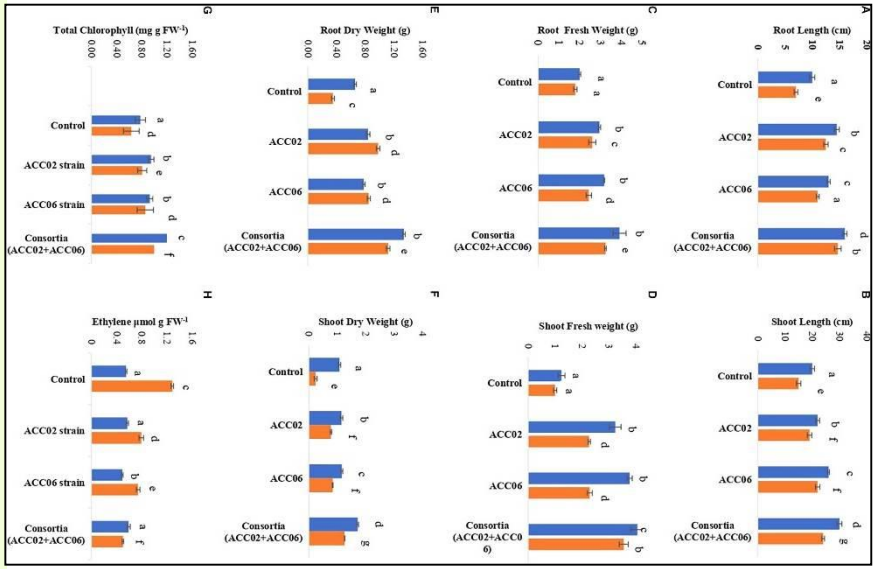
B. normal condition as compared to positive (uninoculated plants growing in normal conditions) and negative control (uninoculated plants growing saline stress conditions).

Bücking *et al.*,2019

Phytohormones

Effects of ACC deaminase producing strains on French bean growth under salt stress(Contd..)

- Effect of potent ACC deaminase isolates on physio-morphology parameters: (A) Root Length; (B) Shoot Length; (C) Root Fresh weight; (D) Shoot Fresh weight; (E) Root Dry weight; (F) Shoot Dry Weight; (G) Total Chlorophyll content and (H) Ethylene content of French beans plants under stress (saline; orange bar) and non-stressed (normal; blue bar) conditions. Control, plants from unbacterized seeds; ACC02, seeds inoculated with *A. aneurinilyticus* strain AIOA1; ACC06, seeds inoculated with *Paenibacillus* sp. strain SG_AIOA2. Columns represent Mean values while bars represent Standard deviation (n = 3). Different letters show statistically significant different values ($P < 0.05$) between treatments as evaluated from Duncan's test.



Bücking *et al.*,2019

Phytohormones

Plant growth-promoting rhizobacteria (PGPR)

acdS gene, encoding ACC deaminase

- The *acdS* gene, encoding ACC deaminase, has been isolated from different species and strains of genera belonging to the:
- *Alphaproteobacteria*,
- *Betaproteobacteria*, and
- *Gammaproteobacteria*, as well as the
- *Firmicutes*, and
- *Actinobacteria.*
- ACCD-producing bacteria are usually grown on minimal media containing ACC as the sole nitrogen source and identified via detection of ACCD activity in bacteria grown on the ACC-media.

Onofre-Lemus *et al.*,2009

Phytohormones

Plant growth-promoting rhizobacteria (PGPR)

Endophytic diazotrophic, and legume-nodulating *Burkholderia* species, as well as of non-N_2-fixing *Burkholderia* strains

- **PCR amplification and sequencing of *acdS* genes in *Burkholderia*.**
- For PCR amplification of partial *acdS* genes, primers 5ACC and 3ACC were used.
- To obtain the complete *acdS* gene sequences, the following two sets of degenerate primers were designed:
- primers F-*acdS* (5AT GAAYCTSCARCGHTTY3) and
- R-*acdS* (5TYARCCGTYSCGRAARRT3);
- and
- primers NF-*acdS* (5ATGAAYCTSCARMRHTTYC3) and
- NR-*acdS* (5TYARCCGTYGCGRAARATV3).

Onofre-Lemus *et al.*,2009

Phytohormones

Plant growth-promoting rhizobacteria (PGPR)

Endophytic diazotrophic, and legume-nodulating *Burkholderia* species, as well as of non-N_2-fixing *Burkholderia* strains

- **PCR amplification and sequencing of *acdS* genes in *Leclercia adecarboxylata* MO1 isolated from tomato rhizosphere.**
- The presence of ACC deaminase responsible gene '*acdS*' were examined by PCR analysis using (5′–3′) primers (Forward: ATCGGCGGCATCCAGWSNAAYCANAC and Reverse: GTGCATCGACTTGCCCTCRTANACNGGRT) as describe by Wang *et al.*,2017.
- Briefly, PCR was carried out for 35 cycles with the initial denaturation at 94°C for 3 min, cyclic denaturation at 94°C for 30 s, annealing 58°C for 30 s and extension at 72°C for 2 min with a final extension of 7 min at 72°C using 50 µL reaction mixture containing 50 ng of DNA, 20 pmoles of each primer, 1.25 units of Taq DNA polymerase, 200 µM of each dNTPs and 1× PCR buffer.
- Moreover the PCR product was examined by agarose gel electrophoresis.

Kang *et al.*,2019

Phytohormones

Plant growth-promoting rhizobacteria (PGPR)

Endophytic diazotrophic, and legume-nodulating *Burkholderia* species, as well as of non-N_2-fixing *Burkholderia* strains

- **PCR amplification and sequencing of *acdS* genes in *Burkholderia*.**
- PCR assays were performed using 50-µl reaction mixtures with PFX polymerase (Invitrogen) under the following conditions:
- Initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C and then a final 5-min elongation at 72°C. The amplified products were cloned into the vector pCR 2.1 (Invitrogen). and the *acdS* gene sequences were determined at the Biotechnology Institute, UNAM (Mexico).

Onofre-Lemus *et al.*,2009

Phytohormones
Proteobacteria and actinobacteria

- Amplification of the *acdS* gene by PCR with degenerate primers has been widely used for molecular identification of ACCD-producing bacteria.
- We designed consensus-degenerate hybrid oligonucleotide primers (acdSf3, acdSr3 and acdSr4) based on differentiating the key residues in ACC deaminases from those of 25 homologs for specific amplification of partial *acdS* genes.
- PCR amplification, sequencing 26 and phylogenetic analysis identified *acdS* genes from a wide range of proteobacteria and 27 actinobacteria.

Li *et al.*,2015

Phytohormones

Proteobacteria and actinobacteria

Primers used in this study

Primer	Sequence (5′ to 3′)	Position in *acdS*[a]	Corresponding amino acid sequences	Length	Number of degeneracy
acdSf3	ATCGGCGGCATCCAGWSNAAYCANAC[b]	217–242	IGGIQSNQT	26	128
acdSr3	GTGCATCGACTTGCCCTCRTANACNGGRT[b]	872–900	DPVYEGKSMH	29	64
acdSr4	GGCACGCCGCCCARRTGNRCRTA[b]	955–977	YAHLGGQP	23	64

[a]Nucleotide position in the *acdS* sequence of *Pseudomonas* sp. UW4.

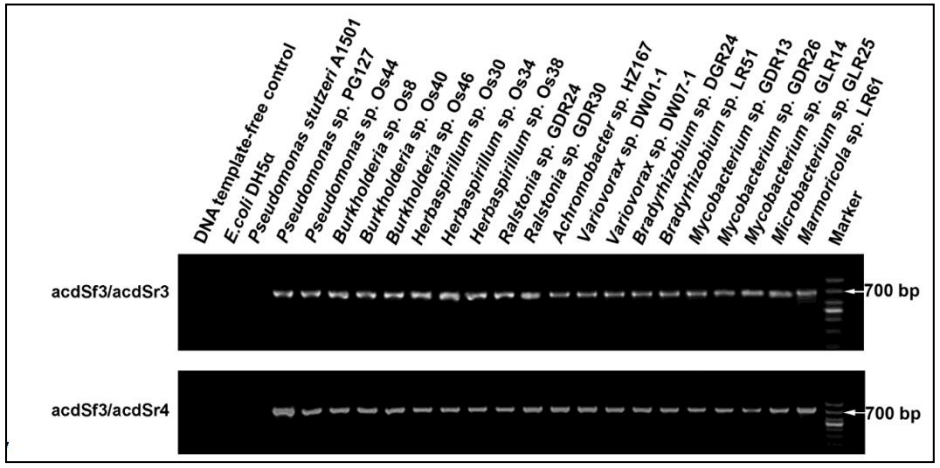
[b]3′ degenerate core regions are underlined.

- The CODEHOP pairs of acdSf3/acdSr3 or acdSf3/acdSr4 were first tested on the 44 known ACCD-producing isolates (18 *Burkholderia*, 10 *Herbaspirillum* and 16 *Pseudomonas*), *P. stutzeri* A1501 containing putative *acdS* genes, and the negative control *Es. coli* DH5α.
- Predicted amplification products (~680 bp with acdSf3/acdSr3 or ~760 bp with acdSf3/acdSr4) were obtained.

Li *et al.*,2015

Phytohormones
Proteobacteria and actinobacteria

1. Agarose gel electrophoresis showing PCR products about 680 bp with Consensus-degenerate hybrid oligonucleotide primers (CODEHOP) acdSf3/acdSr3 run at the annealing temperature 53°C and
2. PCR products about 760 bp with CODEHOP acdSf3/acdSr4 run from 65°C touchdown to the final annealing temperature (55°C).



Li *et al.*,2015

Bioremediation
Utilization of phenanthrene

- Bacteria grew in the presence of phenanthrene, indicating their probable application for bioremediation.
- Many bacterial isolates such as *Pseudomonas* and Bacilli were able to grow in phenanthrene (100 ppm) which is considered to be toxic and carcinogenic compounds that are ubiquitous pollutants in the environment.
- The capacity to degrade a ring compound like phenanthrene in some cultures might be due to the presence of lignin peroxidase enzyme system which can breakdown a number of pollutants like PAH(Polycyclic aromatic hydrocarbons) due to their non-specific action.

Singh *et al.*,2015

Bioremediation
Utilization of phenanthrene

- To evaluate the bioremediation potential, all cultures were grown in 50 ml mineral medium (Miller *et al.*,2004) supplemented with 100 ppm phenanthrene under shaking conditions (140 rpm) at 30°C for 7 days.
- To raise inoculums, all the isolates were grown in Luria broth at 30°C for 24 hrs, centrifuged (6000 rpm for 5 min) and pellet was washed three times with normal saline.
- Pellet was resuspended in normal saline and 2 ml inoculum (OD_{600} 0.8) was added to each flask.
- Any increase in OD_{600} was taken as an indication of bacterial growth and bioremediation potential.

Singh *et al.*,2015

Bioremediation

Utilization of phenanthrene

- Utilization of phenanthrene (100 ppm) by different bacterial isolates.

Isolate		Growth (OD 600 nm)
KC1	+	0.23
KC2	++	0.76
KC3	+	0.20
KC4	-	0.00
KC5	+	0.10
KC6	-	0.00
KC7	++	0.63
KC8	+	0.28
KC9	+	0.31
KC10	+	0.21

Growth was checked by measuring the increase of OD600 nm for 7 days
++: OD600 nm >0.5, +: OD600 nm >0.1, –: No growth

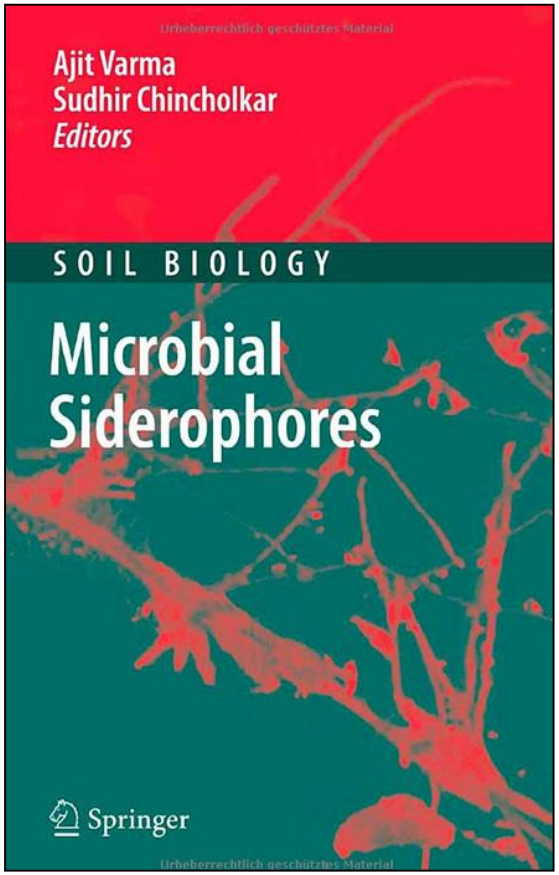
Singh *et al.*,2015

Microbial Siderophores

Bacterial siderophores

Reference Book
Microbial Siderophores

- Varma, A. and Chincholkar, S. (Eds.).
- Publisher: Springer;
- 1 edition (July 31, 2007)
- 248 pages



Siderophores

Plant and microbial siderophores

- Siderophores are metal-chelating agents with low molecular masses (200–2000 Da) that are produced by microorganisms and plants, especially under Fe-limiting conditions.
- Therefore, siderophore production is beneficial when iron is limiting.

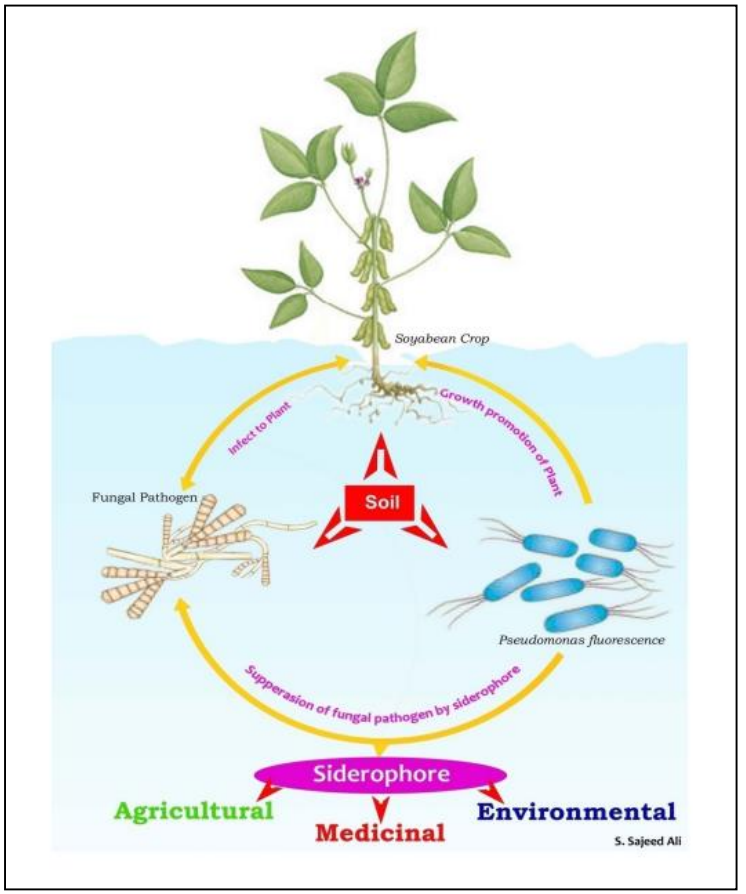
Schwyn and Neilands,1987

Siderophores

Application of siderophore

- Siderophore is biological molecule produced by various bacteria having wide application in various field such as:

1. agriculture to improve soil fertility and biocontrol,
2. environmental application and
3. medicinal application.



Sajeed Ali and Vidhale,2013

Microbial siderophores

What are siderophores in pathogenic bacteria?

- Siderophores are low-weight, high-affinity iron chelating molecules produced in response to iron deficiency by Gram-positive and Gram-negative bacteria which also known as essential virulence factors of bacteria.
- Siderophore producing Pseudmonads play vital role in stimulating the growth of the plant and in controlling phytopathogens.

Microbial siderophores

What are siderophores in pathogenic bacteria?

- Almost all known bacterial species produce siderophores, making their secretion the most prevalent mechanism for iron scavenging in the microbial world.
- Siderophores are a chemically diverse group of secondary metabolites.
- There is an extensive body of work on their chemical structures and the molecular mechanisms of their synthesis, export, uptake, and regulation.

Kramer *et al.*,2021

Bacterial siderophores

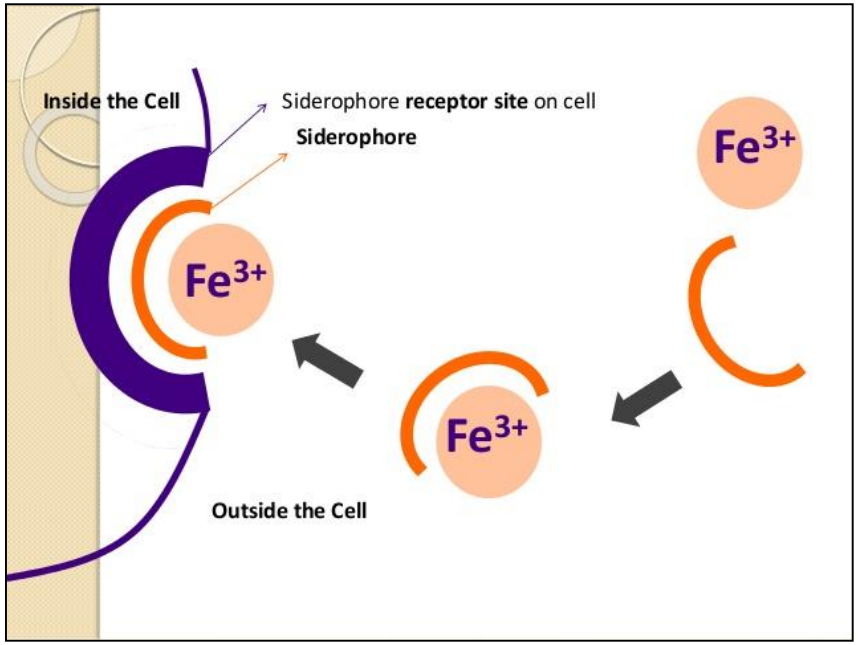
Competition for iron and the role of siderophores

- Iron is extremely limited in the rhizosphere, depending on soil pH.
- In highly oxidized and aerated soil, iron is present in ferric form, which is insoluble in water (pH 7.4) and the concentration may be as low as 10^{-18} M.
- This concentration is too low to support the growth of microorganisms, which generally need concentrations approaching 10^{-6} M.

Siderophore transport in bacteria

The life cycle of siderophore in and outside the bacterial cell

- To survive in such an environment, organisms were found to secrete iron-binding ligands called siderophores having high affinity to sequester iron from the micro-environment.
- Most microorganisms respond to low-iron stress by producing extracellular, low molecular-weight (500-1000 Daltons), iron transport compounds (or siderophones) which bind iron selectively and with great binding power.

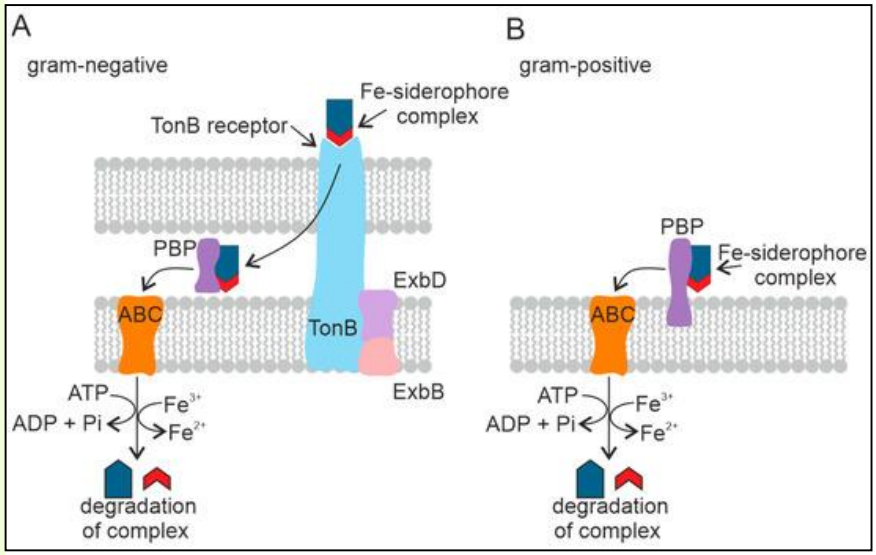


Manju,2014;..

Siderophore transport in bacteria

Mechanism of transport of the Fe-siderophore complex into the cell in Gram-ve and Gram+ve bacteria

- Mechanism of transport of the Fe-siderophore complex into the cell in Gram-negative (A), and Gram-positive (B) plant-growth-promoting bacteria (PGPB).
- Periplasmic binding protein (PBP), ATP-binding cassette transporter (ABC).
- The TonB-dependent receptor is a complex of three proteins.



The transport of siderophores across the outer cell membrane of Gram-negative bacteria is mediated by a complex of three transmembrane proteins: TonB, ExbD, and ExbB. This family of outer membrane transport proteins is called TonB-dependent receptors.

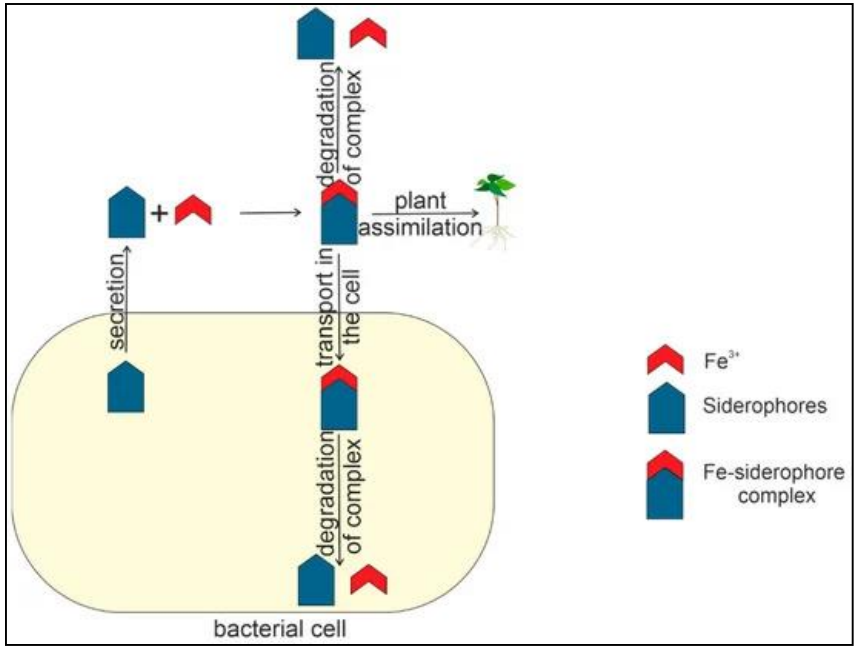
Timofeeva *et al.*,2021

Siderophore transport in bacteria

The fate of the Fe-siderophore complex outside the bacterial cell

- The exact mechanisms of these processes have not been established.
- But two possible ways for plants to obtain Fe from microbial siderophores have been proposed.

1. Fe(III)–siderophores from bacteria are first transported to the plant root apoplast, where siderophore reduction occurs. Thus, Fe(II) is captured by the apoplast, possibly leading to a high local concentration of Fe in the root.
2. The second mechanism is for the bacterial siderophores to chelate Fe from the soil and perform ligand exchange with phyto-siderophores.



Timofeeva *et al.*,2021

Mechanisms of biological control
Siderophores

- Kloepper *et al*.,1980 were the first to demonstrate the importance of siderophore production as a mechanism of biological control of *Erwinia carotovora* by several plant-growth promoting *Pseudomonas fluorescens* strains.

Neilands,1981; Pal and Gardener,2006

Mechanisms of biological control

Siderophores produced by fluorescent pseudomonads

- The capacity to utilize siderophores is important to the growth of bacteria in the rhizosphere and on plant surfaces.
- Specific siderophore-producing *Pseudomonas* strains (PGPR) rapidly colonize plant roots of several crops, and this colonization can result in significant yield increases.
- Enhanced plant growth caused by these strains often is accompanied by reduction in the populations of fungi and other bacteria on the roots.

Penyalver *et al.*,2001

Mechanisms of biological control

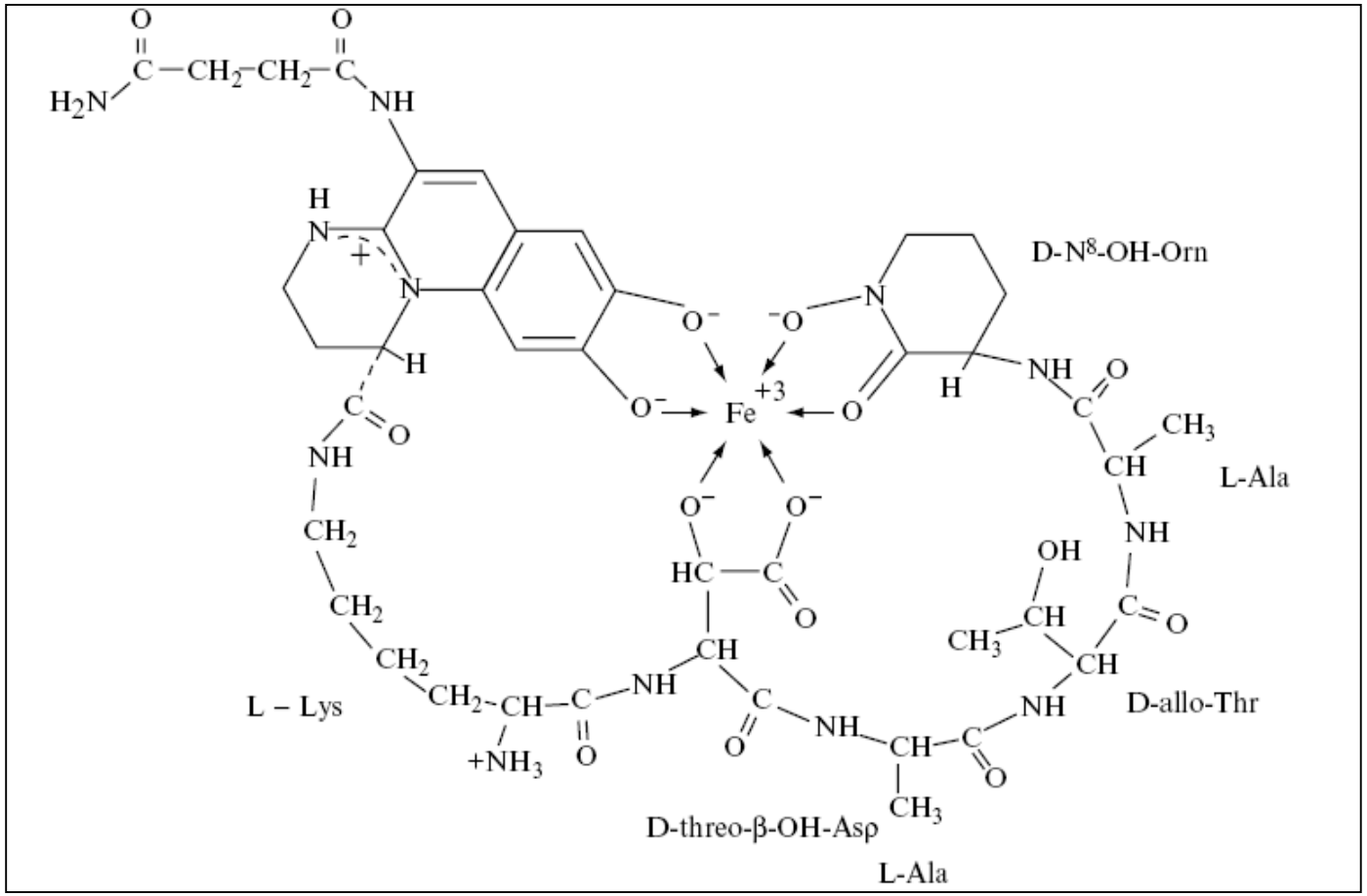
Siderophores produced by fluorescent pseudomonads

- Among fluorescent pseudomonads, such as *P. fluorescens* and *P. putida*, a yellow-green fluorescent pyoverdines act as siderophores.
- The best known of these is pseudobactin, a hexapeptide cyclized through a fluorescent quinoline derivative with a hydroxamic iron-chelating group derived from moieties of ornithine, aspartic acid, and aniline residues.
- Pseudobactin requires several genes for its biosynthesis; at least five gene clusters and a minimum of seven genes are required for biosynthesis of pyoverdine siderophores.

Hagedorn and Lacy

Ferric pseudobactin

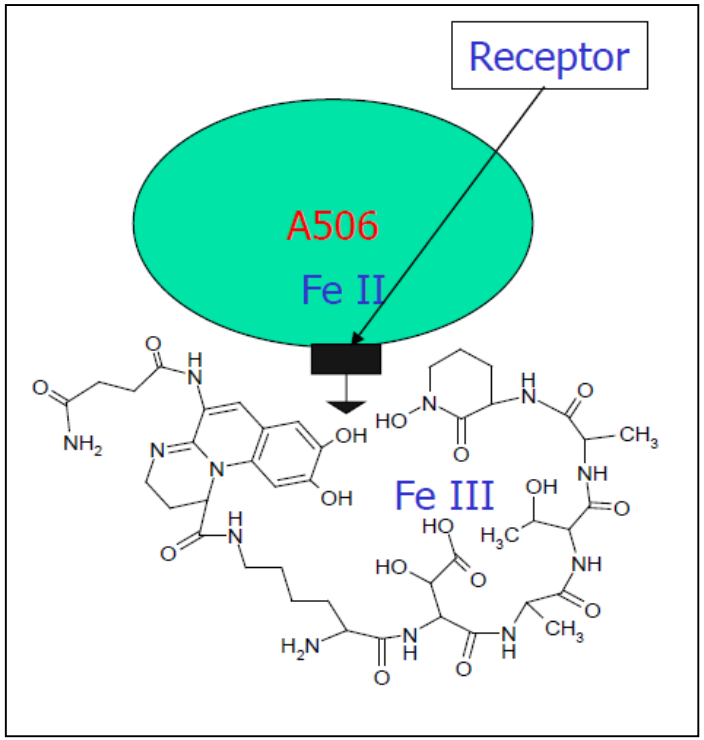
A siderophore produced by fluorescent pseudomonads



Ferric pseudobactin

A siderophore produced by *Pseudomonas fluorescens* A506

- Fluorescence under UV is caused by a pyoverdine.
- Pyoverdines are a class of siderophores (chelating compounds produced by organisms).
- Siderophores are produced in iron-deficient environments, such as aerial plant surfaces.



Kevin Hockett and co-worker

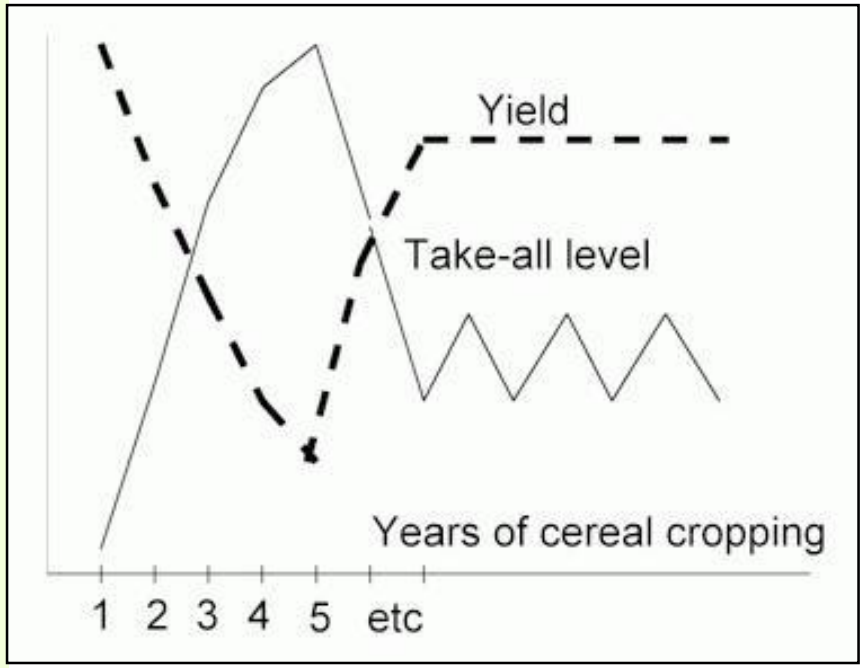
Mechanisms of biological control

Converting conducive soils to suppressive soils

- Suppressive soils are relatively abundant and all have a low pH (<5.5).
- The suppressive nature of the soil may be transmitted to soil that is conducive to the disease by the addition of as little as 1 per cent of the suppressive soil.
- Although siderophores have been implicated as contributors to disease suppression in some instances, in most cases one or more antibiotics are involved.

Take-all disease suppression
Fluorescent pseudomonads

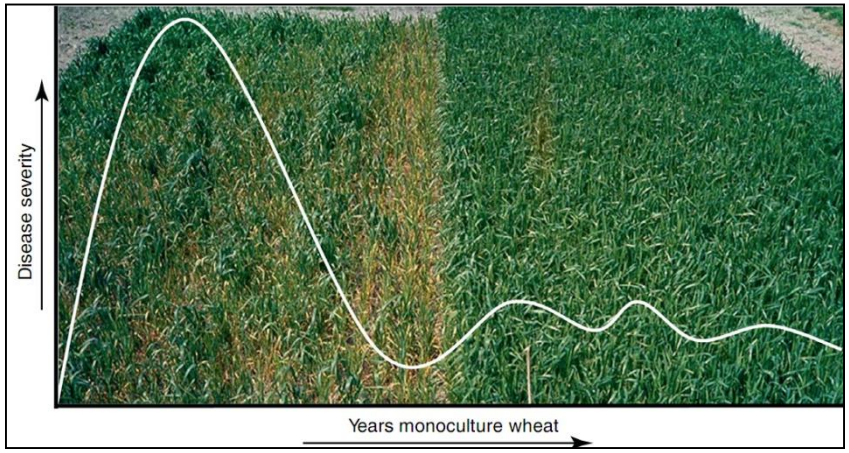
- Take-all infection builds up progressively in the first few years of cereal monocropping.
- Disease severity peaks in 3rd to 5th year and then declines to an economically acceptable level.
- Non-disease causing, fluorescent pseudomonads living as epiphytes on roots are responsible for this dramatic disease decline.



Sonk,2007

Take-all disease suppression
Fluorescent pseudomonads

- Decline of disease severity is typically associated with an increase of Pseudomonas spp. that produce the antifungal 2,4 diacetylphloroglucinol.
- The picture in the background shows an experimental field in which:
- Left, wheat had been grown in rotation with other crops
- Right, in monoculture.
- Following inoculation with G. *graminis* var. *tritici*, less disease developed in the wheat monoculture plot.



Berendsen *et al.*,2012

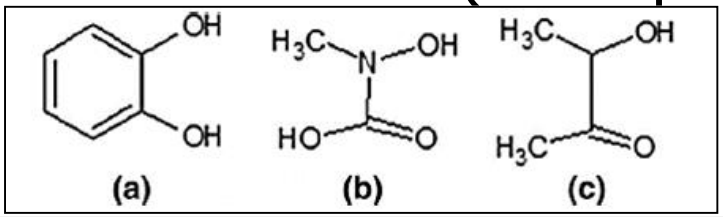
Bacterial siderophores

The two common siderophore types

- Almost all microorganisms produce siderophores, of either the:

1. Catechol type(chelate ferric iron via hydroxyl groups), or
2. Hydroxamate type (chelate ferric iron via a carbonyl group with an adjacent nitrogen).

- Microorganisms, including pathogenic bacteria, use hydroxamate-based entities (siderophores).



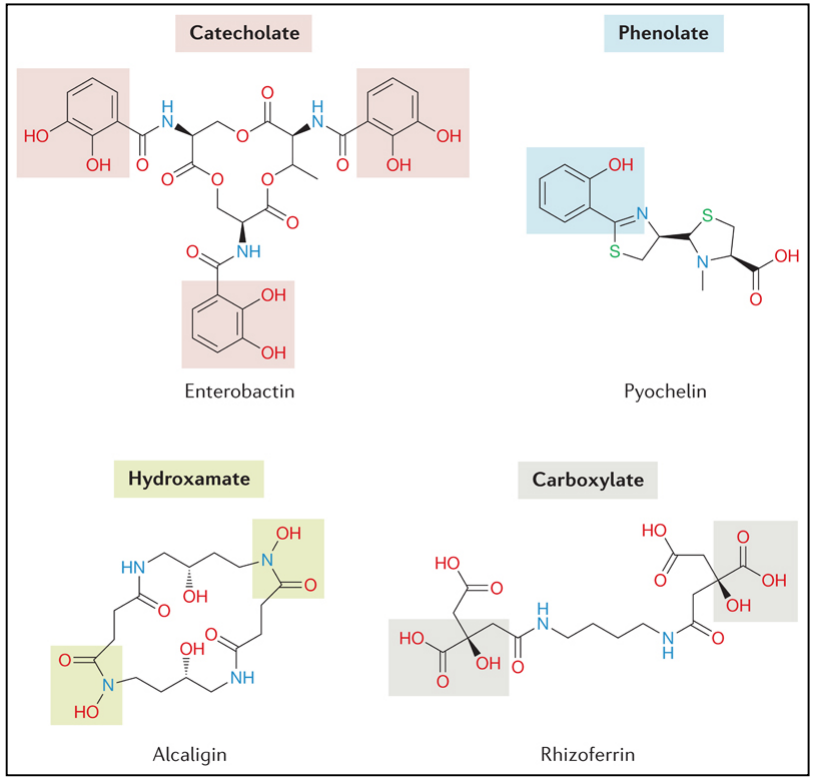
Siderophore functional groups: a) Catecholate, b) Hydroxamate and C) Hydroxy-carboxylate

Neilands,1981; Pal and Gardener,2006; Khasheii *et al.*,2021

Bacterial siderophores

The four main siderophore types produce by bacteria

- The four main types are distinguished based on the moieties involved in iron chelation, which entail catecholate, phenolate, hydroxamate and carboxylate functional groups (grey shadings).
- Siderophores with mixtures of functional groups are also common.
- Representative siderophore examples include enterobactin (which is produced by *Escherichia coli*, for example), pyochelin (which is produced by *Pseudomonas aeruginosa*, for example) featuring a heterocycle (thiazoline) ring, alcaligin (which is produced by *Bordetella pertussis*, for example) and rhizoferrin (which is produced by *Ralstonia pickettii*, for example). Note that the same siderophore can be produced by different species.



Kramer *et al.*,2021

Bacterial siderophores

Major siderophore-producing PGPB for which the siderophore structure has been established

Genus	Strain	Gram	Siderophore	
			Name	Type
Azospirillum	*Azospirillum brasilense*	Negative	Spirilobactin	Catechol
	Azospirillum lipoferum	Negative	2,3-DHB, 3,5-DHB-threonine, 3,5-DHB-lysine	Catechol
Azotobacter	*Azotobacter vinelandii*	Negative	Aminochelin, Azotochelin, Protochelin, 2,3-DHB	Catechol
	Azotobacter vinelandii	Negative	Azotobactin	Mixed
		Negative	Vibrioferrin	Mixed
Bacillus	*Bacillus megaterium*	Positive	Schizokinen, N-schizokinen, N-schizokinen-A	Hydroxamate
	Bacillus subtilis, *Bacillus thuringiensis*	Positive	Itoic acid, Bacillobactin	Catechol
Pantoea	*Pantoea vagans C9-1*	Negative	Enterobactin-like	Catechol
		Negative	Desferrioxamine-like	Hydroxamate
	Pantoea eucalypti M91	Negative	Pyoverdine-like, Pyochelin-like	Mixed
Pseudomonas	*Pseudomonas B10*	Negative	Pseudobactin(s)	Mixed
	Pseudomonas fluorescens, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas aureofaciens*	Negative	Pyoverdine(s)	Mixed
	Pseudomonas fluorescens	Negative	Ferribactin	Mixed
Rhizobium	*Rhizobium radiobacter*	Negative	Agrobactin	Catechol
	R. leguminosarum, *R. phaseoli*	Negative	Vicibactin	Hydroxamate
	Rhizobium leguminosarum	Negative	Schizokinen	Hydroxamate
		Negative	2,3-DHB-threonine	Catechol
	Rhizobium meliloti	Negative	Rhizobactin	Catechol

Timofeeva *et al.*,2021

Bacterial siderophores

List of bacteria which can produce different types of siderophore.

Types of siderophore	Name of siderophore	Siderophore-producing bacteria	References
Hydroxymate	Ferribactin	*Pseudomonas fluorescens*	Maurer and Keller-Schierlein (1968)
	Unknown	*Escherichia coli*	Kannahi and Senbagam (2014)
	Unknown	*Pseudomonas putida*	Sayyed et al. (2005)
	Unknown	*Micrococcus luteus*	Cabaj and Kosakowska (2009)
	Unknown	*Methylobacterium radiotolerans*	Lacava et al. (2008)
	Unknown	*Methylobacterium zatmanii*	Lacava et al. (2008)
	Desferrioxamine B, desferrioxamine	*Streptomyces coelicolor*	Saharan and Nehra (2011)
	Unknown	*Halorubrum saccharovorum*	Dave et al. (2006)
Catecholate	Enterobactin	*Escherichia coli*	Saharan and Nehra (2011)
	Pyoverdine	*Pseudomonas aeruginosa*	Peek et al. (2012)
	Salmochelins	*Salmonella enterica*	Hantke et al. (2003)
	Bacillibactin	*Bacillus anthracis*	Saharan and Nehra (2011)
	Bacillibactin	*Bacillus subtilis*	Saharan and Nehra (2011), and May et al. (2001)
	Petrobactin, Bacillibactin	*Bacillus cereus, Bacillus anthracis*	Wilson et al. (2006)
	Bacillibactin	*Bacillus thuringiensis*	Wilson et al. (2006)
	Vibriobactin	*Vibrio cholera*	Saharan and Nehra (2011), and Griffiths et al. (1984)
	Agrobactin	*Agrobacterium tumefaciens*	Dave et al. (2006)
	Parabactin	*Paracoccus denitrificans*	Dave et al. (2006)
Carboxylate	Rhizobactin	*Rhizobium meloti*	Drechsel et al. (1995)
	Staphyloferrin A	*Staphylococcus hyicus*	Meiwes et al. (1990)
	Staphyloferrin A, Staphyloferrin B	*Staphylococcus aureus*	Beasley et al. (2011)
	Unknown	*Halococcuss accharolyticus*	Dave et al. (2006)
	Unknown	*Halorubrum saccharovorum*	Dave et al. (2006)
	Unknown	*Haloterrigena turkmenica*	Dave et al. (2006)
	Unknown	*Halogeometricum* sp.	Dave et al. (2006)

Pahari *et al.*,2017

Bacterial siderophores

List of bacteria which can produce different types of siderophore.

Organisms	Siderophores
Ustilago sphaerogena	Ferrichrome
Streptomyces pilosus	Desferrioxamines
Escherichia coli	Enterobactin
Aeromonas hydrophila	Amonabactin
Aerobacter aerogens, *Salmonella* sp, *Klebsiella pneumoniae*	Aerobactin
Vibrio cholerae *Vibrio anguillarum*	Vibriobactin (Marine) Anguibactin
Acinetobacter calcoaceticus	Acinetobactin
Mycobacterium tuberculosis	Mycobactin
Pseudomonas aeruginosa	Pyoverdin and Pyochelin
Yersinia pestis	Yersiniabactin
Alteromonas haloplanktis	Bisucabarin (Marine)

Mohandass,2004

Siderophore production assay
In fluorescent *Pseudomonas* isolates

- Different methods are available to evaluate fluorescent *Pseudomonas* isolates for siderophore production:

1. CAS assay-plate screening,
2. CAS assay-spectrophotometric analysis,
3. hydroxyquinoline test,
4. tetrazolium test,
5. Arnow's assay, and
6. $FeCl_3$ test.

Almost all microorganisms produce siderophores, of either the:
1. Catechol type, or
2. Hydroxamate type.

Kotasthane *et al.*,2017

Siderophore production assay

The CAS assay is a universal chemical test

1. The chromeazurol (CAS) agar assay

- CAS is a method that can be used to detect the mobilization of iron. It is a universal test for detection and determination of siderophores, as even 0.02 µm of siderophores can be determined.
- The production of siderophores was tested on:

1. Solid medium by using CAS medium [chrome azurol S, iron(III), hexadecyltrimethylammonium bromide], and
2. In liquid medium (2% sucrose, 0.2% L-asparagine, 0.1 g of K_2HPO_4, 0.05% $MgSO_4.7H_20$;+0.01%$FeCl_3$ $6H_20$) (pH7.0).

Schwyn and Neilands,1987; Gram,1993

Siderophore production assay

The CAS assay is a universal chemical test

The chromeazurol (CAS) agar assay

- All CAS assays in liquid media were done at 25°C at 150 rpm.
- Siderophore-producing strains showed orange halos around the colony.
- The large halos could also be explained by the production of smaller siderophores which diffuse more easily in the agar.

Schwyn and Neilands,1987; Gram,1993

Siderophore production assay

A universal method

Preparation of chromeazurol (CAS) agar

- Briefly, 60.5 mg of CAS was dissolved in 50 mL of deionized water, and mixed with 10 mL of a Fe^{+3} solution (1 mmol L^{-1} $FeCl_3.6H_2O$, 10 mmol L^{-1} HCl).
- While stirring, this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) previously dissolved in 40 mL water.
- The resulting dark-blue solution was autoclaved, cooled to 50/60°C and mixed with 900 mL sterile MM9 (Silva-Stenico *et al.*,2005) containing 15 g L^{-1} agar (also kept at 50/60°C).
- This medium was allowed to gel on Petri dishes, was subsequently inoculated with bacterial strains and incubated in the dark (28°C for 5 days).

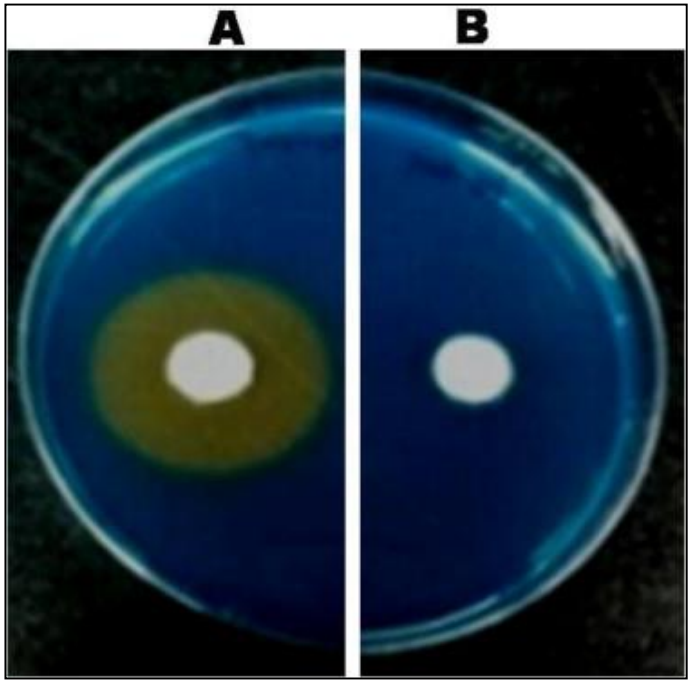
Lacava *et al.*,2008

Siderophore production assay

A universal method

The chromeazurol (CAS) agar assay

- To determine the siderophore-producing ability of these two strains, they were inoculated using sterile toothpicks on the CAS plates and cultivated at a constant temperature incubator at 37°C for 3 days to 5 days and were checked for the presence or absence of orange circles surrounding the bacteria.



Siderophore production assay showing color change from blue to orange halo.

Siderophore production assay

In fluorescent *Pseudomonas* isolates

2. GASN medium

- Siderophores were produced by growing cultures in/on GASN (glucose asparagines) solid/liquid medium in Petri dishes as described (Bultreys & Gheysen,2000; Bultreys *et al*.,2006b).
- GASN medium: For siderophores production, bacterial strains were cultivated at 22°C in GASN medium(2 g/L L-asparagine, 7 g/L glucose, 0.96 g/L Na_2HPO_4, 0.44 g/L KH_2PO_4, and 0.2 g/L $MgSO_4.7H_2O$, pH 7.0).

Bultreys and Gheysen,2008

Siderophore production assay

In fluorescent *Pseudomonas* isolates

Changes in pH during siderophore production

- Bultreys and Gheysen (2000) have reported marked changes in pH during the production of siderophores by *Pseudomonas* strains, grown in different media.
- In a medium containing asparagine, glucose and salts (GASN medium),

1. the pH decreased from 7 to 4.6 after one day,
2. increased to 6.6 on the second day, and
3. rose to 7.5 on the third day.

- The increase in pH resulted in sharp increase in siderophore concentration.

Gaonkar and Bhosle,2013

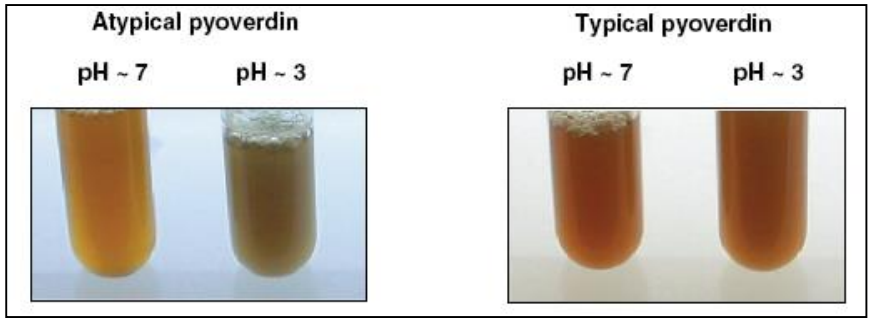
Pyoverdin Tests

Glass tubes assay

Atypical pyoverdins vs. typical pyoverdins

- The atypical pyoverdins of *P. syringae* and *P. cichorii* show a grayish-brown color near pH 3 and a yellow-orange color near pH 7.
- Whereas, the typical pyoverdin of *P. fuscovaginae* and *P. asplenii*, appears similar at pH 7 (remains constant) but shows still a net orange color near pH 3.

Typical pyoverdins, which, most generally, show a constant brown color at both pH 3 and 7



Atypical pyoverdin: Grayish-brown color near pH 3 and a yellow-orange color near pH 7.

Atypical pyoverdin: Net orange color near pH 3.

Bultreys and Gheysen,2008

Siderophore production assay

In fluorescent *Pseudomonas* isolates

3. Hydroxyquinoline mediated siderophore test

- For selection of *Pseudomonas* isolates with high ability to siderophores, isolates were inoculated on King's B medium supplemented with a strong chelater 8-Hydroxyquinoline (50 mg/l) (De Brito *et al.*,1995).
- Organisms growing on this medium were considered positive for siderophore production.

Kotasthane *et al.*,2017

Siderophore production assay

In fluorescent *Pseudomonas* isolates

4. Arnow's test for catecholate siderophores

- The cultures were inoculated in King's B broth medium were centrifuged at 10,000 rpm for 15 min. The cell-free culture filtrate was used for the siderophore production.
- To 1 ml of culture filtrate, 1 ml of 0.5 N HCl, and 1 ml of nitrite molybdate($Na_2MoO_4.2H_2O$) reagent were added. Then 1 ml of 1N NaOH was added.
- Preparation of nitrite molybdate reagent: To 10 g of sodium nitrite was added 10 g of sodium molybdate. The mixture was dissolved in 100 ml of distilled water.
- The formation of red coloured solution which was the indication of the presence of catechol type of siderophore was examined.
- Arnow's assay was used for quantification of catechol type siderophore.

Kotasthane *et al.*,2017

Siderophore production assay

In fluorescent *Pseudomonas* isolates

5. Tetrazolium test for hydroxamate siderophores

- The cultures were inoculated in King's B broth medium were centrifuged at 10,000 rpm for 15 min.
- The cell-free culture filtrate was used for the siderophore production.
- To 0.5 ml of cell-free supernatant, a pinch of tetrazolium salt and a few drops of 2N NaOH were added.
- Instant appearance of a deep red color indicated the presence of a hydroxamate siderophore (Snow, 1954).

Kotasthane *et al.*,2017; Sujatha and Ammani,2013

Siderophore production assay

In fluorescent *Pseudomonas* isolates

6. $FeCl_3$ test for hydroxamate/catecholate type of siderophore

- *Pseudomonas* spp. were inoculated to 20 ml King's B medium in 50 ml tubes and incubated for 3 days at 28 ± 2°C.
- The bacterial cells were removed by centrifugation at 10000 rpm for 5 min.
- One ml of the culture supernatant was mixed with freshly prepared 0.5 ml of 2% aqueous $FeCl_3$ and observed for the presence and absence of deep red colour(sid^+).
- Spectrophotometric assay:
- The resultant mixture was scanned between 400-600 nm.

1. A peak between 420-450 nm in ferrated siderophores indicates a hydroxamate type of siderophore, and
2. A peak at 495 nm indicates a catecholate type of siderophore (Neilands,1981).

Kotasthane *et al.*,2017; Chowdappa *et al.*,2020

Siderophore production assay

Estimation of siderophores in *Bacillus* spp.

Salicylate and catechol type siderophores

- The production of bacterial isolate siderephores was tested as follow:
- The media used is NB.
- One ml of bacterial isolate was added to each flask and incubated at 37°C for 7 days.
- After seven days of incubation, culture of bacterial isolate was centrifuged at 10,000 g for 20 min.
- Supernatant is used to estimate siderophores salicylate type.
- 20 ml of supernatant culture was taken and the pH was set to 2.0 with HCl solution.
- For 20 ml of supernatant was added 20 ml of ethyl acetate and extraction twice.
- Five ml of the test solution were added with 5 ml of the Hathway reagent (1 ml of 0.1 M ferric chloride and 1 ml of 0.1 N HCl added to 100 ml of distilled water and 1 ml of 0.1 M potassium ferricyanide added) and the absorbance was measured at 560 nm with sodium salicylate as a standard for salicylate estimation.
- Standard sodium salicylate is prepared from dilution with a concentration of salicylate nantrium ranging from 0 to 2 mg l^{-1}.
- To measure the concentration of catechol type siderophores, five ml of the test solution was added with five ml of Hathway reagents and absorbance was determined at 700 nm with 2.3 DHBA as standard. The concentration in the culture filtrate was determined and expressed as mg l^{-1}.

Kesaulya *et al.*,2017

Siderophore production assay

Estimation of siderophores in *Bacillus* spp.

Salicylate and catechol type siderophores

- Microbes capable of producing siderophores may affect the biocontrol, virulence and availability of iron nutrients for plants.
- Rhizosphere bacteria can produce various siderophores.
- Generally, siderophores bacteria contain catecholates, and some also contain carboxylates and hydroxamates.
- The results of this research by testing the production of catechol and salicylate showed that the rhizosphere bacteria from the potato plant produced the species of catechol and salicylate siderophores with different concentration.

Bacteria [18]	Type of Siderophore	
	Catechol (mgl^{-1})	Salicylate ($mg\ l^{-1}$)
Bacillus niabensis Strain PT-32-1	2.26	3.23
Bacillus subtilis Strain SWI16b	3.35	1.71
Bacillus subtilis Strain HPC21	2.87	4.21
Bacillus mojavensis Strain JCEN3	4.21	4.12
Bacillus subtilis Strain HPC24	3.03	2.77

Kesaulya *et al.*,2017

Siderophore production assay

In fluorescent *Pseudomonas* isolates

Quantitative assay of hydroxamate siderophores

- Siderophores like lysozymes are the examples of iron binding proteins.
- Among the hydroxamate siderophore producing bacteria, some isolates produced high levels of siderophores that ranged between 32.73 to 24.12 µg hydroxymate/mg protein.
- To estimate this value we should first estimate the Total cell protein.
- Total cell proteins were estimated by Lowry method, 1951.

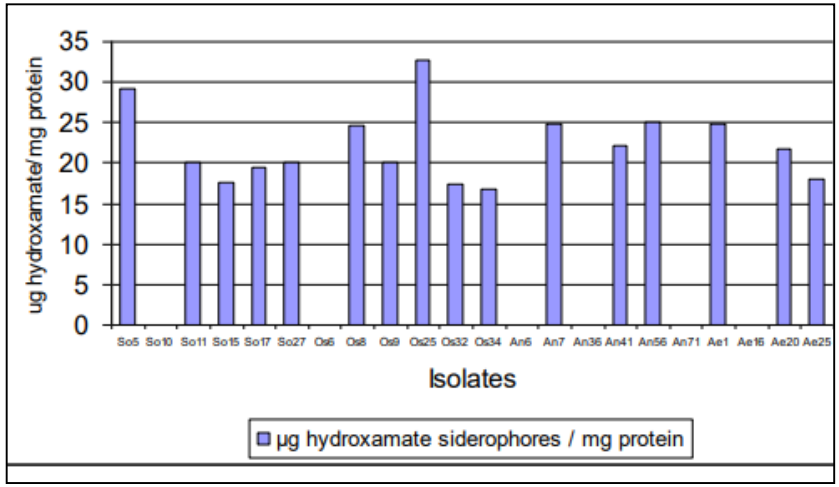
Sujatha and Ammani,2013

Siderophore production assay

In fluorescent *Pseudomonas* isolates

Quantitative assay of hydroxamate siderophores

Isolates	CAS assay	$Fecl_3$ Test	Spectrophotometric assay (nm)	Arnow's test for Catechol	Tetrazolium test for hydroxamate	µg hydroxamate/ mg protein
So5	+	+	430	-	+	29.16
So10	+	+	490	+	-	-
So11	+	+	435	-	+	20.16
So15	+	+	425	-	+	17.52
So17	+	+	440	-	+	19.43
So27	+	+	445	-	+	20.16
Os6	+	+	490	+	-	-
Os8	+	+	430	-	+	24.72
Os9	+	+	435	-	+	20.16
Os25	+	+	430	-	+	32.73
Os32	+	+	445	-	+	17.46
Os34	+	+	425	-	+	16.75
Ah6	+	+	490	+	-	-
Ah7	+	+	430	-	+	24.75
Ah36	+	+	490	+	-	-
Ah41	+	+	435	-	+	22.16
Ah56	+	+	445	-	+	25.12
Ah71	+	+	490	+	-	-
Ae1	+	+	430	-	+	24.79
Ae16	+	+	490	+	-	-
Ae20	+	+	435	-	+	21.67
Ae25	+	+	440	-	+	17.97



Sujatha and Ammani,2013

Siderophore production assay

In fluorescent *Pseudomonas* isolates

Quantitative assay of hydroxamate siderophores

- **Estimation of Total cell protein:**
- Total cell proteins were estimated by Lowry method,1951.
- An aliquot (0.5 ml) of the bacterial suspension was mixed with 0.5 ml of 1 N NaOH and kept in boiling water for 10 min.
- After cooling, 5 ml of copper carbonate reagent (50 ml of 2% sodium carbonate, 1 ml of 0.5 % copper sulphate and 1 ml of 1% sodium potassium tartarate) were added.
- The solution was allowed to stand at room temperature for 10 min. Then 0.5 ml of diluted (1:1 dilution with water) Folin Phenol reagent was added with vigorous shaking.
- After 30 min. the absorbance of the coloured solution was read at 660 nm against reagent blank.
- Bovine serum albumin was used as standard.

Sujatha and Ammani,2013

Siderophore production assays

In other bacteria

Agrobacterium rhizogenes strain K84

- *Agrobacterium rhizogenes* strain K84 two main antiagrobacterial produces:

1. The antibiotic-like substances such as agrocin 84 and ALS84 was proved as a key components in the process of biocontrol by strain K84.
2. A hydroxamate iron chelator siderophore in large amounts.

Penyalver *et al.*,2001

Siderophore production assays

Agrobacterium rhizogenes strain K84

- Production of the hydroxamate siderophores and susceptibility to ALS84 and agrocin 84 under conditions of iron sufficiency and iron deficiency.

Growth medium[a]	Producer strain	Siderophore production[b]	Antibiosis assay[c]				Antibiotic activity produced
			Indicator strain	Assay medium			
				ST	MG	MGF	
ST	K84	−	C58	+	+	+	Agrocin 84
			NT1	−	−	−	
	K84 Agr⁻	−	C58	−	−	−	None
			NT1	−	−	−	
MG or CM9	K84	+	C58	+	+	+	Agrocin 84 + ALS84
			NT1	−	+	−	
	K84 Agr⁻	+	C58	−	+	−	ALS84
			NT1	−	+	−	

[a] ST medium contains nonlimiting amounts of iron; MG and CM9 media contain limiting amounts of iron.
[b] Production of the hydroxamate siderophore was assessed by the CAS and Csàky tests as described in Materials and Methods. +, production; −, no detectable production.
[c] Tested by the plate overlay assay as described in Materials and Methods. +, zone of growth inhibition produced; −, no detectable zone of growth inhibition produced.

Penyalver *et al.*,2001

Siderophore production assay

In vitro screening of antagonistic bacteria against *R. solani*

- Bacterial strains that showed strong antagonistic activity against *R. solani* were tested to evaluate the role of siderophores in fungal growth inhibition.
- Each bacterial strain was spotted on one side of a plate of Kings B media supplemented with $FeCl_3$ at 0, 5, 10, 25 and 50 µg/ml (e.g. 5 µg=0.005 mg, 50 µg=0.05 mg)
- A disc of PDA (5 mm in diameter) of *R. solani* culture was placed on the other side of the plate and incubated at 28 ± 2°C for three days.
- Percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [(1 - \text{Fungal growth})/\text{control growth}] \times 100$$

3 µg	0.003 mg
4 µg	0.004 mg
5 µg	0.005 mg
6 µg	0.006 mg
7 µg	0.007 mg
8 µg	0.008 mg
9 µg	0.009 mg
10 µg	0.01 mg
20 µg	0.02 mg
30 µg	0.03 mg
40 µg	0.04 mg
50 µg	0.05 mg
60 µg	0.06 mg
70 µg	0.07 mg
80 µg	0.08 mg
90 µg	0.09 mg
100 µg	0.1 mg
1000 µg	1 mg

Gull and Hafee,2012

Siderophore production assay

In vitro screening of antagonistic bacteria against *R. solani*

Strains	*R. solani* growth on kings B medium with $FeCl_3$ (µg ml^{-1})				
	0	5	10	25	50
Ms.3y	+	+	++	+/-	-
Mst 8.2	+	+	+++	++	+
Mst 7.4	+	+	+++	++	+
3.1.1 C	+	+	+++	++	+
Z2	+	+	+++	++	+/-
Z5	+	+	+++	++	+/-
Z11	+	+	+++	+	+/-
Z.2.7	+	+	+++	++	+

*indicates approximate size of the zone of inhibition on King's B: + = ≤1.0 cm; ++ = 1 to 2 cm; +++ ≥ 2.0 cm; +/- = no clear inhibition zone; - = no zone.

Gull and Hafee,2012

Siderophore production assay

In vitro screening of antagonistic bacteria against *R. solani*

- To verify the sensitivity of *R. solani* to iron deprivation, 40 µl of a solution (0.1 mg/ml) of ethylenediamine-di-orthohydroxyphenyl acetic acid (EDDA) was added in a well 4 mm in diameter on one side of Kings B agar plate (instead of bacterial culture) and the *R. solani* culture was placed as a disc of PDA 5 mm diameter on the other side of the same plate after 2 days and incubated at 28 ± 2°C for 5 days.
- In the control plate, deionized water (adjusted to pH 9 with NaOH) was used instead of EDDA (Becker and Cook, 1988).

Gull and Hafee,2012

Siderophore production assay

In vitro screening of antagonistic bacteria against *S. rolfsii* and *F. oxysporum*

- Briefly, bacterial isolates (PGPR) were seeded in a 5.0-cm-diameter circle on a 0.1x TSA (Trypticase Soy Agar) plate that was either unamended or amended with 0.1 mM $FeCl_3$(Dissolve 0.01633 g $FeCl_3$ in 1 L water).
- After 24 h at room temperature, a 7-mm plug of each fungus was placed on the center of the circle.
- Plates with *S. rolfsii* and *F. oxysporum* were incubated at 28°C for 6 and 8 d, respectively, and plates with *S. sclerotiorum* were incubated at 22°C for 5 d.
- Fungal growth inhibition was assessed by measuring the mycelial radial growth.

Cattelan *et al.*,1999;..

Siderophore production assays

Pseudomonas spp. against *E. agricola* and *F. oxysporum*

- The antagonistic activity of pseudomonad PGPRs can be tested by measuring their ability to inhibit the growth of *Erwinia agricola* and *F. oxysporum* on low-iron media such as SR medium and SR-Fe^{3+} (20 µg of $FeCl_3$/ml) media.
- Presumably, rhizobacteria(PGPR) which are able to inhibit the test microorganism on SR but not on SR-Fe^{3+} produce extracellular iron-chelating siderophores.

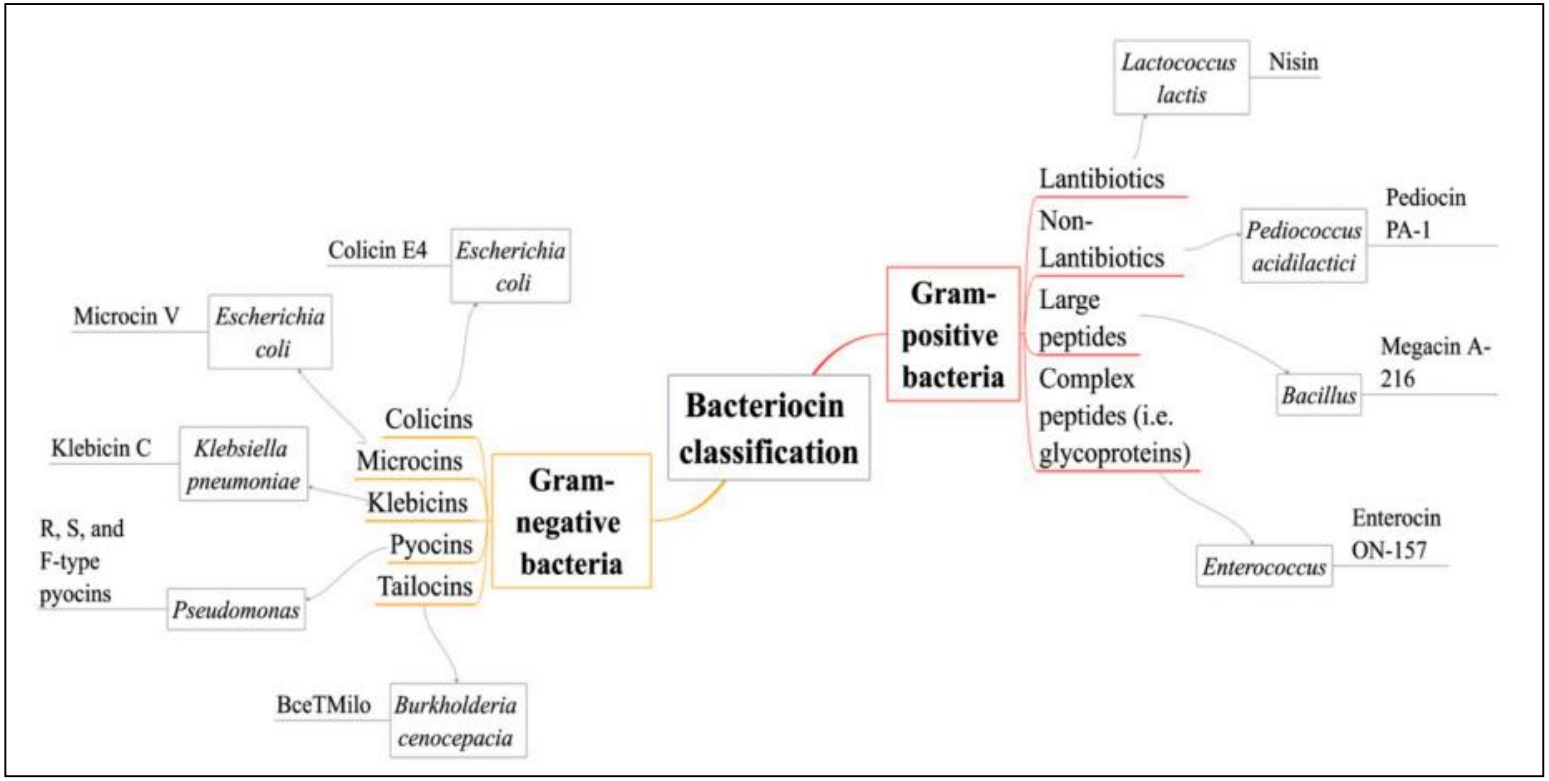
Bashan *et al.*,1993

Mechanisms of biological control

Bacteriocins

- Some bacteria living in a competitive environment secrete proteinaceous toxins, known as bacteriocins, that kill closely related bacteria but not the producer strain itself.
- According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin.
- All major groups of bacteria produce these inhibitors.

Mechanisms of biological control
Bacteriocins

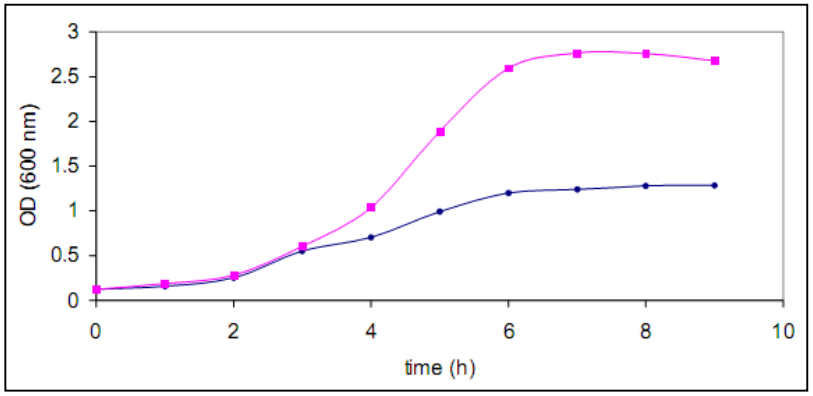


Bacteriocins classification. This figure shows the main examples of bacteriocins produced by Gram-positive and Gram-negative bacteria.

Cesa-Luna *et al.*,2019

Bacteriocin activity
Bacteriostatic against *Lactobacillus casei*

- Effect of bacteriocin produced by *Lactobacillus plantarum* on *L. casei* LHS(indicator strains).
- *L. casei* LHS was grown in BHI broth at 30ºC without bacteriocins (■) and with 6400 AU/ml bacteriocin (●).
- The bacterioin was added after 3h.
- The bacteriocin acted bacteriostatic against *L. casei*.



Todorov *et al.*,2003

Bacteriocin classification

Bacteriocins from plant pathogenic bacteria

- Most plant pathogenic bacteria are Gram-negative bacteria, and almost all known bacteriocins produced by these bacteria are proteins.
- Among bacteria residing in the soil and the rhizosphere as well as among saprophytic bacteria, Gram-positive species are more frequent.
- In Gram-positive bacteria, many peptide bacteriocins, especially from Class I (lantibiotics), have been identified and characterized.

Lantibiotics are defined as peptide antibiotics containing the unusual amino acids mesolanthionine, 3-methyllanthionine, dehydroalanine, and dehydrobutyrine.

Holtsmark *et al.*,2001

Types of bacteriocins

- Two groups of bacteriocins have been described:

1. High-molecular-weight (HMW) bacteriocins,
2. Low-molecular-weight(LMW) bacteriocins.

Wang,2010

Characteristics of HMW bacteriocins

- High-molecular-weight (HMW) bacteriocin bacteriocins are:

1. Thermolabile,
2. Trypsin resistant,
3. Sedimentable by ultracentrifugation,
4. Can be induced by physical or chemical agents, which activate the SOS system.

- HMW bacteriocins have been identified in many enterobacteria, *Pseudomonas* spp., *Rhizobium lupini*, *Bacillus* spp. and *Flavobacterium* spp.

Jabrane *et al.*,2002

Similarities between HMW bacteriocins and bacteriophages

- Similarities between High-molecular-weight (HMW) bacteriocins and bacteriophages have been established on the basis of:

1. Morphology,
2. Antigenic cross-reactivity,
3. Complementation, and
4. DNA hybridization.

- Electron microscopy has revealed that HMW bacteriocins have a structure resembling that of many bacteriophage tails.

Jabrane *et al.*,2002

Similarities between HMW bacteriocins and bacteriophages

- Pyocin F from *Pseudomonas aeruginosa* has sheathless, flexible rod-like structures resembling the lambda phage tail.
- Pyocin R from *Pseudomonas aeruginosa* has a contractile sheath resembling the T-even coliphage tail.
- Serracin P from *Serratia plymithicum* J7 revealed high homology with the Fels-2 prophage of *Salmonella enterica*, the coliphages P2 and 168, the CTX prophage of *Pseudomonas aeruginosa*, and a prophage of *Yersinia pestis*.

Jabrane *et al.*,2002

Dissimilarities of bacteriocins with antibiotics

- Even though both bacteriocin and antibiotics have antimicrobial activity and can be produced by bacteria, there are several differences between them:

1. Bacteriocins are synthesized via ribosome, while antibiotics are synthesized by enzymatic systems.
2. Bacteriocins usually have a narrow spectrum of inhibition, while in general antibiotics have a much broader spectrum.

- Thus antimicrobial peptides produced by bacteria are not necessarily bacteriocins.

Wang,2010

Bacteriocins

Mode of actions

- **Their mode of killing can be either:**
- Membrane pore formation,
- Nonspecific degradation of cellular DNA,
- Cleavage of 16S rRNA and tRNA, or
- Inhibition of peptidoglycan synthesis, resulting in cell lysis.
- In Gram-positive bacteria, Class I (lantibiotics), have been identified and characterized.
- Class I bacteriocins have a range of activities generally resulting in:
- Membrane destabilization,
- Pore formation and/or inhibition of cell-wall synthesis through binding to specific lipids.

Bacteriocins from plant pathogenic bacteria

Number of genes putatively encoding peptide bacteriocins in the genomes of plant pathogenic bacteria

	Number of small protein bacteriocins (size ≤ 100 aa)*
Agrobacterium tumefasciens C58 Cereon	21
Agrobacterium tumefasciens C58 UWash	32
Aster yellows witches broom phytoplasma AYWB	2
Burkholderia cenocepacia AU 1054	15
Burkholderia cenocepacia HI2424	17
Burkholderia cepacia AMMD	15
Clavibacter michiganensis ssp. *michiganensis* NCPPB 382	8†
Pectobacterium athrosepticum SCRI1043	6
Leifsonia xyli ssp. *xyli* CTCB0	5
Onion yellows phytoplasma OY-M	0
Pseudomonas syringae pv. *phaseolicola* 1448A	15
Pseudomonas syringae pv. *syringae* B728a	7
Pseudomonas syringae pv. *tomato* DC3000	7
Ralstonia solanacearum GMI1000	16
Xanthomonas campestris pv. *campestris* ATCC33913	5
Xanthomonas campestris pv. *campestris* 8004	7
Xanthomonas campestris pv. *vesicatoria* 8510	10
Xanthomonas axonopodis pv. *citri* 306	7
Xanthomonas oryzae KACC10331	5
Xanthomonas oryzae MAFF311018	8
Xylella fastidiosa 9a5c	17
Xylella fastidiosa Temecula1	10

*Genome mining for bacteriocins was performed using BAGEL default settings (de Jong *et al.*, 2006). The numbers of putative bacteriocins reported were scored as most significant by the program. In addition, the searches yielded a number of potential bacteriocins with lower scores (results not given).

†Michiganin A (Holtsmark *et al.*, 2006) was among the predicted bacteriocins.

Holtsmark *et al.*,2001

Bacteriocins

Inhibitory substances production was studied by the method of well diffusion assay against various microorganisms/indicator strains

Indicator strain[1]	Source[2]	Culture condition	Strain activity[3]		
			T01	T02	T03
Brochotrix sp. (food isolate)	U12-ES03	APT/20 °C	0	0	0
E. coli XL1-blue	U12-ES03	LB/37 °C	22	22	0
E. coli O157:H7 (food isolate)	U12-ES03	LB/37 °C	22	22	0
Brochotrix thermosphacta 11509	ATCC	APT/20 °C	22	22	0
Pseudomonas sp. (clinical isolate)	U12-ES03	BHI/37 °C	18	18	0
P. aeruginosa 27853	ATCC	BHI/37 °C	22	22	0
P. aeruginosa U12-23 (clinical isolate)	U12-ES03	BHI/37 °C	22	22	0
P. aeruginosa U12-12 (clinical isolate)	U12-ES03	BHI/37 °C	22	22	0
Aeromonas hydrophila (clinical isolate) *U12-33*	U12-ES03	TS agar/22 °C	0	0	0
Yersinia enterocolitica	U12-ES03	TS agar/22 °C	18	18	0
Hafnia sp. (food isolate)	U12-ES03	APT/37 °C	22	22	0
S. enterica	U12-ES03	BHI/37 °C	22	22	0
S. enterica CIP8297	U12-ES03	BHI/37 °C	22	22	0
B. thuringensis	U12-ES03	BHI/37 °C	0	0	0
B. megaterium	U12-ES03	BHI/37 °C	15	15	0
E. faecalis JH-22	U12-ES03	BHI/37 °C	15	15	0
E. faecium MMT21 (food isolate)	U12-ES03	BHI/37 °C	0	0	0
L. ivanovii BUG496	U12-ES03	BHI/30 °C	0	0	0
L. monocytogenes EGDe	Institut Pasteur, Paris	BHI/37 °C	0	0	0
Lb. delbruekii 2011	*DSM*	MRS/30 °C	0	0	0
Lb. bulgaricus Lb340	U12-ES03	MRS/30 °C	0	0	0
Lc. lactis sp. *cremoris 11603*	ATCC	MRS/30 °C	0	0	0
Lc. lactis sp. lactis11454	ATCC	MRS/30 °C	0	0	0
Lc. lactis MMT24 (food isolate)	U12-ES03	MRS/30 °C	0	0	0
M. luteus (food isolate)	U12-ES03	MRS/30 °C	0	0	0
S. aureus 14458	ATCC	BHI/37 °C	0	0	0
Sc. pyogenes (clinical isolate)	U12-ES03	BHI/37 °C	0	0	0
C. tyrobutyricum 4012	ATCC	BHI/37 °C	0	0	0
Saccharomyces cerevisiae	U12-ES03	YPD agar/30 °C	0	0	0
Candida albicans	U12-ES03	YPD agar/30 °C	0	0	0

E., *Escherichia*; S., *Salmonella*; B., *Bacillus*; E., *Enterococcus*; L., *Listeria*; Lc., *Lactococcus*; Lb., *Lactobacillus*; M., *Micrococcus*; S., *Staphylococcus*; Sc., *Streptococcus*; C., *Clostridium*.

Ghrairi *et al.*,2014

Mechanisms of biological control
Bacteriocins against bacterial pathogens

- The best-known bacteriocins produced by Gram-negative bacteria are the colicins produced by *Escherichia coli*.
- Genes for bacteriocin production are also often located on plasmids.
- A bacteriocin of *Agrobacterium radiobacter* is used in biocontrol of the plant pathogenic, tumour-producing bacterium *Agrobacterium tumefaciens*.

Janse,2006

Crown gall disease
Agrobacterium rhizogenes strain K84

- Production of the antiagrobacterial antibiotic agrocin 84, which is coded for by the agrocinogenic plasmid pAgK84, is a key component in the process of biocontrol by strain K84.
- Strain K84 also produces:
- A second antiagrobacterial substance called agrocin 434, affects only *A. rhizogenes* strains.
- A third antibiotic-like substance named ALS84, which inhibits many tumorigenic *Agrobacterium* strains *in vitro*.

Penyalver *et al.*,2001

Bacteriocins production

Well diffusion assay

- The well diffusion assay described by Schillinger and Lucke (1989) was used for the *in vitro* test of antagonistic activities of bacterial isolates against other bacteria.
- Plates containing solidified Nutrient Agar (20 ml) were overlaid with 10 ml of soft Nutrient Agar and inoculated with 0.05 ml of an overnight culture of test isolate.

Agarry *et al.*,2005

Bacteriocin assays

Serracin P against *E. amylovora*

- *Serratia plymithicum* J7 was grown overnight at 30°C in 60 ml of 863 medium (10 g of Bacto Peptone, 10 g of yeast extract, and 10 g of glucose per liter).
- The culture was harvested during exponential growth (A_{450} = 0.7) and divided in two.
- One half was treated with mitomycin C (1 mg/liter).
- This increase the activity of bacteriocins.
- After overnight incubation at 30°C, both the induced and non induced cultures were centrifuged at 7,000 x *g* (20 min, 4°C); the resulting supernatants were filtered through a 0.45-µm filter and stored at 4°C.

Jabrane *et al.*,2002

Bacteriocin assays

Serracin P against *E. amylovora*

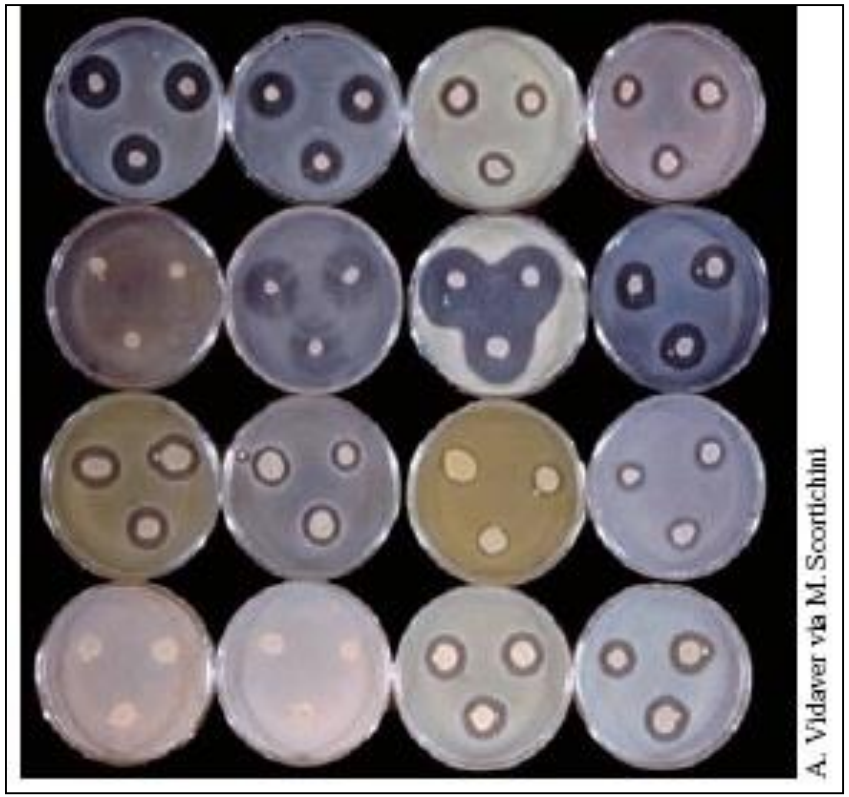
- Then 200 µl of each exponentially growing indicator strain i.e. *E. amylovora* (A_{450}= 0.7) was added to 10 ml of 863 soft agar (10 g of Bacto peptone, 10 g of yeast extract, 10 g of glucose, and 7.5 g of agar per liter) at 55°C, mixed, and plated in petri dishes.
- Then 10 µl of induced *Serratia plymithicum* culture supernatant (contains serracin) was spotted onto the lawn of each indicator strain.
- After overnight incubation at 30°C, a clear zone was taken as indicative of serracin P activity.

Jabrane *et al.*,2002

Bacteriocin assays

Test for production of bacteriocin by *Clavibacter* sp.

- Bacteriocins are toxic, narrow-spectrum protein metabolites of bacteria that inhibit/kill related bacteria.
- Inhibition of growth of (most of the) related bacteria tested, visible as a clear halo (no growth) around the colonies of different *Clavibacter* species.



Janse,2006

Bacteriocin assays

Bacteriocin activities of Tn*5* insertion mutants of *P. carotovorum* subsp.*carotovorum* strains

- *Erwinia* species produce high-molecular-weight bacteriocins (or large bacteriocins), structures similar to those of bacteriophages.
- No genes encoding the low-molecular-weight bacteriocin (or small bacteriocin) of *P. carotovorum* subsp. *carotovorum* have been isolated or characterized.
- The bacteriocin assay for the insertion mutants after transformation indicates a successful recovery of their ability to produce the low-molecular-weight bacteriocin.
- There is strong evidence of the effectiveness of biological control of the soft-rot disease of Chinese cabbage.

Bacteriocin assays

Bacteriocin activity assay of *P. carotovorum* subsp.*carotovorum*

Strain[a]	Inhibition zone due to bacteriocin production	
	Low-mol-wt bacteriocin	High-mol-wt bacteriocin
M-rif-11-2	7 mm	+
TM01A01	None	+
TM01A01/pBYL1	7 mm	+

[a] M-rif-11-2 (parent strain), TM01A01 (Tn*5* insertion mutant), and pBYL-1/TM01A01/pBYL1 (transformed mutant) were used as bacteriocin producers, and strain T-29 was used as an indicator.

Biokeeper

A biological-control agent against Pcc

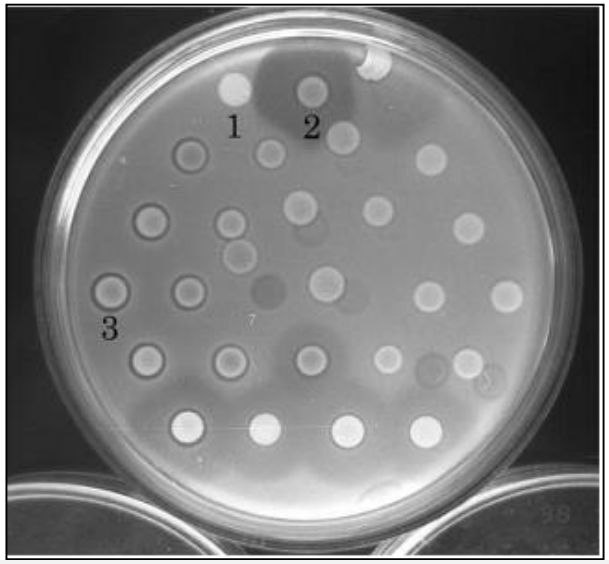
- A biological-control agent with the trade name "Biokeeper" has been developed for the control of soft rot disease caused by *Erwinia carotovora* subsp. *carotovora* in Japan (Central Glass Co., Japan).
- This product is formulated from mutants of *E. carotovora* subsp. *carotovora* producing a low-molecular-weight bacteriocin (small bacteriocin named carocin S1).
- The carocin S1 gene can now be introduced into tobacco, Chinese cabbage, or other plant species by transgenic techniques to protect them against soft rot disease suppression of soft rot disease.

Chuang *et al.*,2007

Bacteriocin assays

Bacteriocin activities of Tn*5* insertion mutants of *P. carotovorum* subsp. *carotovorum* strains

- The parental strain produced an LMW bacteriocin that diffused further from the colony than did the high-molecular weight bacteriocin.
- The zones of inhibition around the putative isolates (insertion mutants) were restricted compared to those of the parent strain.
- This suggested the possibility that transposon Tn*5* had been successfully inserted into the genes of the LMW bacteriocin.



1. *E. coli* 1830/pBJ4JI (containing Tn*5*);
2. H-rif-8-6 (parent); and
3. TH22-10 (insertion mutant).

- The unlabeled strains are all Tn*5* insertion mutants of the H-rif-8-6.
- The indicator was Ea1068.

Chuang *et al.*,2007

Bacteriocin assays

Bacteriocin activities of Tn*5* insertion mutants of *P. carotovorum* subsp. *carotovorum* strains

- Antibacterial activity of Tn*5* insertion mutants of *P. carotovorum* subsp. *carotovorum* Pcc21 against the indicator strain Pcc3.
- Pcc21, *P. carotovorum* subsp. *carotovorum* Pcc21 wild-type strain;
- DK1, -2, -6, -7, and -9, *P. carotovorum* subsp. *carotovorum* Pcc21 mutant strains (loss of low-molecular-weight bacteriocin activity).
- The photo was taken 24 h after the indicator strain had been overlaid.

Roh *et al.*,2010

Bacteriocin assays

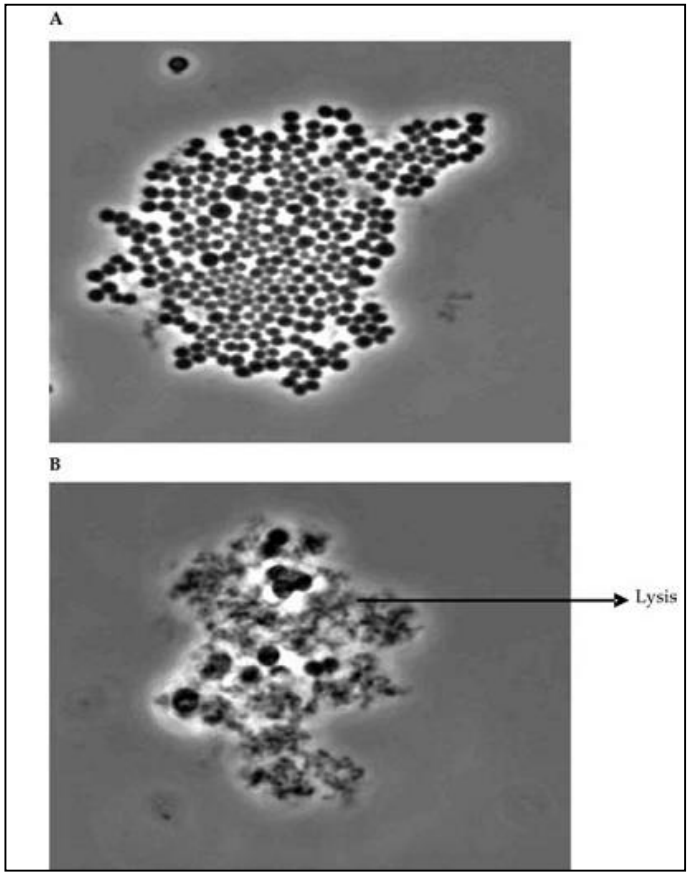
Bactericidal nature of crude extract

- Phase contrast images of live cells of *Staphylococcus aureus*.

A. Control cells without treatment.

B. Cells treated with crude extract of the antagonist *Pseudomonas aeruginosa*.

C. When cells of *S. aureus* were treated with this crude extract, cell lysis was observed immediately after treatment indicating bactericidal nature of this crude extract.



Uzair *et al.*,2009

Bacteriocins production

The effect of physical and chemical factors on the activity of bacteriocins

- The activity of bacteriocin can be affected by different conditions.
- For example,

1. Temperature can affect the activity of some bacteriocins.
2. Some bacteriocins are pH-dependent because pH affects their net charge.
3. Activity of bacteriocins are affected by enzyme treatments such as trypsin, protease K, and pronase.

Wang,2010

Bacteriocins production

Preparation of culture supernatants

- The test antagonist was grown in a nutrient broth (NB) at 25°C for 24 h.
- Bacteria cells were removed by centrifuging the cultures at 3900 rpm for 30 min.
- The supernatant was used immediately.

Agarry *et al.*,2005

Bacteriocins production

Titration of bacteriocins

Quantitative bioassay of inhibitory activity of bacteriocins

- The antimicrobial activity of a bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator strain and is expressed as activity units (AU/mL).
- The titre of bacillocin Bb and pyocin Pa was carried out by two-fold serial dilution in PBS (pH 7) and 0.1 ml of each dilution was placed into wells made on a BHI plates seeded with $1x10^6$ indicator strain.
- Next day zones of inhibition were measured.
- Titration of bacillocin Bb and pyocin Pa produced by *Bacillus brevis* Bb and *Pseudomonas aeruginosa* Pa, were calculated as 5280 and 640 AU/ml, respectively.

Saleem *et al.*,2009

Bacteriocins production

Titration of bacteriocins

Quantitative bioassay of inhibitory activity of bacteriocins

- One AU (arbitrary unit) was defined as reciprocal of the highest serial two fold dilution showing inhibition of the indicator strain and was indicated in AU ml^1.
- The antimicrobial activity of bacteriocins were calculated using the formula:
- 1 AU $ml^{-1} = 2^n$ X (1000 ml/10 µl),
- Where:
- AU ml^{-1} is the arbitrary unit ml^{-1}, and
- n is the reciprocal of the highest dilution showing inhibition.

Prasad *et al.*,2005

Bacteriocins production

Titration of bacteriocins

Quantitative bioassay of inhibitory activity of bacteriocins

- By definition, 1 unit of antimicrobial activity (AU) causes 50% growth inhibition (50% of the turbidity of a control culture without the peptide).
- The total antimicrobial unit was defined as the reciprocal of the highest dilution showing 50% inhibition of the indicator strain and was expressed in activity unit (AU)/ml.
- The antimicrobial activity of bacteriocins were calculated using the formula:
- $1\ AU\ ml^{-1} = 2^n\ X\ (1000\ ml/10\ \mu l)$,
- where
- $AU\ ml^{-1}$ is the arbitrary unit ml^{-1}, and
- n is the reciprocal of the highest dilution showing inhibition.

Prasad *et al.*,2005;..

Bacteriocins production

The effect of physical and chemical factors on the activity of bacteriocins

- The antagonistic effect of lactocin RN78, the bacteriocin produced by *Lactobacillus* RN78 was completely inactivated by proteolytic enzymes such as trypsin and pronase.
- These results reflect the proteinaceous nature of lactocin RN78.
- In contrast, catalase had no effect on the antagonistic agent, which rules out the possibility that inhibitory activity of lactocin RN78 is due to hydrogen peroxide.

No	Agent	Inhibitory activity
1	**Enzymes**	
	Pronase	-
	Trypsin	-
	Catalase	+
2	**Detergents**	
	SDS	+
	EDTA	+
	Tween 80	-
3	**Temperature**	
	80°C	+
	100°C	+
	120°C	+
4	**pH**	
	3.0	-
	5.0	+
	7.0	+
	10.0	+

Mojgani *et al.*,2006

Bacteriocins production

The effect of physical and chemical factors on the activity of bacteriocins against *Lactobacillus* spp.

	Bacteriocin produced by:			
Treatment	L. paracasei	L. pentosus	L. plantarum	L. lactis subsp. lactis
Enzymes (0.1 mg/ml)				
proteinase K	-	-	-	-
Qiagen protease	-	-	-	-
Papain	-	-	-	-
Chymotrypsin	-	-	-	-
Trypsin	-	-	-	-
Pepsin	-	-	-	-
Pronase	-	-	-	-
α-amylase	+	+	+	+
Surfactants (1% final conc.)				
SDS	+	+	+	+
Tween 20	+	+	+	+
Tween 80	+	+	+	+
Urea	+	+	+	+
Triton X-114	-	-	-	-
Triton X-100	-	-	-	-
EDTA (0.1mM, 2.0mM, 5.0mM)	+	+	+	+
PH				
2.0 – 7.0	+	+	+	+
8.0 – 12.0	+	+	+	+
Temperature				
30 – 100°C (90 min)	+	+	+	+
121°C (20 min)	+	+	-	+

No change of activity was recorded after treatment with SDS, urea, Tween 20, Tween 80, EDTA, triton X100 and triton X114.

Todorov *et al.*,2003

Bacteriocin inactivation bioassay
Heat stability

- Partially purified jenseniin P or crude Jenseniin P produced by *Propionibacteria jensenii* , a Gram-positive bacterium commonly found in dairy products, soil and olive fermentations was held in boiling water in eppendorf tubes for 90 min.
- At every 10 min interval, 100 µl of the sample were taken out and placed on ice.
- The amount of bactericidal activity of the sample was measured using the microtiter plate method against *Lactobacillus delbrueckii*, the indicator strain.

Wang,2010

Bacteriocin inactivation bioassay
pH stability

- The effect of pH on the bacteriocin was tested by adjusting the cell-free supernatants from pH 3.0 to 12.0 with sterile 1M HCIA or 4M NaOH.
- After 24 hours of incubation at 37°C, all samples were tested for antimicrobial activity on some Gram positive and Gram negative bacteria such as *B. subtilis*, *Ps. aeruginosa*, *E. coli*, *Kl. pneumoniae*, etc.

Mojgani *et al.*,2006

Bacteriocin inactivation bioassay

Effect of pH on activity of jenseniin P

pH of standard solution	pH of diluted bacteriocin(jenseniin P)	Activity(AU/ml)
1	1.9	128±0
2	4.2	128±0
7	6.4	128±0
12	0.0	128±0
13	12.8	128±0

No significant difference between different samples.

Wang,2010

Bacteriocin inactivation bioassay
SDS, Tween 20 and Tween 80 stability

- The effect of surfactants on the bacteriocin was tested by adding Sodium dodecyl sulphate (SDS), and Tween 20, Tween 80 (1% v/v, final concentration), respectively, to the cell-free supernatant.
- EDTA was added to the cell-free supernatant to yield a final concentration of 0.5 mM and 2.0 mM, respectively.
- Untreated cell-free supernatant and the detergents at these respective concentrations were used as controls.
- All samples were incubated at 37° C for 4 to 6 hours and then tested for antimicrobial activity by agar well diffusion method.

Mojgani *et al.*,2006

Bacteriocin inactivation bioassay
Enzyme treatment

- 25 µl each of 10 mg/ml aqueous solution of trypsin, protease K, pronase, protease XIV, lysozyme, and catalase were loaded onto sterilized disks of 7 mm in diameter on agar plates.
- The distance between the two disks was approximately 1 mm.
- After 1 hour of incubation in 37°C, the plate was overlaid with *Lactobacillus delbrueckii*, the indicator strain in soft agar and incubated in 37°C for overnight.
- The clear zones around the disks were evaluated afterward.

Wang,2010

Bacteriocin inactivation bioassay

Enzyme treatment

Functions of each enzyme

- If the enzyme did not inactivate a bacteriocin, a round, clear inhibition zone around the disks loaded with each bacteriocins was observed.
- Otherwise, one side of the inhibition zone was smaller than the other side.
- **Functions of enzymes:**

1. Trypsin cleaves proteins at the lysine or arginine residues.
2. Protease K is a broad spectrum protease.
3. Pronase can cause complete digestion of proteins.

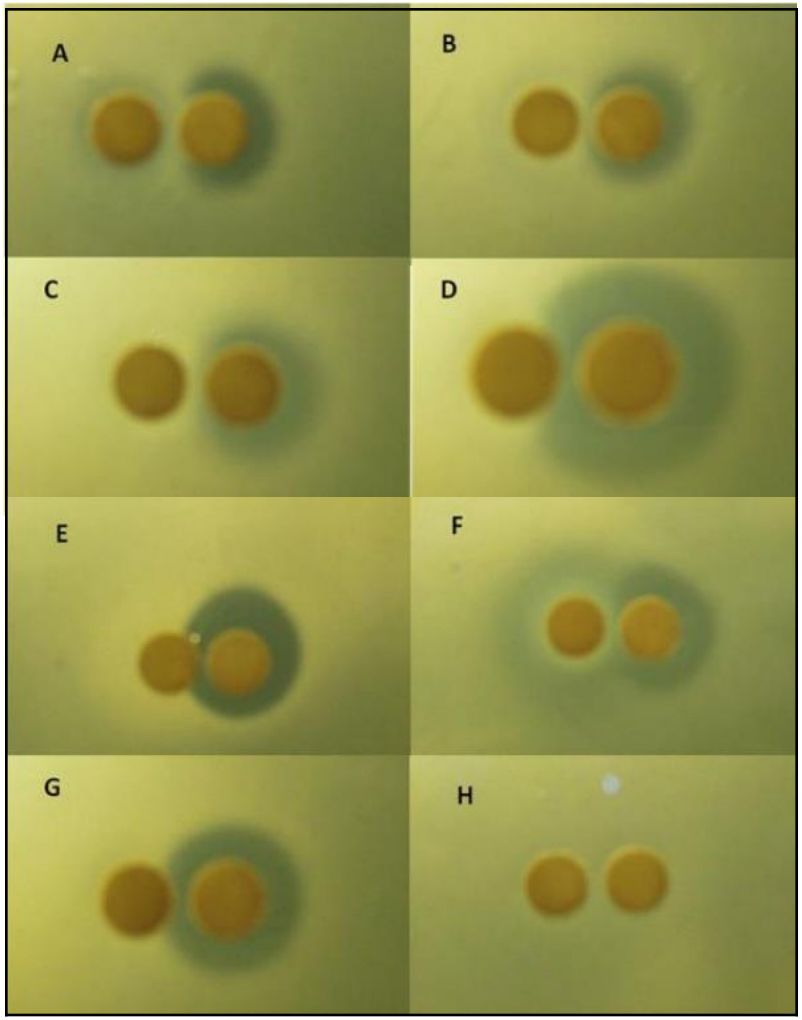
Wang,2010

Bacteriocin inactivation bioassay

Enzyme treatment

Functions of each enzyme on bacteriocin jenseniin P.

- Effect of enzyme treatment on further purified jenseniin P.
- **Left disks:**
- A. trypsin;
- B. protease K;
- C. pronase;
- D. protease XIV;
- E. catalase;
- F. lysozyme.
- G &H. MRS growth broth.
- **Right disks:**
- A-G. Further purified jenseniin P;
- H. 0.05 M sodium phosphate buffer(control).



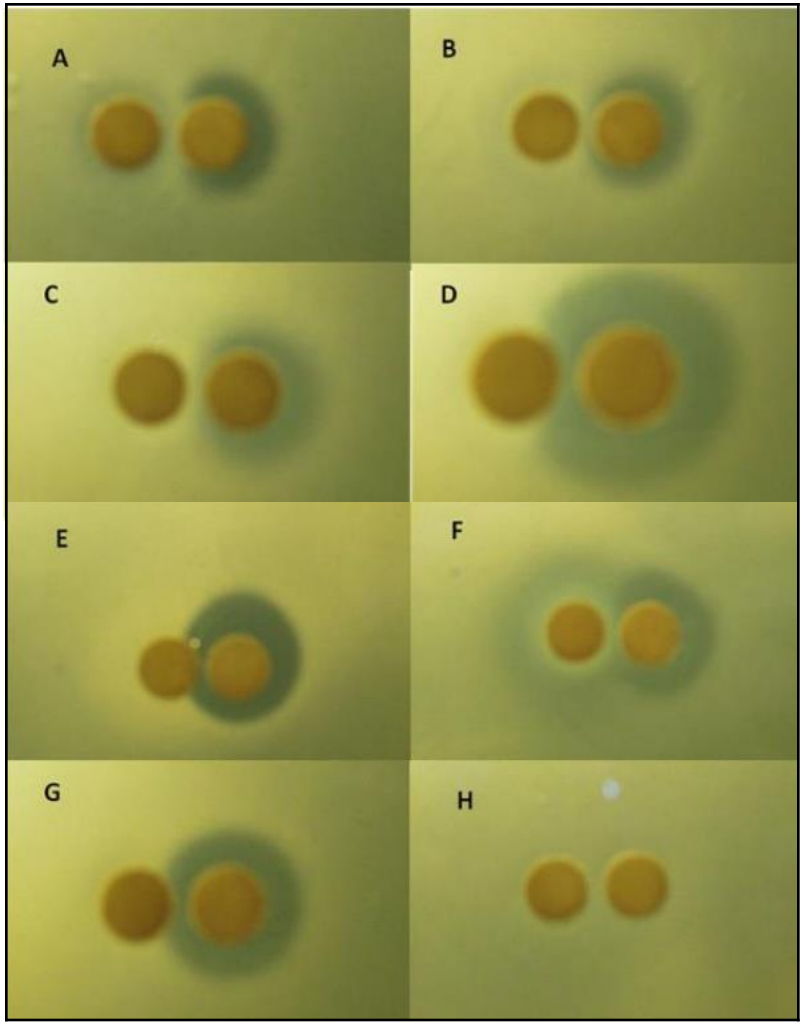
Wang,2010

Bacteriocin inactivation bioassay

Enzyme treatment

Functions of each enzyme on bacteriocin jenseniin P.

- In this present work, activity of jenseniin P was affected by trypsin, protease K, and pronase.
- Whereas, activity of jenseniin P was not affected by protease XIV, lysozyme and catalase.
- If the enzyme did not inactivate jenseniin P, a round, clear inhibition zone around the disks loaded with jenseniin P was observed.
- Otherwise (activated ones), one side of the inhibition zone was smaller than the other side.



Wang,2010

Bacteriocin inactivation bioassay
Plasmid curing and detection

- Lactobacillus RN78 mutant's defective in bacteriocin production were isolated by curing experiments as described by Hirota (1960).
- Acridine orange was used as chemical curing agent at a final concentration of 5-150 µg mL^{-1}
- All the colonies after treatment were picked carefully and checked for inhibitory activity by agar well diffusion method.
- The colonies showing no zone of inhibition were selected as Bac-mutants and screened further for the presence of plasmids.

Mojgani *et al.*,2006

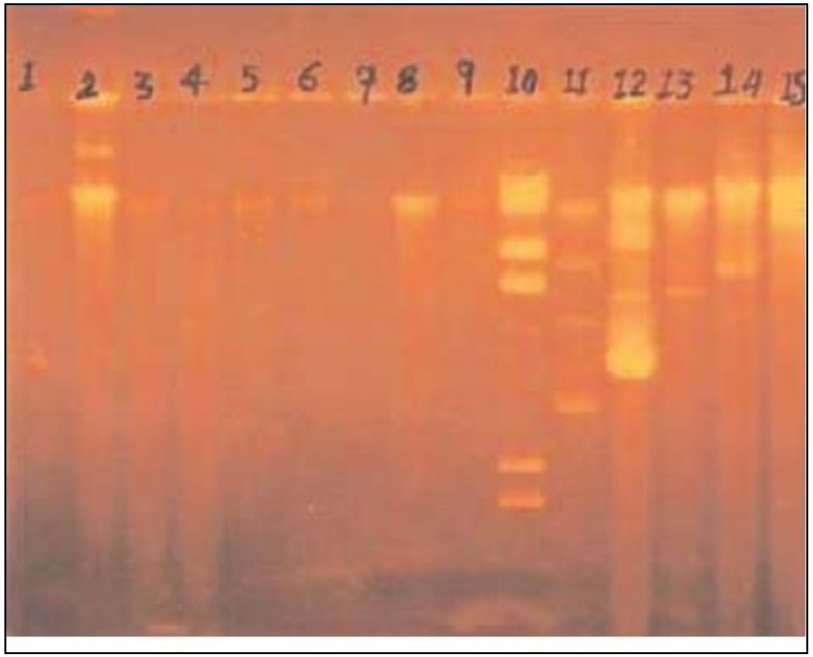
Bacteriocin inactivation bioassay
Plasmid curing and detection

- The presence or absence of plasmid in the wild and Bac-mutants of *lactobacillus* RN78 was analyzed by Echardt gel electrophoresis.
- The cells of the test strain (100-500 µL) were collected by centrifugation and washed with ice cold TNE buffer (10 mM Tris, 100 mM NaCl and 1 mM EDTA).
- All were suspended in 100 µL TNE buffer (10 mM Tris and 1 mM EDTA) and added to the slots made in 0.9% agarose gel.
- 50 µL of lysis solution (25% w/v sucrose solution in TBE, 10 mg/mL^{-1} lysozyme and 1 unit RNase) were added to the suspended cells in the slots and mixed carefully with a sterile toothpick.
- The gel was run for 30 min at 30V and then at 120V for approximately 3 hours and observed under UV.

Mojgani *et al.*,2006

Bacteriocin inactivation bioassay
Plasmid curing and detection

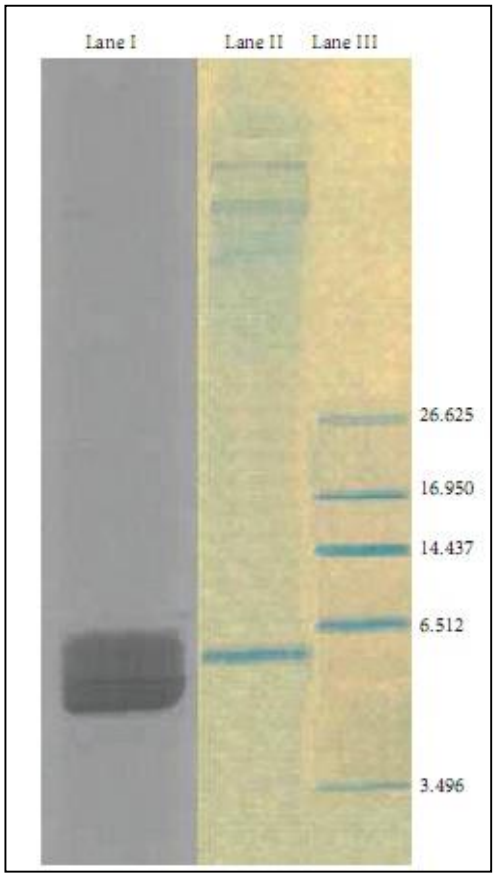
- The plasmid analysis of wild type and cured colonies of *Lactobacillus* RN78 strain.
- The presence of a 42Kb plasmid was verified in the wild producer strain.
- This indicates an early indication of plasmid linked lactocin RN78 production in the test strain.



Mojgani *et al.*,2006

Molecular size of the bacteriocin Lactococcin BZ produced by *Lactococcus lactis* subsp. *lactis* BZ

- Lane I: Inhibition zone of lactococcin BZ (the gel was overlaid with a culture of *Lactobacillus plantarum* in MRS agar and incubated for 24 h at 30°C).
- Lane II: Partially purified lactococcin BZ.
- Lane III: Low molecular weight marker (BioRad).



Mojgani *et al.*,2006

Bacteriocin inactivation bioassay

Stability of bacteriocin during storage

- Lactocin RN78 was analyzed for its stability at different temperatures during long term storage.
- The bacteriocin was incubated in small vials at -20, 4, and 37°C, respectively and the antimicrobial activity on indicator bacteria such as *B. subtilis*, *Ps. aeruginosa* and *E. coli*, determined every month by agar well diffusion method.

Mojgani *et al.*,2006

Bacteriocin inactivation bioassay

Stability of bacteriocin during storage

- [a] *Lactobacillus plantarum* was used as sensitive strain.
- Lactococcin BZ was stable under lyophilization conditions.
- Freeze-dried samples retained their activity during storage at -20 and -80°C for 3 months.
- However, the concentrated lactococcin BZ (10 times) lost 62.5% and 50% of its biological activity after 3 months of storage at -20 and –80°C, respectively.

Şahingil *et al.*,2011

Treatment	Residual activity (%)[a]
Untreated bacteriocin	100
Lyophilization	100
Storage of freeze dried bacteriocin:	
4°C for 3 months	50
–20°C for 3 months	100
–80°C for 3 months	100
Storage of unfreeze dried bacteriocin:	
4°C for 3 months	18.5
–20°C for 3 months	37.5
–80°C for 3 months	50

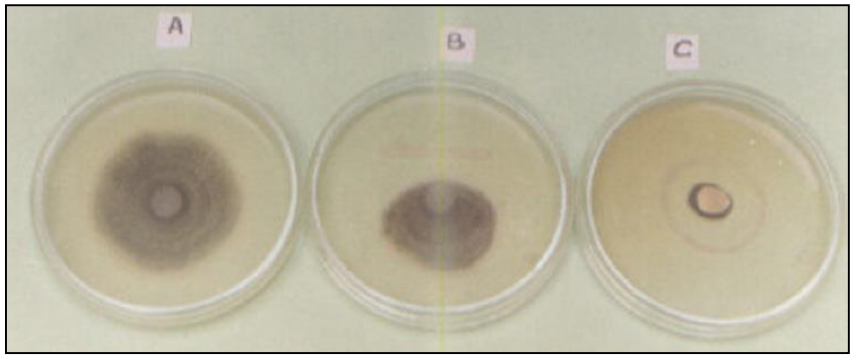
Bacteriocin assays
Against fungal pathogens

- **Agar assay:**
- An agar plug (5 mm in diam) was cut from an actively growing (96 h) fungal culture and placed at the center of the agar plate.
- Simultaneously, the bacterium (24 h grown) to be tested was streaked 2 cm away from the agar plug.
- Plates with only fungus without bacterial culture were used as controls.
- Plates were incubated at 30±1°C until fungal mycelia covered the agar surface of the control plate.

Bacteriocin assays
Against fungal pathogens

- Antagonistic zone between *Bacillus pumilus* and *Aspergillus niger*.
- Photograph taken 5 days after inoculation.
- Codes:

A. Control;
B. Fokkema/Conventional streak method;
C. Concentric/Novel Ring bioassay.



Agarry *et al.*,2005

Bacteriocin assays
Against fungal pathogens

- Growth of pathogen was recorded and percent inhibition of fungus was calculated by using the formula:

$$I = \frac{(C - T)100}{C}$$

- Where:
- I is the percent inhibition of mycelial growth,
- C is the radial growth of fungus in the control plate (mm), and
- T is the radial growth of fungus on the plate inoculated with bacterium (mm).

Hameeda *et al.*,2006

Bacteriocins production
Characterization of inhibitory substances

- To characterize the inhibitory compounds produced by the bacterial isolates, their culture supernatants were assayed for:

1. Organic acids
2. Bacteriocins, and
3. Hydrogen peroxide.

I. For organic acid, adjusted pH of the culture supernatants to 6.5, with the addition of 1 mol NaOH;

1. For bacteriocins detection treat the culture supernatants with trypsin (concentration 1 mg ml^{-1}) for 12 h at 37°C, or

- For hydrogen peroxide production treat the culture supernatants with catalase (concentration 0.5 mg ml^{-1}).

Agarry *et al.*,2005

Bacteriocins production
Characterization of inhibitory substances

- Each treated supernatant (0.1 ml) was transferred into a well at the periphery of the agar and the untreated culture supernatant (0.1 ml) was transferred into the well at the centre.
- The presence or absence of inhibitory zones around the wells was determined after incubation for 24 h at 25°C.
- The assay was conducted in duplicates.

Agarry *et al.*,2005

Bacteriocins production

Characterization of inhibitory substances

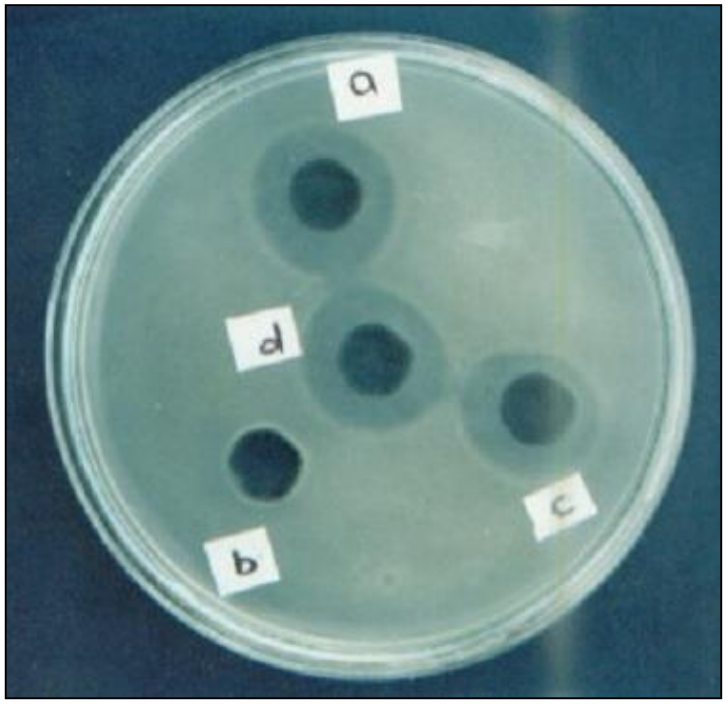
- Inhibition of *Escherichia coli* (indicator strain) by treated supernatant of *Bacillus pulmilus* (antagonist) in a agar well diffusion assay.

a) Well containing supernatant treated with 1 mg/ml trypsin (Inhibition was not due to the production of bacteriocin).

b) Well containing supernatant treated with NaOH (unchanged inhibitory zones indicates no organic acids production).

c) Well containing supernatant treated with 0.5 mg/ml catalase (unchanged inhibitory zones indicates no hydrogen peroxide activity).

d) Well containing untreated culture supernatant (control).



Agarry *et al.*,2005

Mechanisms of biological control
Ammonia production

- Endophytic isolates were tested for the production of ammonia in peptone water.
- Freshly grown cultures were inoculated in 10 ml peptone water separately and incubated for 48-72 h at 36 ± 2°C.
- Nesslers reagent (0.5 ml) was added in each tube.
- Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman,1992).

Prasad and Dagar,2014

Mechanisms of biological control

Detoxification and degradation of virulence factors

- Another mechanism of biological control is the detoxification of pathogen virulence factors.
- For example
- Several different microorganisms, including strains of *B. cepacia* and *Ralstonia solanacearum*, can also hydrolyze fusaric acid, a phytotoxin produced by various *Fusarium* species.
- Recently, it has been discovered that certain PGPB quench pathogen quorum-sensing capacity by degrading autoinducer signals, thereby blocking expression of numerous virulence genes.

See also Quorum sensing

Mechanisms of biological control

Detoxification and degradation of virulence factors

- Since most, if not all, bacterial plant pathogens rely upon autoinducer-mediated quorum-sensing to turn on gene cascades for their key virulence factors (e.g., cell-degrading enzymes and phytotoxins), this approach holds tremendous potential for alleviating disease, even after the onset of infection, in a curative manner.

See also Quorum sensing

Mechanisms of biological control

Detoxification and degradation of virulence factors

- **Albicidin detoxifying bacterium:**
- Leaf scald, caused by *Xanthomonas albilineans*, is a serious disease of sugarcane.
- Certain biocontrol agents are able to detoxify albicidin toxin produced by *Xanthomonas albilineans*. e.g. a gene has been cloned from *Pantoea dispersa* that codes for a peptide which detoxifies albicidin.
- Transformation of sugarcane with the gene confers resistance against the bacterium.

Strange,2003

Mechanisms of biological control

Detoxification and degradation of virulence factors

- **Xanthan-degrading bacterium:**
- The *Microbacterium* sp. XT11, capable of fragmenting xanthan, has been isolated from garden soil sample.
- The xanthan-degrading enzyme produced by the newly isolated XT11 could fragment xanthan to form oligosaccharides.
- It has been shown that xantho-oligosaccharides fragmented from xanthan had both elicitor activity and antibacterial effect against *Xanthomonas campestris* pv. *campestris*.
- Xanthan-degrading products would be useful for potential application in the control of black rot of cruciferous plants caused by *X. campestris* pv. *campestris* and, as an oligosaccharide elicitor, in making these plants resistant to disease.

Qian *et al.*,2007

Biological control of plant diseases

Through induction of host resistance e.g. PR proteins

Mechanisms of biological control

Induction of host resistance

- Plants actively respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability.
- Plants also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes.
- Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens.
- Induction of host defenses can be local and/or systemic in nature, depending on the type, source, and amount of stimuli.

Inducible defenses

The secondary metabolic products

- Inducing systemic resistance (ISR), has opened up a new field of opportunity for enhancing plant health.
- Plant phytohormones such as abscisic acid, jasmonic acid, ethylene and salicylic acid (SA) are important components of different signaling pathways involved in plant defense.
- The inducible defenses include the following:

1. Reactive oxygen species (ROIs),
2. Phytoalexins,
3. Cell wall components (callose, glycine or hydroxyproline-rich proteins),
4. Another group of proteins called pathogenesis-related proteins (PR proteins).

SAR & ISR-induced host resistance

Salicylic acid, jasmonic acid (JA) and/or ethylene

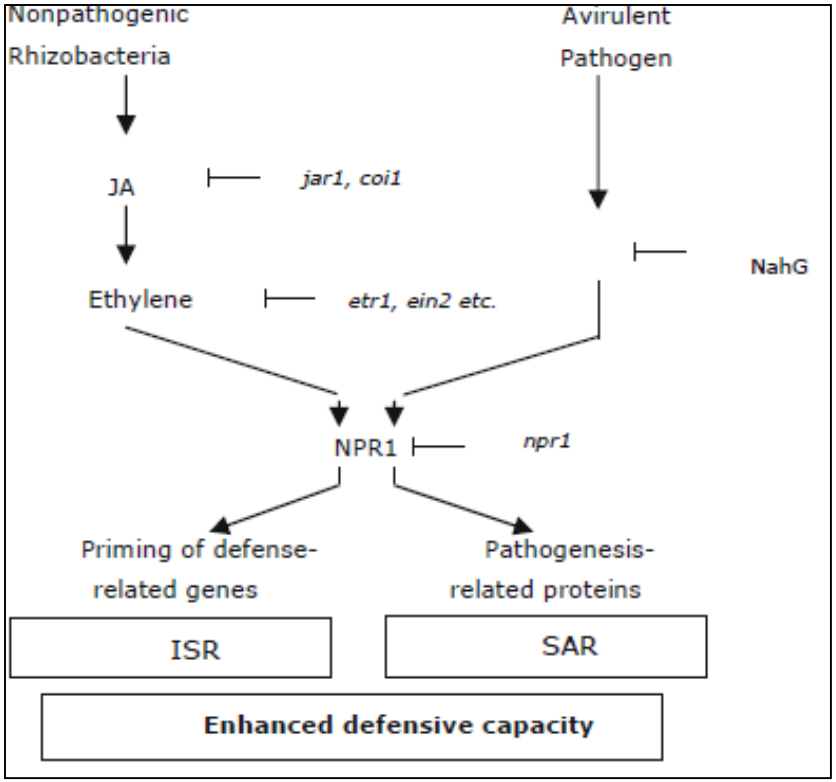
- SA mediates the phenylpropanoid pathway, while as JA mediates the octadecanoid pathway.

1. The first of these pathways, termed systemic acquired resistance (SAR), is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection and typically leads to the expression of pathogenesis-related (PR) proteins.
2. A second phenotype, first referred to as induced systemic resistance (ISR), is mediated by jasmonic acid (JA) and/or ethylene, which are produced following applications of some nonpathogenic rhizobacteria.

See also plant activators for chemically induced host resistance.

Current model of signal-transduction pathways leading to SAR and ISR

- Current model of signal-transduction pathways leading to:
  1. pathogen-induced systemic acquired resistance (SAR) &
  2. rhizobacteria-induced systemic resistance (ISR).
- Some non-pathogenic rhizobacteria may trigger a SA-dependent signalling pathway that leads to a state of induced resistance resembling SAR.



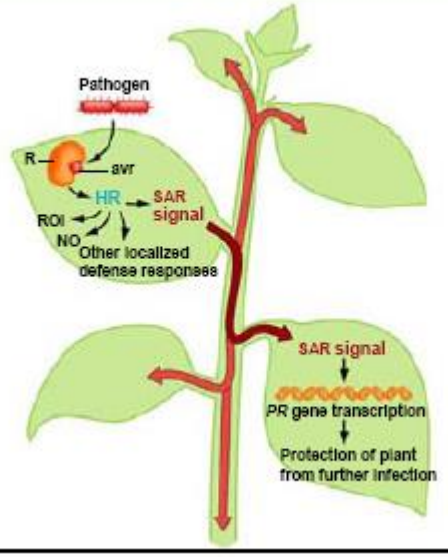
Siddiqui,2006

SAR-induced host resistance

Systemic acquired resistance (SAR)

- SAR is second response
  - Slower wide spread response triggered by HR
  - Provides protection to other plant parts from the pathogen.
- Salicylic acid may be the signal transported from HR site, results are inconclusive.

Figure 37.13



SAR-mediated host resistance

Activation of enzymes and accumulation of defensive compounds

- Salicylic acid (SA), a plant defense hormone regulates the activities of various enzymes such as:

1. peroxidase (POD),
2. polyphenol oxidase, besides the higher accumulation of
3. phenols,
4. H_2O_2, and
5. proteins.

War *et al.*,2011

SAR-mediated host resistance

Enzyme assays

1. Leaf collection

- Newly emerged fully expanded leaves were collected from treated plants and immediately frozen in liquid nitrogen.
- Leaves (0.5 g) were homogenized in 3 ml of ice cold 0.1 M TRIS-HCl buffer (pH 7.5) containing 2-mercaptoethanol(5 mM), 1% polyvinylpyrolidone (PVP) and 0.5 mM EDTA.
- The homogenate was centrifuged at 16,000x g for 25 min and the supernatant was used as enzyme source.
- All spectrophotometric analyses were performed on HITACHI UV-2010 spectrophotometer.

War *et al.*,2011

SAR-mediated host resistance

Enzyme assays

2. Peroxidase (POD) activity measurement

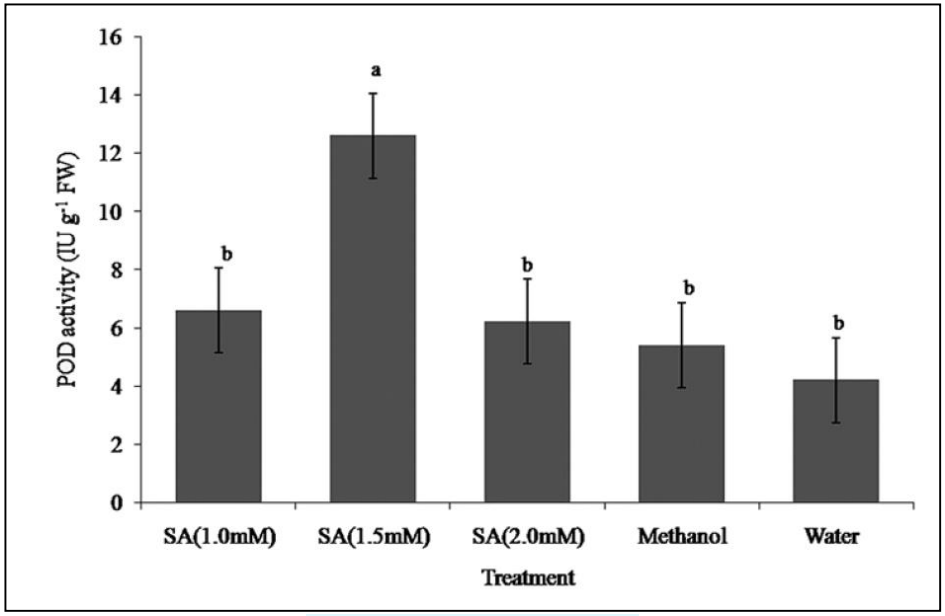
- To the reaction mixture (2.9 ml) containing 0.1 M sodium phosphate buffer (pH 6.5), 0.8 mM H_2O_2 and 5 mM Guaiacol, 0.1 ml of enzyme source was added.
- Absorbance was read at 470 nm for 2 min at 15 sec intervals.
- Enzyme activity was measured as IUg^{-1} FW (International Units g^{-1} FW).
- One unit of POD activity was defined as the change in absorbance by 0.1 units per minute under conditions of assay.

War *et al.*,2011

SAR-mediated host resistance

Peroxidase activity (IUg^{-1} FW) of chickpea plants at 96 h after treatment with SA

- Salicylic acid (SA) regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase, besides the higher accumulation of phenols, H_2O_2 and proteins.



War *et al.*,2011

SAR-mediated host resistance

Enzyme assays

3. Polyphenol oxidase activity measurement

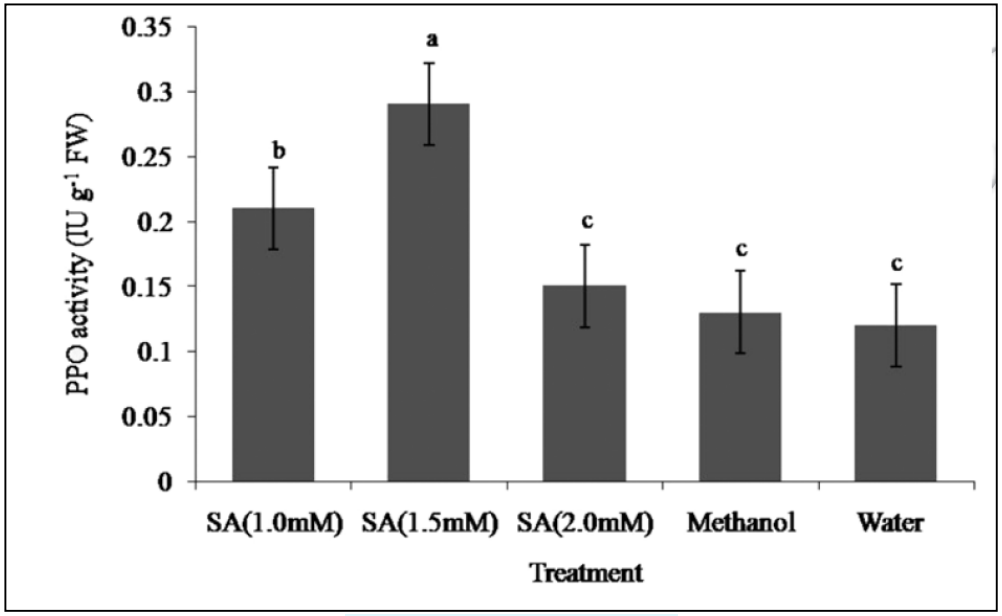
- To the reaction mixture (2.9 ml of 0.1 M sodium phosphate buffer, pH 6.8), 0.1 ml of enzyme source and 0.1 ml of substrate (0.05 M catechol) were added.
- Absorbance was read at 420 nm for 3 min at 30 sec interval.
- Enzyme activity was measured as IUg^{-1} FW.
- One unit of PPO was defined as the change in absorbance by 0.1 units per minute under conditions of assay.

War *et al.*,2011

SAR-mediated host resistance

Polyphenol oxidase activity (IUg^{-1} FW) of chickpea plants at 96 h after treatment with SA

- Salicylic acid (SA) regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase, besides the higher accumulation of phenols, H2O2 and proteins.



War *et al.*,2011

SAR-mediated host resistance

Defensive compounds estimation

4. Phenolic content measurement

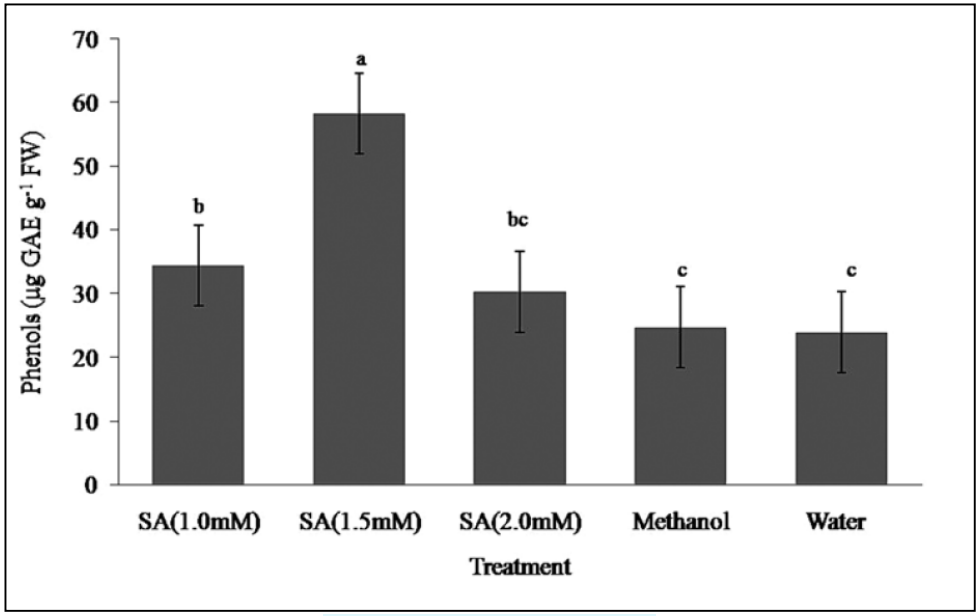
- Fresh leaves (0.5 g) were extracted in 3 ml of 80% methanol and agitated for 15 min at 70°C.
- Briefly, 0.1 ml of methanol extract was added to 2 ml of 2% Na_2CO_3.
- After incubation for 5 min, 0.1 ml of Folin-Ciocalteau reagent was added and the solution was incubated for 10 min at room temperature.
- The absorbance of blue color was measured at 760 nm.
- Gallic acid was used as a standard and a calibration curve was prepared with a range of concentrations.
- Phenolic content was expressed as µg Gallic acid equivalents g^{-1} FW (µg $GAEg^{-1}$ FW).

War *et al.*,2011

SAR-mediated host resistance

Total phenols (µg GAE g^{-1} FW) of chickpea plants at 96 h after treatment with SA

- Salicylic acid (SA) regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase, besides the higher accumulation of phenols, H_2O_2 and proteins.



War *et al.*,2011

SAR-mediated host resistance

Defensive compounds estimation

5. Hydrogen peroxide measurement

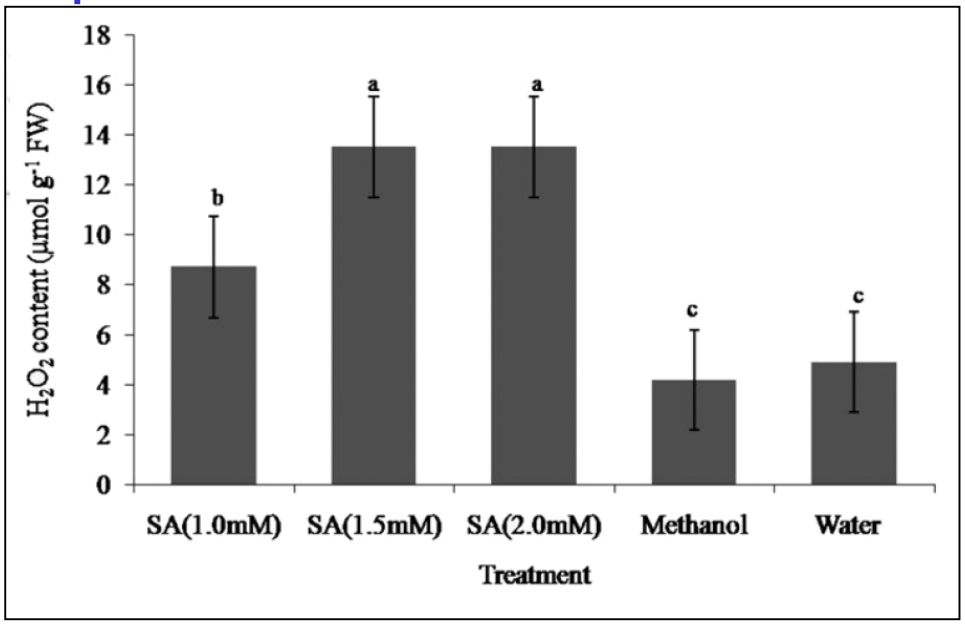
- Fresh leaf tissue (0.1 g) was homogenized in 2 ml of 0.1% (w/v) TCA in a pre-chilled pestle and mortar.
- The homogenate was centrifuged at 12,000x g for 15 min and the supernatant was collected.
- Absorbance of the reaction mixture consisting of 0.5 ml supernatant, 0.5 ml sodium phosphate buffer (pH 7.0) and 1 ml of 1 M KI was read at 390 nm.
- The H_2O_2 content was determined by using an extinction coefficient of 0.28 μMcm^{1} and expressed as $\mu molg^{-1}$ FW.

War *et al.*,2011

SAR-mediated host resistance

Hydrogen peroxide content (µmolg^{1} FW) of chickpea plants at 96 h after treatment with SA

- Salicylic acid (SA) regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase, besides the higher accumulation of phenols, H2O2 and proteins.



War *et al.*,2011

SAR-mediated host resistance

Defensive compounds estimation

5. Protein content measurement

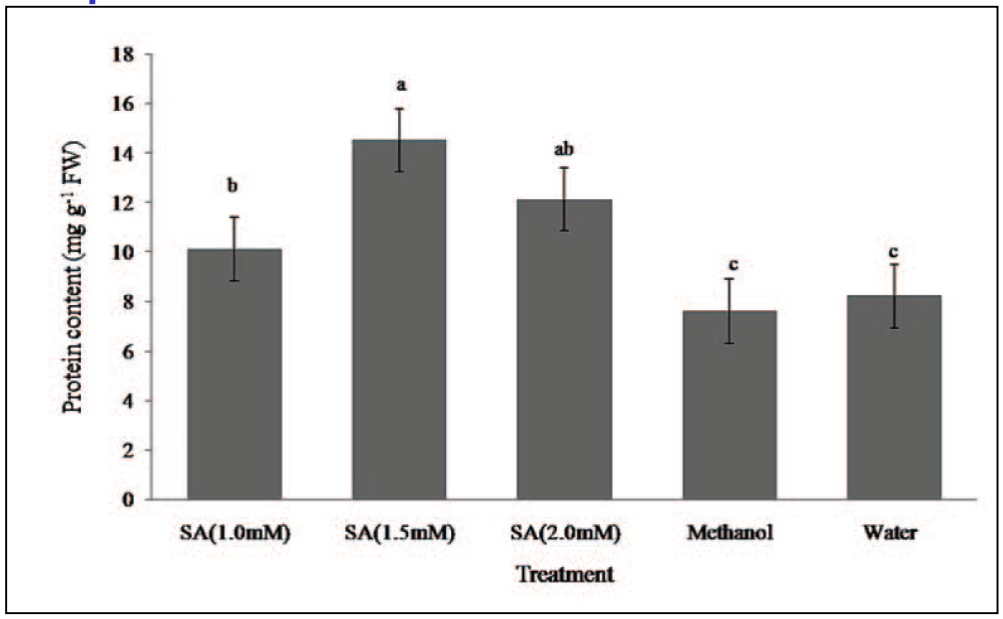
- Protein was determined according to the method of Bradford,1976 with minor modifications, using bovine serum albumin as a standard.
- Protein solution containing 10 to 100 µg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes.
- The volume in the test tube was adjusted to 0.1 ml with appropriate buffer.
- Five milliliters of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing.
- The absorbance at 595 nm was measured after 2 min and before 1 hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent.
- The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.
- **Preparation of protein reagent:**
- Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

War *et al.*,2011

SAR-mediated host resistance

Protein content (mgg^{-1} FW) of chickpea plants at 96 h after treatment with SA

- Salicylic acid (SA) regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase, besides the higher accumulation of phenols, H2O2 and proteins.



War *et al.*,2011

SAR-mediated host resistance

Defensive compounds estimation

6. Quantification of total salicylic acid (SA)

- Salicylic acid (SA) activities were determined in the inoculated and non-inoculated tomato plants.
- One gram of frozen tissue was ground in 3 ml of 90% methanol and centrifuged at 6000 rpm for 15 min.
- The pellet was back extracted with 3 ml of 99.5% methanol and centrifuged as above.
- Methanol extracts were combined and then centrifuged at 1500 to 2000 rpm for 10 min.
- The supernatant was dried at 40°C under vacuum using rotary evaporator (Heidolph.).
- The dried extracts were then resuspended in 3 ml of distilled water at 80°C and an equal volume of 0.2 M sodium acetate buffer, pH 4.5, containing 0.1 mg/ml β-glucosidase (22 unit/ mg, Sigma) was added, then the mixtures were incubated at 37°C overnight. After digestion, mixtures were acidified to pH 1 to 1.5 with HCl.
- SA was extracted by adding (1:2, v: v) of sample: cyclopentan/ ethylacetate /isopropanol (50:50:1).
- The organic extract was dried under nitrogen and analyzed by HPLC.
- One hundred microliters of each sample were injected into Dynamax 60A8 µm guord column (46mm x 1.5cm) linked to 40°C. SA was separated with 23% v/v methanol in 20 mM sodium acetate buffer, pH 5.0 at a flow rate of 1.5 ml min $^{-1}$.
- SA level was determined using standard curve.

Mahdy *et al.*,2010

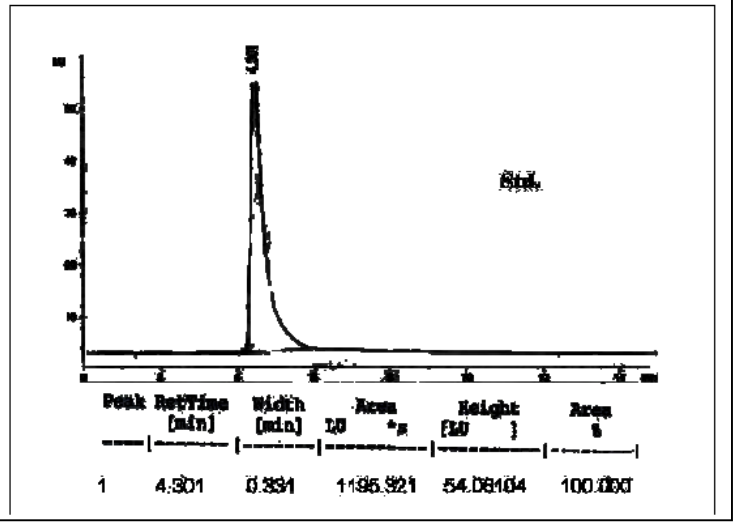
SAR-mediated host resistance

Defensive compounds estimation

6. Quantification of total salicylic acid (SA)

- Quantification of total SA in tomato plants induced with biotic inducers compared with healthy and infected plants.

Treatments	No. peak	Area	Ret. time	Area %	Total SA (µg/g FW)
Standard SA	1	1195.321	4.301	100	-
Inoculated control	2	3861.915	4.951	57.5735	1615.43
M. jalapa(M)	1	17814.2	4.892	70.9815	7451.63
C. inerme(Y)	4	20685.7	4.050	37.1380	8652.78
Mixed (M+Y)	2	7468.814	4.944	57.4512	3124.18
Kombucha	6	22344.2	4.188	38.9318	9346.61
Healthy	4	702.349	4.626	92.5020	293.79



HPLC quantification of free and endogenous SA in induced tomato plants.

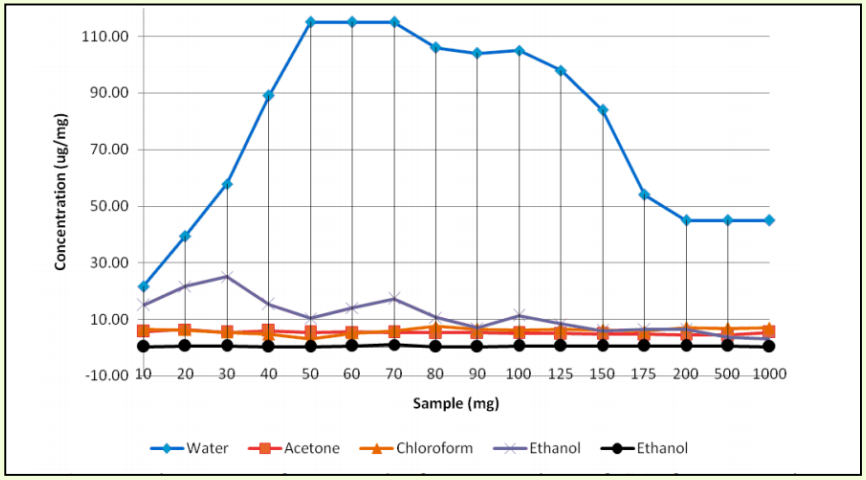
Mahdy *et al.*,2010

SAR-mediated host resistance

Defensive compounds estimation

6. Quantification of total salicylic acid (SA)

- A simple and reliable procedure for spectrophotometric determination of salicylic acid (SA) in Eucalyptus leaves is described.
- The procedure is based on the formation of $Fe(H2O)_6^{3+}$ ion which has an intense violet colour when SA in an aqueous form reacts with Fe (III).
- The absorbance of the complex was measured at 540 nm.
- The procedure was tested with different solvents and varying sample sizes to optimize the extraction protocol and study the interference of oils.



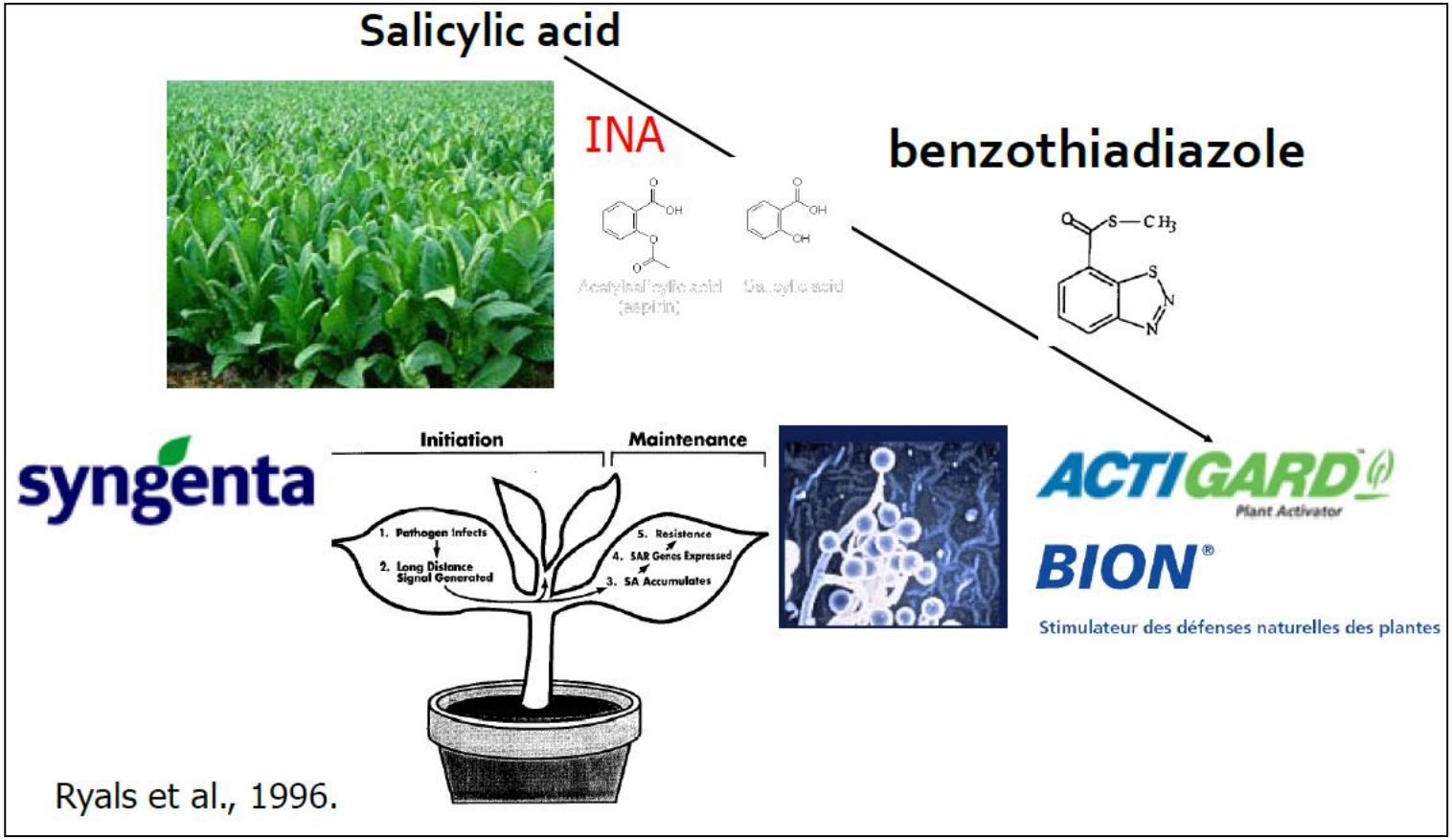
Development of protocols for extraction of SA from Eucalyptus leaves using five different solvents.

Warrier *et al.*,2013

SAR-mediated host resistance

A commercial SAR chemical inducer:

BTH on the market (1999)



The agents of biological control
Criterion for ISR as a tool for biocontrol

- Metagenome and new *bacillus* species can be new genetic resources to be utilized as biological control agents in the field.
- Balancing between plant growth and ISR capacity:

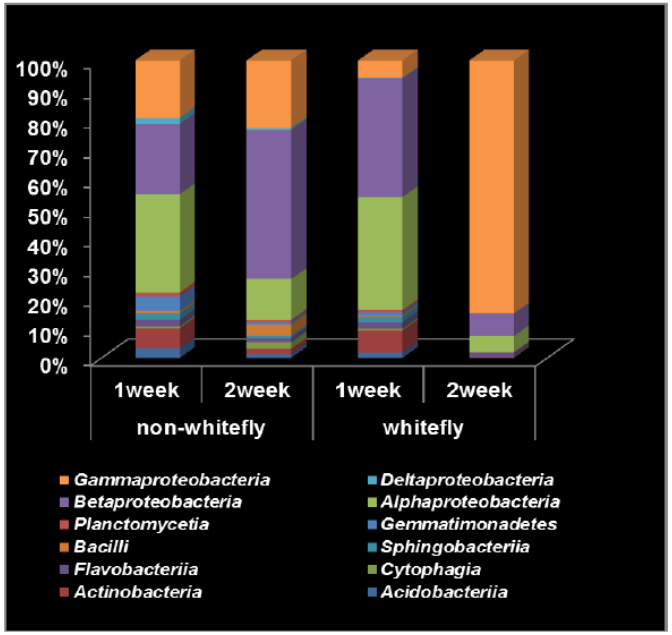
1. Minimizing effect of plant growth, and
2. Maximizing induction of systemic resistance.

- In other word, successful application of ISR requires minimizing plant growth effect by triggers but maximizing ISR capacity.

All the genetic material present in an environmental sample, consisting of the genomes of many individual organisms.
Meta: "after", "beyond", "with", "adjacent", "self".

The agents of biological control
Study of silent majority by metagenome

What's point to know the bacterial community?



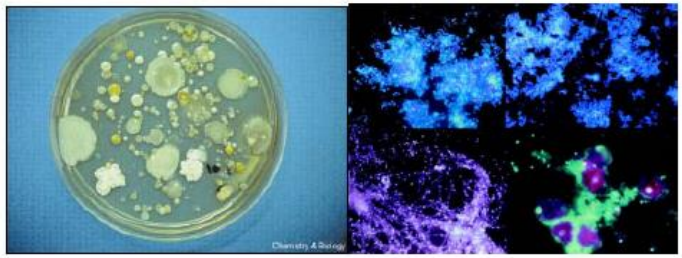
➡ functional metagenome

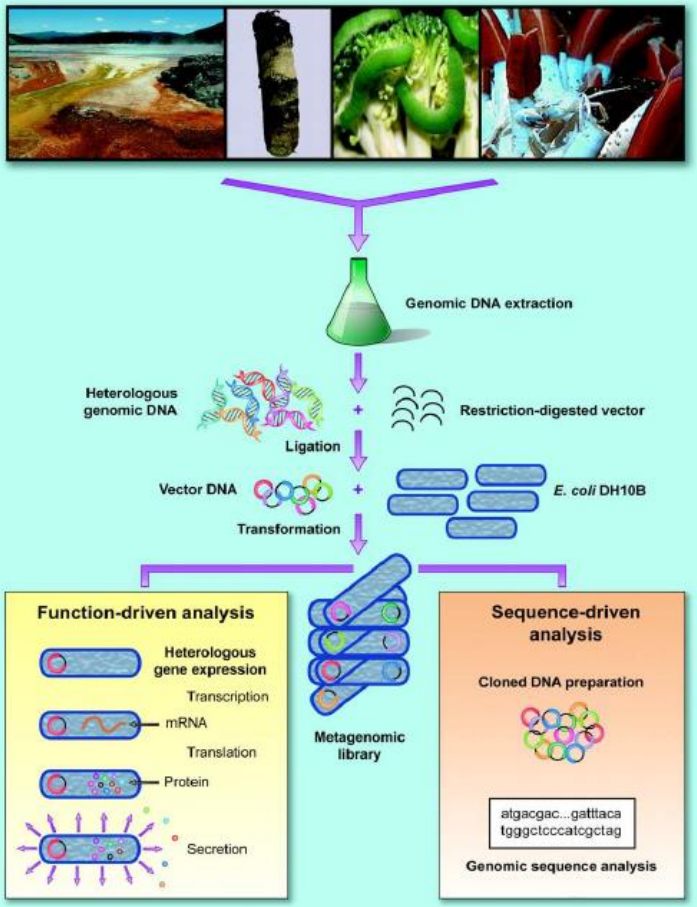
The agents of biological control
Study of silent majority by metagenome

Metagenomics (also **Environmental Genomics** or **Community Genomics**) is the study of genomes recovered from environmental samples as opposed to from clonal cultures.

The term "metagenomics" was first used by Jo Handelsman and others in the University of Wisconsin Department of Plant Pathology, and appeared in publication in 1998.

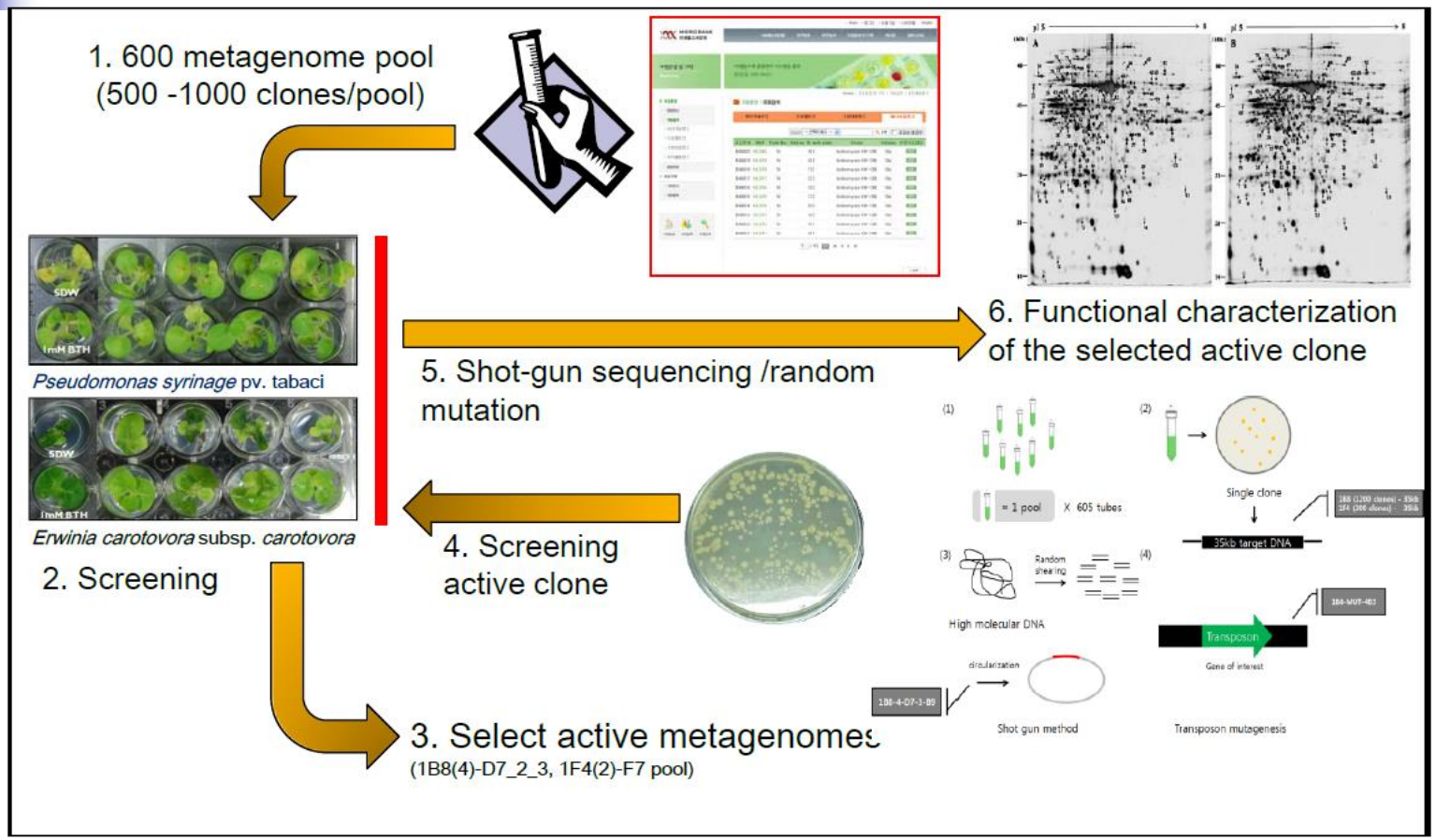
Handelsman et al. (1998). *Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products.* Chemistry Biology 5:R245-R249.





The agents of biological control

Screen active metagenome



Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

BTH and whitefly-elicited resistance against *R. solanacearum*

Whitefly-elicited induced resistance against bacterial wilt

- Soil-borne pathogen: *Rhalstoni*

Whitefly infestation of pepper plants elicits defense responses against bacterial pathogens in leaves and roots and changes the belowground microflora (J Ecology, 2011)

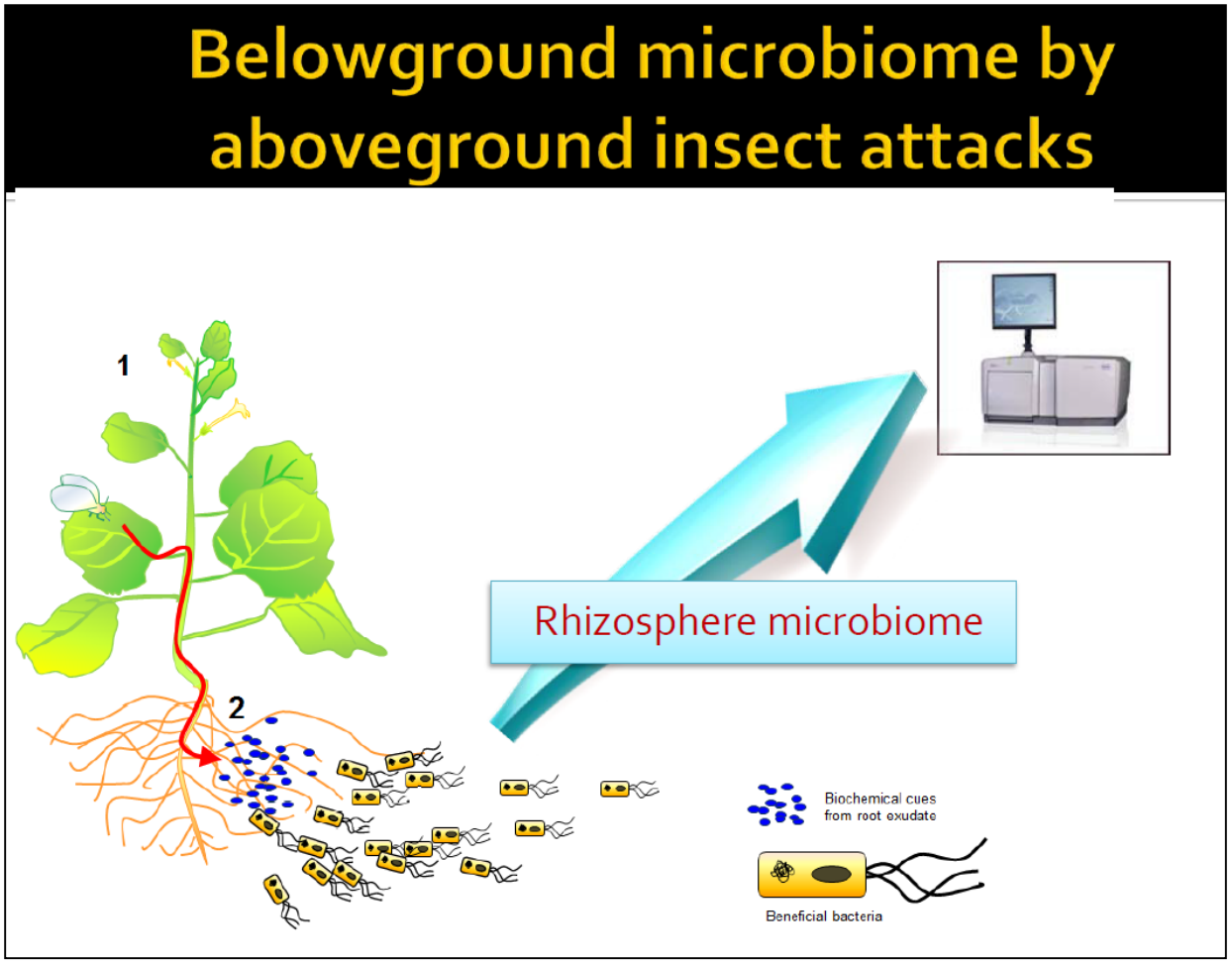
Yang et al., (2011)

Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

Whitefly-elicited induced resistance

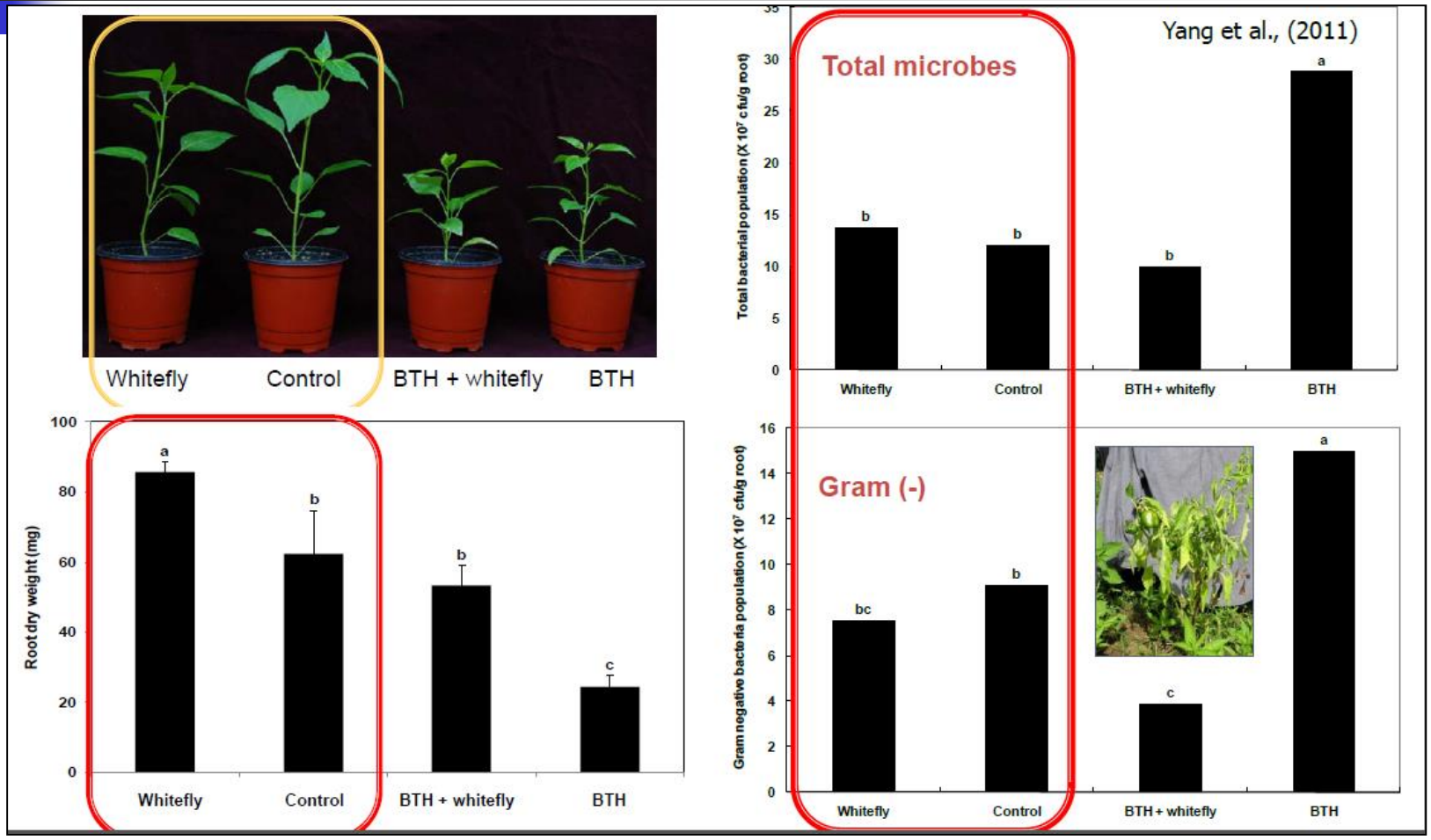
Rhizosphere microbiome



Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

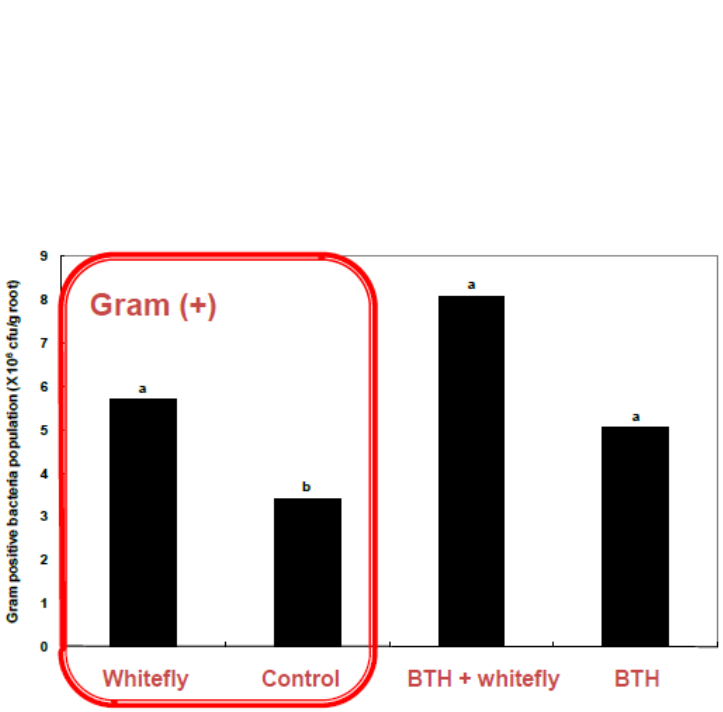
How can we explain the root growth promotions?

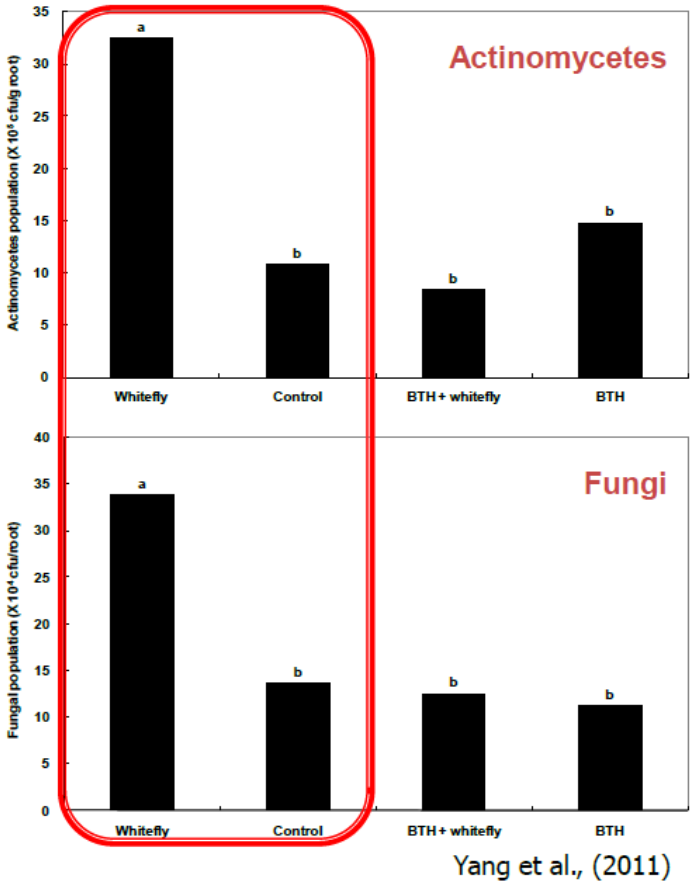


Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

Major players are Gram+ve and fungi

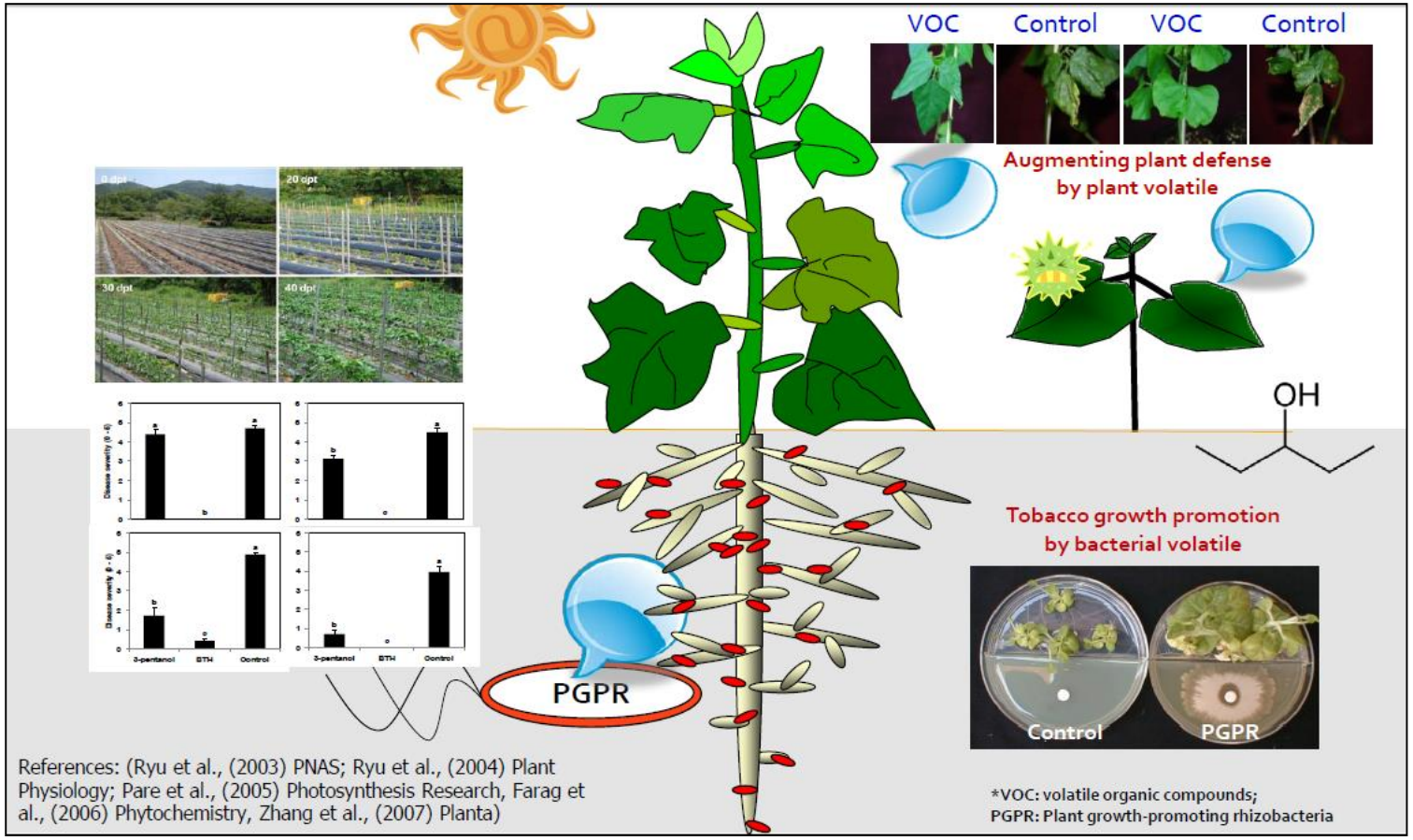




Yang et al., (2011)

ISR-induced host resistance

Volatile-mediated induced resistance

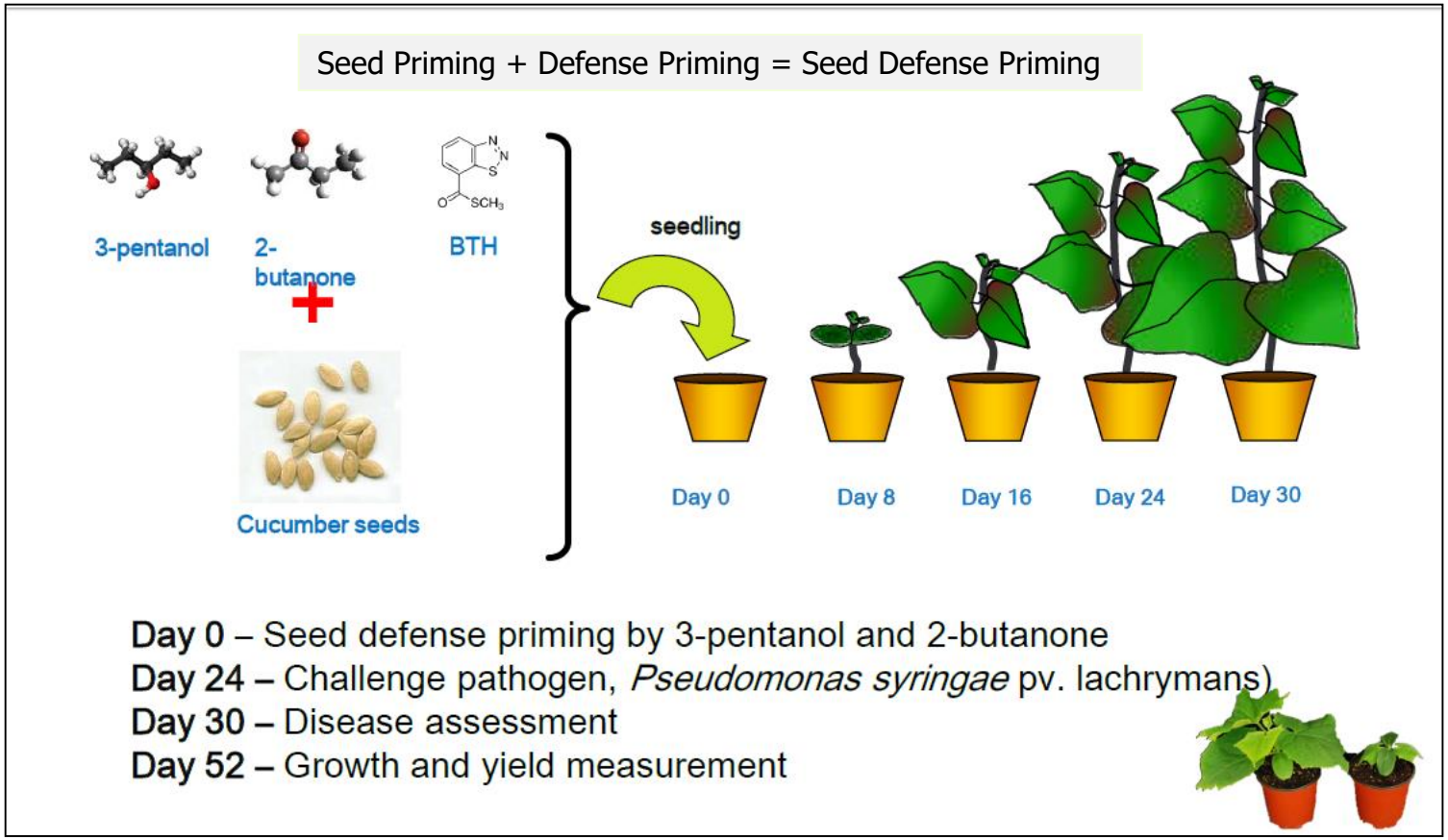


Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

Formulation of metagenome and field application

Optimization using chemical inducers



Choong-Min Ryu, Geun-Cheol Song

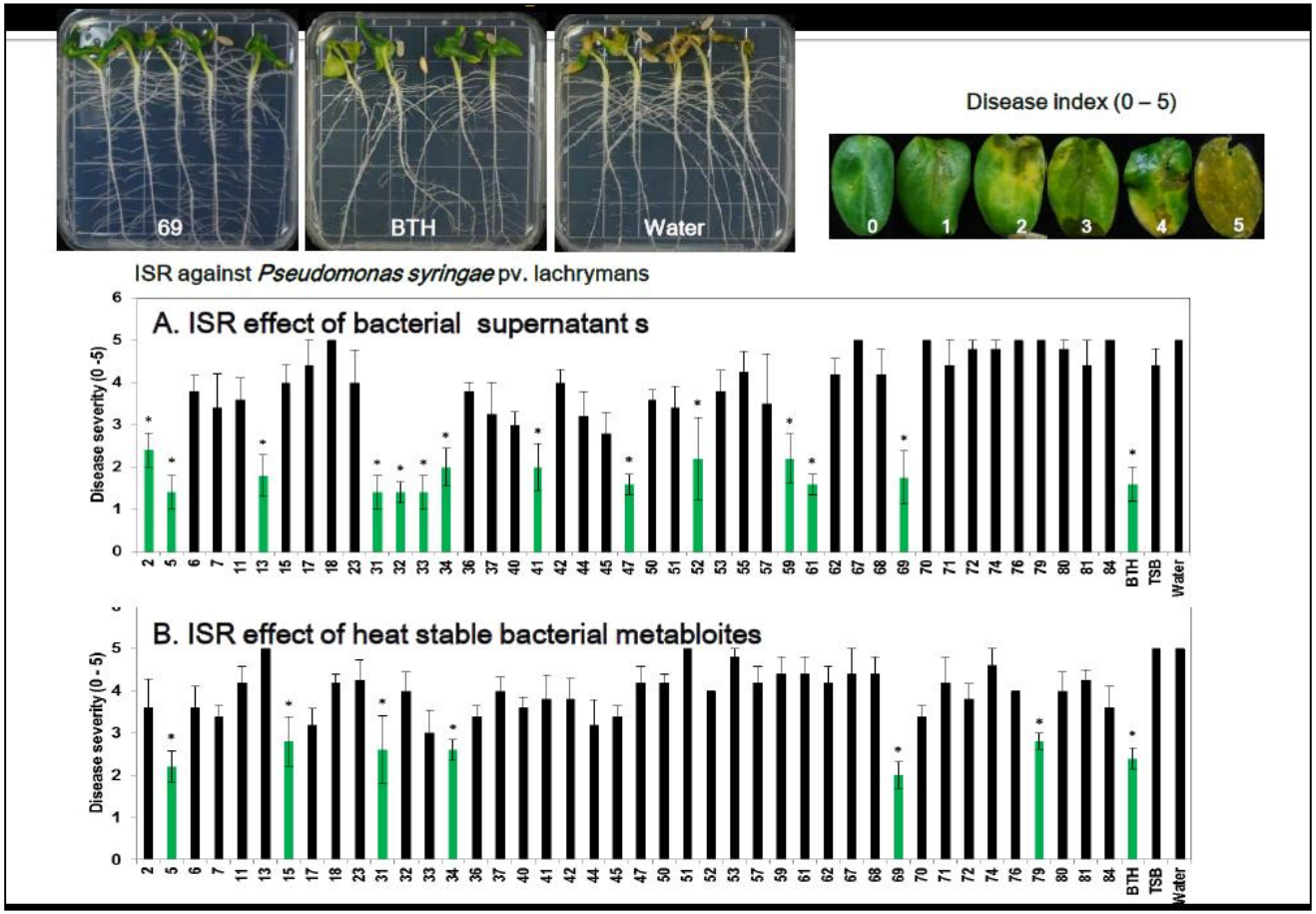
ISR-induced host resistance

List of 85 new bacilli species used as seed defense priming agent

No.	KCTC No.	Strain name	No.	KCTC No.	Strain name	No.	KCTC No.	Strain name
1	3117	*Bacillus laevolacticus*	30	3905	*Bacillus krulwichiae*	59	13202	*Bacillus zhejiangensis*
2	3346	*Bacillus sphaericus*	31	3906	*Bacillus marisflavi*	60	13210	*Bacillus subtangerinus*
3	3347	*Bacillus circulans*	32	3912	*Bacillus salarius*	61	13218	*Bacillus tashidiensis*
4	3393	*Bacillus fastidiosus*	33	3914	*Bacillus koreensis*	62	13219	*Bacillus nitroreducens*
5	3399	*Bacillus psychrosaccharolyticus*	34	3918	*Bacillus taeanensis*	63	13244	*Bacillus trypoxylicola*
6	3447	*Bacillus azotoformans*	35	3947	*Bacillus alkalitelluris*	64	13246	*Bacillus deserti*
7	3449	*Bacillus flexus*	36	3961	*Bacillus odysseyi*	65	13278	*Bacillus rigui*
8	3562	*Bacillus niacini*	37	3969	*Bacillus nealsonii*	66	13297	*Bacillus sorghi*
9	3572	*Bacillus cohnii*	38	13003	*Bacillus shackletonii*	67	13298	*Bacillus sorghicola*
10	3626	*Bacillus firmus*	39	13005	*Bacillus algicola*	68	13318	*Bacillus gaemokensis*
11	3706	*Bacillus mojavensis*	40	13006	*Bacillus asahii*	69	13299	*Bacillus sorghihabitans*
12	3737	*Bacillus insolitus*	41	13007	*Bacillus oshimensis*	70	13565	*Bacillus foraminis*
13	3739	*Bacillus badius*	42	13012	*Bacillus velezensis*	71	13572	*Bacillus soli*
14	3748	*Bacillus ehimensis*	43	13023	*Bacillus acidicola*	72	13573	*Bacillus vireti*
15	3776	*Bacillus oleronius*	44	13024	*Bacillus bataviensis*	73	13604	*Bacillus xyleni*
16	3777	*Bacillus sporothermodurans*	45	13025-1	*Bacillus drentensis*	74	13605	*Bacillus acetophenoni*
17	3793	*Bacillus psychrodurans*	46	13025-2	*Bacillus drentensis*	75	13613	*Bacillus siamensis*
18	3794	*Bacillus psychrotolerans*	47	13026	*Bacillus novalis*	76	13622	*Bacillus tequilensis*
19	3796	*Bacillus funiculus*	48	13078	*Bacillus acidiproducens*	77	13711	*Bacillus hunanensis*
20	3837	*Bacillus carboniphilus*	49	13114	*Bacillus arenosi*	78	13754	*Bacillus isabeliae*
21	3838	*Bacillus horti*	50	13115	*Bacillus arvi*	79	13918	*Bacillus sonorensis*
22	3839	*Bacillus jeotgali*	51	13117	*Bacillus fujiedaensis*	80	13922	*Bacillus endophyticus*
23	3846	*Bacillus luciferensis*	52	13118	*Bacillus shizuokaensis*	81	13929	*Bacillus panaciterrae*
24	3847	*Bacillus vulcani*	53	13178	*Bacillus massiliensis*	82	13930	*Bacillus ginsenggisoli*
25	3850	*Bacillus decolorationis*	54	13180	*Bacillus shandongensis*	83	13934	*Bacillus kribbensis*
26	3853	*Bacillus barbaricus*	55	13181	*Bacillus solisalsi*	84	13943	*Bacillus pocheonensis*
27	3900	*Bacillus schlegelii*	56	13194	*Bacillus basaltis*	85	13944	*Bacillus ginsengihumi*
28	3901	*Bacillus vedderi*	57	13199	*Bacillus anhuiensis*			
29	3903	*Bacillus aquimaris*	58	13201	*Bacillus sinense*			

ISR-induced host resistance

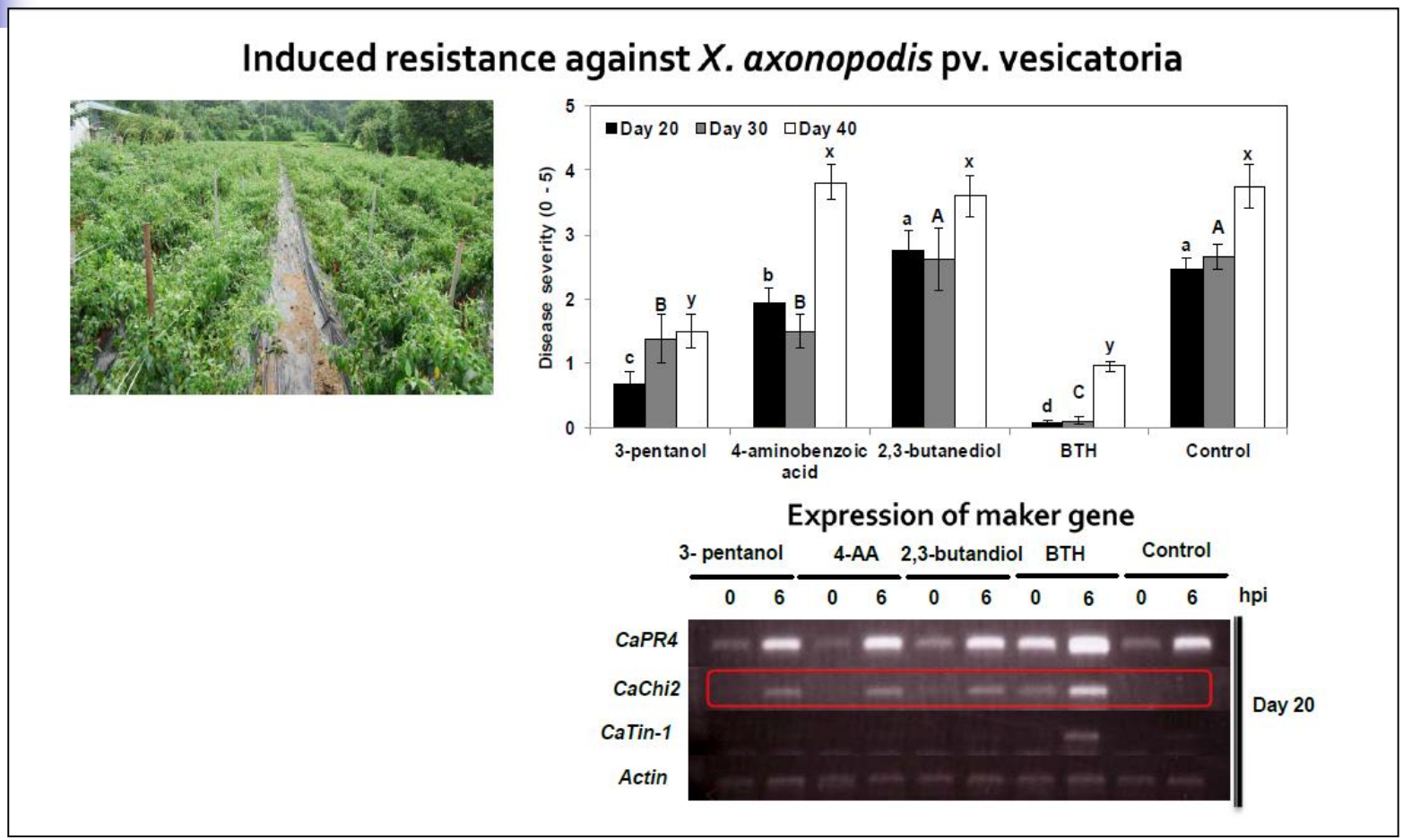
Seed defense priming by *Bacillus* supernatant



Choong-Min Ryu, Geun-Cheol Song

ISR-induced host resistance

Seed defense priming by *Bacillus* supernatant in the field



Choong-Min Ryu, Geun-Cheol Song

Active Defence Mechanisms
Pathogenesis-related proteins (PRs/PRPs)

- Pathogenesis related (PR) proteins are one of the major sources of plant derived allergens.

1. These proteins are induced by the plants as a defense response system in stress conditions like microbial and insect infections, wounding, exposure to harsh chemicals, and atmospheric conditions.
2. However, some plant tissues that are more exposed to environmental conditions like UV irradiation and insect or fungal attacks express these proteins constitutively.

Sinha *et al.*,2014

PR-proteins
Common characteristics

- The PR-proteins were defined as 'proteins coded by the host plant but induced only in pathological or related situations'.
- PRs was proposed based on their grouping into families sharing:

1. The same enzymatic or biological activity,
2. Amino acid sequence similarities,
3. Serological relationships.

Muthukrishnan *et al.*,2000;..

PR-proteins
Distinguished characteristics

- So far 14 distinct groups of PR-proteins (Van Loon and Van Strien,1999) were recognized.
- The PR-proteins of different groups (and often, even within the same group) differ in:

1. Molecular weight,
2. Isoelectric point (bands with different electrophoretic mobilities), and
3. Immunological cross-reactivity.

Biochemical, structural characteristics, cellular and tissue localization of PRs

- The 5 classical groups of PR proteins (PR-1,-2,- 3,-4,-5) has 2 subclasses:

1. A basic subclass found in plant cell vacuole.
2. An acidic subclass found in the extracellular space.

- Thus PRs have dual cellular localization:

1. Vacuolar, and
2. Apoplastics pace,

- The apoplast being the main site of their accumulation.

Sreeremya,2014

Biochemical, structural characteristics, cellular and tissue localization of PRs

- PR proteins are distinguished by specific biochemical properties:

1. Low-molecular proteins (6-43 kDa),
2. Acidic proteins (extractable and stable at low pH (<3),
3. Thermostable, and
4. Highly resistant to proteases(Van Loon, 1999).

- Among the 17 PR protein families already described, at least 9 present enzymatic activity such glucanases (PR-2); osmotins and thaumatins (PR-5); protease inhibitors (PR-6); lysozymes (PR-8); peroxidase (PR-9), ribonucleases (PR10) and chitinases (PR-3, PR-4, PR-8, PR-11).

Menezes *et al.*,2014;..

Induced plant proteins
Pathogenesis-related proteins(PRPs)

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* (‘*Glb*’)]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3*, *Chia*
PR-4	Tobacco ‘R’	Chitinase type I, II	*Ypr4*, *Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6*, *Pis* (‘*Pin*’)
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8*, *Chib*
PR-9	Tobacco “lignin-forming peroxidase”	Peroxidase	*Ypr9*, *Prx*
PR-10	Parsley “PR1”	Ribonuclease-like	*Ypr10*
PR-11	Tobacco “class V” chitinase	Chitinase, type I	*Ypr11*, *Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13*, *Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14*, *Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

Further details can be found at http://www.bio.uu.nl/~fytopath/PR-families.htm.
The PR-8 is an endochitinase that also has lysozyme activity.

Van Loon *et al.*,2006

Different PR-protein families and allergens identified

Family	Proteins	Functions	Allergens identified with source and allergenic symptoms
PR-1	PR-1 a, PR-1 b, and PR-1 c	Antifungal	Cuc m 3 (muskmelon)—oral allergy syndrome
PR-2	β-1,3-Glucanases	Cleaves β-1,3-glucans	Hev b 2 (latex)—contact dermatitis Ole e 9 (olive)—respiratory allergy Mus a 5 (banana)—oral allergy syndrome
PR-3	Chitinase types I, II, IV, V, VI, and VII	Endochitinase	Pers a 1 (avocado)—itchy eyes or nose, asthma, swelling, and so forth. Mus a 2 (banana)—food allergy like swelling of lips, anaphylaxis, and so forth
PR-4	Chitinase types I and II	Antifungal and chitinase	Hev b 6.01, Hev b 6.02, and Hev b 6.03 (latex)—contact dermatitis
PR-5	Thaumatin-like proteins	Antifungal	Jun a 3 (mountain cedar), Cry j 1 (Japanese cedar), and Cup a 3 (Arizona cypress)—rhinitis, conjunctivitis, and asthma Pru av 2 (cherry), Mal d 2 (apple), Cap a 1 (bell pepper), Act d 2 (kiwi), and Mus a 4 (banana)—oral allergy syndrome
PR-6	Tomato proteinase inhibitor I	Proteinase inhibitor	—
PR-7	Tomato endoproteinase P	Endoproteinase	—
PR-8	Cucumber chitinase	Chitinase III	Hevamine (latex)—contact dermatitis. Ziz m 1 (Indian jujube)—oral allergy syndrome Cof a 1 (coffee)—eye and airway allergy
PR-9	Tobacco lignin-forming peroxidase	Peroxidase	—
PR-10	Parsley "PR-1" Bet v 1, Mal d 1, Api g 1, and Dau c 1	Ribonuclease-like	Bet v 1 (birch pollen)— allergic rhinoconjunctivitis and asthma Pru av 1 (cherry), Mal d 1 (apple), Api g 1 (celery), and Dau c 1 (carrot)—oral allergy syndrome Gly m 4 (soy), Vig r 1 (mung bean), Cor a 1 (hazelnut), and Cas s 1 (chestnut)—oral allergy syndrome
PR-11	Tobacco chitinase type V	Chitinase	—
PR-12	Radish Rs-AFP3	Defensin	—
PR-13	*Arabidopsis* THI2.1	Thionin	—
PR-14	Lipid transfer proteins	Shuttling of phospholipids and fatty acids	Par j 1 (weed)—rhinitis and asthma Pru p 3 (peach), Mal d 3 (apple), Pru av 3 (cherry), Pru ar 3 (apricot), Cor a 8 (hazelnut), Cas s 8 (chestnut), and Zea m 14 (maize)—oral allergy syndrome
PR-15	Barley OxOa	Oxalate oxidase	—
PR-16	Barley OxOLP	Oxalate-like oxidase	—
PR-17	Tobacco PRp27	Unknown	—

An allergen is any substance (antigen) that is recognized by the immune system and causes an allergic reaction. E.g. Allergen= Cuc m 3. plant, Muskmelon (*Cucumis melo*).

Sinha *et al.*,2014

Biological role of PR-proteins

- An important common feature of most PRs is:

1. Their antifungal effect;
2. Some PRs exhibited also antibacterial (e.g. PR-8, PR-10, PR-14 proteins), insecticidal, nematicidal, and
3. As recently shown antiviral action.

- Therefore PR proteins are generally used as ISR markers, but no antiviral or antibacterial activity has yet been reported for any PR protein.

Plants are exposed to large number of pathogenic fungi. Although plants do not have an immune system, but they have a defense mechanism including antifungal activity There are hundred's of antifungal peptides & proteins known, with more being discovered almost daily.

See also the genetic engineering plants section, item 2

Some PR proteins function

- The PR-6 proteins were shown to be inhibitors of proteases. Presumably, their targets are insect or microbial proteases.
- The PR-7 proteins are endoproteases.
- The PR-9 proteins are lignin-forming peroxidases.
- Some PR-10 proteins have been shown to have an RNAse activity.
- More recently, the PR-protein classification has been extended to include other inducible proteins (antibacterial peptides), namely:

1. Defensins (PR-12),
2. Thionins (PR-13), and
3. Lipid transfer proteins (PR-14).

Some PR proteins function
PR-1 proteins

- **Example of antifungal effects of PRs:**
- PR-1 proteins were first found to be expressed in tobacco in response to tobacco mosaic virus (TMV) infection having 14 to 17 kDa molecular weights.
- These widely distributed proteins of plant kingdom have antifungal activity at the micromolar level against a number of plant pathogenic fungi including *Uromyces fabae*, *Phytophthora infestans*, *Erysiphe graminis*, but their mechanism of action is not known.

Sinha *et al.*,2014

Biological role of PR-proteins

Lytic enzymes

PR-2, PR-4, PR-8, and PR-11 proteins proteins

- **Example of antifungal effects of PRs:**
- Since most fungi contain *β*-1,3-glucans or chitin in their cell walls, these hydrolytic enzymes can be a tool in weakening and decomposing of fungal cell walls, containing glucans, chitin and proteins.

1. The PR-2 proteins (*β*-glucanses) had an endo 1,3-glucanase activity (Kauffmann *et al.*,1987), whereas
2. The PR-3 proteins (plus PR-4, PR-8, and PR-11 proteins) were shown to have endochitinase activity (Legrand *et al.*,1987).

Biological role of PR-proteins
PR-10, PR-15 and PR-16; PR-16

- **Example of antibacterial and antiviral effects of PRs:**
- PR-10 induced in hot pepper (*Capsicum annuum*) by incompatible interactions with TMVPo and *Xanthomonas vesicatoria*, was shown to function as a ribonuclease.
- Germins and germin-like proteins (GLPs) have been classified as PR-15 and PR-16; PR-16 has been isolated from hot pepper during the resistance response to bacterial and viral infection.

Biological role of PR-proteins
PRs 12, 13 and 14

- **Example of antibacterial and antifungal effects of PRs:**
- Additional families of PRs comprise the pathogen induced plant defensins (PR-12), thionins (PR-13) and Lipid transfer protein, LTPs (PR-14).
- All exhibit antifungal and antibacterial activity, exerting their effect at the level of the plasma membrane of the target micro-organism.
- PR-14 proteins are present in significant amounts in vascular tissue and in the outer cell layers of plants.
- PR-14 proteins are involved in plant defense against bacterial and fungal pathogeneses as well as under different environmental stresses such as drought, heat, cold, or salt.

van Loon and Strien,1999; Sinha *et al.*,2014

Methods

Purification of PR-proteins and isolation of PR-protein genes

- The PR-proteins are identified easily in cell extracts of infected plants (Van Loon and Van Kammen, 1968; Gianinazzi and Vallee, 1969).
- In fact, they are quite prominent in acid extracts of infected plants.
- PRs have dual cellular localization – vacuolar and apoplastic, the apoplast being the main site of their accumulation.
- Presently, PRs are established in all plant organs – leaves, stems, roots, flowers (Van Loon, 1999).

Methods
Assay of chitinase

- Plant e.g. Mango tissues (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0) and the homogenate was centrifuged for 10 min at 10,000× g at 4° C.
- The supernatant was used for the assay of chitinase and β- 1,3-glucanase.
- For the colorimetric assay of chitinase, 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml eppendorf tube.
- After 2 h at 37°C, the reaction was stopped by centrifugation at 1000× g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme (β-helicase) for 1 h.
- The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Boller & Mauch (1988) using internal standards of GlcNAc in the assay mixtures for calculations.
- Enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} fresh weight.

Vivekananthan *et al.*,2004

Methods

Assay of β-1,3-glucanase activity

- β-1,3-glucanase activity was assayed by the laminarin–dinitrosalicylate method (Pan *et al.*,1991).
- Plant e.g. Mango tissues were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4° C using a pestle and mortar.
- The extract was then centrifuged at 12,000 × g for 15 min at 4°C and the supernatant was used in the enzyme assay.
- The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract.
- The reaction was carried out at 40°C for 10 min.
- The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min in a boiling water bath.
- The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined.
- Enzyme activity was expressed as nmol min^{-1} g^{-1} fresh weight.

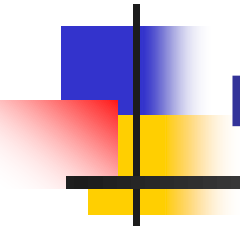
Vivekananthan *et al.*,2004

Methods

Detection of β-1,3-glucanase activity in native PAGE analysis

- β-1,3-glucanase isozymes and protein pattern were detected after a single separation using native polyacrylamide gel electrophoresis (PAGE) by the methods of Pan *et al*. (1991).
- To stain for β-1,3-glucanase, the PAGE gels were washed, then incubated at 40°C for 30 min in a mixture containing 75 ml of 0.05 M sodium acetate (pH 5.0) and 0.6 g of laminarin (Sigma) dissolved in 75 ml of water.
- The gels were then incubated in a mixture of methanol, water and acetic acid (5:5:2) for 5 min, washed with water and stained with 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1.0 M NaOH in a boiling water bath until red bands appeared (approx. 10 min).

Vivekananthan *et al.*,2004



Bacterial control of plant Pests and Diseases

Bacilli-based biological control

See also previous PR proteins section

Bacillus -based biological control agents

- *Bacillus subtilis*
- *Bacillus pumilus*
- *Amyloliquefaciens*
- *Bacillus cereus*
- *Bacillus mycoides*
- *Bacillus thuringiensis* (against insects)

Adoption of Bt crops in USA

From James 1998

	1996 area		1997 area		1998 area	
Crop	ha	%	ha	%	ha	%
Bt corn	300 000	1	2 800 000	9	6 500 000	22
Bt cotton	700 000	13	<1 000 000	17	>1 000 000	20
Bt potato	4 000	1	10 000	2.5	20 000	5

Berkeley *et al.*,2002

Registered *Bt* plant pesticides
Adapted from EPA 1999

Event/ product	Year registered	Expiration date	Toxin	Crop	Company
NewLeaf	May 1995	None	Cry3A	Potato	Monsanto/ NatureMark
NewLeaf Plus	Dec 1998	None	Cry3A + viral resistance gene	Potato	Monsanto/ NatureMark
Bollgard	Oct 1995	Jan 2001	Cry1Ac	Cotton	Monsanto
Event 176 (KnockOut)	Aug 1995/ March 1998	April 2001	Cry1Ab	Field corn/ popcorn	Novartis
Event 176 (NatureGard)	Aug 1995	April 2001	Cry1Ab	Field corn	Mycogen
Bt 11 (YieldGard)	Oct 1996	April 2001	Cry1Ab	Field corn	Novartis
Bt 11 (Attribute)	March 1998	April 2001	Cry1Ab	Sweet corn	Novartis
Mon810 (YieldGard)	Dec 1996	April 2001	Cry1Ab	Field corn	Monsanto
DBT-418 (Bt-Xtra)	March 1997	April 2001	Cry1Ac	Field corn	DeKalb (Monsanto)
CBH-351 (StarLink)	May 1998	April 2001	Cry9C	Field corn	PGS/AgrEvo (Aventis)

Berkeley *et al.*,2002

Bacillus subtilis
Against *Rhizoctonia solani*

- Loeffler and co-workers (1986) found that *Bacillus subtilis* gave good control of *Rhizoctonia solani* in many crops.
- The bacterium produces bacilysin and fengymycin A and B which are composed of a C15-C18 lipid moiety and a peptide moiety of eight amino acid residues.
- Bacilysin inhibits yeasts and bacteria and fengymycin inhibits filamentous fungi.

PGPR

Bacillus subtilis

- Beneficial plant rhizobacteria (PR) or Plant growth-promoting bacteria (PGPB, PGPR) are associated with the surfaces of plant roots and may increase plant yield by mechanisms that impart improved mineral nutrient uptake, disease suppression, or phytohormone.
- One beneficial rhizobacterium is *Bacillus subtilis*, which is ubiquitous in soil, can promote plant growth, protect against fungal pathogen attack.
- The commercial biofungicide, Serenade, which contains a *B. subtilis* strain, is reported to be effective against a variety of pathogenic bacteria, including *Erwina*, *Pseudomonas*, and *Xanthomonas* strains.

Bais *et al.*,2004

Mechanism of antibacterial effect
Bacillus subtilis

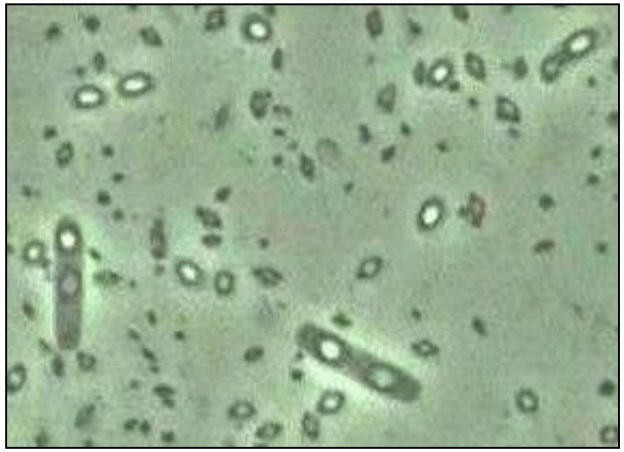
- The mechanism of this antibacterial effect is uncertain, although it is known that *B. subtilis* can produce a variety of antibacterial agents, including a broad spectrum of lipopeptides, such as surfactin, that are potent biosurfactants (Zuber *et al.*,1993).
- General properties: No systemic action, various mode of actions with low resistance potential.

The mechanism antibacterial effect
Bacillus cereus

- *Bacillus cereus* UW85, another successful biocontrol agent which is effective against damping-off and root rot of soybean caused by *Phytophthora sojae*.
- When seed was treated with this isolate and planted in soil known to be infested with the pathogen, emergence was significantly greater than controls.
- The bacterium is now known to produce a novel antibiotic (aminopolyol), zwittermicin A.

Bacillus thuringiensis (Bt)

- Bt, a soil bacterium whose spores contain a crystalline (Cry) protein.
- In the insect gut, the protein breaks down to release a toxin, known as a delta-endotoxin.
- This toxin binds to and creates pores in the intestinal lining, resulting in ion imbalance, paralysis of the digestive system, and after a few days, insect death.
- Different versions of the Cry genes, also known as "Bt genes", have been identified.
- They are effective against different orders of insects, or affect the insect gut in slightly different ways.



The vegetative cells contain endospores (phase bright) and crystals of an insecticidal protein toxin (delta endotoxin).

Insecticidal protein crystals of *Bacillus thuringiensis*

- The spore-forming bacterium *Bacillus thuringiensis* bears plasmids encoding genes for insecticidal proteins typically synthesized during sporulation.
- These proteins crystallize forming large polyhedral parasporal inclusions.
- Two types of crystal proteins are recognized:

1. Cry proteins, the most common and larger proteins(either 60–80 or130–150 kDa), and
2. Cyt proteins approximately 28 kDa, and are unrelated to Cry proteins.

- When ingested by insects and certain other arthropods, these inclusions dissolved, releasing active toxins.
- These endotoxin proteins bind to the host's midgut membrane and kill the host.

Shively,2006

Typical example of sporulated cells of *B. thuringiensis* and parasporal protein crystals

- Transmission electron micrographs of a sporulating cell of *B. thuringiensis* subsp. *israelensis* and parasporal bodies (a crystalline protein that forms around a spore in some bacteria that acts as a toxin precursor when digested)

A. Sporulating cell illustrating the developing spore (Sp) and parasporal body (PB).

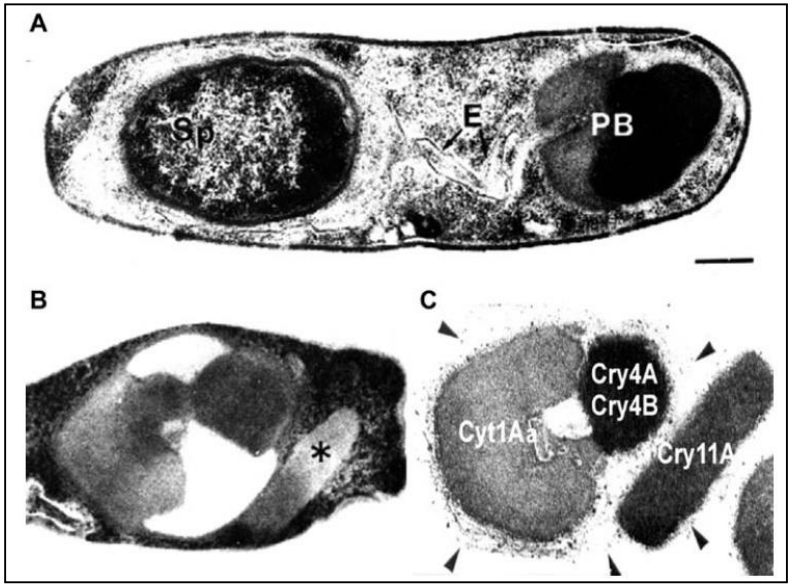
- The parasporal body, composed primarily of Cry4A, Cry4B, Cry11A, and Cyt1A proteins, is assembled outside the exosporium membrane (E).

B. Portion of sporulating cell just prior to lysis.

- The Cry11A crystal (asterisk) lies adjacent to the Cyt1A and Cry4A and Cry4B inclusions.

C. Purified parasproal body showing the components of the parasporal body.

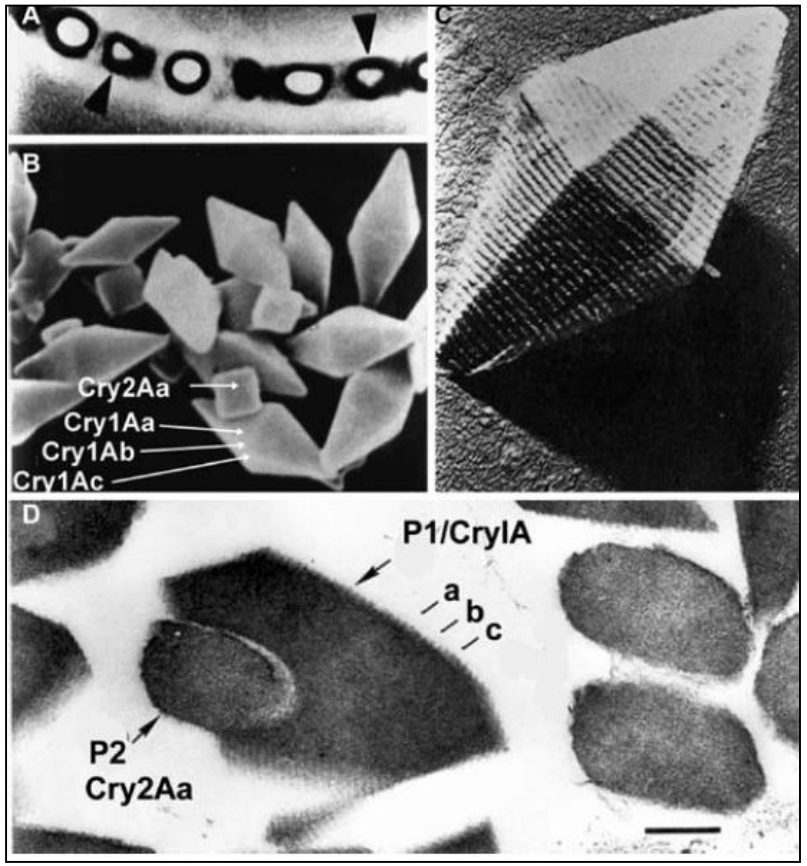
- These crystals are toxic to beetles.



Shively,2006

Sporulated cells of *B. thuringiensis* and parasporal protein crystals

- **A.** Phase-contrast micrograph of cells from a sporulated culture of *B. thuringiensis* just prior to lysis.
- Parasporal protein crystals (*arrowheads*) lie adjacent to oval spores.
- **B.** Scanning electron micrograph of typical Cry1 and Cry2 crystals purified from a sporulated culture of *B. thuringiensis* subsp. *kurstaki*, isolate HD1.
- **C.** Carbon replica of a typical bipyramidal Cry1-type protein crystal exhibiting the lattice of Cry1A molecules that compose the crystal.
- **D.** Transmission electron micrograph through a parasporal body of the HD1 isolate of *B. thuringiensis* subsp. *kurstaki* illustrating the embedment of the cuboidal Cry2A crystal (P2) in the bipyramidal crystal (P1).



Shively,2006

Some commercially available **Bt varieties** and target pests

- There are many different strains of *Bacillus thuringiensis* that attack specific kinds of insects.
- For example:

1. *Bacillus thuringiensis* var. *israeliensis* is used to control mosquitoes,
2. *Bacillus thuringiensis* var. *tenebrionis* is used to treat some pest beetles specie.
3. *Bacillus thuringiensis* var. *kurstaki* (Btk for short) is used to control caterpillar pests such as gypsy moths.

Mechanism of antibacterial effect

Bt toxins and their classification *Bacillus subtilis*

Gene	Crystal shape	Protein size (kDa)	Insect activity
cry I [several subgroups: A(a), A(b), A(c), B, C, D, E, F, G]	bipyramidal	130 -138	lepidoptera larvae
cry II [subgroups A, B, C]	cuboidal	69-71	lepidoptera and diptera
cry III [subgroups A, B, C]	flat/irregular	73-74	coleoptera
cry IV [subgroups A, B, C, D]	bipyramidal	73-134	diptera
cry V-IX	various	35-129	various

Summary of known holotypes of crystal (Cry) and cytolytic (Cyt) proteins

Revised nomenclature of pesticidal crystal proteins

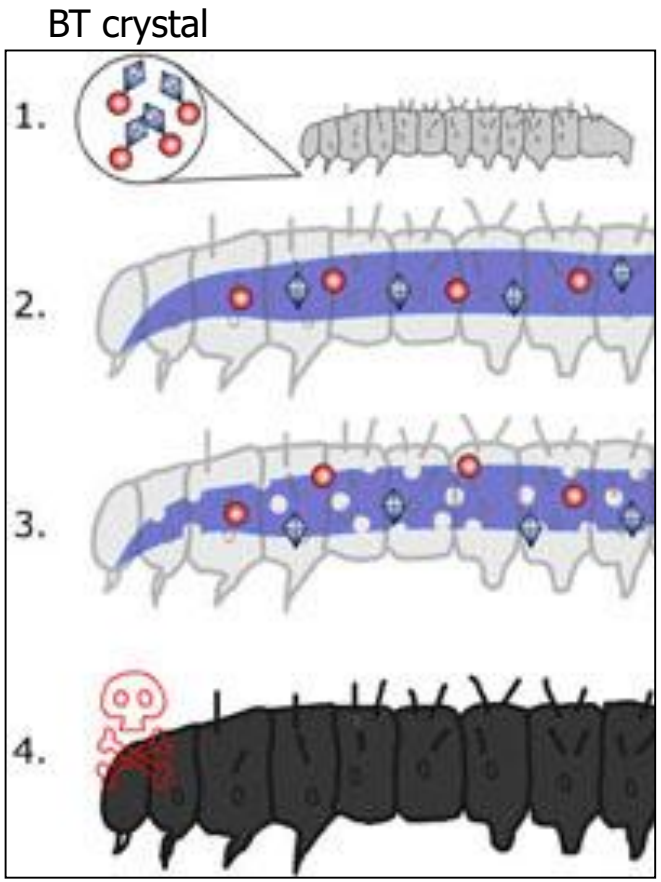
Cry1Aa	Cry1Ha	Cry5Aa	Cry12Aa	Cry23Aa
Cry1Ab	Cry1Hb	Cry5Ab		
Cry1Ac		Cry5Ac	Cry13Aa	Cry24Aa
Cry1Ad	Cry1Ia			
Cry1Ae	Cry1Ib	Cry5Ba	Cry14Aa	Cry25A
sCry1Af	Cry1Ic			
Cry1Ag	Cry1Id	Cry6Aa	Cry15Aa	Cry26Aa
Cry1Ah	Cry1Ie	Cry6Ba		
			Cry16Aa	Cry27Aa
Cry1Ba	Cry1Ja	Cry7Aa		
Cry1Bb	Cry1Jb	Cry7Ab	Cry17Aa	Cry28Aa
Cry1Bc	Cry1Jc			
Cry1Bd		Cry8Aa	Cry18Aa	Cry29Aa
Cry1Be	Cry1Ka	Cry8Ba		
		Cry8Ca	Cry18Ba	Cry30Aa
Cry1Ca	Cry2Aa			
Cry1Cb	Cry2Ab	Cry9Aa	Cry18Ca	Cry31Aa
	Cry2Ac	Cry9Ba		
Cry1Da	Cry2Ad	Cry9Ca	Cry19Aa	Cry32Aa
Cry1Db		Cry9Da		
	Cry3Aa	Cry9Ea	Cry19Ba	
Cry1Ea	Cry3Ba			Cyt1Aa
Cry1Eb	Cry3Bb	Cry10Aa	Cry20Aa	Cyt1Ab
	Cry3Ca			Cyt1Ba
Cry1Fa		Cry11Aa	Cry21Aa	
Cry1Fb	Cry4Aa			Cyt2Aa
		Cry11Ba	Cry22Aa	Cyt2Ba
Cry1Ga	Cry4Ba			Cyt2Bb
Cry1Gb		Cry11Bb		

Berkeley *et al.*,2002

Bacillus thuringiensis

How does *Bt* work?

1. Insect eats *Bt* crystals and spores.
2. The toxin binds to specific receptors in the gut and the insects stops eating.
3. The crystals cause the gut wall to break down, allowing spores and normal gut bacteria to enter the body.
4. The insect dies as spores and gut bacteria proliferate in the body.



Mode of Action

- Helicopter spraying pesticide over wooded area.

Schedule For Spraying *Bacillus thuringiensis* var. *kurstaki* (*Btk*) for Gypsy Moths in Indiana:

	Date of application	Look for:	Target of spray
First *Btk* spray	Late April/early May	First bloom of redbud trees	7-10 day old caterpillars
Second *Btk* spray	Approx. 2 wks after first spray	Redbud trees in full bloom; blooms beginning to fade	Older caterpillars (but still less than 3/8-inch in length)

Jodie A. Ellis

Biological control of nematodes

by fungal and bacterial antagonists

Biological control of nematodes

- Fungi:

1. Trapping fungi *Paecilomyces lilacinus* and *Verticitlium chlamydosporium*
2. Endophytic fungi(non-pathogenic root-infecting fungi and mycorrhizae)
3. Nematophagous fungus *Hirsutella rhossiliensis*

- Bacteria:

1. *Pasteuria* spp.
2. PGPR

Biological control of nematodes
By bacteria

- Compared to the substantial volume of work reported with the use of bacteria as biological control agents of soilborne pathogenic fungi, considerably less work has been done examining the potential of plant-associated bacteria to control phytoparasitic nematodes.
- But In the last two decades there has been large body of literature describing potential uses of PGPR against control of plant parasitic nematodes of different crops.

Biological control of nematodes

Reported bacterial groups with pathogenic activity against nematodes *Pasteuria, Pseudomonas* and *Bacillus*.

Tian *et al.*,2007

Genus and species	Target nematodes	Pathogenic effects on nematodes	Action mode
Four species: *Pasteuria. penetrans* ; *P. thornei* ; *P. nishizowae* ; Candidatus *Pasteuria usgae*	323 nematode species of 116 genera	Major economic important plant-parasitic nematodes have been observed to be parasitized by *Pasteuria*	Parasitism
Bacillus nematocida (*Bacillus* sp. B16); *Brevibacillus laterosporus* . *Bacillus* sp. RH219 etc	*Panogrellus redivius* and *Bursophelenchus xylophilus*	*Br. laterosporus* strain G4 could penetrate the nematode (*Pan. redivius* and *Bu. xylophilus*) cuticles and eventually digest the target organism in the laboratory	Parasitism, production of enzymes and toxin
Distribution in more than 29 genera. *Bacillus* (more than 15 species) and *Pseudomonos* (more than 11 species) are two of the most dominant populations	Reduce nematode populations in soil	Different rhizobacteria showed different degrees of suppression on nematodes in various conditions. Three commercial bionematicides from bacteria all belong to this group	Interfering with recognition, production of toxin, nutrient competition, plant-growth promotion; induction of systemic resistance
Bacillus thuringiensis (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21)	*Trichostrongylus colubriformis* ; *Caenorhabditis elegans* ; and *Nippostrongylus brosiliensis*	These Cry proteins showed toxicity to larval stages of free-living and parasitic nematodes	Cry proteins caused damage to the intestines of nematodes
The majority of rhizobacteria can also be identified as endophytic bacteria	Root-knot nematode and root-lesion nematode etc	Suppress root-knot nematodes and root-lesion nematode etc	Rhizobacteria and endophytic bacteria use some of the same mechanisms
Two genera: *Xenorhabdus* .	*Bursophelenchus xylophilus* ; *M.*	Toxic to juveniles of root-	Toxin production

The advantages and limitations of potential biological control agents with different modes of action against plant-parasitic nematodes

Type of agent	Mode of action	Comments
Obligate parasites *Pasteuria* spp.	Adhesive spores	Advantages: Most isolates highly virulent; infective spores resistant to drying; good shelf-life; reduce infectivity of nematodes as well as fecundity. Limitations: Very difficult to culture *in vitro*; isolates are highly specific; no proliferation in soil in absence of nematodes.
Rhizosphere bacteria	Toxins or modification of root exudates	Advantages: Easy to culture *in vitro*; can be applied as seed treatments; reduce plant damage. Limitations: Effective for a relatively short period; activity affected by crop cultivar and nematode species; little effect on nematode multiplication.

Shelf-life: the length of time for which an item remains usable.

Kerry,1987; Stirling,1991;Sikora,1992

Biological control of nematodes

Mode of action

Nematotoxic activity of rhizobacteria (PGPR) against nematode

- Some rhizosphere-inhabiting bacteria (rhizobacteria) are antagonistic to plant-parasitic nematodes.
- These bacteria inhibit nematode egg hatch and/or penetration of roots.
- The mechanism by which antagonistic bacteria inhibit plant-parasitic nematodes is not known.
- However, several hypotheses have been put forth:
- Production of antibiotics that kill nematode eggs.
- Degradation of the root exudates that the nematode relies on for host location and to stimulate egg hatch.
- Induction of systemic acquired resistance (SAR).

Biological control of nematodes

Mode of action

1. Soil amendments with chitin

- Recent work with several nematode control strategies demonstrates that it is possible to achieve at least a limited induced soil suppressiveness, through shills in microbial community structure and function, by several cultural practices.
- Examples of such practices include:
- The use of organic amendments, inclusion of antagonistic plants in cropping systems, and applications of biorational nematicides.
- Among the many organic amendments, chitin has been perhaps most extensively studied.

Kloepper *et al.*,1999

Biological control of nematodes

Mode of action

1. Soil amendments with chitin

- Amendment of soil with chitin leads to enhanced activity of chitinolytic microorganisms(bacilli, *Arthrobacter* spp., *Burkholderia cepacia*).
- It has been considered that the observed biocontrol results partly from activity of chitinases on nematode eggs and partly from the accumulation of ammonium, which is toxic to nematodes, by microbial deamination of the chitin polymers of acetylglucosamine and chitobiose.
- It was recently reported that soil amendment with 1% chitin led to alterations in the taxonomic structure of the bacterial communities of the soil, rhizosphere and endorhiza (Hallmann *et al.*,1998).

Kloepper *et al.*,1999

Biological control of nematodes

Mode of action

2. Antibiosis of *Pseudomonas fluorescens* F113 against bacteria, fungi and nematodes

- Two of the best-studied antibiotics responsible for the activity of *Pseudomonas* biocontrol agents are the:

1. Phenolic compound 2,4-diacetylphloroglucinol (DAPG), and
2. Phenazines.

- The DAPG biosynthetic locus, *phl*, includes six genes, and
- The phenazine-1- carboxylic acids (PCA) biosynthetic locus, *phz*, includes seven genes.
- The activity of these two antibiotics is broad.
- For example, the DAPG producer *Pseudomonas fluorescens* F113 suppresses:
- Damping-off sugar beet caused by *Pythium ultimum*, and
- Cyst nematode caused by *Globodera rostochiensis*, and
- Soft rot of potato by *Erwinia carotovora*.

Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

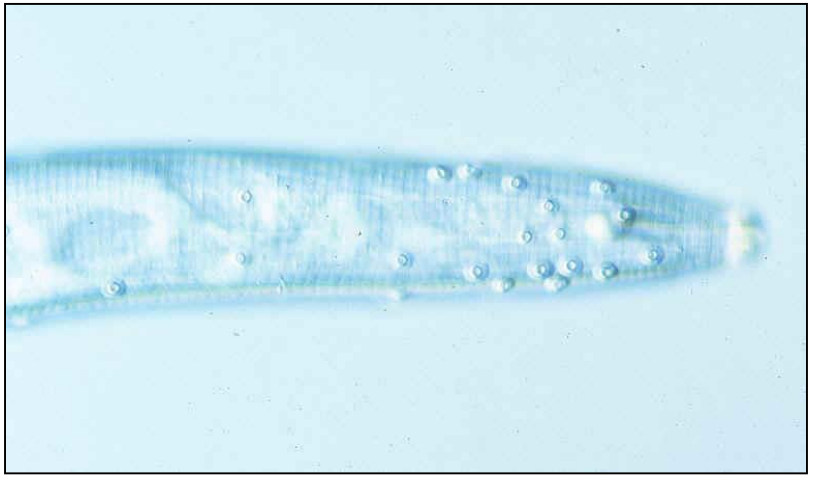
- *Pasteuria* spp. are endospore-forming actinomycetes that are parasites of invertebrates, including nematodes.
- The bacterium is an obligate parasite and cannot be cultured outside the body of the invertebrate host.
- *Pasteuria* spp. are very host specific.
- Generally, populations of this bacterium are only efficient parasites of the nematode species from which they originated.
- There are four described species of *Pasteuria* and several undescribed species.
- Spores of nematophagous species adhere to the cuticle of host nematodes that encounter them while moving through soil.

Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- *Pasteuria* sp. spores adhering to the cuticle of *Hoplolaimus galeatus*.
- Photo by B. A. Jaffee.

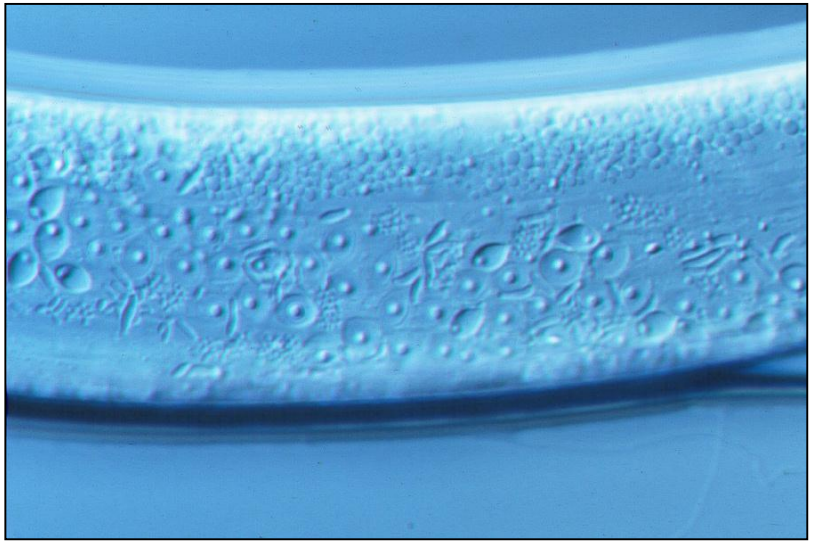


Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- *Pasteuria* sp. in the body cavity of *Xiphinema americanum*.
- Three stages in the life cycle of *Pasteuria* can be seen:
- Microcolonies,
- Separated quartets or doublets, and
- Single sporangia.
- Photo by B. A. Jaffee.

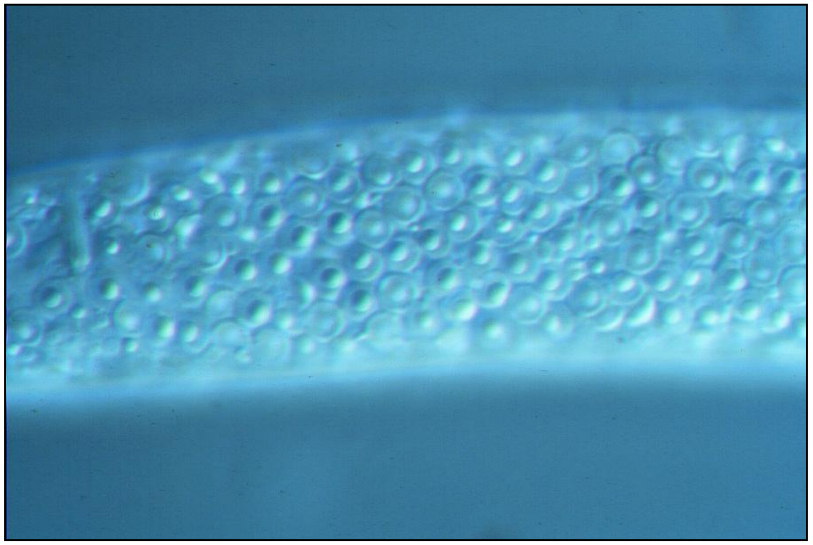


Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- A female of *Pratylenchus penetrans* filled with endospores of *Pasteuria brachyurus*.
- The nematode was still alive at time of photo.
- Photo by P. Timper.

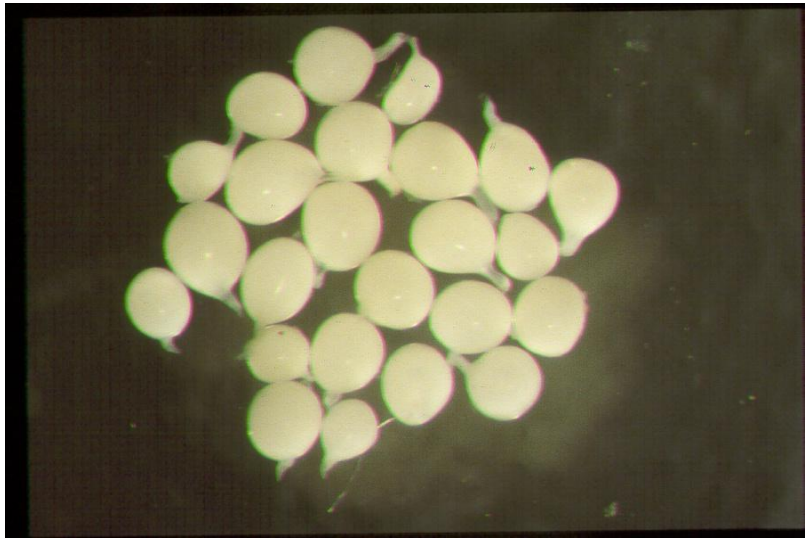


Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- Females of *Meloidogyne javanica* infected with *Pasteuria penetrans*.
- Note the opaque, pearly white appearance of the females.
- Photo by S. Verdejo-Lucas.

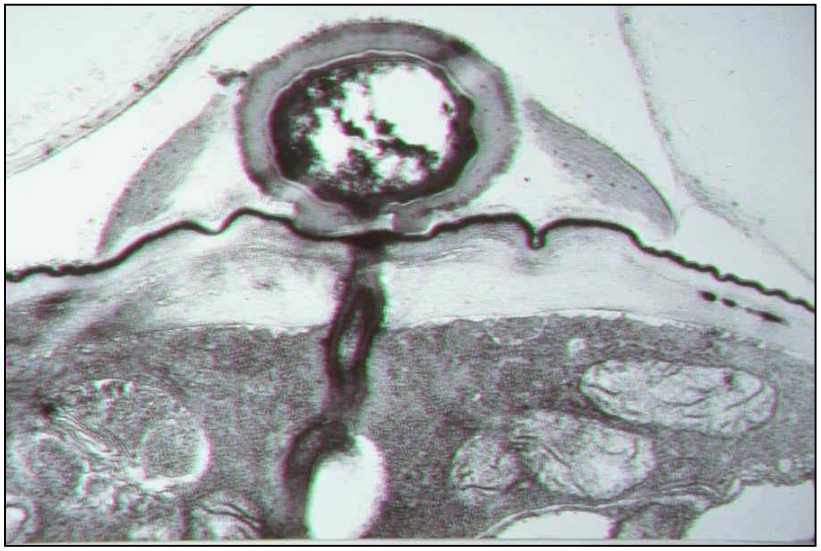


Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- Infection of a root-knot nematode by *Pasteuria penetrans*.
- The spores form a germ tube and penetrate the cuticle of the nematode.
- Photo by R. M. Sayre.

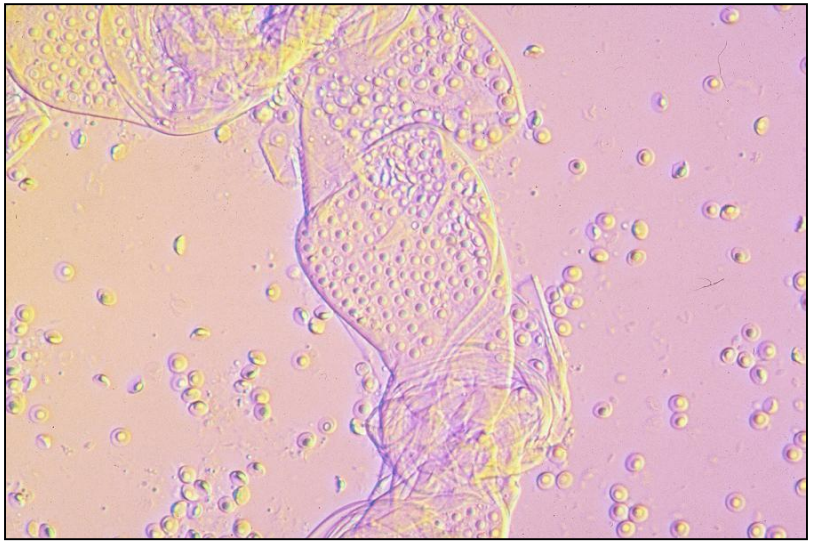


Biological control of nematodes

Mode of action

3. Penetration of nematode's cuticle by *Pasteuria* spp.

- *Pasteuria* spores from ruptured nematode.
- Photo by B. A. Jaffee.



Biological control of nematodes

Mode of action

4. catabolic enzymes,…

- Many studies on mechanism of action of rhizobacteria indicated that:

1. Catabolic enzymes (e.g. proteases, chitinases and glucanases),
2. Peptide antibiotics, or
3. Small molecules secreted by various *Bacillus* species might contribute to their activity against pathogenic nematodes.

See also mechanism of action of lytic enzymes in biological control section.

Biological control of nematodes

Mode of action

4. Proteases

- The rhizobacteria have been extensively studied as agents for the biological control of plant-parasitic nematodes.
- These bacterial species include:
- *Bacillus thuringiensis*
- *Bacillus subtilis*
- *Bacillus amyloliquefaciens*
- *Bacillus cereus*
- *Bacillus licheniformis*
- *Bacillus megaterium*
- *Bacillus mycoides*, and
- *Bacillus pumilus*

Bacteria that colonize the rhizosphere are commonly referred to as rhizobacteria.

Lian *et al.*,2007

Biological control of nematodes
Rhizobacteria
Proteases

- Native populations of *Bacillus* and *Paenobacillus* spp. occur abundantly in most agricultural soils.
- Multiple *Bacillus* spp. can promote crop health in a variety of ways: e.g.

1. They can suppress plant pathogens and pests by producing antibiotic metabolites, or
2. They can directly stimulate plant host defences prior to infection and promote plant growth and health.

- Despite a wealth of new information on the genetics and physiology of *Bacillus* and related species, the microbial mechanism of action against nematode populations remains very limited.

Lian *et al.*,2007

Biological control of nematodes

Mode of action

Proteases

- These have direct antagonistic effects to pathogenic nematodes belonging to the genera:

1. Heterodera, and
2. Meloidogyne.

- The rhizobacteria including plant growth promoting rhizobacteria (PGPR) and plant health promoting rhizobacteria (PHPR) can be isolated from rhizosphere and endosphere of infected crops plants.
- Microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes.
- Selection of potential bioantagonists and their application were done under *in vitro* and in green house conditions.

Qiuhong *et al.*,2006; Lian *et al.*,2007

Biological control of nematodes

Mode of action

Proteases

- Siddiqui *et al.*,2005 also demonstrated that the deletion of a major extracellular protease from *Pseudomonas fluorescens* CHA0 reduced bacterial activity against the root-knot nematode *Meloidogyne incognita*.
- These researches suggested extracellular proteases might play important pathogenic roles in suppressing nematodes in the soil.
- The proteases with cuticle-degrading and nematicidal activities were found to be extensively distributed in *Bacillus* spp.

Lian *et al.*,2007

Biological control of nematodes

Mode of action

Proteases

- Rhizobacteria with nematicidal activity were isolated from soil samples of five root knot nematode-infested farms.
- The nematicidal toxins from *Bacillus* sp. strain RH219 produced an extracellular cuticle-degrading protease Apr219.
- Apr219 is an important pathogenic factor of strain RH219.
- The Apr219 shared high similarity with previously reported cuticle-degrading proteases from *Brevibacillus laterosporus* strain G4 and *Bacillus* sp. B16 (*Bacillus nematocida*).
- In addition to Apr219, a neutral protease Npr219 from *Bacillus* sp. RH219 was also investigated for activity against nematodes.

Qiuhong *et al.*,2006; Lian *et al.*,2007

Biological control of nematodes

Mode of action

Proteases

- These strains presented remarkable nematotoxic activity against target free-living nematode *Panagrellus redivivus*.
- The crude extracellular protein extract from culture supernatant of the bacteria killed about 80% of the tested nematodes within 24 h, suggesting the involvement of extracellular proteases.
- The purified protease can hydrolyze several native proteinaceous substrates, including collagen and nematode cuticle.

Qiuhong *et al.*,2006; Lian *et al.*,2007

Biological control of nematodes

Isolation of rhizobacteria with nematicidal activities

Proteases

- Rhizobacteria were isolated from the rhizosphere of tobacco in five root knot nematode-infested farms in Yunnan province in China.
- To isolate these bacteria, roots were washed in 0.1 mol^{-1} phosphate buffer, and appropriate dilutions were plated on NA agar and incubated at 28°C for 2 days (Fang 1998).
- Nematicidal activities of isolated strains were tested according to the methods described below using the free-living nematode *Panagrellus redivivus* as the target nematode.

Lian *et al.*,2007

Biological control of nematodes

Identification of rhizobacteria with nematicidal activities

Proteases

- The nematicidal bacteria were classified based on their morphological and biochemical characteristics and their 16S rDNA sequences.
- After the genomic DNA of the *Bacillus* strains was extracted, 16S rRNA genes were amplified using the forward (5′-GGTTACCTTGTTACGACTT-3′) and reverse (5′-AGAGTTTGATCCTGGCTCAG-3′) primers as described by Lane (1991).
- The sequenced 16S rRNA genes were compared and analysed using the clustal X 1.83 and mega version 3.1 programs.

Lian *et al.*,2007

Biological control of nematodes

Gene cloning

Proteases

- The gene for the virulence protease was cloned, and the nucleotide sequence was determined.
- To determine the distribution of the cloned cuticle degrading protease genes in *Bacillus* spp. with nematotoxicity, PCR amplification was also conducted for the isolated bacteria using previously designed primers (ap1 and ap2).
- The deduced amino acid sequences from the genes were analysed using the Bioedit software package (Raleigh, NC, USA).

Qiuhong *et al.*,2006; Lian *et al.*,2007

Biological control of nematodes

Storage of rhizobacteria with nematicidal activities

Proteases

- Candidate *Bacillus* spp. were stored in 30% glycerol at -20°C for further assays.

Lian *et al.*,2007

Biological control of nematodes

Bioassay on nematode cuticles

Proteases

- All of the isolated rhizobacterial strains were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YPD(yeast, peptone, glucose) medium each and grown at 28°C with rotary shaking at 220 rev $min)^{-1}$ for 3 days (Dillon *et al.*,1985).
- After centrifugation at 8500 g for 15 min, the culture supernatants were collected for the measurement of nematicidal activity.
- In bioassay, approximately 200 nematodes were added to 300µl culture supernatants in a 1.5 ml Eppendorf tube containing two antibiotics (50 µg ml^{-1} ampicillin and 30 µg ml^{-1} kanamycin).

Lian *et al.*,2007

Biological control of nematodes

Bioassay on nematode cuticles

Proteases

- After incubating the tubes at 28°C for 2-10 h, the numbers of dead nematodes in each treatment were counted under a light microscope.
- The experiments were performed in triplicates and repeated at least three times.
- Controls were incubated with:
- Water,
- YPD medium, and
- The culture supernatant boiled for 15 min.

Lian *et al.*,2007

Biological control of nematodes
Bioassay on nematode cuticles
Proteases

- Mortality of *Panagrellus redivivus* in culture supernatants from *Bacillus* spp.
- The percentage of dead nematodes was determined after 2, 5, and 10 h exposures to the supernatants.

Bacillus strain number	2-h mortality of *P. redivivus* % (SE)	5-h mortality of *P. redivivus* % (SE)	10-h mortality of *P. redivivus* % (SE)
RH219	80 (2·11)^{b,B}	99 (0·50)^{b,B}	100 (0)^{b,B}
A29	47 (1·67)^{b,B}	58 (3·87)^{b,B}	90 (1·41)^{b,B}
A56	61 (2·93)^{b,B}	76 (1·06)^{b,B}	100 (0)^{b,B}
A104	33 (1·19)^{b,B}	75 (2·80)^{b,B}	100 (0)^{b,B}
B101	40 (3·86)^{b,B}	82 (0·15)^{b,B}	100 (0)^{b,B}
Water	5 (0)	6 (0)	12 (0·62)
YPD medium	7 (0)	8 (0)	10 (0·10)

Mortality of nematodes = number of dead nematodes/all tested nematodes x 100%.

Lian *et al.*,2007

Biological control of nematodes

Bioassay on nematode cuticles

Proteases

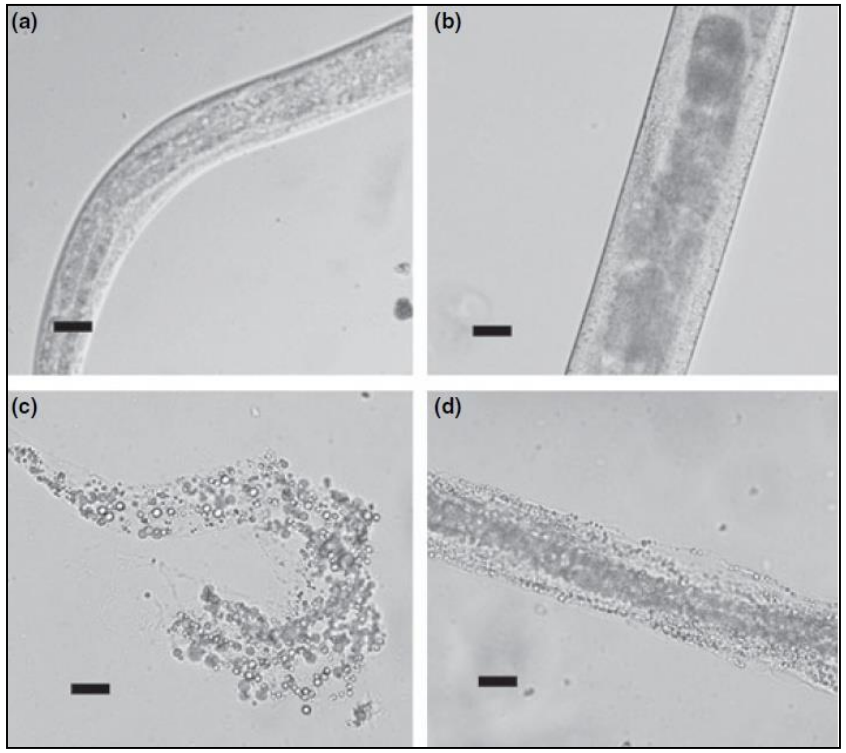
- The action of the proteases Apr219, Npr219 and culture supernatant of *Bacillus* sp. RH219 against *Panagrellus redivivus*.

a) The cuticles of nematodes in the control were intact and smooth within 24 h.

b) The cuticles of nematodes in the Npr219 treatment were also intact within 24 h.

c) The nematode cuticles were degraded and their bodies destroyed after 24 h in the culture supernatant treatments.

d) The nematode cuticles were degraded after 24 h in the Apr219 treatments (bar=10 μm).



Lian *et al.*,2007

Biological control of nematodes
Bioassay on purified nematode cuticles
Proteases

- Fragments of cuticles of the target free-living nematode *Panagrellus redivivus* were purified according to the method described by Cox *et al.*,1981.
- After 200 µl of the purified protease sample (the proteases Npr219 or Apr219) was mixed with nematode cuticles, the degradation process was observed under a light microscope once in every hour.
- For negative controls:

1. 0.1 mol l^{-1} bovine serum albumin (BSA), and
2. The target sample boiled for 15 min were added to the nematode cuticle.

Lian *et al.*,2007

Biological control of nematodes
Bioassay on purified nematode cuticles
Proteases

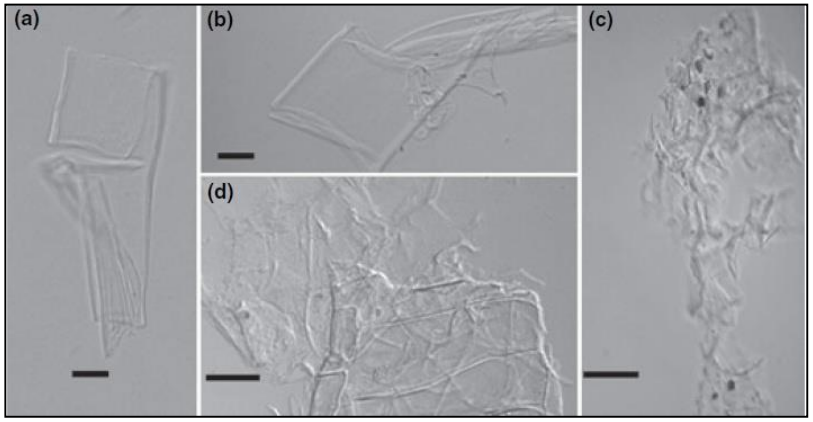
- The action of Apr219, Npr219 and
- culture supernatant of *Bacillus* sp. RH219 against purified nematode cuticles.

a) The purified cuticle of *Panagrellus redivivus*.

b) The cuticle was intact in the Npr219 treatment for 2 h.

(c) and (d) The cuticle was treated with culture supernatant and Apr219 for 2 h, respectively.

- Most extracted cuticles were destroyed and only incomplete and minor fragments were observed (bar=10 μm).



Lian *et al.*,2007

Biological control of nematodes
Purification of proteases
Proteases

- A 500 ml bacterial culture solution for *Bacillus* sp. RH219 was pooled and bacterial cells were removed by centrifugation at 8500 g for 15 min at 4°C.
- The resulting supernatant was salt-out by adding ammonium sulfate to 80% saturation.
- After centrifugation at 8500 g for 20 min again, the precipitated protein was dissolved in 100 ml of 50 mmol l^{-1} sodium phosphate buffer (pH 7)with 1 mol l^{-1} ammonium sulfate.
- The dissolved protein solution was applied to HiPrep 16/10 column and HiTrapTM SP FF column to get purified proteases according to previously described manipulation procedures for protein purification.
- The fractions were pooled and assayed for:

1. Protease activity, and
2. Assayed by 12% SDS PAGE (Laemmli, 1970; Huang *et al.*,2005a).

Lian *et al.*,2007

Biological control of nematodes

Purification of proteases

Proteases

- Characterization of the purified protease revealed:
- The molecular mass of 28 kDa, and
- The optimum activity at pH 10, 50°C.

Qiuhong *et al.*,2006

Biological control of nematodes

Purification of proteases

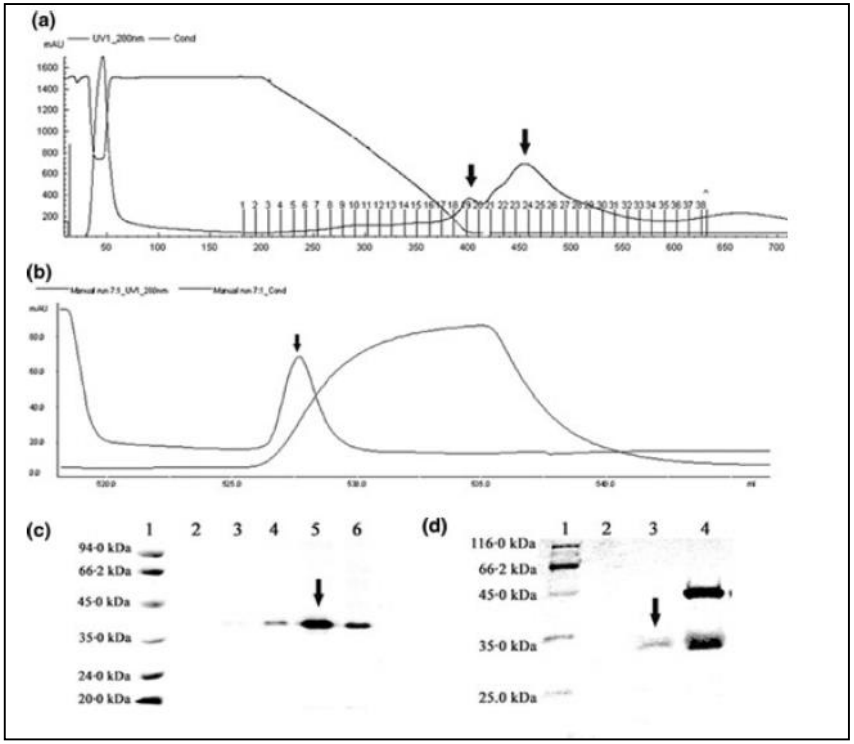
Proteases

a) Result of hydrophobic interaction chromatography.

b) Result of cation-exchange chromatography.

c) Twelve per cent SDS-PAGE assays indicated fractions 22-26 contained a single protein band with a molecular mass about 41 kDa.

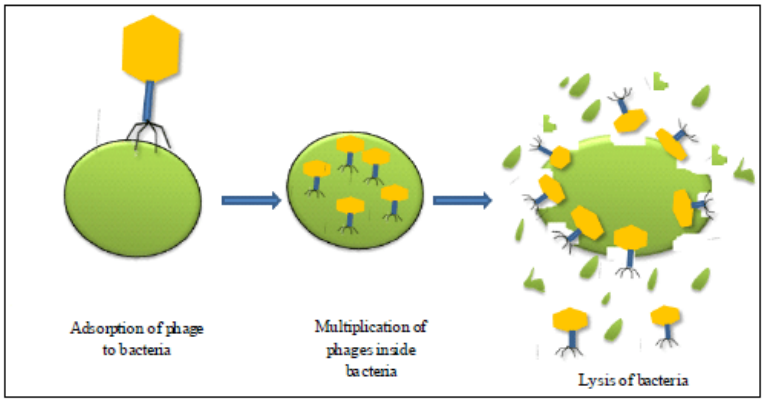
d) After purification by cation-exchange chromatography with tubes 14-20, the fraction containing protease activity only detected one peak.



Lian *et al.*,2007

Bacterial control of plant diseases

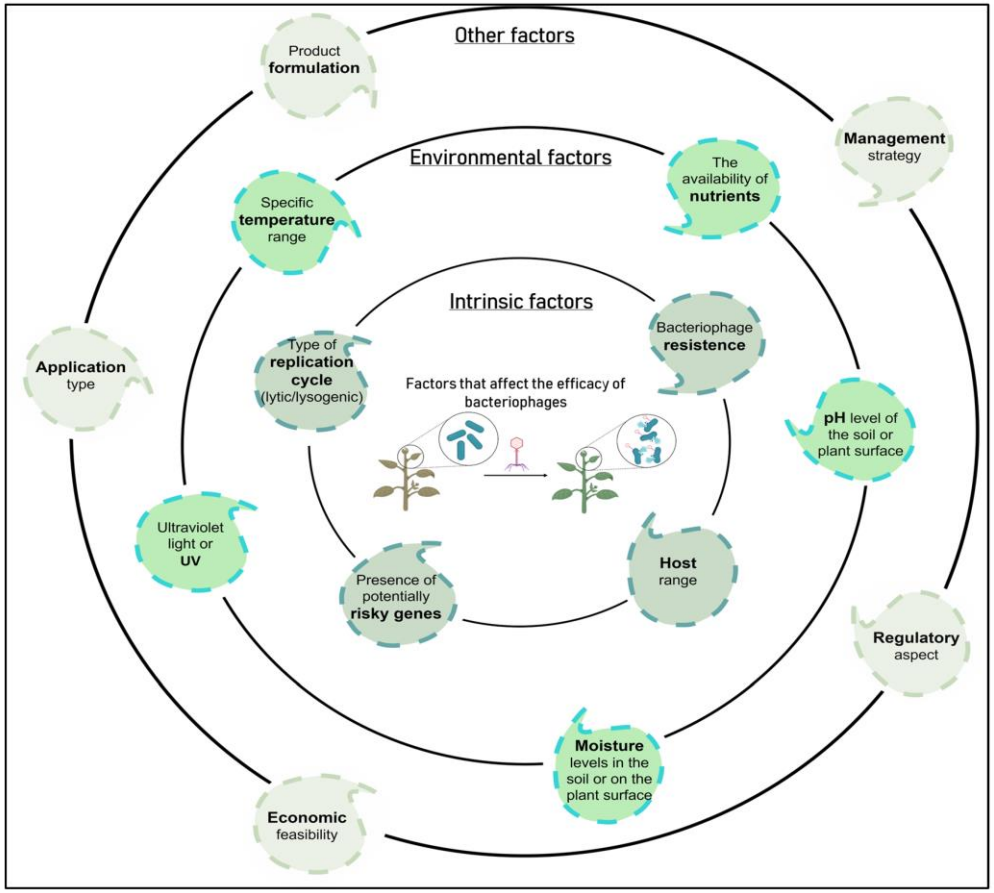
Phage-based biological control



Phage therapy

Levels of factors to consider for the development of a control of phytopathogenic bacteria with bacteriophages

Summarizes the principal considerations that should be taken when implementing a bacteriophage biocontrol program and the possible strategies to overcome these limitations.



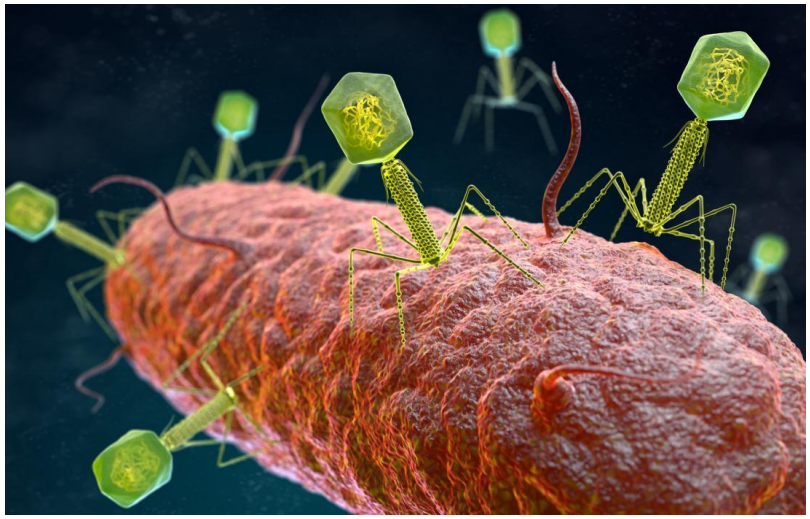
Córdova *et al.*,2023

A bacteriophage

- A bacteriophage is a type of virus that infects bacteria.
- In fact, the word "bacteriophage" literally means "bacteria eater," because bacteriophages destroy their host cells.
- All bacteriophages are composed of a nucleic acid molecule that is surrounded by a protein structure.

Phage therapy
A form of biological control

- Phage therapy is a form of biological control- the use of one organism to suppress another.
- Like other methods of biological control, one advantage of phage therapy is a reduction in the usage of chemical agents against pest species, which, in the case of phage, means a reduction in the usage of chemical antibiotics.



Bacteriophage therapy
The history

- As a century-old technique, bacteriophage (phage) therapy has a high efficiency in targeting and inactivating pathogenic bacteria in different environmental systems.
- Bacteriophages, the viruses that can infect and kill only target bacteria very specifically, have been demonstrated as potential agents, which may have no negative effects on environment and human health.

Ye *et al.*,2019;..

Phage therapy
The history

- Many bacteriophages have been isolated against diverse plant-pathogenic bacteria, and many studies have shown to efficiently manage the disease development in both controlled and open conditions such as greenhouse and field.
- Moreover, the specificity of bacteriophages to certain bacterial species has been applied to develop detection tools for the diagnosis of plant-pathogenic bacteria.

Vu and Oh,2020

Phage therapy
The history

- Bacteriophages or phages are bacterial viruses that were discovered by Twort in 1915 and by d'Herelle in 1917,who independently reported on filterable and transmissible agents of bacterial lysis and coined the term bacteriophage in 1917.
- Bacteriophages, in terms of association with plant pathogenic bacteria, were first discovered by Mallmann and Hemstreet,1942, who demonstrated the inhibited growth of *Xanthomonas campestris* pv. *campestris*, by treatment with filtered decomposed cabbage.

Nagy *et al.*,2012

Phage therapy
The history

- Subsequently, Kotila and Coons,1925, suggested that the isolated bacteriophages could prevent:

1. Soft rot on slices of potato tuber caused by *Pectobacterium atrosepticum*, and
2. Soft rot of carrot caused by *Pectobacterium carotovorum* subsp.*carotovorum*.
3. Bacteriophage effects under open conditions were first shown that bacteriophage-treated corn seeds displayed a reduced incidence of Stewart's wilt disease incited by *Pantoea stewartii* by 16.5% (Thomas,1935).

Vu and Oh,2020

Phage therapy
The history

- However, early scientists found that bacteriophage treatment in the field to control bacterial diseases in plants was less effective than newly discovered broad-spectrum antibiotics (Goto, 2012).
- Due to this reason, the interest in phage therapy was rapidly faded, despite their promising results for disease management.

Vu and Oh,2020

Phage therapy

Potential advantages for using phages in disease control

- The appearance of multi-resistant bacterial strains, as well as the lack of discovering new and effective antibiotics, has resulted in a renewed interest in phage therapy in the field of:

1. medicine, and
2. in modern agriculture.

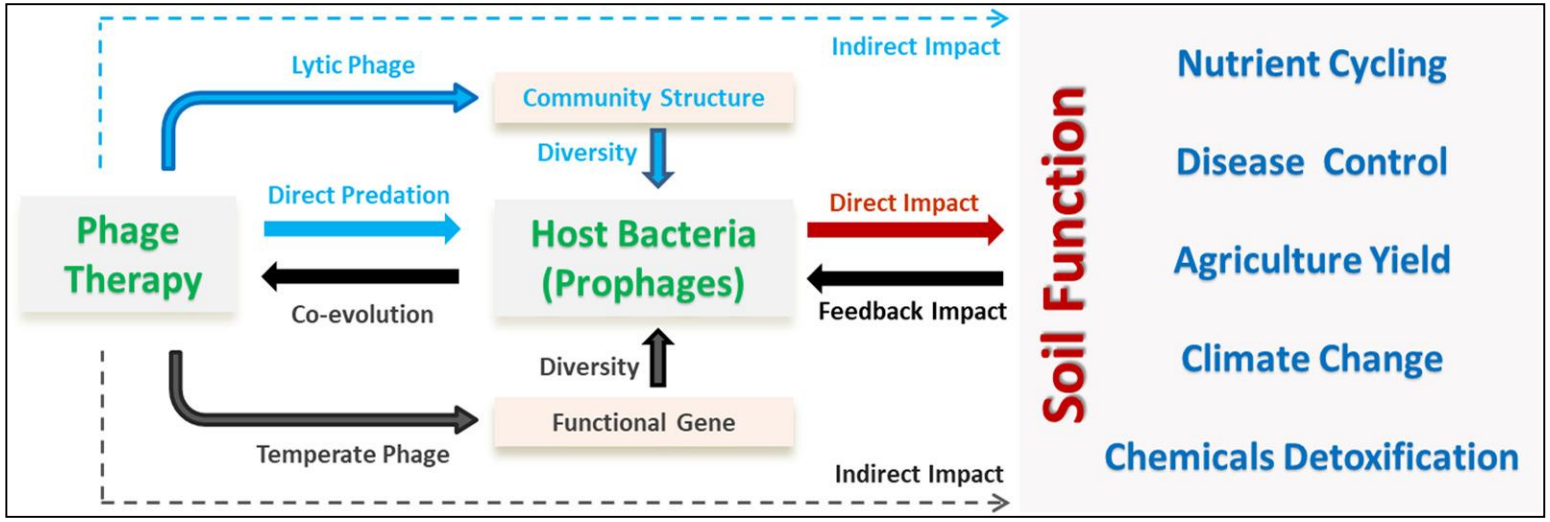
- Since phages are not inhibited by the majority of agrochemicals, they can be tank-mixed with many agrochemicals without significant loss in titer.

Jones *et al.*,2007;Nagy *et al.*,2012

Phage therapy

Impact of phage therapy on the soil function

- The impacts of phage therapy on the environmental functioning can be realized through direct and indirect pathways.
- Comparing with the temperate(lysogenic) phages, lytic phages affect environmental microbial diversity to a greater extent because of their lysis of the host bacteria.



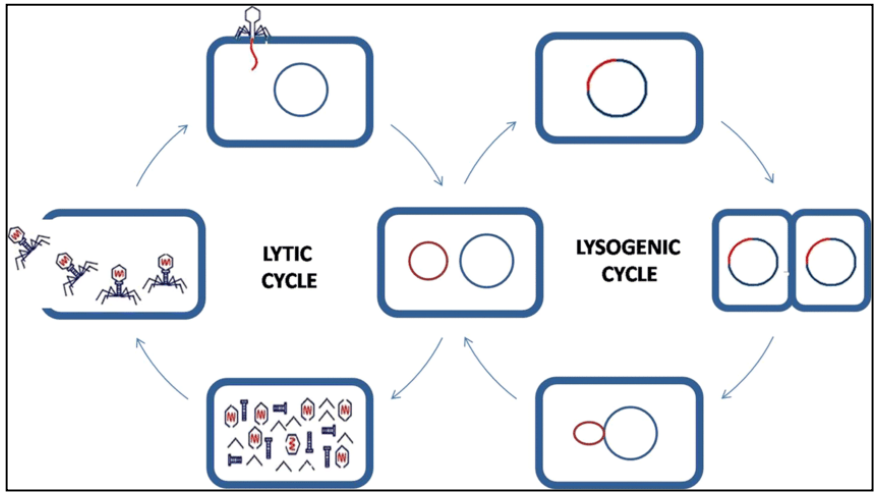
Ye *et al.*,2019

Bacteriophage types and replication cycles

Lytic and lysogenic infections and one intermediate

- Bacteriophages may have:

1. a lytic cycle, or
2. a lysogenic cycle, and
3. a few viruses are capable of carrying out both(lysis-lysogeny).



LibreTexts libraries

Bacteriophage types and replication cycles

Lytic and lysogenic infections and one intermediate

1. The lytic cycle kill the host cell. It involves the reproduction of viruses using a host cell to manufacture more viruses; the viruses then burst out of the cell.
2. The lysogenic cycle does not kill the host cell. It involves the incorporation of the viral genome into the host cell genome, infecting it from within. In the lysogenic cycle, the DNA is only replicated, not translated into proteins.

Bacteriophage types and replication cycles

Lytic and lysogenic infections and and one intermediate

- Bacterial cells can undergo one of two types of infections by viruses:

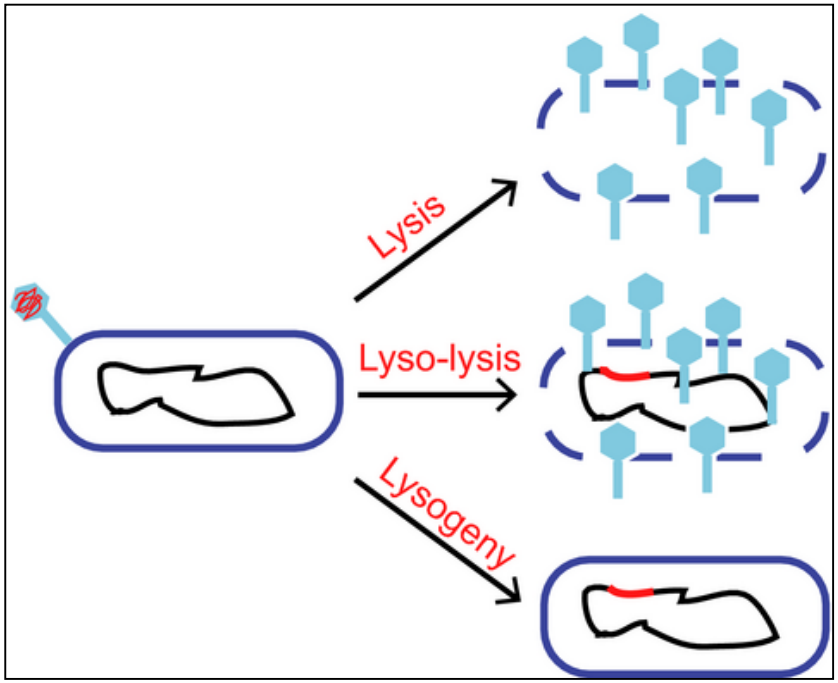
1. lytic (virulent) infections. e.g. T-even phages such as T2, T4 and T6 that kill the host cells.
2. lysogenic (temperate) infections. e.g. phage lambda (phage λ). Temperate phages establish a persistent infection of the cell without killing the host cells.

BC Yang;Todar,2012

Bacteriophage types and replication cycles

Two types of infections and one intermediate

- Historically, it is understood that infection by phage lambda culminates either in:
1. cell lysis and progeny release, or
2. Cell lysogenic and prophage integration and cell growth.
- Surprisingly, we frequently observed an interesting "lyso-lysis" phenomenon in lytic cells, where phage integrates its DNA into the host, a characteristic event of the lysogenic pathway, followed by cell lysis.



Lysis-lysogeny coexistence: prophage integration during lytic development in phage lambda.

Shao *et al.*,2016

Bacteriophage types and replication cycles

Transition from lysogenic to lytic

- If a bacterium containing prophage is exposed to stressors, such as UV light, low nutrient conditions, or chemicals like mitomycin C, prophage may spontaneously extract themselves from the host genome and enter the lytic cycle in a process called induction.
- This process, however, is not perfect and prophage may sometimes leave portions of their DNA behind or take portions of host DNA with them when they re-circularize.

Steward,2021

Bacteriophage types and replication cycles

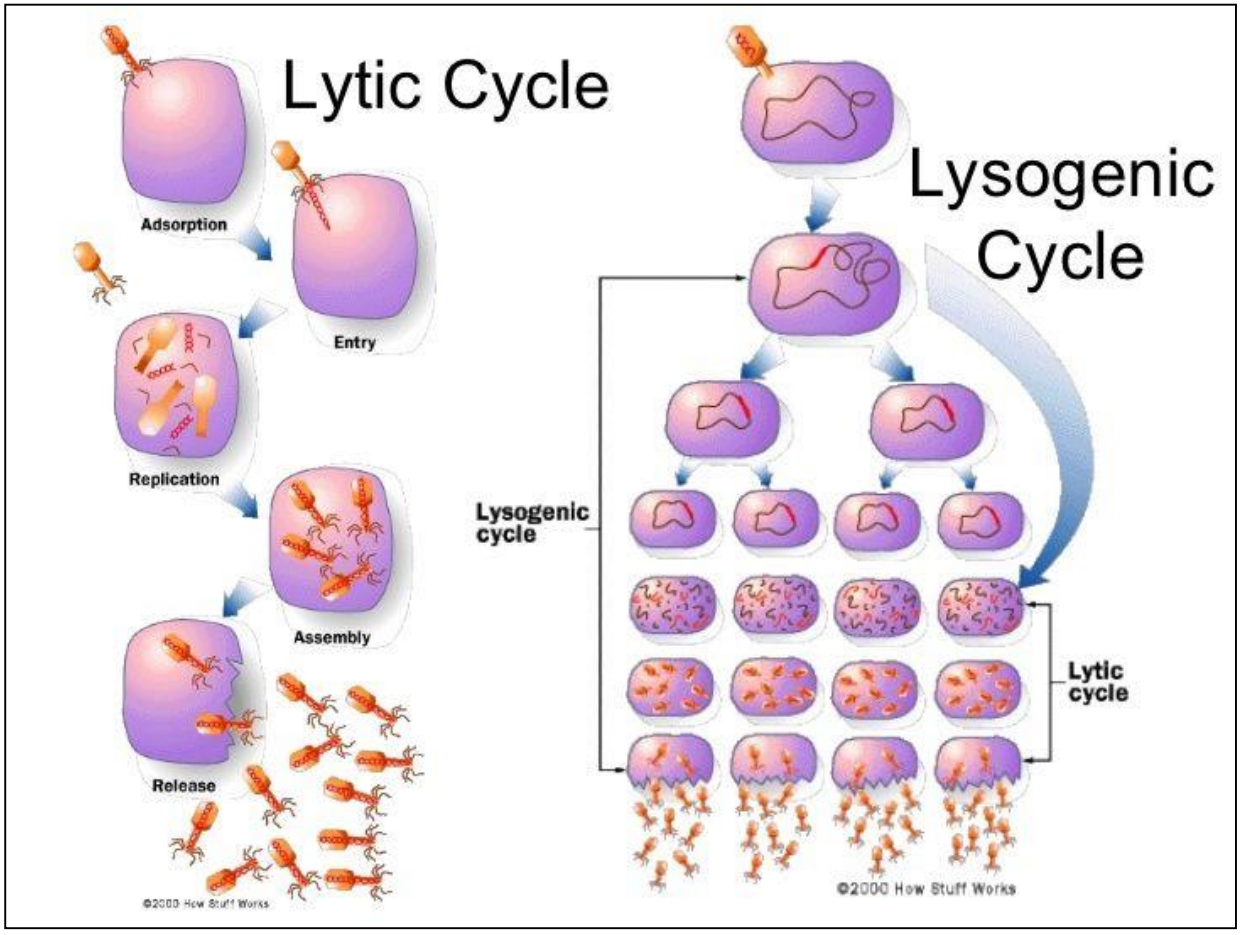
Transition from lysogenic to lytic

- If they then infect a new host cell, they may transport bacterial genes from one strain to another in a process called transduction.
- This is one method by which antibiotic resistance genes, toxin and superantigen-encoding genes and other virulence traits may spread through a bacterial population.
- Recent work has shown that transition between lytic and lysogenic infection is also dependent on the abundance of phage in an area as they are able to produce and sense small peptides in a process akin to quorum sensing.

Steward,2021

Bacteriophage types and replication cycles

Transition from lysogenic to lytic



Pinterest

Classification of Bacteriophages
Three order and 19 families

- Phages are presently classified in a hierarchical and holistic system(a combination of analysis, looking at the 'big picture') with Three order and 19 families.
- The cubic, filamentous and pleomorphic phages are small and well defined.
- They contain ds or ssDNA or RNA.
- The Podoviridae and Myoviridae families of tailed phages.

Holistic system is any set (group) of interdependent or temporally interacting parts.

Ackermann,2011;..

Classification of Bacteriophages

ICTV taxonomic classification of bacteriophage infecting bacteria and archaea

Order	Family	Morphology	Nucleic acid	Examples	Subfamilies	Genera
Caudovirales	Ackermannviridae		dsDNA		2	4
	Myoviridae	Nonenveloped, contractile tail	Linear dsDNA	T4 phage, Mu, PBSX, P1Puna-like, P2, I3, Bcep 1, Bcep 43, Bcep 78	6	41
	Siphoviridae	Nonenveloped, noncontractile tail (long)	Linear dsDNA	λ phage, T5 phage, phi, C2, L5, HK97, N15	11	100
	Podoviridae	Nonenveloped, noncontractile tail (short)	Linear dsDNA	T7 phage, T3 phage, Φ29, P22, P37	3	23
Ligamenvirales	Lipothrixviridae	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentous virus 1		3
	Rudiviridae	Nonenveloped, rod-shaped	Linear dsDNA	Sulfolobus islandicus rod-shaped virus 1		1
Unassigned	Ampullaviridae	Enveloped, bottle-shaped	Linear dsDNA			1
	Bicaudaviridae	Nonenveloped, lemon-shaped	Circular dsDNA			1
	Clavaviridae	Nonenveloped, rod-shaped	Circular dsDNA			1
	Corticoviridae	Nonenveloped, isometric	Circular dsDNA			1
	Cystoviridae	Enveloped, spherical	Segmented dsRNA			1
	Fuselloviridae	Nonenveloped, lemon-shaped	Circular dsDNA			2
	Globuloviridae	Enveloped, isometric	Linear dsDNA			1
	Guttaviridae	Nonenveloped, ovoid	Circular dsDNA			2
	Inoviridae	Nonenveloped, filamentous	Circular ssDNA	M13		7
	Leviviridae	Nonenveloped, isometric	Linear ssRNA	MS2, Qβ		2
	Microviridae	Nonenveloped, isometric	Circular ssDNA	ΦX174	2	6
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA			1
	Tectiviridae	Nonenveloped, isometric	Linear dsDNA			2

Phage are classified by the International Committee on Taxonomy of Viruses (ICTV), as of their 2017 update, there are 19 families of phage that infect bacteria and archaea but as more samples from more remote areas are sequenced this is only likely to grow in the future.

Steward,2021

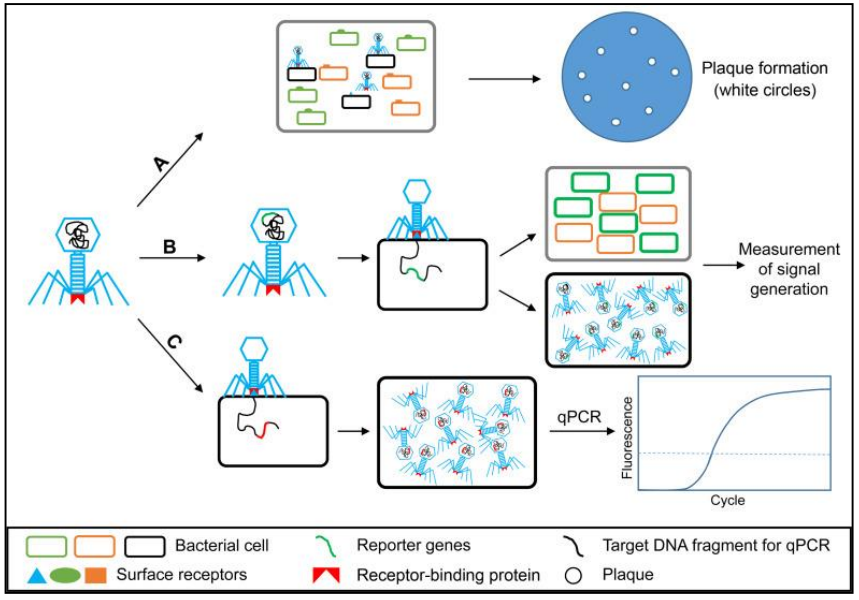
Phage therapy

The working models of bacteriophages to detect plant-pathogenic bacteria

A. Phage typing: traditionally employed a specific phage for identification and characterization of different pathogenic bacteria based on its lysis activity.

B. Reporter phages: engineered phages are used as an importer of marker gene that makes target bacterial cells detectable.

C. Phage progeny-based detection: using specific bacteriophage to generate rapid amplification of progenies before detection by quantitative PCR (qPCR).



Phage progeny release from phage-infected bacterial cells.

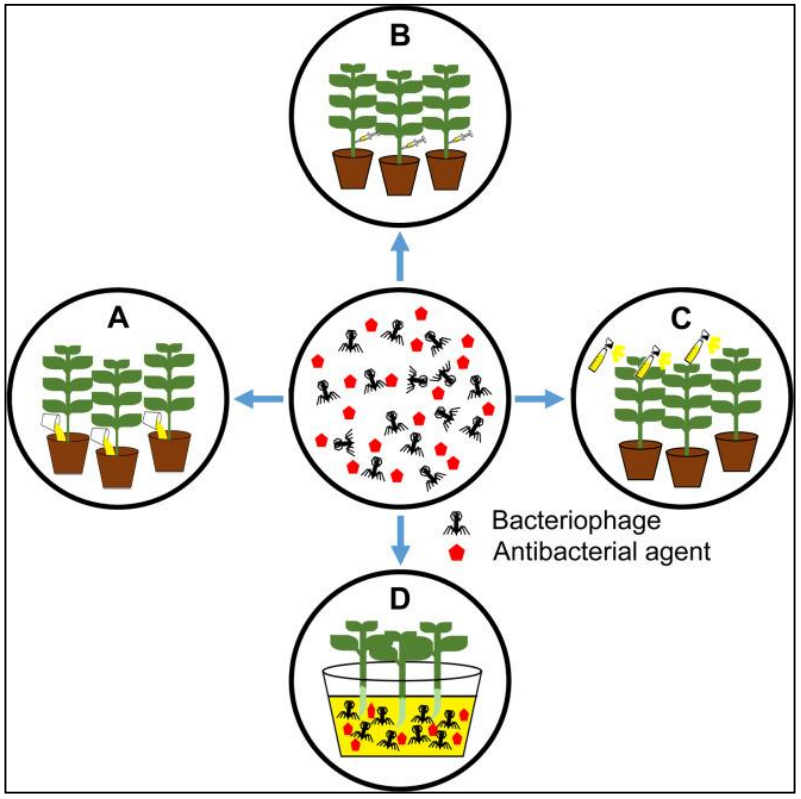
Vu and Oh,2020

Phage therapy

Treatment methods of bacteriophages in a greenhouse or field

A. soil drench: phage solution was directly added into the base of the plant;
B. direct infiltration into plant tissues;
C. foliar application by spraying;
D. immersion: the seedlings or seeds can be immersed into phage solution before planting.

- Red pentagons indicate antibacterial agents like antibiotics and systemic acquired resistance inducers.



It is supposed that phages can be used, alone or in combination with antibiotics, etc.

Vu and Oh,2020

Phage therapy

Application of bacteriophages with other antimicrobial agents

- The application of bacteriophages in combination with acibenzolar-S-methyl (ASM), a well-known plant activator(SAR inducers):

1. suppressed a visible hypersensitive response caused by ASM, and
2. provided excellent control efficacy of tomato-bacterial spot disease incited by *X. campestris* pv. *vesicatoria* in greenhouse experiments (Obradovic *et al.*,2005).

Acibenzolar-S-methyl (**ASM**) activates treated plants to produce pathogenesis-related proteins (PR-proteins) in intercellular spaces and causes systemic acquired resistance.

Vu and Oh,2020

Phage therapy

Application of bacteriophages with other antimicrobial agents

- The treatment with bacteriophage mixture together with acibenzolar-S-methyl (ASM), reduced disease severity by 50% compared to 31% of copper hydroxide-mancozeb treatment against bacterial leaf blight of onion caused by *X. axonopodis* pv. *allii* under field conditions(Lang *et al*.,2007).

Vu and Oh,2020

Phage therapy

Application of bacteriophages with other antimicrobial agents

- The integration of a lytic bacteriophage KΦ1 and copper hydroxide significantly reduced the lesion number on pepper leaves caused by *Xanthomonas euvesicatoria*, approximately 81%, 90%, and 88% for three separate trials in greenhouse condition.

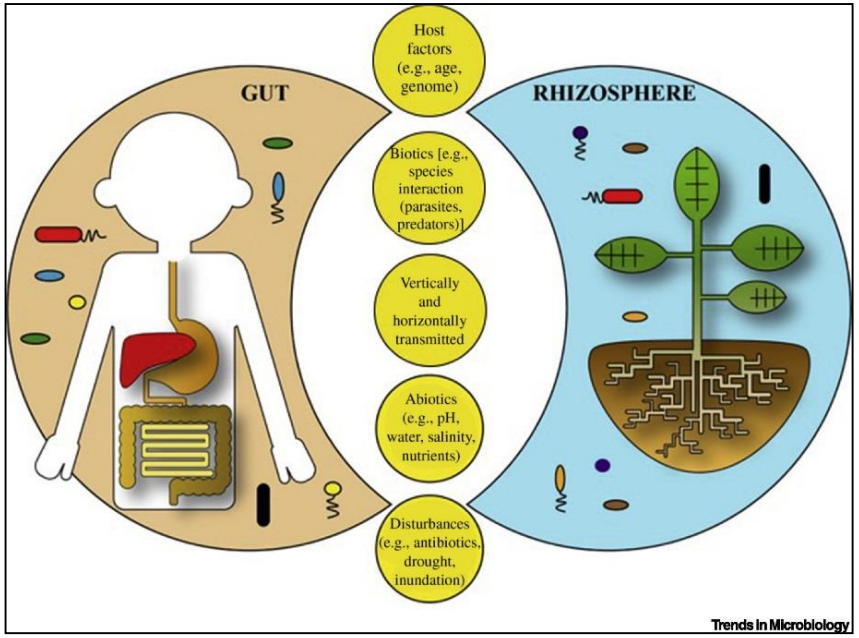
Φ=Phi is the 21st letter of the Greek alphabet.

Vu and Oh,2020

Phage therapy

The role of rhizosphere bacteriophages in plant health

- The rhizosphere harbors a complex microbiome, but little is known about the role of phages in this ecosystem.
- Bacteriophages play an important role in the ecology and evolution of the host-associated microbiome and are directly linked to host fitness.
- Importantly, such understanding might generate strategies to improve plant resistance and resilience in the context of climate change.



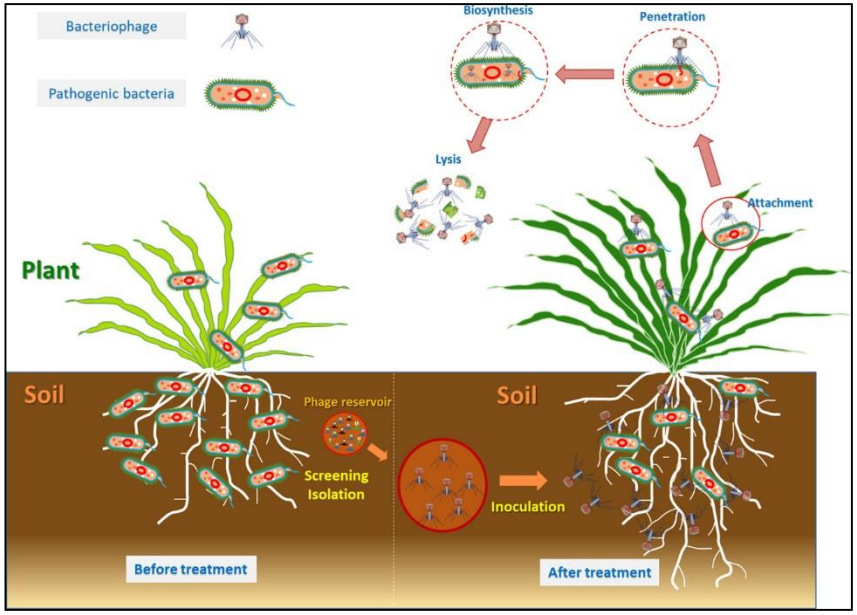
Resilience allows you to face a problem or challenge, overcome it, and get back to life a little bit stronger and a little bit wiser.

Pratama *et al.*,2020

Phage therapy

Bacteriophage therapy for the inactivation of pathogenic bacteria in the soil environment

- Applications of phage therapy in soil-plant and soil-groundwater systems.
- Bacteriophage (phage) therapy has a high efficiency in targeting and inactivating pathogenic bacteria in different environmental systems.

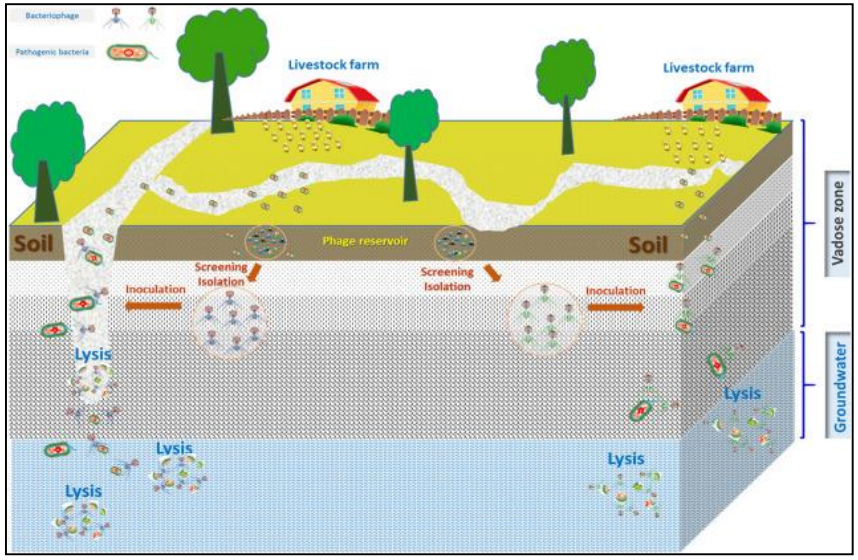


Ye *et al.*,2019

Phage therapy

Phage therapy to control pathogenic bacteria in soil-groundwater systems

- Applications of phage therapy in soil-plant and soil-groundwater systems.
- Bacteriophage (phage) therapy has a high efficiency in targeting and inactivating pathogenic bacteria in different environmental systems.



Ye *et al.*,2019

Phage therapy

Effective for control of several plant pathogenic bacteria

- Bacteriophages have been found to be effective for control of several phytobacteria such as:

1. *Pectobacterium* spp. cause bacterial soft rots,
2. *Erwinia amylovora*, fire blight on apple and pear,
3. *Xanthomonas* spp., which cause bacterial spot of tomato, peach, geranium, citrus, walnut blight, leaf blight of onion and citrus canker,
4. *Ralstonia solanacearum* which causes bacterial wilt of tobacco,
5. *Pseudomonas* spp., which causes bacterial blotch of mushrooms(*P. tolaasii*), and
6. *Streptomyces scabies* which causes potato scab.

Nagy *et al.*,2012;..

Phage Therapy

Application of bacteriophages in the greenhouse or field conditions, which have been conducted since 2010 to present

Target diseases and pathogens	Bacteriophages	Supplements	Hosts	Treatment methods	Test conditions	Control efficacy (reduction of disease incidence, %)	Reference
Bacterial wilt: Ralstonia solanacearum	Single (RsPod1EGY)	None	Tomato	Soil drench	Greenhouse	100	Elhalag et al. (2018)
	Cocktail (NJ-P3, NB-P21, NC-P34, NN-P42)	None	Tomato	Soil drench	Greenhouse	80	Wang et al. (2019)
					Field	80	Wang et al. (2019)
	Cocktail (M5, M8)	None	Banana	Soil treatment	Greenhouse	100	Ramírez et al. (2020)
Bacterial blight: Xanthomonas oryzae pv. oryzae	Cocktail (P4L, P43M, P23M1)	Skim milk	Rice	Spray	Field	50.8	Chae et al. (2014)
Black rot: X. campestris pv. campestris	Single (XcpSFC211)	None	Broccoli	Spray	Greenhouse	60	Nagai et al. (2017)
		Nonpathogenic Xanthomonas sp. strain	Broccoli	Spray	Field	16.7–55	Nagai et al. (2017)
Bacterial spot: X. axonopodis	Cocktail (X. phage -1, X. phage -2)	None	Pepper	Spray	Greenhouse	65	Tewfike and Desoky (2015)
		Skim milk or corn flour	Pepper	Spray	Field	53.3, 66	Tewfike and Desoky (2015)
Bacterial spot: X. euvesicatoria	Single (KΦ1)	None	Pepper	Spray	Greenhouse	50–67	Gašić et al. (2018)
Canker: X. citri subsp. citri	Cocktail	ASM	Citrus	Spray and soil drench	Greenhouse	56.9	Ibrahim et al. (2017)
		ASM	Citrus	Spray and soil drench	Field	42.6–56.9	Ibrahim et al. (2017)
		ASM + Skim milk	Citrus	Spray and soil drench	Field	81.2–86.1	Ibrahim et al. (2017)
Soft rot: Pectobacterium carotovorum subsp. carotovorum	Single (PP1)	None	Cabbage	Spray	Greenhouse	80	Lim et al. (2013)
	Cocktail (φMA11, φMA12, φMA13, φMA14)	None	Onion	Immersion and spray	Field	2.5–15	Zaczek-Moczydłowska et al. (2020)
Soft rot: Dickeya solani	Single (vB_DsoMLIMEstone1)	None	Potato	Spray	Field	5	Adriaenssens et al. (2012)

Vu and Oh,2020

Listing of some of the bacteriophages used as bio-control in controlling plant pathogens.

Disease	Pathogen	Host	Phage used in study
Soft rot	*Dickeya solani*	Potato	ØD1, ØD2, ØD3, ØD4, ØD5, ØD7, ØD9, ØD10, ØD11 (Czajkowski et al. 2014)
Common scab	*Streptomyces scabies*	Potato	ØAS1 (McKenna et al. 2001)
Bacterial wilt	*Ralstonia solanacearum*	Tomato	ØRLS1 (Fujiwara et al. 2011)
Bacterial wilt	*Ralstonia solanacearum*	Tomato	phage PE204 (Fujiwara et al. 2011)
Bacterial spot	*Xnathomonas campestriespv. vesicatoria*	Tomato	Formulated phage cocktails (Bae et al. 2012)
Pierce's disease	*Xylella fastidiosa*	Grapevines	Phage cocktail of Sano, Salvo, Prado and Paz (Das et al. 2015)
Soft rot	*Pectobacterium carotovorum ssp. carotovorum*	Lettuce	Phage PP1 Lim et al., 2013
Common scab	*Streptomyces scabies*	Radish	Phages Stsc1, Stsc3 (Goyer 2005)
Bacterial blight	*Pseudomonas syringae pv. porri*	Leek	phages vB_PsyM_KIL1, vB_PsyM_KIL2, vB_PsyM_KIL3, and vB_PsyM_KIL3b (Rombouts et al. 2016)
Fire blight	*Erwinia amylovora*	Pear	ØEa1337-26, ØEa 2345 (Boulé et al. 2011)
Bacterial spot	*Xanthomonas campestris pv. vesicatoria*	Tomato	Combination of phage and plant activator Obradovic et al., 2004
Soft rot	*Pectobacterium carotovorums sp. carotovorum, P. wasabiae*	Potato	ΦEC2, LIMEstone1, ΦD3, ΦD5, ΦPD10.3, ΦPD23.1, PP1, My1, PM1, PM2, ZF40 (Czajkowski 2015)
Asiatic citrus canker and citrus bacterial spot	*Xanthomonas axonopodis pathovars citri* and *citrumelo*	Citrus	CP2, ΦXac2005-1, ccΦ7, ccΦ13, ΦXacm2004-4, ΦXacm2004-16, ΦX44 Xacm 47, ΦXaacA1 (Balogh et al. 2008)
Asiatic citrus canker disease	*Xanthomonas axonopodis pv. citri*	Citrus	XacF1 (Ahmad et al. 2014)

Das and Jha,2020

Bacteriophage evaluation methods

- Bacteriophages were evaluated based on:

1. Plaque morphology,
2. Chloroform sensitivity,
3. Host range,
4. Genome size,
5. DNA restriction profile, and

- Virion morphology.

The **virion**, that is the complete infectious virus particle, includes a genome comprising one or a few molecules of either DNA or RNA, surrounded by a by a protective coat of protein called a capsid.

Jones *et al.*,2007

Isolation, characterization and genomic analysis of Bacteriophages

- Bacteriophages were evaluated based on:

1. Bacterial isolation
2. Phage isolation
3. Phage purification
4. *In vitro* lytic activity of the phage
5. Determination of the host range
6. Electron microscopy
7. Phage stability(thermal and pH stability tests)
8. DNA genome extraction
9. Proteomics analysis of the phage
10. Whole genome sequence

Yazdi *et al.*,2020

Phage associated host plants

Phages in association with different host plants

- Phage have been found in association with:

1. Buds, leaves, root nodules (leguminous plants), roots, rotting fruit, seeds, stems and straw; crown gall tumors…
2. Healthy or diseased alfalfa, barley, beans, broccoli, Brussels sprouts, buckwheat, clover, cotton, cucumber, lucerne, mulberry, oats peas, peach trees, radish, rutabaga, ryegrass, rye, timothy, tobacco, tomatoes, [and] wheat.

Bacteriophage evaluation methods

Isolation of bacteriophages against *E. amylovora*

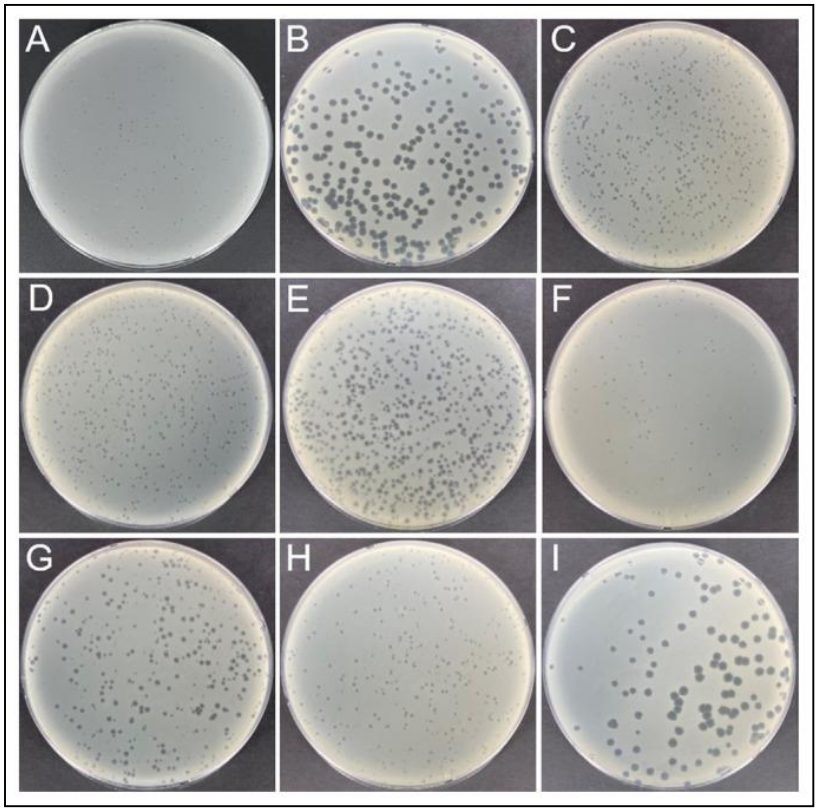
1. Removal of the bacteria by Millipore

- To obtain *E. amylovora*-specific phages in Korea, environmental samples were collected from apple and pear orchards in which fire blight was observed in 2020. Each sample (10 ml or 10 g) was mixed with 10 ml of tryptic soy broth (TSB) and 1 ml of *E. amylovora* strain YKB 14808 (approximately 10^9 colony-forming unit [cfu]/ml).
- The samples mixed with *E. amylovora* were incubated overnight(12 h) in a shaking incubator for enrichment of bacteriophages.
- All samples were centrifuged at 10,000 ×g for 10 min at 4°C and filtered using a 0.22 μm PVDF syringe filter (Millipore, Bedford, MA, USA) for removal of bacteria cells and other debris.

Park *et al.*,2022

Bacteriophage evaluation methods
Plaque morphology

- Plaque morphology of nine bacteriophages against *Erwinia amylovora* strain YKB 1480:
- (A) ΦFifi011, (B) ΦFifi044, (C) ΦFifi051, (D) ΦFifi067, (E) ΦFifi106, (F) ΦFifi287, (G) ΦFifi318, (H) ΦFifi450, (I) ΦFifi451.



Park *et al.*,2022

Bacteriophage evaluation methods
Plaque morphology

- List of soil and water samples for isolation of bacteriophages.

Location	Orchard	Host plant	Sample	Isolated phages
Anseong	A	Pear	Soil	ΦFifi011
	B	Pear	Water	ΦFifi044, ΦFifi450, ΦFifi451
	C	Pear	Soil	ΦFifi106
	D	Pear	Soil	ΦFifi287
Chungju	E	Apple	Soil	ΦFifi051
	F	Apple	Water	ΦFifi067
	G	Apple	Water	ΦFifi318

Park *et al.*,2022

Bacteriophage evaluation methods

Isolation of bacteriophages against *E. amylovora*

2. Removal of the bacteria by Millipore

- Aerial tissues and/or soil from beneath fire blight infected trees were mixed with sterile water.
- Samples were filtered through a 0.45 micron membrane filter and 5 ml of the phage filtrate used to inoculate 5 ml of *E. amylovora* broth cultures that were subsequently incubated at +28°C under agitation.
- After 18 h cultures were centrifuged at 4°C and 8,000 x g for 20 min.
- A mixture of 100 µL of the supernatant with 100 µL of *E. amylovora* hosts was added to 3 mL of soft agar (0.7%) and overlaid to hard agar (1.5%).
- Plates were incubated at +28°C.

Samoilova and Leclerque,2014

Bacteriophage evaluation methods

Isolation of bacteriophages against *E. amylovora*

Plaque formation and removal of the bacteria by chloroform and titration method(continued)

- Lawns were routinely checked for plaque formation after 24 h and 48 h.
- Single plaques were picked, transferred to 1 mL nutrient broth containing 10 µL chloroform and centrifuged at 8,000x g for 5 min.
- Resulting supernatants were transferred to sterile micro-centrifuge tubes and plated in triplicate using the soft agar overlay method.
- Phage preparations were diluted to obtain a titer corresponding to app. 50 phages per plate.

Samoilova and Leclerque,2014

Bacteriophage evaluation methods

Isolation of bacteriophages against *E. amylovora*

Plaque formation and removal of the bacteria by chloroform and titration method(continued)

- Both *E. amylovora* strain Ea179 and further strains isolated in the Republic of Moldova were used as bacterial enrichment hosts.
- Bacteria and phages were cultured on nutrient agar (Obolensk, Russia) with fish meal pancreatic digest at 24 g/l H_2O, NaCl 4.0 g/l, and agar 12.0 g/l, and incubated at 28°C.
- For liquid culture, a nutrient broth with enzymatic peptone digest at 100 g/l H_2O, NaCl 50.0 g/l, KNO_3 1.0 g/l, $NaHCO_3$ 10 g/l, and $Na_2S_2O_5$ 3.5 g/l was used.

Samoilova and Leclerque,2014

Bacteriophage evaluation methods

Isolation of bacteriophages against *E. amylovora*

3. Removal of the bacteria by chloroform

- At each collection site, cuttings were taken from the aerial portions of trees and soil samples were taken from the bases of trees by using a stainless-steel soil corer (diameter, 2 cm; length, 35 cm) driven to a depth of 10 to 20 cm approximately 1 m from the base of the tree.
- All soil and aerial samples were enriched in liquid cultures in a procedure modified from that of Crosse and Hingorani,1958.
- Flasks containing 60 ml of NBSYE were inoculated with 200 µl overnight cultures of each of the six *E. amylovora* propagation hosts.

Gill *et al.*,2003

Bacteriophage evaluation methods

Removal of the bacteria by chloroform(continued)

- Into each flask was placed 50 to 60 g (wet weight) of soil or 10 to 20 g (fresh weight) of aerial tissue and incubated for 18 to 20 h.
- The resulting slurry was agitated thoroughly with 500 µl of chloroform and centrifuged at 4°C and 8,000x *g* for 20 min.
- The supernatant was removed with a pipette and stored at 4°C over chloroform.
- The supernatant was diluted and plated onto six lawns each, seeded with one of the propagation hosts.

Gill *et al.*,2003

Bacteriophage evaluation methods

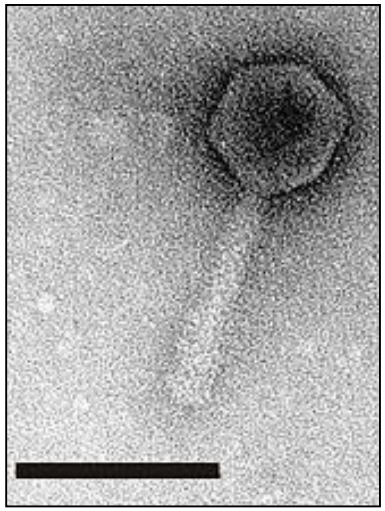
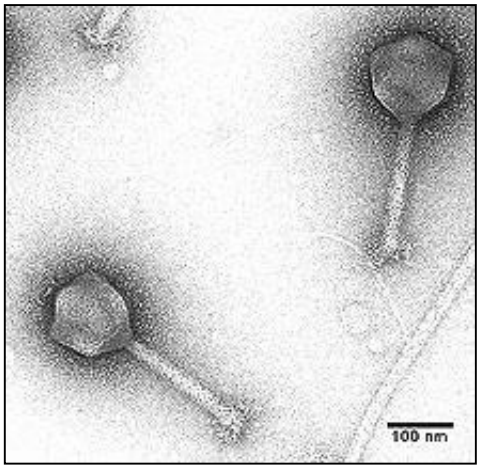
Removal of the bacteria by chloroform(continued)

- Lawns were checked for the formation of plaques after 24 and 48 h.
- Single plaques were picked from these lawns and placed into microcentrifuge tubes containing 1 ml of NBSYE and 2% (vol/vol) chloroform.
- Tubes were centrifuged at 8,000x *g* and stored at 4°C.
- Bacteriophage isolates were purified by passage through this single-plaque isolation procedure three times.
- Bacteriophage PEa1(h) (ATCC 29780-B1) was obtained from the American Type Culture Collection.

Gill *et al.*,2003

Bacteriophage evaluation methods

Plaque morphology of *E. coli*, *Erwinia* and *E. amylovora*

		
LG1 plaques on a wide range of *E. coli*, and a few Enterobacteriaceae.	Phage plaques on a *Erwinia* lawn.	*Erwinia amylovora* phage isolate PEa 31-2.

Gill,2003

Phages of *E. amylovora*
Plaque morphology

- *Erwinia* bacteriophages belonging to the *Myoviridae*.

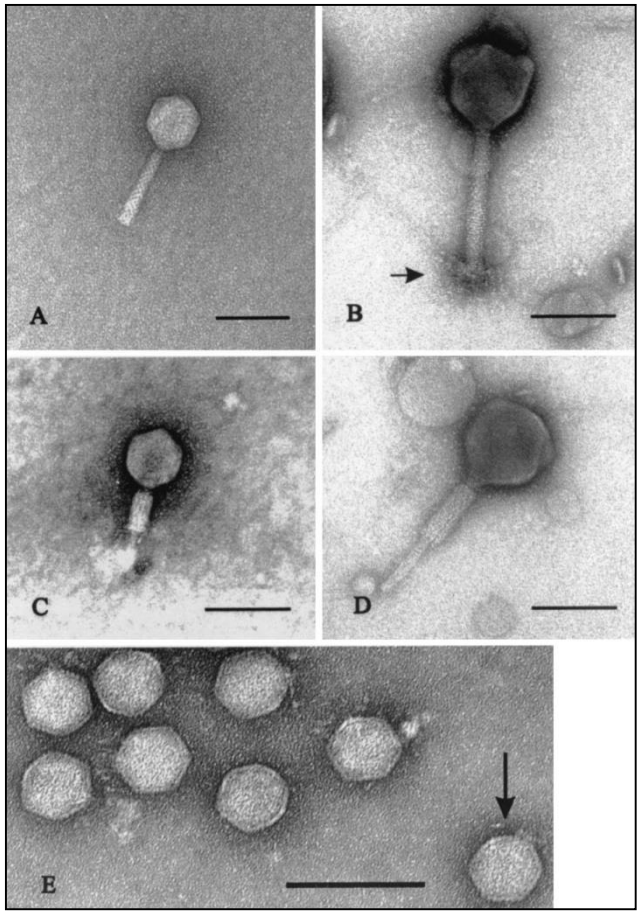
A. Small phage in uncontracted state.

B. Larger phage in uncontracted state. Note the decorations at the bottom of the tail [arrow]).

C. Small phage in the contracted state.

D. Larger phage in the contracted state are shown.

- Panel E shows group 3A phages belonging to the *Podoviridae*.
- The arrow points to the tail region.
- Micron marker, 100 nm.



Gill *et al.*,2003

Bacteriophage evaluation methods

Bacteriophage isolation infecting *X. oryzae*

Soft agar plaque method

- The filtrate(leaf, water or soil) was inoculated in triplicates by agar overlay technique on PSA double-layer plates containing solid (1.5% agar) and semi-solid (0.7% agar) PS medium supplemented with the bacterial host (*Xanthomonas oryzae*) and incubated at 28°C for 5 days until the formation of plaques.
- Plates having plaque formation (clear lysis, appreciable by naked eyes, on Xoo culture lawn) were preserved, and the plaques were confirmed for the presence of phage by secondary streaking.

Jain *et al.*,2023

Bacteriophage evaluation methods

Host range of most phages

- The host range of most phages, i.e., the species that they are capable of productively infecting, consequently is relatively narrow.
- Typically limited to only a single bacterial genus, species, or
- Often, even to only a limited number of strains within a given species.

Bacteriophage evaluation methods

Host range determination of *E. amylovora* phages

Soft agar plaque method

- Host range of all eight bacteriophages for *E. amylovora* was determined using the soft agar plaque method (Hockett and Baltrus,2017).
- For this, 50 µL of bacteriophage lysate dilutions were incubated with 500 µL of bacteria grown overnight for 30 min before plating in top agar. The plates were incubated with the top agar facing up at 25°C overnight for this assay.
- 17 bacterial strains including *E. amylovora* ATCC 29780 as control were used including five other *E. amylovora* strains, *Pantoea agglomerans* E325, *Pantoea vagans* C91, *E. coli* K-12 BW 25113, *Salmonella enterica* LT2, *Yersinia pestis* KIM6, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *Bacillus subtilis* ATCC 6033, *Cronobacter sakazakii* ATCC 29544, standard clinical isolate *Pseudomonas aeruginosa* PA100 and *Pseudomonas chlororaphis* ATCC 13985.
- An average of two readings was taken to obtain bacteriophage titers post-infection.

Sharma *et al.*,2019

Bacteriophage evaluation methods

Host range determination of *E. amylovora* phages

Soft agar plaque method

- The host ranges of all phage isolates were tested against 13 *E. amylovora* strains.
- Host ranges of a limited number of phages were also tested against five bacterial strains representing four species other than *E. amylovora*.
- Bacterial lawns were prepared by seeding 3 ml of top agar with 10^7 CFU bacteria suspended in 10 mM sodium phosphate buffer (pH 6.8).
- Phage lysates were diluted to a concentration of 10^7 PFU/ml, and 10 µl was spotted onto lawns.
- Plates were dried in a laminar flow hood for 10 min and incubated at 26°C for 18 to 20 h.

1. Areas of clearing under points of phage application were scored as positive, while
2. Areas which looked no different than the surrounding untreated lawn were scored as negative.

- Experiments were repeated three times.

Gill *et al.*,2003

Bacteriophage evaluation methods

Host range determination of *E. amylovora* phages

Soft agar plaque method

- The host ranges of all phage isolates were tested against 13 *E. amylovora* strains.
- Host ranges of a limited number of phages were also tested against five bacterial strains representing four species other than *E. amylovora*.
- Bacterial lawns were prepared by seeding 3 ml of top agar with 10^7 CFU bacteria suspended in 10 mM sodium phosphate buffer (pH 6.8).
- Phage lysates were diluted to a concentration of 10^7 PFU/ml, and 10 µl was spotted onto lawns.
- Plates were dried in a laminar flow hood for 10 min and incubated at 26°C for 18 to 20 h.

1. Areas of clearing under points of phage application were scored as positive, while
2. Areas which looked no different than the surrounding untreated lawn were scored as negative.

- Experiments were repeated three times.

Gill *et al.*,2003

Bacteriophage evaluation methods

Host range determination of *Pectobacterium* phages

- Host range pattern of phages on *P. carotovorum* subsp.*carotovorum* and selected bacterial species.

	Group 1			Group 2	
Bacterial isolates	ΦEcc1	ΦEcc3	ΦEcc5	ΦEcc13	ΦEcc14
Erwinia carotovora subsp. *carotovora*					
Ecc1[a]	(+)	(+)	(+)	+	+
Ecc26	–	–	–	–	–
Ecc48	(+)	(+)	(+)	+	+
Ecc71	–	–	–	–	–
Ecc83	–	–	–	–	–
Escherichia coli DH5α	–	–	–	–	–
Erwinia chrysanthemi EchS80	–	–	–	–	–
Erwinia amylovora	–	–	–	–	–
Pantoea agglomerans	–	–	–	–	–

Note: (+), weak plaque formation; +, plaque formation; –, no plaque formation.
[a]Bacterial isolate on which the phages were initially isolated and propagated.

Ravensdale *et al.*,2007

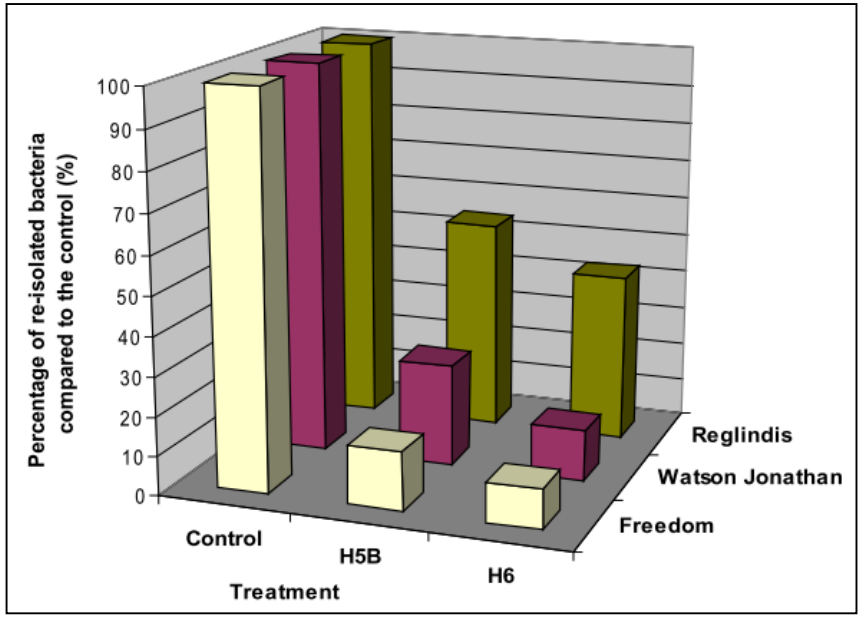
Bacteriophage evaluation methods
DNA restriction profile using the primers specific for bacteriophage PEa1

- Molecular characterization of the *E. amylovora* phages with a combination of PCR and restriction endonuclease digestions (RFLP) showed that six distinct phage types, described as groups 1 to 6, were recovered.
- Using the primers specific for bacteriophage PEa1, phages PEa10-7, PEa10-8, PEa10-9, PEa10-10, PEa10-11, PEa10-13, PEa10-14, PEa10-15, PEa31-3, and PEa46-2 produced a ca. 300-bp PCR product, indicating relatedness to phage PEa1.
- Primer sequences were 5' AATGGGCACCGTAAGCAGT 3' for PEa1-A and 5' TAATGGGTATGATA GAAGGCAGAC 3' for PEa1-B.

Gill *et al.*,2003

Field phage therapy
Fire blight disease
Erwinia amylovora

- Effect of phages on *Erwinia amylovora* infection on flowers of different apple cultivars.
- Hungarian phage isolates (H5B, H6) applied in spray inoculation on flowers (1010 PFU/ml), significantly reduced the number of re-isolated bacteria on all three apple cultivars tested, compared to the untreated control.
- However, a significant difference was not detectable between the effects of the two phages.



Nagy *et al.*,2012

Field phage therapy
Walnut bacterial blight disease
Phage therapy of *Xanthomonas juglandis*

- Alternative control chemicals are few, and even more toxic, while no effective plant resistance has been identified.
- A reliable and manageable biological control is needed.
- Bacteriophages provide highly specific control opportunities for bacterial diseases by specifically infecting and destroying the disease-causing bacteria.
- With no proven alternatives for walnut blight control, investigating the potential for bacteriophage based biocontrol is warranted.

Phage type	Bacterial strain		
	134[2] Lincoln	143 Lincoln	6494 Auckland
$Bp60C_1$	+++	+	+
$Bp60C_2$	+++	+	+++
$Bp60C_3$	+++	+	+++
Bp_{10}	+++	+	-
Bp_{20}	+++	-	-
Bp_{22}	+++	+	-

[1]+++ Reactive (total lysis); - Not reactive; + Reactive (lysogenic).
[2]=Strain used for initial isolation.

Mcneil *et al.*,2001

Field phage therapy

Bacterial canker disease of sweet cherry

Phage therapy of *P. syringae* pv. *syringae*

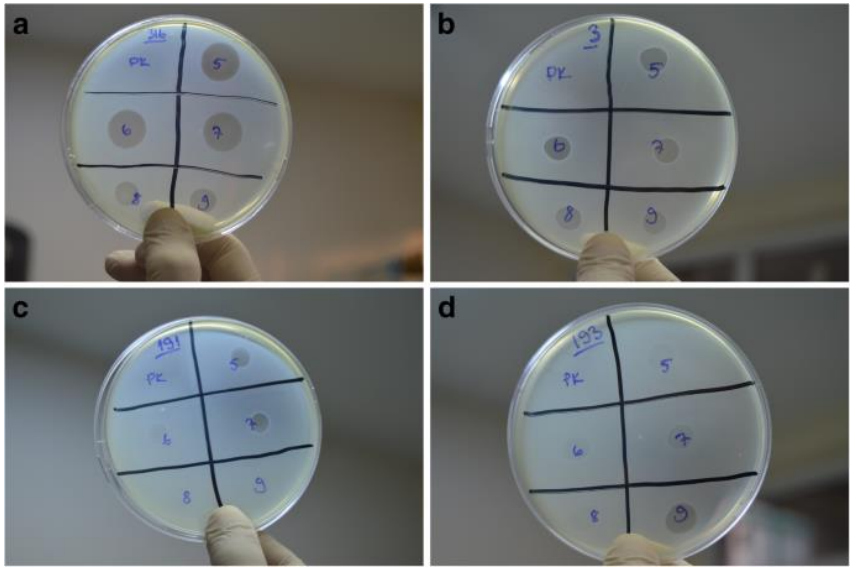
- Lytic effects of bacteriophages Φ1215; Φ1226; Φ137; Φ358 and Φ369 against *Pseudomonas syringae* pathovars.

a. *Pss* strain BY5L316 (the most virulent pathogenic isolate).

b. *Pss* strain BAY3.

c. *Psm* R1 strain 25B.

d. *Psm* R2 strain 732.



Akbaba and Ozaktan,2021

Field phage therapy

Bacterial canker disease of sweet cherry

Phage therapy of *P. syringae* pv. *syringae*

- List of bacteriophages isolated from soil.
- 36 different soil samples were collected and nine different pure phage isolates (Φ911, Φ922, Φ943, Φ954, Φ1215, Φ1226, Φ137,Φ358, and Φ369) were isolated from four locations.

No.	Isolate code	Sample location	pfu/ml (– 80 stock)	Plaque sizes (~mm)	Plaque forms on solid media
1	Φ911	Ören-6	9×10^7	1.15	Smallest and turbid
2	Φ922	Ören-6	2×10^6	0.98	Smallest and clear
3	Φ943	Ören-6	5×10^8	2.62	Medium and turbid
4	Φ954	Ören-6	2×10^6	2.48	Medium and turbid
5	Φ1215	Bagyurdu-7	3×10^8	4.37	Largest and clear
6	Φ1226	Bagyurdu-7	5×10^8	4.50	Largest and clear
7	Φ137	Ören-5	6×10^8	4.22	Largest and clear
8	Φ358	Y. Çobanisa-2	2×10^9	1.00	Smallest and clear
9	Φ369	A. Çobanisa-3	8×10^9	0.95	Smallest and turbid

Plaques of different diameter (Φ) are shown: Φ < 2 mm (smallest), Φ = 2–4 mm (medium), and 4 mm < Φ (largest)

Akbaba and Ozaktan,2021

Field phage therapy

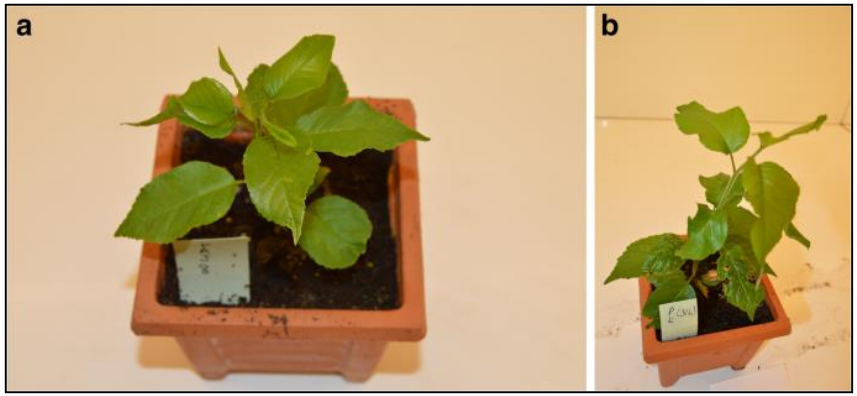
Bacterial canker disease of sweet cherry

Phage therapy of *P. syringae* pv. *syringae*

- Average results of two separate trials demonstrated that at 10 days post-inoculation.

a. 4 out of 6 phage treatments (Φ1226, Φ137, Φ358, Φ369) reduced successfully more than 50% of the disease incidence caused by *Pss* strain BY5L316

b. compared to the control (48.1%).



Φ=Phi is the 21st letter of the Greek alphabet.

Akbaba and Ozaktan,2021

Phage therapy

Commercially available phage-based biopesticides

- In the past twenty years numerous successful experiments have been reported on bacteriophage-based biocontrol measures, and several comprehensive studies have recently been published discussing detailed results of phage application practices in pest management, mainly from North American authors.

1. AgriPhages for bacterial spot or speck of tomatoes and peppers and fire blight of apple and pear trees,
2. Erwiphage for fire blight of apple trees,
3. Biolyses for soft rot disease of potato tubers.

Nagy *et al.*,2012; Vu and Oh,2020

Phage therapy

Future perspectives of bacteriophage usage in plants

- Multiple investigations of the potential application of bacteriophages in the case of bacterial disease control have been studied and observed many promising results.
- However, almost successful applications of bacteriophages were performed in controlled conditions like greenhouses, while agricultural production mainly occurs in an open environment where the environmental factors are constantly changeable and uncontrolled.
- Therefore, more field trials have to perform to fully implement its efficacy in open conditions.

Vu and Oh,2020

Phage therapy

Future perspectives of bacteriophage usage in plants

- Developing standard criteria for selecting bacteriophages is also needed more attention for phage therapy.
- Only lytic bacteriophages have been utilized for plant disease management nowadays, but there is still a big question mark over the potential and risk of temperate(lysogenic) bacteriophages.
- Although the natural-temperate bacteriophages were not ideal as biological agents for plant disease control because of their replication cycle, they can be modified to become virulent or work as a delivery vehicle for genetic elements for restoration of antimicrobial susceptibility or virulence-factor disruption.

Vu and Oh,2020

Phage therapy

Future perspectives of bacteriophage usage in plants

- Although agrochemicals such as antibiotics and copper are still mainly used for control of bacterial plant diseases in field, there is a significant potential of bacteriophage usage:

1. To reduce the amount of agrochemicals, or
2. To replace those agrochemicals for the control of bacterial diseases in plants.

- For this,

1. more bacteriophages should be collected for diverse bacterial pathogens, and
2. more field trials instead of trials in the controlled conditions are necessary.

Vu and Oh,2020

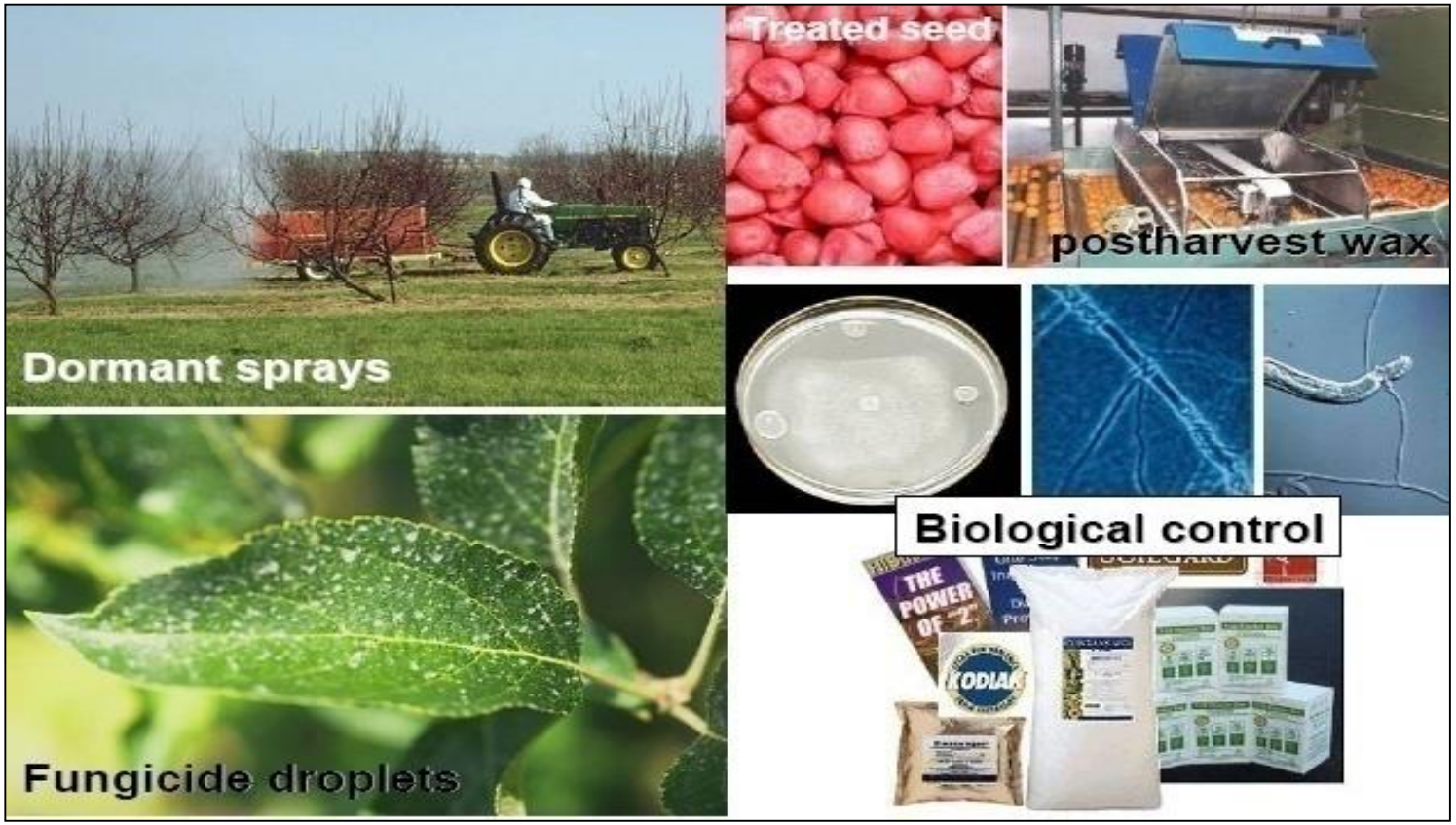
Biopesticides

Production, Formulation and Registration

Safe and effective pest control products

Commercial Products

Bio-products



Biopesticides
Biological Fungicides

- What are Biological fungicides?
- According to USDA, …… they are beneficial Fungi and Bacteria which attack and control plant pathogens and the disease which they cause disease.

Microbial agents

- Bacteria are the microorganisms that are known to produce the most diverse range of antimicrobial compounds.

Biopesticides

Biopesticides Market

- Biological control is an alternative to the application of pesticides in some circumstances and there are about 80 products on the market.
- This is widely seen as a 'green' and more environmentally-friendly method for controlling plant pests and diseases.

Strange,2003

Biopesticides

Bio Pesticides Market Segmentation, Forecasts and Trends

- "Biopesticide Market was defined by:

1. Type (Bioinsecticides, Biofungicides, Bioherbicides, and Bionematicides),
2. Origin (Beneficial Insects, Microbials, Plant-incorporated Protectants, and Biochemicals),
3. Mode of Application,
4. Formulation, &
5. Crop Type - Global Forecast to 2022".

Markets and Markets,2017;..

Biopesticides

Biopesticides are attracting global attention as safer strategy to manage pest and diseases

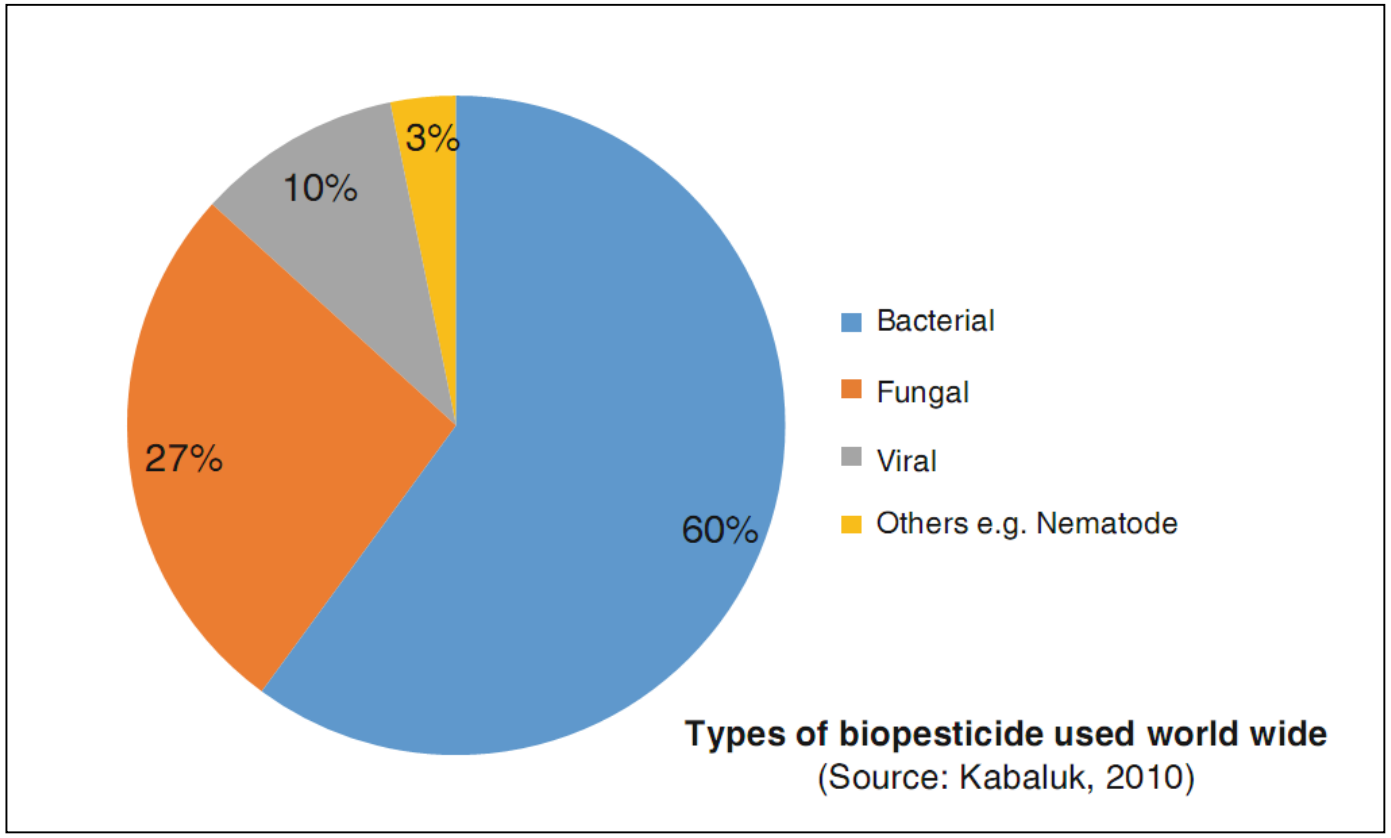
1. Increase in crop loss due to insects and diseases,
2. development of pest-resistance to various chemicals,
3. changing farming practices (from traditional to smart farming), have been of importance to the growth of the global biopesticides market.

- Biopesticides are used for high-quality crop yield for the increasing population and growing organic food demand.
- Hence, biopesticides will be viewed as a business opportunity in the next six years.
- As a result, many multinational players have entered into the production of various types of biopesticides, which can be used for diversified crop types.

Markets and Markets,2017

Biopesticides

Global biopesticide market based on types of microbes used

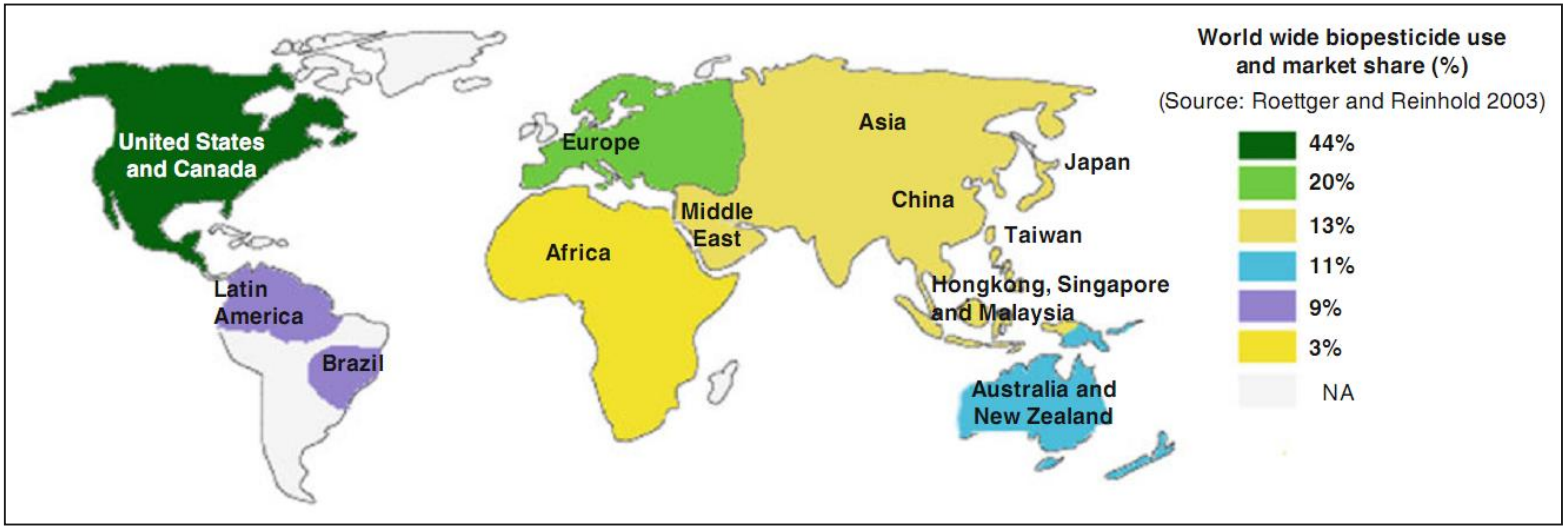


Types of biopesticide used world wide
(Source: Kabaluk, 2010)

Mishra *et al.*,2015

Biopesticides

Global biopesticide use and market



Mishra *et al.*,2015

Biopesticides

Biopesticides Market worth 8.82 Billion USD by 2022

- The global biopesticides market is estimated to be valued at USD 3.36 Billion in 2016 and projected to reach USD 8.82 Billion by 2022, at a CAGR(compound annual growth rate) of 17.4% from 2016.
- The value of the global biopesticide market is expected to reach $4,556.37 Million by 2019, at a compound annual growth rate of 15.30% from 2014 to 2019 (source: Marketsandmarkets.com, 2014; last access 31/03/2015).

Pertot *et al.*,2016

Biopesticides

Worldwide biopesticide sales

Biopesticide	Estimated sales figures (in $US million) North America	Europe	Asia and Australia	Latin America	Africa and Middle East	Total
Total Bt (products based on *B. thuringiensis* serotypes)	72.0	27.57	74.75	30.19	6.28	201.79
Other bacteria	23.94	6.30	14.05	4.56	0.40	49.25
Viruses	5.57	7.47	23.90	3.80	0.48	41.22
Fungi	15.85	5.64	18.85	35.96	0.78	77.08
Nematodes and other	9.4	7.50	0.95	0.16	0.13	18.14
Total	126.76	54.48	132.5	74.67	8.07	396.48

Source: CPL Business Consultants (2010)

Mishra *et al.*,2015

Biopesticides

The fruits & vegetables segment projected to be the fastest-growing market during the forecast period

- The major types of crops on which biopesticides are used are grains & oilseeds, fruits & vegetables, and others, which include turf, plantation, sugar crops, cotton, and ornamental crops.
- The fruits & vegetables segment is projected to be the fastest-growing market from 2016 to 2022.
- Growth in demand for fruits & vegetables, owing to the increasing awareness with regard to their nutritional benefits and the rise in need for enhancement of crop productivity is driving the market for biopesticides in the fruits &vegetables segment.

Markets and Markets,2017

Biopesticides

Increase in need for organic food products, high investment in R&D, and change in farming practices key to success in the North American region

- North America is one of the largest contributors to the global biopesticides due to the rise in use of biopesticides through advanced agricultural techniques and increase in need for organic food security in the North American countries.
- High market penetration by the leading biopesticide companies and growth in need for nutrients for enhancing the agricultural growth and productivity, as well as the change in climatic conditions are the main factors influencing the growth of the biopesticides market in North America.

Markets and Markets,2017

Biopesticides
Leading biopesticide companies

- BASF SE (Germany),
- Bayer CropScience AG (Germany),
- Marrone Bio Innovations Inc. (U.S.),
- Certis USA LLC (U.S.),
- Koppert Biological Systems (Netherlands), and The Dow Chemical Company (U.S.),
- Monsanto Company (U.S.),
- Isagro SPA (Italy),
- Camson Bio Technologies Limited (India),
- Bioworks, Inc. (U.S.),
- W. Neudorff GmbH Kg (Germany), and
- Valent Biosciences Corporation (U.S.).

Markets and Markets,2017

Biopesticides

Advantages of biopesticides

- Reduced synthetic pesticide use and residues
- Greater public acceptance
- Production is relatively inexpensive
- A renewable resource
- High specific activity
- Usually target specific
- Biodegradable

Biopesticides

Disadvantages of biopesticides

- Tend to be more difficult to implement;
- Generally have a narrow target range;
- May not work as quickly as chemicals;
- Do not eradicate pathogen or rescue the host plant;
- May have a shorter shelf-life;
- Generally more expensive;
- May not be compatible with chemical fungicides or bactericides.

Biopesticides

Main problems with biopesticides

- It is worth to mention some biocides are not treated but rather the potential of an alternative, namely biological control.
- It should be recognized that these too may be hazardous.
- For example, HCN, a product of *Pseudomonas fluorescens*, is hardly a benign compound!
- Nevertheless, plants do support a considerable microflora around both their aerial and subterranean organs and it would therefore seem to make sense to exploit this in plant protection.

Biopesticides

Main problems with biopesticides

- Biosafety and environmental concerns are also major limiting factors for microbial pesticide prospects.
- **Main problems with biopesticides are:**

1. Displacement of beneficials including micorhizae or symbiotic rizobacteria.
2. Allergenicity
3. Toxinogenicity: Production of secondary metabolites (bioactive compounds) toxic to plants, animals, or humans).
4. Pathogenicity (to plants or animals) by the agent itself or due to contaminants.

- Problems may also derive from horizontal gene transfer of these characteristics to non-target microorganisms.

Kinds of biopesticides

- Microbials
- Nonviable
- Biochemicals (naturally occuring or synthetic analog compounds with unique mode of action)
- Genetically altered microbials
- Transgenic plants

Supporting data for biopesticides

- Phytotoxicity
- Efficacy
- Yield

Patenting, registration and commercialization of microbial pesticides

- Before the commercial exploitation of a microbial pesticide, its legal protection as a biotechnological invention can be assured by means of a patent.
- A patent is a temporary privilege for industrial or commercial exploitation given by the administration to the owner for 20 years after the application date.
- A patent is not an authorization for commercial use (phytosanitary registration).

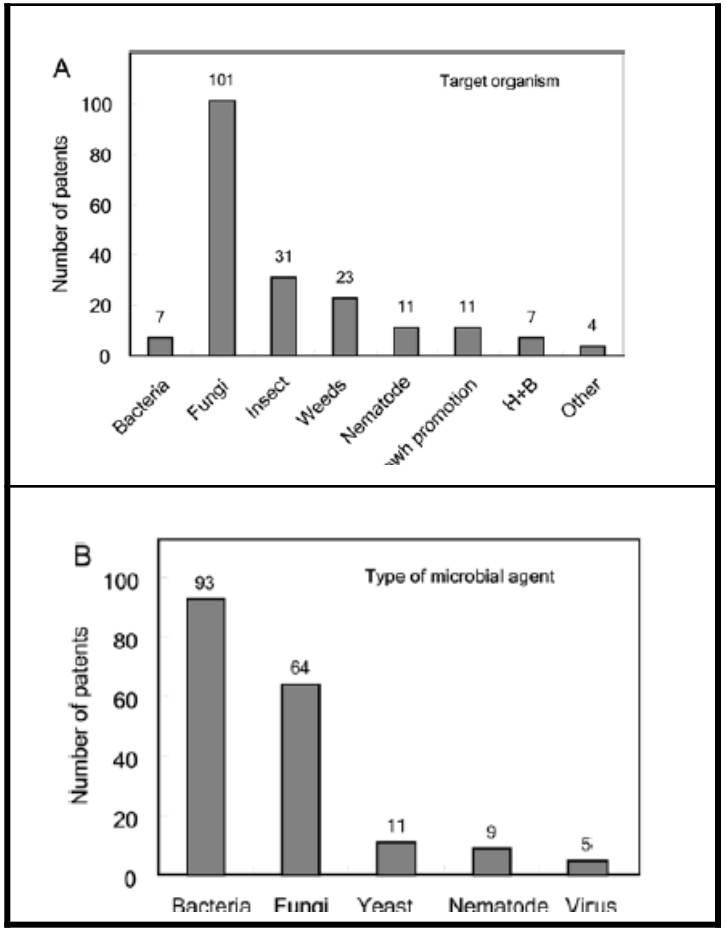
Emilio Montesinos,2003

Number and natures of patents

- Number of patents of microbial pesticides approved in the United States, Europe or world-wide (Patent Cooperation Treaty) from 1979 to 2001.
- Distribution of microbial pesticide patents according to the nature of the

A. Target organism, and

B. The type of microbial agent.



How do biological fungicides work?

- Direct competition
- Antibiosis
- Predation or parasitism
- Induced resistance of host plant.

How do biological fungicides work?

- **Biocontrol agents normally possess several of the following characteristics:**

1. Ability to associate sufficiently closely with the plant to exert an effect on the pathogen, i.e. to be phyllosphere or rhizosphere competent,
2. Ability to compete with the pathogen for nutrients or niches, e.g. infection courts (Competition),
3. Production of antibiotic compounds (Antibiosis),
4. Production of lytic enzymes effective against the pathogen,
5. Ability to parasitize the pathogen (Parasitim),
6. Ability to interfere with the reproduction of the pathogen,
7. Ability to interfere with the virulence mechanisms of the pathogen,
8. The induction of host defence mechanisms.

No one pathogen is likely to have all these properties so it is often advantageous to combine biocontrol agents which exert control by different mechanisms.

Commercial biopesticide production
Bioproducts

- Although the number of bio control products in plant disease management is increasing, these products still represent only 1% of the agricultural control measures while fungicides account for 15% of total chemicals used in agriculture (Friavel *et al.*, 2005).
- In recent years many small and large entrepreneurs have entered into the commercial production of bio control agents resulting into the entry of various bio-control products into the world market.

Junaid *et al.*,2013

Commercial biopesticide production
Bioproducts

- Commercialization of bio-control products is a multi-step process involving a wide range of activities:

1. Isolation of micro- organism from the natural ecosystem.
2. Evaluation of bio-agent both in vitro and under glass house conditions
3. Testing of the best isolate under field conditions
4. Mass production
5. Formulation
6. Delivery
7. Compatibility
8. Registration and release.

Junaid *et al.*,2013

Commercial biopesticide production

List of bio control products

SN	Bio control agent	Product	Target disease/organism	Crop	Manufacturer
1	*Ageobacterium radiobacter strain 84*	Galtrol	*Agrobacterium tumefaciens*	Ornamentals, Fruits, Nuts	AgBioChem, USA
2	*Ageobacterium radiobacter strain* K 1026	Nagol	*Agrobacterium tumefaciens*	Ornamentals, Fruits, Nuts	Bio-care
3	*Bascillus subtillus* strain GB34	GB34	*Rhizoctonia, Fussarium*	Soyabean	Gustafon, USA
4	*Bascillus subtillus* strain GB03	Kodiac, companion	*Rhizoctonia, Aspergillus*	Wheat, barley, peas	Growth products,USA
5	*Pseudomonas aureofaciens* strain TX-1	Bio–jet, spot less	*Pythium, Rhizoctonia solani*	Vegetables and Ornamentals ingreen houses	EcoSoil system
6	*Pseudomonas fluorescence* strain A506	Frostban	Fire blight, bunch rot	Fruit crop, Tomato, Potato	Plant Health Technologies
7	*Streptomycine griseoviridis*	Mycostop	Soil borne pathogens	Ornamentals, Tree seedlings	Kemira Oy, Finland
8	*Trichoderma harzianum* T-22	Root shield, plant shield	Soil borne pathogens	Green house nurseries	Bio works, USA
9	*Trichoderma harzianum* T-39	Trichodex	*Botrytis cinerea*	Most of the food crops	Bio works, USA
10	*Ampelomyces quisquallis* isolate M-10	AQ10	Powdery mildew	Fruits, Ornamentals , Vegetables	Ecogen,USA
11	*Aspergillus flavus* AF36	Alfa guard	*Aspergillus flavus*	Cotton	Circleone globa,USA
12	*Gliocladium catenulatum* strain JI446	Prima stop soil guard	Soil borne pathogens	Vegetables, Herbs , Spices	Kemira Agro Oy, Finland
13	*Gliocladium virensGL-21*		parasitic nematodes	Food, Fibre,	-Do-

Junaid *et al.*,2013

Commercial biopesticide production

Available Bio-Fungicides/Bactericides for Greenhouse use

Galltrol A	***Agrobacterium radiobactor***
Norbac	***Agrobacterium radiobactor***
AQ 10	***Ampelomyces quisqualis***
Epic	***Bacillus subtilis***
Kodiak	***Bacillus subtilis***
Serenade	***Bacillus subtilis***
Deny	***Burkholdia cepacia***

Guy Metzler

Commercial biopesticide production

Available Bio-Fungicides/Bactericides for Greenhouse use

Aspire	***Candida oleophila***
Primastop	***Gliocladium catenulatum***
Actinovate	***Streptomyces lydicus***
Mycostop	***Streptomyces griseovirdis***
Plant Shield	***Trichoderma harzianum***
Trichodex	***Trichoderma harzianum***
SoilGard	***Trichoderma virens***

Guy Metzler

Commercial biopesticide production
Actinovate

- Actinovate is a microbial pesticide registered for suppression of botrytis fruit rot and powdery mildew on banana in the field and greenhouse.
- Repeat Actinovate application in 7 to 14 days interval.
- Use spray mixture of Actinovate within 4 hours of its preparation.
- Store Actinovate at room temperature (21-26°C), but should not be frozen or exposed to very high temperatures.
- Actinovate contains live bacteria and should not be used with bactericides.

List of the active substances active against plant pathogens based on a microbial strain and their status; in bold the microorganisms with claimed or possible activity against soil-borne pathogens and nematodes

FU= fungi;
BA= bacteria;
NE=nematodes,
Pending=awaiting decision.
(http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=homepage&languag e=EN, last accessed on 15/03/2015).

Substance	Category	List (*)	Status under Reg. (EC) No 1107/2009	Date of approval	Expiration of approval
Ampelomyces quisqualis strain AQ10	FU	C	Approved	01/04/2005	31/07/2017
Aureobasidium pullulans (strains DSM 14940 and DSM 14941)	FU, BA	C	Approved	01/02/2014	31/01/2024
Bacillus amyloliquefaciens MBI 600	FU	C	Pending		
Bacillus amyloliquefaciens strain FZB24	FU	C	Pending		
Bacillus amyloliquefaciens subsp. plantarum D747	FU	C	Approved	01/04/2015	31/03/2025
Bacillus firmus I-1582	NE	C	Approved	01/10/2013	30/09/2023
Bacillus pumilus QST 2808	FU	C	Approved	01/09/2014	31/08/2024
Bacillus subtilis str. QST 713	BA, FU	C	Approved	01/02/2007	30/04/2018
Candida oleophila strain O	FU	C	Approved	01/10/2013	30/09/2023
Coniothyrium minitans	FU	C	Approved	01/01/2004	31/10/2016
Gliocladium catenulatum strain J1446	FU	C	Approved	01/04/2005	31/07/2017
Purpureocilium lilacinum strain 251	NE	C	Approved	01/08/2008	31/07/2018
Phlebiopsis gigantea (several strains)	FU	A 4	Approved	01/05/2009	30/04/2019
Pseudomonas chlororaphis strain MA342	FU	C	Approved	01/10/2004	30/04/2017
Pseudomonas sp. Strain DSMZ 13134		C	Approved	01/02/2014	31/01/2024
Pseudozyma flocculosa	FU	C	Pending		
Pythium oligandrum M1	FU	A 4	Approved	01/05/2009	30/04/2019
Saccharomyces cerevisiae strain LAS02	FU	C	Pending		
Streptomyces K61 (formerly S. griseoviridis)	FU	A 4	Approved	01/05/2009	30/04/2019

List of the active substances active against plant pathogens based on a microbial strain and their status; in bold the microorganisms with claimed or possible activity against soil-borne pathogens and nematodes

FU= fungi;
BA= bacteria;
NE=nematodes,
*A: Existing active substances divided into four lists for phased evaluations,
B: Substances not considered in scope of the directive, either because already banned or never notified under directive 91/414/EEC or because not considered as plant protection products,
C: New active substances

Trichoderma asperellum (strain T34)	FU	C	Approved	01/06/2013	31/05/2023
Trichoderma atroviride (formerly T. harzianum) strains IMI 206040 and T11	FU	A 4	Approved	01/05/2009	30/04/2019
Trichoderma atroviride strain I-1237	FU	C	Approved	01/06/2013	31/05/2023
Trichoderma atroviride strain SC1	FU	C	Pending		
Trichoderma gamsii (formerly T. viride) strain ICC080	FU	A 4	Approved	01/05/2009	30/04/2019
Trichoderma harzianum strains T-22 and ITEM 908	FU	A4	Approved	01/05/2009	30/04/2019
Trichoderma polysporum strain IMI 206039	FU	A 4	Approved	01/05/2009	30/04/2019
Verticillium albo-atrum (formerly Verticillium dahliae) strain WCS850	FU	A 4	Approved	01/05/2009	30/04/2019
Zucchini yellow mosaic virus (ZYMV mild strain)	FU	C	Not Approved		
Zucchini Yellow Mosaik Virus, weak strain	FU	C	Approved	01/06/2013	31/05/2023

Pertot *et al.*,2016

Commercial products of PGPR in plant disease management.

Continued table 3.

Victus™ – *P. fluorescens*	*P. tolassii*	Mushrooms	Mauri Foods, Australia
BioJect Spot – less – *P. aureofaciens*	Dollar spot, Anthracnose and *P. aphanidermatum*	Turf and other crops	Eco Soil Systems, San Diego, CA
BioJet™ – *Pseudomonas* sp + *Azospirillum*	Brown batch and Dollar spot disease	Turf and other crops	Eco Soil Systems, San Diego, CA
Deny - *Burkholderia cepacia (Pseudomonas cepacia*)	*Rhizoctonia, Pythium, Fusarium* and diseases caused by lesion, spiral, lance, and sting nematodes.	Alfalfa, Barley, Beans, Clover, Cotton, Peas, Sorghum, Vegetable crops and Wheat	Stine Microbial Products, Shawnee, KS
Intercept ™- *P. cepacia*	*Rhizoctonia solani, Fusarium* sp., *Pythium sp.*	Maize, Vegetables, Cotton	Soil Technologies Corp, USA
Kodiak™, Kodiak HB™, Epic™, Concentrate ™, Quantum 4000 and System 3 ™ – *B. subtilis* GB03	*Rhizoctonia solani, Fusarium spp, Alternaria spp, and Aspergillus spp*	Cotton, Legumes	Gustafson, Inc., Dallas, USA
Bio Yield – Combination of *B. subtilis* and *B.amyloliquefaciens*	Broad spectrum action against greenhouse pathogens	Tomato, Cucumber, Pepper and Tobacco	Gustafson, Inc., Dallas, USA
Rhizo-Plus – *B. subtilis* strain FZB24	Against *R. solani, Fusarium spp., Alternaria spp., Sclerotinia* and *Verticillium.*	Greenhouses grown crops,forest tree seedlings, ornamentals, and shrubs.	KFZB Biotechnik GMBH, Berlin, Germany.
Serenade – *B. subtilis* strain QWT713. Available as wettable powder.	Powdery mildew, Downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight and others.	Cucurbits, Grapes, Hops, Vegetables, Peanuts, Pome fruits, stone fruits and others	AgraQuest, Inc., Davis, USA.

Siddiqui,2006

Commercial products of PGPR in plant disease management

Continued table 3.

Rhapsody – *B. subtilis* strain QST713. Aqueous suspension formulation	Powdery mildew, sour rot, downy mildew, and early leaf spot, early blight, late blight, bacterial spot, and walnut blight diseases.	Cherries, cucurbits, grapes, leafy vegetables,peppers, potatoes, tomatoes, and walnuts.	AgraQuest, Inc., Davis, USA.
Subtilex - *B. subtilis* MB1600	*Fusarium* spp., *Rhizoctonia* spp. and *Pythium* spp.	Ornamental and vegetable crops	Becker Underwood, Ames.
GB 34 Concentrate Biological Fungicide - *B. pumilus*	*Rhizoctonia* and *Fusarium*, which attack developing soybean roots	Soybean	Gustafson LLC1400 Preston Road TX 75093
SonataTM ASO *B. pumilus* strain QST 2808	Fungal pests such as molds, mildews, blights, rusts and to control Oak death syndrome	Used in nurseries, landscapes, oak trees and green house crops	Agra Quest, Inc.,Davis, USA
System 3 - *Bacillus subtilis GB03* and chemical pesticides	Seedling pathogen	Barley, Beans, Cotton, Peanut, Pea, Rice, Soybean	Helena Chemical Co.,Memphis USA
AtEze P. chlororaphis strain 63-28	*Pythium* spp., *Rhizoctonia solani, Fusarium oxysporum*	Ornamentals and vegetables	EcoSoil Systems, Inc., San Diego, CA
Pix plus plant regulator, *B. cereus* BPO1 technical, - *B. cereus* strain UW85	Used as growth regulator	Cotton	Micro Flo Company, Lakeland, FL 33807
Bio-save 10LP, 110 – *P. syringae*	*Botrytis cinerea*, *Penicillium* spp., *Geotrichum candidum*	Pome fruit, Citrus, Cherries and Potatoes	Eco Science Corp., FL 32779.

Siddiqui,2006

Biopesticides
BlightBan A506

- BlightBan A506 against *E. amylovora*.
- Composition: *P. fluorescens* strain A506 (71% by weight and other gradient 29%).
- Potential environmental effects: Dose not harm environment including birds, plants, mammals, aquatic organisms and honeybees.

Biopesticides
Biokeeper

- A biological-control agent with the trade name "Biokeeper" has been developed for the control of soft rot disease caused by *P. carotovorum* subsp. *carotovorum* in Japan (Central Glass Co., Japan).
- This product is formulated from mutants of *P. carotovorum* subsp. *carotovorum* producing a low-molecular-weight bacteriocin, carocin.
- There is strong evidence that avirulent mutant strains of *P. carotovorum* subsp. *carotovorum* effectively control the soft rot disease of Chinese cabbage.

Chuang *et al.*,2007

Biopesticides
Serenade

- Serenade products (Serenade ASO, Serenade MAX) are registered for the suppression of a variety of diseases on fruit crops.
- Serenade, which contains a *B. subtilis* strain, is reported to be effective against a variety of pathogenic fungi and bacteria, including *Erwinia*, *Pseudomonas*, and *Xanthomonas* strains (http://www.agraquest.com).
- Serenade is most effective when applied during the early stages of disease development.

Bais *et al.*,2004

Biopesticides

Three new defense products against fungal diseases

- Three products KeyPlex 250-DP, 350-DP, and 445-DP are being marketed in the United States, and the company is expanding the market to Central America and the Caribbean Basin.
- These products are as effective as fungicides against two diseases that plague citrus:

1. greasy spot, *Mycosphaerella citri*, and
2. postbloom fruit drop, *Colletotrichum acutatum*.

Mayer *et al.*,2007

Grapefruit leaves on the right have been treated with KeyPlex 445-DP, which reduces the incidence of greasy spot ,*Mycosphaerella citri* , shown on left.

KeyPlex products stimulate plants to increase production of their own defensive compounds.

Biopesticides

Bacterial bioherbicides

- Another group of bacteria under intensive investigation for bioherbicidal potential are deleterious rhizobacteria (DRB), which differ from bacterial pathogens in that:

1. They are nonparasitic bacteria colonizing plant roots, and
2. Able to suppress plant growth without invading the root tissues (Kremer and Kennedy,1996).

Kremer,2002

Biopesticides

Bioherbicides from saprophytic strain of *P. syringae*

BioSave™ 100

- The antagonist bacterium, *Pseudomonas syringae*, is a saprophytic strain which was originally isolated from an apple leaf and can be frequently isolated from apple fruit.
- *P. syringae* (strain L-59-66 renamed as strain ESC-11) can control fungi like blue *Penicillium expansum*) gray mold(*B. cinerea*), Mucor rot(*Mucor* spp.) on apple and pear, blue mold(*Penicillium italicum*) and green mold caused by *Penicillium digitatum* on citrus fruit.
- *P. syringae* ESC-11 is sold under the name BioSave™ 110 and is recommended for the control of postharvest decays of pear and apple.
- *P. syringae* ESC-10 is commercially available under the name BioSave™ 100 and is recommended for the control of postharvest decays of citrus fruits.

Commercial biopesticide production
Formulation of Bioagents

- Formulation of the agent has an important role to play in biological control.
- For the commercial development of a microbial pesticide, the biocontrol agent should be produced at the industrial scale (fermentation), preserved, stored and formulated.
- In general and depending of the agent's nature (bacteria, fungi or yeast, nematodes, or viruses), the methods used for industrial scale-up are solid- or liquid-phase fermentation, which can profit from the advanced technology in the pharmaceutical and food industries.

1. Bacteria and yeast are usually produced by liquid fermentation using continuously stirred tank bioreactors, but
2. Many fungi are fermented in a solid state.

Commercial biopesticide production
Characteristics of an ideal formulation

- Should have increased shelf life;
- Should not be phytotoxic to the crop plants;
- Should dissolve well in water and should release the bacteria;
- Should tolerate adverse environmental conditions;
- Should be cost effective and should give reliable control of plant diseases;
- Should be compatible with other agrochemicals;
- Carriers must be cheap and readily available for formulation development.

Siddiqui,2006

Commercial biopesticide production
Characteristics of an ideal formulation

- Formulation is blending of active ingredients such as fungal spores with the inert material such as diluents (water, organic solvents such as 1% neem solution) and surfactants (increase the ability of the pesticide to spread evenly over the surface of a leaf or fruit) in order to alter the physical characteristics of to a more desirable form.
- A final formulation must:

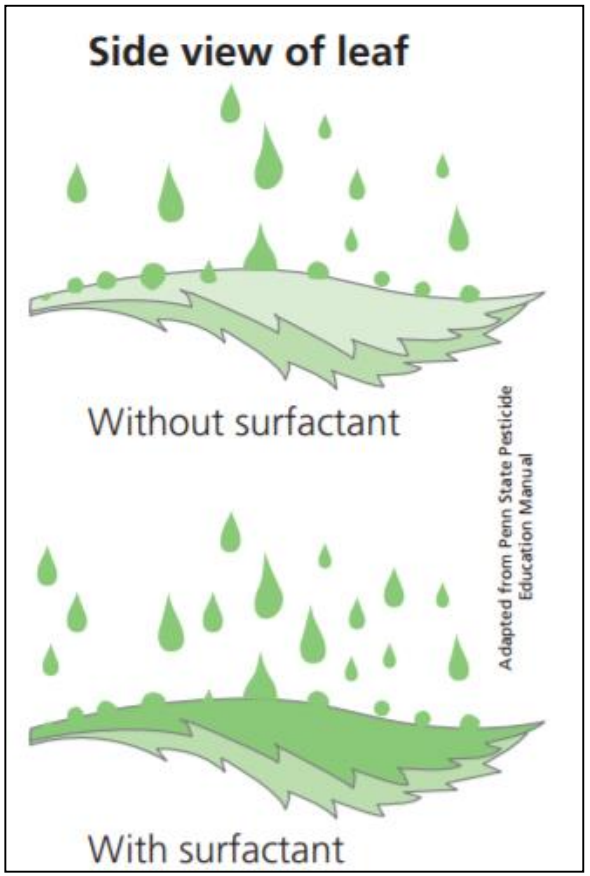
1. Be easy to handle.
2. Be stable over a range of -5 to 35°C.
3. Have a minimum shelf-life of two years at room temperature.

Junaid *et al.*,2013;..

Commercial biopesticide production
Characteristics of an ideal formulation
Surfactants

- Surfactants such as tween 20, tween 80, triton x 100 are classified by how they split apart into charged atoms or molecules, called ions.

1. Anionic surfactants have a negative charge. They are most often used with contact pesticides, which control the pest by direct contact instead of being absorbed systemically.
2. Cationic surfactants have a positive charge. Do not use them as "stand-alone" surfactants- often, they are phytotoxic.
3. Nonionic surfactants have no electrical charge. They are often used with systemic products and help sprays penetrate plant cuticles. They are compatible with most pesticide products.



Commercial biopesticide production
Natural surfactants or biosurfactants
Plant based biosurfactants

- Many plants produce natural soaps (called saponins).
- Natural surfactant or wetting agent to help the solution flow over and stick to foliage.
- Yucca is a natural surfactant and is often a component of compost teas.
- The great majority or other soaps contain detergents that do not break down easily.
- These are natural surfactants, or detergents (natural soapy substances) with distinct foaming characteristics.
- They are found in many plants and are the plant's "immune system".
- These chemicals are toxic to bacteria and fungi and so form part of the plant's protection against disease.

Commercial biopesticide production

Natural surfactants, biosurfactants or green surfactants

Microbial based biosurfactants

- Biosurfactants which are reported to be produced by bacteria, yeasts, and fungi can serve as green surfactants.
- The biosurfactants synthesized by environmental isolates also has promising role in the agricultural industry.
- Many rhizosphere and plant associated microbes produce biosurfactant; these biomolecules play vital role in motility, signaling, and biofilm formation, indicating that biosurfactant governs plant-microbe interaction.
- In agriculture, biosurfactants can be used for plant pathogen elimination and for increasing the bioavailability of nutrient for beneficial plant associated microbes.

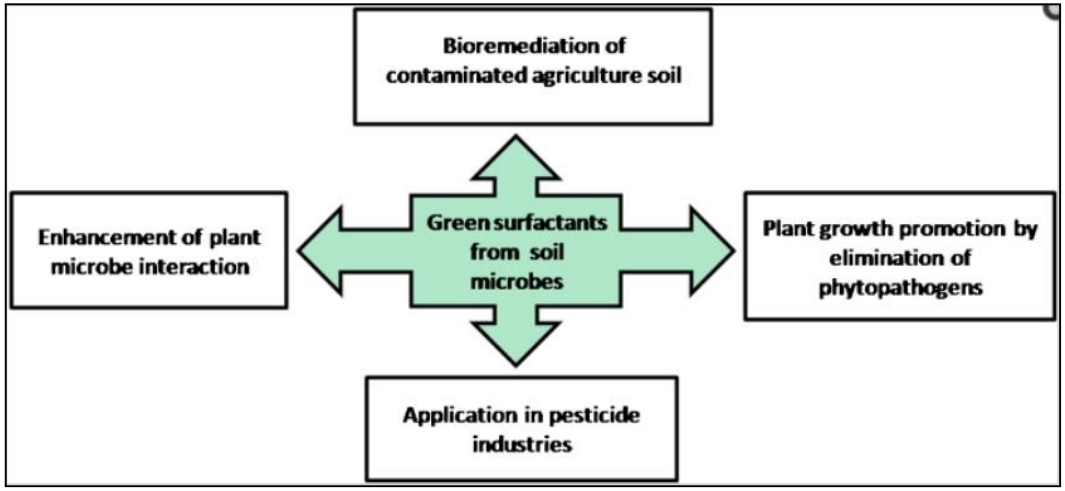
Sachdev and Cameotra,2003

Commercial biopesticide production

Natural surfactants, biosurfactants or green surfactants

Microbial based biosurfactants

- Role of biosurfactants and biosurfactant producing microbes in the most important commercial sector *viz* agriculture.
- Multifunctional prospective of biosurfactants in agriculture:



Sachdev and Cameotra,2003

Commercial biopesticide production

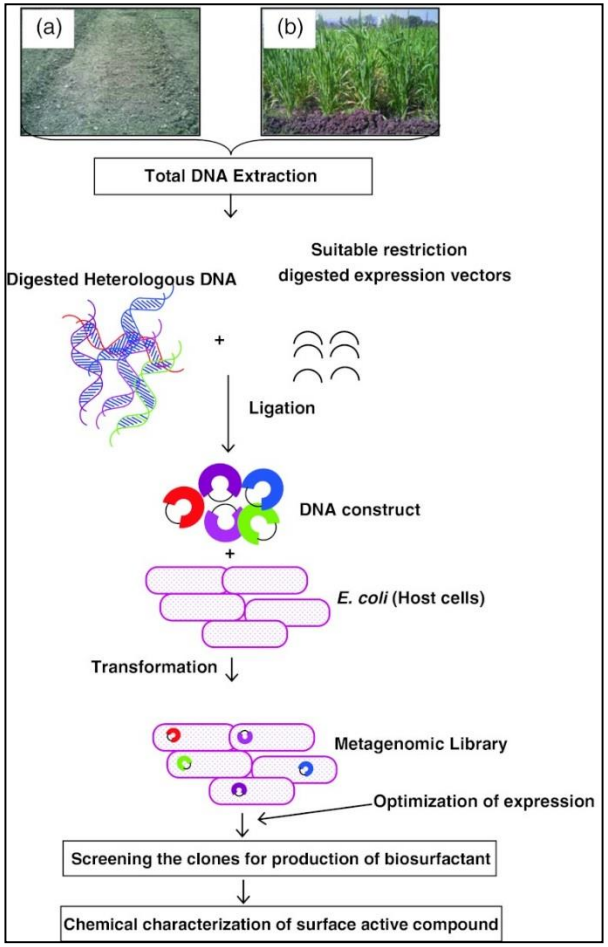
Natural surfactants, biosurfactants or green surfactants

Microbial based biosurfactants

- Schematic representation of construction and screening of metagenomic libraries from:

a. contaminated agriculture soil, and

b. rhizosphere for novel biosurfactant from uncultured bacteria.



Sachdev and Cameotra,2003

Commercial biopesticide production

Natural surfactants, biosurfactants or green surfactants

Microbial based biosurfactants

Microorganism	Source	Reference/s
Pseudomonas aeruginosa	Indigenous flora of apple; petroleum-contaminated soil	Abbasi et al. (2012); Kumar et al. (2012)
Bacillus cereus, B acillus megaterium, B. thuringiensis	Land farming soil	Cerqueira et al. (2012)
Stenotrophomonas maltophilia	Land farming soil	Cerqueira et al. (2012)
Psuedomonas nitroreducens	Petroleum-contaminated soil	Onwosi and Odibo (2012)
Acinetobacter sp.	Petroleum-contaminated soil	Chen et al. (2012)
Staphylococcus sp.	Crude oil-contaminated soil	Eddouaouda et al. (2012)
Pseudomonas sp.	Crude oil-contaminated soil	Hua and Wang (2012)
Pseudomonas aeruginosa, Bacillus cereus	Petrochemical waste-contaminated soil	Cerqueira et al. (2011)
Bacillus subtilis	Endosulfan sprayed cashew plantation soil containing hydrophobic substances	Sekhon et al. (2011)
Serratia marcescens	Hydrocarbon-contaminated soil	Roldán-Carrillo et al. (2011)
Enterobacter cloacae, Pseudomonas sp.	Heavy crude oil-contaminated soil	Darvishi et al. (2011)
Streptomyces rochei	Heavy crude oil-contaminated soil	Chaudhary et al. (2011)
Pseudomonas fluorescens	Rhizosphere of fique	Sastoque-Cala et al. (2010)
Pseudomonas aeruginosa	Petroleum-contaminated soil	Nie et al. (2010)
Rhodococcus fascaians	Antarctic soil	Gesheva et al. (2010)
Bacillus subtilis	Soil	Kim et al. (2010)
Bacillus mojavensis	Endophytic bacteria from maize	Snook et al. (2009)
Sphingomonas paucimobilis	Phenanthrene-contaminated soil microcosm	Coppotelli et al. (2010)

Sachdev and Cameotra,2003

Commercial biopesticide production

Natural surfactants, biosurfactants or green surfactants

Microbial based biosurfactants(continued)

Pseudomonas sp.	Agriculture soil	Singh et al. (2009)
Pseudomonas sp.	Oil-contaminated soil	Cameotra and Singh (2009)
Pseudomonas putida	Rhizosphere of black pepper	Kruijt et al. 2009
Pseudomonas aeruginosa	Oil-contaminated soil	de Lima et al. (2009)
Burkholderia cenocepacia	Fuel oil-contaminated soil	Wattanaphon et al. (2008)
Rhodococcus wratislaviensis	Soil	Tuleva et al. (2008)
Nocardia otitidiscaviarium	Contaminated soil	Zeinali et al. (2007)
Pseudomonas aeuroginosa	Diesel-contaminated soil	Chen et al. (2007)
Pantoea sp.	Ornithogenic soil of Antarctica	Vasileva-Tonkova and Gesheva (2007)
Pseudomonas aeruginosa Bacillus subtilis	Petroleum oil-contaminated soil	Das and Mukherjee (2007)
Pseudomonas sp.	Rhizosphere of white and red cocoyam plants	Perneel et al. (2007)
Pseudomonas chlororaphis	Soil	Gunther et al. (2005)
Acinetobacter junii	Long Beach soil	Menezes Bento et al. (2005)
Pseudomonas fluorescens	Sugar beet rhizosphere	Nielsen and Sørensen (2003)
Flavobacterium sp.	Hydrocarbon/metal-contaminated soil	Bodour et al. (2003)
Bacillus sp.	Soil	Takeyama et al. (2002)
Pseudomonas fluorescens	Petroleum-contaminated soil	Barathi and Vasudevan (2001)

Sachdev and Cameotra,2003

Formulations and shelf life

- Talc formulation
- Peat formulations
- Press mud formulation
- Vermiculite formulation

Shelf-life: the length of time for which an item remains usable. The improper selection of surfactants and diluents decreases shelf life and the effectiveness of biological agents.

Siddiqui,2006

Shelf life of formulations in different carrier materials.

Carrier	Bacteria	Shelf life	Reference
Talc	Rhizobacteria	2 months	Kloepper and Schroth (1981)
Talc	*P. fluorescens* (P7NF, TL3)	12 months (8.4 Log cfu/g)	Caesar and Burr (1991)
Talc	*P. fluorescens* (Pf1)	8 months (1.3 x 10^7cfu/g)	Vidhyasekaran and Muthamilan (1995)
Talc	*B. subtilis*	45 days (1.0 x 10^6 cfu/g)	Amer and Utkhede (2000)
Talc	*P. putida*	45 days (1.0 x 10^3 cfu/g)	Amer and Utkhede (2000)
Talc	*P. putida* strain 30 and 180	6 moths (>1 x 10^8cfu/g) (not estimated during subsequent months)	Bora *et al.* (2004)
Lignite	*P. fluorescens* (Pf1)	4 months (2.8 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Peat	*P. fluorescens* (Pf1)	8 months (7.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Peat supplemente-d with chitin	*B. subtilis*	6 moths (>1 x 10^9cfu/g) (not estimated during subsequent months)	Manjula and Podile (2001)
Peat	*P. chlororaphis* (PA23) and *B. subtilis* (CBE4)	6 moths (>1 x 10^8cfu/g) (not estimated during subsequent months)	Nakkeeran *et al.* (2004)
Vermiculite	*P. fluorescens* (Pf1)	8 months (1.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Vermiculite	*B. subtilis*	45 days (>1.0 x 10^6 cfu/g)	Amer and Utkhede (2000)
Vermiculite	*P. putida*	45 days (>1.0 x 10^3 cfu/g)	Amer and Utkhede (2000)
Farm yard manure	*P. fluorescens* (Pf1)	8 months (1.0 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Kaolinite	*P. fluorescens* (Pf1)	4 months (2.8 x 10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)

Siddiqui,2006

Kaolinite is a clay mineral, part of the group of industrial minerals, with the chemical composition $Al_2Si_2O_5(OH)_4$.

Formulations and shelf life

Formulation of Bioagents

Talc formulation

- Talc is a natural mineral referred as steatite or soapstone composed of various minerals in combination with chloride and carbonate.
- Chemically it is referred as magnesium silicate ($Mg_3Si_4O_{10}(OH)_2$ and available as powder form from industries suited for wide range of application.
- The fluorescent Pseudomonads did not decline in talc mixture with 20% xanthum gum after storage for two months at 4°C.
- *P. fluorescens* isolate Pf1 survived up to 240 days in storage.
- *P. putida* strain 30 and 180 survived up to 6 months in talc based formulations.

Siddiqui,2006

Commercial biopesticide production
Formulation of Bioagents
Talc based preparation

- Talc based preparation of the *Trichoderma virens* conidia retain 82% viability at 5°C in refrigerator after 6 months while at room temperature same level of viability was observed for a period of 3 months.

Junaid *et al.*,2013

Formulations and shelf life
Peat formulation

- Peat (Turf) is a carbonized vegetable tissue formed in wet conditions by decomposition of various plants and mosses.
- It is formed by the slow decay of successive layers of aquatic and semi aquatic plants, e.g., sedges, reeds, rushes, and mosses.
- Peat soils are used as carrier materials to formulate PGPR.
- The shelf life of *P. fluorescens* in peat-based formulation was maintained up to 8 months (2.8×10^6 cfu/g).
- Shelf life of *Pseudomonas chlororaphis* (PA23) and *Bacillus subtilis* (CBE4) in peat carriers was retained for more than six months.

Siddiqui,2006

Formulations and shelf life

Press mud formulation

- Press mud is a byproduct of sugar industries.
- It was composted using vermin-composting technique and later used as a carrier for *Azospirillum* spp.(well known PGPR able to excrete phytohormones such as:

1. Gibberellins,
2. Cytokinins, and
3. Auxins.

- This carrier maximizes the survival of *Azospirillum* spp. than lignite, which is predominantly used as a carrier material in India (Muthukumarasamy *et al.*,1997).

The term "vermin" is used to refer to a wide scope of organisms, including rodents, cockroaches, fleas, termites, lice, bed bugs and white ants.

Siddiqui,2006

Formulations and shelf life

Vermiculite formulation

- Vermiculite is a light mica-like mineral used to improve aeration and moisture retention.
- It is widely used as potting mixture and used as a carrier for the development of formulations for harboring microbial agents.
- Vermiculite based formulation of *P. fluorescens* (PF1) retained shelf life for a period of 8 months.
- The viable load of bacteria in the formulation was $1x10^6$ cfu/g (Vidhyasekaran and Muthamilan,1995).

Mica - a shiny silicate mineral with a layered structure, found as minute scales in granite and other rocks, or as crystals.

Siddiqui,2006

Formulations and shelf life

1% methylcellulose or clay-granule formulations

- In initial field trials of *Bacillus cereus* UW85, the bacterium was added to alfalfa seeds in 1 per cent methylcellulose, CMC (Handelsman *et al.*,1990) but later, Osburn and co-workers (1995) found that clay-granule formulations applied in furrow gave the most consistent results.

Formulations and shelf life

Chitosan

A natural seed treatment

- Chitosan is determined as a non-toxic, a biodegradable and a biocompatible polymer.
- Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit).
- The natural biocontrol active ingredients, chitin/chitosan, are found in the shells of crabs, shrimp and cell walls of fungi.
- It is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi.

Wikipedia,2010

Formulations and shelf life

Chitosan

Acid/water soluble compounds

- Chitosan is relatively insoluble in water, but can be dissolved by dilute acids e.g. in 2 percent acetic acid solution, which would make it a highly-viscous dietary fiber.
- Chitosan originating from crustaceans was also dissolved in 2% (w/v) in 1% HCl by stirring overnight at room temperature and the original solution was then diluted to a series of 0.01, 0.05, 0.1, 0.5 and 1% and pH values of each solution was adjusted to 5.4 with NaOH.
- There is also water soluble chitosan which is off-white flavorless amorphous powder. It is freely soluble in water, the solution is clear and stable.

Liu *et al.*,2006; Wikipedia,2010

Formulations and shelf life

Chitosan

Antibacterial effects

- In agriculture, chitosan is used primarily as a natural seed treatment and plant growth enhancer, and as a ecologically friendly biopesticide substance that boosts the innate ability of plants to defend themselves against fungal infections.
- Incorporating of essential oils and chelating agent (EDTA) with chitosan improve their antibacterial activities.
- EDTA itself enhanced antimicrobial effects of essential oils against both gram-positive and gram-negative bacteria(Hosseini *et al.*,2008).

Ethylenediaminetetraacetic acid(EDTA) is mainly used to sequester metal ions in aqueous solution. It is a powerful chelating agent causes dispersion of LPS molecules in Gram-negative bacteria. In molecular biology assays it was used to deactivate metal-dependent enzymes to suppress damage to DNA or proteins.

Formulations and shelf life

Chitosan

Chitosan based formulations

- Incorporation of commercial chitosan based formulations LS254 (comprising of *Paenobacillus macerans* + *B. pumilus*) and LS255 (comprising of *P. macerans* + *B. subtilis*) into soil at the ratio of 1:40 (Formulation: Soil) increased bio-matter production by increasing both root and shoot length and yield (Vasudevan *et al.*,2002).

Siddiqui,2006

Formulations and shelf life

Chitin bioformulation

- The amendment of chitin thus enhanced its activity in field conditions (Vivekananthan *et al.,*2003).
- It was shown PGPR strains e.g. *P. fluorescens* FP7 with chitin bioformulation significantly suppressed the anthracnose (*Colletotrichum gloeosporioides*) incidence under endemic conditions.
- Seed treatment of groundnut and pigeon pea with peat based formulation of *B. subtilis* supplemented with 0.5% chitin or with 0.5% of sterilized *Aspergillus* mycelium controlled crown rot and wilt of groundnut and pigeon pea respectively.
- Since incorporation of chitin will increase the production cost of biopesticides, identification of cheap and easy available source of chitin is essential.

Formulations and shelf life

Efficacy of PGPR formulations against plant disease and growth promotion

Formulation	Crop	Results	Reference
Talc based *P. fluorescens*	Potato	Significant plant growth promotion.	Kloepper and Scroth (1981)
Talc based *P. fluorescens*	Winter wheat	Significant plant growth promotion.	De Freitas and Germida(1992)
Peat based *P. fluorescens*	Cotton	Significant reduction of cotton seedling diseases.	Hagedorn *et al.* (1993)
Talc based *P. fluorescens*	Chickpea	Significant increase in grain yields and controlled fusarial wilt under field conditions.	Vidhyasekaran and Muthamilan, (1995)
Talc based *P. fluorescens*	Pigeonpea	Control of pigeonpea wilt and significant increase in grain yield.	Vidhyasekaran *et al.* (1997)
Chitosan based *B. pumilus*	Tomato	Induced resistance against *F. oxysporum.*	Benhamou *et al.*(1998)
Methyl cellulose and talc based *P. fluorescens.*	Rice	Suppressed rice blast both in nursery and field conditions.	Krishnamurthy and Gnanamanickm (1998)
B. subtilis strain LS213(commercial product)	Watermelon and muskmelon	Increased plant growth, and improved yield.	Vavrina (1999)

Siddiqui,2006

Formulations and shelf life:

Efficacy of PGPR formulations against plant disease and growth promotion.

Siddiqui,2006

Continued table 2.

B. subtilis Formulations	Cucumber, Watermelon, squash, ornamentals, vegetables, pepper, tobacco, loblolly pine and lodge pine.	Significant induction of resistance against various different pathogens.	Reddy *et al.* (1999); Kenney *et al.* (1999); Martinez- Ochoa *et al.* (1999) ; Ryu *et al.*(1999) ;Yan *et al.* (1999) and Zhang *et al* (1999).
Chitosan based *B. subtilis* strain LS213 (commercial product)	Tomato, tobacco, cucumber and pepper	Reduced the incidence of bacterial spot and late blight of tomato, angular leaf spot of cucumber and blue mold of tobacco.	Reddy *et al.* (1999)
Talc based formulation of *P. fluorescens* (CHAO and Pf1)	Sugarcane	Increased germination of sugarcane seeds, plant growth besides the supper-ssion of damping- off.	Viswanathan and Samiyappan (1999)
Vermiculite based *P. fluorescens*	Sugarbeet	Significant control of damping off	Moenne-Loccoz *et al.* (1999)
Talc based *P. fluorescens*	Rice	Significant reduction of sheath blight under field conditions.	Vidhyasekaran and Muthamilan (1999); Nandakumar *et al.* (2000).
Talc based *P. fluorescens*	Banana	Significant reduction of panama wilt of banana	Raguchander *et al.* (2000)
Vermiculite and Kaolin based *B. subtilis*	Lettuce	Suppressed root rot of lettuce caused by *P. aphanidermatum* and increased fresh weight of lettuce.	Amer and Utkhede (2000)
Vermiculite based *P. putida*	Cucumber	Significantly reduced root rot caused by *Fusarium oxysporum f. sp. cucurbitacearum*	Amer and Utkhede (2000)
Talc based *P. fluorescens* (Pf1)	Urdbean and Sesame	Increased growth promotion and reduced root rot caused by *M. phaseolina.*	Jayashree *et al.*(2000)
Talc based rhizobacterial mixtures of fluorescent pseudomonads	Rice	Significant plant growth promotion and suppression of rice sheath blight.	Nandakumar *et al.* (2001)
Peat based *B. subtilis* supplemented with chitin	Groundnut and pigeon pea	Significant control of groundnut root rot and pigeon pea wilt.	Manjula and Podile (2001)

Formulations and shelf life:

Efficacy of PGPR formulations against plant disease and growth promotion.

Siddiqui,2006

Continued table 2.

Chitosan based mixed formulation of *Paenobacillus macerans* and *B. subtilis* (LS255)	Rice	Increased plant growth and yield in rice cultivars, IR24, IR50 and Jyothi.	Vasudevan *et al.* (2002)
Chitin based formulation of *B. subtilis* strain GB03+*B. pumilus* strain INR7(LS256) and *B. subtilis* strain GB03+*B. subtilis* strain IN937b	Tomato and Pepper	Increased yield of pepper and tomato.	Burelle *et al.* (2002)
Talc based *P. aeruginosa* strain 78	Mung bean	Reduced the incidence of root knot and population density of *Meloidogyne javanica* under field conditions.	Ali *et al.* (2002)
Talc based fluorescent Pseudomonads	Sugarcane	Significant increase in sett germination, increased cane growth and reduced red rot incidence.	Viswanathan and Samiyappan (2002)
Talc based *P. fluorescens*	Rice	Significant reduction of rice sheath blight, leaf folder and increased yield. Beside it also increased the population of insect parasites and predators.	Radja Commare *et al.* (2002)
Talc based *P. fluorescens*	Groundnut	Significant reduction of leaf spot and rust of groundnut.	Meena *et al.* (2002)
Talc based formulation of *B. subtilis* and *P. chlororaphis* (PA23)	Tomato	Increased growth promotion and significant reduction of damping off.	Kavitha *et al.* (2003)
Chitosan based mixed formulation of *B. subtilis* strain GB03+*B. pumilus* strain INR7(LS256) and *B. subtilis* strain GB03+*B. pumilus* strain T4(LS257)	Pearl millet	Reduced downy mildew and increased plant growth promotion in pearl millet.	Niranjan Raj *et al.* (2003)
Talc based *P. fluorescens* FP7 supplemented with chitin.	Mango	Significant reduction of anthracnose coupled with increase in fruit yield and quality.	Vivekananthan *et al.*(2004).

Biopesticides
High cost of registration

- The high cost of registration prevents a large number of potential biocontrol agents to reach the market.
- Since each registered use should be supported by specific efficacy trials, the companies tend to register the products against those pathogens with the largest potential market.
- This results in products potentially active against a large number of soilborne pathogens, but authorised only on the specific one on which the registration trails have been carried out.

Pertot *et al.*,2016

Commercial biopesticide production
Registration of Bioagents

- Two important factors in the registration of bio agents are the:

1. Toxicity, and
2. environmental fate.

- Under the section 9 (3) of pesticide act of India 1968 information required for the registration of any bio pesticide are:

1. Systemic name and common name of the bio control agent
2. Natural occurrence
3. Morphological description of the of the bio agent
4. Details of manufacturing process
5. Mammalian toxicity
6. Environmental toxicity
7. Residual analysis.

Junaid *et al.*,2013

Biopesticides based on bacteria that are registered in some countries. I Insecticide, F fungicide, B bactericide, N nematicide, H herbicide

Species/strain	Type	Target
Bacillus popilliae	I	*Popilla japonica*
B. thuringiensis var. *aizawai*	I	*Galleria melonella*
B. thuringiensis var. *israeliensis*	I	Dipteran larvae
B. thuringiensis var. *kurstaki*	I	Lepidopteran larvae
B. thuringiensis var. *xentari*	I	Lepidopteran larvae
B. thuringiensis var. San Diego	I	Coleopteran larvae
B. thuringiensis var. *tenebrionis*	I	Coleopteran larvae
B. thuringiensis EG2348	I	*Lymantria dispar*
B. thuringiensis EG2371	I	Lepidopteran larvae
B. thuringiensis EG2424	I	Coleopteran larvae
Burkholderia cepacia	F	Soil-borne fungi, nematodes
Pseudomonas fluorescens	F	Soil-borne fungi
P. syringae ESC-10, ESC-11	F	Post-harvest fungi
P. chlororaphis	F	Soil-borne fungi
P. aureofaciens Tx-1	F	Antracnose, soil-borne
Bacillus subtilis	F	Soil-borne fungi
B. subtilis FZB24	F	Soil-borne
B. subtilis GB03	F	Soil-borne and wilt
B. subtilis GB07	F	Soil-borne fungi
Streptomyces griseoviridis K61	F	Various fungi
S lydicus	F	Soil-borne
Agrobacterium radiobacter K84, K1026	B	Crown gall *A. tumefaciens*
Ralstonia solanacearum non-pathogenic	B	Pathogenic *R. solanacearum*
Pseudomonas fluorescens A506	B	Frost damage, fire blight (*E. amylovora*)
Bacillus firmus	N	Nematodes
Pseudomonas syringae pv. *tagetis*	H	*Cirsium arvense*
Xanthomonas campestris pv. *poae*	H	*Poa annua*

Montesinos,2003

Commercial biopesticide production
Biosafety of the microorganism

- Several toxicological tests can be performed to assess biosafety of the microorganism, such as the hypersensitivity reaction test on tobacco plants and the determination of the median lethal dose (LD_{50}) on mammals.
- For the commercial development of a microbial pesticide, the BCA must be mass produced at industrial scale by fermentation processes, preserved and stored by different stabilizing treatments (refrigeration, freezing, lyophilization), and formulated, using biocompatible additives that increase survival and improve application.

Alcals,2007

Commercial biopesticide production
Biosafety of the microorganism

- It is also necessary the development of a traceability method to specifically detect and quantify the BCA once it has been applied.
- These traceability methods are based on specific genotypic markers that can be detected and quantified by quantitative PCR and other molecular techniques (Montesinos, 2003).

Alcals,2007

Molecular techniques

Construction for high potential BA

- **Gene transformation:**
- Considerable effort has been expended on transforming fungi and bacteria with genes that improve the biocontrol properties possessed by the wild type or add to them.

Gene transformation
Construction for high potential BA

- For example, Delany and co-workers (2001) genetically modified strain F113 of *Pseudomonas fluorescens* by introducing two plasmids in separate experiments.
- One genetically modified strain contained the multicopy plasmid, pCU8.3 and the other pCUP9.
- Both plasmids contained the genes for the synthesis of the antimicrobial compound 2,4-diacetylphloroglucinol (phlA, C, B and D) as well as a putative permease gene (phlE).
- The modified strains produced:
- Ssignificant and substantial increases in the antibiotic in the early logarithmic phase of growth and in the stationary phase compared with the wild-type strain and they were as effective as a proprietary fungicide in controlling damping-off of sugarbeet seedlings caused by *Pythium ultimum*.

Molecular techniques
Construction for high potential BA

- *Pseudomonas putida* WCS358r strains genetically engineered to produce phenazine and DAPG displayed improved capacities to suppress plant diseases in field-grown wheat.
- Strain 2-79 produces phenazine carboxylic acid (PCA) and strain Q8r1-96 produces DAPG. Both strains are biocontrol agents of take-all disease of wheat.
- Molecular techniques have been used to construct rhizobacteria with increased or novel antibiotic production capacities to enhance biocontrol activity.
- Strain Z30-97 was constructed by inserting the seven gene *phz* locus into the rifampicin-resistant strain of Q8r1-96.
- The introduction of transgenic, biocontrol *Pseudomonas* strains presents no signifcant a priori risk to non-target bacterial populations in the rhizosphere of treated wheat. This means ecological significance of altering the structure of soil and rhizosphere microbial populations remains unclear.

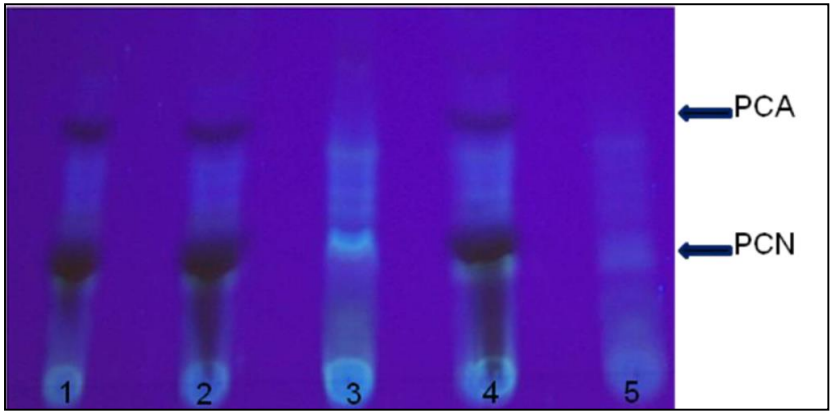
Bankhead *et al.*,2004

Molecular techniques

Detection of phenazines produced by the *Pseudomonas* sp. CMR12a mutants

TLC analysis

- All generated mutant strains were tested for the production of phenazines by TLC analysis.
- Observation of the silica plates under UV light showed phenazine-1-carboxylate(PCA) and phenazine-1-carboxamide (PCN) spots for the wild type strain, CMR12a, together with mutants CMR12a-ΔCLP2 and CMR12a-ΔCLP2-CLP1.
- As expected, the phenazine mutant strains produce neither PCA nor PCN.



Olorunleke *et al.*,2011

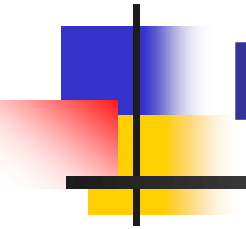
Biopesticides
Application time

- **Antibiosis**
- Antibiosis involves the production of toxins which inhibit the growth of the pathogen.
- Once the pathogen enters the root, antibiosis may have no effect.
- **Direct Competition**
- Before infection by pathogen occurs, BCO (Biological Control Organisms) must become associated with roots; area is called rhizosphere.
- This area is nutrient rich, a food source.
- Generally BCO must be present in large numbers to compete.
  Predation or parasitism
- Predation or parasitism is when the BCO attacks and feeds on the pathogen.
- Again the BCO must be present before the pathogen invades.

Biopesticides

Evaluating efficacy in the greenhouse environment

- Use according to label;
- Compare with properly applied fungicides;
- Leave a control Leave a control;
- Is the BCO (Biological Control Organisms) present and active;
- How long is the BCO on duty?



Plant extracts

As crop protectants in bio-organic agriculture

Plant oils and petroleum oils

- Highly refined petroleum spray oils kill insects and mites through suffocation, are used as adjuvants with conventional pesticides.
- Oil obtained from seeds of several plants such as sunflower, olive, corn, and soybean gave excellent control of powdery mildew of apple when applied from 1 day before to 1 day after inoculation of the plants with the fungus.
- Similarly, several essential oils have been shown to reduce infection of plants by pathogens.
- So far, none of them is used commercially.

Natural products vs. plant diseases

- Plants elaborate a vast array of natural products, many of which have evolved to confer selective advantage against microbial attack.
- Recent advances in molecular technology, aided by the enormous power of large-scale genomics initiatives, are leading to a more complete understanding of the enzymatic machinery that underlies the often complex pathways of plant natural product biosynthesis.
- Meanwhile, genetic and reverse genetic approaches are providing evidence for the importance of natural products in host defence.
- Metabolic engineering of natural product pathways is now a feasible strategy for enhancement of plant disease resistance.

Natural products

Bioactive compounds in plants

Secondary metabolites

- Collectively, plants produce a remarkably diverse array of over 100,000 low-molecular-mass natural products, also known as secondary metabolites (phytochemicals).
- Secondary metabolites are distinct from the components of intermediary (primary) metabolism in that they are generally nonessential for the basic metabolic processes of the plant.

Natural products

Bioactive compounds

Secondary metabolites

- The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds.
- Others are saponinsin, glycosides, flavonoids and proanthocyanidins, mono- and sequi-terpenoids, phenylpropanoids, resins, lignans, proteins and peptides.
- Most are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways.

Natural products

Bioactive compounds

Secondary metabolites

- That plants producing bioactive compounds seem to be the rule rather than the exception.
- Thus, most plants even common food and feed plants are capable of producing such compounds.
- However, the typical poisonous or medicinal plants contain higher concentrations of more potent bioactive compounds than food and feed plants.
- Plants with secondary metabolites eliciting pharmacological or toxicological effects in man and animals and any microrganisms.

Bernhoft,2008

Natural products

Classes of bioactive compounds with examples of compounds, plant food sources and reported biological activities

Compound class	Examples	Typical plant food sources	Examples of reported biological activities
Alk(en)yl-cysteine sulfoxides	*S*-methyl-L-cysteine sulfoxide, *S*-propenyl-L-cysteine sulfoxide	Onions, garlic, leeks	Induction of phase-2 enzymes and apoptosis, arrest of cell cycle
Capsaicinoids	Capsaicin, dihydrocapsaicin	Chilli peppers Sweet peppers	Induction of cellular antioxidant responses, inhibition of LDL-oxidation
Carotenoids	Beta-carotene, lycopene, cryptaxanthine	Tomatoes, carrots, bell peppers	Antioxidant, anti-inflammatory, anti-carcinogenic activities
Coumestans	Coumestan, coumestrol	Soya bean, mung bean	Hepatoprotective, inhibition of Na(+)/K(+)-ATPase
(Dihydro)chalcones	Phloretin	Apples	Inhibition of intestinal glucose uptake
Flavonoids			
Flavones	Apigenin, luteolin	Celery, parsley	Antioxidant, antiproliferative, anti-hypertensive, anti-carcinogenic, anti-thrombotic, cell cycle arrest, induction of phase-2 enzymes, inhibition of phase-1 enzymes, inhibition of LDL-oxidation, improvement of vascular tone
Flavanones	Naringenin, hesperetin	Citrus fruits	
Flavonols	Quercetin, kaempferol	Onions, tea, green beans, tomatoes	
Flavan-3-ols	(+)-Catechin, (−)epicatechin, procyanidin B1, procyanidin B2	Tea, cocoa, apples, berries, certain beans	
Anthocyanidins	Cyanidin, delphinidin, pellargonidin	Blackcurrants, blueberries, strawberries	
Isoflavones	Daidzein, genistein	Soy beans	
Glucosinolates/ isothiocyanates	Glucoraphanin/sulphoraphane	Broccoli, cabbage, Brussel's sprouts	Antiproliferative, cell cycle arrest, induction of phase-2 enzymes, inhibition of carcinogen-induced cancer formation/progression
Lignans	Secoisolariciresinol, matairesinol	Linseed, fruits and vegetables	Estrogenic
Phenolic acids	Ferulic acid, salicylic acid	Coffee, cereal bran, fruits	Anti-inflammatory
Phytosterols	Campostanol, sitostanol	Wheat	Cholesterol lowering
Polyacetylenes	Falcarinol, falcarindiol	Carrots, celery, parsley	Anti-carcinogenic
Stilbenes	Resveratrol, trans-piceid	Grapes, peanuts	Antioxidant, cardio-protective, lifespan extension

Gry *et al.*,2007

Natural products

Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts

Mushroom sample	Total phenols (mg/g)	Flavonoids (mg/g)	Ascorbic acid (mg/g)	β-carotene (μg/g)	Lycopene (μg/g)
L. deliciosus					
Total	17.25 ± 0.65	8.14 ± 0.81	0.24 ± 0.02	90.10 ± 4.76	40.71 ± 3.45
Cap	10.66 ± 0.52	4.76 ± 0.11	0.21 ± 0.01	51.00 ± 3.12	25.83 ± 2.12
Stipe	6.31 ± 0.29	3.59 ± 0.16	0.18 ± 0.01	18.50 ± 1.25	5.69 ± 0.62
S. imbricatus					
Total	3.76 ± 0.11	2.82 ± 0.09	0.50 ± 0.04	20.40 ± 1.33	10.51 ± 1.00
Cap	2.59 ± 0.10	1.72 ± 0.08	0.36 ± 0.02	12.54 ± 1.11	6.03 ± 0.63
Stipe	1.50 ± 0.06	1.46 ± 0.08	0.19 ± 0.01	7.84 ± 0.75	4.48 ± 0.60
T. portentosum					
Total	10.80 ± 0.47	0.40 ± 0.02	0.52 ± 0.04	–	–
Cap	6.57 ± 0.31	–	0.31 ± 0.02	–	–
Stipe	3.91 ± 0.17	–	0.22 ± 0.02	–	–

Note. Each value is expressed as mean ± standard deviation ($n = 3$); (–) Not detected.

Barros *et al.*,2007

Natural products

Essential plant oils

Differences between essential oils and fatty oils

- Essential oils are not truly "oils" at all.
- They are volatile organic compounds (VOC) made up of thousands of nature's chemicals but because they contain no lipids, they are not technically considered oils--at least nutritionally.
- A essential oil 'VOC' is a carbon compound that has a high vapor pressure and low water solubility which evaporates at everyday temperatures.
- The low water solubility is what gives essential oils its 'oily' appearance.
- Vegetable fatty oils generally come from the seed of the plant where essential oils generally come from leaves, roots, bark and stems.

Wiki.answers.com

Natural products
Bioactivity of essential oils

- Volatile Organic Compounds (VOCs) present in essential oils has been reported for their anti-spasmodic, restraining, diuretic, anti-biotical, antimicrobial, antifungal, insectisidal, and anthelmintic efficiency.
- Recent investigations in several countries confirm that some plant essential oils not only repel insects, but have contact and fumigant insecticidal actions against specific pests, and fungicidal actions against some important plant pathogens.

Gyawali and Kim,2012;..

Essential oils

Essential Oils are Stored in Surface Glands

Glandular hair of young leaf of spring sunflower

Lavender (*Lavendula*)

Peppermint (*Mentha*)

BIOL 360 Fall 08

11

Essential oils or etheric oils

- The word "oil" in essential oil is often misleading.
- Essential oils are not greasy and can be used by people with oily skin for therapeutic effects without clogging the pores or adding more oil to the skin.
- Put a drop of essential oil on a piece of paper and check in 2 hours to see that it has evaporated.
- Essential oils are also known as "etheric oils" due to their light, airy, and delicate nature.

The miracle of essential oils

- The essential oils are totally natural and documented in scientific studies.
- IMMUNE STIMULATING
- ANTI-MICROBAL
- ANTI-VIRAL
- ANTISEPTIC
- ANTI-INFECTIOUS
- ANTI-TUMORAL
- ANTI-BACTERIAL
- ANTI-FUNGAL

Antifungal and antibacterial activities of some essential oils

- Antimicrobial agents from plants: antibacterial activity of plant volatile oils
- Basil (*Ocimum basilicum*)
- Coriander (*Corriandum sativum*)
- Lavender (*Lavandula angustifolia)*
- *Neem (Azadirachta indica)*
- Thyme (Thymus vulgaris)
- Several techniques were evaluated to find the Minimum Inhibitory Concentration (MIC) of the oil needed to inhibit the growth of the fungi, these included:
- The droplet technique
- The borehole method
- Disc diffusion method.
- Vapour chambers were constructed to evaluate the fungicidal properties of the volatile components of the oil.

Antifungal and antibacterial activities of some essential oils

Thymus vulgaris

- The major constituents of the essential oil thyme include:
- Phenols, thymol and carvacrol, which make up about 20-25% of the essential oils.
- The other constituents include:
- Linaalool, p-cymol, cymeme, thymeme, pinene, apigenin, leteolin and geraniol (Grieves,1995;Torras et al.,2007; WHO,1999).
- Fabio *et al.*,2007 found potent antibacterial activity of the essential oil against seven various bacteria at a minimum concentration of 0.0002 mL/mL.

Nagy,2010

List of selected essential oils and their properties

Common name	Botanical name (Family)	Properties
Aniseed oil	*Pimpinella anisum* (Umbelliferae)	Carminative, stimulant, expectorant, condiment and flavouring agent.
Calamus oil	*Acorus calamus* (Araceae)	Carminative, bitter stimulant, vermifuge and insect repellent
Camphor oil	*Cinnamomuum camphora* (Lauraceae)	Rubefacient, tooth powder and cosmetic agent.
Cedarwood oil	*Cedrus atlantica* (Coniferae)	Antiseptic, astringent, diuretic, fungicidal, sedative and stimulant.
Cinnamon oil	*Cinnamomum zeylanicum* (Lauraceae)	Carminative, stomachic, astringent, stimulant and antiseptic.
Citronella oil	*Cymbopogon nardus* (Gramineae)	Perfumery, mosquito repellent and flavouring agent.
Clove oil	*Eugenia caryophyllus* (Myrtaceae)	Dental analgesic, carminative, stimulant and antiseptic.
Eucalyptus oil	*Eucalyptus globulus* (Myrtaceae)	Counter-irritant, antiseptic, expectorant, cough reliever.
Geranium oil	*Pelargonium graveolens* (Geraniaceae)	Flavouring agent and stimulant.
Lavender oil	*Lavandula angustifolia* (Labiatae)	Stimulant and flavouring agent.
Lemon oil	*Citrus limon* (Rutaceae)	Carminative, stimulant, perfuming and flavouring agent.
Lemongrass oil	*Cymbopogon citratus* (Graminae)	Flavouring agent, antiseptic and deodorant.
Lime oil	*Citrus aurantium* (Rutaceae)	Stomachic, carminative and flavouring agent.
Nutmeg oil	*Myristica fragrans* (Myristicaceae)	Stimulant, anti rheumatic and carminative.
Orange oil	*Citrus sinensis* (Rutaceae)	Stomachic, carminative and flavouring agent.
Palmarosa oil	*Cymbopogon martini* (Graminae)	Cosmetic, anti rheumatism and insect repellent.
Peppermint oil	*Mentha piperita* (Labiatae)	Digestent, stimulant and tonic.
Rosemary oil	*Rosmarinus officinalis* (Labiatae)	Carminative, stimulant and flavouring agent.
Basil oil	*Ocimum sanctum* (Labiatae)	Antibacterial, insecticidal, stimulant, stomachic and diaphoretic.
Vetiver oil	*Vetiveria zizanioides* (Graminae)	Stimulant, refrigerant, flavouring agent, stomachic and fixative.
Wintergreen oil	*Gaultheria fragrantissima* (Ericaceae)	Irritant, vermicide agent and flavouring agent.

Prabuseenivasan *et al.*,2006

Major components of selected[a]EOs that exhibit antibacterial properties

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Cilantro	*Coriandrum sativum* (immature leaves)	Linalool	26%	(Delaquis et al., 2002)
		E-2-decanal	20%	
Coriander	*Coriandrum sativum* (seeds)	Linalool	70%	(Delaquis et al., 2002)
		E-2-decanal	–	
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	65%	(Lens-Lisbonne et al., 1987)
Oregano	*Origanum vulgare*	Carvacrol	Trace-80%	(Lawrence, 1984; Prudent et al., 1995;
		Thymol	Trace-64%	Charai et al., 1996; Sivropoulou et al., 1996;
		γ-Terpinene	2–52%	Kokkini et al., 1997; Russo et al., 1998;
		p-Cymene	Trace-52%	Daferera et al., 2000; Demetzos and Perdetzoglou, 2001; Marino et al., 2001)
Rosemary	*Rosmarinus officinalis*	α-pinene	2–25%	(Daferera et al., 2000, 2003; Pintore et al., 2002)
		Bornyl acetate	0–17%	
		Camphor	2–14%	
		1,8-cineole	3–89%	
Sage	*Salvia officinalis* L.	Camphor	6–15%	(Marino et al., 2001)
		α-Pinene	4–5%	
		β-pinene	2–10%	
		1,8-cineole	6–14%	
		α-tujone	20–42%	
Clove (bud)	*Syzygium aromaticum*	Eugenol	75–85%	(Bauer et al., 2001)
		Eugenyl acetate	8–15%	
Thyme	*Thymus vulgaris*	Thymol	10–64%	(Lens-Lisbonne et al., 1987;
		Carvacrol	2–11%	McGimpsey et al., 1994;
		γ-Terpinene	2–31%	Cosentino et al., 1999; Marino et al., 1999;
		p-Cymene	10–56%	Daferera et al., 2000; Juliano et al., 2000)

[a] EOs which have been shown to exert antibacterial properties in vitro or in food models and for which the composition could be found in the literature.

[b] Percentages of total volatiles rounded up to the nearest whole number.

Burt,2004

Zones of growth inhibition (mm) showing antibacterial activity for a number of selected plant volatile oils

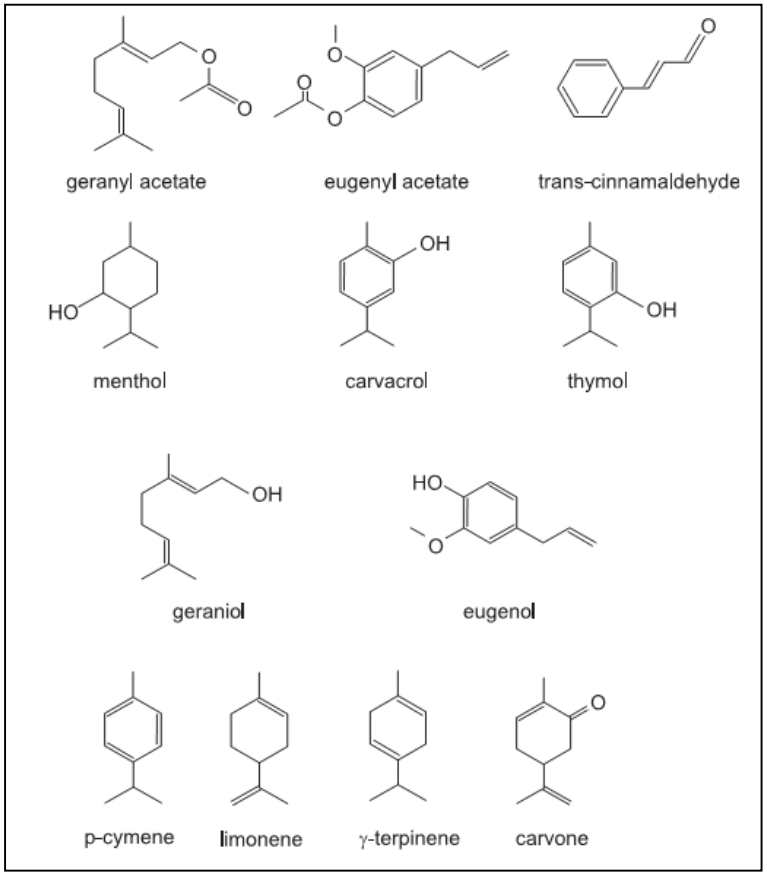
Bacterial strain	Source	*Myristica fragrans*	*Origanum vulgare*	*Pelargonium graveolens*	*Piper nigrum*	*Syzygium aromaticum*	*Thymus vulgaris*
Acinetobacter calcoacetica	NCIB 8250	12.7 ± 1.3	52.2 ± 1.5	13.0 ± 0.3	12.3 ± 2.0	10.3 ± 0.2	30.7 ± 0.5
Bacillus subtilis	NCIB 3610	7.0 ± 0.4	20.5 ± 0.4	11.4 ± 0.6	9.5 ± 0.6	21.1 ± 0.1	23.4 ± 1.2
Clostridium sporogenes	NCIB 10696	No inhibition	>90.0	7.8 ± 0.6	8.7 ± 0.3	13.4 ± 0.5	>90.0
Enterococcus faecalis	NCTC 775	18.5 ± 1.2	17.9 ± 0.8	19.8 ± 2.1	8.8 ± 0.9	15.5 ± 0.6	41.8 ± 0.8
Enterobacter aerogenes	NCTC 10006	No inhibition	14.6 ± 0.1	No inhibition	No inhibition	7.8 ± 1.1	15.2 ± 0.7
Erwinia carotovora	NCPPB 312	14.1 ± 2.6	31.2 ± 1.4	No inhibition	12.9 ± 1.0	11.7 ± 0.4	35.8 ± 4.4
Escherichia coli	NCIB 8879	10.4 ± 0.1	29.5 ± 3.4	No inhibition	7.3 ± 0.4	13.6 ± 0.3	32.4 ± 0.1
Flavobacterium suaveolens	NCIB 8992	16.9 ± 0.9	9.4 ± 0.7	30.9 ± 5.4	10.1 ± 0.1	14.4 ± 0.2	>90.0
Klebsiella pneumoniae	NCIB 418	16.9 ± 0.9	19.0 ± 1.5	13.8 ± 0.2	No inhibition	9.1 ± 0.1	31.8 ± 0.5
Moraxella sp.	NCIB 10762	6.4 ± 0.2	31.4 ± 1.9	No inhibition	5.4 ± 0.2	15.8 ± 0.8	29.0 ± 5.6
Pseudomonas aeruginosa	NCIB 950	No inhibition	>90.0	19.4 ± 0.1	7.7 ± 0.9	14.0 ± 1.9	33.5 ± 2.0
Serratia marcescens	NCIB 1377	8.2 ± 0.3	18.9 ± 0.4	8.5 ± 0.4	7.5 ± 0.4	21.6 ± 0.9	39.1 ± 0.8

Values for zone of growth inhibition are presented as mean ± SEM. **(Dorman and Deans,1999)**

Structural formulae of selected compounds of essential oils (EOs)

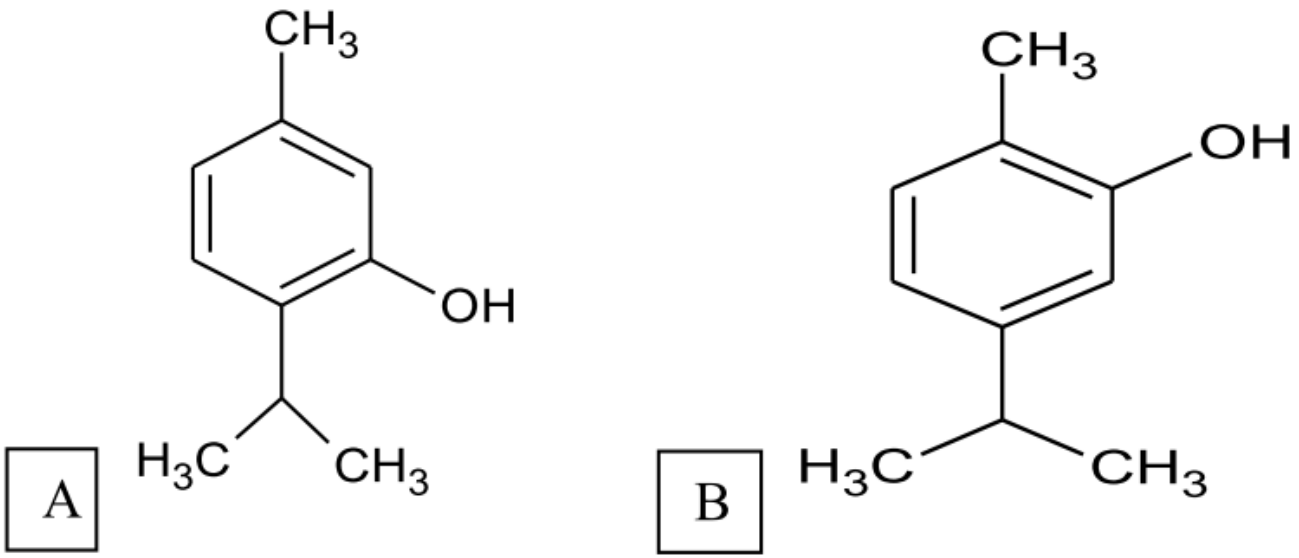
e.g. Geraniol, eugenol, p-cymol, menthol, limonene

- **37 monoterpenes found in essential oils:**
- **The structural formulae of a number of antibacterial components.**
- Monoterpenes are a class of terpenes that consist of two isoprene units and have the molecular formula $C_{10}H_{16}$.



Burt,2004

Structural formulae of selected compounds of essential oils (EOs)



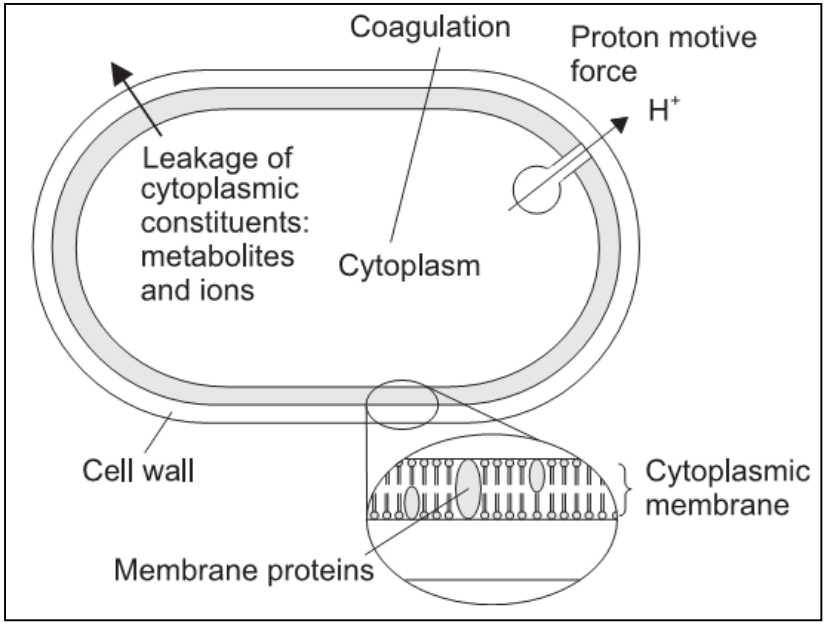
A) Thymol and B) Carvacrol

Nagy,2010

Possible mechanism of action
Essential oils

- Locations and mechanisms in the bacterial cell thought to be sites of action for EO components:

1. degradation of the cell wall
2. damage to cytoplasmic membrane
3. Damage to membrane proteins
4. leakage of cell contents
5. coagulation of cytoplasm and
6. depletion of the proton motive force.



Burt,2004

Antibacterial activities of some essential oils
Bio-organic agriculture

- The essential oils may be useful bactericides for the control of plant bacterial diseases and, in particular, in a bio-organic agriculture.
- Of particular interest is the possibility to use the above substances for seeds treatment.
- The significant antibacterial activity of essential oils toward the bacterial pathogens of mushroom suggest the possibility to use the substances also on this crop.
- Of course, other studies are necessary to evaluate the toxicity of the above substances toward seeds and/or plants and to set the appropriate formulations useful for the purpose.

Comparison of antimicrobial activity (mm) of the essential oils of *Lippia chevalieri* and *Ocimum canum with* antibiotics on agar disc diffusion method

Bacteria	Gentamicin	Neomycin	*L. chevalieri* oil		*O. canum* oil	
			Leaves	Flowers	Leaves	Flowers
Bacillus cereus	26	30	16	8	22	7
Enterococcus faecalis	28	23	30	10	12	10
Escherichia coli	20	17	14	6	10	6
Listeria innocua	30	24	14	7	17	10
Proteus mirabilis	23	16	21	6	8	6
Salmonella enterica	22	23	28	11	10	9
Shigella dysenteriae	25	20	9	6	6	6
Saphylococcus aureus	30	28	15	6	21	6
Saphylococcus camorum	21	22	19	6	22	6

*Diameter of zone of inhibition (mm).

Bassole *et al.*,2005

Medicinal plants used against *Xanthomonas axonopodis* pv. *citri*

Botanical name	Common name	Family	Plant part
Hibiscus subdariffa Linn.	Roselle	Malvaceae	Dry flower
Psidium guajava Linn.	Guava	Myrtaceae	Fresh leaf
Punica granatum Linn.	Pomegranate	Punicaceae	Dry fruit skin
Spondias pinnata (Linn.f.) Kurz	Hog plum	Anacardiaceae	Fresh leaf
Tamarindus indica Linn.	Tamarind	Leguminosae	Fruit pulp

Mueller Hinton Agar (M-H Agar) is recommended for antimicrobial disc diffusion susceptibility testing of common, rapidly growing bacteria, and most human and plant pathogenic bacteria such as *Xanthomons*, *Ralstonia*, etc.

Formula/Liter

Beef Extract ..... 2 g
Acid Hydrolysate of Casein ..... 17.5 g
Starch ..... 1.5 g
Agar ..... 17 g
Final pH 7.3 ± 0.1 at 25°C

Leksomboon *et al.*,2001; Acumedia,2011

Effect of different plant species aqueous extracts on the growth of Xoo by in vitro inhibition assay method

Adhatoda vasica	**4.1c**
Allium sativum	**1.8b**
Lantana camera	**1.3a**
Streptomycin (100 ppm)	**1.4a**

Means followed by the same letters are not significantly different at 0.05 levels according to LSD.

Govindappa *et al.*,2011

Some Thai herbal extracts for inhibiting growth of *Ralstonia solanacearum*, the causal agent of bacterial wilt of tomato

No.	Common name	Scientifics name	Part of use	Inhibition zone
1.	cashew nut	*Anacardium occidentale*	nut shell	-
2.	cogon grass	*Imperata cylindrica*	all part	-
3.	guava	*Psidum guajava*	leaf	+
4.	lemon grass	*Cymbopogon citratus*	all part	-
5.	mangosteen	*Garcinia mangostana*	fruit shell	+
6.	nutsedge	*Cyperus rotundus*	root	+
7.	papaya	*Carica papaya*	leaf	-
8.	pomegranate	*Punica granatum*	fruit shell	+
9.	tangerine	*Citrus maxima*	fruit skin	-
10.	thong-phun-chang	*Rhinacanthus nasutas*	leaf	+
11.	tumeric	*Curcuma longa*	rhizome	+

1/ + inhibition zone shown
\- inhibition zone not shown

Sasitorn Vudhivanich,2003

Health and environmental impacts

Essential oil compounds

LD50 values

- Perhaps the most attractive aspect of using essential oils and/or their constituents as crop protectants is their favorable mammalian toxicity.
- Some of the pure essential oil compounds are slightly toxic, with rat acute oral LD50 values of 2-3 g kg^{-1} (viz. carvacrol, pulegone).
- But an essential oil insecticide consisting of a proprietary mixture of essential oil constituents (EcoSMART Technologies Inc.), resulted in no mortality when fed to rats at 2 g kg^{-1} (Enan, unpublished data), the upper limit required for acute toxicity tests by most pesticide regulatory agencies including the EPA in the United States.

LD50 test: The traditional LD50 (lethal dose 50 percent)test forced animals, often rats and mice, to ingest chemicals to determine the dose that resulted in the death of 50 percent of the animals.

Health and environmental impacts

- Static water toxicity tests using juvenile rainbow trout (*Oncorhynchus mykiss*) indicated that based on 96 h-LC50 values, eugenol is approximately 1500 times less toxic than the botanical insecticide pyrethrum, and 15,000 times less toxic than the organophosphate insecticide azinphosmethyl.

Health and environmental impacts

- Eugenol and other essential oil constituents are:
- non-persistent in fresh water, based on laboratory tests.
- Aso non-persistent in soils: under aerobic conditions at 23°C.
- Eugenol is completely broken down to common organic acids by soil-borne *Pseudomonas* bacteria(Rabenhorst,1996).

Commercialization of essential oil-based pesticides

1. Cinnamite™, an aphidicide/miticide/fungicide for glasshouse and horticultural crops.
2. Valero™, a miticide/fungicide for use in grapes, berry crops, citrus and nuts.

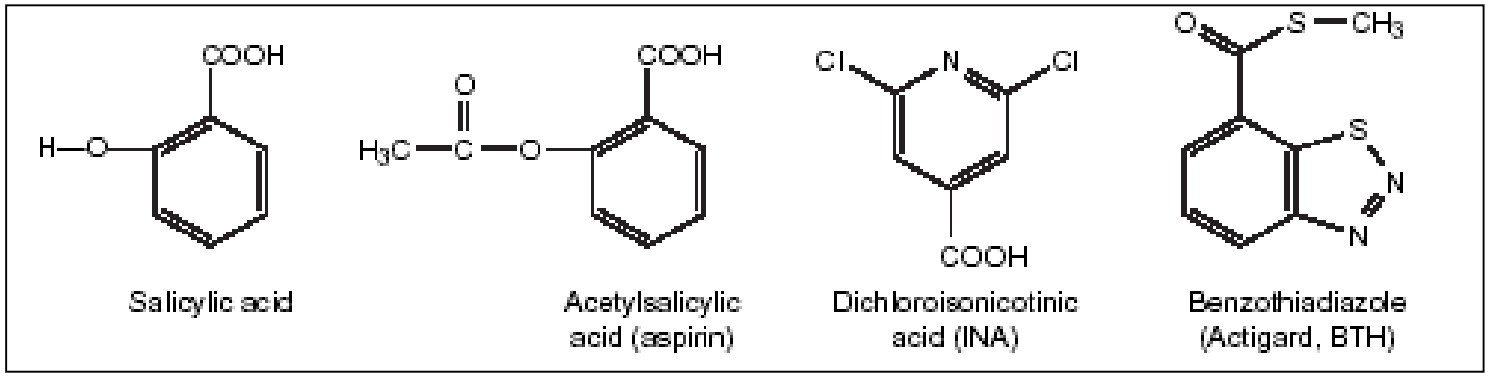
- Both products are based on cinnamon oil, with cinnamaldehyde (30% in EC formulations) as the active ingredient.

Biozell 2000B
Against *E. amylovora*

- Control of fire blight on pome fruits, caused by *Erwinia amylovora*, is of great importance for German and European fruit growing.
- Several industrial and experimental products for biological control have been developed in recent years.
- For example:
- The plant extract from *Hedera helix* revealed a high efficacy in the field in combination with a low concentrated copper compound and metal salt.
- The control of fire blight was comparable with the antibiotic streptomycin under artificial and natural infection conditions.
- Moreover, a natural product based on an etheric oil of *Thymbra spicata* (Biozell 2000B), which was developed together with Turkish scientists from the University of Akdeniz, Antalya, showed good effect under field conditions.

Plant defense activators

- Include salicylic acid (a derivative of which, acetyl salicyclic acid, is the common aspirin) and INA:



Plant activators (synthetic elicitors)

Mode of actions

- Recently, products (synthetic elicitors/activators) called "plant activators" that induce SAR in plants were identified.
- The phenomenon in which a plant can activate protective mechanisms following contact by a pathogen or invader is termed systemic acquired resistance (SAR).
- SAR inducers are potential candidates for controlling several bacterial diseases of many crops such as bacterial spot and bacterial speck of tomato (*PST*) and fire blight of apples (*Ea*).
- One such compound is elicitor acibenzolar-*S*-methyl (ASM; Actigard 50 WG, Syngenta, Basel, Switzerland).

Effective inducers of plant resistance (ISRs)

- A wide range of compounds, such as benzothiadiazoles, salicylic acid and harpin protein, are known to be effective inducers of plant resistance to diseases (Romero *et al.*,2001; Wei *et al.*,1992).
- Several mechanisms for induced systemic resistance (ISR) may operate simultaneously to control the disease, reducing the risk of development of pathogen resistance (Tally *et al.*,1999).

Plant activators

- Plant activators such as 1,2,3-benzothiadiazole (or acibenzolar-S-methyl; also known as Actigard or Bion and probenazole which induce systemic resistance in plants can be used to control bacterial leaf pathogens.
- Louws *et al.*,2001 have shown that Acibenzolar-S-methyl can be integrated as a viable alternative to copper-based bactericides for field management of bacterial speck, caused by *P. syringae* pv. *tomato*, particularly where copper-resistant populations predominate.
- Actigard was also used to control P. *syringae* pv. *tabaci* on tobacco in field trials (Cole,1999).

Höfte & De Vos,2006

Induced resistance

Effective inducers of plant resistance (ISRs)

- The compounds acibenzolar-S-methyl, a benzothiadiazole, registered as 'Actigard' in the USA and 'Bion' in Europe and South America (Syngenta Crop Protection), and harpin protein, a hrp gene product registered as 'Messenger' (Eden Bioscience) are marketed for the control of certain xanthomond diseases.
- ISR activity could potentially be deployed early in the season to slow bacterial growth in rapidly developing leaves to complement the protectant activity of Cu.

Some other synthetic plant activators against bacterial pathogens

Environmentally friendly compounds for bacterial diseases control

benzothiadiazole (**BTH**) (benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester) (CGA-245704) (acibenzolar-S-methyl; **Bion**)	Apple	*Erwinia amylovora*	Website for Bion at Syngenta
BTH	*Arabidopsis thaliana*	*Pseudomonas syringae* pv *tomato*	BTH induced PR-1, PR-2, PR-5 genes
BTH	*Phaseolus vulgaris*	*Xanthomonas campestris* pv. *phaseoli*	BTH induced chitinase, *B*-1,3-glucanase, and peroxidase. Did not induce resistance to *Pseudomonas syringae* pv. *phaseolicola* (halo blight)
N-cyanomethyl-2-chloroisonicotinamide (**NCI**)	Arabidopsis	*Pseudomonas syringae* pv. *tomato*	Website for Bion at Syngenta
DCINA	pepper , rice, pear	*Xanthomonas* spp., *Erwinia*	-

Some other synthetic plant activators against bacterial pathogens

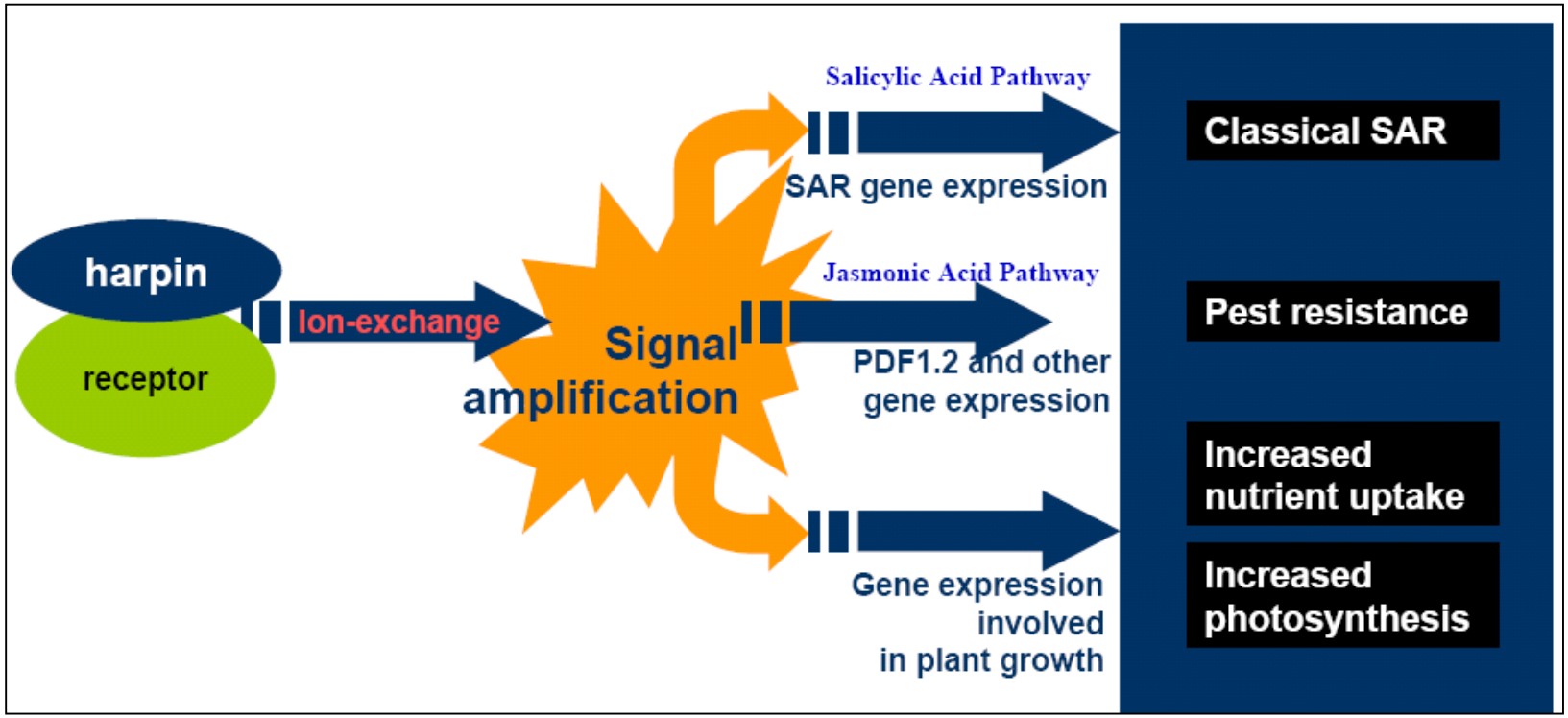
Environmentally friendly compounds for bacterial diseases control

DCINA (2,6-dichloroisonicotinic acid; CGA-41396)	Arabidopsis	*Pseudomonas syringae* pv *tomato*	-
probenazole (Oryzemate) (**PBZ**; 3-allyloxy-1,2-benzisothiazole-1,1-dioxide)	Arabidopsis	*Pseudomonas syringae* pv. *tomato*	PBZ1 mRNA induced
1,2-benzisothiazol-3 (2H)-one 1,1-dioxide (**BIT**)	Arabidopsis	*Pseudomonas syringae* pv. *tomato*	-
VacciPlant (a glucan from brown algae)	Apple trees	*E. amylovora*	Goëmar web site
Messenger® (Based on harpin from bacteria). ▪ General properties: SAR protein with systemic action, unknown resistance risk, host resistance as mode of action.	Tested on more than 45 crops including cotton, citrus, wheat, rice, tomato, peanut, pepper, rose, strawberry, cucumber.	Many fungal and bacterial apthgens such as *E. amylovora*	Eden Bioscience, Bothwell, Washington

What is Harpin?
The commercial product Messenger

- Harpin is a naturally occurring bacterial protein present in a number of species of plant pathogenic bacteria.
- The first harpin protein was isolated from the bacterium *Erwinia amylovora.*
- Early in the characterization of harpin from *Erwinia amylovora*, it was discovered that harpin could elicit disease resistance in plants and, surprisingly, increase plant growth.
- Based on these findings, harpin was developed at EDEN Bioscience into the commercial product Messenger®.

Messenger
Mode of action



Other harpin proteins

- Harpin from *Erwinia amylovora* is one member of a family of related proteins from several different plant pathogenic bacteria.
- Different harpin proteins may be able activate disease resistance to different spectrums of plant pathogens and may have different levels of potency.
- These other harpin proteins are a potential resource for further development of Messenger technology.

Development of Harpin as a Commercial Product

- Injection of purified harpin protein into a plant made that plant resistant to a subsequent pathogen attack.
- Specifically, injection of harpin into a few leaves of the plant resulted in resistance to pathogen attack in the non-injected leaves.
- This result demonstrated that harpin can activate systemic acquired resistance (SAR), a well characterized plant defense mechanism that provides resistance to a variety of viral, bacterial, and fungal pathogens.

Harpin interacts with plants to elicit disease resistance and enhanced growth

- Harpin elicits disease resistance by activating multiple defense signal transduction pathways leading to activation of defense genes.
- Harpin accelerates plant development.
- This includes specific effects on development such as increased root and shoot biomass, early flowering, early fruit set, early fruit maturation, and increased fruit number.
- Harpin affects basic physiological processes that are important for growth including increased photosynthetic activity and nutrient uptake.

Systemic acquired resistance (SAR) Side-/non-effects

- Systemic acquired resistance (SAR) plant inducers have shown activity against bacterial diseases of tomato and pepper (*Xanthomonas vesicatoria*), *Xanthomonas* leaf blight on onion, and fire blight on apple (Maxson-Stein *et al.*,2002).
- Although SAR inducers may reduce disease, they may also have deleterious effects on certain plant species and/or affect yield (Gent & Schwartz,2005; Romero *et al.*,2001).
- Plant inducers have also been ineffective for disease control in some pathosystems such as for control of citrus canker (Graham & Leite,2004).

Jones *et al.*,2007