Asian PGPR 2015 Hanoi Vietnam

Session 6, 1:40 - 4:00 PM, PGPR: Molecular Communication

Induction of systemic resistance and tolerance against biotic and abiotic stress in Chinese cabbage by cyclic peptides producing *Bacillus vallismortis* strain BS07M

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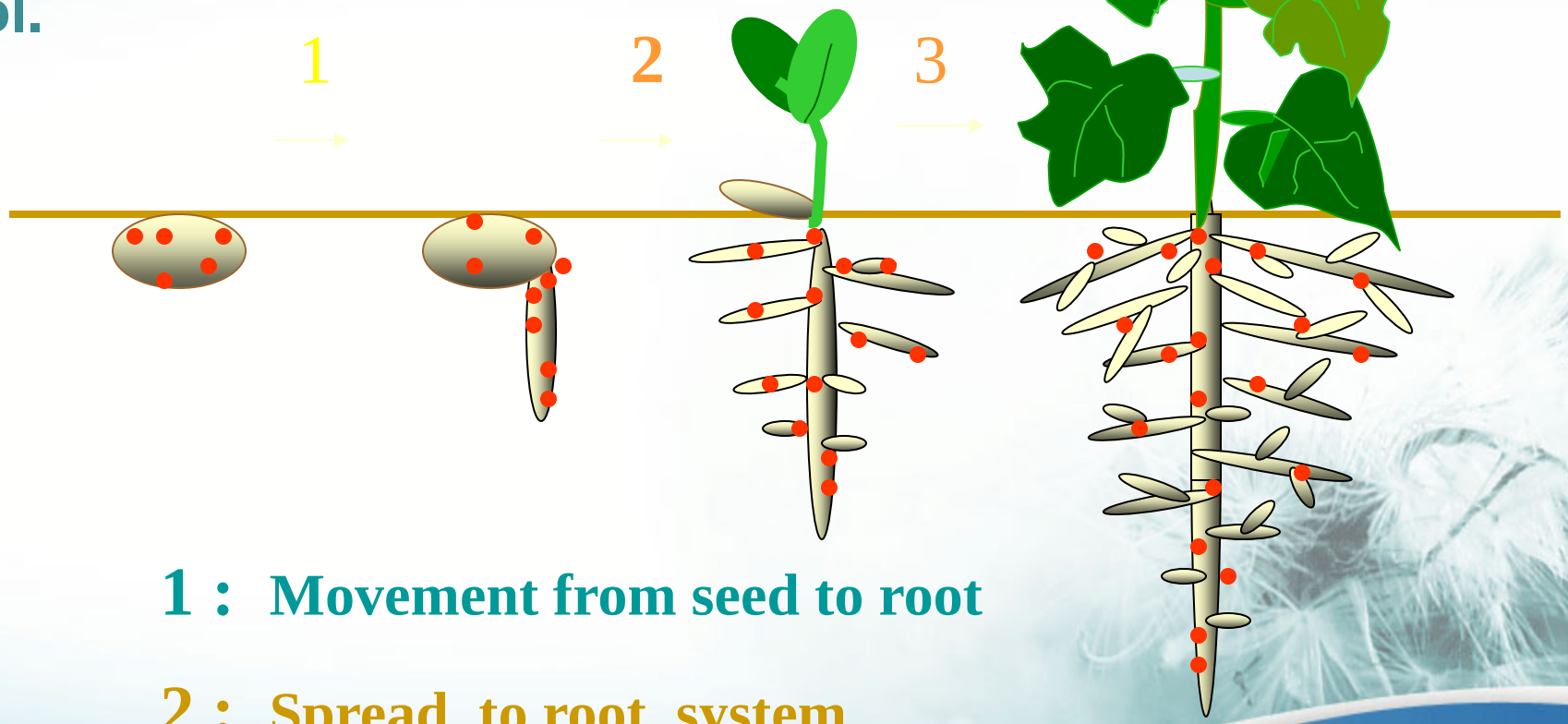
Plant Growth-Promoting Rhizobacteria

Naturally occurring root-colonizing bacteria that benefit plants by growth promotion and biological control.

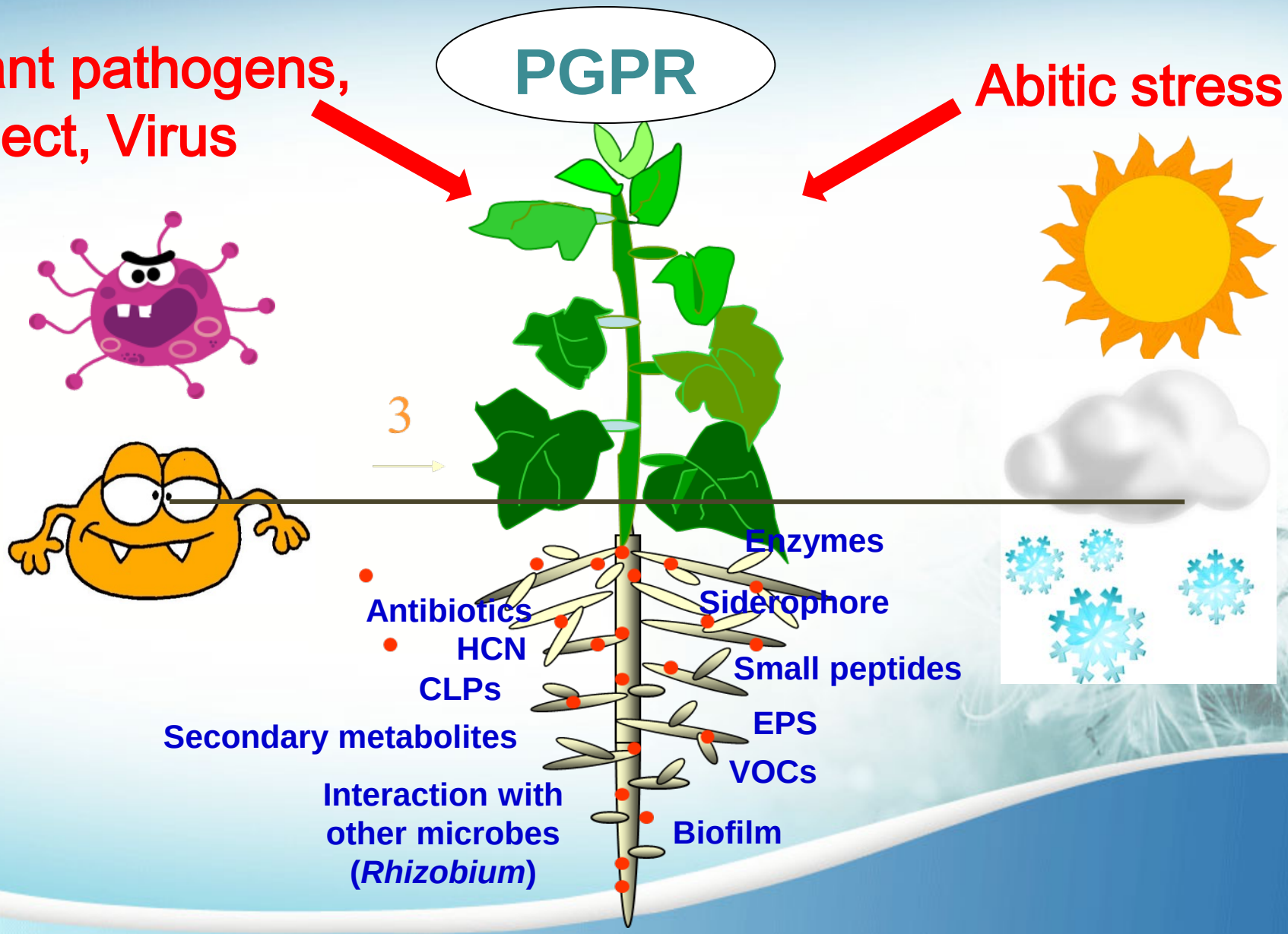


1 : Movement from seed to root

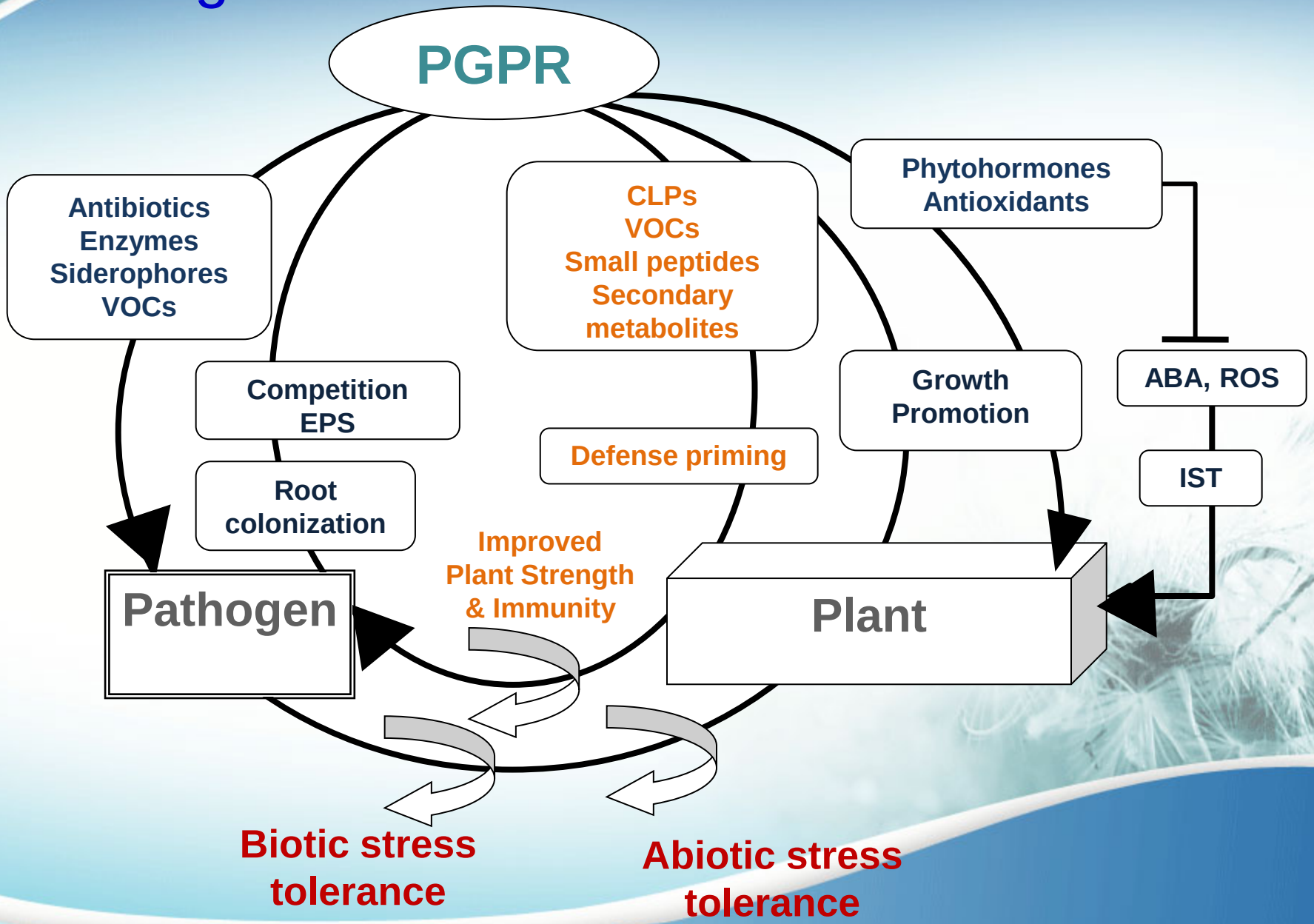
2 : Spread to root system

3 : Maintenance of population

PGPR-defense priming



PGPR against biotic & abiotic stress



Why Bacillus strains have great potential as biological control agents in environmental friendly plant health management?

- *Bacillus* spp. are essentially ubiquitous in agricultural systems.
- Important physiological traits:
  - formation of stress-resistance endospore
  - production of peptide antibiotics
  - production of small peptides elicitor for ISR
  - peptide signal molecules
  - extracellular enzymes etc.

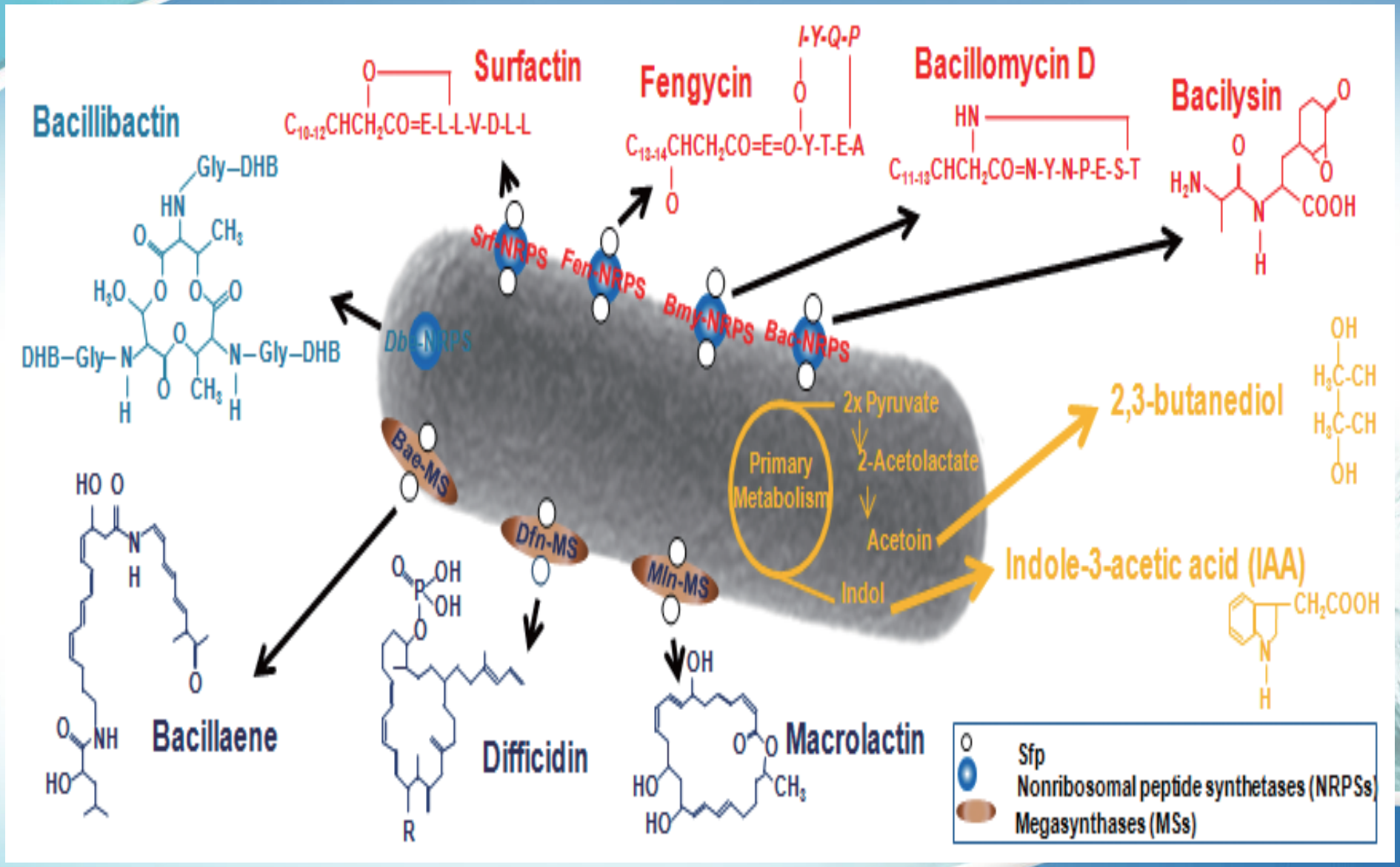


Fig. Bacillus producing metabolites(Rainer Borriss2010)

A rhizobacterium, BS07M was obtained from rhizosphere of Chili-pepper from Korea and was identified as *Bacillus vallismortis* by 16s RNA pattern.

- **Bio screening of ISR activity**
- **Defense mechanisms**
- **Dipeptide Isolation**
- **Field application**

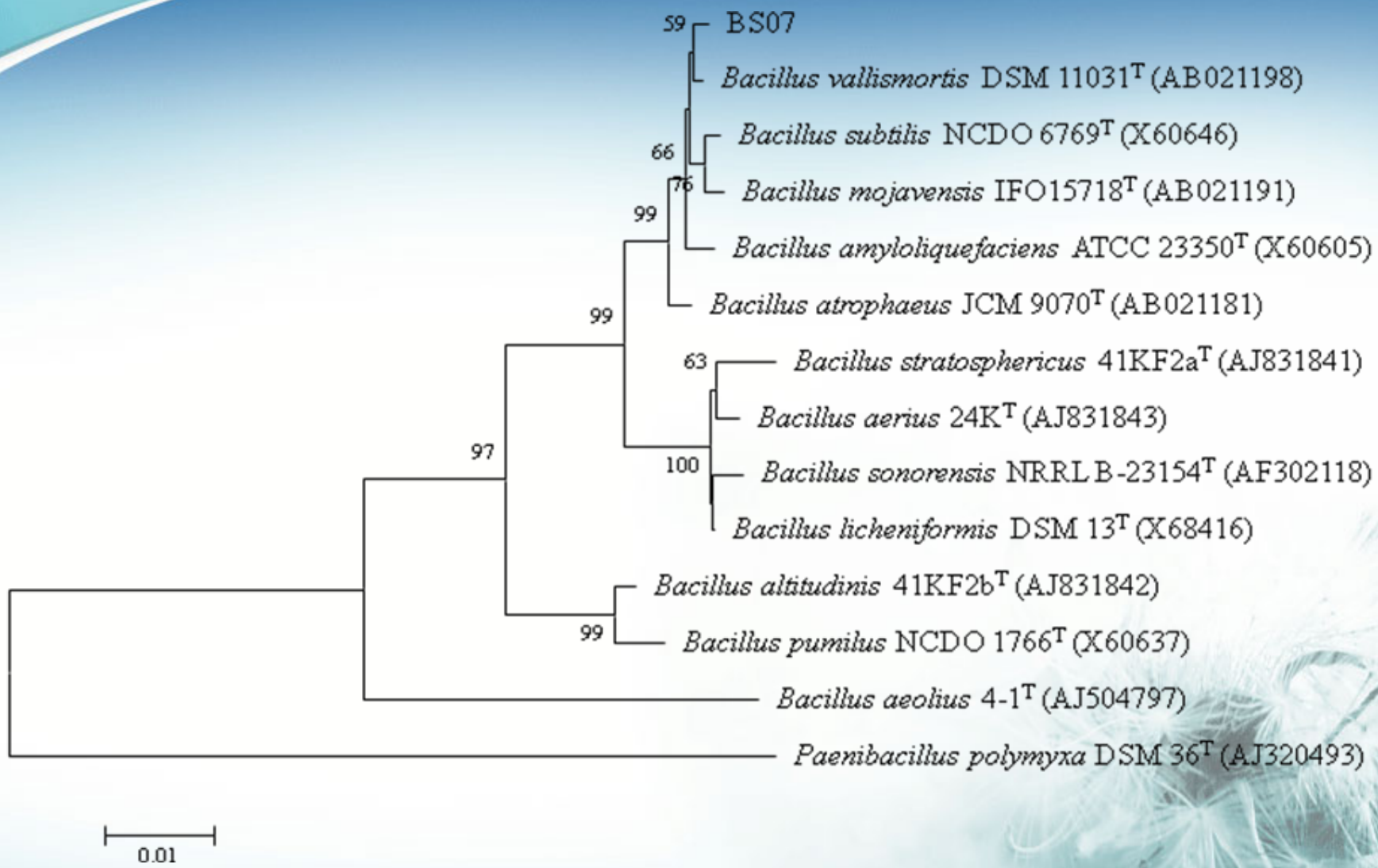


Fig. Phylogenetic dendrogram constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between strain *Bacillus vallismortis* strain BS07M and related *Bacillus* species. **Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points and the species names followed the GeneBank accession numbers. Bar, 1 substitutions per 100 nucleotide positions. *Paenibacillus polymyxa* DSM 36^T (AJ320493) was used as an outgroup.**

Bacillus vallismortis vs. closely related *Bacillus* sp.

- *B. vallismortis* is a close relative to other *Bacillus* sp. like *B. amyloliquefaciens* and *B. subtilis*
- It is distinguished from *B. subtilis* by differences in whole-cell fatty acid compositions, DNA sequences and levels of reassociation of genomic DNA (Roberts et al., 1996)
- The *gry*A sequences, code for DNA gyrase subunit A, can be used for accurate identification of *B. vallismortis* and related taxa including *B. subtilis, B. amyloliquefaciens, B. mojavensis, B. atrophaeus and B. licheniformis* (Earl et al., 2006; Chun and Bae, 2000)
- *B. vallismortis* has not been much studied as a PGPR. However, our studies have shown it to be a very potential strain for growth promotion as well as disease control

Antibiosis

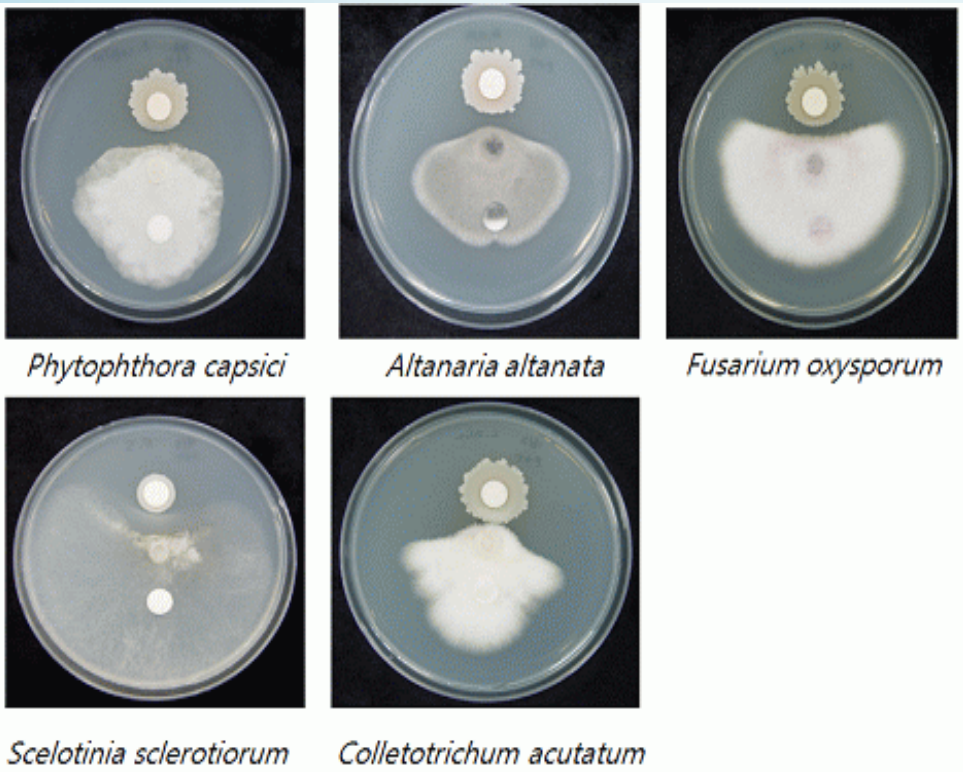
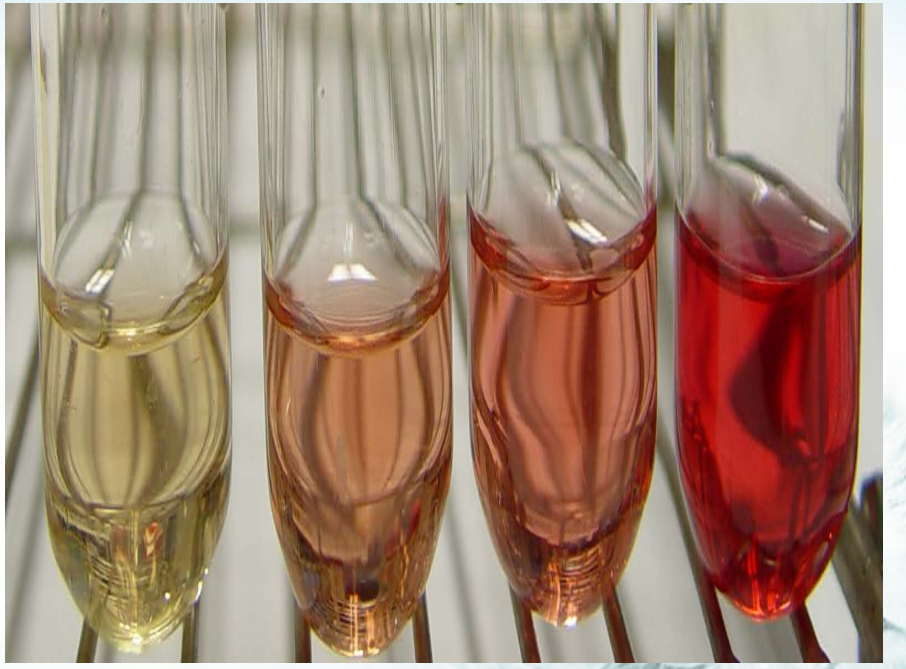


Fig. Antifungal activity against major plant fungal pathogens by dual culture with *Bacillus vallismortis* BS07M

IAA formation



Check EXTN-1 B17 BS07M

Fig. IAA formation by treatment of *Bacillus vallismortis* BS07M

Epidermal cell strengthening

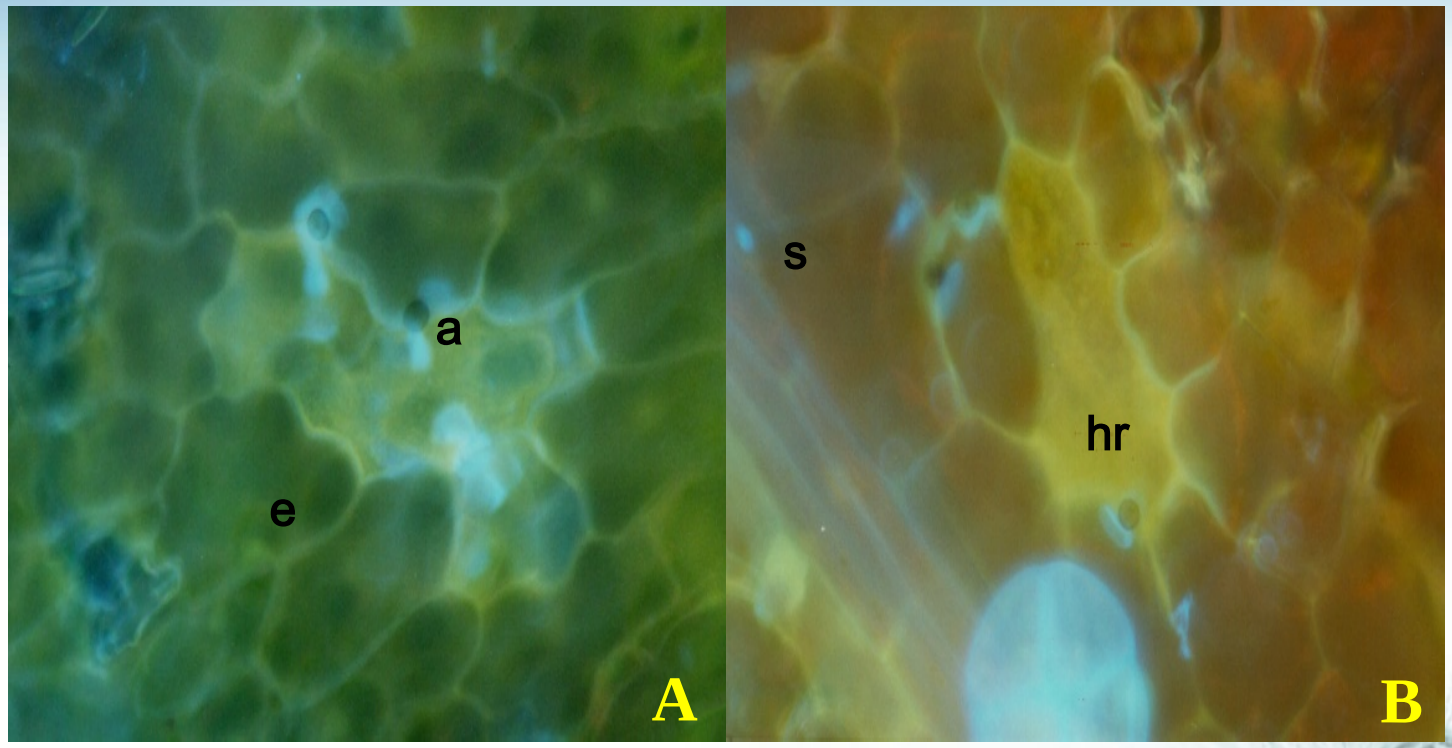


Fig. Penetration sites on the leaves of cucumber plants inoculated with C*olletotrichum orbiculare* 3 days later.

Non-treated control plants(A)and pre-inoculated plants with BS07M (B). All bars = 20 μm. Abb.: a, appressorium; e, epidermal cell; hr, hypersensitive reaction; ih, intercellular hypha; s, spore

Defence gene expression

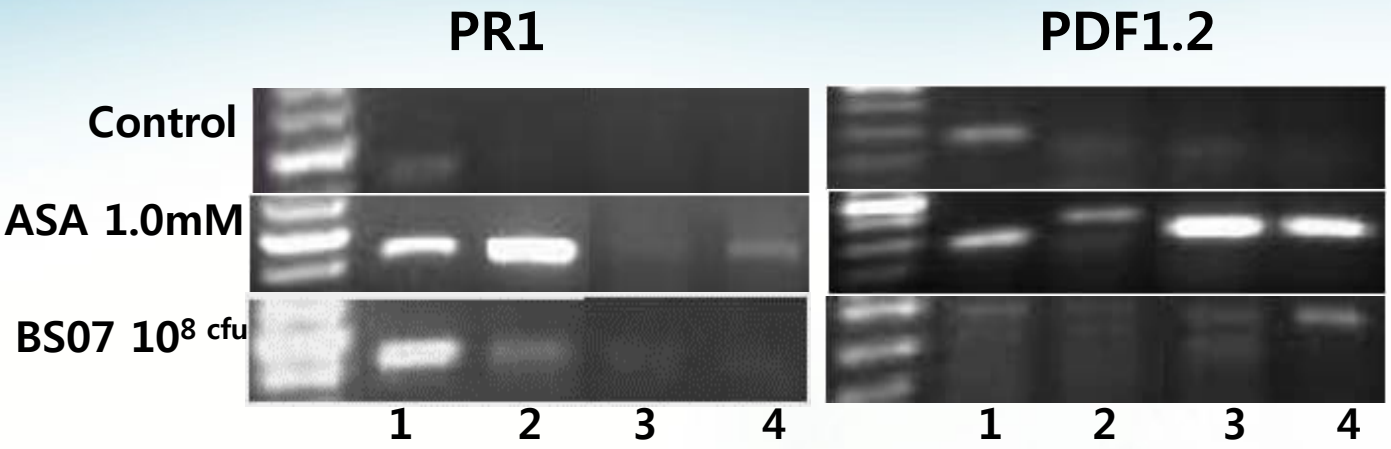
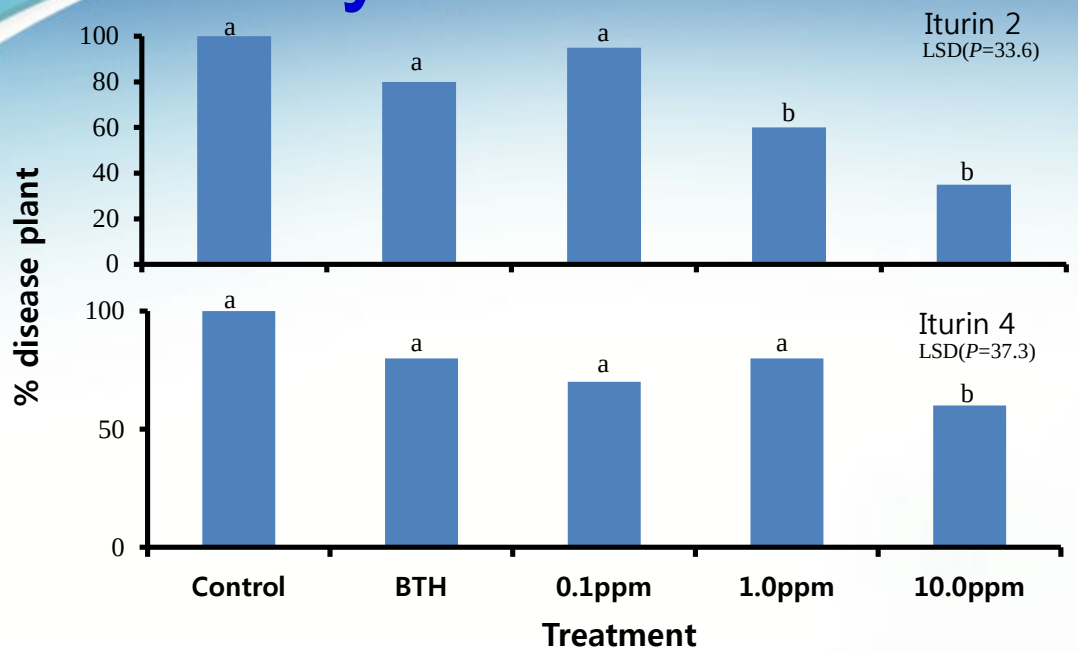


Fig. Elicitation of defense-related gene expression in *Arabidopsis thaliana* mutant lines

(lane 1: *Col-0*, lane 2: *etr3-5*, lane 3: *Nah-G* and lane 4: *npr-1*) after treatment with *Bacillus vallismortis* strain BS07. The expression of PR genes(*PR-1a* and *PDF1.2*) analyzed by RT-PCR was examined after 24 hours inoculation with BS07 on *A. thaliana* mutants.
Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. As a positive control, plants were treated with 1.0 mM ASA (Amino salicylic acid). The experiment was repeated two times obtaining with the same results.

ISR activity



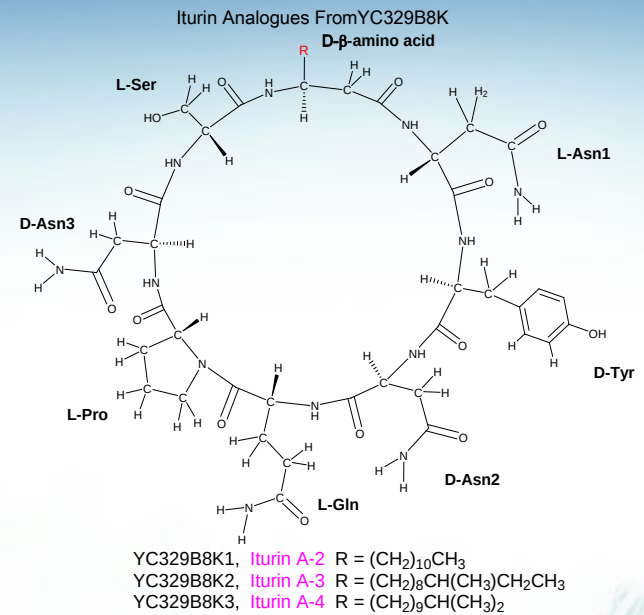


Fig. ISR by treatment of Iturin A2 & A4 from *B. vallismortis* BS07M

Fig. Induced systemic resistance on chili pepper by Iturin A2 from BS07M

ISR activities

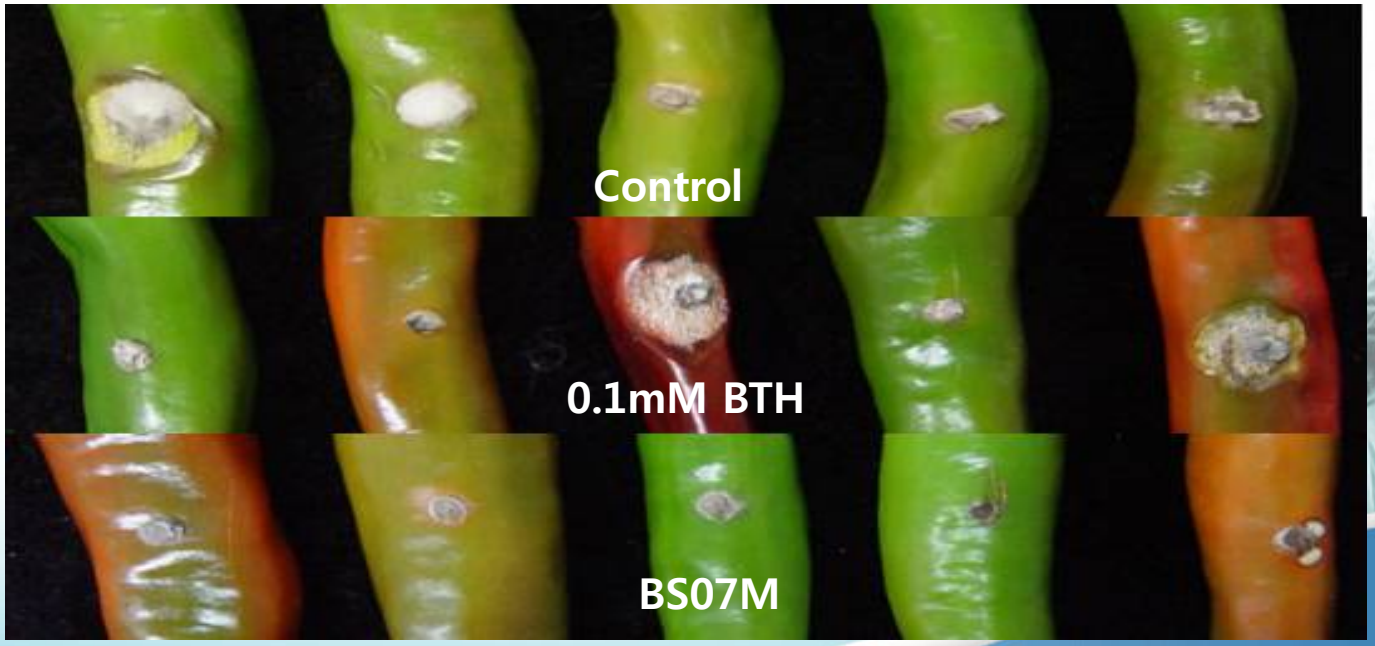


Fig. ISR activity against *P. carotovorum* SCC1 and *C. acutatum* by treatment with *B. vallismortis* BS07M

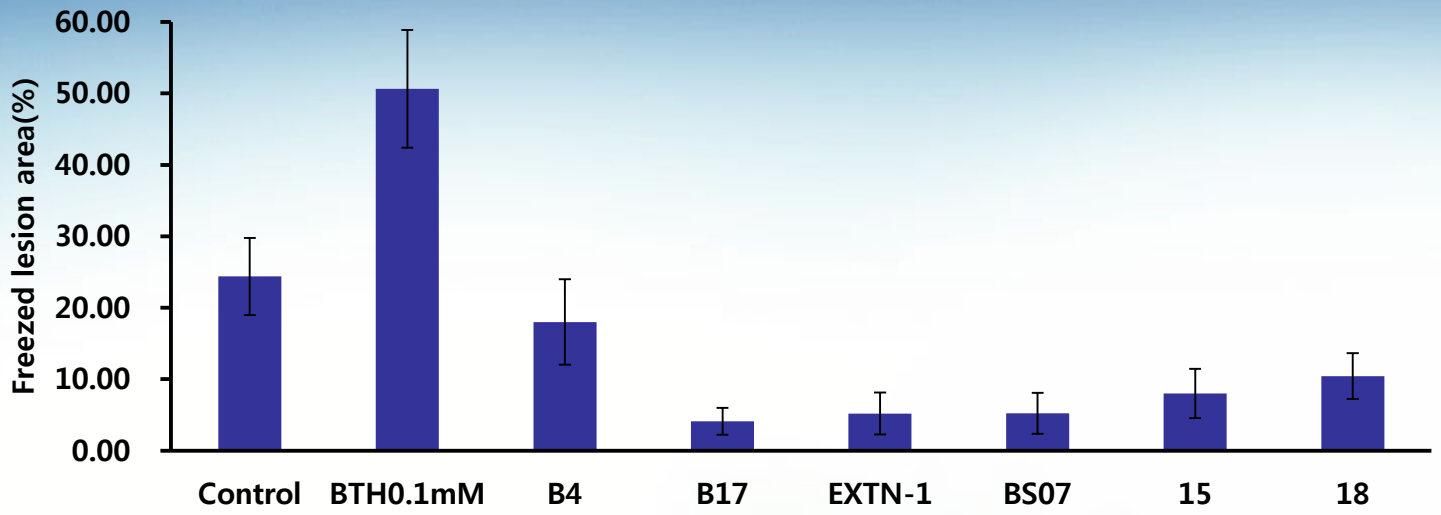


Fig. Cold stress tolerance by spray of *B. vallismortis* BS07M on chinese cabbage

Cyclic dipeptide Isolation and Biological Activity

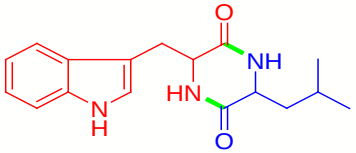
Cyclic dipeptide, Diketopiperazine, DKP Cyclo(AA-AA)

Found in beer, coffee, cocoa, Japanes sake

JAFC, **1997**, *45*, 3183-3189; **2000**, *48*, 3528-3532; **2005**, *53*, 7222-7231; **2007**, *55*, 75-79

Distributed in central nerve system, gastrointestinal tract, and body fluids. Peptides, **1995**, *16*, 151-164

Agricultural Usages



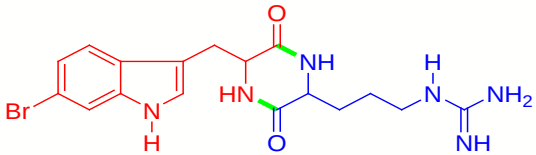
Plant growth regulator comparable to GA3

Isolated from *Penicillium brevicompactum*

Cyclo(Try-Leu) JNP, **2005**, *68*, 237-239

Drought -resistance in rice (water stress caused by sodium chloride)

Phytochem. **1990**, *29*, 35-39

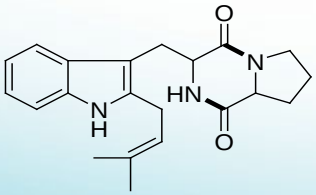


Isolated from marine sponge

Antifouling activity (potent inhibitory effect on the settlement of barnacle larvae), field-tested in antifouling paints for the ships. Ecofriendly attractive candidate to replace tributyltin oxide

Cyclo(Br-Try-Arg) JNP, **2008**, *71*, 330-333

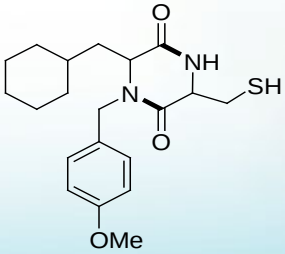
Medicinal Usages



Isolated from *Aspergillus fumigatus*

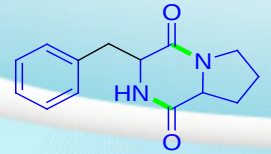
Mammalian cell growht inhibition

JMC, **2002**, *45*, 1559-1562



Collagenase-1 inhibitor (matrix metalloproteases, MMPs) - rheumatoid arthritis

JMC, **1999**, *42*, 1348-1357



Antibiotic against *Vibrio anguiiarum*

Isolated from *Pecten maximus* (marine bacteria)

Cyclo(D-Phe-D-Pro) JNP, **2003**, *66*, 11299-1301

Table 1. Specific examples of biological activity exhibited by cyclic dipeptides

Activity	Cyclic Dipeptide	Reference
Immunomodulation	cyclo(Arg-Lys), cyclo(Asp-Lys)	Blažíčková *et al.*, 1994
Modulation of Glucose Metabolism	cyclo(His-Pro)	Hwang *et al.*, 2003
Hormonal Regulation	cyclo(His-Pro)	Fragner *et al.*, 1997
Anticonvulsant	cyclo(Gly-Phe), cyclo(Ala-Phe)	Szkaradzinska *et al.*, 1994
Antifungal	cyclo(Phe-Pro)	Ström *et al.*, 2002
	cyclo(Trp-Pro), cyclo(Trp-Trp)	Graz *et al.*, 1999
Antibacterial	cyclo(Trp-Trp), cyclo(Trp-Pro), cyclo(Phe-Pro), cyclo(Tyr-Pro)	Graz *et al.*, 1999
Inhibition of Cell Division	Dehydro derivatives of cyclo(Phe-Phe)	Kamzalo *et al.*, 2000
Inhibition of Dietary Fat Intake	cyclo(Asp-Phe)	Lin *et al.*, 1994
Induction of Differentiation in Cancer Cell Lines	cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Pro-Pro), cyclo(Trp-Pro)	Graz *et al.*, 2000
Molecular Recognition by Peptide Hosts†	cyclo(Gly-Leu), cyclo(Leu-Leu)	Christofaro and Chamberlin, 1994

† Enantioselective and Diastereoselective Molecular Recognition of Cyclic Dipeptides by *a* C_2 Macrolactam Host

Preparative HPLC : YMC pac pro C18, 60-80% aqueous MeOH for 120 min, flow 7 mL/min, 220 nm

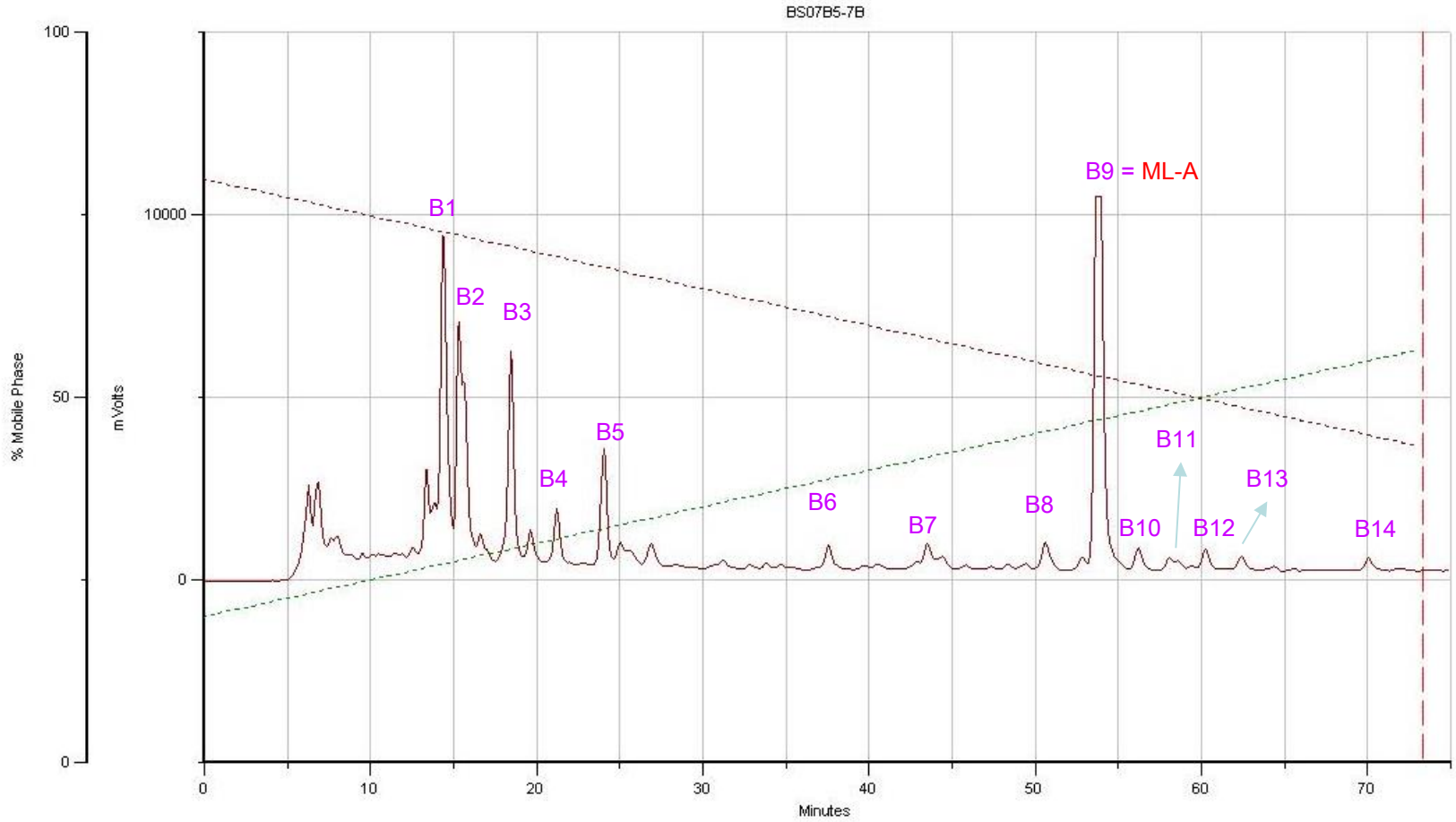
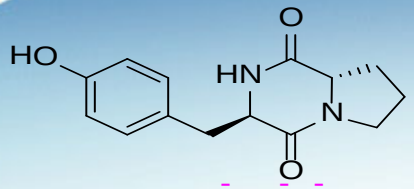
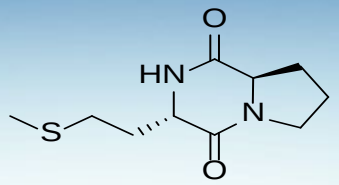


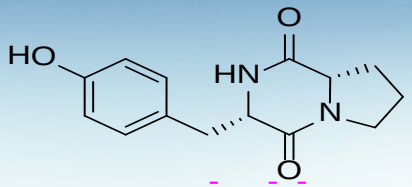
Fig. Purification of Dipeptides from culture filtrates of *B. vallismortis* BS07M by preparative HPLC



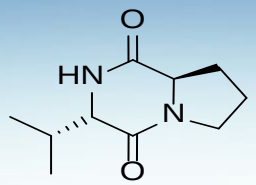
Cyclo(L-Tyr-D-Pro)
BS07M515B3B2



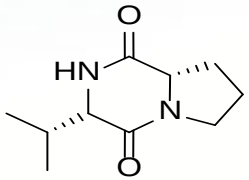
Cyclo(L-Met-D-Pro)
BS07M515B3B3



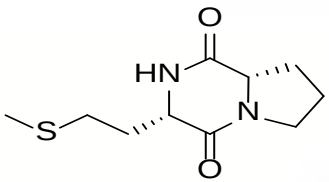
Cyclo(L-Tyr-L-Pro)
BS07M515B3B4



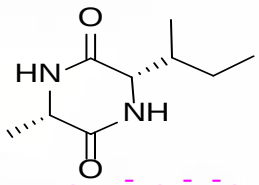
Cyclo(L-Val-D-Pro)
BS07M515B3B5



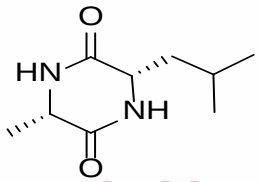
Cyclo(L-Val-L-Pro)
BS07M515B3B6



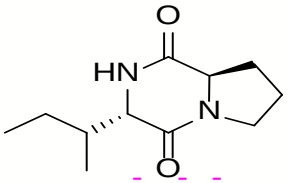
Cyclo(L-Met-L-Pro)
BS07M515B3B7



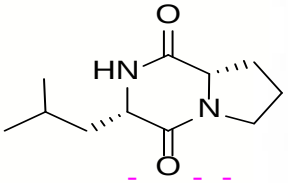
Cyclo(L-Ala-L-Ile)
BS07M515B3B8



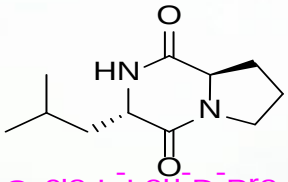
Cyclo(L-Ala-L-Leu)
BS07M515B3B9



Cyclo(L-Ile-D-Pro)
BS07M515B3B10
BS07M515B4B3



Cyclo(L-Leu-L-Pro)
BS07M515B3B12
BS07M515B4B5



Cyclo(L-Leu-D-Pro)
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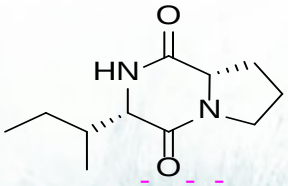

Cyclo(L-Ile-L-Pro)
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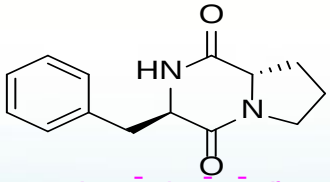
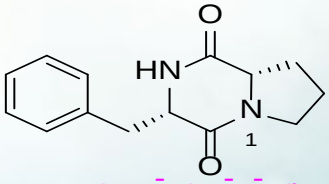


Cyclo(L-Phe-D-Pro)
BS07M515B4B6



Cyclo(L-Phe-L-Pro)
BS07M515B4B8

Fig. Identification of dipeptides from strain BS07M by NMR data

Table. Enhanced cold tolarence by cyclic dipeptides on tobacco plant

Treatments	Cold injured plant(%)		
	1.0	10.0	100 ppm
CON	71.9	71.9	71.9
BTH	84.4	84.4	84.4
P1(Thr-Pro)	46.9*	21.9*	46.9
P4(Gln-Pro)	37.5*	28.1*	78.1
H3(Ala-Pro)	50.0	56.3	65.6
Q3(Gly-Pro)	21.9*	28.1*	37.5*
D52(Cys-Pro)	71.9	75.0	90.6
MIX(P1, P4, H3, Q3, D52)	68.8	71.9	68.8
LSD(*p=0.05*)	24.7	24.5	25.8

R^1, R^6: H, C1~C6 alkyl
R^2, R^3, R^4, R^5: H, hydroxy, mercapto, amino, guadinino, carbamoyl, C1~C6 alkylcarbonyl, C1~C6 alkylthio, tritylthio, acetylamino, phenyl, hydroxyphenyl, indolyl
R^2, R^3: 5~7 membered ring
R^4, R^5: 5~7 membered ring with R6

Soft rot control

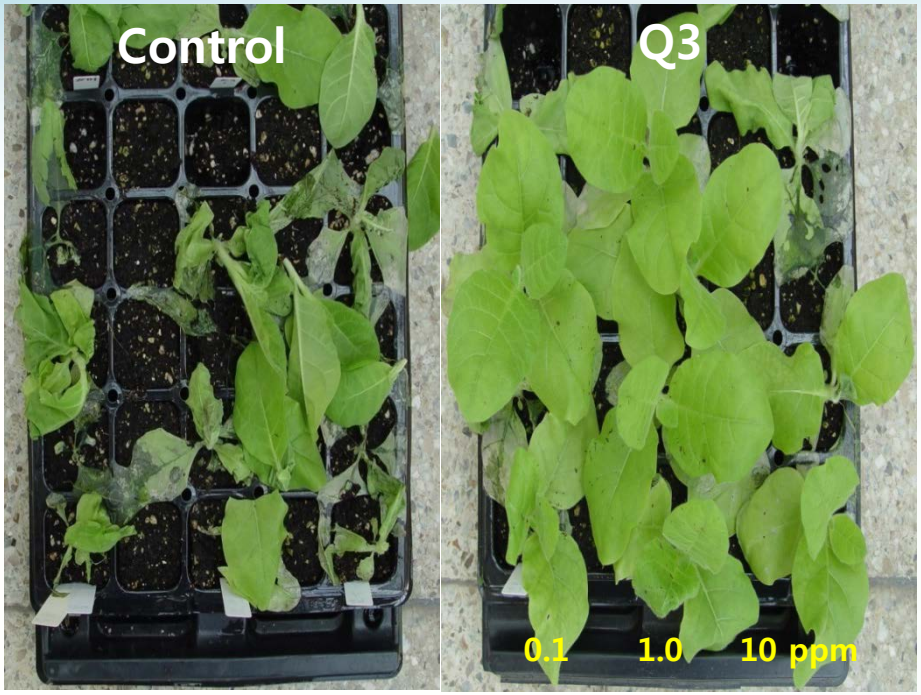


Fig. Soft rot disease control against *P. carotovora* SCC1 on tobacco plant by dipeptide Q3 of *B. vallismortis* BS07M

Cold stress tolerance

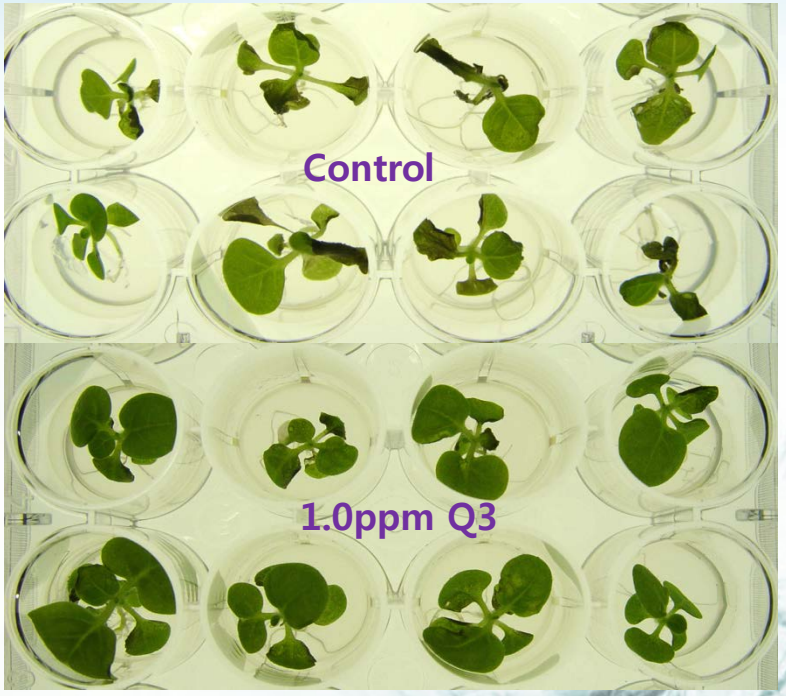


Fig. Cold resistance by cyclic dipeptide Q3 of *Bacillus vallismortis* BS07M on tobacco plant (1.0 C 24h)

Biotic stress

Abiotic stress

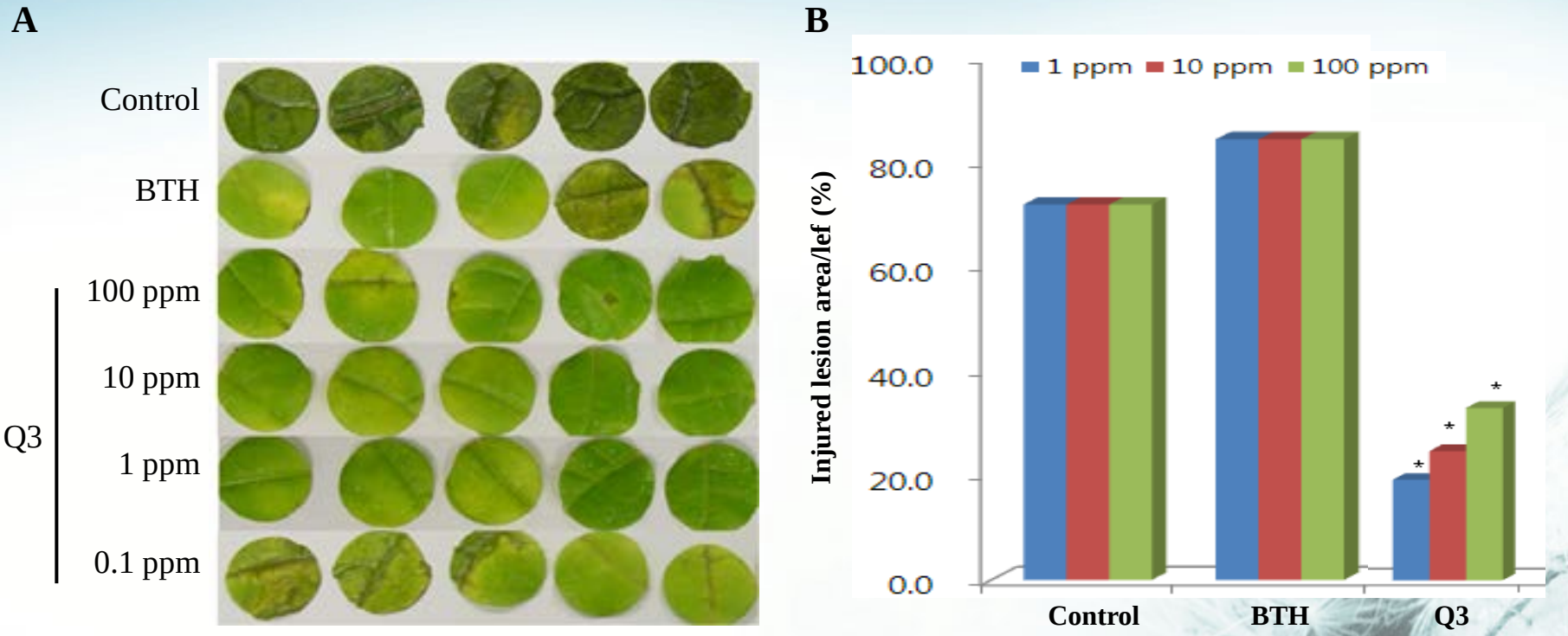


Fig. Evaluation of disease symptom and cold tolerance by synthesized Q3 (cyclic Gly-Pro) in tobacco plants.

(A)Assessment of disease incidence caused by *Pectobacterium carotovara* SCC1. Disease symptom was monitored in Q3 treated tobacco plants.

(B)(B) Q3 treated plants resulted in improvement of cold tolarence. Asterisks indicate the significant difference compared to control (*P = 0.05*).

Cucumber seedlings

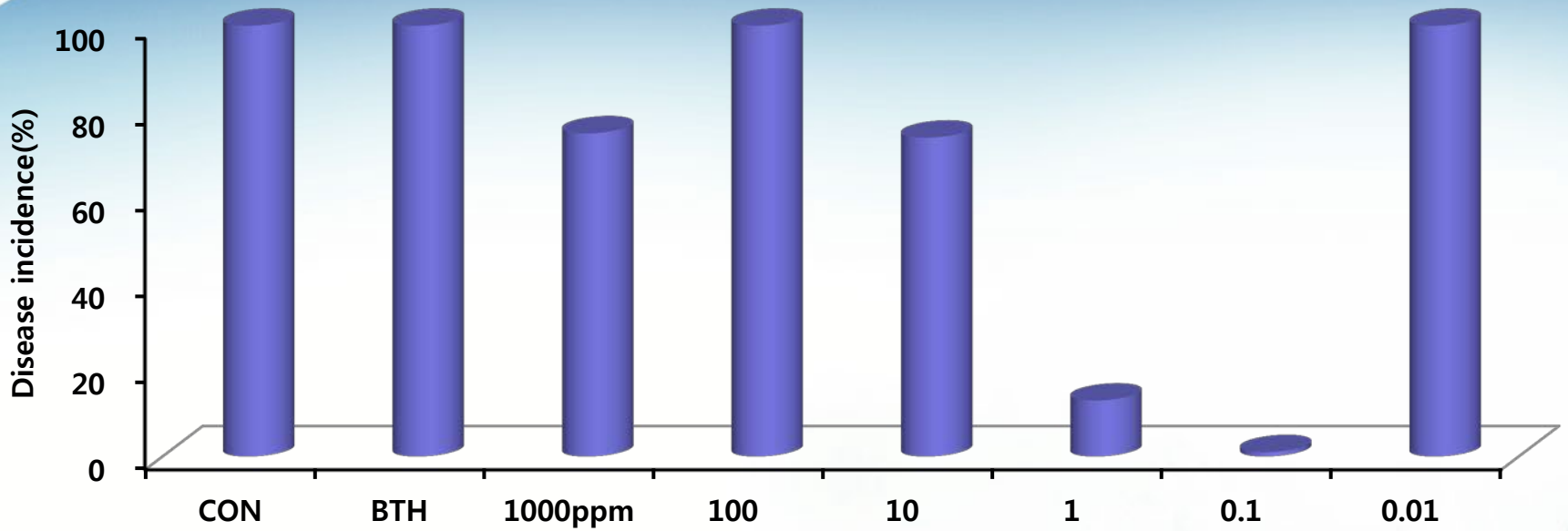
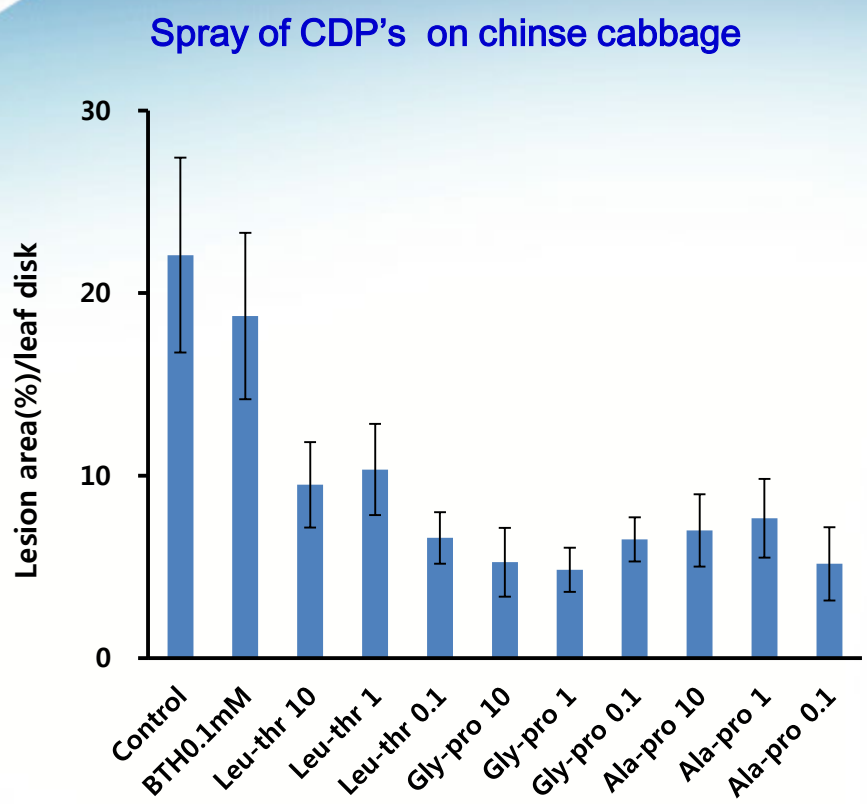


Fig. Cucumber protection against *Pectobacterium carotovora* SCC1 by infiltration of a promising cyclic dipeptide Q3



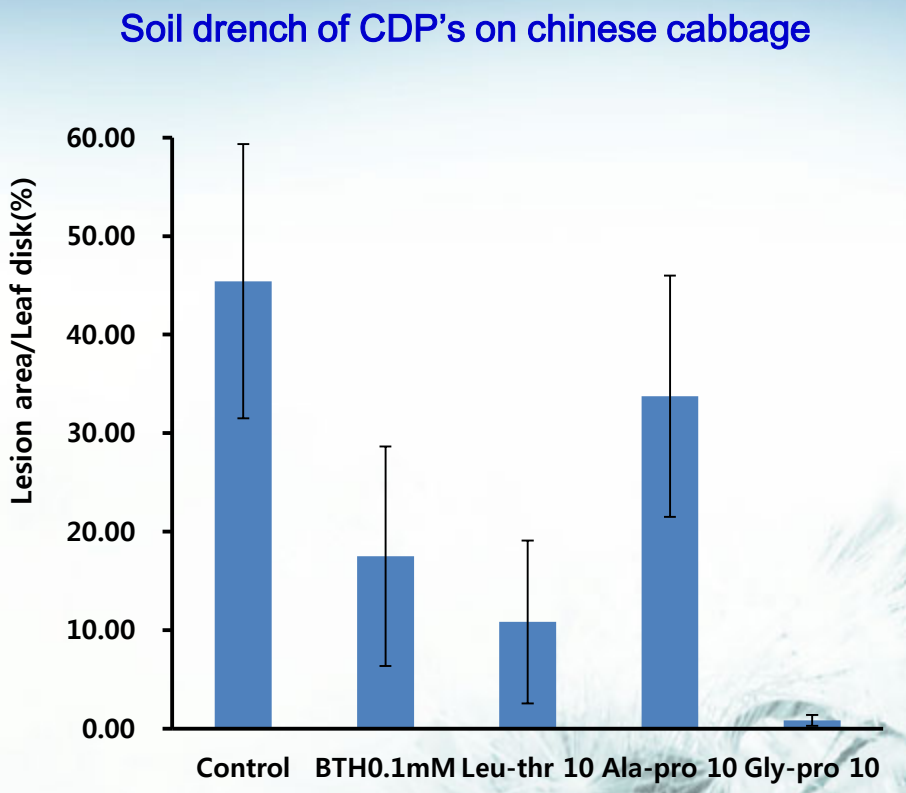
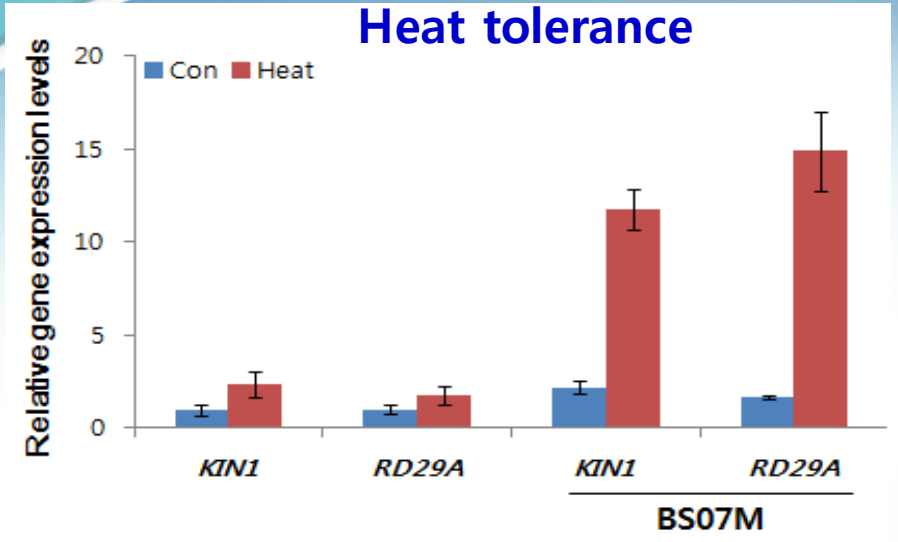
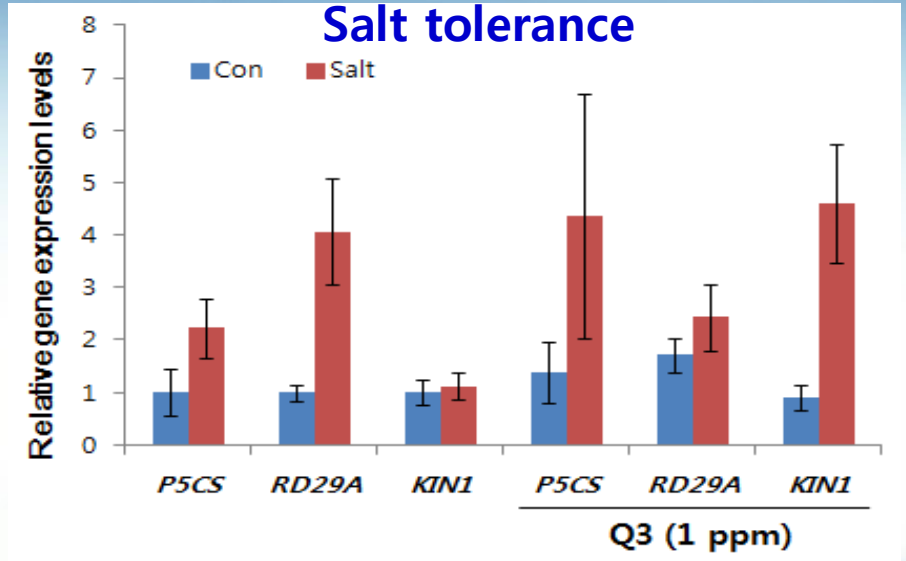


Fig. Suppression of soft rot disease on chinese cabbage by treatment of selected cyclic dipeptides





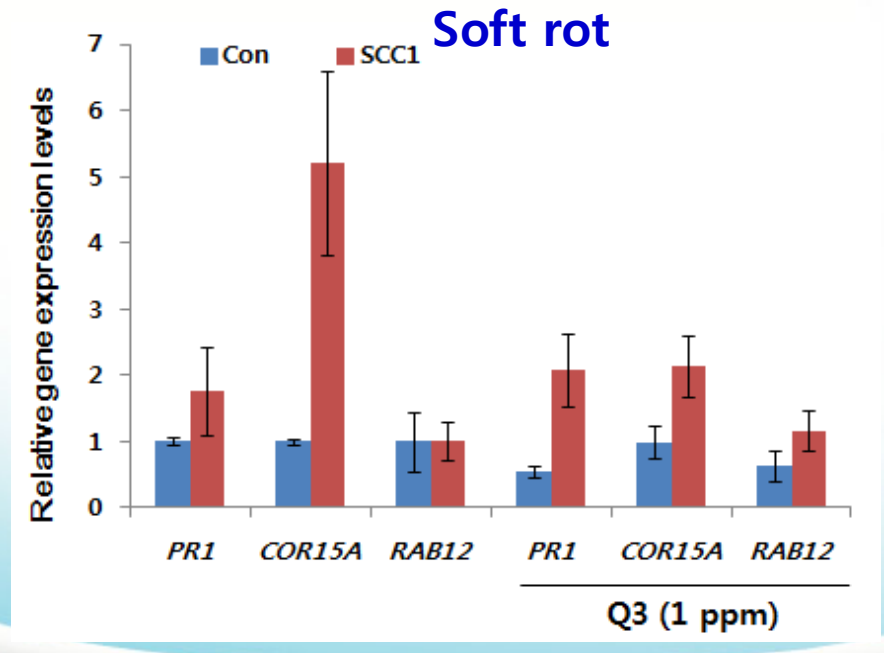


Fig. Biotic abiotic stress tolerance gene activation by treatment of Bacterial strain BS07M & Dipeptide Q3(cylic Gly-Pro) on chinese cabbage

P5CS : DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE1,

RD29A : RESPONSIVE TO DESSICATION 29A,

KIN1 : SNF1 KINASE HOMOLOG 1,

RD22 : RESPONSIVE TO DESSICATION 22,

PR1 : PATHOGENESIS-RELATED GENE 1,

COR15A : COLD-REGULATED 15A,

RAB12 : RESPONSIVE TO DESSICATION 17

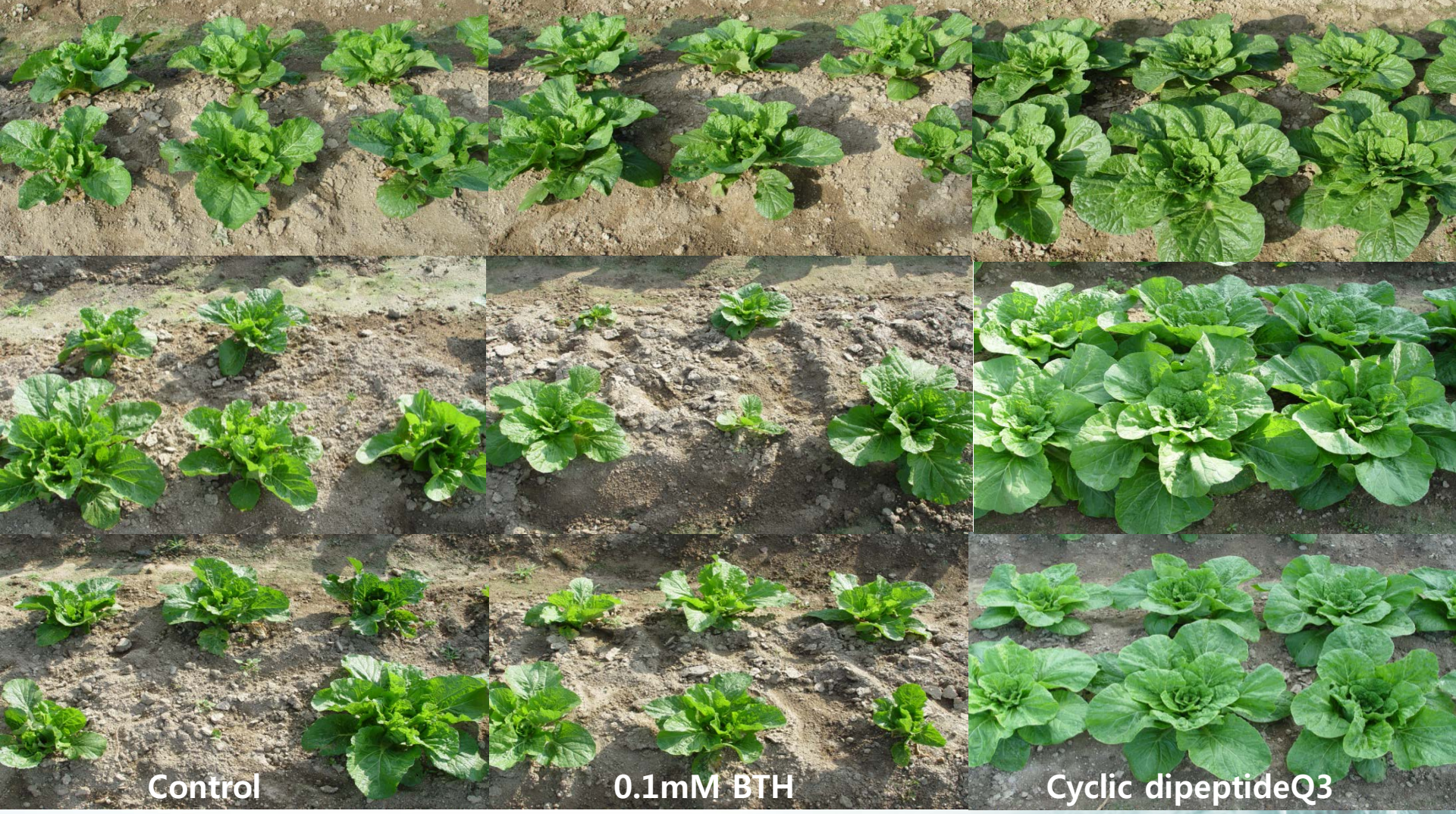


Fig. Chinese cabbage plant growth promotion by seed treatment of Cyclic dipeptide Q3 isolated from *B. vallismortis BS07* in field condition

Field Application of PGPR and Bacterial Dipeptide Q3

Field Aplication of EXTN-1 with Dipeptide Q3

SOFT ROT DISEASE IN CHINESE CABBAGE

- Chinese cabbage (*Brassica campestris* var. *pekinensis*) is a major crop in Korea

- Production of Chinese cabbage is severely affected by high temperature with heavy reduction during summer season
- Control of soft rot disease is difficult due to wide range of hosts, survival of the bacteria in plant debris in soil and host susceptibility
- Farmers opt for highland cultivation which gives geological advantage for cool temperature
- However, conventional culture practices and chemicals are either not efficient enough

PGPR Seed Priming

Application of BS07(107cfu/ml) + Dipeptide Q3(1.0ppm)
-Seed soaking 2 hours before planting
-Soil drench of BS07+Q3 suspension 3 times at weekly interval
-Transplanting of 40 day-old-seedlings in the field

Fig. Soft rot disease control by combination treatment of BS07M and Q3(Cyclic Proline- Glycine)

Fig. Abiotic stress resistance to high temperature by treatment of BS07M and Q3 on chinese cabbage(38℃, 12hrs)

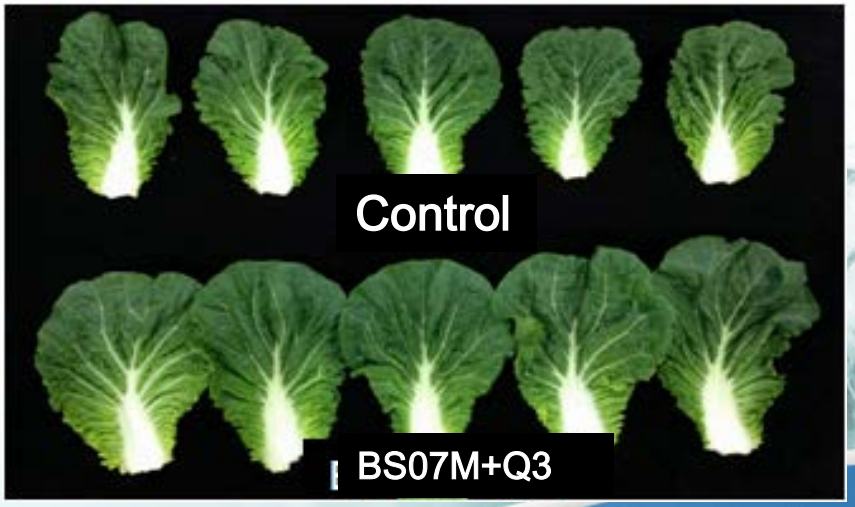
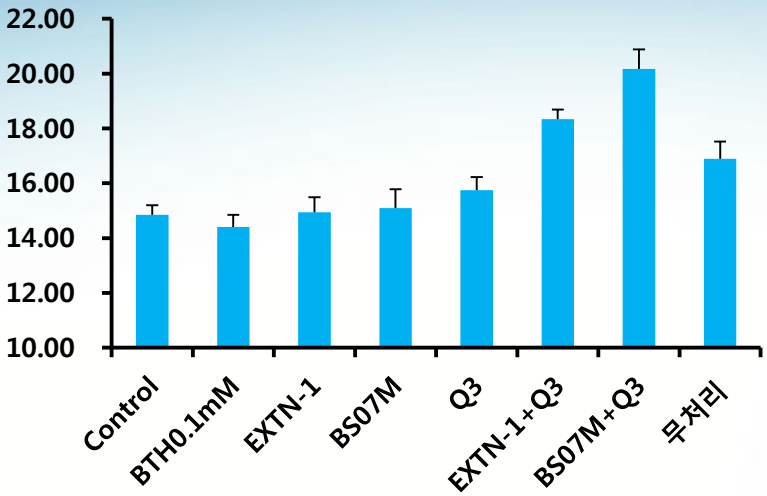
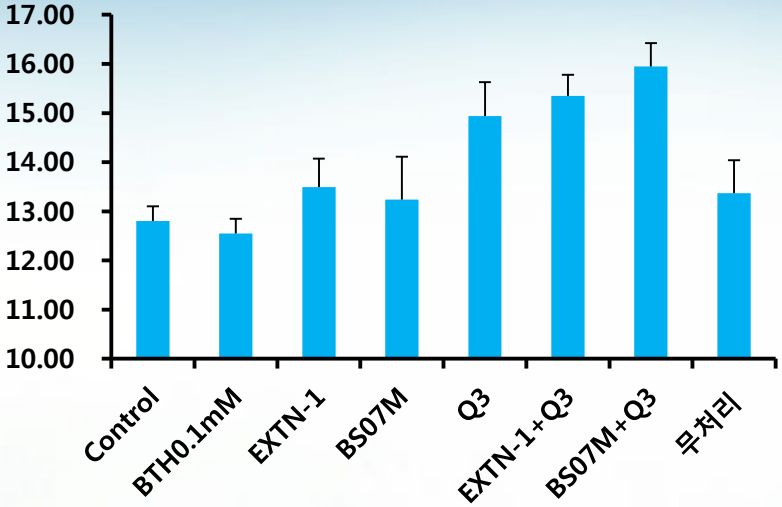


Fig. Plant growth promotion by treatment of BS07M with cyclic dipeptide Q3

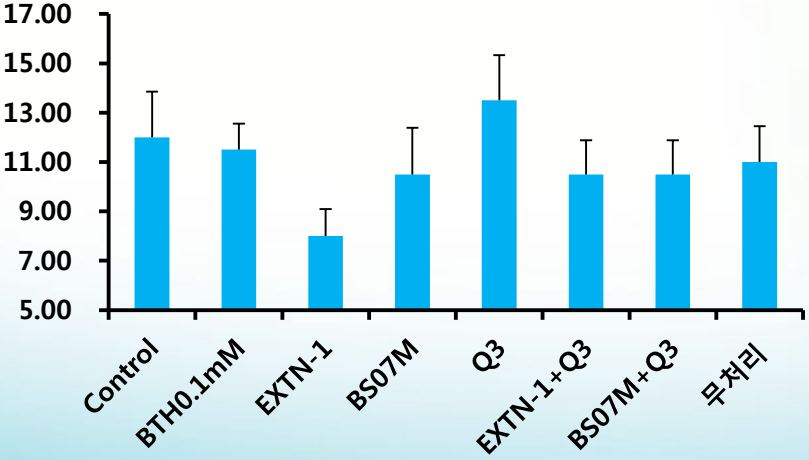
Leaf length(cm)



Leaf width(cm)



Lesion area(%)



Chlorophyll contents (SPAD Value)

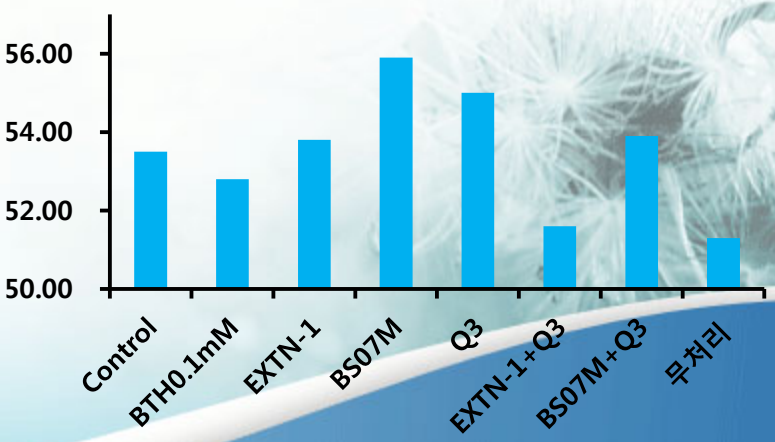


Fig. Disease suppression of bacterial soft rot against *Pectobacterium carotovora* SCC1 (Kangreung Aug. 8 2013)

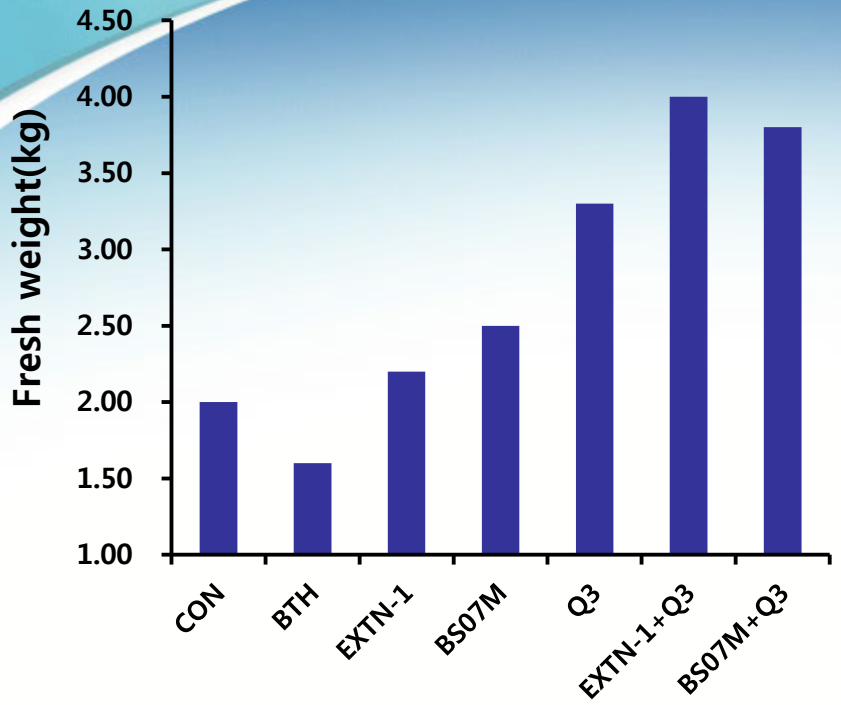
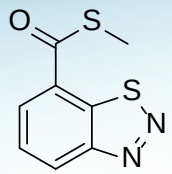
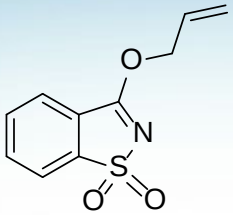


Fig. Plant growth promotion by combination treatment of BS07M with dipeptide Q3 on chinese cabbage

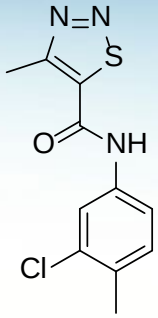
Commercial Product of Plant Defense Activator



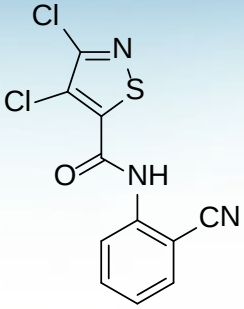
Benzothiadiazole (BTH)
Norvatis



Probenazole (Oryzemate)
Meiji Seika Kaisya



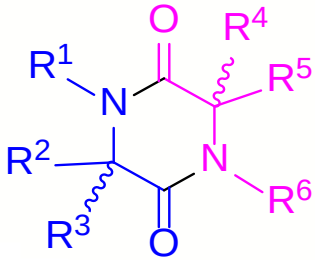
Tiadinil
Nihon Nohyaku



Isotianil
Bayer Crop Science

Fluorothiazole-ester
ECUST

A Promising Plant Dense Activator by RDA



R^{1}, R^{6}: H, C1~C6 alkyl
R^{2}, R^{3}, R^{4}, R^{5}: H, hydroxy, mercapto, amino, guadinino, carbamoyl, C1~C6 alkylcarbonyl, C1~C6 alkylthio, trityltthio, acetylamino, phenyl, hydroxyphenyl, indolyl
R^{2}, R^{3}: 5~7 membered ring
R^{4}, R^{5}: 5~7 membered ring with R6

Patent related

1. Korean patent (10-2010-0039xxx): Defense activator
2. Korean patent (10-2010-0068xxx): ”
3. Korean patent (10-2011-0070xxx): ”
4. Korean patent (10-2011-0110xxx): ”
5. PCT patent (PCT/KR2011/005xxx) : ”
   Japan, EU, USA, China

Waiting mass production and registration of the peptide !

Conclusion

- 14 CDPs were isolated from the culture broth of *B. vallismortis* BS07M and their structure were characterized base on their spectral data

- The results indicate an important role of BS07M on plant growth promotion and induced systemic resistance in plants

- Proline-based dipeptides elicit induced systemic resistance of tobacco & chinese cabbage in response to *P. carotovora* SCC1.

- Induced systemic resistance can be positively regulated by Q3 (cyclic Pro-Gly) application in a dose-dependent manner.

- The biotic & abiotic tolarence is significantly increased by treatment of BS07M plusQ3 on plants than in control plants.

Thank you..