Antifungals from fluorescent pseudomonads: Biosynthesis and regulation

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A group of root-associated bacteria, plant growth-promoting rhizobacteria (PGPR), intimately interact with the plant roots and consequently influence plant health and soil fertility. Among these PGPR, fluorescent pseudomonads occur commonly in the rhizosphere of plants and help suppress disease establishment and spread. Pseudomonads suppress soil-borne fungal pathogens by producing antifungal metabolites such as pyoluteorin, pyrrolnitrin, phenazines, and 2,4-diacetyl phloroglucinol. In addition, pseudomonads can indirectly suppress fungal pathogens by scavenging iron in the rhizosphere environment through the release of siderophores. Considering the global significance of antifungal metabolites in disease suppression and consequent applicability of pseudomonads in biological control strategies, biosynthesis and regulation of these molecules is discussed in this review to highlight new developments in the subject.

FLUORESCENT pseudomonads are ubiquitous bacteria that are common inhabitants of the rhizosphere, and are the most studied group within the genus Pseudomonas. They comprise of P. aeruginosa, the type species of the genus; P. aureofaciens, P. chlororaphis, P. fluorescens (four biotypes), P. putida (two biotypes), and the plant pathogenic species P. cichorii and P. syringae; the latter includes a large number of nomenspecies.

All fluorescent pseudomonads fall into one of the five ‘ribonucleic acid homology groups’ as defined by rRNA–DNA competition experiments. The G+C content ranges from 58 to 68%. Most of the plant-beneficial pseudomonads are quite heterogeneous in that they comprise a collection of non-enteric Gram-negative bacteria, generally aerobes, which are non-fermenting and motile. These fall into one of the three categories: pathogens, biodegraders and root-colonizers/biological control agents. The last category exerts a protective effect on the roots through antagonism towards phytopathogenic fungi and bacteria. Two major mechanisms have been proposed to explain the suppressive and antagonistic effects of fluorescent pseudomonads. According to one, the pathogen is inhibited by competition for iron, since availability of Fe [III] in this soil is low (10⁻¹⁷ M). In general, most microorgan-
isms, including bacteria and fungi excrete molecules under Fe-starvation conditions, known as siderophores, which can trap traces of insoluble iron (III) and form stable complexes. Such complexes are internalized into cells through specific membrane-bound receptors. In this manner, these organisms circumvent the solubility problem in the soil by their efficient uptake mechanisms. Among better-known siderophores of pseudomonads are high-affinity iron chelators such as pyoverdins that help compete out harmful microorganisms by reducing the levels of Fe (III). According to the second mechanism, fluorescent pseudomonads inhibit phytopathogens by producing secondary metabolites with antibiotic activity, e.g., phenazines, pyroles, acetylphloroglucinols and cyanides. However, disease suppression is a multifunctional attribute; hence the two mechanisms are not mutually exclusive. Additional factors such as aggressive root colonization play an important role in rhizosphere competence and associated biocontrol ability of a fluorescent pseudomonad. For example, we have reported that fluorescent Pseudomonas sp. strain PRS and GRP are able to survive even under heavy inoculum pressure of Fusarium oxysporum f. sp. Lini.

Production of antibiotics in several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root pathogens. A number of disease-suppressive antibiotic compounds have been characterized, including N-containing heterocycles such as phenazines, pyrrole-type antibiotics, pyo-compounds and indole derivatives (Table 1). The natural decline in ‘take-all’ disease (TAD) of wheat root caused by Gaeumannomyces graminis tritici (Ggt) during extended monoculture of wheat, is an interesting and extensively studied example of natural biological control phenomenon. Fluorescent pseudomonads are thought to be responsible for the reported biocontrol (Table 2). Furthermore, a minor class of antifungal compounds that does not have nitrogen, 2,4-di-acetylphloroglucinol (DAPG), has been studied extensively in the context of biological control of TAD of wheat. Besides secretion of antifungal molecules, fluorescent pseudomonads such as P. aeruginosa produce rhombo-lipids that have multifarious applications including biocontrol of zoospore plant pathogens. Genes involved in the biosynthesis of these antibiotic molecules have been cloned and characterized. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA) and 2,4-di-acetylphloroglucinol (Phl) are currently the major focus of research in biological control, since they help in competition within the rhizosphere milieu (Table 1). In this review, biosynthesis and regulation of antifungal molecules of fluorescent pseudomonads are described, to lay a suitable background for the development of more versatile and predictable bioinoculants in the future.

**Phl**

A broad-spectrum antibiotic, Phl is a phenolic molecule produced by many fluorescent pseudomonads and exhibits antifungal, antibacterial, antihelminthic and phytotoxic activities. In addition, it shows herbicidal activity resembling 2,4-dichlorophenoxyacetic acid (2,4-D). Phl is

![Phl Structure](image)

Table 1. Major antibiotics of pseudomonads

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Size (kb)</th>
<th>Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>phlABCD</td>
<td>2,4-Diacetyl phloroglucinol</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>phlFABCDEFG</td>
<td>Pyoluteorin</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>prnABCD</td>
<td>Pyrrolnitrin</td>
<td>5.8</td>
<td></td>
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<tr>
<td>phzFARCD</td>
<td>Phenazine</td>
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Table 2. Antifungal spectrum of antibiotics produced by pseudomonads

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>Pathogen</th>
<th>Disease</th>
<th>Producer</th>
<th>References</th>
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<tr>
<td>DAPG</td>
<td>G. graminis tritici</td>
<td>Take-all disease</td>
<td>P. fluorescens CHAO</td>
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<td></td>
<td>Pythium ultimum</td>
<td>Damping-off of sugarbeet</td>
<td>P. fluorescens Q2-87 P. fluorescens F113</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Rhizoctonia solani</td>
<td>Sheath blight</td>
<td>P. fluorescens Pf-5</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Thielaviopsis basicola</td>
<td>Black rot of tobacco</td>
<td>P. fluorescens CHAO</td>
<td>97</td>
</tr>
<tr>
<td>Pyrrolnitrin</td>
<td>Dermatophyte fungi esp.</td>
<td>Skin mycoses</td>
<td>Fluorescent and non-fluorescent pseudomonads</td>
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</tr>
<tr>
<td></td>
<td>Trichophyton</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Bipolaris maydis</td>
<td>Southern maize leaf blight</td>
<td>P. cepacia</td>
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<tr>
<td></td>
<td>Sclerotinia homoeocarpa</td>
<td>Dollar spot of turf grass</td>
<td>P. fluorescens Pf-5</td>
<td>96</td>
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<tr>
<td></td>
<td>Drechslera poae</td>
<td>Spring and fall disease of Kentucky bluegrass</td>
<td>P. fluorescens Pf-5</td>
<td>96</td>
</tr>
<tr>
<td>Pyoluteorin</td>
<td>Members of oomycetes esp.</td>
<td>Damping-off</td>
<td>P. fluorescens Pf-5</td>
<td>71</td>
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<td>Pythium</td>
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<tr>
<td>Phenazines</td>
<td>Various species of bacteria and fungi</td>
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<td>P. fluorescens 2-79, P. aureofaciens 30-84,</td>
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<td>G. graminis tritici</td>
<td>Take-all disease</td>
<td>P. aureofaciens P6112</td>
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</table>

Figure 1. Genomic region for DAPG production.28

A polyketide synthesized by condensation of three molecules of acetyl CoA with one molecule of malonyl CoA to produce the precursor monoacetylphloroglucinol (MAPG), which is subsequently transacylated to generate DAPG (Table 1).

Raaijmakers and Weller29 have demonstrated that root-associated fluorescent Pseudomonas spp. with the capacity to produce Pfl are the key components in biological control of Ggt. Pfl is also a major determinant in the biological control activity of plant growth-promoting rhizobacteria (PGPR), P. fluorescens CHAO against black root rot of tobacco caused by Thielaviopsis basicola and P. fluorescens F113 against damping-off of sugarbeet caused by Pythium ultimum. According to Picard et al.30, spatial and temporal selection in the rhizosphere is responsible for fluctuation in the population of Pfl producers and induction of Pfl producers in exudates of older plants. This means that various biotic and abiotic factors associated with field location and cropping time affect the performance of fluorescent pseudomonads.23-24. Complex biotic factors such as plant species, plant age, host cultivar and infection with the plant pathogen Pythium ultimum, can significantly alter the expression of the gene pflA.25 Among abiotic factors, carbon sources and various minerals influence production of Pfl. Fe2+ and sucrose have been reported to increase the levels of DAPG and MAPG in P. fluorescens F113, whereas in P. fluorescens Pf-5 and CHA0, Pfl was stimulated by glucose.16,26 In P. fluorescens strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon.27 Microelements, such as Zn2+, Cu2+ and Mo2+ have been found to stimulate Pfl production in P. fluorescens CHAO27. The exact mechanism of DAPG action is still unclear, although it is known that disease suppression by this antifungal molecule is a result of interaction of specific root-associated microorganisms and the pathogen. Pfl also appears to cause induced systemic resistance (ISR) in plants. Thus, Pfl-producing bacteria used in biocontrol can serve as specific elicitors of phytoalexins and other similar molecules.

The sequences of the entire biosynthetic loci of pfl are now available for P. fluorescens strain Q2-87 in the EMBL database (accession no. U41818). A total of five complete open reading frames (ORFs) and one partial ORF, within the 6.8 kb segment of DNA, is responsible for biosynthesis of DAPG (Figure 1). The genes pflA, pflC, pflB and pflD are contained within a large transcriptional unit transcribed in the same direction. This is similar to pflE, which is located downstream of pflD.28 New primers and probes have been designed by Shani29 to detect the eventual presence of pflD in fluorescent pseudomonad populations and other bacteria in natural environment. The gene pflE produces a red pigment that is involved in the transport of Pfl out of the cell. In spite of these developments, the precise role of each gene in Pfl biosynthesis is not clear. Another divergently transcribed gene, pflF is located 421 bp upstream of biosynthetic genes and consists of an ORF of 627 bp with a corresponding protein of 209 amino acids, with predicted molecular mass of 23,570 Da (Figure 1). PflF is a repressor molecule that exhibits a helix–turn–helix DNA binding motif which regulates the pfl operon.12,28,29,31

PflF regulates the biosynthesis of Pfl at the transcriptional level (Figure 1). The pflA–pflF intergenic region
displays a complex organization wherein phIA is transcribed from a σ22 RNA pol-dependent promoter that overlaps the promoter of the divergently transcribed phIF gene. Another specific sequence of 30 bp, known as phIG, is located downstream of phIA. Interaction of PhIF repressor protein with this sequence results in repression. This signifies that the repression occurs by inhibition of promoter clearance. Two more regions of 7 and 9 bp are located in the intergenic region of phIA-phIF, which not only bind the repressor but further strengthen the binding. The repression however occurs only during the early log phase, after which it is ineffective because of its interaction with the inducer Phl. Salicylate can interact with PhIF to stabilize its interaction with the phIA promoter, leading to tighter repression of Phl production. Thus, interactions of these molecules with PhIF contribute towards complex regulation of Phl biosynthesis.

Phn

Pyrolnitrin [3-chloro-4-(2’-nitro-3’-chloro-phenyl) pyrrole] is a broad-spectrum antifungal metabolite produced by many fluorescent and non-fluorescent strains of the genus Pseudomonas. It was first described by Arima et al. This highly active metabolite has been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, particularly members of the genus Trichophyton. A phenyl pyrrole derivative of Phn has been developed as an agricultural fungicide. Others like isopyrolyrinnitri, oxyipyrolnitrin and monodechloropyrolnitrin have lower antifungal activities than Phn. Pyrolnitrin persists actively in the soil for at least 30 days. It does not readily diffuse and is released only after lysis of host bacterial cell. This property of slow release facilitates protection against Rhizoctonia solani as the cell dies. The biological control agent, P. fluorescens BL915 contains four gene clusters involved in the biosynthesis of antifungal molecule Phn from the precursor tryptophan.

The prn operon has been completely sequenced; prnABCD spans 5.8 kb DNA which encodes Prn biosynthetic pathway in which four ORFs, prnA, prnB, prnC and prnD are involved (Table 1). Two stem-loop structures, which are similar to σ-independent transcription termination signals, have been identified in this sequence. One gene is located immediately 5’ to the beginning of ORF 1, while the other is located at the end of ORF 4. However, there are no σ-independent transcription termination signals within or between the four ORFs. All four ORFs are located on a single transcriptional unit. The four genes encode proteins which are identical in size. Among these, prnA gene product catalyses the chlorination of L-trp to 7-chloro-L-trp. The prnC gene product chlorinates it at the 3-position to form an amino pyrrolnitrin. The prnD gene product catalyses the oxidation of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Figure 2). The organization of prn genes in the operon is identical to the order in which the reactions are catalysed in the biosynthetic pathway. The regulation of prn operon occurs through the global regulatory gene, gacA that is described later in the review.

Phenazines

Phenazines (Phz) are N-containing heterocyclic pigments synthesized by Brevibacterium, Burkholderia, Pseudomonas and Streptomyces. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism. Growth conditions determine the number and type of Phz synthesized by an individual bacterial strain. For example, P. fluorescens 2-79 produces mainly PCA (phenazine 1-carboxylic acid), whereas P. aureofaciens 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines. The major Phz synthesized by P. aeruginosa is pyoceanin (1-OH-5-methyl Phz). It has been shown that bacterization of wheat seeds by P. fluorescens strains 30-84 and 2-79 provides primary protection against G. graminis tritici on account of release of Phz. Almost all Phz exhibit broad-spectrum activity against bacteria and fungi. In addition to inhibiting fungal pathogenesis, Phz play an important role in microbial competition in rhizosphere, including survival and competence. Use of mutants of strains 30-84 and 2-79 has confirmed their long-term survival in wheat rhizosphere on account of their ability to produce phenazine. The intense colour of this molecule, its antibiotic property and involvement in pathogenic reaction have made it an interesting molecule for study.

Phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid, wherein the amide nitrogen of glutamine serves as the immediate source of N in the heterocyclic nucleus. PCA is the first Phz formed, which gets converted to PCA and acts as the key intermediate in the synthesis of other Phz in fluorescent pseudomonads. The broad-spectrum activity exhibited by Phz compounds against fungi and other bacteria is not well understood. However, it is believed that Phz can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (MnSOD) which causes enhanced production of O2 (superoxide radical), as shown in Figure 3. There is a distinct possibility that the antibiotic action of pyoceanin is actually a result of toxicity of O2 and H2O2 produced in increased amounts in its presence.

Structural and functional analysis shows that seven genes, PhzABCD, are involved in the synthesis of PCA. These are localized within a 6.8 kb fragment in P. fluorescens 2-79 (ref. 51). The Phz biosynthetic loci in P.
**Figure 2.** Proposed biosynthetic pathway for the synthesis of pyrrolnitrin.\(^{17}\)

**Figure 3.** Site of action of Phz.\(^{65}\)

\textit{fluorescens} 2-79 (refs 50 and 51), \textit{P. aeruginosa} PA01 and \textit{P. chlororaphis} PCL 1394 are highly conserved\(^{64}\) (Figures 4 and 5). Each \textit{phz} locus contains a set of seven-gene core operons, regulated in a cell density-dependent manner by homologues of LuxI, and LuxR\(^{64,65}\). In \textit{P. fluorescens} 2-79, \textit{P. aureofaciens} 30-84, and \textit{P. chlororaphis} PCL 1391, these two homologues (\textit{phzI}/\textit{R}) are found directly upstream of the Phz core. Phz production in \textit{P. aeruginosa} is controlled by two sets of regulatory proteins, \textit{rhlI}/\textit{R} and \textit{lasI}/\textit{R} that are located elsewhere in the genome. The core gene products, PhzC, PhzD and PhzE, which are homologous with PhzI, PhzA and PhzB in strain 30-84, are similar to enzymes of shikimic acid and chorismic acid metabolism\(^{66}\) (Figure 7). PhzG is similar to pyridoxamine 5'-phosphate oxidase, which was found to be the source of cofactor for the PCA-synthesizing enzyme(s). Products of PhzA and PhzB genes are highly homologous and appear to be involved in the stabilization of a putative PCA-synthesizing multienzyme complex. Based on the functional analysis of \textit{phz} genes in strains 2-79 and 30-84, it has been suggested that various fluorescent pseudomonads have similar PCA-synthesizing systems\(^{51,67}\). Although \textit{phz} biosynthetic loci of various strains of fluorescent pseudomonads are highly homologous, individual species differ in the range of compounds they produce\(^{66}\). For example, \textit{P. fluorescens} 2-79 produces only PCA, whereas \textit{P. aureofaciens} 30-84 produces, in addition to PCA, lesser amounts of 2-OH-PIHZ-1-carboxylic acid (2-OH-PCA) and small quantities of 2-OH-PIHZ. The conversion of PCA to 2-OH-PCA in strain 30-84 is brought about by a gene \textit{phzO} which is located immediately downstream of the biosynthetic operon in strain 30-84 (ref. 51). PhzO is a non-heme, flavin-diffusible monooxygenase that adds a hydroxyl group to
Molecular mechanism of regulation

The regulation of the production of secondary metabolites such as antifungals is operated in bacteria through various mechanisms acting at transcriptional and post-transcriptional levels. An understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens, is a prerequisite for predicting the environmental conditions under which such bacteria are likely to perform optimally. Three levels of regulation have now been suggested: a primary sensing level which is dependent on the surroundings and a secondary or intermediate level that is responsible for regulation of antibiotic biosynthesis with other metabolic processes through global regulation and cellular homeostasis, and a highly specific tertiary level which requires an involvement of regulatory loci that are linked and divergently transcribed from structural genes for antibiotic biosynthetic genes.

An environmentally regulated, two-component system is now known to be essential for antibiotic production in various Gram-negative bacteria, including *Pseudomonas*. This prokaryotic, two-component regulatory system is a transmembrane protein that functions as a sensory kinase GacS and the cytoplasmic cognate response regulator GacA protein that mediates changes in gene expression in response to sensor signals. As a consequence of interaction with unknown signals, GacS sensor activates GacA response regulator by phosphorylation. The activator GacA, by virtue of its typical C-terminal helix–turn–helix motif, regulates the transcription of the target genes. While the direct GacA targets are not known, GacS/GacA system exerts a positive effect on cell density-dependent gene regulation; the latter is mediated by *N*-acylhomoserine lactone (AHL) in *P. aeruginosa*, *P. syringae* and *P. aureofaciens*. However, a similar system also effectively operates in other Gram-negative bacteria which do not produce AHL, e.g. *P. fluorescens* CHAO; here, GacS/GacA strictly controls the expression of extracellular products such as exoenzymes, antibiotics and HCN when cells are in idiophase, i.e. transition from exponential to stationary phase.

Bacterial populations in their natural habitats have now been reported to communicate with each other through chemical signals that are released in a cell density-dependent manner. This is referred to as quorum sensing (QS), i.e. a minimum cell number, and operates through two...
Figure 6. Proposed mechanism for the production of PCA and 2-OH-PHZ\textsuperscript{31}.

Figure 7. Hypothetical pyoluteorin biosynthetic pathways. X indicates covalent attachment of proline either to CoA or to adenosine. 1, Acyl CoA synthetase; 2, Acyl-CoA dehydrogenase; 3, Halogenase; 4, Polyketide synthetase; 5, Polyketide synthase\textsuperscript{38}.
broad categories of molecules, viz. amino acids and short
peptide hormones commonly involved in Gram-positive
bacteria and fatty acid-derivatives such as AHLs, in
Gram-negative bacteria. On the root surface, many biofilm-
forming bacteria are present, where they can attain high
population densities and accumulate the concentration
of such signal molecules and regulate various physiological
processes. For example, a large family of regulatory systems
has now been described that closely matches the LuxI and LuxR proteins of V. fischeri. QS relies on the
fact that LuxI-type proteins synthesize AHLs (also known
as autoinducers), which diffuse from bacteria that pro-
duce them either passively or by means of active efflux
and accumulate at high population densities. AHL binds
to and activates LuxR-type receptor proteins. These function
either as cytoplasmic transcriptional factors or as
repressors.

Regulation of antifungals operates at another level as well.
This requires involvement of sigma factors that are
otherwise an integral component of regulation of antifun-
gals like Phl and Ph, e.g. P. fluorescens Pf-5. The over-
expression of activator gene rpoD or mutation deletion of
suppressor gene rpoS increases Phl or Pht production.
These genes encode sigma-factor $\sigma^{32}$ and stationary-phase
$\sigma^{35}$ respectively, required during transcription. This
suggests that $\sigma$ factors compete for RNA pol, and any im-
balance either due to excess of $\sigma^{32}$ or lack of $\sigma^{35}$ might
enhance the expression of genes; the expression is driven by
weak $\sigma^{32}$-dependent promoters. In addition, path-
way-specific regulators have been reported to be involved
in the regulation of Phl biosynthesis. For example, Phl biosynthetic gene cluster is negatively regulated by the
repressor Phl F and positively modulated by genetically-
linked PhlH. Furthermore, post-transcriptional control
of Phl production also occurs via small RNA-
binding protein RsmA and an RNA molecule, RsmB.
RsmA is a translational repressor protein. Both, GacA
and RsmA depend on the same specific ‘RBS regions’
(Ribosome Binding Site), which further, enhances
RsmA-mediated translational repression. Another factor,
RsmB exerts a relief to repression. Thus, these molecules
of RNA bind and sequester the repressor proteins. Recent-
ly, prpB encoding a regulatory RNA homologue of RsmB
has been characterized, whose overexpression restores
Phl production in gacA and gacS mutants; this leads to
overproduction of Phl in wild-type P. fluorescens.

Microbial metabolites also play an important role in
the regulation of antifungal molecules, e.g. synthesis of
DAPG is autoinduced and repressed by other bacterial
extracellular metabolites of strain CHAO (ref. 88). Fur-
thermore, salicylate and secondary metabolites (fusaric
acid and Pln) have negative effect on Phl production.
Salicylate interacts with repressor PhlF and stabilizes its
interaction with phlA promoter. This results in tighter
repression of Phl production.

Conclusion

Fluorescent pseudomonads produce highly potent broad-
spectrum antifungal molecules against various phytopath-
genous, thus acting as effective biocontrol agents. They
could serve as promising bioinoculants for agricultural
system to increase productivity since the action of such
bacteria is highly specific, ecofriendly and cost-effective.
However, selecting an appropriate inoculant requires det-
tailed analysis of secondary metabolites of effective bact-
eria, including the antifungals, since both abiotic and
biotic factors regulate their production under in vitro
and in situ conditions. An understanding of the molecular ba-
sis of regulation can further help the introduced isolate to
withstand competition from indigenous flora and esta-
blish itself in an alien environment. It would help if optimum
growth conditions and factors that elevate the level of
antifungal molecules are known, so that effective use of
likely bioinoculants can be made. In addition, current in-
formation relating to QS suggests that build-up of certain
population levels will be necessary to target a phytopa-
thonic population. What is also relevant in the chang-
ing scenario of sustainable production systems is to view
the emerging bioinoculants as a component of the inte-
grated system, besides their stand-alone action within
the ambit of varied abiotic and biotic stresses. The future
of bioinoculants based on fluorescent pseudomonads and
other rhizobacteria appears promising. However, technol-
ogy development based on much-needed scientific data
may help avoid variability and failures in the field. This
would further help in preparing efficient biofertilizers.

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