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Smart biologics for crop protection in agricultural systems

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Abstract: Crop losses caused by insects, pests, and pathogens remain one of the major problems in sustainable agriculture. Environmental and health concerns regarding the overuse of pesticides, and the impacts of climate change on epidemics are immediate pressing issues. In addition, the breakdown of plant resistance by pathogen populations brings limitations to the genetic control of diseases. Biologics can be effective in all types of agricultural systems including organic, sustainable, and conventional. Beneficial microorganisms including *Bacillus* and *Trichoderma* species have been employed as environmentally safe biopesticides. Molecular and proteomic studies on biopesticides have revealed the nature of antibiotics, secreted enzymes, and inhibitory compounds. This review focuses on the current knowledge regarding biological agents and their metabolites including quorum-sensing molecules and volatile compounds, and how they can be used in pest and disease management programs.

Key words: Crop protection, *Bacillus*, *Trichoderma*, volatile compounds, plant growth- promoting bacteria

1. Introduction

Accessing adequate amounts of nutritious, safe, and culturally appropriate foods in an environmentally sustainable manner is important for a growing population (Carvalho, 2006). Producing enough food in the future is possible but care must be taken not to damage the ecosystem and biodiversity. The current approach promotes sustainable intensification, by using less water, fertilizers, and pesticides to obtain greater yield. Although crop loss due to pests and pathogens in a changing environment is still high, frequent use of pesticides in modern agriculture poses some major risks if used improperly. Many pesticides may leave residues in or on treated fruits, vegetables, and grains as well as in soil even if they are used according to the manufacturer's instructions (EEA, 2005). There are also a number of plant diseases for which chemical solutions are not so effective or need high-cost investment compared to the expected income for farmers. Biotechnology contributes to sustainable agricultural productivity for poor and/or small-scale farmers in developing countries (OECD, 2009). Recombinant DNA techniques provide plant breeders with the ability to introduce traits into plants such as enhanced resistance to insect pests or diseases (Gould, 2003). However, potential adverse environmental and/or human health consequences arising from the introduction of genetically engineered or transgenic plants

into the environment led to the development of specific regulatory regimes to assess the safety of these techniques. This, together with an increasing demand for pesticide-free food, has driven the demand for biological control through the use of natural antagonistic microorganisms. Additionally, in recent years in a number of studies, practical crop protection methods based on the concept of induced resistance have also been reported (Walters et al., 2013). On the other hand, the new term biologic is used more broadly to describe a living system such as a microorganism, or plant or animal cells. Many biologics are also produced using recombinant DNA technology (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/default.htm>).

Beneficial microorganisms are considered to be one of the most promising methods for more rational and safe crop-management practices (Selosse et al., 2004). Biocontrol of plant disease involves the use of an organism or organisms to reduce disease. The phenomenon is thought to be biological in nature because fumigation or heat-sterilization of the soil eliminates the suppressive effect, and disease is severe if the pathogen is reintroduced (Weller et al., 2002). Disease suppression has been attributed to an increase in nonpathogenic microorganisms that

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are well adapted to growth on plant roots. Many of these root-colonizing bacteria may also produce antibiotics that further inhibit growth of the pathogen (Baysal et al., 2008). There are 3 main mechanisms by which one microorganism may limit the growth of another microorganism: antibiosis, mycoparasitism, and competition for resources. In recent years, different strategies and concepts have been developed to enhance the success of biocontrol. Introduction of living microorganisms that have a noticeable effect on target pathogens may also augment natural beneficial populations to reduce the damage caused by pathogens (Compant et al., 2010).

This review focuses on the contributions of recent findings from investigations of biocontrol agents on the control of plant pathogens.

2. Concepts of quorum sensing (QS)

Quorum sensing is a regulatory response that coordinates gene expression according to the density of the cell population. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. In gram-positive bacteria such as *Bacillus subtilis*, QS-signaling molecules are generally peptides, except for the universal pheromone LuxS produced by both gram-positive and gram-negative bacteria (Schauder et al., 2001). QS plays a crucial role in bacterial physiology, including regulation of rhizospheric competence factors such as antibiotic production, horizontal gene transfer, and control of functions that are directly or indirectly related to plant-microbe interactions (Whitehead et al., 2001).

N-acyl homoserine lactones (AHLs) are QS molecules produced by gram-negative bacteria that play a major role in sensing the population density and regulating the expression of target genes, including virulence factors. An AHL-degrading enzyme was detected from *B. thuringiensis* that affects the virulence of the gram-negative bacterium *Erwinia carotovora* in the root system of pepper (Park et al., 2008). So far, 2 types of enzymes that inactivate AHLs have been identified in several species/genera of bacteria: the AHL lactonases that cause lactonolysis (opening of the gamma-butyrolactone ring) resulting in acyl-homoserine with reduced biological activity, and the AHL acylases that break the amide linkage of AHLs to produce homoserine lactone and fatty acids with no biological activity (Uroz et al., 2008). Abolishment of the production of the quorum-sensing signals, known as quorum quenching (QQ), results in significantly defective biofilm formation, and thus reduces the ability of the pathogen to colonize the host, resulting in biocontrol. The mechanisms and functions of QQ have been evaluated in order to shed light on the possible applications of this phenomenon in the control of plant diseases and promotion of plant health (Dong et al.,

2007). In this new concept, rhizobacterial volatiles are used as an important alternative to antibiotics in the biocontrol of various plant pathogens and are capable of inhibiting the QS network mediated by AHL signal molecules. The potential of QQ to develop novel biocontrol strategies for plant pathogens has been recognized (Dong et al., 2007). These studies clearly suggest that QQ can be used as a potential weapon for biological control of pathogenic microorganisms by targeting the QS pathway; however, little is known about the ecological aspects of QQ enzymes under in situ conditions. All QQ strategies have so far been developed under in vitro or greenhouse conditions and their efficacy under field conditions remains to be evaluated.

3. Production of 1-amino cyclopropane-1-carboxylic acid (ACC) deaminase and its role in growth regulation

Although ethylene is essential for normal growth and development in plants, at high concentrations it can be harmful since it induces defoliation and other cellular processes leading to reduced crop performance. This occurs particularly in plants under environmental stress. It has been proposed that plant growth-promoting rhizobacteria (PGPR) may enhance plant growth by lowering a plant's ethylene levels. The immediate precursor of ethylene is 1-amino cyclopropane-1-carboxylic acid (ACC). The PGPR can divert ACC from the ethylene biosynthesis pathway using ACC deaminase (Desbrosses et al., 2009). Thus, the rhizobacteria diminish the accumulation of excess ethylene levels and re-establish a healthy root system to deal with environmental stress. The study of rhizospheric bacteria such as *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Rhizobium*, which have ACC deaminase activity (Duan et al., 2009), has demonstrated the expression of the ACC deaminase gene in plants under environmental stress treated with PGPR. *Enterobacter cloacae* and *Pseudomonas putida* expressing ACC deaminase have been associated with an increase in resistance to stress in inoculated tomato seeds (Glick, 2004). ACC deaminase activity in 3 *Bacillus* species, namely *B. circulans*, *B. firmus*, and *B. globisporus*, stimulated root elongation in *Brassica campestris* (Ghosh et al., 2003). In addition, the role of ACC deaminase in the regulation of canola root growth by *Trichoderma asperellum* was demonstrated by silencing the ACCD gene encoding ACC deaminase (Viterbo et al., 2010).

In our recent investigation into the effect of *Bacillus* species on soilborne plant pathogens, we detected an upregulated protein showing similarity to SAM-dependent methyltransferases (Baysal et al., 2013). It has been known that ACC synthase converts S-adenosylmethionine (AdoMet) into ACC, which is thereafter converted to ethylene by ACC oxidase. We suggested that a similar mechanism might exist, which led to the observed

inhibition of the soilborne pathogen *Fusarium oxysporum* f. sp. *radicis lycopersici* (FORL) by *B. subtilis* EU07. It is postulated that AdoMet is involved in an ongoing competition between FORL and EU07. Therefore, it seems incorrect to consider only microorganism antibiotics as a reason for inhibition of pathogens.

4. Volatile organic compounds from beneficial microorganisms enhance plant growth and suppress pathogens

The release of volatile organic compounds (VOCs) constitutes an important mechanism for the elicitation of plant growth by rhizobacteria. Some PGPR strains including *B. subtilis* GB03, *B. amyloliquefaciens* IN937a, and *E. cloacae* JM22 have been recorded as releasing a blend of volatile components, particularly 2,3-butanediol and acetoin, that promoted the growth of *Arabidopsis thaliana*, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon (Ryu et al., 2004). Of these enzymes, the acetoin-forming ones had been identified earlier in certain crops like tobacco, carrot, maize, and rice (Forlani et al., 1999). Although their possible functions in plants had not been properly elucidated at that time, it is now known that the VOCs produced by the rhizobacterial strains can act as signaling molecules to mediate plant-microbe interactions. Volatiles produced by PGPR-colonizing roots are generated at sufficient concentrations to trigger the plant responses (Ryu et al., 2004). Identified low-molecular weight plant volatiles such as terpenes, jasmonates, and green leaf components have been shown to be potent signal molecules for living organisms (Farmer, 2001).

Recent investigations into VOCs have shown that the production of 3-hydroxy-2-butanone (acetoin) by *Bacillus* strains EU07 and FZB24, but not by QST713 (Baysal et al., 2013), can serve as an agent for triggering growth promotion in *Arabidopsis thaliana* (Munimbazi et al., 1998; Zhao, 2006). Previously, we reported a similar observation in which an EU07 treatment of plants resulted in increased plant height in comparison to that observed with QST713 (Baysal et al., 2008). External applications of commercial acetoin and 2,3-butanediol, produced by *Bacillus* species, resulted in a dose-dependent stimulation of plant growth under optimum concentrations (Zuber et al., 1993). In addition, the production of acetoin and 2,3-butanediol PGPR was reported to increase systemic disease resistance and drought tolerance (Han et al., 2006).

VOC production by a nonpathogenic strain (MSA35) of *F. oxysporum*, which is antagonistic to pathogenic strains, suggests a new potential long-distance mechanism for antagonism, mediated by VOCs (Minerdi et al., 2009). Antagonism is also suggested to be the cause of both the reduction of pathogen mycelial growth and the inhibition

of pathogen virulence gene expression (Minerdi et al., 2011).

5. *Bacillus thuringiensis* (Bt) and its disadvantages for further sustainable agriculture

Bt is another biological agent and insecticidal bacterium that is used for control of many important plant pests such as caterpillars of the Lepidoptera (Çakıcı et al., 2014), mosquito larvae, and simuliid blackflies. It is applied to the leaves or other environments where the insect larvae feed. Environmental temperature has an effect on the spore-crystal mixture of *Bacillus thuringiensis* isolates, which affects the toxicity and efficiency (Yılmaz et al., 2013). The toxin genes have also been genetically engineered into several crop plants. Although *Bt* seems to provide effective control, it also possesses some disadvantages. Cry toxins are known to be encoded by genes on plasmids of *Bt*. There are nearly 5 or 6 different plasmids in a single *Bt* strain, and these plasmids encode different toxin genes (Padron et al., 2004). The plasmids can be exchanged between *Bt* strains by a conjugation-like process; therefore, there could be a potentially wide variety of strains with different combinations of Cry toxins. There is also another issue that different combinations of Cry toxins are designed to delay the development of resistance in target pests, but the adverse effect is the simultaneous development of resistance to several different toxins in the target pest (Sakai et al., 2007). The target insects are perpetually exposed to toxins, creating a very strong selection pressure for the development of novel resistance to the toxins. Therefore, to reduce this possibility, various crop management strategies should be improved (Brookes and Barfoot, 2010).

6. Plant growth promoting rhizobacteria (PGPR) inhibit growth of phytopathogens

Rhizobacteria can suppress the growth of pathogens in different ways such as by competing for nutrients and space, limiting the available Fe³⁺ supply through producing siderophores, producing lytic enzymes, and antibiosis (Jing et al., 2007). Competition for nutrients, niche exclusion, induced systemic resistance, and production of antifungal metabolites (AFMs) are the prominent properties of the biocontrol activity of PGPRs (Figure) (Bloemberg and Lugtenberg, 2001). Most of the PGPRs produce AFMs such as phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, viscosinamide, and tensin. Among the PGPRs, the best-characterized biocontrol agent *Pseudomonas fluorescens*, WCS374, is able to suppress *Fusarium* wilt in radish, leading to an average increase of 40% in yield (Bakker et al., 2007). More recently, the biosynthesis of pyoluteorin in *P. fluorescens* strain Pf-5 and 2,4-diacetylphloroglucinol in *P. fluorescens*

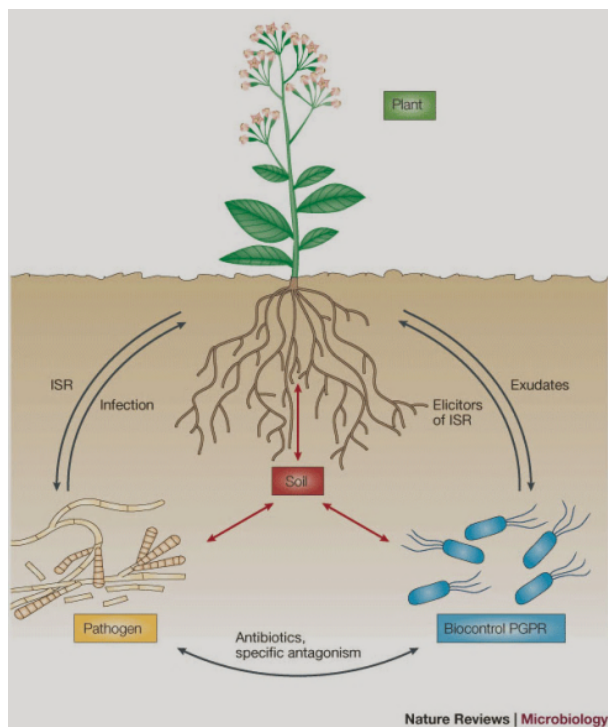


Figure. Interactions between biocontrol plant growth-promoting rhizobacteria (PGPR) plants, pathogens, and soil. Illustration is from Haas and Défago (2005).

Q2-87 have been reported to be effective compounds showing antifungal activity (Kidarsa et al., 2011).

Potential PGPRs are selected for their ability to inhibit the growth of various phytopathogens or miscellaneous rhizospheric bacteria and fungi *in vitro*. Pure cultures of antagonistic rhizobacterial strains are then screened in greenhouse trials by treatment of soil with a bacterial suspension (10^8 CFU/mL) and measurement of the effect on plants (Baysal et al., 2008). The question then arises as to how we can elucidate the reasons for one biocontrol agent's superiority over another in controlling plant pathogens.

7. *Trichoderma* strains as biocontrol agents

Trichoderma species are opportunistic facultative fungi growing in the rhizosphere and are capable of penetrating and internally colonizing plant roots (Harman et al., 2004) as well as boosting plant immunity against pathogens (Shoresh and Harman, 2008; Vargas et al., 2011). The presence of *Trichoderma* triggers different transcriptomic, proteomic, and metabolomic responses in plants (Shoresh and Harman, 2008; Lorito et al., 2010; Moran-Diez et al., 2012), resulting in the production and modulation of hormonal signals in order to facilitate the colonization of roots. For example, auxin production by the fungus promotes root growth, which increases the surface area available for colonization (Contreras-Cornejo et al., 2009).

The secretion of cysteine-rich hydrophobin-like proteins is required for anchoring/attachment to cellulose binding molecules and endopolygalacturonase is used for root penetration (Brotman et al., 2008). *Trichoderma* invasion induces plant responses such as rapid ion fluxes and an oxidative burst, deposition of callose, and synthesis of polyphenols (Shoresh et al., 2010). Even jasmonic acid/ethylene-mediated induced systemic resistance has been associated with the response triggered by PGPR. Recent findings also indicate the triggering of SA-mediated systemic acquired resistance at higher inoculum doses of *Trichoderma*, which is similar to the effect of necrotrophic pathogens (Segarra et al., 2007; Yoshioka et al., 2012). As biofungicides, the major role of *Trichoderma* is to kill or parasitize other fungi. Furthermore, some *Trichoderma* spp. kill nematodes, and so have potential for application as bionematicides (Sharon et al., 2011). Mycoparasitic interactions include different stages: sensing of the host/target fungus, attraction, attachment, coiling around with lysis resulting from the secretion of hydrolytic enzymes, and the production of antibiotic secondary metabolites (Table). Although hydrolytic enzymes and antibiotics play a role in the ability of *Trichoderma* to kill other fungi, the genomes of the mycoparasitic *Trichoderma* spp. are rich in genes encoding enzymes like chitinases and glucanases and those for secondary metabolism (Kubicek et al., 2011). Definitive roles for chitinases, glucanases, and proteases, such as Prb1/Sp1, have been identified in biocontrol during mycoparasitism (Viterbo and Horwitz, 2010).

Many members of these families are expressed before and during contact with the host/prey fungus (Seidl et al., 2009). A recent secretome analysis further revealed that *Trichoderma* may have one of the largest sets of proteases among fungi. The number of subtilisin-like proteases of the S8 family, dipeptidyl and tripeptidyl peptidases, is expanded in the mycoparasites (Druzhinina et al., 2012). These findings indicate the importance of these genes to *Trichoderma* species in attacking and killing fungal pathogens, particularly in view of their antagonistic lifestyle.

8. Biologics in integrated pest management (IPM) programs

The ecological context of agriculture relies on the world food balance and preserving the ecosystem in a stable condition. The dynamics of agro-ecosystems directly depend on climate and agriculture, bioclimatology, soil formation and structure, soil water, and nutrients. These should be conserved for sustainable production, which indirectly inhibits the problems stemming from cultivation, irrigation and fertilization, soil and plant microbiology, maintenance of the soil ecosystem, agricultural pest problems, chemical pest control, and host resistance.

Table. Properties of biologics and their mechanisms of biological control.

Property	Source	Target effect / pathogen	Reference
PGPR strains containing ACC deaminase gene	<i>Methylobacterium fujisawaense</i>	Root elongation	Madhaiyan et al. (2006)
	<i>Bacillus circulans</i> DUC1, <i>B. firmus</i> DUC2, <i>B. globisporus</i> DUC3	Root and shoot elongation	Ghosh et al. (2003)
	<i>Pseudomonas</i> sp. <i>Bradyrhizobium</i> sp.	Promoted nodulation	Shaharoon et al. (2006)
Antibiotics	<i>Bacillus amyloliquefaciens</i> FZB42	<i>Fusarium oxysporum</i>	Koumoutsis et al. (2004)
	<i>Lysobacter</i> sp. strain SB-K88	<i>Aphanomyces cochlioides</i>	Islam et al. (2005)
	<i>B. subtilis</i> QST713	<i>Botrytis cinerea</i> and <i>R. Solani</i>	Paulitz and Belanger (2001), Kloepper et al. (2004)
	<i>B. subtilis</i> BBG100	<i>Pythium aphanidermatum</i>	Leclere et al. (2005)
Lytic enzymes such as chitinases and proteases	<i>P. fluorescens</i> 2-79 and 30-84	<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	Thomashow et al. (1990)
	<i>Bacillus subtilis</i> , EU07	<i>Fusarium oxysporum</i> f. sp. <i>radicis lycopersici</i>	Baysal et al. (2008; 2013)
	<i>Pseudomonas fluorescens</i> strain CHA0	<i>Peronospora parasitica</i>	Iavicoli et al. (2003)
	<i>T. harzianum</i>	<i>Fusarium udum</i>	Rangeshwaran and Prasad (2000)
Volatile organic compounds	<i>T. harzianum</i>	<i>Penicillium expansum</i>	Batta (2004)
	<i>B. subtilis</i>	<i>Fusarium oxysporum</i> f. sp. <i>radicis lycopersici</i>	Baysal et al. (2008; 2013)
	<i>Bacillus subtilis</i> GB03 and IN937a	2,3-butanediol	Ryu et al. (2004)
Phages	<i>Pseudomonas putida</i> WCS 358, BTP1	Lipopolysaccharide, Siderophore, Z,3-hexenal	Meziane et al. (2005) Ongena et al. (2004)
	<i>Bacillus subtilis</i> EU07	2,3-butanediol, acetoin	Baysal et al. (2013)
	<i>Bacillus pumilus</i> 203-6	Peroxidase, β -1,3-glucanase	Bargabus et al. (2004)
Phages	Some testing in plants, promising results in combination with nonpathogenic carrier <i>Pantoea agglomerans</i>	Isolated and characterized in vitro for control of <i>E. amylovora</i> .	Boulé et al. (2011)
	In greenhouse trials, pretreatment of tomato seedlings with RSL1 prevented bacterial wilt in all plants	Isolated and partly characterized in vitro for control of <i>R. solanacearum</i> .	Fujiwara et al. (2011)

Biological control is a component of an IPM strategy, designed to have no adverse effect on sustainable food production. It is defined as the reduction of pest populations by natural enemies. Biological control agents of plant diseases are most often referred to as antagonists. A biologic (a successful natural enemy) should have a high reproductive rate, good searching ability, host specificity, adaptability to different environmental conditions, and be synchronized with its host (pest). A high reproductive rate is important so that populations of the natural enemy can rapidly increase when hosts are available. The natural enemy must be effective at searching for its host and be adapted to the local environment and to the target pest.

8.1 Novel control agents can benefit from new technology

Highly opportunistic microbes are increasingly finding their use in biological control as biopesticides. Their versatile modes of action suggest an evolutionary capacity for adaptation to new hosts. They are therefore promising models to further explore the relationship between host specificity and biological control. Their genomes are easier to engineer than those of parasitoids and predators. Progress in 'omics' and genetic engineering now enables the construction of microbial agents that can be more virulent and specific (Palumba et al., 2005). Furthermore, the production of pathogens that express different levels of virulence, produce specific antipest molecules, or

trigger the activation of a particular mode of action would provide a powerful tool to investigate evolutionary principles and applications of the specificity of biocontrol agents. However, it is imperative that scientists developing opportunistic pathogens continue to rigorously weigh the advantages and hazards posed by these new biocontrol agents and help regulatory authorities make decisions. All biological control strategies have so far been developed under in vitro or greenhouse conditions and their efficacy under field conditions remains to be evaluated. Some of them display no persistent effect in whole tested areas. The assessment of interconnections in the signal molecules using advanced analytical tools and techniques including transcriptomics, proteomics, and metabolomics would provide new insight into interspecies communications in the rhizosphere and their ecological impact on the rhizospheric microbiota.

In addition to microbes, phytopathogen-specific phages have potential for biocontrol. It is necessary to test their efficacy in relation to plant disease before scaling up phage preparations, which requires knowledge about the characteristics and lifestyle of the phages (Ackermann et al., 2004). The table summarizes the results of phage trials that have been performed on a range of phytopathogens including *Erwinia amylovora* and *Ralstonia solanacearum*. However, field trials are biologically complex and the presence of other microbes and pathogens can influence the effectiveness of the phages when introduced into fields (Adriaenssens et al., 2012).

9. Conclusion

Soils are, to some extent, living laboratories where the complex interactions between microorganisms result in disease suppression. Characterization of biological communities in soil has proved to be a formidable challenge, and the nature of disease-suppressive soils remains largely an enigma. Suppressive soils have nevertheless proved to be sources of some important antagonists and they continue to provide important information about biocontrol mechanisms and biocontrol strategies.

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Several biocontrol products are now in widespread use in plant protection. Of the hundreds of pesticides registered by the EU and the US, half are biopesticides, including several microbial biopesticides for plant disease control. There has been a proliferation of many small companies interested in bringing new biocontrol products to the marketplace and many are in collaboration with university researchers and scientists to develop practical alternatives to chemical pesticides.

In conclusion, successful application of biological control strategies requires more knowledge-intensive management. Understanding when and where the biological control of plant pathogens can be profitable requires an appreciation of its place within IPM systems (Santoyo et al., 2012). Newer technologies that directly incorporate genes into biologics’ genomes, commonly referred to as genetic modification or genetic engineering, are bringing new traits into biocontrol agents and biologically based products, such as microbial fungicides, that can be used to interfere with pathogen activities. Registered biofungicides are generally labeled with short reentry intervals and pre-harvest intervals, giving greater flexibility to growers. When living microorganisms having a prominent effect on target pathogens are introduced, they may also augment natural beneficial populations to further reduce the damage caused by pathogens and increase plant fitness (Han et al., 2013).

The use of advanced analytical tools and techniques including transcriptomics, proteomics, and metabolomics will continue to provide new insights into biologics, their mode of action, and their impact on the rhizospheric microbiota. It seems possible that in the near future inhibitory compounds may be mass produced by microorganisms with the required properties and used as replacements for the pesticides which are currently employed.

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