Biological Control of Plant Diseases

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Abstract

Biological control is the control of disease by the application of biological agents to a host animal or plant that prevents the development of disease by a pathogen. With regard to plant diseases the biocontrol agents are usually bacterial or fungal strains isolated from the endosphere or rhizosphere. Viruses can also be used as biocontrol agents and there is a resurgent interest in the use of bacterial viruses for control of plant diseases. The degree of disease suppression achieved with biological agents can be comparable to that achieved with chemicals. Our understanding of the ways in which biocontrol agents protect plants from disease has developed considerably in recent years with the application of genomics and genetic modification techniques. We have uncovered mechanisms by which biocontrol agents interact with the host plant and other members of the microbial community associated with the plant. Understanding these mechanisms is crucial to the isolation of effective biocontrol agents and the development of biocontrol strategies for plant diseases. This review looks at recent developments in our understanding of biocontrol agents for plant diseases and how they work.

Introduction

Plant diseases are a major constraint on crop production in all agricultural and horticultural systems. All crops are susceptible to diseases caused by a variety of pathogens (bacteria, fungi, and viruses). In general losses of crops due to disease amount to 25% of world crop production per annum Lugtenberg (2015). Of course, losses are not distributed evenly but in some cases may be much higher resulting in loss of the entire crop. At the very least this can have severe financial implications at the local, regional or national levels. At worst it can lead to famine with considerable loss of life.

Management of plant diseases is a significant cost component in crop production. Traditionally the approaches to dealing with disease in agricultural ecosystems includes breeding resistant varieties of the crops species, hygiene to prevent the spread of contaminated soil or seed, and fungicides to kill potentially infecting fungi. However increasing concerns about the effects of fungicides in the environment and residues in food have resulted in deregistration of a number of fungicides. The need to replace these has increased interest in biological control of plant diseases in recent years. Biological control is the suppression of disease by the application of a Biocontrol Agent (BCA) usually a fungus, bacterium, or virus, or a mixture of these to the plant or the soil. The BCA acts to prevent infection of the plant by the pathogen, or establishment of the pathogen in the plant. The main advantage of using a BCA is that they are highly specific for a pathogen and hence are considered harmless to non-target species. Over the past decade there have been many reports of the identification of effective BCAs for fungal and bacterial diseases in crops and a number of BCAs are in commercial production (Table 1). In recent years our understanding of how BCAs protect the plant from infection has changed dramatically with the application of genomics. In order to implement an effective biocontrol program it's essential to understand how BCAs work to prevent disease development. The purpose of this article is to review the various mechanisms by which BCAs protect plants against pathogens.

How effective is biocontrol

The level of disease control achieved by application of BCAs to a crop can be close to or equivalent to that achieved by application of a fungicide. Application of a fungicide to *Phytophthora cactorum* infected apple resulted in 100% disease suppression whilst application of various BCAs singly resulted in levels of disease suppression between 79%-98% depending on the BCA (Alexander and Stewart 2001) (Table 2). In another study application of a *Bacillus amyloliquefaciens* BCA to mandarin fruit suppressed *P. digitatum* infection by 77% which compares to 96% after application of the fungicide imazalil (Table 2). The efficacy of a BCA can be enhanced by mixing with a fungicide provided the fungicide does not adversely affect the BCA. Infection of strawberry by *Botrytis cinerea* was reduced to low levels by application of a *Trichoderma atroviridae* BCA, but was eliminated by application of the BCA with a fungicide (Table 2). Interestingly in this case the fungicide alone was less effective than the BCA alone. Nakayama and Sayama (2013) reported a similar enhancement in disease control using a BCA-fungicide mix to inhibit powdery scab of potato (Table 2). Where there are comparative results for disease suppression in glasshouse and field trials, the degree of suppression tends to be lower in the field trials e.g., in the study of Fu et al. (2010) the degree of suppression was 24% lower in the field (Table 2). This is considered to reflect the more diverse environment in the field. A number of studies have demonstrated that biocontrol can also be used effectively against postharvest diseases (Table 2).

Some endophytes protect against multiple pathogens. An endophytic strain G3 with potential as a biocontrol agent was isolated from the stems of *Triticum aestivum* L. It was classified by 16S rDNA sequencing as a member of *Serratia*. Although strain G3 displayed a broad spectrum of antifungal activity *in vitro* against a number of phytopathogens such as *Botrytis cinerea, Cryphonectria parasitica, Rhizoctonia cerealis* and *Valsa sordida* is has not been tested for disease suppression (Liu et al. 2010). A strain of *Bacillus pumilis* isolated from the endosphere of poplar suppressed the growth of three pathogens *Cytospora*

chrysosperma, Phomopsis macrospora and *Fusicoccum aesculi* in greenhouse tests (Ren et al. 2013).

Host Genotype Effect

One of the problems with biocontrol is the lack of consistency in disease suppression by a BCA. Differences in host genotype contribute to differences in responses to a BCA. In control of *Phytophthora meadii* infection of *Hevea brasiliensis* by *Alcaligenes* sp the degree of control differed between two cultivars of the host (Table 2) (Abraham et al. 2013). A cultivar effect was also observed in studies on biocontrol of diseases in strawberry (Card et al. 2009) and pepper (Lee et al. 2008). The specificity effect may be related to the production of plant molecules that activate transcriptional activators of the LuxR family in the bacterium. The products of the LuxR genes act as global regulators controlling such processes as biofilm formation and antibiotic production among others. Although LuxR regulators normally operate in quorum sensing systems whereby bacteria communicate with each other, some such as the PsoR gene of *P. fluorescens* (Subramoni et al. 2011) and the OryR gene of *Xanthomonas oryzae* (Ferluga and Venturi 2009) respond to plant compounds thereby facilitating plant-BCA communication. Alternatively communication could be mediated by secondary metabolites produced by the BCA. Endophytes produce a large array of different types of secondary metabolites many of which have not been detected directly but have been inferred from genomic analysis (Brader et al. 2014). There are examples where the synthesis of secondary metabolites stimulates changes in plant metabolite production and vice-versa (Ludwig-Muller 2015).

Mixtures of BCAs

Several researchers have reported that using mixtures of BCAs has increased the consistency of biocontrol across sites with different conditions. In studies on

infection of potato by *Phytophthora capsici* greater disease control was achieved using a mixture of three bacterial BCAs compared to using the single strains (Kim et al. 2008) (Table 2). Slininger et al. (2001) in their investigation into postharvest dry rot of potato found that formulations of mixed BCAs performed more consistently across 32 storage environments varying in cultivar, washing procedure, temperature, harvest year, and storage time. Enhanced biocontrol using mixtures of BCAs has been reported for control of late blight in potato (Slininger et al. 2007), diseases of poplar (Gyenis et al. 2003), chilli (Muthukumar et al. 2011), and cucumber (Raupach and Kloepper 1998; Roberts et al. 2005). It is also possible that different mixtures may need to be used in different climatic areas. Thus there is a need to identify a number of potential biocontrol agents. Mixtures do not always give increased control. In some cases there may be antagonism between the BCAs that results in reduced control compared to single strains. In evaluating agents for control of fire blight in pear, Stockwell et al. (2011) found that mixtures of *Pseudomonas fluorescens* A506 , *Pantoea vagus* C9-1 and *Pantoea agglomerans* Eh252 were less effective that the individual strains. It was found that the *Pantoea* strains exert their effect through the production of peptide antibiotics. In the mixture these were degraded by an extracellular protease produced by *P. fluorescens* A506. Roberts et al. (2005) have also reported antagonism between BCA strains. They observed that populations of *Trichoderma virens* GL3 or GL321 were both substantially reduced after co-incubation with *Bacillus cepacia* BC-1 or *Serratia marcescens* isolates N1-14 or N2-4 in cucumber rhizospheres. These reports highlight the importance of considering possible antagonism between strains when developing a biocontrol formulation. Co-cultivation *in vitro* can sometimes reveal inhibitory effects (Roberts et al. 2005) but not always. In the study by Stockwell et al. (2011) antagonism between the species in the mixture would not have been evident from co-cultivation of the three species, it would only have been evident if the mixture was tested in a confrontation assay with the pathogen.

Where do BCAs come from.

Most commonly BCAs are isolated by screening organisms from the rhizosphere or endophyte population for inhibition of growth of the target pathogen *in vitro*. Those that show inhibition are assessed further although it should be stressed that *in vitro* inhibition is not always a successful indicator of a successful BCA as there are other mechanisms of disease suppression (stimulation of host growth; induction of host defence; occlusion of pathogen; competition for nutrients; toxin inactivation) that do not involve growth inhibition, and there are other characteristics required for a successful BCA such ability for mass production and persistence under field conditions (Elliott et al. 2009; Martin et al. 2015; Melnick et al. 2008). Prominent among those species of rhizosphere and endophytic bacteria that are effective BCAs are the actinomycetes and species from the genera *Pseudomonas* and *Bacillus*. Among the fungi that constitute effective BCAs species of the genus *Trichoderma* are well represented (Table 2). All of these are capable of synthesizing an array of secondary metabolites.

Actinomycetes make very good BCAs. Endophytic actinobacteria isolated from healthy cereal plants were assessed for their ability to control fungal root pathogens of cereal crops both *in vitro* and *in planta*. Thirty eight strains belonging to the genera *Streptomyces, Microbispora, Micromonospora*, and *Nocardioidies* were assayed for their ability to produce antifungal compounds *in vitro* against *Gaeumannomyces graminis* var. *tritici* (Ggt), the causal agent of take-all disease in wheat, *Rhizoctonia solani* and Pythium spp. Spores of these strains were applied as coatings to wheat seed, with five replicates (25 plants), and assayed for the control of take-all disease in plantain steamed soil. The biocontrol activity of the 17 most active actinobacterial strains was tested further in a field soil naturally infested with take-all and Rhizoctonia. Sixty-four percent of this group of microorganisms exhibited antifungal activity *in vitro*, which is not unexpected as actinobacteria are recognized as prolific producers of bioactive secondary metabolites. Seventeen of the actinobacteria displayed statistically significant activity *in planta* against Ggt in the steamed soil bioassay. The active endophytes included a number of Streptomyces, as well as

Microbispora and Nocardioides spp. and were also able to control the development of disease symptoms in treated plants exposed to Ggt and Rhizoctonia in the field soil (Coombs et al. 2004; Costa et al. 2013; Doumbou et al. 2001) .

Hypovirulent isolates of a pathogen species can also act as BCAs (Sneh 1998). A naturally occurring hypovirulent isolate of *Phytophthora nicotianae* was found to effectively control citrus root rot caused by *P. nicotianae* and *P. palmivora* (Colburn and Graham 2007). In another study binucleate isolates of *Rhizoctonia solani* were effective at controlling damping off diseases in pepper caused by *Rhizoctonia solani* or *Pthyium ultimum* (Harris 2000). Hypovirulent isolates of the Chestnut Blight disease pathogen *Cryphonectria parasitica* were widely and successfully used to control the disease in chestnut trees (Nuss 2005).

Viruses as BCAs

Due to the paucity of effective bactericidal compounds for management of bacterial phytopathogens there is renewed interest in the use of bacterial viruses (bacteriophage or phage) as BCAs for control of bacterial diseases. The history of phage use for management of plant diseases is reviewed in Jones et al. (2007). Recent studies have demonstrated significant reduction in disease severity for a range of pathogens including, *Agrobacterium, Xanthomonas, Ralstonia solanacearum, Erwinia amylovora*, and *Streptomyces* on a variety of crops. The advantages of using phage are: a) ease of production; b) high specificity for the target organism; c) long shelf life. The phage can be grown in the field using an avirulent form of the pathogen infected with the phage applied as a dressing to the crop. The avirulent strain acts as a vehicle for production of the phage but is not able to damage the crop (Diallo et al. 2011). In effect this creates a self-perpetuating biocontrol system in the field. In the studies on the suppression of *Ralstonia* wilt of tomato using phage, infective phage particles were detected four months after treatment (Fujiwara et al. 2011) (Table 2). One problem associated with the use of phage BCAs is the development of resistance in the

host bacterial population. The use of a cocktail containing a number of host range mutants is recommended to overcome this. Such mutants can be evolved in the lab (Jackson 1989). The persistence of phage BCAs in the field may be enhanced by microencapsulation of the BCA in an inert polymer matrix and the slow release of phage from this matrix (Choinska-Pulit et al. 2015; Vonasek et al. 2014).

Fungal viruses (mycoviruses) have also been used as BCAs. Mycoviruses are present in all major taxa of fungi (Ghabrial et al. 2015; Nuss 2005) They do not appear to have mechanisms of tissue infection but rather are transmitted by hyphal anastomosis, and thus can only be exchanged between vegetatively compatible strains. In the majority of cases infection does not appear to cause any symptoms although in some cases mycovirus infection results in a hypovirulent phenotype. The most famous example is the Chestnut Blight pathogen *Cryphonectria parasitica* which has devastated chestnut populations in the USA and Europe (Nuss 2005). Application of virus infected hypovirulent strains to chestnut trees resulted in transmission of the virus to virulent strains by hyphal anastomosis with attenuation of virulence and protection of the trees. Whilst this strategy was successful in Europe, it did not work in the USA because of vegetative incompatibility between the strains prevented transmission of the virus to the pathogenic strains. Hypovirulence inducing mycoviruses with the ability to infect host fungal tissue when applied externally without the need for anastomosis have been identified in the fungal pathogens *Sclerotinia sclerotiorum* and *Rosellinia necatrix*. These are likely to be particularly useful as BCAs as their spread will not be limited by vegetative incompatibility (Ghabrial et al. 2015).

How do endophytes protect plants?

Stimulation of plant growth

A common effect of the application of a rhizospheric or endophytic BCA to a plant is accelerated growth of the plant. Many bacterial and fungal BCAs produce analogues of plant growth regulatory hormones and volatile compounds that stimulate growth (Harman et al. 2004; Taghavi et al. 2009). The growth increase can be quite substantial. In one experiment inoculation of lettuce with growth promoting strains of *Bacillus* resulted in a 30% increase in plant weight two weeks after inoculation (Santoyo et al. 2012). Thus besides disease suppression, another advantage of biocontrol is increased yield even in the absence of disease. Volatiles such as 2,3-butanediol, acetoin, and aldehydes and ketones are produced by bacteria and may play a part in promoting plant growth. Inactivation of genes for synthesis of the volatiles 2,3-butanediol and acetoin in the *B. subtilis* biocontrol strains BSIP1173 and BSIP1174 disrupted stimulation of the host plant growth (Santoyo et al. 2012). Fungal BCAs also stimulate growth of the host plant. *Trichoderma harzianum* produces a butenolide metabolite called harzianolide that both stimulates growth and induces defence mechanisms (Cai et al. 2013).

Analogs of plant hormones produced by endophytic bacteria not only promote growth of the plants but they alleviate other stresses such as drought. For example, abscisic acid and gibberellins produced by the bacterial endophyte *Azospirillum lipoferum* have been shown to be involved in alleviating drought stress symptoms in maize (Brader et al. 2014).

Induction of Host Defence Mechanisms

Another mechanism commonly associated with protection of plants by BCAs is induction of the host defence pathways. This occurs as a result of the release of elicitors (proteins, antibiotics and volatiles) by the BCA that induce expression of the genes of the salicyclic acid pathway or the jasmonic acid/ethylene pathway (Nawrocka and Malolepsza 2013; Pieterse et al. 2014). A different defence mechanism, Induced Systemic Resistance (ISR), characterised by broad spectrum resistance against pathogens of various types as well as abiotic stresses is also

induced (An et al. 2010; Shoresh et al. 2010). Induction of ISR usually involves a primed state for an enhanced reaction to a biotic or abiotic stimulus rather than full induction (Conrath et al. 2006; Pieterse et al. 2014). Because this is not full induction it is considered to require less energy than full induction and consequently have less of a negative impact on growth (Perazzolli et al. 2011). Bacterial volatiles have also been implicated in induction of systemic resistance in the host plant via an ethylene dependent pathway (Kloepper et al. 2004). In addition to volatiles ISR is induced by siderophores and cyclic lipopeptide antibiotics (Jan et al. 2011).

Secretion of polysaccharide degrading enzymes

Secretion of a variety of polysaccharide degrading enzymes including chitinases, glucanases, proteases and cellulases is a common feature of bacterial and fungal BCAs (Jan et al. 2011; Quecine et al. 2008). These enzymes are capable of degrading the cell walls of fungal (or oomycete) hyphae, chlamydospores, oospores, conidia, sporangia, and zoospores resulting in lysis and thus contribute to the protection of the plant. The oligosaccharides released from degradation of the fungal cell walls act as signaling molecules to induce the host defence mechanisms. However the production of enzymes capable of degrading the hyphal cell walls of pathogenic fungi *in vitro* does not constitute proof that these enzymes are responsible for biocontrol activity *in planta*. Michelsen and Stougaard (2011) showed that although *Pseudomonas fluorescens* In5 produces chitinase and beta-1,3-glucanase the biocontrol activity exhibited by this strain is not due to these enzymes but to the production of the non-ribosomal peptide antibiotics nunamycin and nunapeptin (Michelsen et al. 2015). In other studies Kim et al. (2008) found that bacterial chitinase production is not responsible for biocontrol of phytophthora blight of pepper, whilst Worasatit et al. (1994) showed that there was no relationship between the biocontrol activity of *Trichoderma koningii* and the production of chitinase, glucanase, or cellulase by the fungus. However, contrasting results were provided by Chernin et al. (1995)

who showed by gene inactivation that chitinase production is responsible for biocontrol activity of *Enterobacter agglomerans*, and by Downing and Thomson (2000) who transformed a *Pseudomonas* strain with a chitinase gene thus creating a BCA.

Production of antibiotics

Many biocontrol bacteria and fungi produce multiple antibiotics (including biosurfactants with antibiotic properties such as lipopeptides) that confer a competitive advantage by eliminating other bacteria and fungi. Single strains can produce multiple variants of each type (reviewed in Raaijmakers et al. (2010) and Jan et al. (2011)). In addition to their antibiotic properties, lipopeptides are important signalling molecules and affect processes such as motility, induction of host plant defence mechanisms, and formation of microbial biofilms on the inner and outer surfaces of plants. The fungus *Trichoderma* which is widely used as a biocontrol agent and which forms the basis of several commercial products for biocontrol (Table 1) also synthesizes an array of secondary metabolites with antibiotic activity (Druzhinina et al. 2011). Among these are the non-ribosomal peptides which form voltage dependent ion channels in membranes; polyketides of unknown function; isoprenoid derivatives that are highly fungitoxic and phytotoxic; and pyrones with antifungal activity.

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Various studies have attempted to provide evidence for a role for these antibiotics in pathogen suppression by enhancing their synthesis or disrupting the genes for their synthesis. Inactivation of antibiotic synthesis genes in various species of *Pseudomonas*, or *Bacillus* has provided strong evidence for the role of antibiotics in biocontrol by these species (Wu et al. 2015). Initial observations showed that a tryptophan auxotrophic mutant of *P. aeruginosa* deficient in phenazine synthesis was ineffective at supressing infection of cocoyam by *Pythium myriotylum* in contrast to the wild type strain which effectively suppressed infection (Tambong and Höfte 2001). Disruption of rhamnolipid and

phenazine synthesis genes in the species *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis* significantly reduced the ability of this species to suppress *Verticillium microsclerotia.* However it did not completely remove the suppression suggesting that there are other mechanisms of pathogen suppression (Debode et al. 2007). Subsequent experiments in which the *darA* and *darB* genes responsible for the biosynthesis of the antibiotic 2-hexyl, 5-propyl resorcinol (HPR) in *Pseudomonas chlororaphis* were inactivated confirmed the role of the antibiotic in antagonism (Calderon et al. 2013). Similarly, gene disruption was used to provide evidence for roles for fengycin (Yanez-Mendizabal et al. 2012) and iturin in biocontrol of peach and curcubit diseases respectively by strains of *Bacillus subtilis* (Zeriouh et al. 2011) and of iturin in biocontrol of fruit diseases by *Bacillus amyloliquefaciens* (Arrebola et al. 2010). More recent work suggests that different antibiotics from the same strain interact synergistically to achieve disease suppression. A Pseudomonas strain producing phenazine and two types of cyclic lipopeptide antibiotics (sessilins and orfamides) suppressed infection of Chinese cabbage by *R. solani* AG2-1 (Olorunleke et al. 2015). Although production of phenazine alone was sufficient to achieve disease suppression, in the absence of phenazine both sessilins and orfamides were required. In suppression of root rot of bean caused by *R. solani* 4-HG1 both phenazines and either sessilins or orfamides were required. This study also demonstrates that the lack of an effect upon inactivation of the synthesis of a single antibiotic in a biocontrol strain does not preclude a role for that antibiotic in biocontrol.

Despite the evidence produced by the above studies showing that antibiosis is the basis for biocontrol activity in a number of species, a number of studies have produced conflicting results. Poritsanos et al. (2006) reported that a *GacS* mutant of *P. chlororaphis* was greatly reduced in the production of phenazine and showed ten fold reduction in biocontrol efficacy. . However, the *GacS* mutation also affected the production of protease, lipase, and biofilm formation all of which would contribute to biocontrol efficacy. The biocontrol yeast *Pseudozyma flocculosa* (syn: *Sporothrix flocculosa*) is an effective biocontrol agent for control of powdery mildew (Bélanger et al. 2012). The yeast produces

a powerful antibiotic that induces a rapid cell collapse in the pathogen. Despite the initial indications that the antibiotic is responsible for the biocontrol activity, it turned out not to be involved. In contrast to experiments showing that disruption of antibiotic synthesis genes in species of *Pseudomonas* and *Bacillus* reduced biocontrol efficacy (Arrebola et al. 2010; Calderon et al. 2013; Debode et al. 2007; Tambong and Höfte 2001; Yanez-Mendizabal et al. 2012), Mazzola et al. (2007) found that disruption of synthesis of the cyclic lipopeptide antibiotic, massetolide in *Pseudomonas fluorescens* by Tn5 insertion did not affect biocontrol activity. The demonstration of the involvement of antibiosis as a mechanism of biocontrol is complicated by the plethora of antibiotics produced by individual bacterial strains. In addition, many antibiotic synthesis genes are only synthesized at high cell density, or when the bacterium forms part of a biofilm (Rutledge and Challis 2015). Many such cryptic genes have been detected in the genomes of filamentous fungi, in particular *Aspergillus* spp. and actinomycetes. Demonstrating the involvement of antibiosis in biocontrol is further complicated by the fact that antibiotics often have additional roles other than inhibiting the growth of microorganisms. Surfactins for example are important in motility of cells on the plant surfaces, in triggering the formation of biofilms and induction of host defences. Thus inactivation of cyclic lipopeptide antibiotic genes leads not only to decreased antibiosis, and impaired host induction but also decreased ability to form biofilms (Raaijmakers et al. 2010). Thus antibiotics act in multiple ways to suppress pathogens.

Biofilms

On plant surfaces bacteria rarely exist as single cells, but form large multicellular assemblages called biofilms (Bogino et al. 2013; Flemming et al. 2016). Biofilms typically contain multiple bacterial, or mixed bacterial and fungal species (Flemming et al. 2016; Frey-Klett et al. 2011). In a biofilm the cells are covered by a matrix that protects them from desiccation, UV, predation, and bactericidal compounds such as antibiotics. The matrix consists of soluble gel forming polysaccharides, protein, lipid and DNA as well as insoluble amyloids, fimbriae,

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pili and flagella and is permeated by channels that act as a circulatory system for exchange of nutrients, water, enzymes, signalling molecules and removal of toxic metabolites. Biofilms are complex sorbent systems with both anionic and cationic exchangers, which means that a very wide range of substances can be trapped and accumulated for possible consumption by cells in the biofilm. The nutrient capture efficiency of the matrix exceeds that of free living cells (Flemming et al. 2016). Not only nutrients, but also toxic substances, can accumulate in biofilms by binding to the matrix. In this way the matrix soaks up toxic substances that would otherwise be inhibitory to the cells. These substances are either retained in the matrix until it decomposes, or released from the matrix into the water phase and exuded from the matrix. Biofilm formation also facilitates the exchange of genetic information between cells. Conjugation has been shown to be 700 times more efficient in biofilms than in free-living cells (Flemming et al. 2016). Biofilms aid in plant protection by preventing access to the surface of the plant by the pathogen, and by the production of antibiotics, many of which are only produced when growing in a biofilm. Just as biofilms may aid the survival and proliferation of biocontrol species on plant surfaces, so may they aid the survival and proliferation of pathogenic species (Morris and Monier 2003). Additionally, cell wall degrading enzymes secreted by the pathogen bind to the biofilm matrix leading to increased heat tolerance and protection against enzymatic degradation (Flemming et al. 2016).

Biofilm formation initiates with the aggregation of cells on the plant surface a process that is triggered by the secretion of AHLS signaling molecules by neighbouring cells. The aggregation is facilitated by components such as surfactin which modify the surface properties to enhance motility and adhesins (Bogino et al. 2013; Flemming et al. 2016). Once aggregation has initiated the cells synthesize the components for the matrix.

Competition for nutrients

Competition for nutrients on, or proximal to the plant surface (rhizosphere) is another mechanism used to protect plants from pathogens. BCAs compete for sugars on the leaf surfaces or root exudates in the rhizosphere. These same food sources are required for initial establishment of the pathogen prior to infection. By utilising these food sources the BCA prevents establishment of the pathogen (Card et al. 2009; Ellis et al. 1999). For these reasons hypovirulent variants of the pathogen make effective BCAs. They occupy the same niches as the pathogen, utilise the same nutrients, and can occupy entry points to the plant tissues that would be used by the pathogen thereby preventing infection by the pathogen (Sneh 1998). Biocontrol species are able to sequester iron for their own use by the production of iron binding siderophores. This reduces the availability of iron to other organisms such as pathogens (Santoyo et al. 2012). Because bacterial siderophores have a higher affinity for iron than fungal siderophores, they are effective at depriving fungi of iron (Jan et al. 2011).

Inactivation of pathogen phytotoxins.

Many plant pathogens produce phytotoxins that contribute to pathogenicity by disrupting process in the host plant (Strange 2007). These toxins either act as enzyme inhibitors (HC toxin of *Helminthosporium carbonum*), interfere with membrane function (syringomycin of *P. syringae*), or prevent induction of host defences (coronatine of *P. syringae*). BCAs can protect plants from phytotoxins by inactivating them or preventing their production. The potent BCA *Burhholderia heleia* PAK1-2 prevents synthesis of the phytotoxin tropolone by the rice pathogen *Burkholderia plantarii* (Wang et al. 2016). A biocontrol strain of *Bacillus mycoides* inactivates the toxins thaxtomin A(1) and B(2) produced by the potato common scab pathogen *Streptomyces scabei* (King et al. 2000). The rice sheath blight pathogen *R. solani* produces a host specific toxin, the RS toxin that is part of it's pathogenicity. Known biocontrol strains of *T. viridae* that produce an alpha-glucosidase that inactivates the toxin have been isolated (Shanmugam et al. 2001). The alpha glucosidase is different from other known alpha-glucosidases and is specific for the toxin. Strains of *Fusarium* and *Trichoderma* capable of inactivating the toxins Eutypine, 4-

hydroxybenzaldehyde, and 3-phenyllactic acid produced by the pathogens causing Eutypa dieback and esca disease, two trunk diseases of grapevine (*Vitis vinifera*) have been isolated (Christen et al. 2005).

Genetically Modified BCAs

Techniques for genetic engineering of all organisms have been developed to a high degree of precision and have been applied to the improvement of strains of bacteria, and fungi for industrial processes. These techniques can be applied to improve the efficacy of BCAs. In one experiment the transfer of a chitinase gene from *Serratia* to a *Pseudomonas* endophyte created a strain with a greatly increased ability to suppress *R. solani* infection of bean (Downing and Thomson 2000). Similarly the addition of a glucanase gene to *Trichoderma* resulted in a strain that secreted a mixture of glucanases and showed greatly enhanced protection against the pathogens *Pythium, Rhizoctonia*, and *Rhizopus* (Djonovic et al. 2007). Zhou et al. (2005) assembled a 2,4-diacetylphloroglucinol (2,4-DAPG) biosynthesis locus phlACBDE cloned from strain CPF-10 into a mini-Tn5 transposon and introduced into the chromosome of the non 2,4-DAPG producing strain *P fluorescens* P32. The resultant strains provided significantly better protection of wheat against take-all caused by *Gaumannomyces graminis* var *tritici* and tomato against bacterial wilt caused by *Ralstonia solanacearum*. In spite of the results of these studies these newly created BCAs are subject to the regulations that govern the use of organisms that are genetically modified through the use of recombinant DNA. Given the stiff opposition that has faced the use of transgenic plants and the even greater difficulties of containment faced with genetically modified microorganisms it is unlikely that BCAs created by recombinant DNA technology will be approved for general use in the near future.

A more realistic approach would be to use non-recombinant DNA technology to enhance BCAs. Clermont et al. (2011) used genome shuffling to generate

519 improved biocontrol strains of *Streptomyces melanosporofaciens* EF-76. Two
520 rounds of genome shuffling resulted in the isolation of four strains with
521 increased antagonistic activity against the potato pathogens *Streptomyces scabies*
522 and *Phytophthora infestans*. Chemical mutagenesis has been used to enhance
523 biocontrol activity, e.g., nitrosoguanidine mutagenesis of *Pseudomonas*
524 *aurantiaca* B-162 resulted in the isolation of a strain with threefold elevated
525 levels of phenazine production and enhanced biocontrol activity (Feklistova and
526 Maksimova 2008). Marzano et al. (2013) isolated strains of *T. harzianum* with
527 greatly enhanced biocontrol activity after UV mutagenesis. Because the genetic
528 techniques used in these studies do not involve recombinant DNA, they simply
529 mimic what happens naturally they do not fall under the regulations governing
530 the use of genetically modified organisms and hence they should be more
531 acceptable to being used for disease control. However one of the potential
532 problems with such agents is that aside from the desired mutation there may be
533 additional mutations in other genes that can result in undesirable consequences..
534 More recently developed techniques of genome editing can overcome these
535 limitations. Using tools such as Crispr/Cas we can with great precision introduce
536 mutations into specific locations in the genome with great efficiency (Barrangou
537 and van Pijkeren 2016). An additional advantage is that mutations can be
538 induced in multiple genes simultaneously and this will be an advantage is
539 identifying the role of different genes in biocontrol.
540

541 ## Conclusions

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543 The traditional method of searching for a BCA is based on the assumption that
544 the BCA will directly antagonise the pathogen either by antibiotic production or
545 predation. Such antagonism is detected by confrontation assays on agar
546 medium. As we have seen there are multiple mechanisms by which a BCA may
547 protect plants from pathogens, and different BCA's may use different
548 combinations of these, or may use different mechanisms under different
549 circumstances. Only some of these would be detected by confrontation assays.
550 Others require communication between the BCA and the host and other

endophytes or growth of the BCA in biofilms. It is apparent that the confrontation assays are an inadequate screening method as they do not take into account all mechanisms of antagonism, and do not replicate the environment in which the BCA must function. They therefore identify only a subset of possible BCAs. It is considered that the use of inappropriate screening methods is a major contributor to the failure of biocontrol strategies (Pliego et al. 2011). Screening for BCAs must be done with an *in planta* assay or an assay with tissue explant. With the continued application of genomics to identification of genes responsible for maintaining the endophytic state it is possible that we will be able to identify effective BCAs based on a genetic profile (Benítez and McSpadden Gardener 2009). In addition, gene identification opens up possibilities for genetic modification so that instead of screening for new BCAs we simply make new ones by modification of pre-existing ones.

Having identified a suitable BCA the assumption is that it can be produced in liquid culture and used as a seed dressing, soil drench or foliar spray. What is crucial to effective disease control is the persistence of the BCA. It has to compete with other microbial species in the rhizosphere, endosphere and/or phyllosphere so that it can establish and offer protection over a reasonable timeframe. In this regard the method of production is crucial as it determines the type of propagules (spores, conidia, vegetative cells) produced and thus the shelf life, and persistence of the product in the environment (Bisutti et al. 2015; Hanitzsch et al. 2013; Kakvan et al. 2013; Mocellin and Gessler 2007).

Despite the fact that a lot of organisms with biocontrol potential have been identified against a large number of pathogens there have been relatively few developed commercially. To remedy this and take full advantage of the benefits in biological control the research focus needs to shift from identification of antagonistic organisms towards production, formulation and delivery.

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Table 1. Examples of biological control agents in commercial production.

BIOCONTROL AGENT	CROP	SUPPLIER	COUNTRY
Ampelomyces quisqualis M-10	Powdery mildews	EcoGen	USA
B. subtilis MB1600	Fungal pathogens of cotton, Large seeded legumes Soybeans	Beker Underwood	USA
B. subtilis MB1600 + Rhizobium	Fungal pathogens of soybeans, peanut	Beker Underwood	USA
Bacillus amyloliquefaciens GB99+ *B. subtilis* GB122	Bedding plants in potting mixes	Gustafson, Inc.	USA
Bacillus lichenformis SB3086	Turf Grass, Sclerotinia	Novozymes Biologicals, USA	USA
Bacillus pumillus GB34	Soybean fungal diseases	Gustafson, Inc.	USA
Bacillus subtilis GB03	Cotton, legume fungal diseases	Gustafson, Inc.	USA
Bacillus subtilis GB03, other *B. subtilis* *B. lichenformis, B. megaterium*	Fungal pathogens of greenhouse and nursery plants.	Growth Products Ltd	
Bacillus subtilis QST 713	Vegetables, Fruits	AgraQuest	USA
Coniothyrium minitans	Root rot	Prophyta Biologischer	Germany
Coniothyrium minitans	Root rot	Bioved	Hungary
Escherichia coli phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
Fusarium oxysporum non-pathogenic	Wilt	S.I.A.P.A.	Italy
Fusarium oxysporum non-pathogenic	Wilt	Natural Plant Protection	France
Gliocladium catenulatum	Vegetables, Fruits	ArgaQuest	
Gliocladium catenulatum	Root rot wilt	Verdera	Finland
Listeria monocytogenes phage	Bacterial pathogens of fruit and vegetables	Micreos	The Netherlands

Listeria monocytogenes phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
P. fluorescens A506, and 1629RS *P syringae* 742RS	Certain fruits, almond, potato, tomato	Frost Technol Corp.	
Pseudomonas aureofaciens Tx-1	Fungal opathogens of turf Grass	Turf Science Laboratories	
Pseudomonas chlororaphis 63-28	Wilt diseases of ornamentals and vegetables in GH	Turf Science Labs	
Pseudomonas syringae	Pome fruit, citrus, cherries, potatoes	JET Harvest Solutions	
Pseudozyma flocculosa	powdery mildew	Plant Products	Canada
Pythium oligandrum	Root rot	Bioreparaty	Czech Republic
Salmonella phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
Streptomyces griseoviridis	Vegetables, Fruits	ArgaQuest	
Streptomyces griseoviridis K61	Field ornamental, vegetable fungal pathogens	AgBio	
T. harzianum	Grey mold	Makhteshim Chemical Works	Israel
T. harzianum ATCC20476 *Trichoderma polysporum* ATCC20475		Binab	Sweden
T. harzianum T-22	Root rot	Bioworks	USA
Trichoderma harzianum	Vegetables, Fruits	ArgaQuest	
Trichoderma harzianum	Root rot	Efal Agri	Israel
Trichoderma spp	Root rot wilt	Binab	Sweden
Trichoderma spp.	Root rot wilt	Bioplant	Denmark
Trichoderma spp.	Root rot	Agrimm Technologies	New Zealand
Trichoderma virens GL-21	Root rot	Certis	USA

Trichoderma viride	Root rot wilt	Ecosense Laboratories	India
Xanthomonas campestris phage	Bacterial pathogens of fruit and vegetables	Omnilytics	USA
Pseudomonas syrinage phage			

Table 2. Suppression of pathogens on various host species by biological and chemical control agents.

PLANT HOST	PATHOGEN	DISEASE CONTROL AGENT	YEAR	DEGREE OF CONTROL	ASSAY TYPE	SOURCE
Apple	*Phytophthora cactorum*	*Flavobacterium*		79%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	*Oidiodendron*		85%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	*Microsphaeropsis*		98%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	*Trichoderma harzianum*		89%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	*Trichoderma koningii*		93%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	*Paecilomyces*		93%	GH	(Alexander and Stewart 2001)
Apple	*Phytophthora cactorum*	Metalaxyl + Mancozeb		100%	GH	(Alexander and Stewart 2001)
Banana	*Pseudocercospora musae*	*Bacillus subtilis* B106		72%	GH	(Fu et al. 2010)
Banana	*Pseudocercospora musae*	*Bacillus subtilis* B106		48%	F	(Fu et al. 2010)
Chinese cabbage	*Plasmodiophora brassica*	*B subtilis*		>80%	F	(Peng et al. 2011)
Chinese cabbage	*Plasmodiophora brassica*	*Gliocladium catenulatum*		>80%	F	(Peng et al. 2011)
Hevea brasiliensis RRII 105	*Phytophthora meadii*	*Alcaligenes* sp EIL-2		63%	GH	(Abraham et al. 2013)
Hevea brasiliensis RRIM 600	*Phytophthora meadii*	*Alcaligenes* sp EIL-2		30%	GH	(Abraham et al. 2013)
mandarin fruit	*Penicillium digitatum*	*B. amyloliquefaciens* HF-01		77%	PH	(Hao et al. 2011)
mandarin fruit	*Penicillium digitatum*	Imazalil		96%	PH	(Hao et al. 2011)
Pepper	*Phytophthora capsici*	single bacterium		32-73%	F	(Kim et al. 2008)
Pepper	*Phytophthora capsici*	mix of 3 bacteria		99%	F	(Kim et al. 2008)
Pepper	*Phytophthora capsici*	*B. subtilis* R33		87%	F	(Lee et al. 2008)
Pepper	*Phytophthora capsici*	*B. subtilis* R13		71%	F	(Lee et al. 2008)
Potato	*Fusarium sambucinum*	*Serratia plymuthica* 5-6		75%	PH	(Gould et al. 2008)
Potato	*S. subterranea*	*Aspergillius versicolor* lm6-50		70%	T	(Nakayama and Sayama 2013)
Potato	*S. subterranea*	*Aspergillius versicolor* lm6-50 + fluazinam		93%	T	(Nakayama and Sayama 2013)

Strawberry cv Yolo	*B. cinerea* LU829	*Trichoderma atroviridae* LU132	2004	77%	F	(Card et al. 2009)
Strawberry cv Pajero	*B. cinerea* LU829	*Trichoderma atroviridae* LU132	2004	88%	F	(Card et al. 2009)
Strawberry cv Pajero	*B. cinerea* LU829	Fenhexamide	2004	71%	F	(Card et al. 2009)
Strawberry cv Pajero	*B. cinerea* LU829	*Trichoderma atroviridae* LU132 + Fenhexamide	2004	100%	F	(Card et al. 2009)
Tomato	*Ralstonia solanacearum*	Phage PhiRSL1		100%	P	(Fujiwara et al. 2011)

*Assay Type: F, field trial; GH, greenhouse; P, pot; PH, post-harvest; SD, seed dressing; TC, tissue culture