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REVIEW ARTICLE

Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens

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Abstract Over the years, many bacterial isolates have been evaluated as potential biocontrol agents against soilborne fungal phytopathogens. However, few of them were ultimately successful after evaluation in field trials. One of the major reasons for this failure is the lack of appropriate screening procedures to select the most suitable microorganisms for disease control in diverse soil environments. For this reason, the study of bacterial screening has a future that is characterised by many technical and conceptual challenges. In this review, we summarise and discuss the convenience of use of the main screening methods currently applied to select bacterial candidates for biocontrol of fungal and oomycete soilborne phytopathogens. Also, a comparative case study of the application of different screening methods applied to an experimental pathosystem is shown, revealing the success of bacterial candidates selected by different strategies for biocontrol of the phytopathogenic fungus *Rosellinia necatrix* in avocado plants. Screening for antagonism against this fungal pathogen, one of the more straightforward methods used for the selection of bacterial biocontrol agents, was proven to be a valid strategy for this experimental system.

Keywords Selection · Antagonism · Root colonisation · Induction of resistance · PGPR

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Introduction

Soilborne fungal and oomycete plant pathogens are important determinants of the dynamics of plant populations in natural environments and in agricultural environments. Examples of economically important soil-borne fungal and oomycete plant pathogens include *Fusarium* spp., *Gaeumannomyces graminis*, *Verticillium* spp., *Phytophthora* spp., *Pythium* spp. and *Rhizoctonia solani*. Despite low initial densities of inoculum in soil, these pathogens can cause complete destruction of plants, and occasionally, total loss of yield (Deacon 1991; Pal and McSpadden Gardener 2006).

The term "biological control" and its abbreviated synonym "biocontrol" have been used in different fields of biology, but in plant pathology, this term is applied for the use of microbial antagonists (the biological control agent or BCA) to suppress diseases.

However, various definitions of biological control offered in the scientific literature have sometimes caused confusion and controversy; for example, this term has been used to define the cultural practices performed by growers, such as the use of rotations and planting of disease-resistant cultivars. Most narrowly, biological control refers to the suppression of a single pathogen (or pest) by a single antagonist in a single cropping system. The result of the BCA application is decreased incidence and severity of diseases (Haas and DeFago 2005; Pal and McSpadden Gardener 2006).

Microorganisms as BCAs are widely reported, and in some cases, their modes of action against the plant pathogen have been elucidated. Examples of these modes of action by bacterial BCAs in different pathosystems are listed in Table 1. In hyperparasitism, the pathogen is directly attacked by a specific BCA that kills it or its propagules (Leveau and Preston 2008). Many microorganisms produce and release lytic enzymes against compounds such as chitin, proteins, cellulose, hemicellulose and DNA (Table 1), sometimes resulting in the direct suppression of plant pathogenic activities (Kobayashi et al. 2002). Furthermore, some products of lytic enzyme activity may contribute to indirect disease suppression, such as oligosaccharides derived from fungal cell walls, which are known to be potent inducers of plant host defences (Kilic-Ekici and Yuen 2003).

One of the best studied modes of action of bacterial BCAs is the antagonism mediated by different compounds with antifungal properties (Haas and Keel 2003). Most microbes produce and secrete one or more compounds with antibiotic activity (Raaijmakers et al. 2002; Gross and Loper 2009), such as the biocontrol strain *Pseudomonas fluorescens* Pf-5, which produces the antibiotics pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol (Loper et al. 2007), or the strain FZB42 of *Bacillus amyloliquefaciens*, which produces various antifungal lipopeptides (Koumoutsi et al. 2004). Other microbial byproducts (Table 1), such as hydrogen cyanide (HCN, Howell et al. 1988) or ammonia (Voisard et al. 1989), also may contribute to pathogen suppression.

Rhizosphere colonisation is one of the first steps in pathogenesis by soilborne pathogens. For this reason, the trait of some bacteria to colonise the root and to intergere with the biology of the pathogen can be used for biological control of plant diseases (Bloemberg and Lugtenberg 2001, Lugtenberg and Bloemberg

2004; Lugtenberg et al. 2001). To colonise the rhizosphere successfully, a microbe must effectively compete for available nutrients. On plant surfaces, host-supplied nutrients include exudates, leachates, and senesced tissue (Kamilova et al. 2007; Pliego et al. 2008). For example, effective nutrient catabolism in the spermosphere has been identified as a mechanism contributing to *Pythium ultimum* suppression by *Enterobacter cloacae* (van Dijk and Nelson 2000; Kageyama and Nelson 2003).

Other aspects, such as the induction of host resistance, are very important modes of action to protect against fungal diseases. Plants actively respond to a variety of environmental stimuli (including gravity, light, temperature, physical stress, water and nutrient availability) and can also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes (van Loon et al. 1998; Haas and Defago 2005). In several instances, inoculations with plant-growth-promoting rhizobacteria (PGPR) were effective in controlling multiple diseases caused by different pathogens (van Loon et al. 1998; Ongena et al. 2004; Ryu et al. 2004).

The background for screening methods

Some authors separate biocontrol strategies into two broad categories. One strategy, which could be considered preventive, follows a fundamentally ecological approach. This biocontrol strategy pretends to reach a long-time plant protection against the pathogen, and it is mainly based on induction or improvement of suppressive soils. On the other hand, a second strategy, which could be considered curative, uses microorganisms as biopesticides and resembles in some important respects the approach of chemical pesticide treatment, which aims for control at a limited period of time (Knudsen et al. 1997). These differences in control strategy should influence the choice of isolation as well as the screening method (Köhl 2009).

Currently, it is believed that one of the biggest reasons for biocontrol failure is the lack of appropriate screening procedures to select those microorganisms which are most suitable for disease control in diverse soil environments (Merriman and Russell 1990; Folman et al. 2003). Current screening procedures may ignore the influence of biotic and abiotic factors

Table 1 Modes of action displayed by bacterial BCAs against soilborne fungal and oomycete plant pathogens in different pathosystems

Mode of action	Specific mechanism	BCA/Pathogen/Host	References
Hyperparasitism/predation	Disorganization of fungal cell walls and/or cell contents	*Paenibacillus* sp./*Fusarium oxysporum*/Sorghum	Budi et al. 2000
Hyperparasitism/predation	Mycophagy	Many combinations	Leveau and Preston 2008
Lytic enzymes	Chitinases, β-1,3-Glucanases	*Lysobacter enzymogenes/ Fusarium graminearum*/wheat	Li et al. 2008
Lytic enzymes	Chitinases	*Serratia plumuthica/Botrytis cinerea*/many host	Frankowski et al. 2001
Lytic enzymes	Proteases	*Stenotrophomonas maltophilia/ Pythium ultimum*/sugar beet	Dunne et al. 2000
Antibiotics	Bacillomycin, fengycin	*Bacillus amyloliquefaciens/ Fusarium oxysporum*/maize	Koumoutsi et al. 2004
Antibiotics	Agrocin 84	*Agrobacterium radiobacter/ Agrobacterium tumefaciens/many hosts*	Vicedo et al. 1993
Antibiotics	2,4-diacetylphloroglucinol	*Pseudomonas fluorescens/Pythium* spp./sugar beet	Shanahan et al. 1992
Antibiotics	Pyoluteorin, pyrrolnitrin	*Pseudomonas fluorescens/Pythium ultimum*/sugar beet	Howell and Stipanovic 1980
Antibiotics	2-hexyl, 5-propyl resorcinol	*Pseudomonas fluorescens/ Rosellinia necatrix*/avocado	Cazorla et al. 2006
Antibiotics	Phenazines	*Pseudomonas chlororaphis/ Fusarium oxysporum*/tomato	Chin-A-Woeng et al. 2001
Antibiotics	Polyenes	*Streptomyces violaceusniger/Pythium ultimum*/sugar beet	Trejo-Estrada et al. 1998
Antibiotics	Cyclic lipopeptides	*Pseudomonas* sp./*Phytophthora infestans*/tomato	Raaijmakers et al. 2006
Waste products	Ammonia	*Enterobacter cloacae/Pythium* spp./sugar beet	Howell et al. 1988
Waste products	Hydrogen cyanide	*Pseudomonas fluorescens/ Phytophthora infestans*/tomato	Voisard et al. 1989
Physical/chemical interference	Molecular cross-talk confused	*Pseudomonas fluorescens/ Fusarium oxysporum*/wheat	Duffy et al. 2003
Competition	Exudates and/or leachates consumption	*Collimonas fungivorans/Fusarium oxysporum*/tomato	Kamilova et al. 2007
Competition	Physical niche occupation	*Pseudomonas pseudoalcaligenes/ Rosellinia necatrix*/avocado	Pliego et al. 2008
Competition	Iron and siderophore scavenging	*Pseudomonas putida*/different pathogens/cucumber	Loper and Henkels 1999
Induction of host resistance	Detection of pathogen-associated, molecular patterns	Many combinations	Nürnberger and Lipka 2005
Induction of host resistance	Phytohormone-mediated induction	*Pseudomonas putida/Phytophthora infestans*/potato	van Loon 2007

in the rhizosphere. For example, dual culture with fungal pathogens on agar plates has often been used as a screening method (Kloepper and Schroth 1981; Schroth and Hancock 1982). However, the method may be inappropriate because it excludes host-antagonist-pathogen interacting factors and it cannot select biocontrol agents that provide disease control by other mechanisms such as root colonisation, induction of systemic resistance and/or niche competition (Lugtenberg et al. 2001; Bakker et al. 2003; Kamilova et al. 2005; Lugtenberg and Kamilova 2009; Pang et al. 2009). Regardless, it should be

considered that any screening method is selective; therefore, it is to be expected that only a portion of the antagonistic microbiota will be detected.

Although a number of authors have stressed the importance of appropriate screening procedures (Merriman and Russell 1990; Deacon 1991; Campbell 1994; Whipps 1997), only a few studies have been conducted that compared the results of different screening methods in one experimental system (e.g. Kommedahl and Windels 1978; Renwick et al. 1991; Duczek 1994). After testing different screening strategies, Daayf et al. (2003) concluded that whole plant tests were the most convincing strategy, but other tests (in vitro or in detached leaves) also provided an indication of alternative mechanisms of action that could cooperate. On the other hand, Knudsen et al. (1997) found that screening for antagonistic BCAs by different strategies yielded positive results. Thus, it can be concluded that screening methods should be used with caution if candidates with multifactorial or plant-mediated mechanisms of control are to be obtained. A screening strategy can be developed to assess the potential of plant-associated bacteria to control diseases by a hierarchical combination of assays (Faltin et al. 2004).

A valuable background for any screening approach is a thorough knowledge of the aetiology and life cycle of the causal agent to be controlled; particularly, it is important to have knowledge about inoculum transfer, survival, critical inoculum threshold level, the infection process, and climatic conditions favourable for disease outbreak and development. These aspects need to be mirrored in the biological control strategy, time and place of application, and thus, initially in the choice of an appropriate screening method. More often, however, researchers have focused on the mode of action of the antagonist in rather artificial environments with hardly resemble from the field situation. Thus, the search for isolates or strains of microorganisms with antagonistic properties is often performed using pure laboratory methods. As most of these methods have important drawbacks and shortcomings as discussed below, they should be used with great caution.

The first step in all of these studies is the isolation and construction of bacterial collections of isolates from selected sites. Campbell (1986) offered a pragmatic approach for selecting search sites and screening methods for BCAs. It was suggested that culture collections are generally unsuitable because the microorganisms may have high nutritional requirements that are impractical for a commercial product and lack survival abilities in harsh environments. Practical considerations for selecting sites include areas where there is high disease pressure and where the plants show little or no disease symptoms. Disease-suppressive soils offer a logical site for selecting naturally occurring BCAs for soilborne diseases (Haas and DeFago 2005; Schroth and Hancock 1982). It may be practical to isolate microorganisms from agroecosystems under various farm management practices where a crop is being grown. This may aid in choosing microorganisms that are compatible with certain farm management practices such as fertiliser and pesticide applications and tillage practices.

Subsequently, the bacterial collection should be screened using selected strategies. Screening methods can be arranged based on the level of complexity they represent. Methods with low numbers of components (e.g. an antagonist and a pathogen) mainly give information about mechanisms such as antagonism. More complex methods with higher numbers of components—such as antagonist, pathogen, host and environmental factors—may give less exact information about the mechanisms of action, but they may more closely mimic the field situation. The success of the screening process is related to the objectives of the researcher. However, in some cases, particular screening strategies failed in selecting BCA candidates, as it was reported by Folman et al. 2003, who developed a screening procedure based on carbon source oxidation profiles and growth rates of the bacteria as indicators of a partial niche overlap with the pathogen without a positive result.

Types of screening strategies

Introduction

Once the bacterial collection of isolates from roots and soil is established, the choice of the screening procedure will drive the selection of the different bacterial BCA candidates. This decision must be based on the objectives of the work, the type of pathogen, the environmental parameters, the desired

future biocontrol strategy (preventive or curative), difficulties for future formulation, and other factors. In brief, the main screening methods broadly used in the literature can be classified as follows:

In vitro *assays*

The main in vitro screening methods that have been performed previously used plate assays with only one microorganism (mainly searching for lytic enzymes or siderophores production) or with two different micro-organisms (mainly searching for antagonistic or parasitic relationships).

Searching for lytic enzymes is a very easy procedure. This method has been used successfully to select different BCAs (Viterbo et al. 2002). Selection of enzyme-producing microorganisms by an easy plate assay is very rapid and simple and can be performed prior to interactions with the pathogen and/or the plant (Cattelan et al. 1999). The main lytic enzymes produced by bacterial BCA are chitinases, glucanases and proteases; however, although chitinol-ysis is a common trait in bacteria that exhibit antifungal activity (de Boer et al. 2004; Hoster et al. 2005; Ajit et al. 2006), chitinase activity alone appears to be insufficient to account for bacterial lysis of fungal hyphae (Budi et al. 2000; Zhang and Yuen 2000; Kobayashi et al. 2002). The complexity of the fungal cell wall makes it a formidable challenge as a primary target for bacterial attack, as bacteria would need to rapidly produce a wide variety of exoenzymes to degrade cell wall components to the level needed to compromise structural integrity (de Boer et al. 2005) and enhance biocontrol efficacy (Someya et al. 2007). For screening based on non-antibiotic substances, such as siderophore production, specific media should be used (Schwyn and Neilands 1987), which has resulted in selection of bacterial BCAs belonging to the genera *Pseudomonas*, *Bacillus* and *Kocuria* against many fungal soilborne phyto-pathogens such as *Fusarium oxysporum*, *Pyricularia oryzae*, and *Sclerotium* spp. (Chaiharn et al. 2009).

Antagonism, as a mode of action, could be considered as a method of inhibiting phytopathogenic fungi through secretion of substances that interfere with the life cycle of the target microorganism. When an in vitro plant test is the first line of screening, it is generally designed to screen BCAs with antibiotic activity (Burkhead et al. 1995). Finding organisms

with specific enzyme activity or toxin production is the object of some procedures. Antagonistic bacterial-fungal interactions are typically assessed in vitro in terms of an unoccupied "inhibition zone" between a bacterial colony and fungal hyphae cocultured on an agar plate. Two-component screening (e.g. dual cultures of a candidate antagonist and a pathogen on agar) is exclusively related to interaction studies, and potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen expressed by an inhibition zone. The antibiotic-producing strains have been studied for their antagonism in this way, and these antibiotics are known to be active against fungi in vivo. The production of these antago-nistic substances sometimes correlate very well with the biocontrol ability of these bacteria, at least for the antibiotics listed in Table 1, and the dual culture method has performed reasonably well for their screening. However, other authors have found that production of antibiotics in vitro does not correlate with their production in vivo (Fravel 1988; Renwick et al. 1991). One reason for this lack of correlation could be that antibiotics may be induced by specific nutrients. No single medium can, it seems, promote the expression of the full range of antibiotic production of an organism. Studying the antifungal compounds produced by *Pseudomonas* spp., Haas and Keel (2003) found that nutrients played a role in the amount of antibiotic produced, and the authors suggested that caution should be taken when attempting to equate in vitro and in vivo production of these compounds. On the other hand, several studies also demonstrated that toxins presence and environmental conditions (such as pH and temperature) can have an enormous influence on the level of antibiotic produced (Duffy and Defago (1999); van Rij et al. 2005, 2004). Moreover, antibiotic production in the rhizosphere has been confirmed (Thomashow and Weller 1996; Rochat et al. 2010).

This type of approach is most often used to differentiate candidates of a species already known to possess antagonistic potential. Thus, this approach is focused on some facet of the mechanism of antagonism itself, but it has been argued that these screening methods may not be suitable and should be avoided (Campbell 1986). However, screening for this mode of action is easy and inexpensive and permits massive screening of several strains of microorganisms. If the goal is to select microorgan-isms with high capabilities of natural metabolite

production and to develop these natural products for commercial applications, prescreening for antibiosis may be appropriate. Moreover, different works, such as that of Larkin and Fravel (1998), consider that identification of effective antagonist strains represents only the first step toward the development of effective biological control. For biocontrol to be implemented on a practical level, the antagonists must be ecologically fit to survive, become established, and function within the particular conditions of the ecosystem. However, from the applied point of view, antibiotic-producing bacteria could present some difficulties to register.

Assays involving plants

In addition to the in vitro studies, the screening programs could be extended by including plants growing in natural substrates. Thus, other parameters such as induced resistance, plant growth promotion, edaphic or nutritional factors such as root exudates and plant residues could be considered in this experimental system.

In recent years, an increasing number of scientific reports have revealed that several mechanisms may be responsible for a biological control effect (Mercado-Blanco and Bakker 2007). The involvement of various mechanisms and especially the role of competition in biological control have been reviewed (Lugtenberg et al. 2001; Kamilova et al. 2005). Thus, even though antibiosis or mycoparasitism has been shown to occur, it is often competition for nutrients and the ability to compete against other organisms in the rhizosphere, spermosphere, and other areas that are the essential attributes of successful biocontrol organisms (Lugtenberg and Kamilova 2009). Clearly, in the absence of plants, selection for antagonism alone will not provide information concerning the ability of a microorganism to colonise and protect roots and seeds. It is well established that seed and root-infecting pathogens are often highly dependent on exudates to initiate plant infection, and the ability of the antagonist to metabolise these exudated molecules may be an important step in biocontrol processes (Kamilova et al. 2005). Indeed, rhizosphere competence has in some cases been noted as an important prerequisite for obtaining successful bio-control, and specific tests have been devised to select for this characteristic (Lugtenberg et al. 2001). Inoculation with BCAs based on single clonal strains

may result in poor or short-lived colonisation of the rhizosphere. To overcome this problem, Deacon (1994) defended the use of mixtures of ecotypes of a BCA organism or combinations of different BCAs to achieve synergism and more persistent control (Meyers and Roberts 2002; Raupach and Kloepper 1998). However, Roberts et al. (2005) reported bacterial combination less effective that the strains separately. Moreover, compatibility between particular isolate pair varied with the assay and possibly the method of application. Anyway, the best option to use different microorganisms as BCA is to combine different mode of action in order to increase the chances of plant protection (Guetsky et al. 2002).

Screening for plant-growth promoting rhizobacteria

Many root-associated bacteria have a direct positive influence on plant growth and can indirectly stimulate plant health (Compant et al. 2005), which is also an important criterion for a BCA. To test the plant-growth-promoting effect, many plate tests have been developed using different plants with small seeds, such as a microplate assay with strawberry seedlings (Berg et al. 2001). These plant growth promotion assays in microplates are an easier *in planta* test than a whole plant system in terms of time, plant material, and growth facilities. In addition, it has the advantages of allowing many repetitions and high-throughput screening for a large number of bacterial isolates. On the basis of in vitro testing, an assessment system should be developed to select the most efficient BCAs for greenhouse trials. Ideally, greenhouse trials should be followed by field trials under different climatic conditions and diverse soil qualities (Berg 2007).

Screening for colonisation

There is correlation between the efficacy of biocontrol microorganisms against soilborne pathogens and their ability to colonise the root system of the plant to be protected, especially when the mode of action used by these bacterial strains is antibiosis (Chin-A-Woeng et al. 1998) or competition for niches and nutrients (Kamilova et al. 2005; de Weert and Bloemberg 2006; Lugtenberg and Kamilova 2009). Recently, a new strategy for screening bacterial BCAs was developed based in the efficient colonisation of the plant root to

avoid selection by antagonism because future registration of antibiotic-producing strains could be more difficult (Kamilova et al. 2005; Pliego et al. 2007; Egamberdieva and Kucharova 2009). This technique is based on several cycles of inoculation and reisolation of microorganisms from the plant root tip (Kamilova et al. 2005; Pliego et al. 2007). By using this strategy, enhanced root colonisers were isolated, which supported the notion that these bacteria could act through the mechanism of competition for niches and nutrients (Pliego et al. 2008); however, excellent colonisation is not sufficient by itself for excellent biocontrol because it would be also needed the ability to differentially colonize the root zones that could be the target of the pathogen (Kamilova et al. 2005; Pliego et al. 2008).

Another approach was followed by Martínez-Granero et al. (2006), who selected highly motile phenotypic variants of *P. fluorescens* with enhanced competitive colonisation ability to improve biocontrol ability.

Screening for induced resistance

Interaction of some bacteria with the plant roots can result in plants resistant to some pathogenic bacteria, fungi and viruses. This phenomenon is called "induced systemic resistance" (ISR). ISR is dependent on jasmonic acid and ethylene signalling in the plant, and it can be induced by non-pathogenic bacteria in the soil (Kloepper et al. 2004). ISR differs from SAR (systemic acquired resistance), which required accumulation of endogenous salicylic acid, and it can be induced by pathogenic microorganisms (Ryals et al. 1996). As an area of increasing in biocontrol, ISR has been intensively studied in various plant pathosystems using biotic or abiotic inducing agents and is the subject of different works (Kloepper et al. 2004; van Loon 2007). To select bacterial BCAs with this trait, an easy screening method based on PGPR could be developed. Using a plate assay, the increase in growth of root and/or aerial plant (such as tobacco, tomato, lettuce, arabidopsis, etc.) can be measured after exposure to the potential BCA or its exudates incorporated to the media (van Loon 2007). Such screening methods could indicate that multiple defence mechanisms in plants can be activated by, and may be effective against, diseases caused by a broad range of fungi, bacteria and viruses. Subsequently, a second round of experiments, this time including the pathogen, could lead to the selection of potential bacterial BCAs. Naturally, only screening methods employing plants can take advantage of the phenomenon of induced resistance in the evaluation of putative BCAs (Shoresh et al. 2010).

The development of BCAs that act through induced systemic resistance is very attractive because the number of plant pathogens that may be potentially controlled is increased and it may be possible to apply a mixture of ISR-mediated bacterial strains for biological control. Screening of bacterial strains for this mode of action may be somewhat more expensive, time-consuming, and labour-intensive than screening for antibiotic production. Recently, several PGPR have been shown to efficiently help plants overcome biotic stresses by induced systemic resistance (Kloepper et al. 2004).

Screening by plant performance

Screening methods involving plants in which the results are measured as disease severity or disease incidence do not reveal the mechanisms involved. However, from a practical point of view—at least at this stage in the selection process—knowledge about mechanisms is not critically important. *In planta* screening could be conducted in defined media, such as sand, peat soil or in natural soils (Knudsen et al. 1997), or under different conditions (Validov et al. 2009). The ability to protect infection sites in situ may be one of the key attributes of a BCA. Although the period of protection may be more prolonged, a similar attribute is required of antagonists used for protecting seeds and roots from infection by seedborne pathogens (Lugtenberg et al. 2001).

The importance of field screening has already been demonstrated in a number of studies. Lumsden and Lewis (1989), for example, recommended screening in vivo in non-sterile natural soil and preferred field screening for organisms to be used in field crops. However, screening in the field may be difficult to perform due to inconsistent abiotic and biotic parameters in addition to being time- and space-consuming and is also expensive. Because the requirements of a primary screen are often that it is simple, rapid and repeatable, we are often forced to make compromises. One compromise is to attempt to simulate field conditions in pot tests. However, the physical,

chemical and biological characteristics of the soil will not be identical with those in the field. Moreover, sterilised field soil or artificial substrates often are used to avoid compaction and similar cultural problems or to minimise labour. All such simplifications may cause problems in selecting the fittest antagonists for use under field conditions.

Screening of endophytes

Plant bacterial endophytic populations correlate to a certain extent with plant growth performance (Sessitsch et al. 2004). The genera *Bacillus*, *Pseudomonas*, *Serratia*, *Arthrobacter*, *Micrococcus* and *Curtobacterium* include endophytic representatives (Aravind et al. 2009; 2010). For this reason, much effort has been focused recently on the study of such groups of microorganisms, due to their good performance in some experimental systems (Prieto et al. 2009). In these biocontrol experiments, test of endophytes revealed that they preferentially colonise the inner part to the plant roots (Tjamos et al. 2004). To obtain an endophyte candidate for a BCA, isolation of candidate plant root endophytes is usually performed by isolation of microorganisms associated with very well washed and surface-sterilized roots. After obtaining bacterial candidates, authors generally search for antagonistic endophytes by dual-plate assays, similar to that shown before (Sessitsch et al. 2004; Aravind et al. 2009). However, recent molecular techniques permit a genetic screening approach, allowing the exploration of unstudied traits (Wu et al. 2009).

Molecular techniques of screening

Selection and evaluation of microbial strains for their antifungal activity in natural environments is time- and energy-consuming. For this purpose, molecular approaches have been developed. In this sense, Giacomodonato et al. (2001), adapted a PCR-based method to search for peptide-producing microorganisms, resulting in the selection of *Bacillus* strains with antifungal activity against *Sclerotinia sclerotiorum*.

Broadly speaking, there are two distinct culture-independent approaches that can be followed to rapidly discover functionally important microbes such as BCAs. The first approach is to use genetic markers for a functionally important activity such as antibiosis. The theory behind this approach is that natural

variation of such marker genes will reveal concomitant natural variation in functional activity. By isolating a diverse set of genetic variants, one can identify strains or subspecies with various capacities to colonise plant roots and/or suppress pathogens. This approach has been used to identify and recover novel genotypes of 2,4-diacetylphloroglucinol producers from the rhizosphere of fields-grown crop plants (Bergsma-Vlami et al. 2005; Landa et al. 2002). In the absence of knowledge about the mechanisms involved in biocontrol, PCR-based suppressive-subtractive hybridisation can be used to identify new markers (Leveau et al. 2006). The second approach is based on molecular profiling of microbial population structure, an approach sometimes referred to as microbial community profiling. In this approach, ribosomal gene sequences are targeted, amplified from the rhizosphere environment, and analysed. Low-cost, low-resolution techniques, such as terminal restriction fragment (TRF) length polymorphism (T-RFLP) analyses, provide a cost-effective approach to finding generalist populations that consistently contribute to suppression across environments. T-RFLP analyses compare the bacterial community structure in soils differing in their disease-suppressive capacities, revealing the positive association of multiple bacterial populations (marked with different TRFs) with disease suppression (Benitez and McSpadden Gardener 2009). Recently, evolution of molecular tools permitted the development of new screening strategies, such as the development of sequence-based T-RFLP-derived molecular markers to direct the identification and isolation of novel bacteria (Benitez and McSpadden Gardener 2009).

Future prospects

Novel strategies aimed at the screening for biocontrol bacterial take into consideration multitrophic interactions, including the interactions established by biocontrol agents with the root host (Lugtenberg et al. 2001; Kamilova et al. 2005; Pliego et al. 2008), the pathogen (Hogan and Kolter 2002; Pliego et al. 2008), the soil, and the organisms inhabiting the rhizospere (Winding et al. 2004). In relation to bacterial interactions with fungi, the phenomenon of bacterial mycophagy, defined as a set of phenotypic behaviours that enable bacteria to obtain nutrients

from living fungi and thus allowing the conversion of fungal into bacterial biomass (Leveau and Preston 2008), is an open field of study which role as a biocontrol strategy requires more intensive exploration. Bacterial mycophagy do not necessarily implies killing of fungal cells in order to obtain nutrients. In fact, extracellular bacteria are under selection to develop fungi-specific traits that confer a competitive advantage during colonisation of fungal surfaces (de Weert et al. 2004). Recently, the bacterium *Collimonas fungivorans* was shown to be an efficient biocontrol agent of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causative agent of tomato foot and root rot, but it is unclear whether this involved mycophagous behaviour since competition for nutrients and niches appeared to be the main mode of action for this bacterium (Kamilova et al. 2007). It is worth noting that mycophagous BCAs are in essence positive-feedback BCAs because they inhibit the growth of harmful fungi by feeding on them, thus supporting their own growth, which in turn leads to greater biocontrol activity.

Concerning the future of the antagonism screening strategy, it should be taken into consideration that many potential antagonists may be inadvertently disregarded because they demonstrate no inhibition in agar bioassays, and this may exclude the discovery of antagonists that control plant pathogens through other mechanisms (Elad and Chet 1995; Kloepper 1991). For this reason, antagonism should not be considered a phenotype fallen into disuse; however, the combination of diverse methods and the inclusion of the host plant into the screening assays results essential for the selection of antagonistic BCAs acting through modes of action such as induced resistance, competition or parasitism. In addition, improvements are needed to modulate bacterial production in the rhizosphere of antifungal compounds responsible for antagonism (de Werra et al. 2008).

Forthcoming advances in the field of antibiotic-producing bacterial BCAs include the quantification of the production of antibiotics directly on the surface of plant roots or seed coats, which analysis under natural conditions has been impaired due to inadequate methodology (Pal and McSpadden Gardener 2006). For example, rapid quantification of *B. subtilis* antibiotics in the rhizosphere by a method based on solid phase extraction (SPE), high-performance liquid chromatography (HPLC) and mass spectroscopy

(MS) allowed the detection of a few micrograms to almost 0.5 mg of antibiotics per gram of root (Kinsella et al. 2009) or the monitoring of simultaneous root colonisation and gene expression of a bacterial BCA in the rhizosphere (Rochat et al. 2010). In the future, new techniques may be helpful in elucidating the production of specific antibiotics in soil e.g. by immunological methods as shown by Lumsden et al. (1992) or by analysis of the DNA sequence, such as in the case of *P. fluorescens* Pf-5 (Loper and Gross 2007) or *P. fluorescens* CHA0 (Péchy-Tarr et al. 2008), in which different toxic compounds were found after a genomic mining strategy of their sequences.

Design of screening strategies for the selection of BCAs is largely dependent of the specific pathosystem under study. For this reason, extensive knowledge of the biology and epidemiology of the plant pathogen must be clearly obtained priot to bacterial selection. Study of the microbial ecology of the rhizosphere is required to understand the complex interactions of biotic, physical, and chemical processes that occur in the ecosystem and what impact these factors have on the BCA. Multidisciplinary activities in plant pathology and applied microbiology, host-pathogen interactions, epidemiology, molecular technology, and fermentation and formulation technology will advance the development of BCAs to the commercial stage (McSppaden-Gardener and Fravel 2002). In relation to fermentation and BCAs formulation technology, more efforts must be carried out to overcome the lack of adequate methodologies for mass-producing biological agents that have superior efficacy and amenability to the stresses of commercial-scale biomass production. Recently, it has been shown that it is possible to co-culture strains together in one fermetor by a more cost-effectively strategy. This process could stimulate inter-strain activities to boost biocontrol efficacy and consistency beyond that achievable by the more costly method of growing strains in separate fermentations and mixing just prior to addition to the plant (Slininger et al. 2010).

Although bacterial BCAs are naturally occurring microbes, some of them can cause disease in humans. For successful registration, it is necessary to test potentially adverse effects on the health of at-risk candidates. Existing pathogenicity assays are cost-intensive and time-consuming and furthermore, they are often inappropriate for facultative pathogens. For this purpose, a new fast and inexpensive bioassay was

recently developed that is based on the nematode *Caenorhabditis elegans*, which is a well-accepted model organism to study bacterial pathogenicity. Studying survival, motility and reproductive behaviour of nematodes exposed to strains will prevent the selection of potentially pathogenic BCA (Zachow et al. 2009). The *C. elegans* assay can be integrated into initial screenings for BCAs and is a new tool to identify effects against eukaryotes in a very early stage of product development.

## A case study: screening for BCAs of avocado white root rot

Our current work is focused on developing BCAs against avocado white root rot. Avocado (*Persea americana* Mill.) is an important tropical and subtropical crop worldwide, and the most important diseases affecting this crop are root rots primarily caused by the oomycete *Phytophthora cinnamomi* and the ascomycete fungus *Rosellinia necatrix* (Sztejnberg and Madar 1980). *R. necatrix* causes white root rot, and it has been recognised to cause losses in many economically important crops and to cause the destruction of both ornamental and fruit trees (Ten Hoopen and Krauss 2006; Sztejnberg and Madar 1980). Disease development in avocado trees infected by *R. necatrix* is usually rapid, killing the trees within a few weeks after the first foliar symptoms are visible, and is sometimes favoured by the high susceptibility of cv. Topa-Topa, the rootstock most frequently used in avocado orchards in most avocado-producing areas (López-Herrera et al. 1998). Recent studies revealed that fungal penetration of avocado roots occurs simultaneously at several random sites, preferentially in the crown region. *R. necatrix* hyphae were also able to penetrate the primary and secondary xylem (Pliego et al. 2009). Control of avocado white root rot poses difficulties, and several approaches have been used over the past two decades to control *R. necatrix* before planting, such as soil fumigation using methyl bromide (Sztejnberg et al. 1983), fungicides such as fluazinam (Kanadani et al. 1998), soil solarisation (López-Herrera et al. 1998), and biological control using the antagonistic fungus *Trichoderma harzianum* (Sztejnberg et al. 1987).

The aim of our research was to find bacterial BCAs against this soilborne phytopathogenic fungus of avocado. We used different approaches to screen for bacterial BCA candidates, which is summarised in Fig. 1. The first approach used the dual plate assay, searching for antagonistic bacteria that could be used as BCAs (Cazorla et al. 2001, 2006, 2007). Our first studies reported the isolation of antagonistic bacteria against *R. necatrix* (Cazorla et al. 2006, 2007). The success of this strategy was dependent on the presence of typical culturable microorganisms (mainly belonging to *Pseudomonas* and *Bacillus* genera) from the avocado root, as well as the place of *R. necatrix* attack. *Pseudomonas fluorescens* PCL1606 strain and the *Bacillus subtilis* PCL1608 strain were chosen for their high antagonistic activity, which is related to their antibiotic production and biocontrol activity on avocado plantlets. *Pseudomonas fluorescens* PCL1606 produces the antifungal antibiotic 2-hexyl, 5-propyl resorcinol (HPR), which has an important role in biocontrol against white root rot (Cazorla et al. 2006). *Bacillus subtilis* PCL1608 produced iturin and fengicin (Cazorla et al. 2007), antibiotics that are also crucial in the biocontrol of pathogenic fungi in other systems (Romero et al. 2007). Colonisation features of *P. fluorescens* PCL1606 and *B. subtilis* PCL1608 were higher than those of the other related isolates (Cazorla et al. 2006, 2007), demonstrating in this case a correlation between biocontrol and colonisation. Furthermore, despite the antifungal activities shown by most of these isolates on agar plates, some strains do not show biocontrol activity in the rhizosphere environment, probably due to poor colonisation and therefore poor delivery of antifungal metabolites along the root system. To gain insight in the colonising aspects and to improve the selection of potential BCAs, a novel procedure based on selection of competitive root tip colonisers (Kamilova et al. 2005) was recently applied to generate a collection of bacterial isolates that efficiently colonise the roots of avocado plantlets (Pliego et al. 2007). This strategy yielded rhizobacterial strains with efficient colonisation traits, especially the strain *Pseudomonas pseudoalcaligenes* AVO110, which is a non-antibiotic-producing strain that is antagonistic to *R. necatrix*, with biocontrol activity in the avocado/*Rosellinia* test system competition for niches and nutrients as its mode of action (Pliego et al. 2008).

Both screening strategies (antagonism and colonisation) are the most frequently used to select bacterial

Fig. 1 Diagram of the different steps followed in the screening program to isolate candidate bacterial BCAs by three different selective strategies: antagonism (*blue arrows*), colonization (*green arrows*) and direct plant protection (*red arrows*). *Black arrows* were common steps in the screening procedures. KB: King's B agar; NA: nutrient agar; PDA: potato dextrose agar
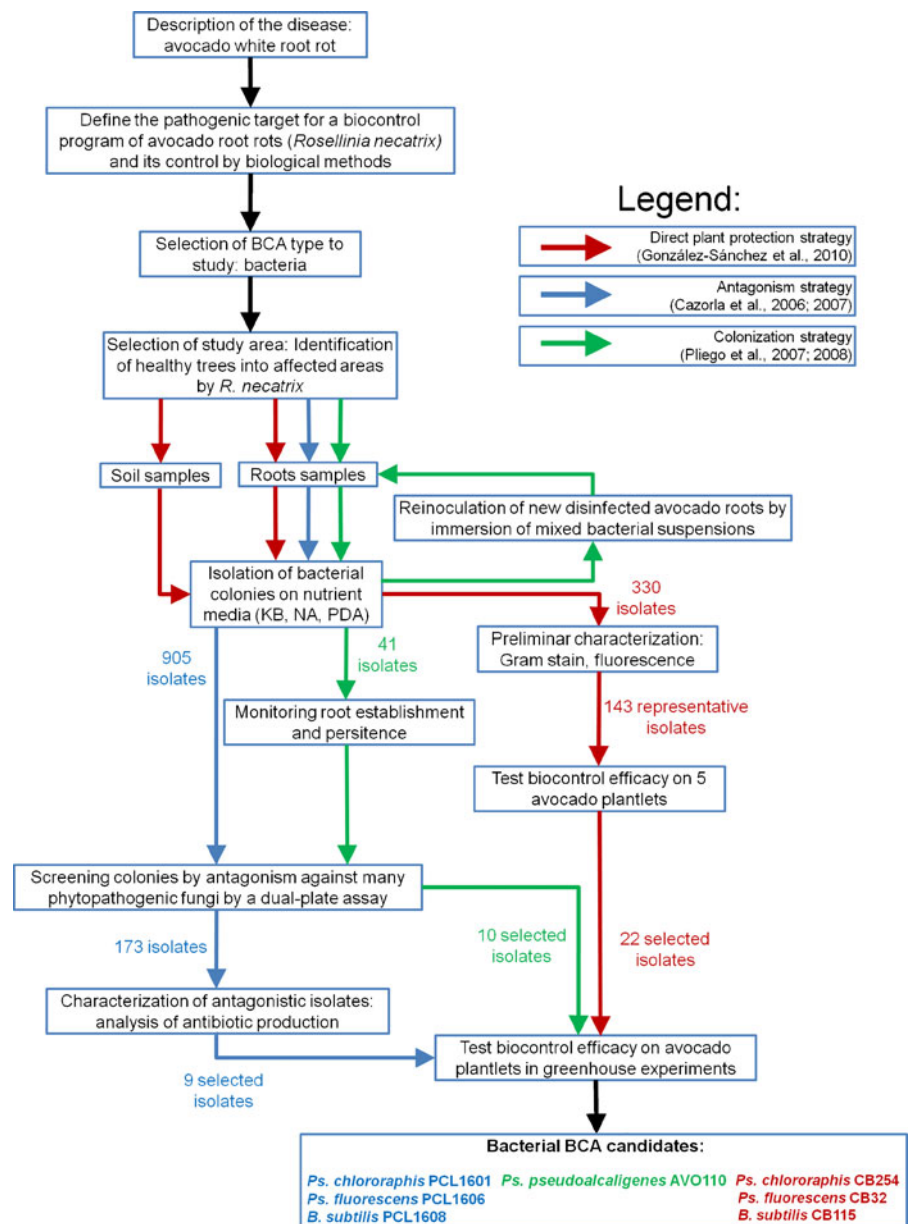
candidates for biocontrol. However, these previous strategies did not take into account the influence of biotic and abiotic factors in the rhizosphere of *Rosellinia*-infected plants, and they introduced a bias, selecting mainly those strains with biocontrol activity based on the mechanism used in the selection method, such as production of antifungal compounds (Cazorla et al. 2006, 2007) or root colonisation (Pliego et al. 2007). For this reason, a direct plant-protection strategy was developed to screen for candidate BCAs

that considered all potential biocontrol traits (red arrow in Fig. 1). This screening method, without a dominant selection pressure (e.g. colonisation abilities), expands the variety of strains selected and the different mechanisms of biocontrol against avocado root rot. Bacterial isolates from avocado root and soil samples were analyzed, and after recording which bacterial strains were protective against the white root rot, the selected strains were characterised. These potential BCA strains belonged to the genera *Pseu-*

domonas and *Bacillus*. All of the protective strains showed different biocontrol traits, but antagonism was identified as a generalised and relevant trait in the biocontrol activity of *Pseudomonas* and *Bacillus* strains against the white root rot of avocado (González-Sánchez et al. 2010).

In summary, our study suggest that in the avocado/*Rosellinia* test system, antagonism appeared to play a key role as pathogen major mode of action to biologically control *R. necatrix* because all of the microorganisms selected by the different screening strategies showed this ability but had no other trait in common. However, colonisation and induction of plant resistance could have also important roles in the final protection of the avocado roots because this has been demonstrated previously. Based on these studies, we support the use of antagonism as a valid screening strategy to select candidate BCAs in the avocado/*Rosellinia* test system because antagonism is a prevalent trait in all of the selected bacterial BCAs.

In conclusion

Identification of biocontrol bacteria based exclusively on their performance on laboratory media usually biases the selection to organisms functioning by antibiosis or hyperparasitism, but with some possible exceptions discussed above, these tests overlook organisms that act by competition or induced host plant resistance. Screening for antagonists in pots containing test plants and the pathogen *via* the disease to be controlled in a more soil-like substrate than agar increases the chances of selecting bacterial agents showing a broader spectrum of desirable biocontrol properties. However, the rather uniform environmental conditions in which pot tests are performed, compared to most field situations, usually leads to an overestimation of antagonistic strains. Nevertheless, and based on our results obtained for the pathosystem avocado plants (*Rosellinia necatrix*), direct selection of antagonistic bacteria might offer an easily performed strategy for some plant/pathogen systems.

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