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Biological control of plant pathogens: advantages and limitations seen through the case study of *Pythium oligandrum*

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Abstract The management of certain plant beneficial microorganisms [biological control agents (BCAs)] seems to be a promising and environmental friendly method to control plant pathogens. However, applications are still limited because of the lack of consistency of BCAs when they are applied in the field. In the present paper, the advantages and limitations of BCAs are seen through the example of *Pythium oligandrum*, an oomycete that has received much attention in the last decade. The biological control exerted by *P. oligandrum* is the result of a complex process, which includes direct effects through the control of pathogens and/or indirect effects mediated by *P. oligandrum*, i.e. induction of resistance and growth promotion. *P. oligandrum* antagonism is a multifaceted and target fungus-dependent process. Interestingly, it does not seem to disrupt microflora biodiversity on the roots. *P. oligandrum* has an atypical relationship with the plant because it rapidly penetrates into the root tissues but it cannot stay alive in planta. After root colonisation, because of the elicitation by *P. oligandrum* of the plant-defence system, plants are protected from a range of pathogens. The management of BCAs, here *P. oligandrum*, is discussed with regard to its interactions with the incredibly complex agrosystems.

Keywords Antibiosis · Biocontrol agent · Biocontrol efficacy · Induced resistance · Microbial communities · Mycoparasitism · Plant growth promotion

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Introduction

Chemical pesticides used to control plant pathogens are frequently known for the negative impacts they may have on the environment and even on the consumers. This issue has become a matter of growing concern and societal pressure for healthy food, free of pesticides residues, compelling the research to rapidly develop environmental friendly methods. The Golden Age of chemical pesticides now seems to have gone with these concerns, and studies on biological control agents (BCAs) have increased significantly over the last years. However, in spite of the urgent need to find alternatives to pesticides, in Europe, only 14 BCAs genus of fungi, oomycete and bacteria microorganisms, such as *Trichoderma* spp., *Pythium oligandrum* and *Bacillus* spp., are currently registered in European regulation no. 1107/2009 (Table 1). Equally, *Pseudomonas* spp., *Trichoderma* spp. and *Fusarium oxysporum*, which are the three most studied groups of BCAs, others, like *P. oligandrum*, have received growing interest in the last decade.

Table 1 Fungal, oomycete, and bacterial biocontrol agents registered in European regulation no. 1107/2009 (according to EU pesticides database http://ec.europa.eu/sanco_pesticides:)

Biocontrol agent	Category	Status under registration	Date of approval	Authorised	Authorisation in progress
Ampelomyces quisqualis strain AQ10	Fungicide	Approved	01/04/05	BG, CY, DE, EL, ES, FR, IT, SI, UK	NL
Bacillus amyloliquefaciens subsp. *plantarum* D747	Fungicide	Pending			ES, IT
Bacillus firmus I-1582	Nematicide	Pending			ES
Bacillus pumilus QST 2808	Fungicide	Pending			ES
Bacillus subtilis str. QST 713	Fungicide+bactericide	Approved	01/02/07	DE, ES, FR, IE, IT, SI, UK	EL
Bacillus thuringiensis subsp. Aizawai strains ABTS-1857 and GC-91	Insecticide	Approved	01/05/09	BE, CY, DE, EL, ES, FI, FR, IT, LU, NL, PT, SE	AT
Bacillus thuringiensis subsp. *israeliensis* (serotype H-14) strain AM65-52	Insecticide	Approved	01/05/09	ES, FR, SE	
Bacillus thuringiensis subsp. kurstaki strains ABTS 351, PB 54, SA 11, SA12 and EG 2348	Insecticide	Approved	01/05/09	AT, BG, CY, CZ, DE, DK, EL, ES, FR, HU, IT, LT, LU, MT, NL, PL, PT, RO, SI, SK, UK	IE
Bacillus thuringiensis subsp. *tenebrionis* strain NB 176 (TM 14 1)	Insecticide	Approved	01/05/09	AT, EL, ES, FR, HU, IT	
Beauveria bassiana strains ATCC 74040 and GHA	Insecticide	Approved	01/05/09	BE, CY, EL, ES, FR, HU, IE, IT, NL, SE, SI, UK	
Aureobasidium pullulans	Fungicide+bactericide	Pending		AT, BE, FR, HU	ES, IT, SI, SK
Candida oleophila strain O	Fungicide	Pending		FR, UK	ES
Paecilomyces fumosoroseus Apopka strain 97	Fungicide	Approved	01/07/01	BE, FI, LU, NL, SE	AT
Paecilomyces fumosoroseus strain Fe9901	Nematicide	Pending		BE, BG, FR	ES, IT
Paecilomyces lilacinus strain 251	Nematicide	Approved	01/08/08	BG, IT	
Pseudomonas chlororaphis strain MA342	Fungicide	Approved	01/10/04	AT, BE, DE, DK, ES, FI, FR, IT, LT, LU, NL, SE, UK	PT
Pseudomonas sp. strain DSMZ 13134	Fungicide	Pending		NL, SE	AT, BE, ES
Pseudozyma flocculosa	Fungicide	Pending			ES
Pythium oligandrum M1	Fungicide	Approved	01/05/09	CZ, HU, PL, SK	AT
Streptomyces K61 (formerly *S. griseoviridis*)	Fungicide	Approved	01/05/09	BE, CY, EE, EL, ES, FI, HU, IT, LT, NL, SE	
Streptomyces lydicus WYEC 108	Fungicide+bactericide	Pending			ES
Trichoderma asperellum (formerly *T. harzianum*) *strains ICC012, T25 and TV1*	Fungicide	Approved	01/05/09	ES, FR, IT, SI	
Trichoderma asperellum (strain T34)	Fungicide	Approved	01/06/13		ES
Trichoderma atroviride (formerly *T. harzianum*) strains IMI 206040 and T11	Fungicide	Approved	01/05/09	ES, IT, SE	
Trichoderma atroviride strain I-1237	Fungicide	Approved	01/06/13	FR	ES
Trichoderma gamsii (formerly *T. viride*) strain ICC080	Fungicide	Approved	01/05/09	ES, SI	
Trichoderma harzianum strains T-22 and ITEM 908	Fungicide	Approved	01/05/09	BE, ES, FR, NL	SE
Trichoderma polysporum strain IMI 206039	Fungicide	Approved	01/05/09	ES, SE	
	Fungicide	Approved	01/05/09	NL	

Table 1 (continued)

Biocontrol agent	Category	Status under registration	Date of approval	Authorised	Authorisation in progress
Verticillium albo-atrum (formerly *Verticillium dahliae*) strain WCS850					
Coniothyrium minitans	Fungicide	Approved	01/01/04	AT, BE, CZ, DE, DK, EL, ES, FR, HU, IE, IT, LU, NL, PL, SE, SK, UK	PT
Lecanicillium muscarium (formerly *Verticillium lecanii*) strain Ve6	Insecticide	Approved	01/05/09	EL, FI, FR, NL, UK	BE

DE Germany, *AT* Austria, *BE* Belgium, *CY* Cyprus, *DK* Denmark, *ES* Spain, *FI* Finland, *FR* France, *EL* Greece, *HU* Hungary, *IE* Ireland, *IT* Italy, *LT* Lithuania, *LU* Luxembourg, *MT* Malta, *CZ* Czech, *NL* Netherlands, *PL* Poland, *PT* Portugal, *RO* Romania, *UK* United Kingdom, *SK* Slovakia, *SE* Sweden

The first description by Dreschler of the oomycete, *P. oligandrum*, dates back to 1930. It was long considered as a non-pathogenic microorganism (Al-Hamdani and Cooke 1983; Kilpatrick 1968; Martin and Hancock 1986), and, over time, several reports have convincingly demonstrated its biocontrol properties (Table 2). Since 1986, at least 44 publications have shown that *P. oligandrum* acts either directly or indirectly to protect the plants. It interacts directly with the fungal pathogens through distinct or combined mode of actions, such as mycoparasitism, antibiosis, nutrient and space competition, and/or indirectly by inducing resistance in the plants. An impressive number of diseases of various plants can be controlled by *P. oligandrum*, the reduction of pathogenic attacks varying from 15 to 100 %, depending on the host plant, the target pathogen and the application method. Some experiments have been made in the field, but most of them were carried out in greenhouses or at the laboratory scale.

Despite the hope raised by the use of BCAs and the research currently being done, the application of microbiological control by farmers is still limited (Alabouvette et al. 2006). This is usually explained by the lack of consistency when BCAs are applied in the field (Alabouvette et al. 2009). As regards *P. oligandrum*, our objective in this review is to describe its modes of action and relationships with the plant and the microflora. Since it shares many of the benefits and limitations of BCAs, it will certainly lead to an improved understanding of what we can expect in the future with this method of controlling plants.

P. oligandrum relationships within agrosystem

Knowledge of the relationship between a biocontrol agent and its environment, including microbial communities and the host plants, is essential to improve BCAs field efficacy. Determining how these complex systems interact will also be helpful in managing plant protection using BCAs (Edel-Hermann et al. 2009; Savazzini et al. 2009).

P. oligandrum relationships with plants

The oomycete *P. oligandrum* has been isolated from the rhizosphere of many plants (Ali-Shtayeh 1985; Klemmer and Nakano 1964; Kobayasi et al. 1977; Martin and Hancock 1986; Mulligan and Deacon 1992; Plaats-Niterink 1981; Ribiero and Butler 1992; Schmitthenner 1962; Vaartaja and Bumbieris 1964), and the relationships with roots have been particularly regarded for tomato. According to Le Floch et al. (2005) and Rey et al. (1998a), the relationship was qualified as an atypical interaction because it differed from all the plant/*Pythium* and plant/BCAs relationships previously described (Benhamou et al. 2012; Rey et al. 2008). Usually, non-pathogenic *Pythium* species do not penetrate into the root tissues, or else, they are immediately halted at the epidermis or the first layers of cortical root cells (Rey et al. 1998b). Although *P. oligandrum* does not damage plants, it can penetrate rapidly into the root tissues, and its ingress is even as rapid as pathogenic *Pythium*; but conversely to them, it does not cause damage in the plant tissues (Rey et al. 1996, 1998b). After root inoculation with *P. oligandrum*, electron microscopic observations and cytochemical labelling revealed that the oomycete first colonised rapidly and deeply the cortical area of tomato roots in <12 h, then the hyphae started to degenerate, while only a few host reactions appeared. Finally, after 48 h, most hyphae were empty, with only oospores being located in the root tissues, but these structures also died, as shown by their typical empty shell appearance. An important finding is that further to this interaction, in addition to the lack of root symptoms, induced resistance was observed in the plant, as well as plant growth promotion.

Table 2 List of publications on *P. oligandrum* biological control of plant pathogens

Mechanisms described	Crop	Pathogen	Protection level (%)	Year	Author	Experiment
Mycoparasitism	Agaricus bisporus	–	–	1990	Fletcher et al.	In vitro
Mycoparasitism	Peas	*Fusarium solani* f. sp. *pisi*, *Phoma medicaginis* var. *pinodella* and *Mycosphaerella pinodes*	–	1991	Bradshaw-Smith et al.	In vitro
Mycoparasitism	Tomato	*Rhizoctonia solani* AG-4, *Pythium ultimum*, *Pythium spinosum* and *Pythium irregulare*	≈70	1992	He et al.	-
Mycoparasitism	Wheat	*Pythium ultimum*	100	1997	Abdelzaher et al.	In vitro
Mycoparasitism	-	Sclerotia of *Sclerotinia sclerotiorum*	≈50	1999	Madsen and Neergaard	In vitro, field
Mycoparasitism	Cucumber	*Pythium ultimum*	≈37	1999	Ali-Shtayeh and Saleh	In vitro
Mycoparasitism	–	*Phytophtora parasitica*	–	2000a	Picard et al.	In vitro
Mycoparasitism	–	Sclerotia of *Botrytis cinerea* and *Sclerotinia minor*	–	2005	Rey et al.	In vitro
Mycoparasitism	–	*Pythium ultimum*, *Fusarium oxysporum*	–	2006	El-Katatny et al.	In vitro
Mycoparasitism	–	*Phytophtora parasitica*	–	2012	Horner et al.	In vitro
Mycoparasitism	Sugar beet, cress	*Pythium* spp.	≈26–33	1990, 1992, 1998	McQuilken et al.	Greenhouse
Mycoparasitism, antibiosis	–	*Pythium ultimum*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Rhizoctonia*, *Phytophthora megasperma*, *Verticillium albo-atrum*	–	1999	Benahmou et al.	In vitro
Mycoparasitism, plant growth promoting	Pepper	*Verticillium dahliae*	–	1998	Al-Rawahi and Hancock	In vitro, greenhouse
Mycoparasitism, plant growth promoting	Pepper	*Verticillium dahliae*	67	2007	Rekanovic et al.	Greenhouse
Mycoparasitism, plant growth promoting	Tomato	*Pythium dissotocum*	–	2009	Vallance et al.	Greenhouse
Induced resistance, mycoparasitism	Tomato	*Fusarium oxysporum* f. sp. *Radicis-lycopersici*	–	1997	Benhmou et al.	In vitro, greenhouse
Mycoparasitism, induced resistance	Potato	*Rhizoctonia solani* AG-3	46–87	2012	Ikeda et al.	In vitro, field
Nutrient and/or space competition	Cress	*Pythium ultimum*	–	1983	Al-hamdani et al.	Field
Nutrient and/or space competition	Sugar beet +cress	*Pythium ultimum* and *Aphanomyces cochlioides*	–	2001	Whipps and McQuilken	Greenhouse
Nutrient and/or space competition	Sugar beet	*Aphanomyces cochlioides*	≈50	2013	Takenaka and Ishikawa	Greenhouse, field
Nutrient and/or space competition, mycoparasitism	Cotton	*Pythium ultimum*	32–66	1986	Martin and Hancock	In vitro
Nutrient and/or space competition, mycoparasitism	Sugar beat	*Pythium ultimum*	≈88	1987	Martin and Hancock	Greenhouse
Nutrient and/or space competition, mycoparasitism	Tomato	Pathogen communities of soil	≈15	2012	Cwalina-Ambroziak and Nowak	Greenhouse
Plant growth promotion	Rice	–	–	1993	Cother and Gilbert	Greenhouse

Table 2 (continued)

Mechanisms described	Crop	Pathogen	Protection level (%)	Year	Author	Experiment
Plant growth promotion	Cucumber	–	–	1994	Kratka et al.	-
Plant growth promotion	Cucumber	–	–	1998	Wulff et al.	In vitro
Induced resistance	Tomato	*Phytophthora parasitica*	60	2000a, b	Picard et al.	Greenhouse
Induced resistance	Tomato	*Ralstonia solanacearum*	≈33	2008	Takenaka et al.	In vitro
Induced resistance	Sugar beet	*Aphanomyces cochlioides*	≈33	2006	Takenaka et al.	In vitro
Induced resistance	Tobacco	*Phytoplasma*	≈40	2003	Lherminier et al.	Greenhouse
Induced resistance	Tomato	*Ralstonia solanacearum*	–	2009	Masunaka et al.	In vitro
Induced resistance	Sugar beet	*Cercospora beticola*	12–52	2009	Takenaka et al.	In vitro, field
Induced resistance	Strawberry	*Botrytis cinerea*+*Sphaerotheca macularis*+*Mycosphaerella fragariae*	43–70	2010	Meszka and Bielenin	Field
Induced resistance	Tomato	*Botrytis cinerea*	≈30	2009	Le Floch et al.	Greenhouse
Induced resistance	Grapevine	*Botrytis cinerea*	75	2007	Mohamed et al.	In vitro
Induced resistance	Tomato	*Botrytis cinerea*	79	2011	Lou et al.	Greenhouse
Induced resistance	Tomato	*Fusarium oxysporum* f. sp. *Radicis-lycopersici*	84	2001	Benhamou and Garand	Greenhouse
Induced resistance	*Arabidopsis thaliana*	*Ralstonia solanacearum*, *Pseudomonas syringae*	87	2009	Kawamura et al.	Greenhouse
Induced resistance	Sugar beet and wheat	*Rhizoctonia solani* AG-2.2, *Fusarium graminearum*	34	2003	Takenaka et al.	In vitro
Induced resistance	Tomato	*Ralstonia solanacearum*	≈40	2006, 2008	Hase et al.	In vitro
Induced resistance, plant growth promotion	Tomtato	*Botrytis cinerea*	≈50	2003a, b	Le Floch et al.	Greenhouse

P. oligandrum plant relationships differed also from those of BCAs, such as *Trichoderma* spp. and *F. oxysporum* (Fo47) (Alabouvette et al. 2009; Howell 2003) because (1) the root colonisation of the two fungi was restricted to the surface or the upper cortical layers and (2) intense host reactions were set up once the fungi attempted to or managed to penetrate inside these root layers (Benhamou et al. 2001, 2002; Harman et al. 2004; Olivain and Alabouvette 1997, 1999; Olivain et al. 2006; Yedidia et al. 1999). In 2009, Le Floch et al. compared Fo47, *P. oligandrum* and *Trichoderma harzianum* interactions with tomato roots. Among the three-biocontrol agents, the *T. harzianum* strain they used was not able to penetrate into the root tissues. Fo47 hyphae penetrated the outer cortical root cells but *P. oligandrum* ingress was faster and deeper. A degenerative process was observed for the two microorganisms consecutively to cells invasion, but with Fo47, it was concomitant with the hyphae ingress in the plant, whereas with *P. oligandrum*, it appeared suddenly once the oomycete hyphae were deeply located in the inner cortical tissue. Thus, BCA interaction with the same host plant can differ significantly. We currently do not know whether the ability to deeply penetrate into the roots provides advantages over the other BCAs that are localised only at the root surface, but, as shown by Benhamou et al. (1997), it can lead to mycoparasitism in the inner roots. However, as the lifetime of *P. oligandrum* hyphae in the plant is short (<12 h), this phenomenon is only transient and cannot be considered as a biocontrol mechanism of the uttermost importance. As subsequent induced resistance occurred after plant interaction with *P. oligandrum*, one can assume that hyphae penetration inside the plants may help and promote the plant to react more rapidly and significantly to pathogenic infections. Nevertheless, experiments have to be done to verify this specific point.

P. oligandrum relationships with fungi

Many interactions between microorganisms occurred in the rhizosphere. They have been reproduced many times in vitro by confronting two microorganisms in Petri dishes. A BCA is assumed to control pathogens through various modes of action. In the literature, reports have shown that *P. oligandrum* is able to directly attack several fungal pathogens, using different mechanisms (Benhamou et al. 1997,

1999; Picard et al. 2000a; Rey et al. 2005). Depending on the fungal target, these include mycoparasitism and antibiosis. Some particular interactions with fungi that produced defence reactions to prevent *P. oligandrum* attacks have also been observed. In addition, the various types of interaction with sclerotia underline the multifaceted relationship that the oomycete established with fungi.

Mycoparasitism

P. oligandrum mycoparasitism is characterised by active growth along the host hyphae and the production of enzymes that degrade or break the host cell wall. *P. oligandrum* penetration inside the host cells was associated with the complete destruction of the cytoplasm of host cells, with the host finally dying. This process can be observed, for instance, in the interaction with *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) or *Pythium ultimum*. In these interactions, hydrolytic enzymes such as chitinases and cellulases are clearly involved (Benhamou et al. 1997, 1999). Against another oomycetes, for instance, *Phytophthora megasperma*, *P. oligandrum* is able to produce great quantities of cellulolytic enzymes as shown by Picard et al. (2000a). Recently, the nature of *P. oligandrum* mycoparasitism was studied at the molecular level by Horner et al. (2012). A complementary DNA library was constructed, and transcripts encoding proteases, protease inhibitors, glucanases, putative effectors and elicitors were identified during *P. oligandrum* interaction with heat-killed *P. infestans* hyphae. This set of proteins may act during mycoparasitism, but further investigations are needed to determine the role of each of the identified proteins. It was noticed that the level of mycoparasitism can be dependent on nutrient stress. Butler (1957) studied *Rhizoctonia solani* mycoparasitism on several fungal hosts, i.e. mainly mucorales species (Phycomycetes) and concluded that regular growth depended on temperature, nutrition and light but that this growth was a prerequisite for heavy parasitism. Thus, we can hypothesise that, in harsh environments, this mode of action is consistently attenuated.

Antibiosis

Antibiosis has long being known and is defined as the specific interaction in which the prey is destroyed by toxic secondary metabolites produced by antagonistic microorganisms (Alabouvette et al. 2009; Baker 1968; Fravel 1988; Haas and Défago 2005; Wright 1956). As regards *P. oligandrum*, this phenomenon was studied by Bradshaw-Smith et al. (1991) against the three major footrot pathogens of pea: *Fusarium solani* f. sp. *pisi*, *Phoma medicaginis* var. *pinodella* and *Mycosphaerella pinodes*. A volatile antibiotic compound produced by *P. oligandrum* reduced the growth rate of *P. medicaginis* and *M. pinodes*, but this molecule has not been yet purified and identified. Another typical phenomenon of antibiosis was reported by Benhamou et al. (1999) when they observed the degradation of *P. megasperma* without physical contact with *P. oligandrum* hyphae. Note that nutrient stress can impact these antagonistic performances and that the production of such compounds is also dependent on abiotic factors.

As mentioned by Rey et al. (2008), mycoparasitism and antibiosis can be observed during the same interaction with a fungal host. Microscopic observations made by Benhamou et al. (1997, 1999) led to the conclusion that antibiosis might precede mycoparasitism, which is associated with hydrolytic enzyme production against *F. oxysporum*. We can hypothesise that *P. oligandrum* modulates the production of antifungal compounds depending on the target pathogen, leading to two strategies: either mycoparastism, associated with hydrolytic enzymes, or parasitism via antibiosis. The former strategy seems more frequent than antibiosis. These results indicate that one of the main advantages in using BCAs is the variety of interactions that they can establish with the fungal hosts. *P. oligandrum* seems to adapt to the fungal pathogens and attempts to destroy them by mycoparastism, antibiosis, or a combination of the two processes (Benhamou et al. 2012). This reflects the multiplicity of interactions that exist in nature.

Nutrient and space competition

As proposed by Alabouvette et al. (2006), this is defined as a general phenomenon regulating the population dynamics of microorganisms sharing the same ecological niche and physiological requirements when the resources are limited. As regards *P. oligandrum*, nutrient and space competition was thought to be involved in at least one case. Martin and Hancock (1986) observed that elevated concentration of chloride raised propagule densities of *P. oligandrum*, allowing successful competition with *P. ultimum*, leading to soil suppressiveness against the pre-emergence damping off of cotton. A relatively similar mode of action was reported for *T. harzianum* against *R. solani* on radish (Lui and Baker 1980) and *F. oxysporum* against fusarium wilt on melon (Alabouvette et al. 1979, 1983). Even if this mode of action is the principal one in microbial antagonism for suppressing pathogens causing the decay of harvested fruits and vegetables (Sharma et al. 2009), this mechanism is considered difficult to exploit for biological control (Alabouvette et al. 2006), in particular for soilborne pathogens whose interactions are numerous. Benhamou et al. (2012) reported that French and Japanese studies on *P. oligandrum* described mycoparasitism and/or induced resistance as the main modes of action. Thus, the competition for

space and nutrients is probably a minor mechanism used for biological control by *P. oligandrum*.

Fungal defence reactions against *P. oligandrum* mycoparasitism

Plant defence reactions are induced after recognition of microbial effectors. Some fungi are able to activate structural defence reactions after microbial recognition. Currently, little is known about this kind of reaction, but two studies on *P. oligandrum* report this kind of finding. Picard et al. (2000a) observed in *P. oligandrum–Phytophthora parasitica* confrontation a pathogen defence reaction to the biocontrol agent prior to direct contact. It was characterised by a global production of cellulose-enriched material around all the hyphae wall of *P. parasitica*. Benhamou et al. (1999) found that in *P. oligandrum–R. solani* interaction, a chitin-rich deposit accumulated in the wall of the pathogen at potential sites of penetration by the biocontrol agent. These two studies showed that two patterns of fungal defence response can be set up on the cell wall of fungi, with one being localised and the other more generalised to the entire hyphae. These pathogen defence reactions were, in the two cases, not strong enough to stop *P. oligandrum* penetration and invasion of the reacting host cell, thus demonstrating that the great ability of *P. oligandrum* to produce large amount of cell wall degrading enzymes is of major importance. Evidence was provided that host defence reactions were initiated by molecules secreted by *P. oligandrum*, but so far, no molecules have been identified.

Microbial antagonists of *P. oligandrum*

P. oligandrum can also be attacked by some fungi. Many examples of cell damage to the oomycete were observed after *P. oligandrum* interaction with *R. solani* (Benhamou et al. 1999). Both microorganisms, the pathogen and the antagonist, were marked by morphological alterations, and 3 days after the start of the interaction, *R. solani* clearly collapsed and the hyphae cells of *P. oligandrum* became disorganised. Other fungi are known to attack *P. oligandrum*. Le Floch et al. (2009) observed that, in Petri dishes, *F. oxysporum* (Fo47) and *T. harzianum* destroyed *P. oligandrum* cells, mainly through a combination of antibiosis and mycoparasitism. It could also happen that a strain belonging to the same plant pathogenic species, e.g. *Pythium aphanidermatum*, can be mycoparasited by *P. oligandrum*, while another strain displayed mycoparasite-like ability against *P. oligandrum* (Benhamou et al. 1999; Jones and Deacon 1995). These results indicate the complexity of the *P. oligandrum*/host interactions in nature partly explaining why some biocontrol experiments aimed at protecting plants fail or are successful.

P. oligandrum and fungal resting structures

P. oligandrum is also able to attack resting structures such as sclerotia (Foley and Deacon 1986). Madsen and Neergaard (1999) reported that, in comparison with the control treatment, survival of *Sclerotinia sclerotiorum* sclerotia was reduced by 50 % after *P. oligandrum* soil treatment. The same result was observed when sclerotia were treated with *P. oligandrum* culture filtrate. The oomycete is able to use sclerotia as sole nutrient source to complete its entire life cycle. It produces cell wall degrading enzymes, endochitinase, protease, ß-glucanase, ß-glucosidase and cellobiohydrolase to attack sclerotia. Foley and Deacon (1986) previously described the ability for *P. oligandrum* to feed from sclerotia exudates. These results underlined that *P. oligandrum* has a great array of degradative enzymes able to penetrate into resting structures like sclerotia and that it can be useful for biological control strategy aimed at reducing sclerotia (primary inoculum). Certain sclerotia seem, however, to be resistant to *P. oligandrum* attacks. For instance, Rey et al. (2005) demonstrated that *Botrytis cinerea* sclerotia were sensitive, while the *S. minor* ones were not. Melanin, a compound that entirely covers *S. minor* sclerotia, apparently protects the rind cells against enzymes from antagonist microorganisms (Bull 1970). In this case, *P. oligandrum* attack failed, and it could not penetrate inside the *S. minor* sclerotia. To support this assumption, Rey et al. (2005) reported that *P. oligandrum* entered *B. cinerea* sclerotia but only through breaches, at the junction of rind cells, that also corresponded to gaps in melanin deposits.

Taken together, these observations show that *P. oligandrum* is able to mycoparasite a broad range of pathogens and, depending on the host, can adjust its attack. For BCA registration, it is worth considering various pathogenic targets. The broad spectrum of *P. oligandrum* hosts suggests that it is important to manage the great variety of interactions that can lower or increase the efficiency of biological control.

P. oligandrum **relationships with the environment**

Natural ecosystems are incredibly complex, and the relationship of a BCA with the environment is still difficult to predict and manage. Interactions occur not only with biotic factors but also with abiotic ones.

Rhizosphere colonisation ability

As mentioned in the literature, *P. oligandrum* is rhizospheric competent (Al-Rawahi and Hancock 1998; Le Floch et al. 2003a; McQuilken et al. 1990; Takenaka et al. 2008). Successful plant protection by a BCA needs its establishment in

the field over the whole span of the culture. In a hydroponic greenhouse, Le Floch et al. (2003a, 2007) reported that optimal protection was obtained when tomato roots were heavily colonised by *P. oligandrum*. In soilless culture, *P. oligandrum* root colonisation depended on the nature of substrates. For instance, Rey et al. (1999) pointed out that a higher level of colonisation was observed in an organic substrate (peat) than in an inorganic one (rockwool). Thus, inoculation strategies must be adapted to the type of culture. To improve *P. oligandrum* colonisation and persistence, Vallance et al. (2009) selected three strains on the basis of their ability to form oospores, to produce an elicitor as well as an auxin-like compound. Finally, the advantage of using a combination of three *P. oligandrum* strains to increase persistence of the strains was shown. However, this study also demonstrated that the screening procedure is of the utmost importance in selecting strains. The experiment made in the laboratory to select the strains did not necessarily reflect their ability to colonise roots in greenhouse conditions. At the present time, this point is a major limitation for the selection of *P. oligandrum* strains for biological control. On the same basis, it is worth noting that the right method needs to be used in order to count and check whether the BCA does or does not colonise the plant. Le Floch et al. (2007) showed that molecular (quantitative real-time PCR and DNA macroarray) and culture-dependent methods to monitor *P. oligandrum* populations provided contradictory results. When a selective medium was used, *P. oligandrum* persisted for 3 months, but, according to quantitative real-time PCR and DNA macroarray, the oomycete was able to persist on roots for 6 months, suggesting that one treatment was sufficient. Weaver et al. (2005) obtained the same type of variation between molecular and cultural analyses when they monitored *Trichoderma virens* populations in soil. Thus, particular attention has to be paid in selecting the methods for monitoring the colonisation of a given BCA.

Impact on microflora

To protect plants, as a great number of BCAs cells are introduced in the culture, the risk assessment of the use of BCAs on indigenous microflora has to be determined. Currently, few studies deal with the impact of BCA on microbial communities. Vallance et al. (2009, 2012) studied the impact of *P. oligandrum* on the fungal and bacterial communities colonising the rhizosphere of tomato plants. A fingerprinting analysis made by single-strand conformation polymorphism revealed that, 6 months after root inoculation with *P. oligandrum*, native fungal communities were similar in tomato roots treated or not with *P. oligandrum*. Investigations on bacterial communities showed transient perturbations in rhizospheric indigenous bacterial communities for the plant treated with *P. oligandrum*. However, this shift did not persist until the end of the cropping season. In fact, in the two studies, bacterial and fungal community shifts were observed over the growing season, but the biocontrol agent had no or little influence on the indigenous microflora. Savazzini et al. (2009) reported that a transient shift in bacterial and fungal communities colonising the vineyards soils was observed only during the first 2 weeks following inoculation with *Trichoderma atroviride* SC1. Edel-Hermann et al. (2009) obtained similar conclusions concerning *F. oxysporum* introduction in soil. Terminal restriction fragment length polymorphism analysis showed that bacterial and fungal communities were not significantly affected by the BCA, but evolved over time.

Consequently, the environmental conditions had a greater influence on the communities than the biocontrol agent application. Concerning the biodiversity of the microflora, those results are favourable to fungal or oomycete BCA regarding the weak impact that they have on the native microbial communities colonising the roots.

Influence of abiotic factors on *P. oligandrum*

The abiotic factors, in association with the biotic ones, certainly have an important role on BCAs behaviours in the field. Little is known about the influence of abiotic factors on biocontrol efficacy. *P. oligandrum* is a common inhabitant of soils (Rey et al. 2008), and recently, strains were isolated (Gerbore, unpublished data) from the vineyards planted in soils with various physico-chemical properties. This capacity to colonise plants growing in various soils is important for a BCA; it means that it could be applied in various environments. Nevertheless, many questions have still to be addressed, such as the impact of cultural practices on BCAs populations. All these factors can have a significant impact on the level of protection.

P. oligandrum plant induction of resistance and plant-growth promotion

Biocontrol agents such as *T. harzianum*, *F. oxysporum* strain Fo47, and *P. oligandrum* also have an indirect effect on diseases through the induction of plant disease resistance. Interestingly, another beneficial effect, the growth promotion of plants, is associated with the colonisation of roots (Benitez et al. 2004; Fravel et al. 2003; Le Floch et al. 2003b; Rey et al. 2008).

Induced plant resistance

Several experiments have reported that treatment of plants with *P. oligandrum* hyphae or its elicitors, oligandrin and

cell wall proteins (CWPs), induced plant resistance (Benhamou et al. 1997; Le Floch et al. 2003a; Lherminier et al. 2003; Masunaka et al. 2010; Mohamed et al. 2007; Picard et al. 2000b; Takenaka et al. 2003, 2006, 2008).

Induction of plant resistance by *P. oligandrum hyphae*

After the introduction of *P. oligandrum* in the rhizosphere, the oomycete induces resistance at the local level, i.e. in the roots but, as shown by Le Floch et al. (2003a), this effect is also systemic, extending to all the parts of the plant. Using transmission electron microscopy (TEM) observations, Benhamou et al. (1997) first described locally induced resistance in tomato roots challenged with pathogen FORL. The host resistance was characterised by an enhanced response to pathogenic attack in comparison to the non-challenged *P. oligandrum* infected plants. TEM observation showed wide accumulation of phenolic and callose in *Fusarium*-challenged–*P. oligandrum*-inoculated tomato roots.

In the plants, resistance to various plant pathogenic fungi, i.e. *FORL*, *B. cinerea*, *P. ultimum* and *R. solani*, are induced by *P. oligandrum* (Benhamou et al. 1997; Brozova 2002; Le Floch et al. 2003a). This broad spectrum of pathogenic attack control is in favour of a non-specific plant stimulation of defences. As regards the systemic resistance, Le Floch et al. (2003a), showed an increased induction of PR proteins, i.e. PR-3b, PR-5a and a new isoform of PR-3b, in tomato plants colonised at root level by *P. oligandrum*, and then infected on leaves with *B. cinerea*. Interestingly, the synthesis of PR proteins was only triggered in *P. oligandrum*-treated plants when the leaves were attacked by the pathogen. This phenomenon, called "priming", corresponds to a particular physiological status in which plants trigger their defence mechanisms more rapidly and at higher level when attacked by a given pathogen (Conrath et al. 2002; Conrath 2009; Jung et al. 2009).

Other studies reported that systemic resistance is induced by many other BCAs, e.g. *Trichoderma* spp. and *F. oxysporum* (Harman et al. 2004; Veloso and Diaz 2012) on a broad range of host plant. A biocontrol agent is, thus, not necessarily specific to a host plant and can trigger general defence responses to control several pathogenic attacks. This argues in favour of the registration of these microorganisms to control not only one disease or a few diseases, as it is the case for chemical control methods, but also against various plant pathogenic microorganisms, fungi as well bacteria.

P. oligandrum elicitors

P. oligandrum produces two types of elicitor, either secreted by the oomycete or extracted from its cell wall. Masunaka et al. (2010) reported that these two elicitors, e.g. oligandrin and CWPs, are specific to this oomycete.

Picard et al. (2000b) discovered an extracellular protein from culture filtrate of the oomycete, named oligandrin. This 10 kDa protein was classified as an elicitin-like protein because of the similarity it shared with the classical elicitin "signature" described by Ponchet et al. (1999). Oligandrin has been successfully used to induce systemic resistance in tobacco (Lherminier et al. 2003) against phytoplasma infection, in tomato against *P. parasitica*, *B. cinerea* and FORL (Benhamou et al. 2001; Lou et al. 2011; Picard et al. 2000b) and in grapevine against *B. cinerea* (Mohamed et al. 2007). This protein induces defence responses but without triggering the hypersensitive reaction (HR) associated with necrotic response (Picard et al. 2000b). Mohamed et al. (2007) compared the application of oligandrin alone or of *P. oligandrum* oospore inoculum on grapevine roots to control *B. cinerea*. The protection level in plant pre-treated leaves reached 75 %, and no significant differences were observed after treatment either with the biocontrol agent, or its elicitor.

The second type of elicitor, classified as an elicitin-like protein (Takenaka et al. 2006), corresponds to cell wall proteins (CWPs), coded POD-1 and POD-2 by Takenaka et al. (2003). Protection via induced resistance was obtained against bacterial and fungal pathogens, i.e. *Ralstonia solanacearum* and *Pseudomonas syringae* on *Arabidopsis* (Kawamura et al. 2009); *Cercospora beticola*, *Rhizoctonia solanacearum* and *Aphanomyces cochlioides* on sugar beet (Takenaka et al. 2006, 2003; Takenaka and Tamagake 2009); and against *Fusarium graminearum* on wheat (Takenaka et al. 2003). Regarding the protection obtained after either POD-1 or POD-2 treatment of plants, Takenaka et al. (2006) obtained equivalent disease protection of sugar beet against *A. cochlioides* with the two CWPs. Nevertheless, distinctness was observed in the number of defence-related genes induced, five genes for POD-1 and three for POD-2. The authors assumed that the two elicitors may induce distinct defence reactions, even if the same protection level is observed.

Combining the two CWPs and oligandrin to stimulate more genes could provide an opportunity to increase plant protection. Another interesting point about these proteins is that the two types of elicitor are not specific to plant species, which is consistent with results obtained with *P. oligandrum* hyphae.

Induced plant growth promotion

Microorganisms promoting plant growth are the object of numerous studies, as shown by the literature dedicated to plant growth promoting rhizobacteria and fungi (Bloemberg and

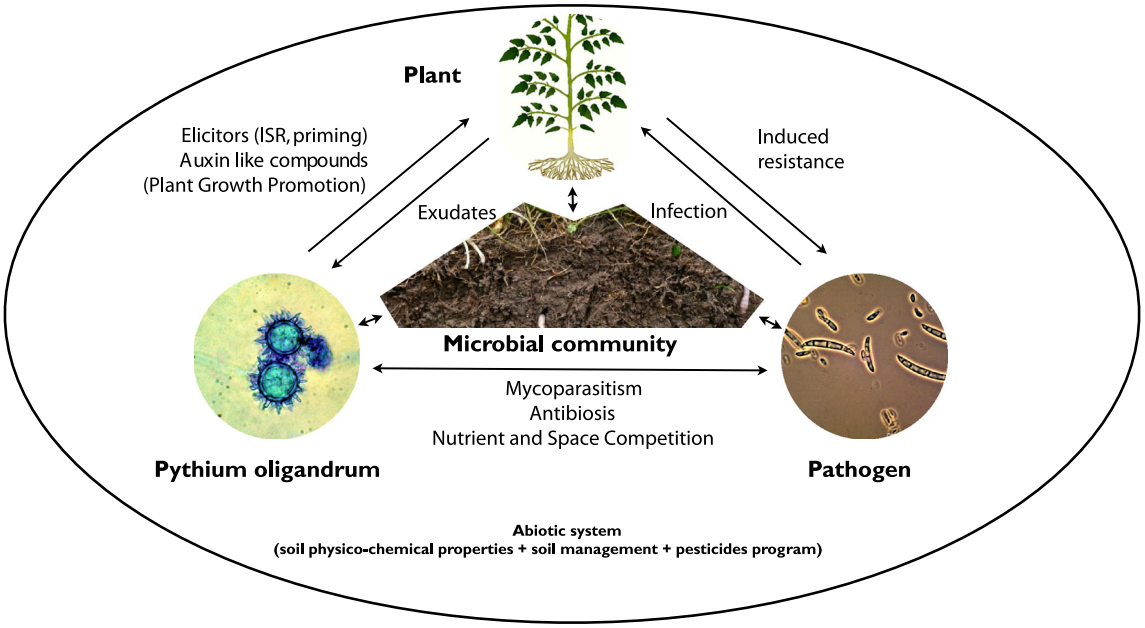

Fig. 1 Summary of *Pythium oligandrum* interactions with its natural environment. *ISR* induced systemic resistance

Lugtenberg 2001; Lugtenberg and Kamilova 2009; Hyakumachi and Kubota 2003; Mallik and Williams 2008). This phenomenon is frequently associated with the microbial production of phytohormones and secondary metabolites by the microorganisms (Helman et al. 2011; Hermosa et al. 2012; Kloepper et al. 1991; Lifshitz et al. 1987). *P. oligandrum* induction of plant growth promotion has also been the subject of studies performed by various authors (Al-Rawahi and Hancock 1998; Cother and Gilbert 1993; Kratka et al. 1994; Le FLoch et al. 2003b; Rekanovic et al. 2007; Wulff et al. 1998). Plant growth promotion occurs after what seems to be a latent period. For instance, Wulff et al. (1998) observed this phenomenon when they applied *P. oligandrum* zoospores on cucumber seedlings. The first 2 days, *P. oligandrum* inoculation caused adverse effects on root seedlings; then, root elongation was stimulated. Rey et al. (1998a) proposed an explanation for these two phases. The first phase is assumed to correspond to the first stage of *P. oligandrum* hyphae penetration in the root tissues; it induces a slight defence response, which is cost effective in terms of energy. After this initial phase, plant growth is promoted. In some cases, this beneficial effect could persist for several months, as shown for tomato plants growing in soilless culture (Le Floch et al. 2003a). Plant growth promotion was associated with the production of auxinic compounds. Le Floch et al. (2003b) reported that the tryptamine pathway exists in the oomycete hyphae. *P. oligandrum* is able to metabolise an auxin-compound, tryptamine (TNH2), from tryptophan and indole-3-acetaldehyde. TNH2 was absorbed by the root system and secondary roots subsequently developed. The tryptamine pathway is known in other non-pathogenic species such as *Aspergillus*, *Penicillium* and *Rhizopus* (Frankenberger and Arshad 1995), but *P. oligandrum* differs from them because it cannot transform TNH2 into indole-3-acetic acid (IAA). Interestingly, TNH2 influx can boost IAA synthesis in tomato, even if it is not the major endogenous precursor of IAA in this plant (Cooney and Nonhebel 1991).

Production of this auxin-like compound (TNH2) in the rhizosphere can promote plant growth promotion of tomato plants, so a slight but frequent release of TNH2 by *P. oligandrum* can be beneficial for plant development. Interestingly, roots are sensitive to very low concentrations of auxins (Taiz and Zeiger 1998), but the quantity of precursors such as tryptophan, which is naturally present in root exudates, can limit this phenomenon (Rybicka 1981). Note that this observation has been made for tomato, but whether this phenomenon occurs in other plants is still unknown.

Biocontrol agent combinations

The idea of combining BCAs came from observations of suppressive soils, in which suppressiveness was attributed to microflora (Louvet et al. 1976; Rouxel et al. 1979). Alabouvette and Lemanceau (1999) reported that soil suppressiveness to *Fusarium* wilts was attributed to the action of fluorescent *Pseudomonas*, saprophytic *Fusarium* such as *F. solani* and non-pathogenic *F. oxysporum*, in concert with abiotic factors, i.e. carbon and iron availabilities.

Consequently, in order to improve biocontrol consistency, one strategy could be to reproduce what occurs in certain suppressive soils. That would consist in opposing the diversity of pathogenic strains to another diversity made up of BCAs in a single inoculum. Such combinations can be made

either by combining BCAs strains of the same species or of several species. To increase rhizosphere colonisation, inoculum with several strains from the same species would probably be the best option. Combining several species of BCAs would provide the opportunity to associate various biocontrol modes of action and would certainly enhance plant protection.

In a 6-month experiment made in a soilless culture, Le Floch et al. (2003a) showed that tomato yield was increased after root colonisation by one strain of *P. oligandrum*. In order to enhance this positive effect on the plants, Le Floch et al. (2007) inoculated three selected strains of *P. oligandrum* on roots of tomatoes grown in the same cultural conditions. They showed that colonisation was effectively improved, but it was not associated with increased tomato yield, suggesting that this positive effect was not always easily reproduced.

Another experiment conducted by Le Floch et al. (2009) aimed at comparing tomato grey mould disease severity for plants treated with various BCAs inoculum. *P. oligandrum* was either alone or in combination with Fo47 or a combination with *T. harzianum*; a combination of the three BCAs was also tested. Even if in dual plate tests, Fo47 and *T. harzianum* destroyed *P. oligandrum* hyphae, the three microorganisms persisted when introduced together in the rhizosphere of tomatoes. Transmission electronic microscopy analysis showed that *P. oligandrum* was attacked by *T. harzianum* or Fo47 only when they were in close contact; otherwise, the three fungi persisted in the rhizosphere. This result underlined that complex interactions in the environment are difficult to predict even if laboratory studies provide relevant indications on the modes of action of each BCA.

Literature studies reported increased efficacy against *B. cinerea* on strawberry using yeast (*Pichia guilermondii*) in combination with a bacterium (*Bacillus mycoides*; Guetsky et al. 2001 and 2002). However, this kind of result was not always obtained, as reported in numerical studies (Xu et al. 2010, 2011a) using models (Jeger et al. 2009) to determine the better efficient biocontrol strategies. The main conclusions to be drawn suggest first that a BCA with a single main mechanism is less effective in suppressing diseases than a BCA, which combines two mechanisms. Second, combining two BCAs with distinct modes of action is generally not better than applying a single BCA. Xu et al. (2011a) concluded that synergic effects between BCAs are difficult to predict and need a clear knowledge of their interactions. These results confirmed the global literature data review of Xu et al. (2011b) based on 36 publications concerning the use of combined BCAs. BCA associations can lead either to an increased, similar or sometimes lower protection efficacy. As regards *P. oligandrum*, Le Floch et al. (2009) demonstrated that for all the BCA treatments, plant-induced resistance to *B. cinerea* was observed, but that no significant differences were obtained after application of *P. oligandrum* on its own or in combination with Fo47 and/or T. *harzianum*. Finally, synergetic effects among BCAs would certainly be of interest for managing plant colonisation, but that does not necessarily increase the level of plant protection. Furthermore, dual culture tests of BCAs provided pieces of information on their ability to grow together, but it did not correlate with their ability to survive in the field, where various ecological niches exist.

Conclusion

This paper on the advantages and limitations of *P. oligandrum* biocontrol stresses the main points to be considered in order to achieve efficient crop protection with a BCA. Figure 1 summarises the interactions of a biocontrol agent, here *P. oligandrum*, within an agrosystem. To colonise and persist in the rhizosphere, the oomycete must adapt to abiotic factors, e.g. soil physico-chemical and structural properties combined with soil management, pesticide programmes and also with biotic factors, e.g. plant hosts, microbial communities and plant pathogens. If the biocontrol agent is established in the soil, biological control can directly affect the pathogens via mycoparasitism, antibiosis and/or indirectly effect, via induced resistance to plant diseases with or without induced plant growth promotion. The fitness of strains is also very important. One of the main advantages in the use of BCAs comes from the minor impact that they have on microflora biodiversity. The impact of long-term biological control strategies will, however, have to be addressed to anticipate possible negative effects on environment. Finally, a major challenge for the scientific community in the coming years will be to (1) focus on field experiments, which, although they are time consuming, are of the utmost importance, and (2) make growers sensitive to these new kinds of control products.

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