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Original article

Enhancement of development and induction of resistance in tomato plants by the antagonist, *Pythium oligandrum*

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Abstract – To exert an optimal biological control, *P. oligandrum* must colonise roots and persist in the rhizosphere of plants throughout the cultural season. The present study demonstrated that, after inoculation of root system by *P. oligandrum*, it colonised 20 to 40% of roots of tomato plants grown in hydroponic system. Constant presence of the introduced fungus in the rhizosphere over the cultural season is correlated with an increase in tomato yield. The combination of several factors likely explains this *P. oligandrum*-mediated increase. Among them, one may cite mycoparasitism; however, though *P. oligandrum* can parasitize other pathogenic *Pythium* species; root colonisation by the antagonist was not associated with significant reduction in *Pythium* spp. populations. In the present case, the induction of plant resistance seems more prevalent. Indeed, root colonisation with *P. oligandrum* induced systemic resistance. Interestingly, *P. oligandrum*-inoculated plants triggered and amplified PR proteins synthesis only when leaves had been attacked by the pathogen, *Botrytis cinerea*.

mycoparasitism / yield increase / induced resistance / PR proteins / biocontrol agent

Résumé – Augmentation du développement et induction de résistance chez des plants de tomate par l'agent antagoniste, *Pythium oligandrum*. Pour que *Pythium oligandrum* exerce un contrôle biologique optimal, il doit coloniser et persister dans la rhizosphère des plantes durant toute la saison culturale. La présente étude montre qu'après inoculation du système racinaire par *Pythium oligandrum*, celui-ci colonise 20 à 40 % des racines de plants de tomate cultivés dans un système hydroponique. La présence constante du champignon dans la rhizosphère tout au long de la saison culturale est corrélée avec une augmentation de rendement en tomates. La combinaison de plusieurs facteurs explique certainement l'augmentation induite par *P. oligandrum*. Parmi ceux-ci, on peut citer le mycoparasitisme. Bien que *P. oligandrum* puisse mycoparasiter d'autres espèces de *Pythium* pathogènes, la colonisation racinaire par l'antagoniste n'est pas associée avec une réduction significative des populations de *Pythium* spp. Dans le cas présent, l'induction de résistance chez la plante semble prévaloir. En effet, la colonisation des racines par *P. oligandrum* induit une résistance systémique. D'une façon remarquable, les plantes inoculées par *P. oligandrum* déclenchent et amplifient la synthèse de protéines PR seulement quand les feuilles sont attaquées par l'agent pathogène, *Botrytis cinerea*.

mycoparasitisme / augmentation de rendement / résistance induite / protéines PR / agent de lutte biologique

1. INTRODUCTION

In soilless culture, although pathogenic fungal species are relatively few in number compared to those observed in conventional cultures, root diseases are frequently noticed and diseases are occasionally more severe than in soil [6, 9, 27]. Additionally, emergence of infecting pathogens specific to hydroponics has been frequently pointed out. For instance, some fungi which are of little or unrecognised importance under field conditions, e.g. *Pythium* group F [19, 21] and *P. dissotocum* [26], may become of economic importance in hydroponic cultures. Once introduced in the greenhouse, infections can reach an important level because of pathogen development and spreading to the whole cultural system. Several methods are now available to prevent attacks; among them, one can cite: (i) nutrient solution disinfection [5, 22, 25]; (ii) resistant cultivars [27] or (iii) fungicides application [27]. But, each of them has its limits. Nutrient disinfection is mainly effective when used as preventive application. Resistant cultivars against *Pythium* spp. do not exist and few are resistant to the other most frequent pathogens. Additionally, when resistant cultivars are available, they are not always of economical

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interest for growers. Application of some fungicides to the recirculating nutrient solution is relatively easy, but, only few of them are registered.

This is why the interest for developing alternative pathogen-control methods based on the management of microorganisms has been growing in recent years [11, 12, 14, 24]. Such methods may consist of the introduction of antagonists into the plant nutrient solution or favour the development of indigenous suppressive microflora in the rhizosphere [15, 16]. Rafin et al. [17] pointed out that antagonistic-*Pythium* are naturally present, though at low level, in soilless cultures. Previous experiments performed by our group with one of them, *Pythium oligandrum*, have shown that it can protect plants through a tripartite interaction between the biocontrol agent, the pathogen and the plant. It is generally considered that such a complex process includes three different effects: (i) the control of pathogens in the rhizosphere by mycoparasitism and/or antibiosis [2]; (ii) plant-induced resistance, because tomatoes inoculated with *P. oligandrum* or a proteinaceous elicitor produced by the fungus, sensitise plants to respond more rapidly and efficiently to pathogen attacks [1, 3, 13] and (iii) plant growth promotion associated with cucumber [10, 31] or tomato root colonisation by *P. oligandrum*. Nevertheless, these beneficial effects are assumed to occur only when the biocontrol microorganism is colonising plants and persists in the rhizosphere. In the literature, it has been reported that, often, a lack of survival of the introduced microorganisms resulted in an insufficient biocontrol effect [16].

To overcome this problem, our aim was to introduce *P. oligandrum* in an experimental hydroponic greenhouse and to assess its ability to colonise roots throughout the cultural season. To verify the positive impact of *P. oligandrum*-root colonisation both on plant development and resistance, tomato yield was studied and compared to those of non-inoculated plants. The control of *Botrytis cinerea*, one among the most important agent responsible for foliar diseases observed in greenhouse-cultivated tomatoes [4], was concomitantly investigated.

2. MATERIALS AND METHODS

2.1. Plant culture

2.1.1. Assessment of tomato root colonisation by P. oligandrum *and consequence on yield*

Experiments were performed with seeds of tomato cv. Daniela (Hazera) and cv. Tradiro (De Ruiter) sown in rockwool-cubes and fertilised daily with a nutrient solution containing in meq. 14.5 NO_3; 1.8 H_2PO_4; 7.5 K; 8.8 Ca; 4.0 Mg and 0.5 NH_4. After 4 weeks in a nursery greenhouse, the plants were installed in the gulleys of a greenhouse as soon as the first flowercluster appeared. In this N.F.T. (Nutrient Film Technic) system, roots developed freely in the nutrient solution described above. The pH of the nutrient solution and the greenhouse temperature were regularly monitored and ranged from 5.5 to 6.2 and 18 to 22 °C, respectively. Eight independent units were used, 4 per cultivar. For each cultivar, 2 units (34 plants per unit) were inoculated with *P. oligandrum,* whereas 2 others were used as control. Tomato fruits were collected every week from March to September and the yield per m^2 was assessed. Data were analysed with StatGraphics and means separated with the Student's *t*-Test.

2.1.2. Assessment of tomato protection by P. oligandrum *against* B. cinerea

Tomato seeds, cv Prisca, (Novartis), were sterilised by immersion in 70% ethanol for 5 minutes, soaked in 5% aqueous sodium hypochlorite for 10 minutes and thoroughly rinsed 3 times in sterile distilled water. Seeds were, then, placed for one week on water-soaked filter paper (Whatmann No. 1) in Petri dishes in the dark at 25 °C. Then, they were put for another week in a plastic cuvette filled with vermiculite, then transferred into pots (10 × 10 × 11 cm) filled with peat and vermiculite (50/50) and grown for 5 weeks in a greenhouse under a 14-h-light 16 °C/10-h-dark 25 °C photoperiod. Plants were regularly fed with Solufeed (ICI Agrochemicals) nutrient solution.

2.2. Plant inoculations with fungi

2.2.1. Root inoculation with P. oligandrum

P. oligandrum Dreschler, Strain No. 1133, was kindly provided by Professor John Hockenhull, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. It was grown on yeast malt agar at 24 °C in the dark and regularly subcultured. For the production of inoculum, *P. oligandrum* was cultured in a liquid medium containing 1.02 g KH_2PO_4; 0.17 g K_2HPO_4; 0.5 g $MgSO_4$ $7H_2O$; 30 g cane molasses and 30 mg ergosterol per liter of distilled water. Plastic bottles containing 150 ml of culture medium autoclaved at 121 °C for 15 minutes were inoculated with 4 disks of *P. oligandrum* (10 cm in diameter), then incubated in the dark for 14 days at 25 °C. Mycelial mats were then removed and fragmented into distilled water using a Waring blender.

For experiment in N.F.T. greenhouse, *P. oligandrum* inoculum consisted of oospores-mycelium homogenate (30 000 oospores·ml^{-1}). Twenty ml of the inoculum were deposited at the collar level of each plant on January. First inoculation was performed with 10-week-old plants. A second inoculation was carried out 4 weeks later using 20 ml of the inoculum poured in the gulleys on each side of the plants.

For the experiment conducted to control *B. cinerea*, plants were inoculated 3 times with the *P. oligandrum* oospores-mycelium homogenate (50 000 oospores·ml^{-1}). The first inoculation was done in a plastic cuvette, whereas the two others were performed 3 and 4 weeks after plant transfer to pots.

2.2.2. Leaf inoculation with B. cinerea

The isolate of *B. cinerea* (Collection of Laboratoire de Microbiologie, ESMISAB, France) was grown on yeast malt agar at 24 °C in the dark and regularly subcultured. Inoculation with the pathogen was performed by depositing 20-µl droplets of conidial suspension onto the upper surface of each leaf (500 spores·$droplet^{-1}$, 10 droplets·$leaf^{-1}$). Infections were scored according to a disease index with a relative scale of 0 to 10 built on the basis of necrotic-lesion development. The lesions restricted to the deposition area were scored from 0 to 4; those spreading out of it from 5 to 10.

2.3. Root sampling

2.3.1. Assessment of P. oligandrum*-root colonisation*

Tomato (cv. Daniela and cv. Tradiro) roots from control and *P. oligandrum*-inoculated plants were sampled monthly in N.F.T. gulleys from February to August in 6 randomly-selected sites per unit. For each sample, roots taken from all parts of the root system were cut into 5-mm segments and cultured onto a selective *Pythium* isolation medium coded CMA-PARP at 25 °C in the dark. Sixty root segments were plated per unit (10 per sites). After 32 to 48 h, *P. oligandrum* thalles were counted, and results were expressed as the percentage of root pieces from which *P. oligandrum* thalles were recovered.

2.3.2. Assessment of Pythium *spp. root colonisation*

Similarly from above, tomato (cv. Daniela and cv. Tradiro) roots from control and *P. oligandrum*-inoculated plants were sampled monthly in N.F.T. gulleys from February to August in 3 randomly-selected sites per unit. Ninety root segments were plated per unit (30 per sites). The method previously described was used to count and to determine *Pythium* spp.

2.4. Tomato plant protection against *B. cinerea*: PR-proteins analysis

Acid-soluble proteins extracted from infected leaves as described by Renault et al. [18] were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein extracts were mixed with equal volumes of SDS sample buffer; the mixtures were boiled for 3 minutes and subjected to electrophoresis. Samples were applied to stacking and resolving gels usually containing 5 and 13.5% (w/v) of polyacrylamide, respectively. After electrophoresis, proteins were directly transferred onto 0.2-µm nitrocellulose filters (Schleider and Schüll, IKA-Filtrax) in electrophoresis buffer supplemented with methanol (Tris 25 mM pH 8.3, Glycine 192 mM, SDS 0.1%, methanol 20%) at 200 mA for 30 min. After electrotransfer and subsequent incubation of the blots with tobacco PR-3b (Q) and PR-5a (S) antisera, the antigen-first antibody complexes were detected with a second antibody phosphatase-conjugated goat anti-rabbit. The different polyclonal antibodies raised against PR-3b (Q) and PR-5a (S) purified from tobacco plants, were kindly provided to us by Pr. Fritig (IMBP, Strasbourg, France). A high degree of homology between PR proteins from tobacco and tomato plants has been documented by Van Loon and Van Strien [30].

3. RESULTS

3.1. *P. oligandrum*-root inoculation of plants grown in N.F.T. system

3.1.1. Assessment of root colonization by P. oligandrum

Figure 1 clearly shows a substantial colonisation of the roots of both tomato cultivars by *P. oligandrum* further to its introduction inside the greenhouse. Root colonisation by

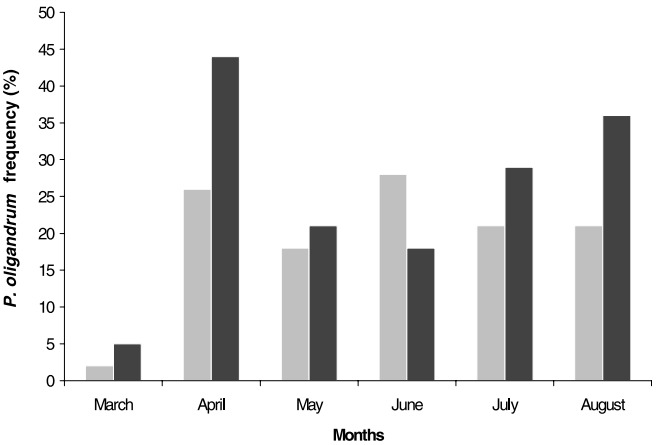

Figure 1. Root colonisation by *P. oligandrum* of tomato plants (cv. Daniela and cv. Tradiro) grown in hydroponic greenhouse (N.F.T. system). Data are expressed in percentage of colonised roots (60 roots per sample). Light grey bars: cv. Tradiro; black bars: cv. Daniela.

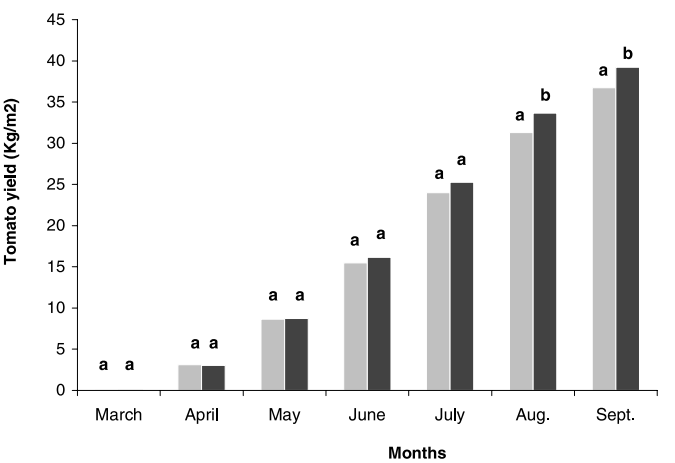

Figure 2. Tomato yield of plants (cv. Daniela) inoculated or not with *P. oligandrum*. Data correspond to the means of each month, the bars with the same letter do not differ significantly at $P \geq 0.05$ determined by Student's *t*-Test. Light grey bars: control plants; black bars: *P. oligandrum* inoculated plants.

P. oligandrum remained relatively stable over the whole experimental period, i.e. April–September. For the tomato cultivars, Tradiro and Daniela, *P.oligandrum* was detected from about 20 to 30% of the roots sampled and 20 to 40%, respectively.

3.1.2. Tomato yield of plants inoculated or not with P. oligandrum

Except for the first month of tomato production, when roots were colonised by *P. oligandrum* there was a yield increase for both cultivars (Fig. 2). This increase in yield was statistically significant over the last months of the cultural season, i.e. August and September for Daniela cultivar (Fig. 2), and July and September for Tradiro one (data not shown).

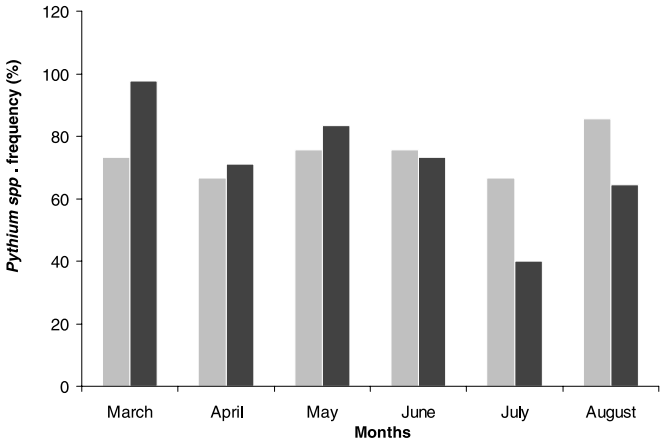

Figure 3. *Pythium* spp. colonisation on tomato roots inoculated or not with *P. oligandrum*. Data are expressed in percentage of roots colonised by *Pythium* spp. (90 roots per sample). Light grey bars: control plants; black bars: *P. oligandrum* inoculated plants (cv. Daniela).

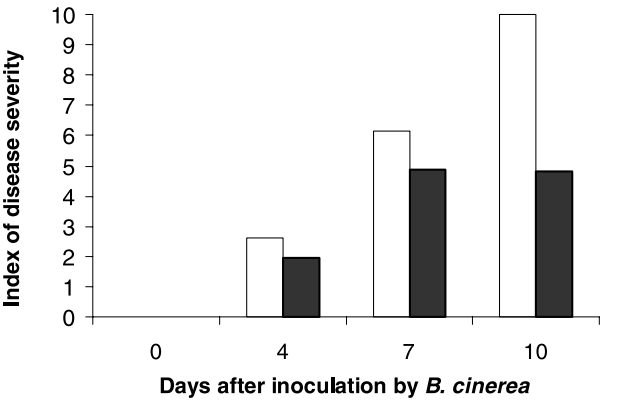

Figure 4. Effect of tomato root colonisation by *P. oligandrum* on severity of disease caused by *B. cinerea* 1 to 10 days after inoculation. Data correspond to the means of disease index noted on 10 plants. Open bars: *B. cinerea* inoculated plants; black bars: plants treated first with *P. oligandrum*, then with *B. cinerea*.

3.1.3. Assessment of Pythium *spp. on roots colonised or not with* P. oligandrum

On *P. oligandrum*-inoculated or control roots, *Pythium* spp. thalles were consistently and regularly isolated from samples of roots of both cultivars. No clear relation was found between the extent of root colonisation by *Pythium* spp. and the type of treatment undergone. Colonisation by *Pythium* spp. is relatively equal on roots treated or not with the antagonist fungus (Fig. 3) (for Tradiro roots, data are not shown).

3.2. *P. oligandrum*-mediated protection against *B. cinerea* attacks

3.2.1. Assessment of leaf protection against B. cinerea

About 80% of the roots from plants inoculated with *P. oligandrum* were colonised by the fungus. Ten days after inoculation of tomato leaves with *B. cinerea* alone, an important necrosis leading to complete senescence was recorded, and then leaves detached from the stem (Fig. 4). Pre-inoculation of tomato roots with *P. oligandrum* before *B. cinerea* challenge on the leaves significantly reduced grey mould severity; in addition, defoliation was not observed**.**

3.2.2. Analysis of PR-3b proteins in leaves of tomato plants

Western blotting revealed that the leaves of both control and *P. oligandrum*-inoculated plants (Figs. 5, 6a) synthesised only a 27-kDa protein. This result was observed with each tested sample (Fig. 6a). When plants were inoculated with *B. cinerea* alone, one additional 30-kDa band was detected (Figs. 5, 6b). The synthesis of this PR-3b protein was enhanced in the plants pre-inoculated with *P. oligandrum* and challenged by *B. cinerea*; whereas a third protein of about 32 kDa was detected (Figs. 5, 6c).

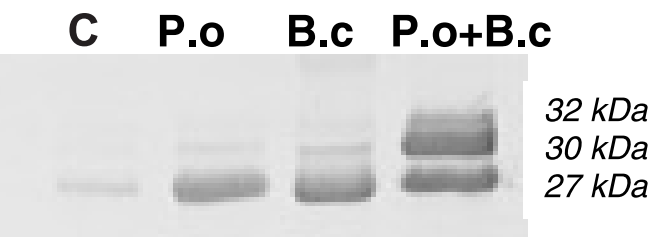

Figure 5. Accumulation of PR-3b proteins in different plants. The experiment was carried out 10 days after inoculation of plants with *B. cinerea.* C: control plants; *P.o*: *P. oligandrum*-treated plant; *B.c*: *B. cinerea*-inoculated plant; *P.o* + *B.c*: plants treated first with *P. oligandrum*, then with *B. cinerea.*

3.2.3. Analysis of PR-5 proteins in leaves of tomato plants

PR-5a proteins were not monitored in the leaves of both control and *P. oligandrum*-colonised plants (data not shown). A 24-kDa protein was detected in *B. cinerea*-infected leaves. Its synthesis was enhanced in infected leaves upon pre-treatment with *P. oligandrum* (Fig. 7).

4. DISCUSSION

In recent years, numerous studies have highlighted different aspects of *P. oligandrum* interactions with plants or pathogenic microorganisms in appropriate environments [1, 2, 3, 10, 13, 20, 31]. The present paper reports on a crucial point for plant protection by *P. oligandrum*: root colonisation and persistence of the antagonist at potential infection sites. During the whole cultural season, the antagonist was detected on about 20 to 40% of roots of the plants grown in greenhouse hydroponics, here the N.F.T. system. This relative constant colonisation is achieved in a cultural system known for its high

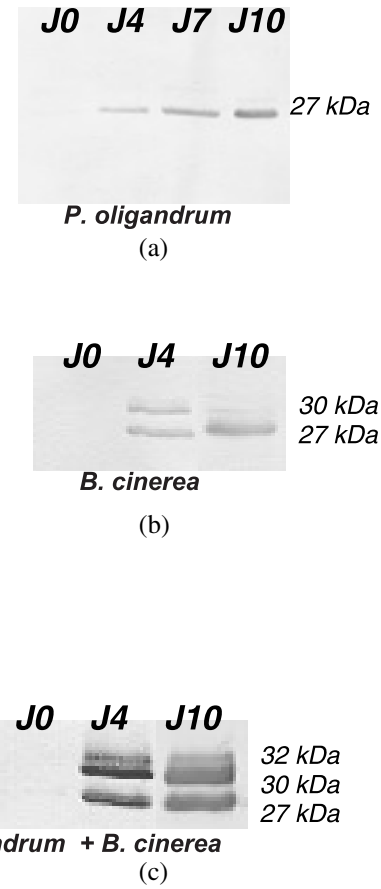

Figure 6. (a) Time course induction of PR-3b protein in leaves of plants treated three times with *P. oligandrum* alone. J0 to J10 correspond to 0 to 10 days after the last inoculation with *P. oligandrum.* (b) Time course induction of PR-3b protein in leaves of plants inoculated with *B. cinerea* alone. (c) Time course induction of PR-3b protein in leaves of plants treated three times with *P. oligandrum,* then *B. cinerea*.

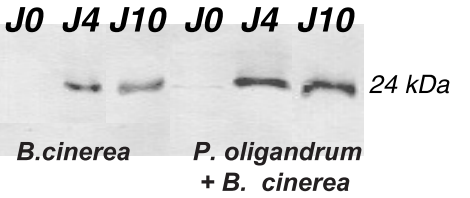

Figure 7. Time course induction of PR-5a proteins in plants treated with either the sole *B. cinerea* or *P. oligandrum,* then *B. cinerea* at 0, 4 and 10 days after inoculation with *B. cinerea*.

receptivity to *Pythium* spp. [6, 19, 22, 27]. Indeed, within hydroponics, several environmental conditions are controlled in such a way that they facilitate spreading and development of *P. oligandrum*. For instance, water abundance favours dispersion of *P. oligandrum* zoospores. Wulff et al. [31] reported on the accumulation of *P. oligandrum* zoospores on the roots of cucumber; but they were less numerous than those of pathogenic *Pythium* species. However, alike other zoosporic fungi, once produced, zoospores can spread within the whole system via the nutrient solution [27]. In addition, in greenhouse the temperature is relatively constant [27]; generally, it is close to the one required for *P. oligandrum* growth. It facilitates the development of the fungus in the rhizosphere of tomatoes.

In this context of relative constant root colonisation by *P. oligandrum*, increased tomato yield production was noticed. Root growth stimulation has already been described on young cucumber plants [10, 31]. Nevertheless, the present study pointed out that, in appropriate conditions, the beneficial effect exerted on young plants can persist throughout the growing season as demonstrated by the yield increased obtained at the end of the growing season. Three among the factors that may partially explain the *P. oligandrum*-mediated growth response should be considered: enhanced plant hormone production, minor pathogen control or induced plant resistance. (i) Regarding the first point, it has been convincingly shown that pathogenic *Pythium* species [23], but also *P. oligandrum,* produce auxin compounds. Only with the antagonist, absorption of fungal-auxin compound in appropriate concentrations by the root system was associated with increased plant growth (unpublished data). However, the level of implication of these molecules on yield increase still remains to be determined. (ii) Although *P. oligandrum* mycoparasitism against pathogenic *Pythium* species has been convincingly demonstrated by Benhamou et al. [2], it does not seem to be the main factor in the present case: root colonisation by *Pythium* spp. was not different in roots treated or not by *P. oligandrum*. In fact, as the level of *Pythium* spp. population is always high in hydroponics [19, 22, 27], one can assume that *P. oligandrum* parasitizes other *Pythium* species, but cannot destroy them to significantly reduce their population. It is also noticeable that the minor pathogenic *Pythium* group F, which accounts for 75 to 90% of all *Pythium* isolates in hydroponics [19], has no negative impact on plant growth. One can speculate that (iii) induced resistance is the prevalent phenomenon in our experiments and that minor pathogens cannot penetrate in the roots to induce their deleterious effect on plants. Previous experiments have supported this assumption because it was shown that roots colonised by *P. oligandrum* developed resistance against *Fusarium* root rot [1].

In conclusion, the investigations reported here demonstrate that under specific and favourable cultural conditions provided by hydroponic systems, *P. oligandrum* can colonise and exert its beneficial effect on plants throughout the cultural season. Further studies are needed to assess its positive impact on plants in different cultural systems. Similarly, the present study demonstrates that under conditions favourable for root colonisation by *P. oligandrum*, plant systemic resistance against the foliar pathogen, *B. cinerea* is also induced. In systemic resistance, the expression of PR-protein genes is considered as a marker of late defence genes [28]. Interestingly, the synthesis of PR-proteins was enhanced in leaves pre-inoculated with *P. oligandrum*, and challenged by *B. cinerea*. Only for this condition, synthesis of PR-3b and

PR-5a proteins was increased concomitantly with the induction of a new PR-3b protein. However, PR-proteins are not always induced by antagonist microorganism as a system of defence [7, 8]. In a relevant manner, it has been demonstrated that induction of systemic resistance in tomato plants or radish by a biocontrol agent, *Penicillium oxalicum* [7] or by a non-pathogenic rhizobacterium, *Pseudomonas fluorescens* [8] is not associated with accumulation of PR-proteins. Considering the existence of different signalling pathways of systemic resistance within plants, one can assume that, depending on the biocontrol agent concerned together with the elicitors it produces, the synthesis of PR-proteins is induced or not. As reported above, pre-inoculation of tomato plants with *P. oligandrum* triggered PR-protein synthesis, which was amplified upon pathogen attack by *B. cinerea*. On the one hand, it suggests that such a defence system is very efficient in terms of reduction of energy costs for tomato plants. One the other hand, it is likely that accumulation of PR-proteins is not a pre-requisite for the induction of resistance though they contribute to the protective state [29].

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