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Biocontrol management in soilless culture: impact of the antagonist *Pythium oligandrum* on native fungal populations

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Abstract: Fungal populations and their dynamics were investigated in relation to the introduction of the biocontrol agent *Pythium oligandrum* in the rhizosphere of tomato plants grown in greenhouses. The research was done by inoculating roots of tomato plants grown in hydroponic culture by three selected strains of *P. oligandrum*. After introduction, these strains were monitored over time to evaluate their persistence and their effect on the microflora. *P. oligandrum* was detected at high rates throughout the 8-months experiment by Real-Time PCR but seemed to have a slight influence on the root distribution of the other *Pythium* species, especially on *Pythium* group F, ubiquitous tomato root minor pathogens. Inter Simple Sequence Repeat analysis performed on *P. oligandrum* isolates collected at the end of the growing season, showed that 90% of the samples belonged to only one of the three selected strains. Single-Strand Conformational Polymorphism analyses pointed out that the introduction of the antagonistic fungus only had a slight influence on the native fungal community and its dynamics.

Key words: Hydroponics, microbial ecology, oomycetes, rhizosphere

Introduction

Soilless culture is popular worldwide because of its potential for improved control of the root environment and avoidance of diseases prevalent in soil-based growing media. But, evidence exists that when pathogens do manage to enter recirculating systems, dissemination and disease epidemics can occur rapidly (Paulitz and Bélanger, 2001). Thus, numerous minor pathogens, such as *Pythium* spp. and *Phytophthora* spp., commonly found in conventional cultures, may become of economically importance.

Different approaches can be used to reduce risks to plant health: (i) preventives measures (resistant cultivars or sanitation measures) or (ii) systematic disinfection of the nutrient solutions (ozonisation, UV, chlorination, thermodisinfection, fungicides…). But, although disinfection methods are effective, they destroy not only pathogenic microorganisms but also species, which could be beneficial. This is why more interest has been focused on the management of microorganisms in soilless cultures.

In that context, the introduction in the rhizosphere of the biocontrol agent *P. oligandrum* presents numerous advantages (Le Floch et al, 2003a, Le Floch et al., 2003b). Because an enhanced rhizosphere competence by *P. oligandrum* is a prerequisite for an efficient plant protection strategy, the aim of the present study was to optimise and to characterise the persistence of the antagonist agent while evaluating its impact on the roots native fungal microflora. Plate counting and real-time PCR were used for the detection and the quantification of *P. oligandrum*, as well as *Pythium* group F (Rey *et al.*, 1998), a common tomato root pathogen endemic in this trial. Inter Simple Sequence Repeat (ISSR) method was used to identify which *P. oligandrum* strains have colonised the rhizosphere. And fingerprints of the fungal community obtained by CE-Single-Strand Conformational Polymorphism (SSCP) allowed investigations on the influence of *P. oligandrum* on the rhizosphere colonising-fungal populations together with the dynamic of these fungi throughout the cultural season.

Material and Methods

Plant material and Pythium oligandrum inoculations

Seeds of tomato *Lycopersicon esculentum* Mill. cv Durinta (Western Seed, France) were grown in coco-fibre slabs (4 plants per slabs) in two commercial greenhouses. Each slab was wrapped in a plastic bag to be isolated from the others. The culture conditions were the same in each greenhouse, except that in the second greenhouse the difference of temperatures between night and day was higher than in the first one.

Inoculation with three *P. oligandrum* selected strains was performed as previously described using an oospore-mycelium homogenate (Le Floch *et al*. 2007).

Sampling and DNA extraction

Roots samples were collected twice a month throughout the cultural season, from March to October 2006. DNA was extracted by using the Fast DNA Spin Kit (MP Biomedicals) procedure with extraction buffer, CLS-VF, required for processing infected plant tissues as described by the manufacturer.

Assessment of root colonization by Pythium spp. using plate counting method

Root colonization by *Pyhtium* spp. was monitored from March to October for each modality (inoculated or control plant) through direct plating of non-disinfected root fragments on selective medium and incubation at 25°C in the dark.

Assessment of root colonisation by Pythium spp. using real-time PCR

Real-time quantifications were performed using primers pairs and probes, whose sequences were based on oligonucleotides oli 142 and dis183 spotted on the *Pythium* array designed by Tambong *et al.* (2006), and tested for specificity to the targeted *Pythium* species. Real-time reactions were carried out on a MiniOpticon thermocycler (Bio-Rad) as described by Le Floch *et al.* (2007).

ISSR amplification of Pythium oligandrum DNA

The 90 isolates of *P. oligandrum* investigated in this study were isolated at the end of the cultural season from the tomato roots inoculated as described above. After plate counting, *P. oligandrum* strains, identified by the presence of echinulated oospores, were isolated, purified, transferred into liquid medium and after 15 days of culture, DNA was extracted. Inter Simple Sequence Repeat (ISSR) analysis was performed using the primer $[GACA]_4$ as described by Vasseur *et al.* (2005).

Analysis of fungal populations by SSCP

The fluorescently labeled (6-FAM) primers used for universal fungal amplification were U1 and U2 (Sandhu *et al*., 1995). SSCP analyses were performed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems).

The co-migration of samples with the fluorescent size standard (GeneScan-400 Rox) was used to compare migration profiles between samples and aligning them with the program SAFUM (Zemb *et al*., 2007) before Principal Component Analysis (PCA) with the software Statbox version 6.6 Pro (Grimmersoft).

Results and discussion

Assessment of the persistence of P. oligandrum by plate counting and Real Time-PCR

After application of the *P. oligandrum* strains mixture on the roots of tomato plants, real-time PCR pointed out that the antagonistic oomycete persisted at high rates in the rhizosphere throughout the cultural season. Plate counting method indicated that at least 50% of the root system was colonized by *P. oligandrum*. These high values had never been obtained during previous trials (Le Floch *et al.*, 2003b and 2007). According to Le Floch et al. (2007) results obtained with cultural methods can introduce a bias since fast growing *Pythium, i.e. Pythium* group F, can grow first on the selective medium and hamper the development of the antagonistic oomycete. In this condition, one can assume that the *P. oligandrum* rhizosphere colonization may be underestimated. These data also suggest that the use of a strain mixture is a must to favour *P. oligandrum* persistence on roots.

Discrimination of Pythium oligandrum strains by ISSR

The three selected antagonistic strains inoculated, were detected and identified by ISSR analysis at different rates in the root samples collected at the end of the cultural season. ISSR analysis performed on the *P. oligandrum* strains have shown that 90% belonged to the strain producing the less oospores; the other two having persisted in the rhizosphere at only 5%. In the experimental conditions of this study, for unknown reasons, only one *P. oligandrum* strain among the three ones seemed to be particularly well adapted to the environment encountered in tomato soilless culture. Furthermore this experiment adds support to the assumption that there is no evidence of direct correlation between oospores germination *in vitro* and what takes place in the rhizosphere.

Effect of the introduction of P. oligandrum on the genetic structure of fungal communities of the rhizosphere

In the present experiment, *P. oligandrum* seemed to have a slight influence on the *Pythium* group F populations; it delayed from one-month their development in the inoculated plants but had no significant influence on their populations over the whole cultural season. Several explanations can be pointed out: (i) according to Vasseur *et al.* (2005), a huge genetic diversity was detected in strains from the *Pythium* group F. As a consequence, interactions between *Pythium* group F and *P. oligandrum* strains are likely very diverse. Therefore, controlling this minor pathogenic group seems to be very complex. (ii) *P. oligandrum* concentrations were too limited to have a significant influence on other oomycetes or fungi. This last assumption was verified when the dynamics of the genetic structure of the rhizosphere fungal communities were characterised by SSCP.

One of the main interests of SSCP fingerprinting technique is to study the rapid changes in microbial communities in the absence of prior knowledge of their composition. The genetic structure of the fungal community in the rhizosphere of the tomato plants was similar in both treated and non treated plants as no differences were observed between the SSCP profiles at a same date of sampling. However, the number of peaks and their areas increase throughout the

cultural season indicating a complexification and a quantitative evolution of the microflora with time. Therefore, this study pointed out that under optimal condition for plant growth the introduction of the antagonist *P. oligandrum* do not induce microbial shifts in the rhizosphere.

In conclusion, this work pointed out a successful implantation of the three *P. oligandrum* selected strains, but only one of them seems particularly adapted to colonize the rhizosphere of plants grown in hydroponics. The *in vitro* selection got some limitations; for instance, the experiments have demonstrated that the strain producing the lowest quantity of oospores was the most well-established in the rhizosphere. And then that *P. oligandrum* was able to colonize and persist in complex ecosystems without inducing modifications of the indigenous populations.

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