In situ Impact of the Antagonistic Fungal Strain, Trichoderma gamsii T30 on the Plant Pathogenic Fungus, Rhizoctonia solani in Soil

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Abstract

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the major mechanisms may include antibiotics, production of lytic enzymes, physical intervention including mycoparasitism and the niche competition (Anees et al. 2010c). Out of the more than 100 known species of *Trichoderma*, a few have been reported as the putative biocontrol agents. For example, strains of *T. virens* and *T. harzianum* were reported as the antifungals against *Armillaria mellea* (Asef et al. 2008). The disease caused by *R. solani* was inhibited by different strains of *T. gamsii* (Anees et al. 2010c). Different strains of *T. asperellum*, and *T. pseudokoningii* were effective against foliar pathogens of agricultural crops (Prabhakaran et al. 2015), like *Alternaria solani*, *Bipolaris oryzae*, *Pyricularia oryzae* and *Sclerotinia sciriorum*. *Trichoderma longibrachiatum* strains were reported as antagonistic against *Fusarium oxysporum* (Anees et al. 2018). Kumar et al. (2018) reported *T. viride* effective against different fungal pathogens. Here, we have used strain T30 of *T. gamsii* that has previously shown antagonistic activity against *R. solani* AG 2-2 on culture plates (Anees et al. 2010c).

The present study was designed to evaluate the *in situ* impact of the biocontrol strain on the population density of the plant pathogenic *R. solani* AG 2-2 strain G6 in the soil. For this purpose, microcosms containing the autoclaved or non-autoclaved soils were used. The microcosms were inoculated with both the pathogen (*R. solani* AG 2-2 strain G6) and the antagonist (*T. gamsii* strain T30) in different ratios. The population densities of the pathogenic fungus were followed up to day 25 using real-time polymerase chain reaction (qPCR) by the help of specific primers designed for detection of *R. solani* AG 2-2 strain G6 based on its ITS region (Edel-Harmann et al. 2009).

**Experimental**

**Materials and Methods**

**The fungal inocula.** Sand inocula of *R. solani* AG 2-2 strain G6 and *T. gamsii* strain T30 were prepared. Autoclaved sand (700 g; 0.5 to 1.2 mm) was mixed with a sterile malt broth (230 g/l; 91 ml). The fungi were cultured onto the malt extract agar (MEA) plates for 4 days and five culture plugs were added to the sand medium followed by incubation for two weeks at 25°C. The bottles with sand inoculum were shaken daily to break the pellets. After two weeks, each inoculum was sampled in triplicates to measure the population density of the inoculated fungi by qPCR as given below.

**Soil microcosms.** The soil microcosms were prepared in 100 ml cylindrical sterile aluminum boxes covered with lids. Each box contained 50 g of soil sampled from the French National Institute for Agromonic Research (INRA) experimental unit at Epoisses, Côte d’Or, from a field of sugar beet that had no recent *Rhizoctonia* disease report. The soil was a silty clay containing 6% sand, 58% silt, and 36% clay. The soil was sieved using a 4 mm sieve and dried in air at 20°C for 4 days. The microcosms were prepared with the autoclaved or non-autoclaved soils. The former was autoclaved at 105°C for 1 h each at three consecutive days. The moisture in the microcosms was adjusted to 20% (based on the dry weight) three days before inoculation using sterile water. The inoculation was performed using different ratios i.e. R1/T1, R1/T0.1, R1/T0.01, R1/T0 and R0/T0 of *R. solani*/*T. gamsii* where ‘1’ denotes the 10⁴ target DNA copies/g of soil. The ratios were found using amounts determined by qPCR assays as explained below (Edel-Harmann et al. 2009; Anees et al. 2010b). The sand inocula were evenly spread over the soil surface in the microcosms at the given ratios and incubated at 20°C. The microcosms were sampled at three different periods i.e. on days 2, 11, and 25 after inoculation in triplicate. Hence, nine independent microcosms were prepared for each experimental treatment, i.e. three microcosms per treatment for each sampling date. Both sets of microcosms (autoclaved and non-autoclaved) were inoculated on the same day.

**Preparation of standard curve for the qPCR.** To generate a standard curve, the internal transcribed spacer (ITS; the target DNA) region of *R. solani* strain G6 or *T. gamsii* strain T30, respectively, were cloned into the plasmid DNA (Anees et al. 2010b). The plasmid DNA was then diluted making ten-fold dilution series that contained the cloned ITS regions from 10⁴ to 10⁶ copies of the target DNA for each PCR reaction. The curve hence generated was then used for quantification of the target DNA in the different DNA samples (Fig. S1).

**Quantification of R. solani by qPCR.** Sand samples of 1 g were used for the extraction of genomic DNA (Edel-Harmann et al. 2004). The procedure used for the DNA extraction included chemical as well as physical processes as previously described (Anees et al. 2010b). The extracted DNA was purified using the standard procedures (Anees et al. 2010b). The extraction was done from each sample in triplicate. The extracted DNA samples were quantified by a Biophotometer (Eppendorf, Hamburg, Germany) with an optical density adjusted at 260 nm. The qPCR technique was used for quantification of *R. solani* AG 2-2 using primers G6-F2 and G6-R2 that have been specifically designed to target the ITS region rDNA gene of the pathogen (Gardes et al. 1993). The qPCR assay was performed and analyzed as previously explained (Anees et al. 2010b). The data were analyzed using analysis of variance (ANOVA) and Fisher LSD tests (*p* = 0.05) using XLSTAT – Version 2007.5 (Addinsoft).
Results

Population density of *R. solani* strain G6 in the autoclaved soil. The population density of *R. solani* strain G6 in the autoclaved soil inoculated only with *R. solani* increased significantly from $1 \times 10^5$ to $6.5 \times 10^6$ copies/g of soil at day 25 after inoculation (Fig. 1). The different doses of *T. gamsii* strain T30 affected the population densities of *R. solani* as given in Fig. 1.

At a ratio of R1/T0.01 of *R. solani* G6 and *T. gamsii* T30, the number of copies of target DNA increased in a similar way as in the absence of *Trichoderma* at day 11 after inoculation; however, the population density decreased significantly by five times at day 25 as compared to the control (1/0). The higher doses (R1/T0.1 and R1/T1 of *R. solani* G6/*T. gamsii* T30), significantly reduced the number of target DNA copies to $2.1 \times 10^6$ and $7.55 \times 10^5$/g of soil, respectively at day 11 after inoculation. Whatever the inoculated dose of *T. gamsii* was in the microcosm, the target DNA copies of *R. solani* G6 decreased to similar levels that were significantly lower than in the absence of *T. gamsii*. When the two fungi were introduced in the microcosms at the same dose (R1/T1), the population density was 17 times lower than in the control samples, not treated with *Trichoderma*.

Population density of *R. solani* strain G6 in the non-autoclaved soil. The effect of the different ratios of T30 on the population densities of *R. solani* G6 in the non-autoclaved soil is given in Fig. 2. In the control samples, not treated with *Trichoderma*, the number of target DNA copies/g of soil was determined to be equal to $1.2 \times 10^5$ at day 11 after inoculation, which was not significantly different from the initial density, and then the density decreased significantly contrary to what was observed in the autoclaved soil.

On the other hand, for the ratios (R1/T0.1 and R1/T1), the number of target DNA copies of *R. solani* was significantly lower than in the control (R1/T0) at day 2 after inoculation. At the ratios R1/T0.1 and R1/T1, the number of target DNA copies had increased at day 11 and then decreased significantly at day 25. However, there was no effect of the ratio R1/T0.01 on the population densities of the pathogen. Overall, the population densities of *R. solani* were lower than those observed in the autoclaved soil.

The DNA copies of *R. solani* remained undetected in the non-inoculated (0/0) autoclaved or non-autoclaved soils.

Discussion

The soil is a complex medium, which makes studies of the fungal growth and interactions between different fungi difficult (Schmidt et al. 2015). This is especially true for fungi that occur in low densities such
As R. solani (Ogoshi et al. 1996). In such cases, qPCR offers a viable and feasible way to explore and follow the pathogens and their antagonists quantitatively (Edel-Harmann et al. 2004; Gerin et al. 2019). However, most of the previous reports used either plant tissues or other derived media for assessment of the fungal interactions. For instance, the interaction of endophytic fungi and the pathogen has recently been reported inside plant tissues using the qPCR technique (Chow et al. 2018). The interaction of T. gamsii and Fusarium spp. in the plant-based substrata such as rice kernels and wheat haulms was studied in the artificial conditions using the same technique (Matarese et al. 2012). The interaction of two pathogens, Aphanomyces euteiches and Fusarium spp. causing root rot disease in field pea was also recently investigated in plant tissues using multiplex qPCR (Willsley et al. 2018). Hypothetically, the antagonistic fungi are supposed to control the pathogenic fungi in soil, however, the interaction is further complicated by the presence of the general microbiome that may entail several antagonistic microorganisms (Steinberg et al. 2007). Trichoderma spp. are known for their antagonistic effects using multiple mechanisms. Here, we reported the impact of T. gamsii strain T30, a biocontrol strain, on the population density of R. solani G6 in different ratios in soil under in situ conditions in the absence of a plant host. The assays were conducted in the artificially prepared microcosms containing the autoclaved or non-autoclaved soils, respectively.

The present study demonstrated that T. gamsii T30 inhibited the growth of the pathogenic fungus in the autoclaved soil in situ. In addition to this, the present study showed the direct control of the pathogen by T30 and, moreover, a dose-dependent response was also observed i.e. with the increase in T30 inoculum, the inhibition of G6 was also stronger. The inhibition of R. solani by T30 in the present study may have been due to the water-soluble metabolites produced by the antagonist, as reported previously (Anees et al. 2010b). The same strain inhibited the disease caused by R. solani in carrots in the bioassays performed in the controlled climatic chambers, which may further reinforce the present results (Anees et al. 2010c).

In the autoclaved soil, the population density of R. solani increased significantly when it was alone until day 25 with a linear growth showing its saprophytic ability in the absence of competitors. This fact is consistent with earlier reports of the higher intrinsic saprophytic ability of the fungus (Ogoshi et al. 1996). However, the presence of the natural microbiome significantly reduced the growth of fungus in the presence or absence of T. gamsii T30. The highest number of target DNA copies detected in the non-autoclaved soil was 100 times lower than the highest number in the autoclaved soil at day 25 after inoculation. This shows the poor competitiveness of R. solani strain G6 as compared to the general microbiome in the natural soils. That may be why the naturally occurring soil inoculum of

![Fig. 2. The influence of different doses of T. gamsii T30 on the R. solani G6 growth in the non-autoclaved soil (R1/T0 represents R. solani G6/T. gamsii T30 in the ratio 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1 = 1/0.1 ratio, R1/T1 = 1/1 ratio; with 1 = 10^5 copies of the target DNA per g of soil). The significant differences in the number of DNA copies are shown using different letters (p≤0.05). Different small letters show significant differences among different treatments for a given sampling time. Different capital letters indicate the significant differences among different sampling times for a given treatment. If the letters happen to be identical at a given data point (either small or capital), they are written only once for the sake of clarity.](image-url)
R. solani is too small to be detected and often occurs as sclerotia (1 sclerotic/10 g of soil; Rodriguez-Molina et al. 2000). The situation varies in case of ascomycetous pathogens such as F. oxysporum which occurs generally as 100–1000 propagules/g of soil (Neate and Schneider 1996). With the small density in soil, R. solani faces a severe competitive pressure of the general microbiome in the soil, as depicted in the present study which is generally referred to as a general microbial suppression (Weller et al. 2002). The competition for nutrients or niches can be the reason for the general microbial suppression because a robust increase in density was observed upon amendment with the buckwheat meal (Steinberg et al. 2007). R. solani, therefore, needs a host plant or a source of nutrients for growth and further dispersal (Steinberg et al. 2007).

In conclusion, the present study for the first time gave an insight into the in situ control of R. solani G6 by T. gamssii T30 in the soil in absence of the plant host in the artificially prepared microcosms. However, future studies of the interaction of the two fungi in the presence of host would also be interesting. The present study also explained the poor competitiveness of R. solani, which may be the reason for the generally low densities of R. solani in the soil.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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