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

Rhizoctonia solani is a soil-borne fungus causing a wide range of plants diseases. *Trichoderma gamsii* strain T30 has previously been reported as antagonistic against *R. solani*. Although there are a few studies about the influence of *Trichoderma* strains on the *R. solani* density in a pathosystem in the presence of plant hosts, this report for the first time comprehensively describes *in situ* effects of a *T. gamsii* strain on the population density of *R. solani* in the soil microcosmic conditions. The population dynamics of *R. solani* were followed in the auto-claved and non-autoclaved soils in artificially prepared microcosms up to day 25 after co-inoculation with *T. gamsii* in the variable ratios (R1/T1; R1/T0.1; R1/T0.01 of *R. solani/T. gamsii*). The population density of *R. solani* was evaluated by qPCR. In the autoclaved soil, target DNA copies of *R. solani* increased in the control samples from 1×10^5 to 6.5×10^6. At R1/T0.01, the number of target DNA copies were not significantly changed until day 11; however, it decreased by around five times at day 25. At R1/T0.1 and R1/T1, the number of DNA copies was reduced to 2.1×10^6 and 7.6×10^5 at day 11, respectively and the reduction was as much as 17 times at day 25. In the non-autoclaved soil, the number of the fungal cells decreased at day 25 whether inoculated or not with *Trichoderma* indicating a general suppression by the soil microbiome. In brief, *T. gamsii* significantly inhibited the growth of *R. solani* in the soil *in situ* and there was a general suppressive effect of the natural microbiome.

K e y w o r d s: *Rhizoctonia solani*, *in situ* study, population density, antagonism, real-time PCR

Introduction

Rhizoctonia solani is a soil-borne plant pathogenic cosmopolitan fungus. It is present in a very low density in soils, but still capable of causing considerable losses in crops of economic importance (Anees et al. 2010a). It is a fungus having a broad host spectrum, generally causing damping off and root rot diseases in a variety of crops. The diseases caused by this fungus commonly occur in patches of various sizes that are spatially mobile i.e. the patch size and location change from one season to another (Schneider et al. 1997; Anees et al. 2010a). The increased suppressiveness inside patches was hypothesized as the probable reason behind the patch mobility because of the development of the antagonistic microbiota (Anees et al. 2010b). However, because of the intrinsic complexity of the soil medium and the low density of *R. solani*, the *in situ* exposure of the pathogen towards the antagonists has not been studied in natural soils in the absence of the plant hosts although there are some reports of the *in vitro* interaction between *Rhizoctonia* and *Trichoderma* (Zhang et al. 2015).

Trichoderma species are cosmopolitan ascomycetous saprotrophic fungi present in soil and are well known for their biocontrol potential (Anees et al. 2018). *Trichoderma* species reportedly use a few different mechanisms to control the plant pathogenic fungi;

the major mechanisms may include antibiosis, 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 mellae* (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 scierotiorum*. *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 Agronomic 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 10^5 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 10^2 to 10^9 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 1×10^5 to 6.5×10^6 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 2.1×10^6 and 7.55×10^5/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 1.2×10^5 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
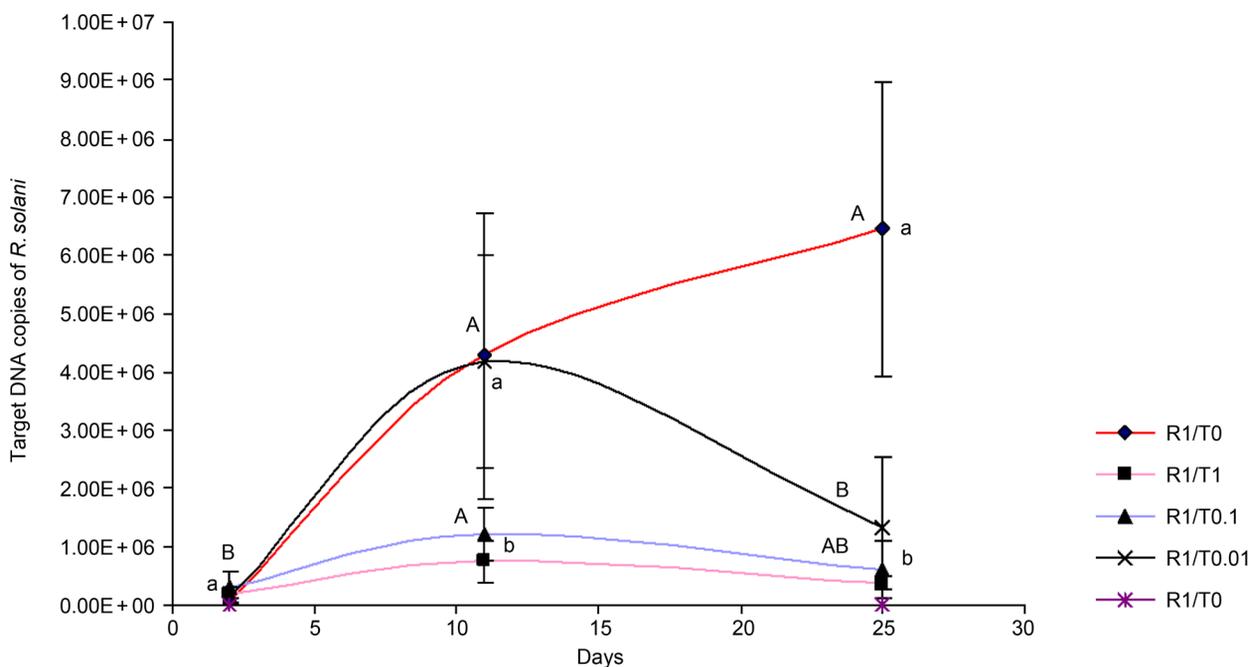
Fig. 1. The influence of different doses of *T. gamsii* T30 on the *R. solani* G6 growth in the autoclaved soil (R1/T0 represents the *R. solani* G6/*T. gamsii* T30 in the ratios 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1 = 1/0.1 ratio, and R1/T1 = 1/1 ratio; with $1 = 10^5$ copies of the targeted DNA per g of soil). The significant differences in the number of DNA copies are shown using different letters ($p \leq 0.05$). Different small letters show the significant differences among different treatments for a given sampling time. Different capital letters indicate 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.
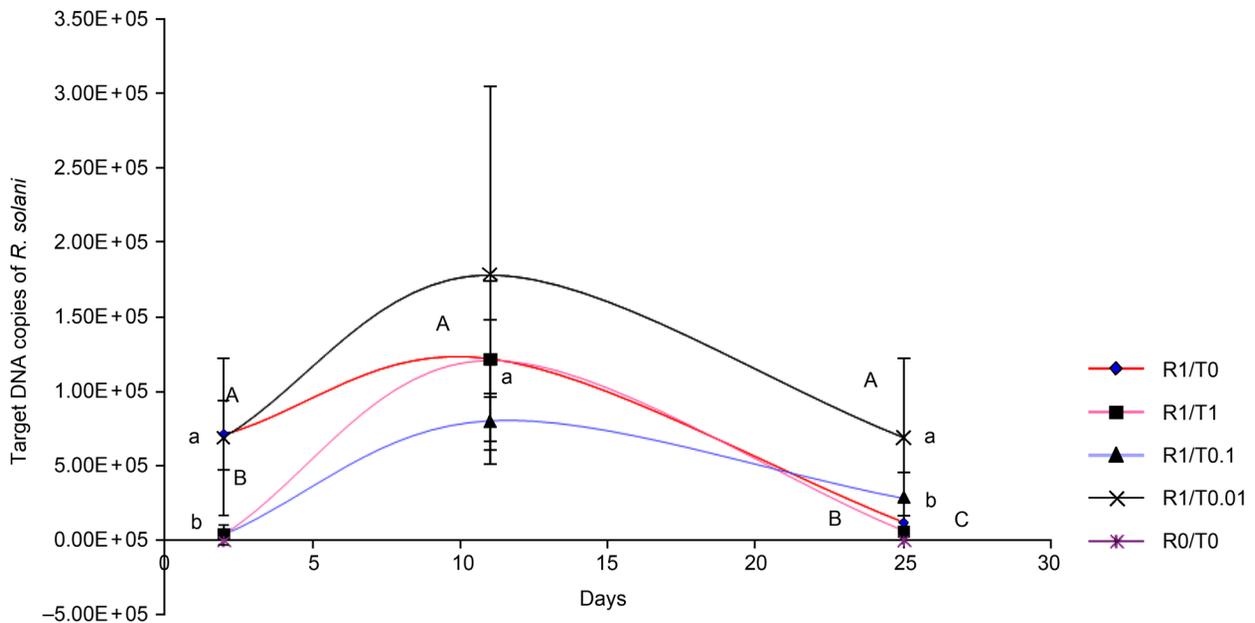
Fig. 2. The influence of different doses of *T. gamsii* T30 on the *R. solani* G6 growth in the non-autoclaved soil (R1/T0 represents *R. solani* G6/*T. gamsii* T30 in the ratio 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1 = 1/0.1 ratio, R1/T1 = 1/1 ratio; with 1 = 10^5 copies of the target DNA per g of soil). The significant differences in the number of DNA copies are shown using different letters ($p \leq 0.05$). 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.

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 (Willsey 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

R. solani is too small to be detected and often occurs as sclerotia (1 sclerotium/10 g of soil; Rodríguez-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. gamsii* 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.

Literature

Anees M, Edel-Hermann V, Steinberg C. Build up of patches caused by *Rhizoctonia solani*. Soil Biol Biochem. 2010a Oct;42(10): 1661–1672. doi:10.1016/j.soilbio.2010.05.013

Anees M, Tronsmo A, Edel-Hermann V, Gautheron N, Faloya V, Steinberg C. Biotic changes in relation to local decrease in soil conduciveness to disease caused by *Rhizoctonia solani*. Eur J Plant Pathol. 2010b Jan;126(1):29–41. doi:10.1007/s10658-009-9517-0

Anees M, Tronsmo A, Edel-Hermann V, Hjeljord LG, Héraud C, Steinberg C. Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. Fungal Biol. 2010c Sep;114(9): 691–701. doi:10.1016/j.funbio.2010.05.007 Medline

Asef M, Goltapeh E, Danesh Y. Antagonistic effects of *Trichoderma* species in biocontrol of *Armillaria mellea* in fruit trees in Iran. J Plant Prot Res. 2008 Jun 1;48(2):213–222. doi:10.2478/v10045-008-0025-6

Chow YY, Rahman S, Ting ASY. Interaction dynamics between endophytic biocontrol agents and pathogen in the host plant studied via quantitative real-time polymerase chain reaction (qPCR) approach. Biol Control. 2018 Oct;125:44–49. doi:10.1016/j.biocontrol.2018.06.010

Edel-Hermann V, Dreumont C, Pérez-Piqueres A, Steinberg C. Terminal restriction fragment length polymorphism analysis of ribosomal RNA genes to assess changes in fungal community structure in soils. FEMS Microbiol Ecol. 2004;47(3):397–404. doi:10.1016/S0168-6496(04)00002-9 Medline

Edel-Hermann V, Jobard M, Gautheron N, Friberg H, Steinberg C. A real-time PCR assay for the identification and the quantification of *Rhizoctonia solani* AG-2-2 in soil. Multitrophic Interactions in Soil. Bulletin IOBC/WPRS. 2009;42:41–46.

Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol. 1993 Apr;2(2):113–118. doi:10.1111/j.1365-294X.1993.tb00005.x Medline

Gerin D, Pollastro S, Raguseo C, De Miccolis Angelini RM, Faretra F. A ready-to-use single-and duplex-TaqMan-qPCR assay to detect and quantify the biocontrol agents *Trichoderma asperellum* and *Trichoderma gamsii*. Front Microbiol. 2018 Aug 31;9:2073. doi:10.3389/fmicb.2018.02073 Medline

Kumar V, Kumar Dubedi Anal A, Nath V. Biocontrol fitness of an indigenous *Trichoderma viride*, isolate NRCL T-01 against *Fusarium solani* and *Alternaria alternata* causing diseases in litchi (*Litchi chinensis*). Int J Curr Microbiol Appl Sci. 2018 Mar 20;7(03):2647–2662. doi:10.20546/ijcmas.2018.703.306

Matarese F, Sarrocco S, Gruber S, Seidl-Seiboth V, Vannacci G. Biocontrol of Fusarium head blight: interactions between *Trichoderma* and mycotoxigenic *Fusarium*. Microbiology. 2012 Jan 01; 158(1):98–106. doi:10.1099/mic.0.052639-0 Medline

Neate SM, Schneider HM. Sampling and quantification of *Rhizoctonia solani* in soil. In: Sneh B, Jabaji-Hare S, Neate S, Dijst D, editors. *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and disease control. Dordrecht (Netherlands): Kluwer Academic Publishers; 1996. p. 185–195.

Ogoshi A. Introduction – the genus *Rhizoctonia*. In: Sneh B, Jabaji-Hare S, Neate S, Dijst G, editors. *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and disease control. Dordrecht (Netherlands): Kluwer Academic Publishers; 1996. p. 1–9.

Prabhakaran N, Prameeladevi T, Sathiyabama M, Kamil D. Screening of different *Trichoderma* species against agriculturally important foliar plant pathogens. J Environ Biol. 2015 Jan;36(1): 191–198. Medline

Rodríguez-Molina MC, Tello-Marquina JC, Torres-Vila LM, Bielza-Lino P. Micro-scale systematic sampling of soil: heterogeneity in populations of *Fusarium oxysporum*, *F. solani*, *F. roseum* and *F. moniliforme*. J Phytopathol. 2000 Dec;148(11-12):609–614. doi:10.1046/j.1439-0434.2000.00575.x

Schmidt R, Cordovez V, de Boer W, Raaijmakers J, Garbeva P. Volatile affairs in microbial interactions. ISME J. 2015 Nov;9(11): 2329–2335. doi:10.1038/ismej.2015.42 Medline

Schneider JHM, Schilder MT, Dijst G. Characterization of *Rhizoctonia solani* AG 2 isolates causing bare patch in field grown tulips in the Netherlands. Eur J Plant Pathol. 1997;103(3):265–279. doi:10.1023/A:1008643311984

Steinberg C, Edel-Hermann V, Alabouvette C, Lemanceau P. Soil suppressiveness to plant diseases. In: Van Elsas JD, Jansson J, Trevors JT, editors. Modern soil microbiology. New York (USA): CRC Press; 2007. p. 455–478.

Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol. 2002 Sep;40(1):309–348. doi:10.1146/annurev.phyto.40.030402.110010 Medline

Willsey TL, Chatterton S, Heynen M, Erickson A. Detection of interactions between the pea root rot pathogens *Aphanomyces euteiches* and *Fusarium* spp. using a multiplex qPCR assay. Plant Pathol. 2018 Dec;67(9):1912–1923. doi:10.1111/ppa.12895

Zhang X, Harvey PR, Stummer BE, Warren RA, Zhang G, Guo K, Li J, Yang H. Antibiosis functions during interactions of *Trichoderma afroharzianum* and *Trichoderma gamsii* with plant pathogenic *Rhizoctonia* and *Pythium*. Funct Integr Genomics. 2015 Sep;15(5):599–610.

doi:10.1007/s10142-015-0456-x Medline

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