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Effect of *Trichoderma asperellum* on growth of *Rhizobium leguminosarum in vitro*

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Abstract

Trichoderma asperellum has been fronted as a biocontrol agent of root-knot nematodes on tomato and French beans in Kenya. Research on the effect of *T. asperellum* on growth of *Rhizobium* species is lacking yet the two interact within the rhizosphere particularly when *T. asperellum* is applied as a biocontrol agent. Legume roots and *Rhizobium* species have symbiotic relationship resulting in biological nitrogen fixation. The plant supplies carbon for the energy-dependent reduction of nitrogen and protects the oxygen-sensitive nitrogenase enzyme. Nitrogen is an important primary nutrient element in plant physiology and is a key factor in agricultural production. *Trichoderma* spp. is increasingly being used as a biological control agent against soil-borne pathogens. The type of interaction between *Trichoderma* spp. and *Rhizobium* spp. could affect nodulation and the symbiotic nitrogen fixation process. In this study, the type of interaction between *Trichoderma* spp. and *Rhizobium* spp. was established by culturing them on yeast extract mannitol agar (YEMA) which was found to support the growth of both organisms. The effect of *T. asperellum* on growth of *Rhizobium* spp. was evaluated by dual culture technique. The means for the radial growth of *Rhizobium* spp. in presence of *T. asperellum* were highly significant (p=0.00). The growth on day 3 was the highest with a mean growth of 29.93 mm followed by the growth on day 5 with a mean of 16.53 mm. There was no rhizobial growth visible on day 7. The *in vitro* research output indicated that *T. asperellum* inhibited the growth of *Rhizobium* spp. by 49.7 to 100 % between day 3 and day 7. The results of this study point towards an antogonistic effect of the biocontrol agent *T. asperellem* on the beneficial *Rhizobium* spp and should be further investigated.

Key words: *T. asperellum, Rhizobium, biocontrol agent, invitro*

1. Introduction

Trichoderma spp. are endophytic fungi present in all soils. Many species in this genus can be characterized as opportunistic avirulent plant symbionts (Harman *et al*., 2004). The genus comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms (Benitez *et al*., 2004). The mycoparasitic activity of *T. asperellum* depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall and proteases (Xiaoxue *et al*., 2013). *Trichoderma* spp. exerts biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms (Hafez *et al*., 2013). The over usage of chemical pesticides present special challenges especially for the export market of locally produced French beans exported to European market which is subject to maximum residual levels (MRL). As such there has been a concerted effort to replace these chemical pesticides with biological agents. A number of *Trichoderma* spp. have been used as biocontrol agents of soil-borne pathogens such as *Fusarium, Pythium,* and *Rhizoctonia* spp. in French beans (Kariuki *et al*., 2010) among other crops. The *Trichoderma* spp. cultures are typically fast growing at 25-30 °C. The antagonistic and mycoparasitic potential of the *T. asperellum* strains were evaluated *invitro* through dual culture and interaction tests (Kucuk and Kivanc, 2003).

Rhizobium spp. is a nitrogen-fixing bacterium that form root nodules on legume plants. Most of these bacterial species are in the Rhizobiacae family in the alpha-proteobacteria (Weir, 2012). However, recent research has shown that there are many other rhizobial species in addition to these. In the laboratory, rhizobia are grown on yeast-mannitol agar (YMA). They are grouped into either fast-growing (e.g. *Rhizobium* spp.). or slow-growing (e.g. *Bradyrhizobium* spp.) (Beck *et al*., 1998). When cultured on YMA, the *Rhizobium* spp. produce visible growth in two to three days. They produce an acid growth reaction, which can be detected by adding a pH indicator, bromothymol blue (BTB), to the medium (Somasegaran and Hoben, 1994). The process by which rhizobia produce nitrogen for the legume is called biological nitrogen fixation (Beck *et al*., 1998).

2. Materials and Methods

2.1. Inoculation and culturing of *T. asperellum*

Trichoderma asperellum was inoculated and cultured on potato dextrose agar medium (PDA). The medium was prepared as described by Beck *et al*. (1993). Inoculation of *T. asperellum* was carried out using a flame sterilized wire loop. The cultures of *T. asperellum* were incubated for seven days at 27 ^{o}C.

2.2 Preliminary laboratory tests

2.2.1 Isolation and identification of Rhizobium spp. *isolates*

Nodulated French beans at their onset of flowering were collected from Mwea, Kirinyaga County, a French bean growing area in Kenya. Mwea area is in the low midland (LM5) agro-ecological zone (AEZ), (Jaetzold and Schmidt 1983). The roots were cut off and placed in a polythene bag before being transported to the laboratory where they were washed under tap water and thereafter the nodules detached from the plant. The sterilization, isolation and culturing of *R. leguminosarum* was carried out as per the procedure described by Beck *et al*. (1993).

2.2.2 Presumptive tests of Rhizobium spp. *isolates*

Presumptive tests were carried out on the isolates of *Rhizobium* spp. to establish their characteristics. The tests carried out were gram staining, growth on yeast extract mannitol agar (YEMA) containing Congo red (CR) dye and growth on YEMA containing Bromothymol blue (Somasegaran and Hoben (1994). A flame sterilized wire loop was used to scoop a loopful of each of the pure culture which was then streaked on a Petri dish containing sterilized YEMA in CR and another one streaked on Petri dish containing sterilized YEMA in BTB. The cultures were incubated at 27 ^{o}C for four days

2.3 Authentication of *Rhizobium* spp. strains in the greenhouse

Authentication was carried out in the Greenhouse of the Department of Plant Sciences, Kenyatta University to confirm that the isolates obtained were actually *Rhizobium* spp. Modified Leonard jar assemblies and sterilized vermiculite were used in the authentication (Somasegaran and Hoben 1994). Vermiculite was washed to obtain a constant pH of 6.8. It was then placed in the upper half of the modified Leonard jar assembly and the top covered with aluminum foil. Sterilized nitrogen-free plant nutrient was placed in the lower reservoir of the modified Leonard jar. The whole set up was covered with light proof paper secured with heat resistant tape before being steam-sterilized for 15 minutes.

Certified French bean seeds were selected for pre-germination. They were sterilized by dipping in 95 % alcohol and then in 3 % NaOCl solution for 3 minutes. The seeds were then rinsed in several changes of sterile distilled water and left in the final change of sterile water until they were fully imbibed. The sterilized seeds were then transferred using sterile forceps to the surface of sterilized 2 % water agar in a Petri dish for pre-germination. The plates were incubated at room temperature for five days. The radicals were allowed to grow until they attained a length of about 1 cm. Using flame-sterilized forceps, a hole was made at the centre of the vermiculite in the Leonard jar assembly. One pre-germinated seedling was transferred to the hole in the vermiculite of each sterile Leonard jar (Somasegaran and Hoben 1994). After three days when the cotyledons opened, the seedlings were inoculated with 1 ml of a sterile yeast extract mannitol broth (YEMB) containing *Rhizobium* spp. culture which had been grown for three days. The seedlings were allowed to grow for 8 weeks and their growth monitored.

2.4 Effect of T. asperellum on growth of R. leguminosarum in vitro

The relationship between *Trichoderma* spp. *and Rhizobium* spp. was established by culturing them on suitable medium that supports the growth of both organisms. Initially, *R. leguminosarum* and *T. asperellum* were cultured separately on their preferential media, which are YEMA for *Rhizobium* spp. and Potato dextrose agar (PDA) for *T. asperellum*. Trials were carried out on YEMA and PDA to establish which of the media can support the growth of both organisms. Eventually, YEMA was found to be the most suitable medium for culturing both *Trichoderma* spp. and *Rhizobium* spp. The YEMA medium was prepared, sterilized and allowed to cool before placing 25 ml of the medium in sterile Petri dish. A 5 mm disc of *T. asperellum* was obtained from a mature culture of *Trichoderma* spp. using a sterilized cork borer and was placed in the middle of the PDA medium. Radial streaks were then made using a culture of *Rhizobium* spp. The set up was incubated at 27^{o}C for seven days. The growth of the two cultures was observed and monitored. The interaction was categorized according to the modified five types of interaction grades proposed for fungi by Prince and Prabakaran (2011) as follows:-

1. Mutual intermingling without any macroscopic signs of interaction-Grade 1
2. Mutual intermingling growth, where the growth of fungus is ceased by growth of opposed organism-Grade 2.
3. Intermingling growth, where the fungus under observation is growing on the opposed organism either above or below - Grade 3.
4. Slight inhibition of both the interacting organisms with narrow demarcation line-Grade 4.
5. Mutual inhibition of growth at a distance of > 2mm - grade 5.

2.4.1 Dual culture technique

The *T. asperellum* isolates were evaluated against *Rhizobium* spp. by dual culture technique as described by Kucuk and Kivanc (2004). A mycelial disc measuring 5 mm in diameter was taken from the margin of seven day old *Trichoderma* spp. culture using a flame sterilized cork borer. This was inoculated at the centre of a Petri dish containing 25 ml of YEMA medium. Radial streaking was then carried out on the same plate using a sterile loop. The loop was loaded with 48 hour old culture of *Rhizobium* spp. grown at 27 ^{o}C on YEMA. The radial streaking was carried out starting from the centre of the plate where the disc of *T. asperellum* culture had been placed, to the peripheral of the plate. The wire loop was sterilized each time after carrying out one radial streak and before proceeding with the next radial streaking. The dual culture was incubated at 27 ^{o}C and observed for seven days. The zone of inhibition was measured and recorded on the third, fifth and seventh day. The experimental design was randomized complete block design with 4 replicates. The control had no *T. asperellum*; instead a 5 mm sterile disc of agar which had been cut using a flame sterilized cork borer was placed at the centre of the plate containing sterilized YEMA. The radial streaks of *Rhizobium* spp. were carried out starting from the centre and extending outward. The wire loop for streaking was sterilized each time before carrying out subsequent streaking on the agar plate. The inoculated plates were incubated at 27 ^{o}C and the

measurements taken on the third fifth and seventh day on the radial growth of *Rhizobium*. The percentage inhibition of the average radial growth was calculated in relation to growth of the controls (Johnson and Sekhar 2012) as follows:-

$$L = \frac{[C - T] \times 100}{C}$$

Key:
L Percentage inhibition of rhizobia radial growth (percentage inhibition)
C Radial growth measurement of the rhizobia in control (radial growth in control)
T Radial growth of rhizobia in the presence of *Trichoderma* (radial growth in the treatment)

2.5 Data analysis
Analysis of variance (ANOVA) was carried out and the differences between means were separated using Tukey's Honest Significant Difference (HSD) at 5% probability level.

3. Results
3.1 Preliminary laboratory tests
The colonies of *Rhizobium* spp. isolates started to appear on the second day after inoculation and by the fifth day they had fully matured. The colonies varied in diameter between 1-3mm, had smooth margins, convex elevation and had mucoid and firm texture. The colour ranged from milky white to cream yellow.

3.2. Presumptive and authentication tests on Rhizobium spp. isolates
The isolates of *Rhizobium* spp. were gram negative rods. The colonies were milky white to translucent and showed no absorption of the Congo red dye. When the *Rhizobium* spp. isolates were cultured in YEMA containing BTB the media turned yellow in colour. All the isolates formed nodules on the roots of the French beans.

3.3 Establishment of T. asperellum culture
As the *T. asperellum* colony grew in PDA medium, it formed concentric rings and changed colour from white to green as it matured and sporulated. The conidia formed within seven days in compact tufts of white and green shades (Plate 3.1). A yellow pigment was secreted into the agar. The concentric rings of *T. asperellum* changed to a green colour by the seventh day, when the *T. asperellum* culture became fully mature and sporulated.

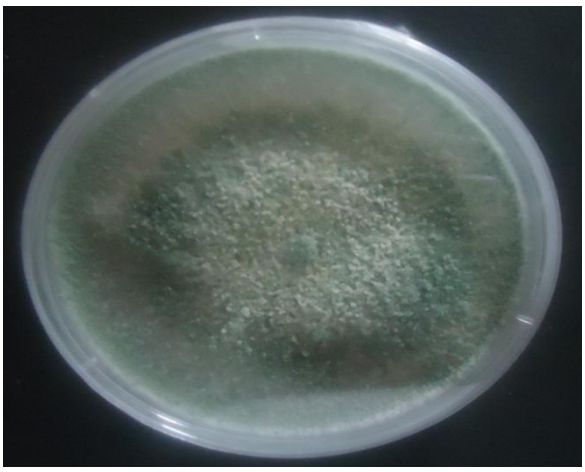

Plate 3.1 *T. asperellum* culture at day 7

3.4 Effect of T. asperellum on growth of R. leguminosarum invitro
Yeast extract mannitol agar was found to be the best medium for use in the dual culture technique. Plates 3.3 below show the inhibitory effects exhibited by *T. asperellum* on the growth of *Rhizobium* spp. culture.

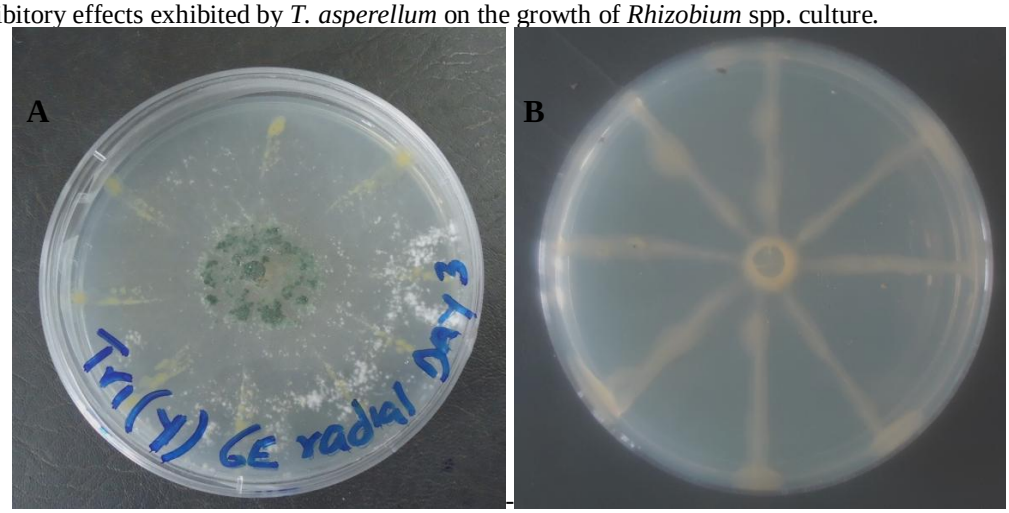

Plate 3.3: Dual culture technique of *T. asperellum* and *Rhizobium* spp. on day 3

A: Radial growth of *Rhizobium* spp. on YEMA in presence of *T. asperellum*. B: Radial growth of *Rhizobium* spp. where the *T. asperellum* is replaced by agar disc, (control).

3.5.1 Dual culture technique

On the third day, the *Rhizobium* spp. and *T. asperellum* cultures were well established. *Trichoderma* spp. was found to inhibit the growth of *Rhizobium* spp. at between 49.7 % to 100 %. On the third day the average inhibition of *Rhizobium* spp. was the lowest and only a little amount of it could be seen on the petri plate by the fifth day (Table 3.1). On the seventh day no *Rhizobium* spp. cultures were visible.

Table 3.1: *Rhizobium* spp. radial growth and percentage inhibition at day 3, 5 and 7

Day and replicates	**Radial growth in control (C) (mm)**	**Average. radial growth in test petri plate (T) (mm)***	**Percentage (%) inhibition (L)****
Day 3	60	29.7	50.5
Day 3	60	30.2	49.7
Day 3	60	29.7	50.5
Day 3	60	30.1	49.8
Day 5	60	16.0	73.0
Day 5	60	16.7	72.2
Day 5	60	16.4	72.7
Day 5	60	17.1	71.5
Day 7	60	0.0	100
Day 7	60	0.0	100
Day 7	60	0.0	100
Day 7	60	0.0	100

*Data are the average radial growth (mm) of *Rhizobium* spp. in the control and in the dual culture technique at day 3, 5 and 7 of four replicates (n=4). **Percentage inhibition calculated according to Johnson and Sekhar (2012).

3.5.2 Radial growth of Rhizobium spp. *in presence of T. asperellum*

The means of the radial growth of *Rhizobium* spp. in presence of *T. asperellum* was highly significant (p=0.00). The growth on day 3 was the highest followed by the growth on day 5 while day 7 had no visible rhizobial growth (Table 3.2).

Table 3.2: Radial growth of *Rhizobium* spp. in presence of *T. asperellum*

Measurement day	**Mean ± SE***
Day 3	29.93 ± 0.13^{c}
Day 5	16.53 ± 0.21^{b}
Day 7	0.00 ± 0.00^{a}

*Data are the mean ± standard error (SE) of the radial growth of *Rhizobium* spp. in presence of *T. asperellum* obtained from four replicates (n=4) which were measured on day 3, 5 and day 7. Means followed by the same letter are not significantly different according to Tukey's Honest Significant Difference at 5% level.

3.5.3 Percentage inhibition of Rhizobium spp.

The means for percentage inhibition were highly significanf (p=0.00). Radial growth on day 7 gave the highest inhibition while day 3 had the least inhibition (Table 3.3).

Table 3.3: Percentage inhibition of *Rhizobium* spp. by *T. asperellum*

Day	Mean ± SE*
3	50.13 ± 0.22^{a}
5	72.35 ± 0.33^{b}
7	100 ± 0.00^{c}

*Data are the means ± standard error (SE) of the % inhibition obtained from four replicates (n=4) which were measured on day 3, 5 and day 7. Means followed by the same letter are not significantly different according to Tukey's Honest Significant Difference at 5% level.

4. Discussion

All the *Rhizobium* spp. isolates had the morphological characteristics typical of the genus. The colour of YEMA containing Bromothymol blue indicator turned from green to yellow indicative of acid production by the bacteria which is a characteristic associated with fast growing *Rhizobium* spp. The bacterial colonies did not absorb Congo red dye. This is an important characteristic associated with rhizobia (Somasegaran and Hoben, 1994).

Previous studies have shown that *Trichoderma* spp. has antagonistic effects on most microorganisms (Paulitz and Belanger, 2001) and especially on plant pathogenic fungi such as *Fusarium oxysporium* and *Rhizoctonia solani* (Hafez *et al*., 2013). The inhibition of *Rhizobium* spp. by *Trichoderma* spp. was found to be between 49.7% and 100% which

indicates that *Trichoderma* spp. was highly inhibitive on *Rhizobium* spp. The inhibitory effects of *T. asperellum* on *Rhizobium* spp. had suppressed the *Rhizobium* spp. culture so that by the third day, *Trichoderma* spp. had grown and almost covered half of the *Rhizobium* spp. culture. The inhibitory interaction observed between *T. asperellum* and *Rhizobium* spp. can be categorized into grade 4 according to the modified five types of interaction grades proposed for fungi interaction by Prince *et al.*, 2011. *Trichoderma* spp. had a more aggressive growth and by the seventh day, it had established on the whole plate and no traces of *Rhizobium* spp. were visible on the Petri plate.

Biological Nitrogen fixation provides plants with nitrogen for photosynthetic activity and also avails nitrates for subsequent crops (Emerich and Hari, 2009). The research findings indicate that *T. asperellum* compromises nodulation in French beans. The farmer may therefore need to spend more money buying nitrogenous fertilizers to boost crop yield in order to meet the increasing demand for French beans. French beans are an important export horticultural crop to United Kingdom and France and the demand has been increasing annually, (H.C.D.A, 2010). Any measures that promote increased production would benefit not only the farmer but also the country at large in terms of foreign exchange earnings.

Root-knot nematodes (RKN) constitute a very significant threat to French bean production in Kenya (Kariuki *et al.*, 2011). RKN can be managed through crop rotation and only recently through biological control. The use of the conventional nematicides is expensive to the farmers and at the same time detrimental to the environment. Furadan (carbofuran) was one of the previously used nematicides but was withdrawn from the market due to residues found in the French beans (Monda *et al.*, 2003). *Trichoderma* spp. also has the potential to control some of the fungal diseases in French beans. However, even if this was to be successful, the fact that *Trichoderma* spp. seems to have antagonistic effect on *Rhizobium* spp. may compromise nodulation and nitrogen fixation in French beans and this will consequently affect the yield of the crop. *Trichoderma asperellum* had an antagonistic relationship with *Rhizobium* spp. inhibiting the growth of *Rhizobium* spp. *in vitro*.

5. Conclusion

The relationship between *Trichoderma* spp. and *Rhizobium* spp. was antagonistic with *Trichoderma* spp. inhibiting the growth of *Rhizobium* spp. *in vitro*. In light of these findings, it is recommended that farmers need to increase the amount of nitrogenous fertilizers used to top dress French beans that have been treated with *Trichoderma* spp. especially in nitrogen deficient soils.

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Competing Interests

Authors have declared that no competing interests exist.

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