



Efficacy of *Bacillus subtilis* and *Trichoderma asperellum* against *Pythium aphanidermatum* in tomatoes



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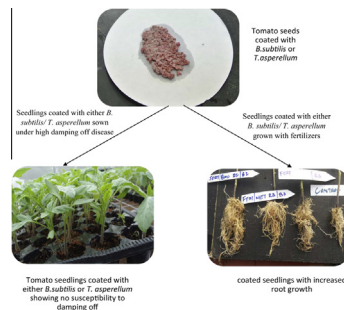
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HIGHLIGHTS

- Coating seeds with *B. subtilis* and *T. asperellum* offers protection of the seedlings against damping off disease.
- Coating of tomato seeds with *B. subtilis* and *T. asperellum* results in improved growth.
- The interaction between the seeds coated with *B. subtilis* and *T. asperellum* fertilizers leads to improved growth.

GRAPHICAL ABSTRACT



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ABSTRACT

Seedling damping-off disease caused by *Pythium aphanidermatum* is the most important seedling disease in tomato production in Kenya. The disease causes seedling losses of up to 30%. Greenhouse trials were conducted to evaluate the application of *Bacillus subtilis* and *Trichoderma asperellum*, as seed coating for management of damping-off in tomato from April 2011 to August 2014. Tomato seeds (var. Rio Grande) were coated with either *B. subtilis* or *T. asperellum* at a concentration of 10^6 CFU/ml. The interaction between the two biocontrol agents and NPK fertilizer was assessed. To simulate the effect of high disease pressure, the coated seeds were planted in *P. aphanidermatum* inoculated media. The post-emergence seedling damping-off on seeds coated with *B. subtilis* and *T. asperellum* was 20.19% and 24.07% respectively while the control (non-coated) had 65.89% seedling mortality. A combination of NPK fertilizer and biocontrols in seedling management resulted to a significantly higher dry mass compared to the use of either biocontrol agent or fertilizer alone ($P \leq 0.001$). This study indicates that coating of tomato seeds with *B. subtilis* and *T. asperellum* may be useful in the management of damping-off disease.

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1. Introduction

Tomato is an important vegetable crop in Kenya with production levels increasing by 31% over the last four years (FAO, 2014). The production however, is threatened by various pests and diseases. The important disease at nursery stage is damping-off

caused by *Pythium* spp. (Babalola and Glick, 2012). The disease results in mass seedling destruction leading to huge yield losses above 30% in Kenya (Muriungi et al., 2014).

Damping-off is managed mainly by application of synthetic pesticides since no tomato cultivar in Kenya has known resistance (Muriungi et al., 2014). Concerns about environmental hazards associated with the use of synthetic pesticides in vegetable production have increased the need for alternative control methods (Rosenzweig et al., 2001). Biological control agents have been

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effectively used in controlling various diseases caused by *Pythium* spp. and *Rhizoctonia solani* (Cubeta and Echandi, 1991). For instance, *Trichoderma* spp. have been shown to reduce a range of crop pathogens such as *Fusarium oxysporum* and *R. solani* (Okoth et al., 2011) while *Bacillus subtilis* produced metabolites with antibiotic activity that suppresses damping-off diseases in tomatoes caused by *R. solani* (Asaka and Shoda, 1996). *Pseudomonas fluorescens* F113 have been used to coat onions seeds to reduce damping-off disease (O'Callaghan et al., 2006). The technology provides timely control of seed and soil-borne disease such as *Pythium aphanidermatum* (Jensen et al., 2004). In addition to managing plant diseases microbial agents can also promotes plant growth (Antoun and Prevost, 2006). The microorganisms promote plant growth by enhancing uptake of micro- and macronutrients (Banerjee and Yesmin, 2002 and Unno et al., 2005). *Bacillus* spp. and *Trichoderma* spp. have been known to have growth promoting properties (Bacon and White, 2000; Kloepper and Mariano, 2000). However, there is no information on the efficiency of *B. subtilis* and *Trichoderma asperellum* applied as seed dress in controlling damping-off diseases in tomatoes. The information on the interaction between these microorganisms with synthetic fertilizers in plant growth promotion is largely lacking.

In this study the efficacy of *B. subtilis* and *T. asperellum* isolates applied as a seed dress against *P. aphanidermatum* was determined. The interaction between the isolates and fertilizer in enhancing growth in tomatoes grown in greenhouse was assessed.

2. Materials and methods

Isolates of *T. asperellum* TRC 900 and *B. subtilis* BS 01 used in the study were obtained from REAL IPM (Thika, Kenya). The isolates were stored in potato dextrose agar (PDA) slants at 4 °C. The pathogen, *P. aphanidermatum*, was isolated from infected tomato roots and stored in sterile water at 25 °C. PDA, Nutrient broth and Nutrient agar (NA) (HiMedia Laboratory, Pvt. Ltd., India) were used to start the working cultures of the stored isolates. Tomato seeds (cultivar “Rio Grande”) and the foliar fertilizer (NPK 17:17:17) were obtained from the Kenya Seed Company (Nairobi, Kenya). Artificial media (Coco peat) used to grow the tomato seedling was obtained from Oshwal Chemical Company (Nairobi, Kenya).

2.1. Site description

Three greenhouse trials were conducted yearly between 2011 and 2014 at the Jomo Kenyatta University of Agriculture and Technology (JKUAT; 1537 MASL; 01°05'25.6"S, 037°00'45.5"E).

2.2. Biocontrol agent and pathogen inoculum preparation

An isolate of *T. asperellum* stored in sterile soil slants was started on PDA and incubated for 4 days at 20 °C in the dark to induce sporulation. One ml of sterile water was then poured onto the 4 day old culture and fungal spores were scrapped using sterile glass rods into 20 ml sterile bottles containing nutrient broth. The bottles with the spores were then shaken in a reciprocal shaker (Taiyo Recipro shaker SR-1, Tokyo Japan) for 3 days to dislodge the cells from the media. Spore concentration of 10^6 spores/ml was then formulated by serial dilution in distilled water and final concentration determined using a hemocytometer.

B. subtilis was started in NA and transferred to nutrient broth after four days. The suspension was shaken in a reciprocal shaker for 72 h at 25 °C and concentration adjusted to approximately 10^6 CFU/ml.

Isolates of *P. aphanidermatum* stored in distilled water were started in PDA at 20 °C and kept in the dark for 4 days. A spore

concentration of 10^4 spores/ml was formulated as described above in the formulation of *T. asperellum* inoculum.

2.3. Seed coating

Six grams of tomato seeds were dipped in distilled water in a beaker for 2 min and water drained off immediately by straining with a sieve. Three grams of the wet seeds were mixed by stirring with the known concentration of either *B. subtilis* or *T. asperellum* suspensions. The coated seeds were then spread on open plastic trays and stored at 25 °C away from direct sunlight to dry for 24 h.

2.4. Determination of efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum*

Three greenhouse trials were conducted annually between 2011 and 2014. The experiment consisted of four treatments (Table 1) replicated three times. The treatments were set out in randomised block design on the greenhouse benches. For each treatment, plastic seedling trays (plug size 3 × 3 × 4 cm, 66 plugs/tray) were filled with coco peat. Treatment groups 1, 2 and 3, were then inoculated with *P. aphanidermatum* by drenching 1 ml of pre-formulated 10^4 spores/ml *P. aphanidermatum* suspension into each plug cell. Two tomato seeds were sown per plug for each treatment. The pre-emergence seedling mortality was assessed daily up to 14th day after sowing. The percentage pre-emergence damping-off was calculated based on the positive control as follows:

$$\text{Pre-emergence damping-off} = ((N - X)/N) \times 100$$

where *N* maximum germination in +ve control and *X* maximum germination in the treatment.

The post-emergence damping-off was assessed from the day of germination to the 28th day based on characteristic symptoms of damping-off infection (stem girdling). The percentage post-emergence damping-off was the calculated using the following formula:

$$\text{Percentage damping-off} = ((X - N)/X) \times 100$$

where *X* number of seedlings in the treatment and *N* number of seedling with damping-off symptoms.

2.5. Effect of the interaction between fertilizer and seedlings coated with *B. subtilis* and *T. asperellum* on tomato growth

The trials consisting of six treatments (Table 2) replicated three times and repeated annually between 2011 and 2014. Plastic seedling plug trays were filled with coco peat and two tomato seeds were sown per plug. After germination, the seedlings in the treatment groups 1, 3 and 5 were drenched with 200 ml/tray of NPK fertilizer (400 ppm) for a period of three days interspersed by a day of watering with no fertilizers. The treatment groups 2, 4 and 6 were

Table 1

Treatments for determination of efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum*.

Treatment	Description
1	<i>B. subtilis</i> coated seeds grown in coco peat with <i>P. aphanidermatum</i>
2	<i>T. asperellum</i> coated seeds grown in coco peat with <i>P. aphanidermatum</i>
3	Seeds not coated grown in coco peat with <i>P. aphanidermatum</i> (–ve control)
4	Seeds not coated grown in coco peat without <i>P. aphanidermatum</i> (+ve control)

The of description of the treatments used in the determination of efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum*.

Table 2

Treatments for determination of the effect of the interaction between fertilizer and seedlings coated with *B. subtilis* and *T. asperellum* on growth.

Treatment	Description
1	Seedlings coated with <i>T. asperellum</i> were grown with fertilizer
2	Seedlings coated with <i>T. asperellum</i> without fertilizers
3	Seedlings coated with <i>B. subtilis</i> were grown with fertilizers
4	Seedlings coated with <i>B. subtilis</i> were grown without fertilizers
5	Non-coated seedlings grown with fertilizers (–ve control)
6	Non-coated seedlings grown without fertilizers (+ve control)

watered daily with no fertilizer application for the experimental period of 28 days. On the 28th day a sample of 30 seedlings was obtained from each treatment and washed in running tap water for 15 min to remove any coco peat residues. The seedlings were then wrapped in old newspapers and oven dried (EYELA windy oven, Tokyo) at 70 °C for 48 h. The weight of the dried seedlings was assessed using a weighing balance (model Traveller TA 302, OHAUS CORPORATION, USA).

2.6. Data analysis

Percentage seedling mortality data were arcsine square root transformed. Lervene test was performed to test for normality before subjecting to generalised linear models procedure of SAS. Factor interaction was assessed and whenever there is a significant factor interaction, analysis was performed for the interacting factor at each level of the other factor. Means were compared using the Tukey test. All tests were performed at 5% level of significance using SAS 9.2. (SAS Institute Inc., Cary, NC).

3. Results

3.1. Efficacy of *B. subtilis* and *T. asperellum* against *P. aphanidermatum*

Coating seeds with either *B. subtilis* or *T. asperellum* in *P. aphanidermatum* inoculated media resulted in a significant reduction in the percentage pre-emergence damping-off compared to the treatment with non-coated seeds (Table 3). The pre-emergence damping-off between the seedlings coated with *B. subtilis* and *T. asperellum* was not significantly different (Table 3). *P. aphanidermatum* symptoms were observed between the 14th and 25th day after sowing. Significantly high percentage of post-emergence damping-off was recorded in the non-coated seedlings growing in *P. aphanidermatum* inoculated media (63.9%) compared to the coated seedlings ($P > 0.05$) (Table 3). The seedlings coated with *B. subtilis* had significantly lower post-emergence damping-off infection compared to those coated with *T. asperellum* (15.3%) (Table 3).

3.2. Effect of the interaction between fertilizer and seedlings coated with *B. subtilis* and *T. asperellum* on growth

The interaction between the fertiliser and the biocontrol agents in promoting tomato seedling growth was significant ($P < 0.001$)

Table 3

Efficacy of *B. subtilis* and *T. asperellum* on seedling damping-off caused by *P. aphanidermatum*.

Treatment	% pre-emergence	% post emergence
<i>B. subtilis</i>	22.81b ± 2.16	10.87a ± 0.013
–ve control	37.12c ± 1.52	63.9c ± 1.25
<i>T. asperellum</i>	21.04b ± 2.01	15.3b ± 0.02

Same letter in each column indicate no significance difference in the values. The significance level is at the 0.05. % pre-emergence refers to the % of the seedlings that succumbed to damping off before emergence from the coco peat. The % post-emergence refer to the seedlings that succumbed to the damping after germination.

(Table 4). Seedlings coated with either of the biocontrol agent and fertilized regularly had a significantly higher dry mass by the 28th day after sowing compared to fertilized but non-coated seedlings (Table 4). A significant difference was also observed between the dry masses of coated seedlings grown with fertilizers with *B. subtilis* dry mass higher than *T. asperellum* ($P = 0.001$) (Table 4). No significant difference was recorded between the dry masses of coated and non-coated seedlings grown without fertilizers.

4. Discussion

The significantly ($P \leq 0.05$) lower percentage pre-emergence damping-off of the seeds coated with either *B. subtilis* or *T. asperellum* under high disease pressure suggests that coating of seeds with *B. subtilis* or *T. asperellum* is effective against *P. aphanidermatum* damping-off. The results of this study corroborates with those obtained by Khare and Upadhyay (2009) in which 72.0% of seedlings coated with *Trichoderma viride* 1433 were not affected by pre-emergence damping-off. An application of *Trichoderma harzianum* and *Trichoderma koningii* conidia to pea seeds reduced the incidence of pre-emergence damping-off by 50% and 66.7% respectively (Lifshitz et al., 1986). Several studies have also documented the efficacy of seed coating in reducing cases of post-emergence damping-off (Adekunle et al., 2006; Abdelzaher, 2004; Berger et al., 1996; Mukhopadhyay et al., 1986; Harman et al., 1980).

The observations from this study indicate that seeds coated with *B. subtilis* have significantly ($P \leq 0.05$) fewer cases of post-emergence damping-off compared to those coated with *T. asperellum*. This suggests a possibly higher efficacy of *B. subtilis* in controlling *P. aphanidermatum* on tomatoes when compared to *T. asperellum*. The variation could be due to the mechanism through which *B. subtilis* or *T. asperellum* antagonize the pathogen. The ability of *B. subtilis* to compete favourably with germinated *Pythium* oospores for soluble carbon and nitrogen sources from root exudates have been reported to greatly reduce the establishment of *Pythium* (Weller, 1988). *Bacillus subtilis* also been shown to have the ability to produce antibiotics and other metabolites against *Pythium* (Haas and Défago, 2005; Paulitz and Belanger, 2001).

The significantly ($P = 0.001$) high dry mass of the seedling coated with *B. subtilis* or *T. asperellum* and grown with fertilizer compared to the non-coated grown with fertilizer indicates a positive interaction between fertilizer and coated seeds in promoting growth ($P < 0.001$). This suggestion is furthered by the significantly low dry mass of coated seedlings grown without fertilizer. These observations indicate that a combination of seed coating and fertilizer application enhances the growth of the seedlings. Rhizospheric microorganisms have been reported to increase the uptake of nutrients by the plant and subsequent increase in plant growth (Douds et al., 2005). The arbuscular mycorrhizal fungi have been observed to increase the plant uptake of potassium, nitrogen and zinc leading to increased plant growth (George, 2000). The present data confirms the increase of plant growth in seedlings coated with

Table 4

Effect of coating tomato seeds with *T. asperellum* and *B. subtilis* on total dry mass of tomato seedlings.

Treatment	Dry mass (g)	
	With fertilizer	Without fertilizers
<i>B. subtilis</i>	2.32a ± 0.019	0.10a ± 0.006
<i>T. asperellum</i>	1.77b ± 0.013	0.08a ± 0.008
Non-coated	1.63c ± 0.006	0.08a ± 0.011

The treatments represent the different groups of seedlings subject to different condition. The dry mass represents means of 30 seedlings in each treatment. Same letter in each column indicate no significance difference in the values. The significance level is at the 0.05.

biological control agent and grown with fertilizer as compared to non-coated seedlings grown under similar conditions.

5. Conclusion

Damping-off diseases of greenhouse grown tomatoes can be reduced by coating of the seeds with either *T. asperellum* TRC 900 or *B. subtilis* BS 01. The coated seedlings have a greater chance to survive pre-and post-emergence damping-off disease cause by *P. aphanidermatum*. In addition to controlling damping-off, *B. subtilis* is also a good growth promoter. The use of *B. subtilis* along with fertilizer leads to increased plant growth. More studies should also be conducted to investigate possibility of lowered fertilizer use through augmentation with biocontrol agents.

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