Plant Pathology & Quarantine 8(1): 37–46 (2018) ISSN 2229-2217
www.ppqjournal.org **Article**

Doi 10.5943/ppq/8/1/5

Augmented maize seed germination and seedling growth under water stress using *Trichoderma harzianum* from semi-arid soils

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Chepsergon J, Mwamburi LA, Kiprop KE 2018 – Augmented maize seed germination and seedling growth under water stress using *Trichoderma harzianum* from semi-arid soils. Plant Pathology & Quarantine 8(1), 37–46, Doi 10.5943/ppq/8/1/5

Abstract

The present study sought to assess the effect of *Trichoderma harzianum* from semiarid soils on maize seed germination and seedling growth under water stress. *Trichoderma harzianum* from semiarid soils was isolated and identified using macro- and micro-morphological characteristics. A three-factor factorial (2×3×4) design was employed, arranged in a completely randomized design with three replications. Maize seeds treated with *T. harzianum* had higher germination than untreated seeds. Maximum germination (96%) was recorded in both treated and untreated maize seeds when grown under water stress free condition (0MPa). However, seeds treated with *T. harzianum* showed a significant higher germination than untreated seeds grown under -0.3, -0.6 and -0.9 MPa among three varieties of maize. Under extreme water stress (-0.9 MPa), shoot dry weight of maize seedlings increased significantly from 0.05–0.06 mg/seedling in control to 0.17–0.19 mg/seedling in treated seedlings. Similarly, root dry weight increased significantly from 0.05–0.06 mg/seedling in control to 0.39–0.42 mg/seedling in treated maize seedlings at -0.9MPa. Under normal conditions (0MPa), *T. harzianum* did not enhance either maize seed germination or seedling growth. Taken together, the study recommends that for enhanced maize seed germination and seedling growth, 10^7 spores/ml of *T. harzianum* isolated from semiarid soils should be used as seed treatment regardless of the maize variety.

Key words – root growth – shoot growth – spores – *Trichoderma harzianum*

Introduction

Maize (*Zea mays* L.) is an important cereal crop grown all over the world (Verma et al. 2012) and is central to the world's food security. Despite its economic importance, the production of maize crop has lagged behind human population growth, leading to a huge discrepancy between food supply and demand (Khush 1999). Low yields have resulted from numerous production constraints including abiotic factors such as recurrent adverse weather conditions such as water stress, high temperature and salinity (Mohammed & Tarpley 2009). These abiotic factors have been recently catalyzed by global climate change that has been observed over the past decades and is anticipated to continue in the future (IPCC 2007). Water stress or drought stress is an inevitable and recurring feature of global agriculture. It is one of the most devastating environmental stresses.

Submitted 29 January 2018, Accepted 31 March 2018, Published 24 April 2018
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Water stress limits growth and productivity of main crop species, reducing yields to less than half (Bayoumi et al. 2008). It has been reported that about one-third of the world's potentially arable land suffers from water shortage (Kramer 1980).

Seed germination is the first stage of plant growth and, therefore, this stage must perform well since effects that occur early in the life of the plant continue throughout the life of most annual plants. In addition, during this stage, plants have a high vulnerability to injury, disease and environmental stress (Rajjou et al. 2012). Lack of adequate soil moisture during planting leads to poor and unsynchronized seedling emergence, poor establishment of crop stand, a reduction in crop yield and/or total crop failure per unit area (Khan et al. 2004). This effect has been seen even in maize crops, where the dehydration of seedlings was associated with the relative small size of their roots (Casanovas et al. 2002).

Trichoderma spp. are plant symbiont opportunistic avirulent organisms, able to colonize plant roots and to produce compounds that stimulate growth and plant defense mechanisms under suboptimal conditions (Harman et al. 2004). *Trichoderma* spp. are the most common fungi to be used as inoculants, mostly as biocontrol agents. However, in recent years, they have also become popular as plant growth promoters (Hermosa et al. 2012). For *Trichoderma* to effectively augment plant development, it must be able to establish in the spermosphere of germinating seeds, distribute on the emerging radicle and colonize the developing roots (Orr & Knudsen 2004). Studies clearly show that seeds do respond to *T. harzianum* very early in germination, that is, before the radicle protrudes (Hermosa et al. 2012). Harman (2000) reported that adding conidia of *T. harzianum* strain T22 as a seed treatment, profits the seed by enhancing phase III imbibition (cell elongation, followed by radicle protrusion). He also revealed that response of seeds to the fungus is hasty and it is said to begin before the fungus penetrates into the living portions of the seed. Microbes that grow in harsh environments become adapted to such stressed conditions, thus developing tolerance and further, they can be isolated and used as inoculum to support crops grown in correspondingly stressed environments (Khan et al. 2012). In that connection, they can protect plants against harmful effects of different environmental stresses to which crop plants are sporadically exposed.

Although seed treatment with *Trichoderma* spp. provides an innovative, cost-effective, low toxicity and environmentally friendly means of increasing crop yields, through improving seedling emergence and growth under drought stress, to date, related studies on maize are few. Curiously, the effect of fungi from adverse environments is an issue that remains to be addressed. Thus, an investigation of the system composed of *Trichoderma* spp. from semi-arid soils and maize seedling was worth carrying out to explore the influence of the fungus on maize seedling emergence and growth under drought, through laboratory analysis of some maize plant growth parameters such as germination percentage, dry weight of roots and shoots.

Materials & Methods

Study area

Soil samples were collected from the semi-arid rangeland of Marigat area, Baringo County. Much of the region receives low to average annual rainfall. Rainfall variability is very high in Marigat area with only one rainy season from April to August, and a prolonged dry season (Wasonga et al. 2011).

Collection of soil samples

Soil samples were collected in January 2014 from six sites of the entire Marigat rangeland. The rangeland was divided into two areas (A and B) based on the density of vegetation. Area A was characterized by low density of vegetation while area B was categorized by high density of grass plants. Six soil samples (10 g each) were collected, of which three were randomly obtained from rhizosphere of grass plants in area A. The other three samples were randomly obtained from bare soil 10 cm deep under area B using a sterile soil auger. The soil samples were then transferred

in separate, sterile polyethylene bags and transported to the Laboratory of Microbiology, University of Eldoret within 24 hours of collection. These samples were used for isolation of *T. harzianum*.

Isolation of T. *harzianum* from the soil

Isolation of *Trichoderma harzianum* from soil followed a modified method of Papavizas & Lumsden (1982). Ten grams of the six different soil samples were thoroughly mixed together to make a composite (60 g) and thereafter made up to 1000 ml using sterile distilled water in a sterile conical flask. The soil suspension was left for one hour at room temperature to release conidia and hyphae adhering to soil particles. Serial dilutions up to 10^{-3} were prepared. One ml portions were spread onto potato dextrose agar (PDA) medium supplemented with 50 mg/l of streptomycin antibiotic to inhibit bacterial growth. The plates were then incubated at 28^0C and 35^0C for 7 days. Distinct colonies of *T. harzianum* were picked based on their morphological characteristics as described by Rifai (1969). Microscopic examination and measurements of conidiophores and conidia were made from slide preparations stained with lactophenol-cotton blue and observed under a light microscope under 400×. Pure cultures of *T. harzianum* were then taken to KARI Njoro for confirmation.

Inoculum production of *Trichoderma* spp.

The procedure of Hassan et al. (2014) was adopted for production of *T. harzianum* inoculum. However, a slight modification was made to suit the present study. The pure cultures obtained were sub-cultured aseptically in eight 90 mm diameter Petri plates each containing 15 ml of a freshly autoclaved PDA media. Incubation of the eight plates was done at 28°C for 10 days. On the tenth day, spore suspensions from the fungus inoculum were prepared by flooding the surface of the agar slant with 10 ml sterile distilled water and the culture surface gently scraped to extricate the spores. The spore suspensions derived from the eight Petri plates were transferred separately to 500 ml flask containing 400 ml distilled water. Flasks were then shaken for 2 minutes to ensure that the spores are mixed. Two concentrations of the fungal spores were prepared (0 and 1×10^7 spores/ml) using a haemocytometer. The control was made up of autoclaved spores of *T. harzianum*.

Seed selection and treatment

Maize seeds with no cracks or any visible deformations were obtained from Kenya Seed Company Kitale. It is the leading seed company in Kenya and many farmers acquire their seeds from here. Maize varieties (H614, H629 and H6210) were used in the study because they have been reported to be highly susceptible to drought stress. More so, these varieties are being planted by most farmers within Uasin Gishu and Trans Nzoia counties, which are the main maize producing counties in Kenya. The seed was surface sterilized for 5 minutes with 1% sodium hypochlorite solution, followed by three rinses in distilled water and finally air dried. Wet seed treatment method was adopted, whereby seed 2% of starch was applied as an adhesive onto the maize seeds. Subsequently, the seeds were dipped in the seed coating suspensions of 0 (autoclaved *T. harzianum* suspension) and 1×10^7 spores/ml of *T. harzianum* for 2 minutes.

Preparation of polyethylene glycol concentrations

Polyethylene glycol 6000 (PEG) at different concentrations was prepared to establish different levels of osmotic potential. Approximately 0, 143.18, 213.64 and 267.97 g of PEG were dissolved in 1000 ml distilled water to generate four osmotic stress levels (0, -0.3, -0.6 and -0.9 MPa, respectively) (Guo et al. 2013). The control was made up of only distilled water with no PEG.

Determination of seedling emergence

The experimental design was a three-factor factorial (2×3×4) design, arranged in a completely randomized design with three replications each. The first factor was the concentration of *Trichoderma harzianum* (0 and 1×10^7/ml). The second factor was the maize seed varieties

(H614, H629 and H6210), and the third factor was the osmotic potential levels (0, -0.3, -0.6 and -0.9 MPa).

Seedling emergence assays was performed following Achakzai (2009). Sterilized maize seeds belonging to H614, H629 and H6210 varieties were treated with *Trichoderma harzianum* at (0 and 1×10^7spores/ml) concentrations for 2 minutes. Ten seeds were evenly distributed in each sterile Petri dish lined with two layers of Whatmann filter paper saturated with 8 ml of PEG solution to mimic drought stress. The plates were then incubated at 25^{o}C. The plates were kept moist throughout the experiment by adding 8 ml of the appropriate concentration of PEG to each plate after every 48 hours. Observations regarding germination were made after every 24 hours and continued till the completion of germination. The emergence of radical and plumule was taken as an indicator or measure of germination. After 7 days the percentage germination was determined using the formula by Achakzai (2009).

Determination of seedling growth

After 10 days of germination, three seedlings from each Petri dish were randomly selected and gently washed Roots and shoots were separately dried in an oven at 65±5°C for 72 hours and then weighed.

Statistical analysis

Maize seed germination and seedling growth experiments had factorial designs with three factors (concentration of *T. harzianum*, maize variety and osmotic potential). Analysis of variance (ANOVA) on maize seed germination percentage data was performed using statgraphics programme, after transforming the percent germination data to logarithm for normal distribution and homogeneity of variances. Both transformed and untransformed data were used in statistical analysis. For maize seedling length, fresh weight, shoot dry weight and root dry weight, data was subjected to ANOVA using statgraphics programme without transformation. Means for both seed germination and seedling growth were separated using Tukey's test.

Results

Isolation of *T. harzianum*

Cultural characteristics

After 7 days of growth, the fungus displayed green conidia, at both 28 and 35^{o}C. Conidia production was dense at the center and towards the margins of the colonies (Fig. 1). Conidia production did not differ at 28 and 35^{o}C.

Micro-morphological characteristics

Microscopic examination using a light microscope at 400× magnifications of 10-day-old cultures of *T. harzianum* grown on PDA showed globose to subglobose conidiophores. Conidia aggregated at the ends of the conidiophores (Fig. 2).

Maize seed germination and seedling growth

The effects of main factors and their interactions on maize seed germination and seedling growth are summarized in Table 1. Concentration of *T. harzianum* spores and osmotic potential had a significant ($p<0.05$) effect on maize seed germination and shoot and root dry weights. Interactions between concentration of *T. harzianum* by osmotic potential and maize variety by osmotic potential also had a significant effect on early seedling growth parameters. However, maize variety, maize variety by *T. harzianum* concentration and maize variety by *T. harzianum* concentration by osmotic potential interactions had no significant effect ($p>0.05$) on seed germination and seedling growth parameters.

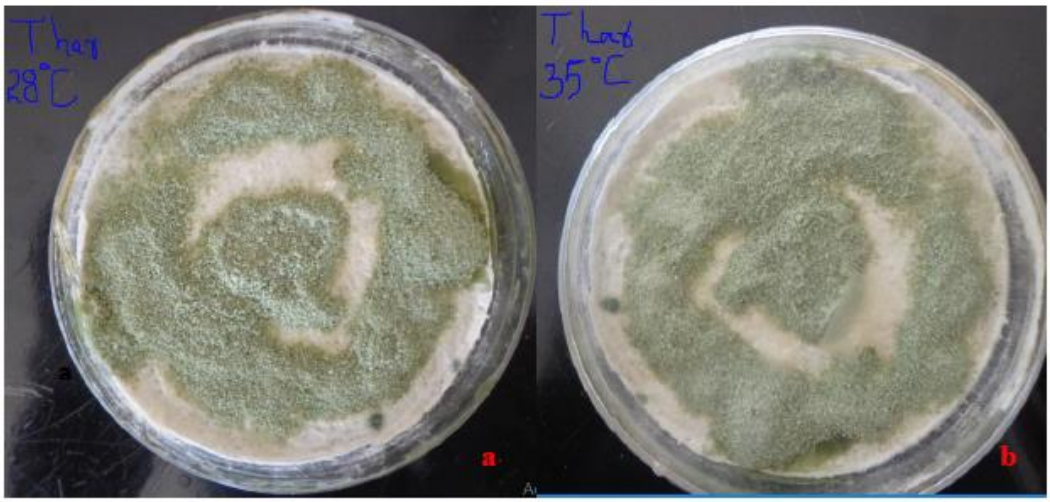

Fig. 1 – Conidia of 7-day-old *T. harzianum* on PDA at (a) 28ºC and (b) 35ºC.

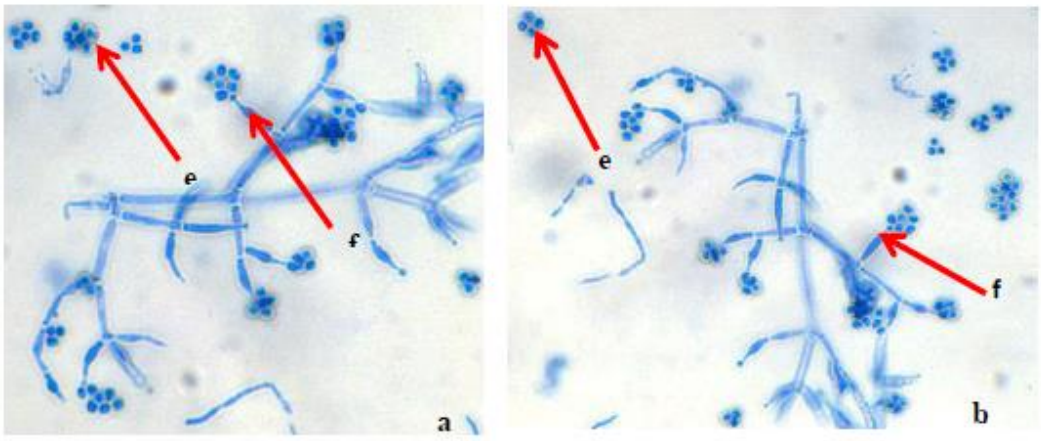

Fig. 2 – Conidia (e) and conidiophores (f) of *T. harzianum* as seen using a light microscope at a 28ºC and b 35ºC under ×400 magnifications.

Table1 Effects of main factors and their interactions on maize seed germination and seedling growth parameters.

	Germination %			**Shoot dry weight (g/seedling)**			**Root dry weight (g/seedling)**		
Variation	**F-ratio**	**P-value**	**Effect**	**F-ratio**	**P-value**	**Effect**	**F-ratio**	**P-value**	**Effect**
Concentration of *T.harzianum*(CT)	100.8	<0.05	**	1378.3	<0.05	**	121.3	<0.05	**
Osmotic potential (OP)	854.9	<0.05	**	7462.9	<0.05	**	580.7	<0.05	**
Maize variety (V)	0.4	>0.05	NS	0.7	>0.05	NS	7.6	>0.05	NS
CT×OP	25.2	<0.05	**	123.5	<0.05	**	4.7	<0.05	**
CT×V	2.4	>0.05	NS	2.0	>0.05	NS	1.1	>0.05	NS
OP×V	1.2	<0.05	**	4.9	<0.05	**	8.4	<0.05	**
CT×OP×V	0.6	>0.05	NS	1.5	>0.05	NS	1.2	>0.05	NS

**Significant at p < 0.05. NS not significant at P< 0.05

Maize seed germination

Generally, percentage germination decreased as osmotic potential increased. However, treated seeds had a higher germination percentage than untreated seeds (Fig. 3). At 0MPa, optimum percentage germination (95%) was recorded in both *T. harzianum* treated and untreated maize seeds, but it decreased as the moisture stress increased in all three varieties of maize.

Fig. 3 – Germination of maize seeds treated with 0 spores/ml of *T. harzianum* (a) and those treated with 10^7 spores/ml (b) at -0.9MPa.

At -0.9MPa, germination percentage reduced significantly from 54% (treated seeds) to 13% (untreated seeds).

Table 2 Effect of *T. harzianum* on germination percentage of three maize seed varieties under different osmotic potentials.

Concentration of *T. harzianum* (spores/ml)	**Osmotic potential (MPa)**	**Germination of maize seeds (%)**		
		Maize variety		
		H614	**H629**	**H6210**
0	0	95.5(1.98)f	95.2(1.97)f	96.7(1.98)f
	-0.3	85.4(1.93)e	85.3(1.93)e	85.1(1.93)e
	-0.6	24.1(1.38)b	23.9(1.37)b	23.7(1.37)b
	-0.9	13.4(1.12)a	13.7(1.13)a	13.3(1.12)a
10^7	0	95.1(1.98)f	96.1(1.98)f	96.8(1.98)f
	-0.3	91.3(1.96)ef	91.7(1.96)ef	90.7(1.95)e
	-0.6	61.0(1.78)d	60.9(1.78)d	62.3(1.78)d
	-0.9	54.3(1.73)c	54.8(1.73)c	54.4(1.73)c

**Values in parenthesis are log transformed values. Means followed by the same letter within the same column are not significantly different at $p < 0.05$

Shoot and root dry weights

Increase in PEG concentrations significantly decreased both shoot dry weight) and root dry weight in seedlings grown beyond -0.3 MPa in both treated and untreated seedlings across the three varieties of maize (Table 3). Shoot dry weight increased significantly ($p<0.05$) from 0.43–0.45 mg/seedling in control to 0.53–0.57 mg/seedling in seedlings treated with 10^7 spores/ml of *T. harzianum*.

At -0.9 MPa, SDW increased significantly ($p<0.05$) from 0.05–0.06mg/seedling in control to 0.17–0.19 mg/seedling in treated seedlings a cross the three varieties of maize as shown in Table 3 below.

Table 3 Effect of *T. harzianum* on shoot and root dry weight of three maize seed varieties under different osmotic potentials.

		Shoot Dry Weight			Root Dry Weight		
(spore/ml)	Osmotic potential	H614	H629	H6210	H614	H629	H6210
0	0	0.34±0.002d	0.31±0.001d	0.33±0.003d	0.41±0.017c	0.40±0.015c	0.42±0.021c
	-0.3	0.44±0.001g	0.43±0.001g	0.45±0.006g	0.86±0.009f	0.85±0.002f	0.81±0.019f
	-0.6	0.13±0.002b	0.11±0.003b	0.13±0.009b	0.30±0.005b	0.28±0.016b	0.25±0.002b
	-0.9	0.05±0.004a	0.05±0.001a	0.06±0.001a	0.06±0.016a	0.05±0.009a	0.05±0.020a
107	0	0.42±0.003f	0.41±0.003f	0.43±0.003f	0.69±0.005e	0.65±0.017e	0.61±0.004e
	-0.3	0.55±0.006h	0.53±0.003h	0.57±0.007h	1.41±0.019g	1.41±0.009g	1.39±0.021g
	-0.6	0.38±0.002e	0.37±0.007e	0.38±0.003e	0.54±0.002d	0.52±0.012d	0.57±0.012d
	-0.9	0.17±0.004c	0.18±0.001c	0.19±0.005c	0.40±0.007c	0.42±0.015c	0.39±0.017c

Means followed by the same letter within the same column are not significantly different at $P<0.05$. ** denotes significant at $p<0.05$

Similarly, irrespective of maize variety, RDW significantly ($p<0.05$) increased from 0.81–0.86 mg/seedling in control to 1.39–1.41g/ seedling in treated seedlings. Likewise, RDW was significantly reduced at -0.9MPa in both treated and untreated seedlings in all the three varieties of maize Nevertheless, RDW increased significantly ($p<0.05$) from 0.05–0.06 mg/ seedling in control to 0.39–0.42mg/ seedling in treated maize seedlings as shown in Table 3.

Discussion

T. harzianum grew well and uniformly at both 28 and 35°C. There is no doubt that the isolated fungus was *T. harzianum* since growth at 35^{o}C was recorded. Samuels et al. (2002) found that the ability of *T. harzianum* to grow at 35°C is useful in distinguishing it from other *Trichoderma* species. Furthermore, morphological characterization has been conventionally used in the identification of *T. harzianum* and it remains a method to identify *Trichoderma* species (Samuels et al. 2002, Bissett et al. 2003, Anees et al. 2010).

Reduction in germination with water stress is attributed to lower infusibility of water through the seed coat, and initial water imbibition of the seed under stress condition and decreased external water potential (Bahrami & Razmjoo 2012). Decrease in seed germination with increase in water stress could also be due to metabolic disorders such as slow hydrolysis of substrate compounds in endosperm or cotyledons and/or slower transportation of hydrolyzed material to developing embryo axis (Ayaz et al. 2000). Studies have shown that water stress sways more or less all aspects of plant physiology, biochemistry and growth metabolism (Li et al. 2007). Similar results have been reported by several authors. For example, a study carried out by Gupta et al. (2003) recorded a decrease in maize seed germination under water stress. Another study on tomato seedlings showed reduced plant growth under water stress (Mastouri et al. 2010). Further, an experiment on vetch reported that water stress decreased all germination and early seedling features (De & Kar 1995).

Three maize varieties showed no significant effect for seed germination percentage and early seedling growth under water stress. This finding disagrees with Ashagre et al. (2014) who observed significant differences in response to water stress on germination and seedling growth of maize cultivars. The present results could be due to the fact that the three varieties used belong to the six

series family and hence genotypic variation is presumed to be minimal. Seeds treated with *T. harzianum* showed significant difference in germination from control with water stress. For example, at -0.9MPa, seeds treated with 10^7 spores/ml of *T. harzianum* recorded significant germination (54%) compared to the control (13%). This finding is attributed to the fact that seeds do respond to *T. harzianum* very early in germination, even before the radicle protrudes (Harman et al. 2004). Also, *Trichoderma* spp. have been shown to augment seed germination by enhancing phase III imbibition (cell elongation, followed by radicle protrusion) (Harman et al. 2004). The present results are in agreement with those of Mastouri et al. (2010) who found that tomato seeds treated with *T. harzianum* (T22) showed higher germination than untreated tomato seeds.

The rationale of *T. harzianum* enhancing early seedling growth under stress could be due to its ability to induce synthesis of hormones, mainly indole acetic acids that promote growth in plants, probably through the up-regulation of plant genes for hormone biosynthesis or the down-regulation of the genes involved in hormone catabolism (Martínez-Medina et al. 2011). Another reason could be due to the fact *T. harzianum* decreases the synthesis of abscisic acids hormone (Aroca et al. 2013). This hormone inhibits plant growth during water stress in its mechanism to enable plants to withstand abiotic stresses (Aroca et al. 2013). Moreover, *T. harzianum* has been reported to produce ACC deaminase, which reduces the availability of the ACC necessary for ET biosynthesis, which results in inhibited plant root growth (Viterbo et al. 2010). Similar results have been reported by several authors. For instance, Harman (2000) found that *Trichoderma* spp. conferred tolerance to water stress at least in part through promotion of deeper root penetration into the soil profile. In another report by Bae et al. (2009), *T. hamatum* increased tolerance of cocoa plants to water deficit through increasing root growth that provided greater water resources to treated plants and delayed the onset of water deficit in these plants. Yildirim et al. (2006) also showed that squash plants treated with T22 or other showed enhanced root and shoot growth under abiotic stresses.

Treatment of maize seeds with *T. harzianum* isolated from semi-arid soils has beneficial effect on seed germination and seedling growth under water stress. Seedling growth at severe water stress was most probably through enhanced root development which resulted from enhanced phyto-hormones production. The study also showed that optimum activity of *T. harzianum* was maximum -0.3MPa, concluding that *Trichoderma* spp. promote plant growth mainly under stressed conditions.

Acknowledgement

The financial support by National Council of Science Technology and Innovation (NACOSTI) of Kenya to the project is hereby acknowledged.

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