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Acclimatization and growth of tissue cultured banana co-inoculated with microbiological and chemical commercial products in different soils in Kenya

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ABSTRACT

The efficacy of co-inoculation of microbiological products and combining them with a chemical stimulant in enhancing survival and growth of tissue culture banana under different soil conditions was investigated in the greenhouse. Tissue cultured banana (Gros Mitchel *cv.*) was inoculated with products containing *Bacillus* spp., arbuscular mycorrhizal fungi (AMF), *Trichoderma harzianum*, Myconate (chemical stimulant) and their combinations. Two soil types (Rhodic Ferralsol and Vertisol) were used at the hardening and potting phases and inoculation of plants was done at both phases. Plant growth was assessed at an interval of two weeks. Destructive harvesting was done and mycorrhizal colonization, root and shoot biomass and shoot nutrient uptake were assessed at the end of nursery phase. The effect of the products on the measured parameters depended on soil type with Vertisol being most receptive to inoculation. The combination of *Bacillus* spp. with AMF or Myconate or *T. harzianum* in the Vertisol gave the most significant increase in plant growth by over 28, 24 and 14%, respectively and in plant biomass accumulation by over 34, 46 and 33%, respectively compared to the control. Mycorrhizal colonization was not significantly affected by product inoculation in the two soils. In the Rhodic Ferralsol, the combination of arbuscular mycorrhizal fungi with *T. harzianum* promoted the highest uptake of zinc, boron, magnesium and phosphorus; *T. harzianum*+ *Bacillus* spp. promoted the highest uptake of potassium, while AMF+ Myconate promoted the highest uptake of calcium. In the Vertisol, the combination of *Bacillus* spp. with arbuscular mycorrhiza fungi gave the highest uptake of phosphorus, magnesium, calcium and boron; *Bacillus* spp.+ Myconate gave the highest uptake of potassium, while singly applied AMF gave the highest uptake of zinc. Results demonstrate that tissue cultured bananas' survival and growth can benefit from co-inoculation of arbuscular mycorrhizal fungi and *Trichoderma or Bacillus* or a chemical stimulant during the nursery phase. The effect of co-inoculation is however depended on soil type.

Key words: Microbiological and chemical products, co-inoculation, TC banana, nursery phase.

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INTRODUCTION

Disease and soil fertility have, largely, constrained production of important food crops such as banana (Akyeampong and Escalant, 1998; Frison and Sharrock, 1999). Tissue culture bananas are of a guaranteed disease-free quality, mature uniformly and ensure high quality marketable fruits. Nevertheless, these plants present some challenges such as somaclonal variations, poor physiology and the lack of any beneficial microbial inhabitants of roots

such as arbuscular mycorrhizal fungi (AMF) and Plant Growth-Promoting Rhizobacteria (PGPR) (George, 1996). This makes the acclimatization phase the most critical phase in the tissue culture process (Vestberg et al., 2002).

Symbiotic microorganisms such as AMF and PGPR play a fundamental role in sustainable agro ecosystems. They improve plant performance under environmental stress conditions and may facilitate plant adaptation at the nursery phase (Smith and Read, 1997; Nowak, 1998). AMF are obligate symbionts that colonize the roots of most cultivated plant species. Mycorrhizae are ubiquitous and over 80% of plant species form this symbiosis naturally (Smith and Read, 1997). This association occurs naturally when plantlets are transplanted into the field; it favors plant establishment, enhances nutrient uptake and offers protection against cultural and environmental stresses (Barea et al., 1997). *Bacillus* spp. are well known for solubilizing phosphorus (e.g. *B. megaterium, B. circulans, B. subtilis and Pseudomonas strait*a) and potassium (*B. mucilaginous* (Wu et al., 2004). *Trichorderma* spp. are well documented as effective biological control agents of plant diseases caused by soil borne fungi (Coley–Smith et al., 1991) through antagonism (Küçük, 2000), competition (Whipps, 1987) and plant growth promotion (Inbar et al., 1994). Myconate is a chemical product that stimulates the occurrence of the mycorrhizae.

A wide range of products exists in the market whereas, the conditions for their effective functioning are variable (Kloepper, 1996; Smith and Read, 1997; Nowak, 1998). However, most studies on commercial products have evaluated them singly (Carletti, 2000; Kavoo et al., 2013) and yet combination of the products may result to better performance of plants because of the combined functions of the microorganisms. It has been proposed that a consortium of biological agents with different individual traits should rather be applied (Raupach and Kloepper, 1998; Roberts et al., 2005). It has been proposed that plants must be mycorrhizal to thrive in degraded nutrient-poor and arid soils (Barea, 1991, 2000) and that mycorrhizal effect can be improved by co-inoculation with mycorrhiza-helper bacteria, which can play an important role in stressed areas (Requena et al., 1997; Barea, 1997). *In vitro* experiments in which saprotrophic fungi were paired with spores of *Glomus mosseae* or *Gigaspora rosea* showed a direct effect of *Trichoderma pseudokoningii* on the germination of spores of both AMF. The results suggest a direct interaction between the mycorrhizal fungus and the saprotrophic fungi in the pre-symbiotic phase of the former. Similar interactions have been proposed for other saprotrophic fungi (McAllister et al., 1994). It is known that effects of the genus *Trichoderma* on AMF spore germination may differ with the species used (Rouseau et al., 1996; Siddiqui and Mohmood 1996; Fracchia et al., 1998; Godeas et al., 1999; Green et al., 1999). The importance of soluble exudates and volatile substances produced by saprotrophic fungi in interactions with AMF has been demonstrated (McAllister et al., 1994, 1995).

Therefore, the hypothesis that co-inoculation of microbiological and chemical commercial products enhances survival, growth, biomass accumulation and nutrient uptake of TC banana (Gros Mitchel *cv.*) was tested in this study.

MATERIALS AND METHODS

Source of tissue culture material and initial soil characterization

Tissue cultured banana plantlets cv. Gros Mitchel were obtained from Jomo Kenyatta University of Agriculture and Technology Biotechnology laboratory. Two soil types; Rhodic Ferralsol and Vertisol were sampled from two agro ecological zones in Kenya where bananas are grown, that is, western Kenya in Nyanza (Vertisol) and coastal Kenya in Kilifi (Rhodic Ferralsol) at a depth of 0 to 20 cm and used for hardening and potting of tissue culture plantlets (Table 1). The 0 to 20 cm soil depth was chosen for mycological considerations since it contains the majority of soil microbiota (Skujins, 1984).

Initial chemical and physical analysis of soils was done before planting for nutrient composition (especially N, P, K, C, Mg, Ca, Na) and Cation Exchange Capacity (CEC), pH and soil texture composition (% Clay, % Sand and % Silt). This was done according to the procedures of Anderson and Ingram (1993) and Okalebo et al. (2002).

Experimental design

The study was carried out under greenhouse conditions. A factorial design experiment consisting of four commercial products (mycorrhizae, *Trichoderma, Bacillus* and chemical based) including Rhizatech (Dudutech Ltd., Kenya, *Glomus mosseae*, G. *etunicatum*, G. *intraradices* and G. *aggregatum*), ECOT (*Trichoderma harzianum* strain Rifai KRL AG2, Plant Health Care Inc., USA), PHC Biopak (Plant Health Care Inc., USA, Various *Bacillus* spp.) and Myconate were used for inoculation (Table 2). Application of products was done per recommendations of the manufacturers. ECO-T was applied at 1.25 g/L, Rhizatech at 2 g/plant, Biopak at 0.8 g/plant and Myconate at 0.02 g/plant. The products were selected based on their content that is known to promote banana growth, nutrition and yield by colonizing the root system, thus causing proliferation of roots, enhanced nutrient uptake (micro and macro nutrients), water uptake and production of plant growth regulators. The products were added to two soil types: Coast soil (Rhodic Ferralsol) and Nyanza soil (Vertisol). Three replicates consisting of 3 plants per replicate were considered per treatment. The experimental units were disposed in a completely randomized design in a green house.

Table 1. Initial soil characteristics (0–20 cm) of Rhodic Ferralsol and Vertisol.

Measurements	Units	Rhodic Ferralsol	Vertisol
pH (H_20)	-	6.98	5.79
Organic C	(%)	1.05	3.45
Total N	(%)	0.08	0.21
Olsen-extractable P	(mg P kg^{-1})	13.86	6.64
Exchangeable Ca	($cmol_c kg^{-1}$)	3.83	26.43
Exchangeable Mg	($cmol_c kg^{-1}$)	1.24	11.64
Exchangeable K	($cmol_c kg^{-1}$)	0.28	0.72
Exchangeable Na	($cmol_c kg^{-1}$)	0.20	0.38
ECEC	($cmol_c kg^{-1}$)	6.00	54.00
Sand	(%)	76.34	20.30
Silt	(%)	3.98	24.94
Clay	(%)	19.67	54.71
Textural class	-	Sandy clay	Clay

Table 2. Description of microorganisms based commercial products used in experiment.

Product	Manufacturer	Composition	Dose	Mode of application
Eco T	Plant Health Care Inc., USA	*Trichoderma harzianum* strain Rifai KRL AG2	0.25 g per 3 m^2	Dry powder applied to soil
Rhizatech	Dudutech Ltd., Kenya	*Glomus mosseae*, *G. claroideum, G. etunicatum, G. geosporum* and *G. intraradices* and ECMF (ectomycorrhizal fungi)	60 kg ha^{-1}	Granules applied to soil
PHC Biopak	Plant Health Care Inc., USA	*Bacillus licheniformis, B. megaterium, B. polymyxa, B. subtilis, B. thuringiensis* and *Paenibacillus azotofixans* (7.5×109 CFU/g each)	2 kg ha^{-1}	Drench application to planting soil
Myconate HB	Plant Health Care Inc., USA	Myconate HB-formononetin	40-90 g/ha	Dry powder applied to soil

Inoculation process

Inoculation of plantlets with products was done at the hardening and subsequently at potting phase. Plantlets with three fully developed leaves and well-developed root system formed *in vitro* using MS culture medium (Murashige and Skoog, 1962) were transferred to small planting pots (8 cm^3). Pasteurized soil from the three sites was used at the hardening stage. Pasteurization was done by autoclaving the soils twice for 30 min at 80°C and 15 Pa following the Mycorrhizal Training Manual prepared from the Centre for Ecology and Hydrology, Peniciuk, UK.

Hardening of plantlets was done in trays under mist conditions in humidity boxes. To avoid contamination, each tray contained banana plantlets inoculated by one treatment. One humidity box was required to hold 6 trays which were used. The position of the trays within the humidity boxes was randomized and changed on a regular basis. In each tray, 36 banana TC plantlets were grown. The trays were divided in 3 parts, containing three soil types. The location of the soils within the tray was randomized. Every hole within the tray contained a separate replicate banana plantlet. After a month, the boxes were gradually opened for a period of two weeks after which the cover was completely removed so as to harden the plants in preparation for potting. The humidity boxes were maintained at a temperature range of 24 to 30°C and a humidity range of 50 to 60%. The hardening stage lasted 10 weeks after which the plants were transferred into 1 L plastic containers with draining holes. Non-sterile soils of medium fertility level similar to the ones used at the hardening phase were used to provide optimal conditions for the functioning of the microorganisms. The plantlets were supplied with water to field capacity. The growth of plantlets was monitored for a period of 10 weeks, after which a destructive harvest was done. A temperature range of 18.9 to 34.4°C was recorded during the potting stage (Figure 1).

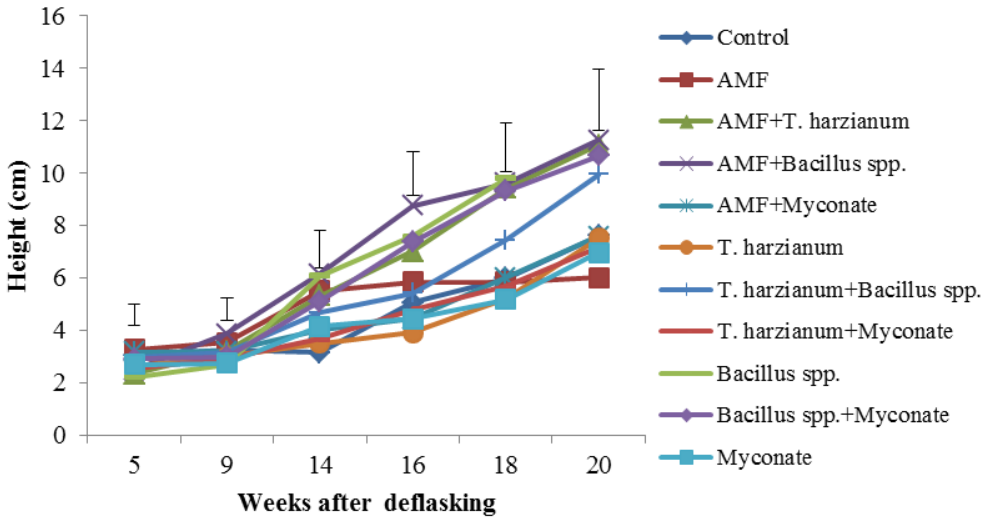

Figure 1. Effect of treatments on the height of banana plantlets in the Rhodic Ferralsol. Error bars are standard error of differences between means for each time of height measurement.

Assessment of growth of banana plantlets

Plant growth parameters (plant height, number of functional leaves, width of the youngest leaf, leaf length and chlorophyll content) were assessed at 5, 9, 14, 16, 18 and 20 weeks after inoculation under greenhouse conditions. Plant girth was measured at 20 weeks of growth. Number of leaves per plant was obtained through physical counting, while plant height, leaf width and length were measured using a ruler each round of data taking. Leaf width was measured across the broadest point of the youngest leaf whereas leaf length was measured along the midrib from the end of stalk to the tip of the same leaf.

Assessment of shoot and root biomass

Three plants were destructively harvested from each treatment at 20 weeks after deflasking (end of potting stage). The plants were assessed as aerial parts (fresh and dry shoot weight), root properties (root fresh and dry weight), number and length of secondary roots and tertiary roots and percent frequency and intensity of AMF colonization and shoot nutrient levels of macro (Potassium, Phosphorous, Calcium and Magnesium) and micronutrients (Zinc and Boron). The roots were separated from the shoots using a sterile scalpel, rinsed carefully and excess water removed using paper towel before recording the number of secondary roots, total root length and total fresh root weight. A portion of the roots was preserved in 70% ethanol for mycorrhizal colonization assessment. The root length was done using WinRHIZO Pro software program (a computerized version of the method of Tennant, 1975). A sub sample of the root was oven dried at 70°C for 72 h to obtain root dry weight. The dry weights of the sub samples were used to calculate the total root dry weights.

Assessment of mycorrhizal colonization

The portion of the root preserved in ethanol was processed for mycorrhiza colonization according to procedures of Koske and Gemma (1989). Estimation of the percentage root AMF colonization frequency and intensity was done using the subjective visual technique by Kormanik and McGraw (1982) commonly referred to as the slide method. The roots were cleared with 2.5% KOH (25 g KOH in 1000 ml water) by heating in an oven at 70°C for one hour and then rinsed with tap water. To remove phenolic substances, alkaline hydrogen peroxide (60 ml of 28-30% NH_4OH, 90 ml of 30% H_2O_2 and 840 ml distilled water) was added and roots placed in the oven at 70ºC for 20 minutes. The process was repeated for all the samples. The roots were rinsed with tap water and acidified with 1% HCl, then left for 30 min. The HCl was decanted and without rinsing the roots, 0.05% trypan blue in acid glycerol staining reagent (500 ml glycerol, 450 ml water, 50 ml of 1% HCl and 0.5 g trypan blue) was added and the roots placed in the oven at 70°C for 1 h. The stain was decanted and a de-staining solution comprising of acid glycerol (500 ml glycerol, 450 ml water, and 50 ml of 1% HCl) was added. Fine root segments were cut into 1 cm-long pieces and 30 pieces randomly picked, mounted on slides and observed under a compound microscope to assess the frequency and intensity of AMF colonization. The Presence of arbuscules, vesicles, internal and external hyphae were examined. The

Table 3. Effect of treatments on survival rates (%) of TC banana at the end of the nursery stage: 20 weeks after deflasking.

Treatments	Rhodic Ferralsol	Vertisol
Control	44.4ab	55.6abc
AMF	66.7ab	77.8bc
AMF+ *T. harzianum*	44.4ab	88.9c
AMF+ *Bacillus* spp	22.2a	77.8bc
AMF+ Myconate	77.8bc	77.8bc
T. harzianum	100c	66.7b
T. harzianum+ *Bacillus* spp	66.7ab	22.2a
T. harzianum+ Myconate	22.2a	11.1a
Bacillus spp	88.9bc	44.4abc
Bacillus spp+ Myconate	66.7ab	66.7b
Myconate	55.6ab	55.6abc
p value	**0.0340**	**0.0580**

Means within the same column with the same letter are not significantly different (Tukey test) at p≤0.05.

frequency of AMF was recorded as the number of root fragments infected with AMF and expressed as a percentage of total number of root fragments observed. The intensity of AMF colonization was recorded as percentage cover of AMF infective propagules in each 1 cm root fragment.

Assessment of nutrient uptake by banana plantlets

Shoots (pseudostems and leaves) were oven dried at 60°C for 48 h before being ground in a ball mill and analyzed for macro, secondary and micronutrient contents. Nutrient analysis was done at K. U. Leuven, Belgium using Inductively Coupled Plasma-Optical Omission Spectrometry (ICP-OES).

Data analysis

Data on survival were scored as a percentage proportion of the total number of surviving plants per product. Analyses of variance were performed on all measured variables using Proc MIXED in the SAS statistical software (SAS Institute 2006) to assess the effects of products and soils. Treatment means that were found to be significant at p≤ 0.05 were subsequently separated using the Studentized Tukey's test.

RESULTS

Survival of plantlets at the end of the nursery phase: 20 weeks after deflasking

Biological hardening is recommended for TC plantlets to reduce shock and loss since they are delicate and prone to shock after transplanting. Treatments on survival of TC bananas in three soil types were evaluated and their ability to sustain plantlets with or without the treatments was indicated (Table 3).

In non-treated Vertisol, the treatments performed were significantly better than those in the non-treated Rhodic Ferralsol by 11.2%, depicting the ability of the Vertisol to support banana growth more than the Rhodic Ferralsol without treatment. The effect of products applied singly or in combination was variable.

In Rhodic Ferralsol, *T. harzianum,* AMF+ Myconate, AMF+ *T. harzianum,* AMF+ *Bacillus* spp., AMF and Myconate increased the survival rate of the banana plantlets by 55.6, 33.3, 33.3, 22.2, 22.2 and 11.1%, respectively relative to the control. *T. harzianum* gave 100% survival rate of the banana plantlets, performing better compared to all other treatments. The effect of single application of Myconate and its combination with AMF or *Bacillus* spp. was greater than its combination with *T. harzianum* by 33.4, 44.5 and 55.6%, respectively. *Bacillus* spp. applied singly gave better results than its combination with AMF, *T. harzianum* and Myconate by 66.7, 22.2 and 22.2%, respectively. AMF+ Myconate performed better than the single application of AMF (11.1%) and its combination with *T. harzianum* (33.4%) and *Bacillus* spp. (55.5%). The single application was also better than the two combinations by 22.3 and 44.5%, respectively. In Vertisol, AMF+ *T. harzianum*, AMF+ *Bacillus*, AMF+ Myconate, AMF, *Bacillus* spp. + Myconate and *T. harzianum* increased the survival rate of the banana plantlets by 37.5, 28.5, 28.5, 28.5, 16.6 and 16.6%, respectively as compared to the control, but only the effect of *T. harzianum* and AMF+ *T. harzianum* were significant (p≤0.05). *T. harzianum* gave 100% survival rate of the banana plantlets by performing better than all other

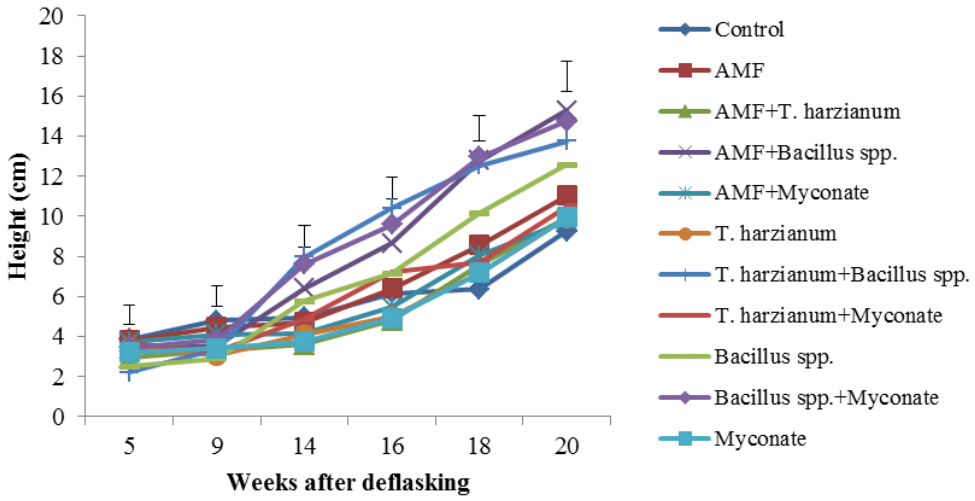

Figure 2. Effect of treatments on the height of banana plantlets in the Vertisol. Error bars are standard error of differences between means for each time of height measurement.

treatments. AMF+ *T. harzianum* performed better than single application of AMF and its combination with *Bacillus* spp. and Myconate by 11.1%. The effect of single application of *Bacillus* spp. was greater than its combination with AMF, *T. harzianum* and Myconate by 11.1, 66.7 and 22.3%, respectively.

Plant growth at the end of the nursery phase: 20 weeks after deflasking

The parameters evaluated for growth were height, girth, leaf length and width, number of functional leaves and leaf chlorophyll content.

Height of plantlets at hardening and potting stages

The effect of treatments on plant height was more pronounced in both soils. In the Rhodic Ferralsol, inoculation with *Bacillus* spp., AMF+ *Bacillus* spp. and AMF+ *T. harzianum* increased the height of plantlets by 33.7, 32.4 and 31.4%, respectively when compared to the control (Figure 1). In the Vertisol, significant increase in height was observed on plants treated with AMF+ *Bacillus* spp., *Bacillus* spp. + Myconate, *T. harzianum+ Bacillus* spp. and *Bacillus* spp. to magnitudes of 39.4, 32.5, 27.6 and 26.4%, respectively relative to the control (Figure 2).

Plant girth

Girth of the banana plantlets was affected by treatments in the two soils (Table 4). The girth of the banana plantlets inoculated with *T. harzianum* + *Bacillus* spp., AMF+ *Bacillus* spp., *Bacillus* spp. + Myconate, *Bacillus* spp., AMF, *T. harzianum*+ Myconate and AMF + Myconate when compared to the control was significantly increased by 42.5, 32.2, 31.1, 29.2, 23.1, 18.2 and 15.6%, respectively in the Vertisol. In Rhodic Ferralsol, combined application of *Bacillus* spp. + Myconate and single *Bacillus* spp. increased the girth of the banana plantlets by 15.0 and 14.7%, respectively compared to the control.

Number of functional leaves

Production of leaves was not significantly affected by treatments across the two soil types (Table 4). However, AMF + Myconate and *Bacillus* spp.+ Myconate in Rhodic Ferralsol and AMF, *T. harzianum+ Bacillus spp.* and *Bacillus* spp. in Vertisol had higher numbers of leaves in the respective soils. Only *T. harzianum+ Bacillus* spp. in the Vertisol significantly increased the number of leaves over the control by 22.2%. The number of leaves produced across all soil types ranged from 5 to 11 leaves irrespective of the treatment.

Leaf length

Treatment effect on leaf length was evident in the two soils (Table 4). In the Vertisol, AMF+ *Bacillus* spp., *Bacillus* spp. + Myconate, and *T. harzianum + Bacillus* spp. significantly increased leaf length of the banana plantlets over the control by 28.1, 27.1, and 14.0%, respectively.

Leaf width

The effect of the treatments on leaf width was only evident in Vertisol. AMF+ *Bacillus* spp., *T. harzianum+ Bacillus* spp.,

Table 4. Effect of treatments on TC banana plantlets growth at the end of the nursery phase: 20 weeks after deflasking.

Rhodic Ferralsol	Treatment	PG	NOL	LL	LW	CC
	Control	3.7bc	8.0ab	18.4b	6.4bc	50.3c
	AMF	2.3a	5.0a	12.2a	2.8a	46.8abc
	AMF+ *T. harzianum*	4.3c	7.0ab	19.1b	8.1c	45.9abc
	AMF+ *Bacillus* spp	4.3c	6.0a	20.8b	8.9c	47.7bc
	AMF+ Myconate	3.0ab	10.0b	14.9ab	4.6ab	50.1c
	T. harzianum	2.9ab	6.0a	16.5ab	4.3ab	50.6c
	T. harzianum+ *Bacillus* spp	3.8bc	7.0ab	21.8b	7.4b	42.3ab
	T. harzianum+ Myconate	3.0ab	7.0ab	16.6ab	4.8ab	46.7abc
	Bacillus spp	4.3c	7.0ab	21.1b	8.1c	46.9abc
	Bacillus spp+ Myconate	4.3c	10.0b	21.0b	7.4bc	40.3a
	Myconate	2.9ab	7.0ab	17.0ab	4.3a	48.0bc
p value		**<.0001**	**0.1767**	**0.0254**	**0.1005**	**0.0123**
Vertisol	Control	3.6a	9.0ab	21.0a	8.6a	47.3ab
	AMF	4.6bcd	10.0bc	23.1ab	10.4bc	50.8b
	AMF+ *T. harzianum*	3.9ab	9.0ab	21.9ab	9.4ab	50.7b
	AMF+ *Bacillus* spp	5.2d	8.0a	29.2c	11.9d	49.7ab
	AMF+ Myconate	4.2b	9.0ab	24.1b	9.3ab	51.2b
	T. harzianum	4.1b	9.0ab	21.2ab	8.7a	49.2ab
	T. harzianum+ *Bacillus* spp	6.1e	11.0c	24.4b	11.8d	52.1b
	T. harzianum+ Myconate	4.4bc	9.0ab	23.1ab	9.8ab	40.5a
	Bacillus spp	5.0cd	10.0bc	21.3ab	10.3bc	49.6ab
	Bacillus spp+ Myconate	5.1d	9.0ab	28.8c	11.3cd	50.0b
	Myconate	3.9ab	9.0ab	22.4ab	9.4ab	50.0b
p value		**<.0001**	**0.0752**	**<.0001**	**<.0001**	**0.7203**
Treatment		<.0001	0.8207	0.0056	0.0985	<.0001
Soil		0.0041	0.0092	0.0019	0.0026	0.0339
Treatment*Soil		0.0001	0.2047	0.0514	0.0454	0.0001

PG= girth of plantlets (cm); LL=leaf length (cm); LW= leaf width (cm), CC= chlorophyll content (%). Means within the same column with the same letter are not significantly different (Tukey test) at p≤0.05.

Bacillus spp. + Myconate, AMF and *Bacillus* spp. significantly increased leaf width of the banana plantlets by 28.2, 28.1, 24.3, 18.0 and 17.4%, respectively compared to the control (Table 4).

Leaf chlorophyll content (%)

The treatment effect on leaf chlorophyll content was not significant across the two soils (Table 4).

Plant biomass accumulation

Number of secondary roots

The number of secondary roots was highly affected by treatments in Rhodic Ferralsol followed by Vertisol (Table 5). The highest number of secondary roots (18) in Rhodic Ferralsol was produced by AMF+ *T. harzianum.* In the Vertisol, AMF+ *Bacillus* spp. and *Bacillus* spp. + Myconate produced the highest number of roots (22) and significantly increased the number of roots by 34.3 and 33.3%, respectively compared to the control.

Root length

The effect of treatments on the root length was highly significant in Rhodic Ferralsol (Table 5). AMF + *T. harzianum, Bacillus* spp., AMF + *Bacillus* spp. and *Bacillus* spp. + Myconate in Rhodic Ferralsol increased the root length of banana plantlets by 71.4, 69.6, 64.8 and 58.4%, respectively when compared to the control.

Root dry matter

Treatment effect on the root dry matter was high in Vertisol

Table 5. Effect of treatments on TC banana root and shoot growth at the end of the nursery phase: 20 weeks after deflasking.

Soil	Treatment	NOR	RL	RDM	SDM
Rhodic Ferralsol	Control	13.0bc	10.0a	0.5a	3.2ab
	AMF	6.0a	2.40a	0.1a	0.3a
	AMF+ *T. harzianum*	18.0d	35.0b	1.6b	8.1c
	AMF+ *Bacillus* spp	17.0d	28.4b	1.2b	5.0bc
	AMF+ Myconate	11.0b	80.6d	0.4a	1.7ab
	T. harzianum	10.0ab	65.8c	0.3a	1.2ab
	T. harzianum+ *Bacillus* spp	13.0bc	16.6ab	0.9ab	5.8bc
	T. harzianum+ Myconate	11.0b	15.9ab	0.6a	4.7bc
	Bacillus spp	17.0d	32.9b	1.7b	7.0bc
	Bacillus spp+ Myconate	14.0bc	24.1b	1.3b	6.0bc
	Myconate	11.0b	95.9d	0.5ab	2.1ab
p value		**0.0004**	**0.0128**	**0.0131**	**0.0084**
Vertisol	Control	15.0ab	9.3a	0.6a	3.5a
	AMF	18.0bc	20.7b	1.2ab	6.5b
	AMF+ *T. harzianum*	11.0a	82.7c	1.2ab	6.0ab
	AMF+ *Bacillus* spp	22.0c	13.3ab	1.6bc	11.4c
	AMF+ Myconate	20.0bc	23.1b	1.4b	6.5b
	T. harzianum	15.0ab	10.0a	0.8a	3.8a
	T. harzianum+ *Bacillus* spp	20.0bc	15.0ab	1.9bc	18.3d
	T. harzianum+ Myconate	-	-	-	-
	Bacillus spp	19.0bc	18.2ab	1.8bc	10.1c
	Bacillus spp+ Myconate	22.0c	18.7ab	2.0c	12.1c
	Myconate	15.0ab	9.7a	0.7a	4.1a
p value		**0.0289**	**0.0584**	**0.7399**	**<.0001**
Treatment		<.0001	<.0001	<.0001	<.0001
Site		0.0011	0.0016	0.0034	0.0014
Treatment*Site		0.0117	0.0100	0.1163	0.0007

NOR, number of secondary roots; RL, root length (m); RDM, root dry matter (g); SDM, shoot dry matter (g). Means within the same column with the same letter are not significantly different (Tukey test) at p≤0.05. (-) means missing value (plantlets died during hardening).

followed by Rhodic Ferralsol (Table 5). All treatments when compared to the control increased the root dry matter of banana plantlets in Vertisol. However, only *Bacillus* spp. + Myconate and *T. harzianum* + *Bacillus* spp. gave significant increases of 70.0 and 68.4%, respectively. In Rhodic Ferralsol, *Bacillus* spp., AMF + *T. harzianum*, *Bacillus* spp. + Myconate, AMF + *Bacillus* spp. and *T. harzianum* + *Bacillus* spp. when compared to the control increased banana root dry matter by 64.7, 62.5, 53.8, 50.0 and 33.3%, respectively.

Shoot dry matter

The effect of the treatments on shoot dry matter was more pronounced in Vertisol followed by Rhodic Ferralsol (Table 5). Inoculation with *T. harzianum* + *Bacillus* spp., *Bacillus* spp. + Myconate, AMF + *Bacillus* spp. and *Bacillus* spp. significantly increased the shoot dry matter of banana plantlets by a magnitude of 80.9, 71.1, 69.3 and 65.5%, respectively compared to the control in the Vertisol. The application of AMF + *T. harzianum, Bacillus* spp., *Bacillus* spp. + Myconate, *T. harzianum* + *Bacillus* spp., AMF + *Bacillus* spp. and *T. harzianum* + Myconate in the Rhodic Ferralsol increased the banana shoot dry matter by 60.5, 54.3, 46.7, 44.8, 36.0 and 31.9%, respectively.

Mycorrhizal colonization

The effect of the treatments on the intensity and frequency of root AMF colonization was not significant (p≤0.05) in the two soils (Table 6). However, the application of AMF + Myconate, AMF, *T. harzianum*, AMF + *T. harzianum* and AMF + *Bacillus* spp. increased mycorrhizal colonization intensity

Table 6. Effect of treatments on root mycorrhizal colonization: 20 weeks after deflasking.

Treatment	Rhodic Ferralsol		Vertisol	
	%Intensity	%Frequency	%Intensity	%Frequency
Control	34.6	12.9	61.1ab	25.1ab
AMF	76.1	31.9	68.3ab	28.9ab
AMF+ *T. harzianum*	41.8	27.5	53.5ab	18.0ab
AMF+ *Bacillus* spp.	71.7	27.4	73.3ab	28.0ab
AMF+ Myconate	67.4	30.7	92.1b	40.9b
T. harzianum	78.9	29.2	63.3ab	16.2a
T. harzianum+ *Bacillus* spp.	51.9	19.5	83.2b	26.1ab
T. harzianum+ Myconate	57.3	19.0	-	-
Bacillus spp.	50.5	14.5	87.4b	37.8b
Bacillus spp. + Myconate	72.6	27.5	62.4ab	19.3a
Myconate	27.8	18.7	33.3a	14.2a
p value	**0.7940**	**0.6089**	**0.3204**	**0.1855**

Means within the same column with the same letter are not significantly different (Tukey test) at p≤0.05.

in the banana roots by 72.0, 60.0, 55.9, 53.2 and 52.9%, respectively compared to the control in Rhodic Ferralsol. In Vertisol, AMF + Myconate and *Bacillus* spp. increased the mycorrhizal colonization intensity in the banana roots by 38.6 and 33.6%, respectively compared to the control.

The mycorrhizal colonization frequency in the plantlets inoculated with AMF + Myconate, AMF, AMF + *Bacillus* spp. and *Bacillus* spp. + Myconate in Rhodic Ferralsol, increased the mycorrhizal colonization frequency of the banana roots by 62.9, 56.3, 53.6 and 52.4%, respectively relative to the control. The application of AMF + Myconate and *Bacillus* spp. in Vertisol increased the mycorrhizal colonization frequency of the banana roots by 33.7 and 30.1%, respectively over the control.

Plant nutrient uptake

The effect of treatments on nutrient uptake in Rhodic Ferralsol when compared to the control was as follows: The co-inoculation of AMF + *T. harzianum* and the combined application of AMF + Myconate increased the uptake of Mg by 69.1 and 68.2%, respectively. AMF + *T. harzianum,* AMF + Myconate and *T. harzianum*+ *Bacillus* spp. increased the uptake of Zn by 64.5, 60.4 and 60.1%, respectively compared to the control. *Trichoderma harzianum* + *Bacillus* spp. and AMF + Myconate increased the uptake of K by 67.6 and 67.2%, respectively. AMF + Myconate and AMF + *T. harzianum* increased the uptake of Ca by 77.2 and 61.9%, respectively compared to the control. AMF + Myconate, AMF + *T. harzianum* and *T. harzianum*+ *Bacillus* spp. increased the uptake of B by 75.8, 66.8 and 63.8%, respectively. The effect of treatments when compared to the control in the Vertisol was significant on the uptake of P, K, Mg, Ca and Zn. AMF + *Bacillus* spp., *Bacillus* spp.+ Myconate, *Bacillus* spp. and *T. harzianum*+ Myconate increased the P uptake by 65.2, 63.9, 57.1 and 50.4%, respectively. *Bacillus* spp. + Myconate, AMF + *Bacillus* spp., *Bacillus* spp., *T. harzianum*+ Myconate, AMF and AMF + Myconate increased the uptake of K by 67.6, 66.4, 64.1, 54.1 42.2 and 40.9%, respectively. AMF + *Bacillus* spp., *Bacillus* spp. + Myconate, *Bacillus* spp. and *T. harzianum*+ Myconate increased the uptake of Mg by 66.6, 65.9, 60.5 and 50.4%, respectively. AMF + *Bacillus* spp., *Bacillus* spp. + Myconate, *Bacillus* spp. and *T. harzianum*+ Myconate increased the uptake of Ca by 70.8, 68.6, 67.1 and 55.3%, respectively. AMF + *Bacillus* spp., *Bacillus* spp.+ Myconate, *Bacillus* spp. and *T. harzianum*+ Myconate increased the uptake of B by 71.8, 67.8, 59.6 and 54.7%, respectively compared to the control (Table 7)

DISCUSSION

Survival of plantlets at the end of the nursery phase

The survival of plantlets was dependent on type of treatment and was influenced by soil types. However, the combined treatments performed better than the singly applied treatments. The improved performance of the AMF combined with *Bacillus* spp. supports the report of Requena et al. (1997) and Barea (1997) that mycorrhizal effect can be improved by co-inoculation with mycorrhiza-helper bacteria, which can play an important role in stressed conditions. The co-inoculation of AMF and *T. harzianum* suggest a direct interaction between the fungi. McAllister et al. (1994) reported a direct effect of *T. pseudokoningii* on the germination of spores of *G. mosseae* or *G. rosea*.

Table 7. Effect of treatments on nutrient uptake in TC banana plantlets at the end of the nursery phase: 20 weeks after deflasking.

Soil	Treatment	P	K	Mg	Ca	Zn	B
Rhodic Ferralsol	Control	5.8ab	120.1a	22.7ab	30.6a	75.4ab	35.9ab
	AMF	-	-	-	-	-	-
	AMF+ *T. harzianum*	15.7c	162.2ab	73.5c	80.3bc	212.1c	108.1d
	AMF+ *Bacillus* spp	4.7ab	171.4abc	20.0ab	25.3a	106.2abc	46.9ab
	AMF+ Myconate	12.7bc	366.3d	71.4c	134.4c	190.2c	148.3d
	T. harzianum	1.8a	42.8a	9.4 a	9.2a	46.3a	14.6a
	T. harzianum+ *Bacillus* spp	15.3bc	370.5d	47.3abc	56.0ab	189.0c	99.2cd
	T. harzianum+ Myconate	10.7bc	173.4abc	56.8bc	69.0b	101.4abc	51.4abc
	Bacillus spp	7.3ab	205.3cd	26.2ab	30.7a	140.4abc	77.4abc
	Bacillus spp+ Myconate	7.5ab	219.1cd	26.4ab	27.2a	157.1abc	61.8abc
	Myconate	4.5ab	76.3a	17.2ab	20.9a	59.1ab	25.2ab
p value		**0.1192**	**0.2248**	**0.0096**	**0.0055**	**0.0888**	**0.0156**
Vertisol	Control	5.7a	186.6a	23.1a	28.0ab	90.1ab	42.2a
	AMF	9.2ab	322.8cd	33.3ab	41.5ab	185.1b	78.3ab
	AMF+ *T. harzianum*	7.6ab	259.6abc	28.1a	37.4ab	122.1ab	67.1ab
	AMF+ *Bacillus* spp	16.4d	554.7e	69.2d	95.9e	180.8b	149.7d
	AMF+ Myconate	9.1ab	315.8cd	37.0ab	44.8ab	167.4b	74.1ab
	T. harzianum	5.8a	193.5ab	23.2a	26.5a	16.4a	43.2a
	T. harzianum+ *Bacillus* spp	-	-	-	-	-	-
	T. harzianum+ Myconate	11.5c	406.7d	46.6bc	62.6c	171.8b	93.2bc
	Bacillus spp	13.3cd	520.5e	58.5cd	85.0de	182.9b	104.4bc
	Bacillus spp+ Myconate	15.8cd	576.5e	67.7d	89.2de	144.8ab	130.9cd
	Myconate	6.7ab	224.3abc	26.9a	31.6ab	118.8ab	43.6a
p value		**0.0038**	**0.014**	**0.0204**	**0.0134**	**0.0131**	**0.7120**
Treatment		0.0007	0.3779	<.0001	0.0003	<.0001	0.1920
Site		0.2016	0.0126	0.0029	0.0802	<.0001	0.0394
Treatment*Site		0.1014	0.0025	0.0005	<.0001	0.0040	0.0078

P=phosphorus (g/kg), K=potassium (g/kg), Ca=calcium (g/kg), Mg=magnesium (g/kg), Zn=zinc (mg/kg) and B=boron (mg/kg) Means within the same column with the same letter are not significantly different (Tukey test) at p≤0.05; (-) means missing value (plantlets died during hardening).

However, the effects of *Trichoderma* spp. on AMF spore germination may differ with the species used (Rouseau et al., 1996; Siddiqui and Mohmood, 1996; Fracchia et al., 1998; Godeas et al., 1999; Green et al., 1999). AMF or *Bacillus* spp. combined with Myconate (a chemical stimulant) significantly improved plantlet survival. This could be attributed to Myconate's ability to stimulate the multiplication of either introduced or indigenous AMF. Myconate applied singly may have led to increased AMF spore population, hence its improved performance in the Vertisol; AMF may promoted improved uptake of nutrients (van der Heijden et al., 1998; Harikumar and Potty, 2002; Rillig, 2004; van der Heijden et al., *2*006; Hu and Rufty, 2007), while *T. harzianum* may have suppressed soil borne disease causing fungi (Whipps, 1987; Coley–Smith et al., 1991; Küçük, 2000).

Plant growth

Treatments had variable effects on growth parameters and biomass accumulation of banana plantlets depending on the product composition and soil physico-chemical properties. The application of single products or their combinations had positive effects on banana growth. Although plantlets inoculated with combined treatments showed the highest significant increases, there were cases where single products, that is, *Bacillus* spp. and AMF significantly increased the growth and biomass accumulation of banana plantlets more than the combined ones. This is in line with the results of the interaction of *G. manihotis* +*Bacillus* sp. treatment on banana growth parameters as compared with the controls, but did not differ from *Bacillus* spp. (Rodriguez et al., 2003).

The combination of *Bacillus* spp. with AMF, *T. harzianum* or Myconate gave the most significant increase on plant growth measurements (height of banana plantlets, plant girth, leaf length and leaf width) in the Vertisol. The effect of the combination on biomass accumulation (number of secondary roots, root length, root dry matter and shoot dry matter) was also significant in Vertisol. The key advantages of *Bacillus* based products are their ability to sporulate. Endospores produced by *Bacillus* spp. are resilient structures capable of surviving desiccation, heat, oxidizing agents, UV and γ radiation (Jacobsen and Douglas, 2002; Wuytack et al., 1999). It has been proposed that plants must be mycorrhizal to thrive in degraded nutrient-poor and arid soils (Barea, 1991, 2000) and that mycorrhizal effect can be improved by co-inoculation with mycorrhiza-helper bacteria, which can play an important role in stressed areas (Requena et al., 1997; Barea, 1997). Myconate may have stimulated the functioning of the mycorrhizal product, hence the significant performance of the combination.

The combination of *T. harzianum* with either AMF or Myconate had an insignificant increase on growth of banana plantlets and biomass accumulation across the four soils. This suggests a direct interaction between the mycorrhizal fungus and the saprotrophic fungi in the pre-symbiotic phase of the former. *In vitro* experiments in which saprotrophic fungi were paired with spores of *G. mosseae* or *G. rosea* showed a direct effect of *T. pseudokoningii* on the germination of spores of both AMF and similar interactions have been proposed for other saprotrophic fungi (McAllister et al., 1994). It is known that the effects of the genus *Trichoderma* on AMF spore germination may differ with the species used (Rouseau et al., 1996; Siddiqui and Mohmood, 1996; Fracchia et al., 1998; Godeas et al., 1999; Green et al., 1999). The importance of soluble exudates and volatile substances produced by saprotrophic fungi in interactions with AMF has been demonstrated (McAllister et al., 1994, 1995). Thus, it is reasonable to assume that soluble or volatile substances produced by the *T. harzianum* could have inhibited the functioning of AMF, hence the insignificant effect of the combination.

Focusing on the combined effect of treatments as compared with the controls and single treatments, increases were observed in all the growth and plant biomass measurements. However, just a few treatment combinations gave significant increases in the Vertisol. Some authors have reported the synergistic beneficial effect in plant development promoted by the fungi-rhizobacteria association (Dhillion, 1992; Singh and Kapoor, 1998). Dual inoculation with both soil microorganisms also induced higher biomass and yield (Dhillion, 1992; Singh and Kapoor, 1998), and even higher nutrient uptake (Singh and Kapoor, 1998). When plant roots were colonized by a mycorrhizal fungus, *Paenibacillus* spp. were preferentially isolated from the mycorrhizosphere when compared to non-mycorrhizal plant roots (Arturrson et al., 2005; Mansfeld-Giese et al., 2002). This suggests a close relation between mycorrhizal fungi and soil bacteria. However, diversity in the response depending on the microbial combination has also been described (Ravnskov and Jakobsen, 1999). This diversity in response can be explained according to the high specificity involved in the rhizosphere microbial interactions (Azcón, 1989; Kloepper, 1996).

In this study, the negative effect of combined treatments was detected, since *T. harzianum* had the highest increase on chlorophyll content in Rhodic Ferralsol soil. *Bacillus* spp. gave greater increases than those of combined treatments on the growth and biomass accumulation of the banana plantlets in Rhodic Ferralsol and Vertisol soils. However, the increases were only significant on height of banana plantlets, leaf length and shoot dry matter in the Vertisol. The control gave better results than single or combined treatments in some cases, although the differences were not significant. The situation of negative effect has been described (Germida and Walley, 1997). The reasons for this phenomenon must be observed again under the high specificity of microbial interactions (Azcón, 1989; Kloepper, 1996).

Mycorrhizal colonization

Combined applications of either Bacillus spp. or Myconate with AMF enhanced mycorrhizal colonization in the two soils (that is, Rhodic Ferralsol and Vertisol), confirming the advantage of co-inoculation over single applications. The performance of AMF + Bacillus spp. confirms the results of Barea et al. (2004) and Budi et al. (1999) that some of the bacteria associated with AMF can improve the mycorrhizal colonization. The application of AMF together with Myconate had the best performance in the two soils because Myconate may have stimulated the multiplication of AMF spores, hence more colonization in the treated banana plants.

It has been shown that increased inoculum dosage results in increased colonization rate (Daft and Nicholson, 1969) and increased percentage root colonization (Johnson, 1977). The low percentage colonization observed in this study could be attributed to the low amount of inocula used in this experiment.

Plant nutrient uptake

Zinc availability is dependent on the kind and intensity of weathering of the parent material and the interaction with other nutrients such as P, Cu, Fe, and Mn. Soils with high concentration of P are deficient in available zinc, hence the application of high amounts of P can induce Zn deficiency in plants and the availability of Zn is highest below pH 7 (Sa and Israel, 1991; Mengel and Kirkby, 2001). This confirms

the low P concentration in the soils, as such, low P concentration in banana shoot tissue and the high Zn concentration in the banana shoot tissue is favored by the low soil pH (5.8-7.0). The fact that the soils had low P content, therefore low mycorrhizal colonization recorded (Sastry, 2000) helps to explain why P uptake was low in all the soils. Sometime, leaves are very conservative in their P concentration. When more P becomes available, they produce greater surface area and thus maintain the same tissue concentration levels (Smith and Read, 1997). However, in this analysis, the uptake of P was low hence resulting in the moderate growth of the banana plantlets. The poor AMF enhancement of plant growth could also be attributed to the low uptake of K, Ca, Mg and B. The lower uptake of the selected nutrients may also be due to the lower uptake of P by the banana plantlets, that is, the influence of higher P uptake on the subsequent greater uptake of Fe is documented (Jones et al., 1991).

CONCLUSION

The results of this study confirm the suitability of the co-inoculation of commercial microbial and chemical products to improve banana growth during the nursery phase; especially the combination of *Bacillus* spp. with AMF, *T. harzianum* or Myconate which gave increases on plant growth measurements and biomass accumulation in the two soils. The suitable management of these symbionts represents a feasible strategy for the tissue culture process in the banana-producing regions. Tissue cultured plants would be carried to the field with an established microbial rhizosphere which would contribute to an easier adaptation to field conditions. However, due to the high specificity in terms of functional compatibility involved in these types of interactions, screening to select the best treatment-host plant combination should be done in order to optimize the results.

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