Interaction between VAM Rate and *Radopholus similis* (Cobb) Inoculation Density on the Growth of Micropropagated Cavendish Banana, Root Damage, and Nematode Reproduction Suppression

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This study was done to determine the interaction between different application rates of VAM and various nematode inoculation densities of *Radopholus similis (Rs)* on micropropagated Cavendish banana cv. Grand Naine. A 4x3 factorial analysis was first done to determine significant interactions of four levels of VAM rates (0, 0.5, 1 and 2 RR) and three nematode inoculation levels (0, 1000, 2000 *Rs*/pot) to aboveground growth parameters, root growth and quality, nematode parameters, and mycorrhizal colonization. Application of mycorrhiza was done at nursery stage where it was able to successfully colonize roots of Grand Naine plantlets prior to inoculation of pathogens. Significant interactions of VAM rates and nematode levels were noted for primary root weight and total root weight. Moreover, VAM at one and two times the recommended rate resulted to significantly lower nematode counts. This study demonstrated the bioprotection potential of mycorrhiza against *Radopholus similis* in Cavendish bananas.

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INTRODUCTION

Banana remains to be an important export crop in the Philippines with its major production found in Mindanao island with a total production of 9.2 M metric tons in 2017 (PSA, 2018). However, there are several parasitic microorganisms that limit banana production. One of them is *Radopholus similis* (Cobb), a migratory endoparasitic nematode considered as the most destructive pathogen of banana (Gowen et al., 2005). Commonly known as the burrowing nematode, *R. similis* causes necrosis on cortical tissues which results to altered uptake of water and nutrients (Gowen et al., 2005; Sarah et al., 1996) resulting to poor root growth, reduced bunch weight and lengthening of the cropping cycle (Araya and Moens, 2003; Felde et al, 2006).

More importantly, anchorage of the plant is also affected which may be severed by secondary infection caused by fungal pathogens which infects the stele (Duncan and Moens, 2006; Stover, 1972). This event eventually leads to toppling over of banana plants especially those bearing with fruits (Gowen et al., 2005; Sarah et al., 1996). In the Philippines, *R. similis* poses a threat to Cavendish bananas. *R. similis* was reported as the most destructive and widely distributed nematodes in Northern Mindanao (Davide, 1988).

Several methods have been developed to protect banana against *R. similis* such as the use of nematicides and fumigants (Sarah et al., 1996), paring of corms (O' Bannon, 1977; Sarah et al., 1996), and other cultural methods such as crop rotation, fallowing, use of organic amendments, and irrigation (Felde et al., 2006; Sarah et al., 1996) are commonly practiced.

The application of mycorrhizal fungi as biocontrol against several pathogens such as *R. similis* has been the subject of many studies. These biotrophic beneficial fungi form symbiotic relationships with majority of plants. All mycorrhizal fungi are members of Phylum Zygomycota containing one order, the Glomales. There are 149 species under this order which are distributed into six genera namely *Glomus*, *Gigaspora*, *Sclerocystis*, *Acaulospora*, *Entrophopora*, and *Scutellospora* (Pal and Gardener, 2006). In mycorrhizal association, the plants provide the fungi with organic compounds and in exchange, the fungi absorb nutrients from the soil for plant use (Koide and Mosse, 2004). Vesicular arbuscular mycorrhiza (VAM) have vesicles which serve as storage organs of the fungus and arbuscules which act as exchange points between the host plant and the fungi. This partnership results to improved growth of the plant and was attributed to the enhanced capability of the roots to absorb soil minerals particularly phosphorus, zinc, sulfur, and copper (Mosse, 1981; Koide and Mosse, 2004).

Mycorrhizal associations are also reported to suppress various pathogens and decrease disease infections. For instance, application of *G. intraradices* significantly reduced penetration and reproduction of *M. incognita* in cucumber plants (Zhang et al., 2009) while a mixture of mycorrhiza reduced disease severity and incidence of *Fusarium* root rot in common bean (Al-Askar and Rashad, 2010). In another experiment, mycorrhization of tomato plants with *G. mosseae* reduced disease severity of *Phytophthora parasitica* (Pozo et al., 2002). The bioprotection of mycorrhiza can be localized or systemic and its efficacy is influenced by several factors like host genotype, mycorrhizal species, degree of mycorrhization, and environmental conditions (Mosse, 1981; Koide and Mosse, 2004). The source of mycorrhiza used in this study is a mixture of propagated units of vesicular forming mycorrhiza species *Glomus mosseae* and *G. fasciculatum* in fine corn root pieces formulated as VAMri (Vesicular Arbuscular Mycorrhiza Root Inoculant). This product is being mass produced by the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, Los Baños, Laguna for commercial use especially by traditional farmers. To avoid confusion with other related literatures, VAMri will be referred to as VAM hereafter unless stated otherwise.

Tissue-cultured Cavendish bananas are commonly used as planting materials to assure the distribution of disease-free plants. However, they are generally observed to be very susceptible to damage by *R. similis* than those grown from conventional planting materials, probably because of succulent root tissues. It is therefore very important that plantlets are protected from soil-borne pathogens. The bioprotection offered by mycorrhiza may enhance resistance and/or tolerance of micropropagated bananas when planted in the field and eventually suppress *R. similis*. This research generally intends to determine the effectiveness of locally available VAM in micropropagated bananas under Philippine conditions. Specifically, it aims to 1) study the interaction between application rate of VAM and inoculum level of *Radopholus similis* on the growth of micropropagated Cavendish banana cv. Grand Naine; and 2) evaluate the bioprotection activity of VAM against *R. similis* in screen house experiments.

MATERIALS AND METHODS

A 4x3 experiment was performed to determine the interaction of VAM rate at 0, 12.5, 25, 50 mg/seedling representing 0, 0.5, 1, and 2 times the recommended rate (RR) and nematode inoculation level (*Pi*) at 0, 1000, and 2000 *Rs*/plant. VAM was applied to the meriplants during planting in the nursery. Six weeks after VAM application, plants were transferred to bigger potting bags with 4 kg sterilized garden soil. Nematode inoculation at designated levels was done 2 weeks after transplanting. Nematode inoculum collected from Davao del Norte was extracted from carrot discs using maceration-sieving method (Hooper et al., 2005). The experiment was arranged in randomized complete block design (RCBD) with six replications per treatment.

Data Collection

Aboveground growth parameters. Measurements of aboveground parts were done at the time of termination. Shoot height was measured from the wooden peg in the soil up to the lower base of the youngest fully opened leaf of the plant while girth diameter was determined using a Vernier caliper. Leaf area, on the other hand, was obtained by multiplying the length and the width of the second youngest fully opened leaf.

Root growth and quality parameters. Root weights were determined after the destructive sampling of test plants 8 weeks after nematode inoculation. The feeder roots, composed of the secondary and tertiary roots, were separated from the primary roots. The feeder roots were classified as all healthy, mostly healthy, all dead, and mostly dead. After classifying, feeder roots were then placed in vials containing ethyl alcohol and were then set aside for VAM staining. Primary roots, on the other hand, were classified as dead or functional. Dead roots are completely rotten while functional roots show at least some healthy tissue (Speijer and De Waele, 1997). However, in this experiment, a third group was added to give weight to the primary roots that were almost totally dead but show a small part of healthy tissue or roots that were almost totally functional but show small part of dead tissue. These roots were classified as damaged but functional. The addition of the third group led to the revision of the functional group which is therefore defined as roots that are completely clean. In summary, the classification groups for primary roots were (1) functional or completely clean, (2) damaged but functional, and (3) dead or completely rotten. Primary roots were then weighed according to classification group.

Root necrosis, nematode population and nematode reproduction. Percent root necrosis (PRN) was determined by scoring randomly selected five 10-cm fragments of primary roots that were cut longitudinally into halves (Speijer and De Waele, 1997). After root parameters were obtained, *R. similis* was then extracted by maceration and sieving (Hooper et al., 2005). All primary roots were weighed and chopped into 1 cm pieces and were mixed inside a polyethylene plastic container. A subsample of 25 g was then obtained per root system. Roots were then macerated using a warring blender in a series of three rounds of 10 second-cycle with 5-second interval each round. After maceration, roots were placed in a container and were

Table 1: Shoot height, girth diameter, and leaf area of Cavendish banana cv. Grand Naine as affected by VAM rate and Rs inoculation density, 10 weeks after transplanting.

Radopholus similis (Rs) INOCULATION DENSITY	VAM RATE Recommended Rate (RR)	ABOVEGROUND GROWTH MEASUREMENTS[1]		
		Shoot Height (mm)	Girth Diameter (mm)	Leaf Area (mm^2)
0	0	36.22	9.17	796.90
0	0.5	35.78	8.95	843.78
0	1.0	36.73	9.67	963.02
0	2.0	35.50	9.02	870.13
1000	0	33.38	8.83	783.00
1000	0.5	31.30	8.48	738.86
1000	1.0	33.27	8.55	814.68
1000	2.0	34.93	9.28	727.30
2000	0	33.43	8.97	903.78
2000	0.5	33.27	8.77	785.79
2000	1.0	30.08	8.45	680.70
2000	2.0	36.93	9.23	885.63
P-value (*Rs* Inoculation Density x VAM Recommended Rate)[2]		0.1927	0.1131	0.0778
P-value (*Rs* Inoculation Density)		**0.0198**	0.0626	0.0879
P-value (VAM Recommended Rate)		0.1740	0.2042	0.9175

[1]Numbers were means of eight replicates
[2]*P*-values from 3x4 factorial ANOVA

incubated overnight. The next day, macerated roots were sieved in mesh no. 60, 120, 230, and 500. Using a pressurized faucet, *R. similis* was collected from 230 and 500-mesh sieves in a 250-ml beaker. Nematode suspension was then brought to 200 ml. Using a pipette, air was blown to assure homogenize the suspension. A subsample of 5 ml was finally collected and was placed in a properly labeled vial.

From the 5 ml suspension, 1 ml was collected, placed in a counting dish. All females and juveniles were counted under a compound microscope at 40 to 100x magnification. The nematode count from the 1 ml suspension was multiplied by 5 to get the number of nematodes in 5 ml suspension. The result was multiplied by 40 in order to obtain the total nematodes in the 200 ml suspension collected from 25 g of root. This represents the total nematodes per 25 g of root. The product was then divided by 25 to get total nematode per gram of root. The result was finally multiplied by primary root weight to get the total number of *R. similis* per root system (*Pf*). Reproduction factor was then computed by dividing the final population density with initial population (*Pf*)/(*Pi*). The equation for calculating total nematode population was presented below:

Total number of nematodes per root system =
[((nematode count from 1 ml suspension x 5) x 40)/25] x
primary root weight

Mycorrhizal colonization. Feeder roots were stained following the modified ink-vinegar staining technique described by Vierheilig and Piché (1998). The technique utilizes vinegar instead of lactophenol (Phillips and Hayman, 1970) and acidic glycerol solution (Koske and Gemma, 1989). The acidification step with HCl was also omitted. This method is also applicable to other soil-inhabiting fungal pathogens.

Subsamples of 3 g feeder roots per replicate were rinsed with tap water to remove soil. After washing, roots were cleared by boiling for 3 min in 10% (w/v) KOH. This clearing step was first demonstrated by Phillips and Hayman (1970). After rinsing several times with tap water, the cleared roots were boiled for another 3 min in a stain solution of 0.05% tryphan blue and 5% vinegar. Thereafter, the stain was removed or destained from the roots by washing with tap water for 20 min (Vierheilig and Piché, 1998). After roots have been cleared, stained and destained, 20 pieces of 1-cm fine root segments were mounted on slides and observed under a light microscope. The frequency of VAM colonization (%F) was calculated as the percentage of root segments colonized by either hyphae or vesicles or arbuscules (Elsen et al., 2008). The intensity of colonization (%I) which is the abundance of hyphae, arbuscules, and vesicles in each mycorrhizal root was also estimated (Plenchette and Morel, 1996).

Data Analysis
Data were run in Analysis of Variance (ANOVA) after checking if the assumptions of normality and variance homogeneity were satisfied. Syntax was designed following the 3 x 4 factorial arranged in RCBD. Since both VAM rate and nematode inoculation density were quantitative, parametric tests were done. For parameters where interactions were significant, ANOVA results were presented. For those with insignificant interactions, VAM rate and nematode density were analyzed separately. Regression and trend analyses, respectively, were done for VAM rate with unequal intervals and nematode density with equally spaced levels. Orthogonal contrasts were also conducted in all parameters to evaluate differences between two subsequent groups of treatments having the same VAM rate and/or nematode density. Data were transformed appropriately whenever needed. Treatment means were separated by Tukey's HSD test. Analysis was done using SAS System for Windows version 9.0. Description of feeder roots was not analyzed because it is non-parametric.

RESULTS AND DISCUSSION

Effect of Nematode Inoculation Density and VAM Rate on Aboveground Growth Parameters
Significant interactions were not detected for aboveground growth parameters. Inoculation of various levels of nematode density resulted to significant differences in shoot height while application of different rates of VAM did not have a significant effect on all of these parameters (Table 1).

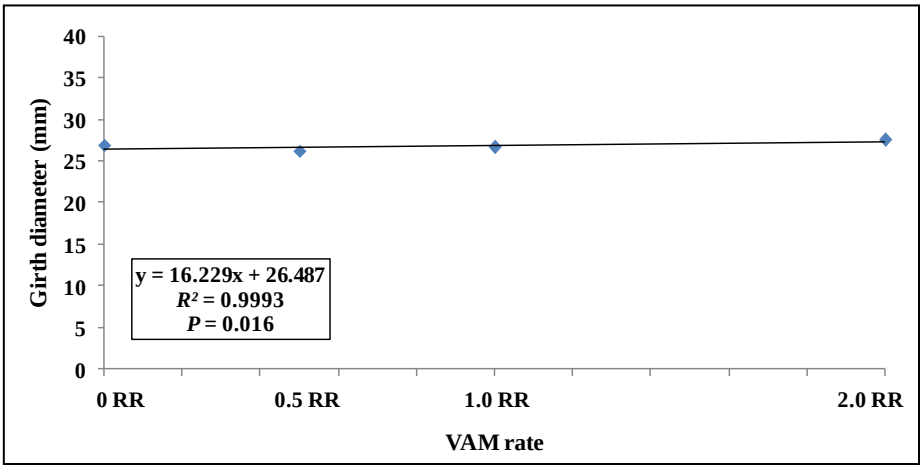

Figure 1: Significant linear model relating recommended rates (RR) of VAM and girth diameter

Table 2: *P*-values of orthogonal contrasts between two subsequent levels of terms (VAM rate and *Rs* inoculation density) on aboveground growth parameters of Cavendish bananas cv. Grand Naine, 10 weeks after transplanting.

CONTRASTS	*P*-VALUES		
	Shoot Height	Girth Diameter	Leaf Area
***Radopholus similis (Rs)* Inoculation Density**			
0 *Rs* vs 1000 *Rs*	**0.0395**	0.0594	0.0757
1000 *Rs* vs 2000 *Rs*	0.9394	0.7853	0.3423
VAM Recommended Rate (RR)			
0 RR vs 0.5 RR	0.5377	0.2984	0.6010
0.5 RR vs 1 RR	0.4335	0.6240	0.8629
1 RR vs 2 RR	0.1052	0.2196	0.8648

For insignificant interactions, two separate analyses were performed. Trend and regression analyses were done for nematode inoculation density and VAM rates, respectively. At various nematode inoculation levels, aboveground growth parameters did not have any significant trend. At different rates of VAM, regression analysis detected a significant linear model for girth diameter ($P = 0.016$). Moreover, R^2 values indicate that VAM rate accounts for almost 100% of the variation in girth diameter (Figure 1).

As identified by 4x3 factorial analyses, there were differences in shoot height at various nematode inoculation densities. Shoot heights of plants inoculated with 0 and 1000 *Rs* differed according to orthogonal contrasts while plants inoculated with 1000 *Rs* per plant were shorter by 4% than uninoculated plants (Table 2).

Most studies illustrated the positive effects of mycorrhiza on aboveground growth parameters of banana plants. Mycorrhized bananas had higher shoot weight and had larger foliar surface than non-mycorrhized ones (Jaizme-Vega et al., 2002; Elsen et al., 2003a, b). Results of this study, however, were different from the previous reports since improved aboveground growth parameters of mycorrhized Grand Naine bananas were not evident.

Effect of Nematode Inoculation Density and VAM Rate on Root Growth and Quality

Significant interactions of nematode inoculation density x VAM rate were recorded for primary root weight and total root weight (Table 3). At 1 RR, lowest root weights were recorded from inoculation of 2000 *R. similis* which were significantly different from 0 and 1000 inoculation levels (Figure 2 A, B). At 0, 0.5, and 2 RR, however, root weights did not vary significantly among nematode levels. The same graph shows the trend on nematode levels which conveys that for 0 *Rs*, highest root weights were observed at 1 RR which then decreased at 2 RR (Figure 2 A, B). For 1000 *Rs*, the roots more or less increased as VAM rate also increases. For 2000 *Rs*, however, root weights were highest with the application of AMF at 0.5 RR rather than the two higher rates. Nevertheless, as VAM rate increased from 1 to 2 RR, root weights also increased.

For different rates of VAM at fixed levels of nematode inoculation density, no significant differences were noted on root weights at 0 and 1000 *Rs* (Figure 3 A, B). For 2000 *Rs*, lowest root weights were recorded from the application of 1 RR which was significant from other VAM rates. Across VAM rates, root weights generally decreased with increasing nematode density at 0, 1, and 2 RR while application of 0.5 RR resulted to heavier roots even with the inoculation of 2000 *Rs*.

In 4x3 factorial analyses, significant differences between various nematode inoculation densities were observed from root weights except for feeder roots (Table 3). Dead, damaged, and functional primary root weights of uninoculated plants were significantly different from plants inoculated with 1000 and 2000 *Rs* (Figure 4 A-C). On the other hand, primary root weight and total root weight were highest in uninoculated plants and lowest in plants inoculated with 2000 *Rs* (Figure 5 A, B). Plants inoculated with 1000 *Rs* had comparable primary root weight and total root weight with plants inoculated with 0 and 2000 *Rs*.

No significant trends were noted for root growth and quality to increasing nematode inoculation density. On the other hand, application of different rates of VAM resulted to a significant effect in functional primary root weight ($P = 0.0267$) based on regression analysis. An increasing linear trend was further detected for this parameter which means that as VAM rate

Table 3: Root weight of mycorrhizal Cavendish banana cv. Grand Naine as affected by VAM rate and Rs inoculation density, 10 weeks after transplanting.

Radopholus similis (Rs) INOCULATION DENSITY	VAM RECOMMENDED RATE (RR)	ROOT WEIGHT (g)					
		Primary Root Classification			Primary Roots	Feeder Roots	Total Roots
		functional	damaged	dead			
0	0	35.22	11.08	0.02	43.32	7.65	53.97
0	0.5	40.12	4.32	0.02	44.45	4.00	48.46
0	1.0	39.82	12.28	0.13	52.23	5.53	57.76
0	2.0	36.12	5.53	0.00	41.65	4.15	45.80
1000	0	4.92	33.68	1.10	39.70	4.77	44.47
1000	0.5	3.68	33.63	0.58	37.90	6.73	44.62
1000	1.0	10.48	23.38	2.50	38.37	3.78	40.14
1000	2.0	17.25	26.87	1.92	46.03	7.90	53.94
2000	0	9.20	25.43	1.47	36.10	5.78	41.88
2000	0.5	0.97	41.60	5.87	48.43	4.53	52.97
2000	1.0	1.60	22.22	1.43	25.25	5.13	30.38
2000	2.0	3.52	35.68	1.10	40.30	6.75	47.05
P-value (*Rs* Inoculation Density x VAM Recommended Rate)[2]		0.6104	0.4150	0.4661	**0.0281**	0.5000	**0.0343**
P-value (*Rs* Inoculation Density)		**<0.0001**	**<0.0001**	**<0.0001**	**0.0149**	0.8981	**0.0439**
P-value (VAM Recommended Rate)		0.4596	0.9496	0.6655	0.4569	0.2904	0.4341

[1]Numbers were means of eight replicates
[2]*P*-values from 3x4 factorial ANOVA

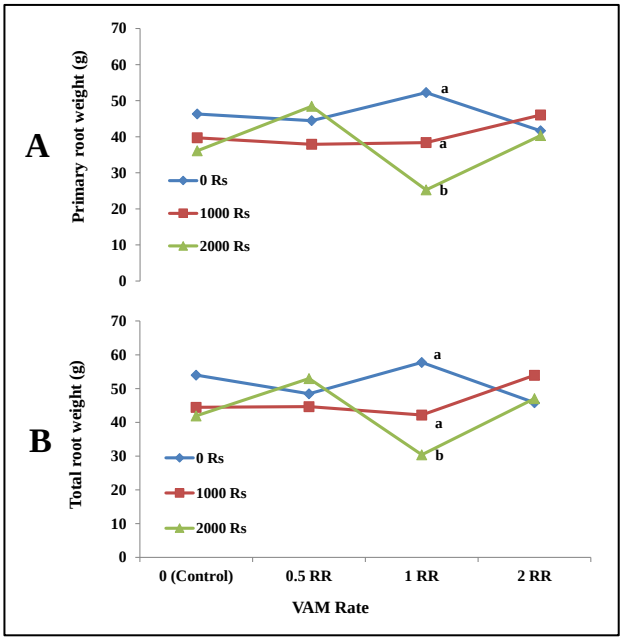

Figure 2: Primary root weight (A) and total root weight (B) as affected by *Radopholus similis (Rs)* inoculation density at recommended rates (RR) of VAM. Letters indicate mean separation at *P*<0.05 by Tukey's HSD.

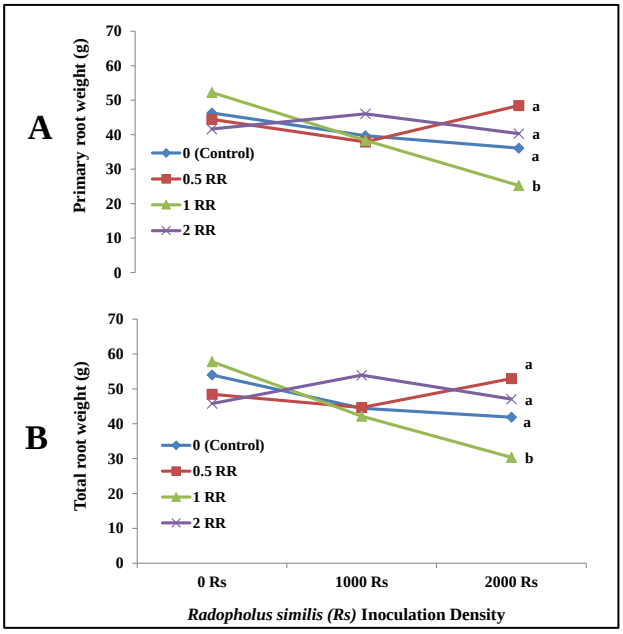

Figure 3: Primary root weight (A) and total root weight (B) as affected by recommended rates (RR) of VAM at fixed levels of *Radopholus similis (Rs)* inoculation density. Letters indicate mean separation at *P*<0.05 by Tukey's HSD.

increases, functional primary root weight also increases (Figure 6).

Significant differences were noted for 0 *Rs* vs 1000 *Rs* of functional, damaged, and dead primary roots and 1000 *Rs* vs 2000 *Rs* of functional primary root based on orthogonal contrasts (Table 4). It was further shown that inoculation of 1000 *Rs* resulted to lower functional root weight and higher damaged and dead root weights than 0 *Rs* inoculation. Functional primary roots of plants inoculated with 1000 *Rs* were about 60% lower than uninoculated plants. In addition, damaged and dead roots were significantly higher in inoculated plants by 60% and 95%, respectively, than uninoculated plants. Functional primary root weight was about 40% higher in plants inoculated with 1000 *Rs* than 2000 *Rs* which indicates that the quality of roots decreases when *Rs* population density is high.

As a migratory endoparasitic root parasite, the burrowing nematode alters the normal physiological processes of the roots.

Table 4: *P*-values of orthogonal contrasts between two subsequent levels of terms (VAM rate and *Rs* inoculation density) on root weight of Cavendish bananas cv. Grand Naine, 10 weeks after transplanting.

CONTRASTS	*P*-VALUES					
	Primary Root Classification			Primary Roots	Feeder Roots	Total Roots
	functional	damaged	dead			
***Radopholus similis (Rs)* Inoculation Density**						
0 *Rs* vs 1000 *Rs*	**<0.0001**	**<0.0001**	**0.0004**	0.2014	0.7476	0.2907
1000 *Rs* vs 2000 *Rs*	**0.0250**	0.9911	0.4793	0.1869	0.9554	0.2536
VAM Recommended Rate (RR)						
0 RR vs 0.5 RR	0.6952	0.8486	0.4979	0.1477	0.1946	0.1563
0.5 RR vs 1 RR	0.9764	0.9994	0.3986	0.1246	0.1566	0.1762
1 RR vs 2 RR	0.5390	0.8051	0.5546	0.3013	0.2426	0.3503

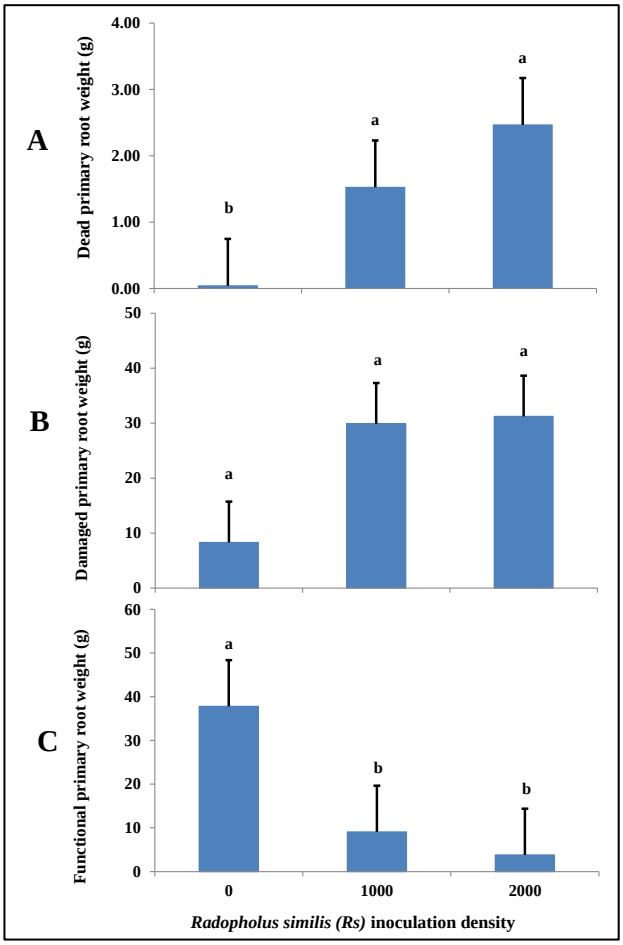

Figure 4: Dead primary root weight (A), damaged primary root weight (B), and functional primary root weight (C) at fixed rates of *Rs* inoculation density. Letters indicate mean separation at *P*=0.05 by Tukey's HSD.

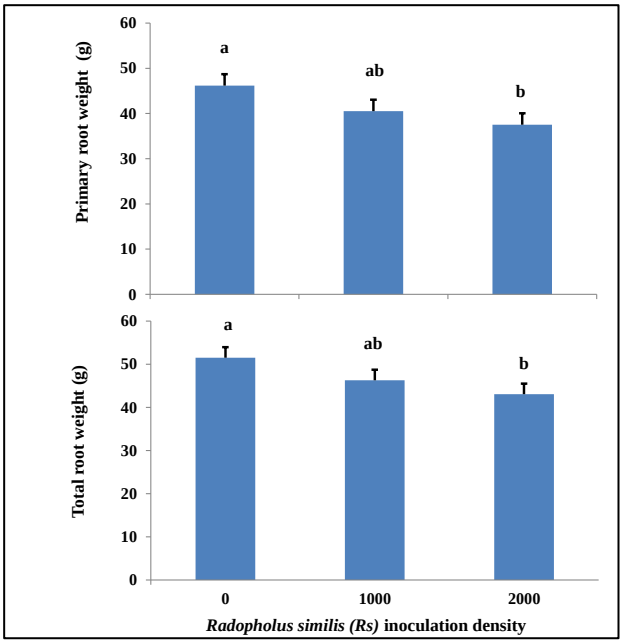

Figure 5: Primary root weight (A) and total root weight (B) at fixed rates of *Rs* inoculation density. Letters indicate mean separation at *P*=0.05 by Tukey's HSD.

By constant migration and feeding, *R. similis* makes the roots weak and light (Sarah et al., 1996). In this study, it was demonstrated that inoculation of *R. similis* reduces root weight and quality. When nematode inoculation density was increased from 1000 *Rs* to 2000 *Rs*, functional primary root weight was reduced. It was further noted that the negative effects of *R. similis* were somehow compensated by the positive effects of VAM in terms of functional primary roots. Moreover, increasing VAM rate resulted to increased functional primary roots even in the presence of *R. similis*.

These findings aligned with the results of other studies involving AMF and *Musa* species. Mycorrhized bananas had greater root weights than non-mycorrhized ones. In particular, fresh root weight was highest in Pisang Jari Buaya bananas colonized with *G. mosseae* and inoculated with *R. similis* (Elsen et al., 2003a). The same observation was noted in Grand Naine bananas inoculated with *P. coffeae* (Elsen et al., 2003b).

Effect of Nematode Inoculation Density and VAM Rate on Nematode Parameters

Interactions of nematode inoculation density and VAM rates were not significant in all nematode parameters. For nematode inoculation densities, significant differences were noted in the number of *Rs* per gram of primary root (P = 0.0109) and total number of *Rs* in primary roots (P = 0.0273) (Table 5). Since, zero inoculation was not included in 3x4 factorial analysis, the variations found indicate significant differences between 1000 and 2000 *Rs*. More nematodes were extracted from plants inoculated with 2000 *Rs* than 1000 *Rs*. Essentially, a higher initial nematode population will mean more initial sources for reproduction.

For VAM rates, significant effects were recorded for number of *R. similis* per gram of root, total number of *R. similis* per root system, and reproduction ratio (Table 5). Application of VAM at 2 RR resulted to significantly lower values than 0 and 0.5 RR while application of VAM at 1 RR resulted to means comparable to 0 and 2 RR (Fig. 7 A-C).

Since no significant interaction was noted in all nematode parameters, trend analysis was made for nematode inoculation

Table 5: Percent root necrosis, nematode population, and nematode reproduction rate of *Radopholus similis* on mycorrhizal Cavendish bananas cv. Grand Naine as affected by VAM rate and *Rs* inoculation density, 10 weeks after transplanting.

Radopholus similis (Rs) INOCULATION DENSITY	VAM RECOMMENDED RATE (RR)	ROOT NECROSIS (%)[1]	NUM. *Rs* PER GRAM OF PRIMARY ROOT	TOTAL NUM. *Rs* IN PRIMARY ROOTS	REPRODUCTION FACTOR[3]
0	0	/	/	/	/
0	0.5	/	/	/	/
0	1.0	/	/	/	/
0	2.0	/	/	/	/
1000	0	23.83	1,125	45, 961	45.96
1000	0.5	30.33	973	36, 404	36.40
1000	1.0	21.83	776	27, 067	27.07
1000	2.0	27.67	637	24, 722	24.72
2000	0	34.00	1,472	50, 319	25.16
2000	0.5	34.33	1,433	73, 619	36.81
2000	1.0	40.17	1,112	28, 206	14.10
2000	2.0	29.00	928	34, 888	17.44
P-value (*Rs* Inoculation Density x VAM Recommended Rate)[2]		0.7002	0.4351	0.3835	0.3835
P-value (*Rs* Inoculation Density)		0.0563	**0.0109**	**0.0273**	0.2878
P-value (VAM Recommended Rate)		0.7895	**0.0185**	**0.0082**	**0.0082**

[1]Numbers were means of eight replicates.
[2]*P*-values were generated by 3x4 factorial ANOVA.
[3]Based on nematode count in root system only. Computed using the formula Rf = *Pf*/*Pi* when *Pi* = 0, 1,000, and 2000.

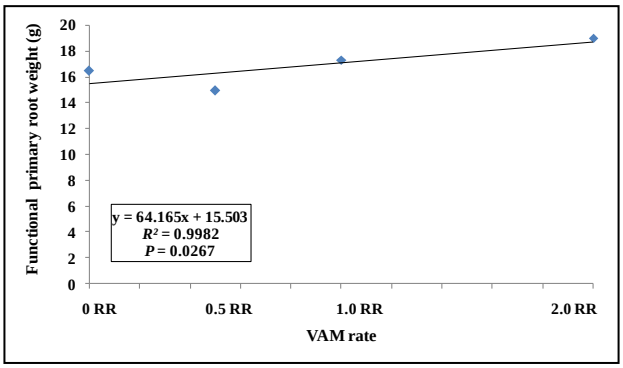

Figure 6: Significant linear model relating recommended rates (RR) of VAM and functional primary root weight.

density. The analysis identified linear trends for three parameters namely *Rs* per gram of primary root, total *Rs* in primary roots, and reproduction factor. These trends were further validated by running regression analysis by which R^2 indicated that 66%, 78%, and 77% of the total variation in *Rs* per gram root, total *Rs* per root system, and reproduction factor, respectively, are attributed to the linear response of these three parameters to increasing levels of nematode density. For simplicity, means for each VAM rate at fixed levels of nematode inoculation density were used for graphical presentation (Figure 8).

Increasing the levels of nematode density also resulted to higher number of *R. similis* per gram of root and per root system (Figure 9 A, B). Basically, an initial inoculum level of 2000 nematodes will multiply faster at a given time compared with 1000 *R. similis* only. However, in contrast to the first two parameters, reproduction factor decreased as the nematode inoculum level increases (Figure 9 C). This is because at high inoculum density, more nematodes will compete for space and food, therefore limiting their reproduction (Gowen et al., 2005).

Regression analysis was also performed for insignificant interactions to determine the effect of increasing VAM rates on nematode parameters. A significant linear model was predicted for root necrosis (P= 0.0346) as a response to increasing rates of

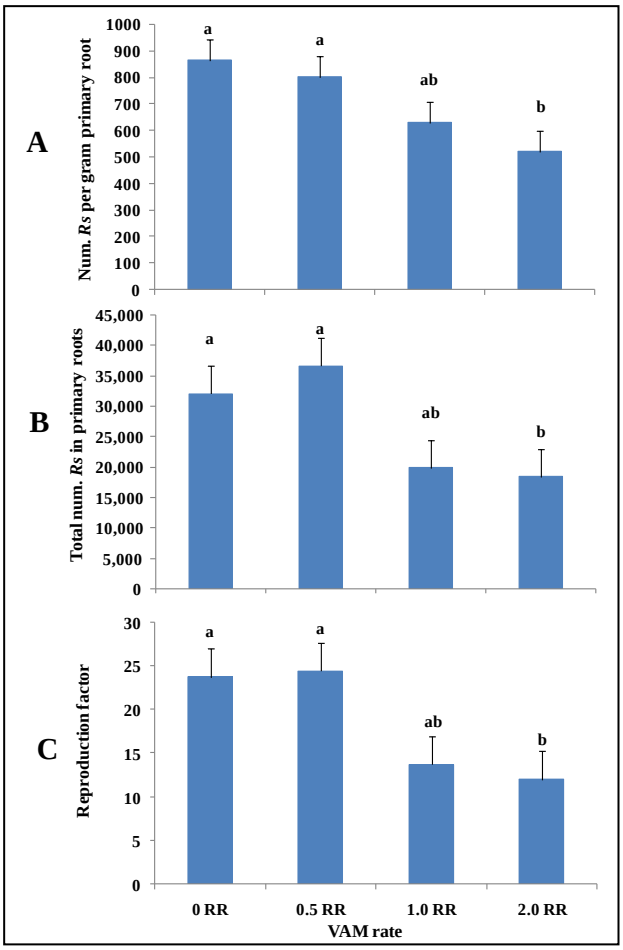

Figure 7: Number of *Radopholus similis (Rs)* per gram of root (A), per root system (B), and its reproduction factor (C) at recommended rates (RR) of VAM. Letters indicate mean separation at *P*=0.05 by Tukey's HSD.

VAM. Furthermore, intercept and parameter estimates were statistically significant. R^2 values indicate that VAM rate accounts for almost 100% of the variation in root necrosis. For

Table 6: ***P*-values of orthogonal contrasts between two subsequent levels of terms (VAM rate and *Rs* inoculation density) on nematode parameters on Cavendish bananas cv. Grand Naine, 10 weeks after transplanting.**

CONTRASTS	*P*-VALUES			
	Root Necrosis	Num. *Rs* Per Gram Of Primary Roots	Total Num. *Rs* In Primary Roots	Reproduction Factor
***Radopholus similis (Rs)* Inoculation Density**				
0 *Rs* vs 1000 *Rs*	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
1000 *Rs* vs 2000 *Rs*	**0.0134**	**0.0050**	**0.0265**	0.2312
VAM Recommended Rate (RR)				
0 RR vs 0.5 RR	0.7362	0.9612	0.9933	0.9793
0.5 RR vs 1 RR	0.9378	0.7723	0.7823	0.3992
1 RR vs 2 RR	0.7382	0.7956	0.9608	0.8786

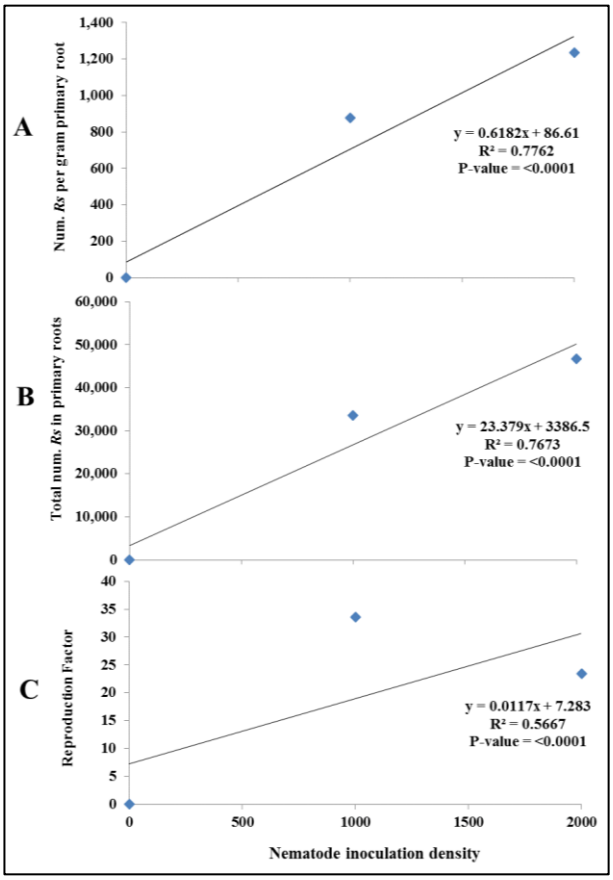

Figure 8: Regression model relating nematode inoculation density and number of *Radopholus similis (Rs)* per gram primary root (A), total *Rs* in primary roots (B), and reproduction factor (C).

simplicity, means for each VAM rate were used for graphical presentation (Figure 9). There was a weak negative correlation detected for root necrosis as a function of VAM rate. For a unit increase in VAM rate, a slight decrease in root necrosis was observed. This observation was consistent with the results generated from 3x4 factorial analyses where nematode population densities were significantly reduced at higher rates of VAM (Figure 7). At low *R. similis* inoculation level, there is less migration and feeding activities of the nematodes in the roots, hence, root necrosis is reduced.

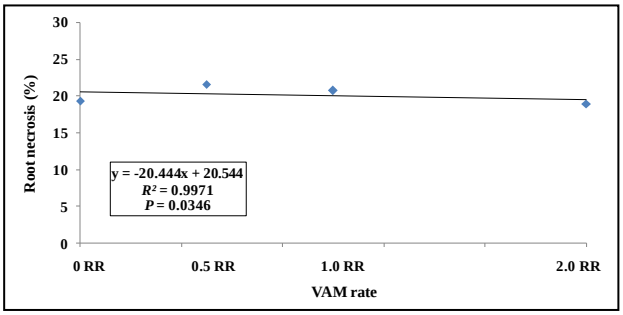

Figure 9: Significant linear model relating recommended rates (RR) of VAM and percent root necrosis.

P-values of orthogonal contrasts between two subsequent levels of terms (VAM rates and nematode density) were shown in Table 6. Contrast analysis of 0 *Rs* vs 1000 *Rs* was significant in all nematode parameters. Clearly, these parameters were recorded in plants inoculated with 1000 *Rs* but not in uninoculated plants (figures not shown). On the other hand, inoculation of 2000 *Rs* resulted to significantly higher root necrosis, more *Rs* per gram of root, and increase in total number of *Rs* in primary roots than in 1000 *Rs* (Table 6). Basically, a high initial inoculum density means more nematodes migrating and feeding in roots causing greater necrotic damage. However, inoculation of 1000 *Rs* and 2000 *Rs* have no significant effect on nematode reproduction.

Contrast analysis of various levels of VAM rates did not result to a significant effect on nematode parameters. This means that the effect of two subsequent levels of VAM rates have no effect on root necrosis, nematode population, and nematode reproduction. But when 3x4 analysis was done across VAM rates, a significant effect was noted on these parameters as shown by Figure 7.

Mycorrhizal fungi have been reported to reduce damage of soil-borne pathogens including nematodes (Linderman, 1994; Siddiqui and Mahmood, 1995). Root damage in Grand Naine and Pisang Jari Buaya bananas due to *P. coffeae* parasitism was reduced by 24% and 4% in mycorrhized plants, respectively (Elsen et al., 2003a). In contrast to other studies, high root necrosis was observed in mycorrhizal bananas cv. Obino l'Ewai inoculated with Ugandan and Indonesian *R. similis* population even though nematode counts were significantly reduced. On the average, root damage reached 30% in non-mycorrhizal bananas and 35.5% in mycorrhizal plantlets (Elsen et al., 2003b). This phenomenon, as reported by the authors, remains difficult to explain. In the present study, the application of VAM decreased root necrosis by 1% and only at the highest rate of mycorrhiza as shown by regression analysis (Figure 9). Therefore, the effect of mycorrhiza on root damage caused by nematodes is variable and may not be a good parameter for determining the efficacy of the former against the latter.

Mycorrhiza is also known to suppress nematode population and nematode reproduction in vascular plants. These two parameters provide a quantitative basis in determining the effect of VAM against plant parasitic nematodes. Several studies demonstrated the bioprotective action of VAM against plant parasitic nematodes in *Musa* species. For instance, Cavendish bananas cv. 'Williams' colonized with two *Glomus* species namely *G. caledonium* and *G. macrocarpum* had significantly fewer number of root galls compared with non-mycorrhized plants. There were only 8 to 45 galls per 5 g of root in mycorrhized 'Williams' and 29 to 53 galls were recorded from bananas not colonized by the fungus (Elsen et al., 2002). In a more related study involving mycorrhiza and migratory endoparasitic nematodes, *G. mossae* significantly suppressed population of Ugandan population of *R. similis* in Grand Naine and Pisang Jari

Table 7: Mycorrhizal colonization on Cavendish bananas cv. Grand Naine as affected by VAM rate and *Rs* inoculation density, 10 weeks after transplanting.

Radopholus similis (Rs) INOCULATION DENSITY	VAM RECOMMENDED RATE (RR)	FREQUENCY (%)[1]	INTENSITY (%)
0	0	/	/
0	0.5	66.11	37.88
0	1.0	64.44	36.17
0	2.0	66.11	43.06
1000	0	/	/
1000	0.5	70.56	36.61
1000	1.0	59.44	36.90
1000	2.0	43.89	29.54
2000	0	/	/
2000	0.5	68.33	39.99
2000	1.0	58.89	32.06
2000	2.0	68.33	36.29
P-value (*Rs* Inoculation Density x VAM Rate)[2]		0.1618	0.3097
P-value (*Rs* Inoculation Density)		0.2995	0.3372
P-value (VAM Recommended Rate)		0.3060	0.6576

[1]Numbers were means of eight replicates.
[2]*P*-values were generated by 3x4 factorial ANOVA.

Buaya cultivars (Elsen et al., 2003a). In particular, nematode count per root system reached 10, 425 for non-mycorrhized bananas but with the application of VAM, nematode population were significantly reduced to only 1,080. *R. similis* per gram of root was also reduced in mycorrhized Obina l'Ewai cultivars inoculated with both Ugandan and Indonesian nematode isolates. Without VAM, 1,336 and 218 nematodes were recorded but with mycorrhization, only 314 and 74 nematodes were counted for Ugandan and Indonesian population, respectively (Elsen et al., 2003b). These studies provide information of the effect of mycorrhiza against plant parasitic nematodes. However, studies involving rates of mycorrhiza were scarce. Most studies reported the use of various mycorrhiza species and their effects on plant growth and nematode suppression but no study determined the effect of these species at various rates (Bagyaraj, 1984).

Effect of Nematode Inoculation Density and VAM Rate on Mycorrhizal Colonization

In terms of mycorrhizal colonization, the statistical procedures 3x4 factorial ANOVA and orthogonal contrasts presented two different results. In ANOVA, both VAM rate and nematode inoculation density did not have significant effect on frequency and intensity of mycorrhizal colonization (Table 7). In orthogonal contrasts, however, direct comparisons of two levels of each factor gave significant differences (Table 5). The reason for the dissimilarities was that in ANOVA, data for 0 *Rs* and 0 RR were not included in the analysis. Hence, in general, the frequency and intensity of mycorrhizal colonization was not affected by either nematode density or VAM rate (Table 7). But when two levels of each factor were directly compared by contrast analysis, significant differences were noted (Table 8). For instance, there were significant differences in the intensity of colonization between inoculations of 2000 *Rs* and 1000 *Rs* while the frequency of colonization remained insignificant (Table 9). Furthermore, the intensity of mycorrhization was estimated to be 3% higher in plants inoculated with 2000 *Rs* than 1000 *Rs*. In this study, *R. similis* did not influence mycorrhizal colonization. But at increased rates, VAM slightly increased its activity in response to more root stresses caused by *R. similis* (Brown pers. comm). Limited studies were reported in terms of the use of mycorrhizal fungi at various rates. But in most mycorrhiza-nematode studies evaluated by Bagyaraj (1984),

Table 8: *P*-values of orthogonal contrasts between two subsequent levels of terms (VAM rate and *Rs* inoculation density) on mycorrhizal colonization on Cavendish bananas cv. Grand Naine, 10 weeks after transplanting.

CONTRASTS	*P*-VALUES	
	Frequency (%)	Intensity (%)
Radopholus similis (Rs)		
Inoculation Density		
0 *Rs* vs 1000 *Rs*	0.5920	0.6521
1000 *Rs* vs 2000 *Rs*	0.6684	**0.0250**
VAM Recommended Rate (RR)		
0 RR vs 0.5 RR	**<0.0001**	**<0.0001**
0.5 RR vs 1 RR	**<0.0001**	**<0.0001**
1 RR vs 2 RR	0.9857	0.7604

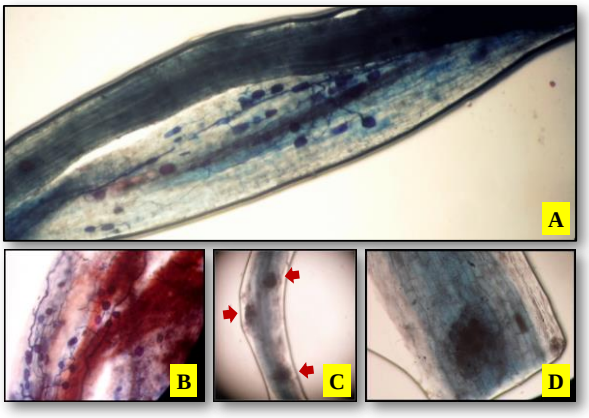

Figure 10: Feeder roots of Grand Naine bananas showing active mycorrhizal colonization as exhibited by hyphae and vesicles (A and B) and arbuscules (C-in red arrows, D).

nematodes had little or no influence on mycorrhizal activity. This is supported by several experiments in which the presence of nematode did not influence colonization of mycorrhiza particularly in various banana cultivars especially in the interaction *G. mosseae* and *P. coffeae* in Obino l'Ewai (*Musa* AAB group), *G. mosseae* and *R. similis* in Calcutta 4 (*Musa* AA group) and *G. mosseae* and *P. coffeae* in Calcutta 4 (*Musa* AA group) (Elsen et al., 2003b). The same observations were reported in the interaction of *G. mosseae* and *R. similis* in Grand Naine (*Musa* AAA group), *G. mosseae* and *P. coffeae* in Grand Naine (*Musa* AAA group) and *G. mosseae* and *P. coffeae* in Pisang Jari Buaya (*Musa* AA group) (Elsen et al., 2003a). Very few studies indicated the effect of nematodes on mycorrhization. At the very least, nematodes only affected the frequency but not the intensity of colonization. For instance, the frequency of colonization of *G. mosseae* was reduced by *R. similis* (Ugandan population) in Obino l'Ewai (*Musa* AAB group) by 22% (Elsen et al., 2003b) and by the *R. similis* (Ugandan population) and *R. similis* (Indonesian population) in Pisang Jari Buaya (*Musa* AA group) by 19% and 34%, respectively (Elsen et al., 2003a).

On the other hand, VAM applications of 0 RR vs 0.5 RR and 0.5 RR vs 1 RR significantly affected the frequency and intensity of mycorrhization (Table 5). For obvious reasons, application of 0.5 RR resulted to higher mycorrhization than 0 RR. However, mycorrhization was still higher with the application of 0.5 RR than 1 RR. From these results, it appeared that mycorrhizal colonization was inversely proportional with VAM application rate. The same observation was noted in citrus plants where root infection of *Glomus fasciculatus* reached 15% and 27% as the density increased from 200 to 500 mycorrhizal spores,

respectively. However, colonization decreased to 13% at 1000 spores and 15% at 2000 spores (Daniels and Menge, 1981). In other studies, researchers also indicated that increased amount of mycorrhizal inoculum, resulted to increased colonization (Carling et al., 1979; Daft and Nicolson, 1969; Daniels and Menge, 1981). But once the upper limit is reached, increase in the amount of inoculum will no longer result to increased colonization (Carling et al., 1979).

Active mycorrhizal colonization was obtained in all studies observed as hyphae, arbuscules, and vesicles present in stained roots (Figure 10). The application method of VAM may have an effect on the low frequency of mycorrhizal colonization. The mycorrhizal inocula were only applied on one side of the plantlets but the determination of frequency (%) was done randomly in all feeder roots of the plant. Although this may be the case, the effect of VAM on nematode population and reproduction factor was still exhibited because the mode of action of mycorrhiza, particularly against migratory endoparasitic nematodes, was reported to be systemic (Elsen et al., 2008). Additionally, the low intensity of mycorrhizal colonization may be attributed to the still early stage of mycorrhization colonization as what Elsen et al. (2003a, b) also reported in their study.

CONCLUSION

These results indicate that increasing VAM rate counteracts the effect of increasing nematode (inoculation) levels by producing numerous and heavy roots. The results of this study showed that VAM, especially at increased rates, suppressed nematode reproduction in roots leading to reduction in root necrosis. Further, it was shown in this study that *R. similis* did not influence mycorrhizal activity hence at increasing nematode levels, increase in VAM rate is recommended. Therefore it can be concluded that VAM offer some level of bioprotection of micropropagated Cavendish bananas cv. Grand Naine against *Radopholus similis*.

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CONTRIBUTIONS OF THE AUTHORS

AMRN did the experiment and wrote the manuscript of the article. NMC and TTR gave inputs on plant physiology and plant nematology, respectively, while JIO and MBB served as the major advisers of the first author.

CONFLICT OF INTEREST

The authors affirm that the study was done without any financial or commercial relationships that could be interpreted as a potential conflict of interest.

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