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Inoculation, colonization and distribution of fungal endophytes in *Musa* tissue culture plants

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Abstract

The use of mutualistic fungal endophytes to control the banana weevil (*Cosmopolites sordidus*) and banana parasitic nematodes (*Radopholus similis, Pratylenchus goodeyi* and *Helicotylenchus multicinctus*) is currently being investigated at the International Institute of Tropical Agriculture. Fungal endophytes are microorganisms that colonize the plant through the root system and for a part or whole of their life cycle live symptomlessly within the plant. Such organisms in some instances have been known to act as antagonists against pests and diseases. For an endophyte strain to be a good antagonist against target pests, it must be present in the plant tissues at the time the plants are attacked by the pests. They therefore need to be artificially inoculated in tissue culture banana plants, must occur at high frequencies in the plant and be able to persist in the plant after inoculation. It is also critical that an endophyte successfully colonizes and persists from the time of inoculation onwards. Screen house studies where two *Fusarium oxysporum* strains, *V2w2* and *III4w1*, were artificially inoculated into two banana cultivars, Nabusa and Kibuzi (*Musa*, AAA-EA), using different inoculation methods revealed that tissue colonization depended on the method of endophyte inoculation and differed for the different tissues investigated. Studies using the same two strains and same banana cultivars showed that colonization persistence also depended on inoculation methods and was different among the types of tissue within the banana plant. Plant tissue colonization varied by cultivar and strain combinations, indicating the need for identifying suitable cultivar-strain combinations.

Key words: Antagonistics, artificial inoculation, banana pests and diseases

Introduction

One of the major constraints to highland cooking banana (*Musa* spp., genome group AAA-EA) production in Uganda is the high level of pests. The most important pests in the region are the banana weevil *Cosmopolites sordidus* (Gold *et al*., 2001) and a complex of plant parasitic root nematodes (*Radopholus similis*, *Pratylenchus goodeyi* and *Helicotylenchus multicinctus*) (Speijer and Ssango, 1999). These attack the root and corm, reducing the stability of the banana plant and interfering with the uptake of nutrients and water. Their combined damage results in plant loss through toppling and snapping, reduced bunch weights and shortened plantation life. Microbial control of banana weevil and nematodes has been reviewed by Gold *et al.* (2001, 2003) and Niere (2001). The use of endophytic fungi is a novel yet promising biological control strategy that can be used against the banana weevil (Griesbach, 2000) and parasitic nematodes (Niere, 2001). Endophytes inhabit the interior of plants for some part or whole of their life cycle. In some instances they are known develop mutualistic relationships with such plants, and may act as antagonists to pests and diseases (Clay, 1991; Azevedo, 1998). In banana systems, endophytes have the advantage of targeting the destructive stages of banana weevil and nematodes that occur within the plant. For instance, the influence of fungal endophytes on herbivores was demonstrated by Baker *et al.* (1984) in a laboratory choice-test involving the larvae of stem weevils. He observed that several insects preferred endophyte-free perennial ryegrass (*Lolium perenne*). Besides acting as antagonists to pests and diseases, fungal endophytes have been reported to increase biomass production in inoculated plants. Latch *et al.* (1985) reported a significant increase in leaf area for perennial ryegrass inoculated with the fungal endophyte *Acremonium lolii*. Griesbach (2000) reported increased banana biomass production in field plants of the cultivars Nakyetengu and Nfuuka (AAA-EA) inoculated with the fungal endophyte *Fusarium concentricum*. Griesbach (2000) obtained 200 strains of endophytes from highland banana (AAA-EA) and Kayinja (Pisang awak subgroup, ABB). In a related study, Niere (2001) found that

22% of the fungi species most frequently colonizing healthy banana corms and roots in Uganda were *Fusarium oxysporum*. Griesbach (2000) and Niere (2001) reported activity of some of these strains against banana weevil and nematodes in *in vitro* tests. Successful inoculation of tissue culture banana plants with fungal endophytes (including *Acremonium* sp., *F. concentricum, F. oxysporum, F. solani* and *Geotrichum candidum*) was reported by Griesbach (2000), Niere (2001) and Paparu et al. (2004). Preliminary results for the effect of the inoculated endophytes on the two pests have however been quite varied. Griesbach (2000) identified *Fusarium* and *Acremonium* species as the most promising fungal endophytes for the control of banana weevil, with a 25% reduction of larval size and 70% reduction of corm damage, as compared to the uninoculated control. Nematode numbers in endophyte-inoculated plants were reduced by 42-79% and root necrosis by 30-40% (Niere, 2001).

For an endophyte strain to be a good antagonist against target pests, it must be present in the plant tissues at the time the plants are attacked by the pests. Endophytes therefore need to occur at high frequencies and be able to persist in the plant after inoculation. Niere (2001) recorded colonization rates in roots and corms of more than 50% one month after inoculation of tissue culture banana plants, but obtained much lower colonization rates five months after inoculation. For an endophyte strain to provide sustained protection against banana pests, it is critical that the fungus successfully colonizes and persists from the time of inoculation through the screen house period to the time of field planting.

The objectives of this study therefore were to determine inoculation methods that lead to increased fungal endophyte colonization and determine the persistence of two endophytic *F. oxysporum* strains, *V2w2* and *III4w1*, in tissue culture highland cooking banana plants before field planting. These two strains were selected on the basis of their performance in an experiment where several strains were screened against banana weevil eggs (Paparu *et al.*, unpublished data).

Materials and methods

Determination of optimal inoculation methods

A four-week screenhouse trial comprising four and six plants per treatment in experiment 1 and 2, respectively, was conducted at the International Institute of Tropical Agriculture (IITA) Sendusu farm, located 28 km northeast of Kampala. Plants of two *Musa* AAA-EA banana cultivars, Kibuzi (K) and Nabusa (N), were each inoculated with *F. oxysporum* strains *V2w2* or *III4w1* using three different inoculation methods. In the second experiment, some plants in each cultivar did not receive endophyte treatment and acted as a negative control. Strains *V2w2* and *III4w1* were previously isolated from Ugandan banana roots by Schuster et al. (1995) and stored at -5°C in soil slants. The three inoculation methods used included root and corm dip in spore suspensions of concentration 1.50-1.75 x 10^6 spores ml^{-1} (CC), root and corm dip in spore suspensions of concentration 4.19-4.80 x 10^6 spores ml^{-1} (IC) and the use of a solid substrate inoculum (SS).

A fungal spore suspension was prepared by inoculating four 0.5 cm^3 synthetic nutrient agar (SNA) (Niere, 2001) blocks containing fungal mycelia and spores in 200 ml of sterile potato dextrose broth (PDB) (Niere, 2001) in Erlenmeyer flasks and incubating for 7 days during which they were shaken once daily. The broth was then filtrated through a 1 mm diameter sieve to obtain a spore suspension. Spore concentration of the suspensions was estimated using a hemocytometer. A solid substrate inoculum was obtained by autoclaving 200 g of maize bran in 500 ml Erlenmeyer flasks (121°C for 1 hr) and adding 25 ml of a spore suspension (1.50-1.75 x 10^6 spores ml^{-1}) of *F. oxysporum* strains *V2w2* or *III4w1*. The bran was then left to stand on the bench and shaken once daily to ensure even spore growth. After 14 days, the bran was completely colonized by the strains and the spore concentration of the suspensions was estimated using a hemocytometer.

Tissue culture plants were propagated using a standard shoot-tip culture protocol for banana (Vuylsteke, 1998). At weaning, plants were removed from the rooting media and their roots thoroughly washed with tap water and cut to a length of 2 cm. Plants of each cultivar were sorted according to size and randomly assigned to the treatments. For the two inoculation methods where the roots and corms were dipped in spore suspensions, endophyte-treated plants were inoculated by dipping their roots for 90 min in spore suspensions of the respective endophyte strains, whereas untreated plants were dipped in sterile PDB. Where solid substrate inoculum was used, 2 g of maize bran containing fungal mycelia and spores was mixed in soil around the roots and the corm. Non-inoculated plants were planted with sterile maize bran mixed in soil around the roots and the corm. All plants were planted in steam-sterilized soil in 200 ml cups and kept at near 100% relative humidity in a humidity chamber in the screenhouse for 4 weeks, after which plant tissue colonization by the endophytes was assessed.

At four weeks after inoculation, all four plants in each treatment were harvested through destructive sampling and their roots and corm thoroughly washed free of soil with tap water. Three roots of each plant were removed and, together with the whole corm, sterilized by dipping for 1 min in 5% sodium hypochlorite and for 1 min in 75% EtOH. The samples were then washed thrice in sterile distilled water. For experiment 1, four cubes of corm (0.25 cm^3) and, for each root, four pieces of root (0.25 cm long) were then incubated in petri dishes containing SNA media with penicillin G (0.1 g L^{-1}), streptomycin sulphate (0.2 g L^{-1}) and chlortetracycline

(0.05 g L^{-1}). For experiment 2, six pieces of root and six cubes of corm were assessed. The plates were incubated for 7-10 days, after which emerging fungi were identified by the characteristic macroconidia, short phialides and chlamydospores (Niere, 2001).

Determination of endophyte colonization

A 16-week screenhouse study comprising 21 plants per treatment was conducted to determine the persistence of two *F. oxysporum* strains, *V2w2* and *III4w1*, in the cultivars Kibuzi and Nabusa. The cultivars were each inoculated with one of the two strains and some plants of each cultivar received no endophyte treatment and acted as negative controls. Spore suspensions of concentration 1.50-1.75 x 10^6 spores ml^{-1} were prepared as above and tissue culture plants inoculated by dipping their roots and corms for 90 min in spore suspensions of the respective endophyte strains, whereas untreated plants were dipped in sterile PDB. All plants were kept in the humidity chamber for four weeks, after which they were transferred into polythene potting bags containing 3 kg of steam-sterilized soil in the screenhouse for 12 weeks. All plants were watered daily.

Plant tissue colonization by the endophytes was assessed at 4, 8, 12 and 16 weeks after inoculation. At each sampling date, four plants per treatment were harvested through destructive random sampling. The re-isolation procedure used was similar to the one described above, except that five pieces per root and corm were used. Fungi were identified according to the methods described above and, after identification, percentage colonization was assessed.

Data analysis

Except for experiment 2 of the inoculation method trial where data were squareroot transformed, percentage tissue colonization was arcsine-squareroot transformed prior to statistical analysis in all experiments. Percentage tissue colonization was subjected to analysis of variance (ANOVA) separately for corms and roots. Percentage tissue colonization between treatment groups was compared using linear contrasts, whereas multiple mean comparisons were performed using Tukey's studentized range test (SAS institute, 1989).

Results

Determination of optimal inoculation methods

In experiment 1, percentage root tissue colonization was significantly influenced by the method of endophyte inoculation ($P < 0.0001$) (Fig. 1). However, for each inoculation method there was no statistical difference between cultivars or between endophyte strains. The highest percentage root tissue colonization (62.5 ± 12.5%) was by strain *III4w1* in the cultivar Nabusa inoculated using a solid substrate inoculum and the lowest percentage root tissue colonization (4.16 ± 4.16%) was by strain *V2w2* in the cultivar Nabusa inoculated using root and corm dip with spore concentration 1.50-1.75 x 10^6 spores ml^{-1}. Where solid substrate inoculum was used, percentage root tissue colonization ranged between 37.5 ± 11.16% and 62.5 ± 12.5%. The lowest root tissue colonization obtained using a solid substrate inoculum was still higher than the highest root tissue colonization obtained using the root dip method ($P < 0.05$). Percentage corm tissue colonization was not influenced by the method of endophyte inoculation ($P = 0.33$) and colonization between cultivars and endophyte did not differ among inoculation methods used. Percentage corm tissue colonization ranged between 37.5 ± 16.14% and 81.25 ± 6.25%.

In experiment 2, percentage root tissue colonization was significantly influenced by the method of endophyte inoculation ($P < 0.0001$) (Fig 2). It was also noted that the inoculation method used significantly influenced tissue colonization by a particular endophyte strain ($P < 0.026$). The highest percentage root tissue colonization (84.44 ± 7.74%) was by strain *III4w1* in the cultivar Kibuzi inoculated using a solid substrate inoculum and the lowest (15.74 ± 5.45%) was by strain *V2w2* in the cultivar Nabusa inoculated using root dip with spore concentration 4.19-4.80 x 10^6 spores ml^{-1}. Where solid substrate inoculum was used, percentage root tissue colonization ranged between 71.39 ± 6.39% and 84.44 ± 7.74%. Again, the lowest root tissue colonization obtained using a solid substrate inoculum was still higher than the highest root tissue colonization obtained using the root dip method ($P < 0.05$). Percentage corm tissue colonization by both endophyte strains was not influenced by the method of endophyte inoculation, and did not differ between cultivars or endophyte strains. Percentage corm tissue colonization ranged between 88.89 ± 5.56% and 100.00 ± 0.00%. Though not inoculated, both root and corm tissue colonization by *F. oxysporum* was noted in the control plants. Percentage root tissue colonization for untreated plants ranged between 5.56 ± 3.51% (Kibuzi dipped in sterile broth) and 10.19 ± 3.63% (Nabusa planted with sterile maize bran around the roots), while percentage corm tissue colonization between 5.56 ± 5.56% (Kibuzi planted with sterile maize bran around the roots) and 19.44 ± 9.04% (Nabusa dipped in sterile broth). However, percentage root and corm tissue colonization by *F. oxysporum* in all inoculated plants was significantly higher than tissue colonization in untreated plants ($P < 0.0001$).

Determination of endophyte colonization

Percentage tissue colonization in both roots and corms was greater in endophyte-inoculated plants than in untreated plants. Percentage tissue colonization was greater ($P < 0.05$) in corms (33.67 ± 4.22%) (Fig. 3) than in roots (17.84 ± 2.18%) (Fig. 4). Whereas percentage root tissue colonization did not vary significantly for all inoculated plants over the weeks, corm tissue colonization differed significantly over time ($P < 0.0001$). After 16 weeks, percentage corm tissue colonization dropped to zero except for Kibuzi inoculated with strain *III4w1* (6.67 ± 4.22%).

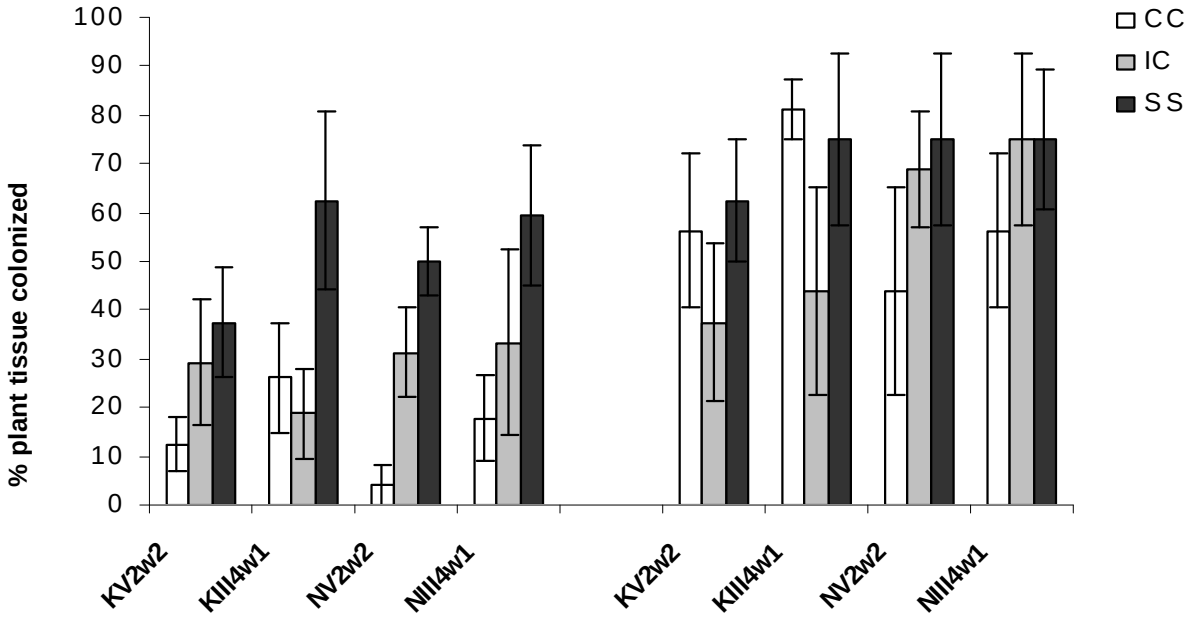

Figure 1. Experiment 1: the effect of three different inoculation methods on percentage root tissue colonization by endophytic *Fusarium oxysporum* in banana cultivars Kibuzi and Nabusa (*Musa* sp., AAA-EA).

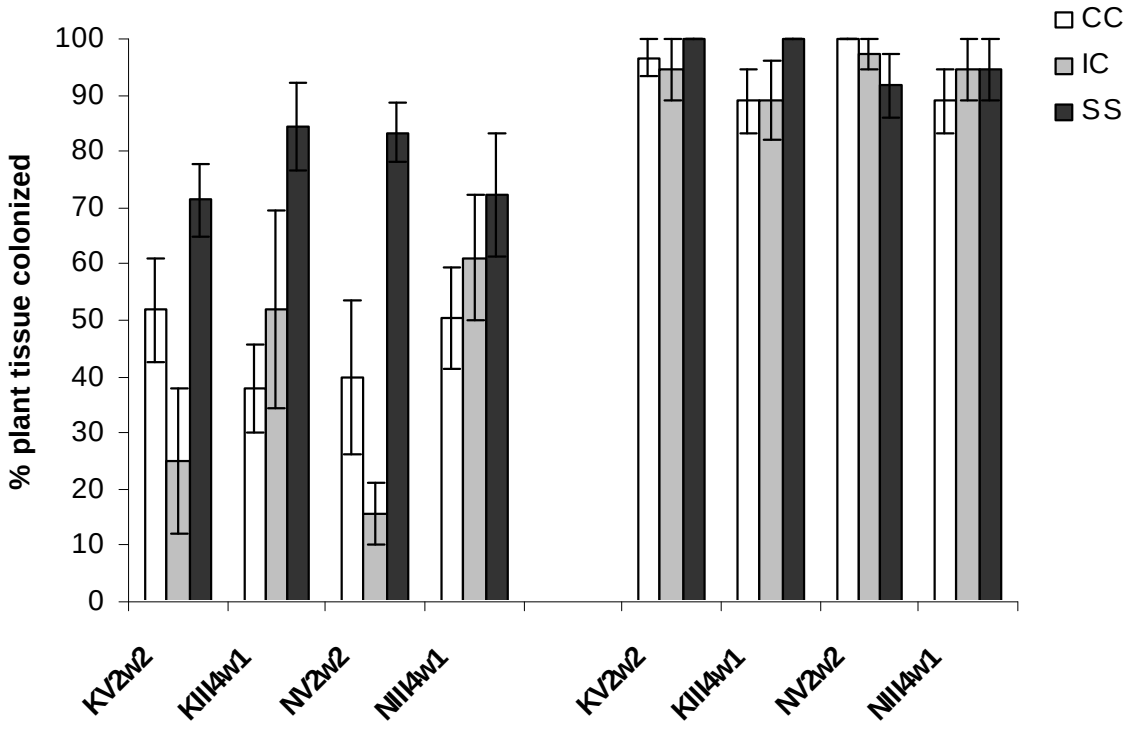

Figure 2. Experiment 2: the effect of three different inoculation methods on percentage root tissue colonization by endophytic *Fusarium oxysporum* in banana cultivars Kibuzi and Nabusa (*Musa* sp., AAA-EA).

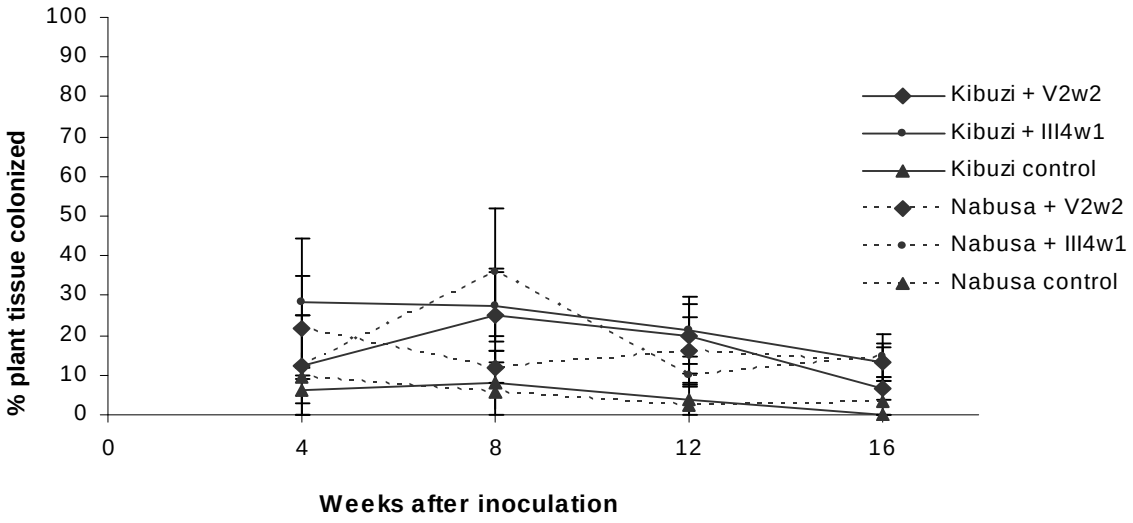

Figure 3. Percentage root tissue colonization by endophytic *Fusarium oxysporum* in banana cultivars Kibuzi and Nabusa (*Musa* sp., AAA-EA).

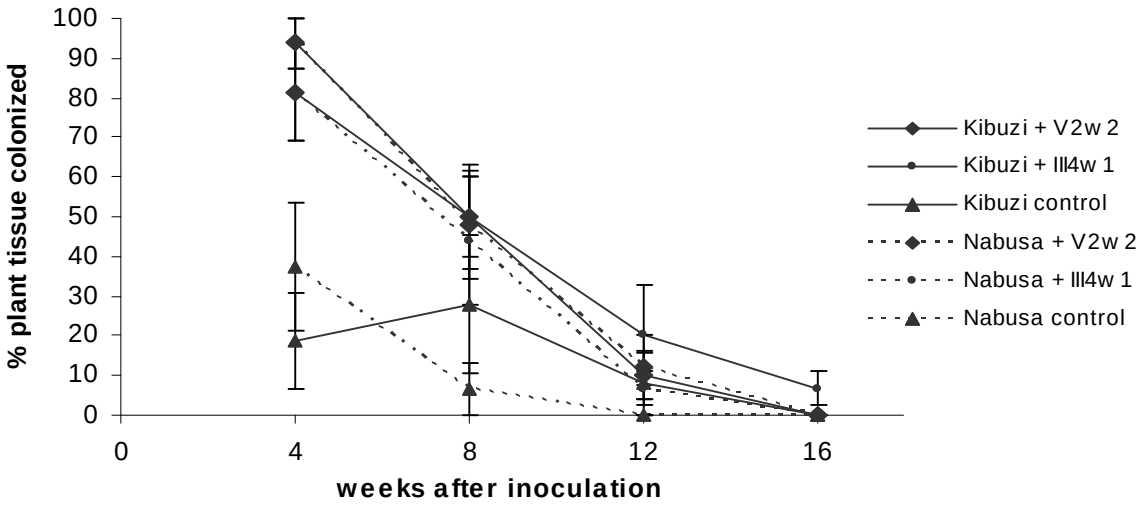

Figure 3. Percentage corm tissue colonization by endophytic *Fusarium oxysporum* in banana cultivars Kibuzi and Nabusa (*Musa* sp., AAA-EA).

Four weeks after inoculation (WAI), percentage endophytic colonization of root tissue in inoculated plants was between 12.5 ± 12.5% and 28.13 ± 16.44% (Fig. 3), with the highest percentage colonization for strain *III4w1* in the cultivar Kibuzi. The highest root tissue colonization was recorded at 8 WAI for strain *III4w1* in the cultivar Nabusa (36.00 ± 16.00%). Root tissue colonization in untreated plants (4.73 ± 1.51%) was significantly lower than in inoculated plants ($P < 0.0001$). Percentage corm tissue colonization by endophytes 4 WAI in inoculated plants was between 81.25 ± 11.97% and 93.75 ± 6.25% (Fig. 4), with the highest percentage corm tissue colonization in cultivar Nabusa inoculated with the strain *V2w2* and Kibuzi inoculated with *III4w1*. At 8 WAI, the highest corm tissue colonization (50.00 ± 10.00%) was recorded in the cultivar Kibuzi inoculated with strain *III4w1*, and this was significantly different ($P < 0.05$) from tissue colonization in untreated plants. Though control plants were not inoculated, 28.13 ± 9.95% tissue colonization by *F. oxysporum* was recorded. However, tissue colonization for inoculated plants was still significantly higher ($P < 0.0001$).

Discussion

The results indicate successful colonization of roots and corms of tissue culture banana by nonpathogenic *F. oxysporum*. Similar results were obtained by Griesbach (2000), Niere (2001) and Paparu *et al.* (2004). Root tissue colonization varied with the method of endophyte inoculation and was particularly higher where a solid substrate inoculum was used. This may be an indication that root tissue colonization is affected by the period of root exposure to fungal endophyte inoculum. Root tissue colonization using a root dip method may therefore be improved if the period of root and corm dip is increased.

The decreasing corm tissue colonization by *F. oxysporum* over the weeks may be due to the failure to re-isolate the strains from the corms of sampled plants since not the whole corm but a sub sample was used for re-isolation, or it may be an indication of the absence of the fungus from the tissues studied. Paparu *et al.* (2004) similarly obtained results that showed a general decrease in corm tissue colonization over the weeks after endophyte inoculation. Whereas there was a significant reduction in corm tissue colonization over the weeks, root tissue colonization did not vary significantly between 4 and 16 weeks after inoculation. This result is also in accordance with Paparu *et al.* (2004). The ability of the two strains to persist in the roots up to 16 weeks after inoculation may be explained by the fact the strains were previously isolated from roots, and not corms, of mature banana plants. These strains might be adapted to particular microecological and physiological conditions present in the roots. Similarly, Caroll *et al.* (1977) observed that most fungal endophytes isolated from petioles of European conifers were restricted to that part and were rarely detected in distal portions of the needle. There is therefore need to identify endophyte strains isolated from roots and corms and test their persistence in these tissues to determine the existence of tissue-specificity among banana fungal endophytes

The required time for field planting of tissue culture plants is between 2-4 months (Vuylsteke and Talengera, 1998), but the persistence studies presented in this paper only looked at the changes in plant tissue colonization by fungal endophytes in plants in a screenhouse. There is need to establish persistence in field plants and the possibility of endophyte transmission from mother plants to suckers. Re-isolation of *F. oxysporum* from non-inoculated banana plants has been reported. Niere (2001) and Paparu et al. (2004) recorded up to 30 and 12.5% isolation of *F. oxysporum*, respectively, from roots and corms of non-inoculated plants. The re-isolation of *F. oxysporum* from non-inoculated plants is possible because certain strains of the species are free living. To establish actual plant tissue colonization by fungal endophytes, there may be need to use molecular techniques that will distinguish the re-isolated endophytes from those previously present in the banana plant.

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References

Azevedo, J. L., 1998. Endophytic microorganisms. In: Melo, I. S. and Azevedo, J. L. (Editors), *Ecological Microbiana, Editoria EMPRAPA*. Sao Paulo, Brazil.

Baker, G. M., Pottinger, R. P., Addison, P. J. and Prestidge, R.A., 1984. Effect of *Lolium* endophyte fungus infections on behavior of adult Argentine stem weevil. *New Zealand Journal of Agricultural Research*, 27, 271-277.

Caroll, F. E., Muller, E. and Sutton, B. C. 1977. Preliminary studies on the incidence of needle endophytes in some European conifers. *Sydonia*, 29, 87-103.

Clay, K. 1991. Endophytes as antagonists of plant pests. In: Andrews, J.H. and Hirano, S.S. (Editors), *Microbial ecology of leaves*. Springer-Verlag, New York, USA.

Gold, C. S., Pena, J. E. and Karamura, E. B. 2001. Biology and integrated pest management for the banana weevil *Cosmopolites sordidus* (Germar) (*Coleoptera*: *Curculionidae*). *Integrated Pest Management Reviews*, 6, 79-155.

Gold, C. S., Nankinga, C., Niere, B. and Godonou, I. 2003. Integrated pest management of the banana weevil in Africa with emphasis on microbial control. In: P. Neuenschwander, P. *et al.* (Editors), *Biological control in IPM systems in Africa*. CABI. Silwood Park, United Kingdom.

Griesbach, M., 2000. Occurrence of mutualistic fungal endophytes in bananas (*Musa* spp.) and their potential as biocontrol agents of banana weevil *Cosmopolites sordidus* (Germar) in Uganda. Ph.D Thesis, University of Bonn, Bonn, Germany.

Latch, G. C. M., Hunt, W. F. and Musgrave, D. R., 1985. Endophytic fungi affect growth of perennial ryegrass. *New Zealand Journal of Agricultural Research*, 28, 165-168.

Niere, B., 2001. Significance of non-pathogenic isolates of *Fusarium oxysporum* Schlecht: Fries for the biological control of the burrowing nematode *Radopholus similis* (Cobb) Thorne on tissue cultured banana. Ph.D Thesis, University of Bonn, Bonn, Germany.

Paparu, P., Niere, B., Gold, C. S., Coyne, D., Adipala, E. and Dubois, T., 2004. Colonization of banana by fungal endophytes. In press.

Schuster, R.-P., Sikora, R. A. and Amin, N. 1995. Potential of endophytic fungi for the biological control of plant parasitic nematodes. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent*, 60, 1047-1052.

Speijer, P. R. and Ssango, F., 1999. Evaluation of *Musa* host plant response using nematode densities and damage indices. *Nematropica*, 29, 185-192.

Vuylsteke, D. 1998. Shoot-tip culture for the Propagation, Conservation, and Distribution of *Musa* germplasm. International Institute of Tropical Agriculture, Ibadan, Nigeria.

Vuylsteke, D. and Talengera, D., 1998. Postflask Management of Micropropagated Bananas and Plantains. International Institute of Tropical Agriculture, Ibadan, Nigeria.