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RESPONSE OF *MELOIDOGYNE HAPLA* TO MYCORRHIZA FUNGI INOCULATION ON PYRETHRUM

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ABSTRACT: *Five arbuscular mycorrhiza fungi (AMF) isolated from pyrethrum were screened in the greenhouse for efficacy in improving pyrethrum growth and in suppressing a root-knot nematode, Meloidogyne hapla. The fungi screened were Glomus spp. (isolates LM61, ML34 and ML35), Scutellospora sp. (isolate KS74) and Gigaspora sp. (isolate LM83). A 20g mixed fungal inoculum was incorporated into sterilized sand-soil mixture before transplanting 6-week-old pyrethrum seedlings. The inoculum consisted of the growth medium, spores, external mycelia and infected root segments. The plants were inoculated with 6000 M. hapla second stage juvenile (J-2) 3 months after fungal inoculation. Dry shoot weights, fresh root weights, percent root colonization by the fungi, nematode gall indices, number of eggs and females in the root system and number of J-2 in the soil were determined at the end of the experiment, two months after nematode inoculation. Glomus LM61 and Scutellospora KS74 significantly improved top biomasses of fungus-treated and fungus-nematode-treated plants. Glomus LM61was more effective (33% top biomass increase). Glomus ML34 and ML35 and Gigaspora LM83 improved top biomasses of fungus-nematode-treated plants. Scutellospora KS74 and Glomus ML34 significantly increased fresh root weights of pyrethrum by 45% and 50%, respectively. Glomus LM61, Scutellospora KS74 and Gigaspora LM83 caused 86%, 32% and 37% nematode suppression, respectively. All the fungal isolates significantly reduced the number of females and J-2. The presence of nematodes in fungus-treated plants did not affect root colonization by the fungi except in plants treated with Glomus ML34 and ML35.*

Key words: *Meloidogyne hapla, Mycorrhiza fungi, pyrethrum*

INTRODUCTION

Kenya is the leading pyrethrum (*Chrysanthemum cinerariefolium* Vis.) producer worldwide. Currently, Kenya supplies 67 - 80% of the world pyrethrum requirements (Pers. Comm. Pyrethrum Board of Kenya (PBK)). The dried flowers are a source of pyrethrins, which are active ingredients of natural insecticides.

Meloidogyne hapla Chitwood, a root-knot nematode, accounts for 95% of the plant-parasitic nematode populations associated with pyrethrum in Kenya (Parlevliet and Brewer, 1971). The nematode causes 20-30% pyrethrum yield losses, a decrease in flower size and pyrethrin content, stunting, chlorosis, wilting and predisposes infected plants to infection by root-rot and wilt fungal pathogens (Warui *et al*., 1991).

The high cost of nematicides, and environmental and health hazards posed by their use in the management of *M. hapla*, as well as the relatively low economic value of recommended rotational crops (maize) involving the long rotational periods (3-4 years)(Wanjala, 1992) the development of new *M. hapla* races pathogenic to hitherto resistant pyrethrum clones (Triantaphyllou, 1985) make imperative a search for viable alternatives in the management of the nematode.

Components of a viable control package should be cost effective and environmentally safe. Arbuscular mycorrhiza fungi (AMF), obligate endophytic symbionts have the potential of suppressing pathogens and associated diseases (Ingham, 1988; Francl, 1993; Lindermann, 1994) and could therefore provide such an alternative.

Glomus mosseae (Nicol. and Gerd.) Gerd. and Trappe, *G. macrocarpum* Tul and Tul and *G. fasciculatum* Walker and Koske have been reported to reduce disease severity and suppress development of *Meloidogyne incognita* (Kofoid and White) Chitwood and *M. hapla* (Sikora, 1979; Kellam

and Schenck, 1980). Besides suppressing disease, AMF promote plant growth and yield, enhance plant water relations, improve soil aggregation and structure and ameliorate aluminium and iron toxicity (Harley and Smith, 1983; Janos, 1987). Use of AMF in the long term would thus favour an agricultural system that is both production and protection oriented thus enhancing stabilization of agroecosystems.

In spite of all these benefits associated with AMF, a literature search revealed that no work has been done to assess interaction between *M. hapla* and AMF that are indigenous to pyrethrum fields in Kenya. This study was, therefore, designed to screen AMF that are indigenous to pyrethrum fields for efficacy in suppressing *M. hapla* and in improving pyrethrum growth.

MATERIALS AND METHODS

Inoculum production and inoculation procedure

Arbuscular mycorrhiza fungi

Greenhouse tests were conducted to investigate effects of five AMF isolates in improving pyrethrum growth and in suppressing *M. hapla*. The fungi screened were *Glomus* spp. (isolates LM61, ML34 and ML35), *Scutellospora* sp. (isolate KS74) and *Gigaspora* sp. (isolate LM83). The fungi were obtained from soils sampled from the rhizosphere of pyrethrum growing in three main pyrethrum growing zones; Kisii (KS), Limuru (LM) and Molo (ML). The areas represented low (1800-2100m), mid (2100-2600m) and high altitude (2600-3100m) zones, respectively.

Soil samples from each study site were thoroughly mixed before taking three 300g sub-samples for physico-chemical characteristic analysis and five 50g sub-samples for AMF spore extraction. The mineral content analysis of the soil was done at National Agricultural Research Laboratories (NARL). About 2kg of soil was used to initiate soil trap cultures for maintaining the fungal populations. The remainder of the soil was used as a growth medium in the experimental trials for the respective fungal isolates.

Arbuscular mycorrhiza fungal spores were extracted from soil using a combination of wet-sieving and decanting (Gerdeman and Nicolson, 1963) and sucrose-centrifugation techniques (Jenkins, 1964). The spores were characterized following Morton (1988), Schenck and Perez (1987) and Walker (1983) and identified to generic taxon. The isolates were cultured on tropical kudzu (*Pueraria phasioloides* Benth), an obligate mycotrophic legume. Healthy spores of each isolate were placed on root tips of *P. phasioloides* seedlings freshly germinated in the greenhouse on autoclaved sand. Before spore placement, the seedling was placed in a 2-cm-depression made in sterilized growth medium contained in a 7-cm-diameter plastic pot. After spore placement, the depression was gently covered with the growth medium taking care not to dislodge the spore from the root tip. Plants were placed in the greenhouse and watered appropriately. Plants were supplied with Hoagland's solution minus phosphorus. General greenhouse sanitation was maintained to ensure purity of the cultures. Cultures were checked 6 weeks after spore placement and monthly thereafter for sporulation. The cultures were then maintained on pyrethrum and served as sources of AMF inocula.

Twenty grams of the mixed fungal inoculum obtained from a 3month-old culture was placed in a 3-cm-depression made in sterilized soil contained in a 15-cm-diameter plastic pot. Soils from which AM fungal isolates were obtained served as the growth media for the respective fungal isolates. The soils from Kisii (pH4.7, clay loam, P=7ppm) and Molo (pH5, clay loam, P=6ppm) sampling sites unlike those from Limuru (pH5.5, loam, P=26-68ppm) were mixed with sand (4:1) to improve soil texture. The soil: sand mixture was sterilized by autoclaving for two separate hour periods at 121C at 103Kpa. The inoculum consisted of the growth medium, spore, external mycelia and infected root segments. After inoculum placement, 6-week-old-pyrethrum seedlings were planted so that the inoculum was directly below the pyrethrum root systems. Uninoculated plants served as the controls. Pyrethrum variety P4 recommended for it's high flower yield and pyrethrin content (Ikahu and Ngugi, 1989) was used as the test plant throughout the study. The pyrethrum seeds were germinated in sterile sand: soil (1:4) mixture and transplanted after six weeks. The plants were watered as required and supplied with 0.3% Wuxal nutrient solution (12%N, 4% P_2O_5, 6%K_2O, 0.02% Boron and 0.01% Copper) monthly.

Meloidogyne hapla

Meloidogyne hapla used in this study was obtained from galled pyrethrum roots sampled from pyrethrum fields where the fungi were obtained. The nematodes were maintained on tomato (*Lycopersicon esculentum* Mill. cv. Money maker) growing in sterile sand: soil (2:1) mixture in the greenhouse.

Nematode inoculum was prepared by extracting *M. hapla* eggs from galled tomato roots using sodium hypochlorite technique (Hussey and Baker, 1973). The egg suspension was aerated for 10-14 days at room temperature to facilitate hatching of eggs into J-2. Plants were inoculated with a

nematode suspension containing 6000 J-2 by dispensing the nematodes into a 3-cm-wide depression made around pyrethrum's root system. The depression was then covered with soil.

Plants without the fungus and the nematode served as controls. The treatments were arranged in a randomized complete design with six replications. The tests were terminated 2 months after nematode inoculation.

Data collection

Plant performance

Dry shoot and fresh root weights were obtained at the end of the experimental period. Shoot systems were dried at 80C for 48 hours before obtaining their weights.

Root colonization by the fungus

Thoroughly washed fine roots were cut into 1-cm-long segments and thoroughly mixed. Five 1g fresh root samples per experimental unit were taken for assessment of fungal colonization. The roots were cleared and stained using Walker's Cold Staining technique, a modification of the technique of Phillip and Hayman (1970). The roots were observed under a dissecting microscope (Mag. x40) for the presence of aseptate intraradical hyphae, vesicles, arbuscules, spores, extraradical hyphae and auxillary cells. Root colonization was assessed using a Grid-Line Intersect Method (Giovanneti and Mosse, 1980). Colonized root length was expressed as a percentage of total root length.

Nematode disease assessment

To assess nematode damage on pyrethrum, the following parameters were obtained:

i) Galling index as a measure of disease severity. Roots were gently washed and rated for galling using a 0-4 galling scale where 0=no galls, 1=1-25%, 2=26-50%, 3=51-75% and 4=76-100% of root system galled (Krusberg and Nelson, 1958)
ii) Number of females within the roots. A one-gram fresh root sample per experimental unit was cleared and stained using a modified NaOCl-Acid fuchsin technique (Byrd *et al*., 1983). The number of females within the 1-cm-long root segments was determined under a dissecting microscope (Mag x40).
iii) Number of eggs within the root systems. Eggs were extracted using the technique of Hussey and Baker (1973). The egg suspension was adjusted to 50ml and the number of eggs in 1ml of the suspension was enumerated in a Hawksley's Slide Counter (Hawksley and Sons Ltd., West Sussex, England) under a microscope.
iv) Number of J-2 in soil. The J-2s were extracted from 100cm^3 of thoroughly mixed soil per experimental unit using the method of Jenkins (1964). The nematodes were enumerated in a Hawksley's Slide Counter.

Data analysis

Treatment effects were assessed by Analysis of Variance (ANOVA) using Statistical Analysis Systems (SAS) computer package. Treatment means were separated using Least Significant Difference (LSD).

RESULTS

There were highly significant differences ($P<0.001$) in dry shoot and fresh root weights among treatments (Table 1). Plants inoculated with *Glomus* LM61 and *Scutellospora* KS74 had the heaviest and second heaviest top dry biomasses, respectively while those inoculated with the nematodes alone had the lightest top dry biomass (Table 1). *Glomus* LM61 and *Scutellospora* KS74 significantly improved pyrethrum's top dry biomass by 33% and 31%, respectively, (Table 1). Plants treated with *Glomus* ML34 and ML35 and *Gigaspora* LM83 had top dry biomasses that did not differ significantly from each other or from the control (Table 1). Plants treated with both the fungal isolates and the nematodes performed significantly better than those treated with the nematodes alone (Table 1). The fungal isolates improved top dry biomasses of nematode- treated pyrethrum by over 100% except for *Glomus* ML34 which improved top dry biomass of nematode-treated plants by 87% (Table 1).

Nematode significantly reduced pyrethrum's top dry biomass by 49% relative to the control (Table 1). The presence of the nematodes did not, however, significantly affect the performance of plants treated with the fungal isolates except in *Glomus* ML35-treated plants (Table 1). This was revealed by the non- significant differences between plants treated with both the fungal isolates and the nematodes and those treated with the respective fungal isolates alone (Table 1).

Table 1: Mean dry shoot weights (DSW), fresh root weights (FRW) and percent root colonization (%RC) of pyrethrum treated with AM fungal isolates and/or nematode

Treatment	DSW (g)	FRW (g)	%RC
***Scutellospora* KS74 + N**	10.17cde¶	18.69cd	42.4bcde
***Gigaspora* LM83 + N**	8.7bc	20.97def	38bc
***Glomus* ML35+ N**	10.65e	18.3bcd	14.9a
***Glomus* ML34+ N**	7.67b	18.2bcd	16a
***Glomus* LM61+ N**	10.32de	16.57bc	51.61e
***Scutellospora* KS74**	10.55e	22.97ef	48.4cde
***Gigaspora* LM83**	8.06b	19.38cde	40.2bcd
***Glomus* ML35**	7.53b	14.43ab	33.9b
***Glomus* ML34**	8.9bcd	23.75f	34b
***Glomus* LM61**	10.72e	16.58bc	48.7de
Nematode (N)	4.1a	10.43a	-
Control	8.04b	15.85bc	-
LSD	1.57	4.25	10.7

Data are means of six replicates.
¶ Means in the same column that are followed by the same letter(s) are not significantly different (P>0.05) using LSD.

Plants treated with *Glomus* ML34 and *Scutellospora* KS74 had the heaviest and second heaviest fresh roots, respectively, that differed significantly (P<0.001) from the control (Table 1). Colonization of pyrethrum by these fungal isolates increased root weights by 50% and 45%, respectively, as compared to the control (Table 1). The other isolates did not, however, significantly improve root weights as was revealed by non-significant differences between the fungi-treated plants and the control (Table 1). Roots of fungus-nematode treated plants were significantly (P<0.001) heavier than those of nematode-treated plants (Table 1). *Glomus* LM61, *Gigaspora* LM83, *Scutellospora* KS74, *Glomus* ML34 and *Glomus* ML35 increased root weights of nematode-treated plants by 59%, 101%, 79%, 75% and 75%, respectively (Table 1). Nematode–treated plants had significantly lighter roots than all the other plants (Table 1). In presence of the fungal isolates *Glomus* LM61 and ML35 or *Gigaspora* LM83, however, nematodes did not have significant effects on root weights (Table 1). Plants treated with both the nematodes and fungal isolates *Glomus* ML34 or *Scutellospora* KS74 had, however, significantly lighter fresh roots (loss of 31% and 23%, respectively) relative to those of plants treated with the respective fungus alone (Table 1).

Root colonization by AMF differed significantly (P<0.05) among treatments (Table 1). Roots of plants treated with *Glomus* LM61 or *Scutellospora* KS74 alone or plus the nematodes were among those that were heavily colonized while those of plants treated with either *Glomus* ML34 or ML35 plus the nematodes were among those that were lightly colonized (Table 1).

There were highly significant differences (P<0.001) in gall indices (disease severity), number of eggs, females and J–2s among treatments (Table 2).

Table 2: Mean gall indices (GI), eggs, females and J-2 in pyrethrum treated with AM fungal isolates and /or nematodes

Treatment	GI†	Eggs/ml	Females/g	J2/100ml
***Scutellospora* KS74 + N**	2.5b¶	1544b	198.2b	597.17b
***Gigaspora* LM83 + N**	2.33b	1471b	226.2bc	472.17ab
***Glomus* ML35+ N**	3.17c	1847.8c	234.7cd	741.84c
***Glomus* ML34+ N**	3.17c	1812.67c	242.3cd	785.5c
***Glomus* LM61+ N**	0.5a	292.7a	33.3a	385.5a
Nematode (N)	3.68c	2003d	260.2d	1270.34d
Control	-	-	-	-
LSD	0.65	190.3	30.3	127.4

Data are means of six replicates.
† Gall indices based on a 0-4 gall rating scale; where 0=no galls, 1=1-25% 2=26-50%, 3=51-75% and 4=76-100% of root system galled.
¶ Means in the same column that are followed by the same letter(s) are not significantly different (P>0.05) using LSD

Plants treated with nematodes had significantly higher gall indices, number of eggs, females and J–2s than those treated with *Glomus* LM61, *Scutellospora* KS74 and *Gigaspora* LM83 (Table 2). Number of eggs and J-2s in plants treated with *Glomus* ML34 and ML35 also differed significantly from those of plants treated with the nematodes alone (Table 2). *Glomus* LM61 reduced galling, number of eggs and, females and J–2 by 86%, 85%, 87% and 70%, respectively (Table 2). *Gigaspora* LM83, on the other hand, reduced galling, number of eggs, females and J–2 by 37%, 27%, 13% and 63%, respectively, while *Scutellospora* KS74 reduced the disease parameters by 32%, 23%, 23% and 53%, respectively (Table 2). *Glomus* ML35 reduced the number of eggs and J–2 by 8% and 42%, respectively, while *Glomus* ML34 reduced the number of eggs by 10% and J–2 by 38% (Table 2).

DISCUSSION

The ability of AM *Glomus* LM61 and *Scutellospora* KS74 to significantly improve pyrethrum top dry biomasses (Table 1) confirms previous reports on the ability of AMF to enhance plant growth. All the fungal isolates significantly improved top dry biomasses of nematode-treated plants (Table 1). As with other AMF, the fungi might have enhanced growth of untreated and nematode-infected plants through enhanced nutrient uptake and

synthesis of plant growth promoting hormones, in particular, auxins, cytokinins and gibberellins (Allen *et al*., 1980, 1982). Improved nutrient uptake results from increased absorptive surface of the root system by AMF external mycelia. As external mycelia ramificate through the soil beyond the nutrient depletion zone they absorb and translocate mineral elements mainly phosphorus, zinc, iron, copper, boron and molybdenum to the root system (Linderman, 1994). The fungi might have in addition, improved water uptake from the growth medium (Hardie, 1985). Improved water uptake in AMF-colonized plant results indirectly from improved plant nutritional status (Harley and Smith, 1983), increased cytokinin levels (Allen *et al*., 1982) and/or increased number of vascular bundles (Daft and Okusanya, 1973). The particular mechanisms involved are being investigated.

The differences in efficacy of AMF isolates to improve pyrethrum growth might be explained by differences in their effectiveness to infect and colonize pyrethrum and their ability to grow in the soil and enhance nutrient uptake. Species and strains of AM fungi have been reported to differ in their effectiveness to increase nutrient uptake and plant growth. The ability of AMF to increase nutrient uptake is related to their ability to form extensive and well distributed hyphae in soil and throughout the developing root system. The ability of AMF hyphae to absorb mineral nutrients from the soil solution and the distance from the soil via the hyphae into the root also influences fungal effectiveness. In addition, the host-fungus compatibility, the mycorrhizal dependency of pyrethrum, fungal inoculum density and potential play a significant role in influencing fungal effectiveness (Abbott and Robson, 1985). Fungal species that are effective in improving plant growth have been reported to infect and colonize the host plant rapidly (Sanders *et al*., 1977). *Scutellospora* spp. for example, has been reported to infect and colonize plants relatively slowly as compared to the *Glomus* spp. (Abbott and Robson, 1985).

Fungal isolates had no significant effects on root weights except *Scutellospora* KS74 and *Glomus* ML34 (Table 1). The improved root weights might have been due to greater secondary cell wall development caused by fungal colonization rather than an increase in root biomass. Plant colonization by some AM fungi leads to an increase in the proportion of lignified and suberized higher order roots (Rajapakse and Miller, 1992).

The significantly lower gall indices, fewer females, eggs and J-2 in nematode-AMF treated plants, in most cases (Table 2), confirm previous reports on the ability of AMF to suppress phytonematodes (Ingham, 1988; Francl, 1993; Linderman, 1994). *Glomus* LM61 was the most effective in reducing nematode disease severity (gall index) followed by *Scutellospora* KS74 (Table 2). *Glomus* ML34, and ML35 did not, however, significantly reduce nematode damage on pyrethrum except for egg production and soil infestation by J-2 (Table 2). Significantly reduced egg production, gall indices and populations of *M. incognita* was reported on tomato inoculated with *Glomus intraradices* Schenck and Smith (Suresh *et al*., 1985) and on cotton inoculated with *G. fasciculatum* (Saleh and Sikora, 1984; Smith *et al*., 1986). Similar results were obtained by Sivaprasad *et al.* (1990) on *P. nigrum* inoculated with *Glomus etunicatum* Becker and Gerd. and by Carling *et al*. (1989) on soybean inoculated with *G. margarita* Becker and Hall. Development of *M. incognita* on cotton and *M. hapla* on onion from J-2 to adults was delayed after inoculation with *G. intraradices* (Smith *et al*., 1986) and *G. fasciculatum* (Mac Guidwin *et al*., 1985), respectively. Inoculating chickpea with *Glomus manihotis* suppressed reproduction of *M. javanica* (Diederichs, 1987).

The fungi might have increased plant resistance or tolerance to *M. hapla* infection through improved plant growth and vigour (Bagyaraj, 1984), increased production of phenolic compounds (Dehne and Schonbeck, 1979), phytoalexins (Morandi, 1987) and lignin (Schonbeck, 1979). Increase in lignin and phenols in mycorrhizal roots were implicated in the reduction of *M. javanica* reproduction in tomatoes (Suresh *et al*., 1985). An increase in amount of vascular bundle (Daft and Okusanya, 1973; Ratnayake *et al*., 1978)in AMF-colonized plants might have compensated for the nematode-damaged xylem tissues and alleviated nematode's detrimental effects on water and mineral uptake and translocation.

The fungi might have, in addition, altered chemotatic attraction of *M. hapla* to pyrethrum roots by altering the quantity and quality of root exudation (Francl, 1993). Besides affecting the attractiveness of roots to the nematode, change in root exudates might have altered the microbial population and composition in the mycorrhizosphere to the detriment of the nematodes. In addition, alteration of protein metabolism frequently reported in mycorrhizal plants might have led to increases in amino acids such as serine, phenylalanine and arginine which have nemastatic properties (Ingham, 1988). These biochemical and structural changes might have negatively influenced nematode penetration, survival and development. The speculated mechanisms of *M. hapla* suppression by the AM isolates will be verifiedin future studies.

Although *Glomus* ML34 and ML35 improved growth of nematode-infected plants, the presence of *M. hapla* affected growth and development of the fungal isolates as indicated by the reduced percent root colonization and consequently their stimulatory effects on pyrethrum growth (Table 1). The presence of the nematode did not, however, significantly affect root colonization by the other fungal isolates (Table 1). Nematodes may increase, decrease or have no effect on root colonization by AM fungi (Francl, 1993). The effect depends on the specific fungal species involved. Decreased sporulation of *G. etunicatum* on soybean by *M. incognita* (Carling *et al*., 1989) and decreased vesicle formation and mycelial growth in citrus by *Radopholus similis* (Cobb) Thorne (O' Bannon and Nemec, 1979) have been reported. Kellam and Schenck (1980), on the other hand, reported that *M. incognita* had no significant effect on soybean root colonization by *G. macrocarpum.*

It appears that in *Glomus* ML34- or ML35-nematode interaction there was mutual inhibition between the fungi and the nematode possibly through competition for host photosynthates (Francl, 1993). Arbuscular mycorrhiza fungi depend on the host for their carbohydrate requirements (Harley and Smith, 1983) while nematodes utilize host's photosynthates for their growth and development (Bird, 1974). Whereas AMF increase photosynthesis through enhanced mineral translocation, water uptake and synthesis of phytohormones (Harley and Smith, 1983), root - knot nematodes decrease photosynthesis through the reverse mechanisms; reduced uptake and translocation of mineral salts and water (Bird, 1974) and inhibition of phytohormone production and translocation ((McClure, 1977).

In addition to decreasing photosynthesis, nematodes act as metabolic sinks (McClure, 1977). The increased metabolic activities of giant cells stimulate mobilization of photosynthates from shoots to roots where they are removed and utilized by the feeding nematodes. By diverting photosynthates, altering nutrient flow patterns in the plant tissue and retarding root growth, nematodes enhance competition with fungus thereby affecting fungal growth and development. This may account for the decreased percent root colonization by *Glomus* ML34 and ML35 and their decreased stimulatory effects on pyrethrum growth by the nematode (Table 1).

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