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Research Application Summary

Efficacy of indigenous fungi isolates against root-knot nematodes (*Meloidogyne* spp) in-vito

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Abstract

Root knot nematodes (*Meloidogyne* spp.) are a serious problem in smallholder tomato farms causing 90-100% yield loss in tomato crop in Kenya. For sustainable food production, effective management of nematodes is primarily dependent on the application of chemical nematicides. Chemical nematicides though very effective are expensive and also environmentally unfriendly due to their residual toxicity and pollution of the environment. The aim of this study was to evaluate the efficacy of indigenous antagonistic fungi in management of Root Knot Nematodes (RKN). Soil and root samples were obtained from Kirinyaga County. Fungal isolates were isolated from healthy tomato roots and *Meloidogyne* eggs by direct plating techniques. Root Knot Nematodes inoculum was extracted by Baermann's technique from soil and through root maceration method from heavily galled tomato roots. A total of 45 fungal isolates were isolated from tomato roots and RKN eggs. The fungal isolates were identified to belong to the various genera (*Trichoderma*, *Fusarium, Paecilomyces*, *Aspergillus* and *Penicilium* spp.). Some genera are yet to be clearly identified. *Trichoderma* spp. were the most prevalent (33.3%) followed by *Fusarium* spp. (28.9%). An experiment was conducted in the Kenyatta University agricultural laboratory with 45 treatments (isolates) replicated three times in a Completely Randomized Design. Data were subjected to Analysis of Variance (ANOVA) using SAS software and Means Separation done using Fisher's Least Significance Difference (LSD) at 5% level of significance. The fungal isolates caused RKN juvenile mortality that was significantly higher than the control. The highest juvenile mortality was recorded with *Trichoderma* spp 13. (88.33%) followed by *Paecilomyces* spp. and *Trichoderma* spp 1. (86.67%) which was significantly different from the untreated (1.67%). The results of this study show that indigenous fungal isolates have the potential of controlling root knot nematodes in vitro.

Keywords: Bio-control products, *Meloidogyne* spp., second stage juvenile

Résumé

Les nématodes à galles (*Meloidogyne* spp.) sont un grave problème dans les petites exploitations de tomates, entraînant une perte de rendement de 90 à 100% dans la culture de tomates au Kenya. Pour une production alimentaire durable, une gestion efficace des nématodes dépend principalement de l'application de nématicides chimiques. Les nématicides chimiques, bien que très efficaces, sont coûteux et également peu respectueux de l'environnement en raison de leur toxicité résiduelle

et de la pollution de l'environnement. Le but de cette étude était d'évaluer l'efficacité des champignons antagonistes indigènes dans la gestion des nématodes à galles (GNG). Des échantillons de sol et de racines ont été obtenus du comté de Kirinyaga. Des isolats fongiques ont été isolés de racines de tomates saines et d'œufs de *Meloidogyne* par des techniques de placage direct. L'inoculum de nématodes à nœuds racinaires a été extrait par la technique de Baermann du sol et par la méthode de macération des racines à partir de racines de tomates fortement écorchées. Au total, 45 isolats fongiques ont été isolés des racines de tomates et des œufs de RKN. Les isolats fongiques appartenaient aux différents genres (*Trichoderma, Fusarium, Paecilomyces, Aspergillus* et *Penicilium* spp.). Certains genres doivent encore être clairement identifiés. *Trichoderma* spp. étaient les plus répandus (33,3%), suivis de *Fusarium* spp. (28,9%). Une expérience a été menée dans le laboratoire agricole de l'Université Kenyatta avec 45 traitements (isolats) répliqués trois fois dans un plan complètement aléatoire. Les données ont été soumises à une analyse de variance (ANOVA) à l'aide du logiciel SAS et à une séparation des moyennes effectuée à l'aide de la plus petite différence significative de Fisher (PDS) à un niveau de signification de 5%. Les isolats fongiques ont causé une mortalité juvénile RKN qui était significativement plus élevée que celle du témoin. La mortalité juvénile la plus élevée a été enregistrée avec *Trichoderma* spp 13. (88,33%) suivi de *Paecilomyces* spp. et *Trichoderma* spp 1. (86,67%), qui était significativement différent du non traité (1,67%). Les résultats de cette étude montrent que les isolats fongiques indigènes ont le potentiel de contrôler les nématodes à galles in vitro.

Mots-clés: Produits de lutte biologique, *Meloidogyne* spp., Juvénile de deuxième stade

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Background

Tomato (*Solanum lycopersicum* L.) is considered an important vegetable crop grown in Kenya and in the world (Waiganjo *et al.,* 2013). It is of economic value as the crop provides domestic and nutritional requirement, generation of income, foreign exchange earnings and creation of employment (Sigei *et al.,* 2014). Tomato accounts for 14 % of the total vegetable products and 6.72% of the total horticultural crops in terms of production (MOA, 2012). The production however, faces the challenge of pests and diseases (Maerere *et al.,* 2006). Plant parasitic nematodes cause severe damage to vegetables leading to yield losses all over the world (Karssen and Moens, 2006). Tomato industry in Kenya has faced a major drawback through infestation by parasitic nematodes with *Meloidogyne* spp. alone causing 90-100% yield loss in tomato crop (Olabiyi, 2008). Furthermore RKN (Root knot nematodes) form disease complexes with pathogenic fungi and bacteria hence increase the damage that lead to great yield losses.

The most effective methods for control of *Meloidogyne* spp. in the past have been the use of nematicides (Onkendi *et al.,* 2014). However, high costs of nematicides in the market and their harmful effects on numerous beneficial microbes found in the soil limit their

use (Faruk *et al.,* 2011). Chemical nematicides have also been reported to have high toxicity to humans and animals and also cause environmental pollution (Nofal, 2009) causing serious threat to the ecosystem. These concerns over chemical pesticides in respect of groundwater contamination, residues on food, and development of resistance to pests have prompted research for safer management alternatives such as biological control (Hussaini, 2014).

Nematophagous fungi including *Aspergillus* sp., *Paecilomyces lilacinu*s (Goswami and Mittal, 2004; Mukhtar *et al.,* 2013), *Pochonia chlamydosporia* (Mukhtar *et al.,* 2013) and *Trichoderma* sp. (Mukhtar *et al.,* 2013; Al-Hazmi *et al.*, 2016) have showed suppressive effects against nematodes including Meloidogyne genus. Most of these fungal antagonists can be found in most agricultural soils and provide an inexpensive environmental friendly technique for management of parasitic nematodes. Most of the research on the application of biological control methods has been conducted outside Africa. Some bio-control agents have been isolated from the local conditions and have shown potential in managing RKN. More research however needs to be conducted to identify appropriate local biological agents or to introduce agents from outside and then test for efficacy and survival under local conditions. This study focused on isolation and identification of fungal antagonists associated with tomato and eggs of RKN and evaluated their efficacy against RKN in vitro and later in vivo.

Materials and methods

Collection of samples. Rhizosphere soils and tomato root samples were collected from 20 randomly selected tomato growing farmers' in Mwea, Kirinyaga County. Three samples of rhizosphere soil and tomato roots (healthy and galled) were randomly sampled from each farm in a zigzag pattern and dug to a depth of approximately 15-20 cm. The rhizosphere soils adhering to the tomato roots system were separated and taken to the laboratory for processing.

Isolation of fungi from healthy tomato roots. The healthy tomato roots were washed under tap water and then surface sterilized by dipping them in 0.5% sodium hypochlorite for three minutes followed by rinsing in sterile distilled water. The roots were then air dried and cut into 1cm sections which were aseptically plated on PDA medium and the plates sealed with parafilm. The plates were incubated at room temperature and fungal growth observed and sub cultured unto fresh PDA medium to obtain the pure cultures.

Isolation of fungal isolates from egg masses. The eggs collected through root maceration and egg masses handpicked using forceps from the galled roots under dissecting microscope were placed aseptically in Petri dishes containing PDA amended with Streptomycin. The Petri dishes were then incubated for seven days. Pure cultures were obtained by sub culturing. All the pure cultures were maintained in PDA slants stored under refrigeration for further use.

RKN inoculum preparation. The second stage juveniles used in this study were extracted from heavily galled roots through root maceration where eggs collected were incubated to hatch into the J2s. The juveniles were used immediately for bioassays and some stored under refrigeration.

Fungal identification. The isolated fungi were identified through morphological characterization. Macroscopic characterization involved observation with naked eyes where the fungal isolates were differentiated through colony appearance, color and growth rate. Microscopic characterization involved slide culture technique where the fungal isolates were further identified up to the genera level based on shape of conidia, presence or absence of micro and macro-conidia and type of fungal mycelium. Molecular identification of the most active isolates is ongoing.

Effect of fungal isolates on Juvenile mortality. An experiment was laid in the Kenyatta University agricultural laboratory in a completely randomized design with 45 treatments replicated three times. To test for effects of the fungal isolates on juvenile mortality, 1ml containing twenty juveniles of *Meloidogyne* sp. was transferred into 5 cm Petri dishes. The plates were then inoculated with fungal spore suspension and water as control then incubated at 28°C. Spore suspension used was prepared by flooding a 14 days old pure culture with sterile distilled water then spores were dislodged using sterile glass slide and passed through a three layer muslin cloth. The spore concentration was subsquently determined using a haemacytometer slide under microscope. After 7 days of incubation, juveniles were observed under microscope. Juveniles were considered dead if they became rigid and fail to exhibit any response after probing the tail with a mounting pin. Treatments were replicated three times and the percentage of death per each treatment calculated according to the following formula:

$$\text{Juveniles mortality} = \frac{100 \times \text{dead J2}}{\text{total no. of J2}}$$

Results

Fungal isolates. A total of 45 fungal isolates were obtained, 27 isolates from the tomato roots and 18 isolates from RKN eggs collected from Kirinyaga County. The fungal isolates included *Fusarium* spp., *Trichoderma* spp., and *Penicilium* spp., from both roots and eggs while *Paecilomyces* spp. was only isolated from RKN eggs. *Trichoderma* spp. (33.3%) was the most abundant followed by *Fusarium* spp. (28.9%) and the least abundant were *Penicillium* spp. (2.2 %) and *Paecilomyces* spp. (2.2 %).

Trichoderma spp. These had rapid growth since the colony diameter after five days was full plate (9.0 cm). The colonies first appeared white on PDA medium three days after initial plating but later grew yellowish green colonies ether arranged in concentric rings or the green conidia would be distributed throughout the plate (Plate 1). The reverse side appeared pale. Conidia were abundant, one celled and ovoid while the conidiophores were extensively branched.

Plate 1. *Trichoderma* spp.

Fusarium spp. They grew as a cream white, pink or purple colony while the reverse side was colorless, pink or purple and cottony on PDA medium. They produced numerous single or double celled micro conidia which were sickle shaped with slightly curved ends. Their growth was considered moderate since the colony diameter got to full plate after seven days of incubation (Plate 2.).

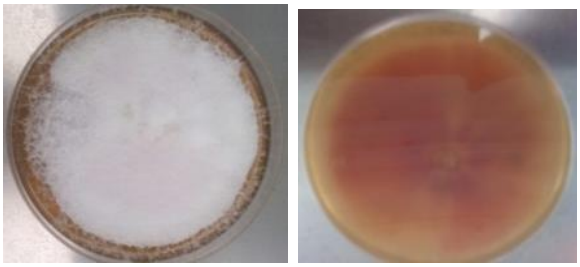

Plate 2. *Fusarium* spp.

Paecilomyces spp. These grew as a white colony but turned pinkish upon sporulation while the reverse was cream white or pale (Plate 3). They had more divergent conidiophores and branches. These had a slow growth with colony diameter of 5.0 cm after 14 days of incubation.

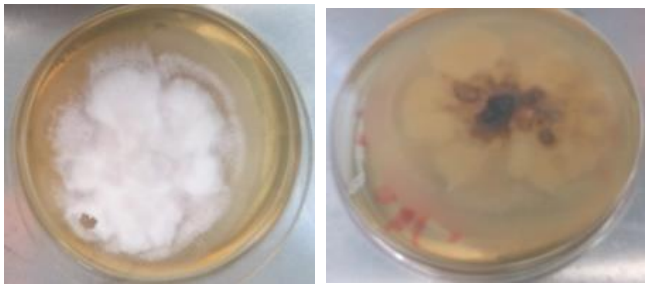

Plate 3. *Paecilomyces* spp.

Effect of fungal isolates on mortality of second stage juveniles. The effect of the fungal isolates on juvenile (J2) mortality showed significant difference (P≤ 0.05) compared to the control. The isolates were considered effective if they achieved 50% J2 mortality after the 7 days. The highest J2 mortality observed was 88.3%± 6.01 with *Trichoderrna* spp 13. isolate followed by 86.67 % ± 6.67 by the *Trichoderma* spp 1. and *Paecilomyce*s spp. after seven days (Table 1).

Discussion

Potential antagonistic fungi were isolated from tomato roots and RKN eggs sampled from Kirinyaga County. These isolates included *Trichoderma* spp., *Paecilomyce*s spp., *Penicillium* spp., and *Fusarium* spp. some of these have been isolated from field grown tomatoes by other researchers (Kariuki *et al.,* 2012). The results agree with studies on fungal endophytes associated with tomatoes which include *Paecilomyces* spp., *Verticillium* spp., *Fusarium* spp., *Aspergillus* spp., *Trichoderma* spp., and *Colletotrichum* spp. (Dorcas *et al.,* 2010).

Table 1. Effect of fungal isolates on J2 mortality rate (%)

Isolate code	Fungal genera	Juvenile 2 mortality (%)
MR8	*Trichoderma* spp 13.	88.33 ± 10.41 a
NCI	*Paecilomyces* spp.	86.67 ± 11.55 ab
MR7	*Trichoderma* spp 1.	86.67 ± 5.77 ab
ME1	To be identified	81.67 ± 7.64 abc
MR2	*Fusarium* spp 12.	80.00 ± 5.00 abc
MR19	*Trichoderma* spp 2.	80.00 ± 13.23 abc
MF3	*Aspergillus* spp. 1.	80.00 ± 5.00 abc
MR9	*Trichoderma* spp 14.	75.00 ± 25.98 abcd
MR17	To be identified	75.00 ± 5.00 abcd
MR11	*Fusarium* spp 1.	75.00 ± 30.41 abcd
M4	To be identified	65.00 ± 10.00 bcdef
MF4	*Aspergillus* spp 2.	63.33 ± 20.82 cdef
MR6	*Trichoderma* spp 3.	63.33 ± 16.07 cdef
MR16	*Fusarium* spp 2.	61.67 ± 22.55 cdef
MR18	To be identified	60.00 ± 18.03 cdefg
MR15	*Fusarium* spp.	56.67 ± 25.66 defgh
MF5	*Aspergillus* spp 3.	56.67 ± 7.64 defgh
MR3	*Fusarium* spp 3.	55.00 ± 5.00 defgh
MR4	*Trichoderma* spp 4.	55.00 ± 8.66 defgh
MR13	*Fusarium* spp 13.	51.67 ± 7.64 efghi
ME9	*Trichoderma* spp 15.	51.67 ± 12.58 efghi
Control	Untreated (water)	1.67 ± 2.89 m
L.S.D	-	21.805

Means in the same column followed by the same letters are not significantly different (P>0.05) according to Fisher's LSD test. Each value is the mean of three replicates (n=3). LSD: Least significant difference.

In this study the isolated fungi; *Trichoderma* spp., *Paecilomyces* spp., *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. showed significant nematicidal activity through mortality of the *Meloidogyne* spp., second stage juveniles. Similar results were reported by Kibunja (2010) where indigenous isolates of *Trichoderma* spp. and *Aspergillus* spp. from Coastal region of Kenya had significantly higher juvenile mortality rate when compared to the control. The 1.67% mortality recorded in the control could be attributed to presence of dead juveniles before starting the experiment and shock to J2 due to disruption during the experiment.

These results are in line with other findings by Singh and Mthur (2010) who reported that fungi isolated from egg masses of RKN including *Trichoderma* spp., *Aspergillus* spp. and *Paecilomyces* spp., caused mortality of second stage juveniles *in vitro.*

The significant juvenile mortality could be due to various antagonism mechanisms exhibited by the fungal isolates. For example, *Trichoderma* spp., particularly *T. harzianum* is reported to act through antibiosis, mycoparasitism and secretion of cell wall degrading enzymes such as cellulose and chitinase to suppress *Meloidogyne* spp. (Harman *et al.,* 2004). *Fusarium* spp. has been reported to have toxic effect on nematodes through egg hatching inhibition and J2 killing (Singh and Mthur, 2010).

Conclusion

According to the results of this study, indigenous fungal isolates including *Trichoderma* spp., *Aspergillus* spp. and *Paecilomyces* spp. have the potential of controlling root knot nematodes in vitro.

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