

1. This manuscript cannot be accepted in the present form and can be submitted after revisions.

2. Authors need to address following points and resubmit.

1. The manuscript seems very interesting and contains a huge data set and also can be a good contribution to the scientific community. But it cannot be accepted in the present form.

2. I strongly recommend the revision of the manuscript including abstract, methodology, results and also discussion. As it contains a huge data set on various aspects. It is creating confusions and unable to put precise and significant outcomes.

3. Abstract needs to be rewritten as it given very detail as well as incomplete results. Methodology needs to incorporate some parameters included in the results. Discussion is not precise, clear and parallel with the results.

4. A literature review supporting the earlier reports of the same studies needs to be incorporated.

5. Grammatical mistakes need to be improved.

Original Research Article

COMPATIBILITY OF ENDOPHYTIC *Colletotrichum nigrum* WITH VELUM® (SYNTHETIC NEMATOCIDE) AGAINST ROOT-KNOT NEMATODES ON TREE TOMATO

ABSTRACT

Root-knot nematodes (RKNs) hinder agricultural production worldwide. Despite many methods of control, management of RKNs involving combined application of endophytic fungi and Velum® is sought to leverage on their synergistic antagonism. This study was carried out between September 2022 to February 2023 to evaluate compatibility of endophytic *Colletotrichum nigrum* isolated from roots of tree tomato and commercial *Trichoderma asperellum* with Velum® to control RKNs *in vitro* and in the greenhouse on tree tomato. Leading discs from the colony of *C. nigrum* and *T. asperellum* were removed and inoculated onto PDA amended with Velum® and incubated for seven days at 25°C. Un-amended media served as control. Forty milliliters of 1×10^6 spores/ml of *C. nigrum* and *T. asperellum* alone and in combinations with Velum® (1ml/L) were tested for their efficacy against second

Comment [s1]: Abstract seems to be very elaborative and lengthy. It needs to be reframed with significant outcomes and results. Methodology part needs to be removed from abstract. A detailed methodology is not needed here.

Comment [s2]: Sentence is very confusing, grammatically incorrect and unable to explain the proper methodology.

Comment [s3]: If *T. asperellum* is used then why not mentioned in the title of the research paper?

stage juveniles (J2s) of RKN in the greenhouse. *In vitro* and greenhouse tests were replicated four times in Completely Randomized Design. The positive control was inoculated with J2s only while the negative control had no J2s and no treatments. Data was analyzed by Anova SAS, 9.2 and means separated by Tukey's Honestly Significant Differences test at $P \leq 0.05$. There was no significant inhibition in the colony growth of *C.nigrum* and *T. asperellum* by Velum® *in vitro* and therefore were found to be compatible. *Colletotrichum nigrum* combined with Velum® significantly ($P \leq 0.05$) reduced the RKN J2s, nematode reproduction factor, galling and egg mass indices in both experiments more than when applied alone. There was also a significant increase in the plant growth parameters of tree tomato treated with *C. nigrum* combined with Velum®. This study has shown that combining *C. nigrum* and *T. asperellum* with Velum® successfully controlled RKNs on tree tomato. These findings could be used to promote integrated nematode management using less toxic nematicides and biocontrol agents.

Key words: Root-knot nematodes; second stage juveniles (J2s), *Colletotrichum nigrum*; Velum®; *Trichoderma asperellum*, Endophytic fungi

1.3. INTRODUCTION

Tree tomato belongs to family solanaceae, a perennial crop that is grown in tropical and subtropical climatic conditions (Ramírez-Gil, 2017). In Kenya, it is an economically important horticultural crop that contributes to rural income generation (Muriithi *et al.*, 2013) and has high nutritional value (AFA, 2021) as well as contributing over 8 million USD annually. The fruits are oval with thick, smooth and shiny covering. When ripe, the fruits are consumed raw or turned into juice. Iron, phosphorus, potassium, calcium, antioxidants, and vitamins A, B, C, and E are abundant in the fruits (Pedrosa, 2016). These nutritional benefits make it to be popularly cultivated around the world.

However, the roots of this crop are seriously attacked by root-knot nematodes (RKNs) causing losses and lowering the yields (Waswaet *et al.*, 2020). Root-knot nematodes are a major threat to horticultural crops across the world (Sikora and Fernandez, 2005). The RKNs cause serious damage to roots and severely reduce yields of many crops such as tree tomatoes, tomatoes, bananas (Jones *et al.*, 2013). Root-knot nematodes are widely distributed in most agricultural soils attacking over 3000 plants globally and causing more than 100 USD billion losses each year threatening development of sustainable agriculture (Abad *et al.*, 2003, Huang *et al.*, 2006; Jones *et al.*, 2013). Infestation with root-knot nematode leads to stunted growth, chlorosis and root shortening. They also cause root galls which interfere with the plants' ability to absorb water and mineral nutrients. The RKNs also cause above-ground symptoms that are usually confused with mineral deficiencies and other diseases (Dahlinet *et al.*, 2019).

Managing RKNs is difficult because of their high rate of multiplication and short life cycle (Trudgil and Blok, 2001). Several methods have been used to control RKNs. Previously; RKNs have been controlled significantly by broad spectrum chemicals that are harmful to users and the environment. Therefore, alternative methods of management are required to reduce the usage of harmful chemicals (Huang *et al.*, 2018). With the increasing demand to meet the challenges of food and nutritional security, management of RKNs is no longer a single approach but integrative strategies (D'erricoet *et al.*, 2019). Going forward, the control of RKNs should not only focus on sustainable methods of management but also by wise use of chemical nematicides for holistic management (Chen *et al.*, 2020; Orlando *et al.*, 2020). Potential biocontrol agents like endophytic fungi that are antagonistic to RKNs are important. Endophytic fungi have shown efficacy against nematodes through production of toxic secondary metabolites, sticky hyphae and

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colonization of nematode eggs and bodies (Li *et al.*, 2015). Endophytic fungi present a significant strategy of managing RKNs in promoting sustainable agriculture. There has been no single method that has been able to eradicate RKNs and BCAS e.g. *Penicillium lilacinum* have shown inconsistencies in their performance against RKNs in the greenhouse and in the field (Gine and Sorribas, 2017). Their potential in the management of RKNs can be improved by combining with nematicides which have low toxicity profiles such as Velum[®] (active ingredient = fluopyram) to leverage on their synergistic effects. Application of endophytic fungi in combination with new chemical nematicides could be a more attractive approach than applying each one of them alone or singly (Dahlinet *et al.*, 2019). Velum[®] has been effectively used against RKNs ((Singh *et al.*, 2017; Dahlinet *et al.*, 2019). In consideration, non-chemical management of RKNs is a cost effective method. However, they are sometimes less effective than chemical nematicides. Thus, use of non-chemical approaches in combination with compatible chemicals could be used in comprehensive management of RKNs (Chen *et al.*, 2020). However, there has been no documentation on the efficacy of combining endophytic *Colletotrichum nigrum* from roots of tree tomato with Velum[®] against RKNs. Thus, using new chemicals that have low toxicities such as Velum[®] in combination with endophytic fungi is currently preferred (Chen *et al.*, 2020; Dahlinet *et al.*, 2019). Therefore, combining such chemicals with endophytic *C. nigrum* to manage RKNs might be a better strategy than applying each of them individually. There has also been no study documenting the effects of Velum[®] has on endophytic *C. nigrum* performance against RKNs on tree tomato and hence the importance of this study.

The main objective of this study was to evaluate the compatibility of endophytic *Colletotrichum nigrum* with chemical nematicide, Velum[®] to demonstrate their synergistic effect in the management of RKNs *in vitro* and in the greenhouse.

2.4. METHODOLOGY

2.1 Sampling

Tree tomato (*Solanum betaceum* Cav.) root samples were collected from thirty farms randomly selected using purposive sampling technique. Ten fresh healthy roots were collected from each farm in Kinangop sub-county (-5.546 ° S, 36.5536 ° E) located in Nyandarua County lying at 0.1804°S and 36.5230°E in Kenya. The roots were put into zip lock bags and transported to Kenyatta University Agriculture Laboratory for processing. The *in vitro* and greenhouse experiments were carried out at Kenyatta University main campus (1.1805° S and 36.9348° E).

2.2 Isolation of endophytic fungi from tree tomato roots

Sterilization of roots was done according to Dababat *et al.* (2008) protocol. The roots were cut into 5 cm length, thoroughly washed with tap water and sterilized with 70 % ethanol for 3 minutes to remove surface epiphytes. The roots were then sterilized in 1.5 % NaOCl for three minutes. Sterilized roots were rinsed three times with sterile distilled water; blot dried using sterile blotting papers and cut into 0.5 cm length using sterile scalpel blades (Dababat *et al.*, 2008). The 0.5 cm root pieces were evenly placed on sterilized potato dextrose agar (PDA) media amended with 150 mg/l each of streptomycin-sulphate to inhibit bacteria contamination. The PDA plates were sealed with parafilm and incubated at 25 °C until endophytic fungi emerged. To evaluate the integrity of sterilization, water from the last rinse was plated onto a fresh PDA media and incubated at 25 °C for seven days. To obtain pure cultures, fungal cultures of the isolate were sub-cultured on a fresh PDA media using discs from the leading mycelia margins taken by flame sterilized 5 mm cork borer and incubated for two weeks.

Comment [s6]: Sentences needs to be reframed

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The endophytic fungus was identified morphologically using macroscopic characteristics of the colonies (colour, margin, texture, density and margin- Plate 1: A, B) and microscopic features of conidia and mycelia under the microscope stained by Lactophenol cotton blue (Plate 1: C).

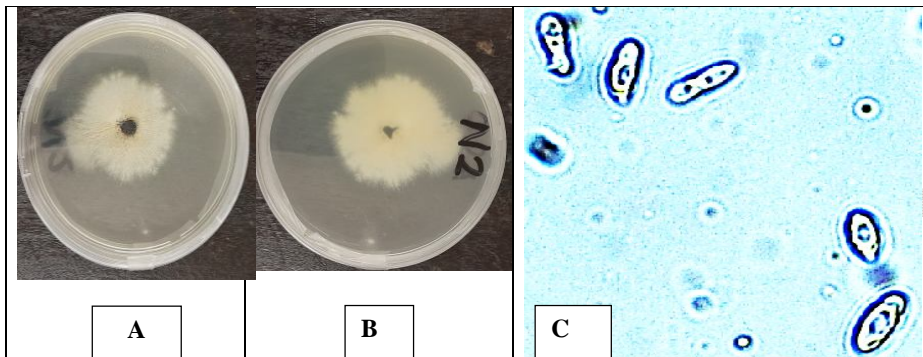


Plate 1: A: *C. nigrum* colony Reverse, B: *C. nigrum* colony Front and C: *C. nigrum* conidia (x1000)

2.3 *In vitro* experiment

One milliliter of Velum[®] suspension was dispensed into a 1 L conical flask containing 1 L sterilized molten PDA media and gently agitated for even mixing. The media was amended with 150 mg/l each of streptomycin-sulphate. The mixture was then poured into 90 mm petri dishes and allowed to cool and solidify. The discs from actively growing margin of the colony of endophytic *Colletotrichum nigrum* or *Trichoderma asperellum* were removed using a sterile 5 mm cork borer and inoculated onto PDA media amended with Velum[®]. Control plates contained un-amended sterile PDA media. The PDA plates were sealed with parafilm and incubated for seven days at 25 degrees Celsius. The treatments were replicated four times each and arranged in CRD. The treatments included:

- i) Plates amended with Velum[®] + endophytic *Colletotrichum nigrum*
- ii) Plates amended with Velum[®] + Commercial *Trichoderma asperellum*
- iii) Un-amended Plates treated with *C. nigrum*
- iv) Un-amended Plates treated with *T. asperellum*

The colony diameter from radial growth was measured from the underside of each petri dish. The percentage inhibition of growth over the control was calculated using the formula:

$$I = \frac{C - T}{C} \times 100 \text{ (Behdani et al., 2012)}$$

Where **I** = percent inhibition, **C** = colony diameter in control plate, and **T** = colony diameter in treated plate. The experiment was repeated once for accuracy.

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Comment [s10]: The purpose of using *T. asperellum* should be mentioned in methodology

2.4 Greenhouse experiment

2.4.1 Mass multiplication of endophytic fungi

The spores of the fungi were mass-multiplied using sorghum grains following the procedures of Cumagun and Moosavi. (2015). The grains were soaked in water, sterilized in an autoclave and inoculated with four 5 mm discs of 5 day old cultures per 200g and incubated in black polythene bags for 14 days. The grains were dried, blended into powder and passed through 50µm sieve. The spore powder was then mixed thoroughly with sterile talc powder (carrier material) in 1:2 ratio and Carboxymethyl cellulose (sticking agent) at 5 g/kg of the product (Sing *et al.*, 2016). Accurate spore density (1×10^6 spores/ml) of the fungi was determined by a hemocytometer.

2.4.2 Preparation of nematode inoculum

Root-knot nematode J2s were reared on a susceptible tomato variety (Cal-J) for three months in the Agriculture greenhouse at Kenyatta University. Galled roots were used to obtain nematode inoculum. An egg mass from a single female was picked using a needle to establish pure cultures for the experiments. The RKN egg mass was put in the sterile 2 sand: 1 soil mixture in pots (12 cm diameter) at the root zone of four week old transplanted susceptible tomato plants of cultivar Cal- J. and maintained in the greenhouse. After three months, plants were uprooted, galled roots were washed, chopped into 1cm, macerated in 1.5 % NaOCl solution in a blender and the suspension passed through 500 µm, 106 µm and 20 µm sieves into a beaker (Hooper *et al.*, 2005). The resultant suspension containing RKN eggs was incubated on plates lined with a serviette in darkness for 14 days and freshly hatched J2s were collected from fourth day every two days (Coyne *et al.*, 2007). The numbers of freshly hatched J2s per ml were determined under the microscope at $\times 40$ using grid-49 nematode counting dish (Hussey and Barker, 1973). The nematode suspension was adjusted with sterile distilled water to 2000 J2s/ml for use in the greenhouse.

2.4.3 Nursery establishment

A certified nursery (Kenya Agriculture, Livestock and Research Organization at Horticulture Research Institute) provided the susceptible cultivar of tree tomato seeds, which were then immersed in sterilized distilled water for a day. After soaking, the seeds were placed in sterile petri plates, lined with moist tissue paper and wrapped with Parafilm. The petri plates were covered with aluminum foil and kept on the benches in the greenhouse for 12 to 15 days. Following germination, the seeds were placed in sterile media-filled germination trays and maintained for four weeks before being transplanted into pots.

2.4.4.4.4. Preparation of growth media for greenhouse experiments

A 2:1 proportion of sand to soil was thoroughly mixed and heated-sterilized to 100 degrees Celsius for 48 hours. To prevent contamination, 2 kilograms of the sterilized media were placed in thoroughly cleaned plastic pots (12-cm-diameter) that had been sterilized with 1.5% of NaOCl.

2.4.5 Greenhouse treatments

Sterile soil media (2 sand: 1 soil) was placed in sterile plastic pots in the greenhouse. At transplanting, 40 ml of 1 ml/L Velum[®] nematicide was applied to the soil in pots. One four-week old tree tomato seedling was then transplanted per pot. Seedlings were treated with 40 ml of 1×10^6 spores/ml of either endophytic *Colletotrichum nigrum* commercial *Trichoderma asperellum* soil drench at transplanting. Pots were inoculated with 2000 J2s of RKN each. Pots inoculated with J2s of RKN only served as positive control while un-treated pots

without nematode J2s served as negative control (absolute control). The treatments had four replicates each and arranged in CRD. The treatments were as follows:

- i) *Colletotrichumnigrum* + Velum[®] + Nematodes
- ii) *C. nigrum* + Velum[®] - Nematodes
- iii) *Trichodermaasperellum* + Velum[®] + Nematodes
- iv) *T. asperellum*+ Velum[®] - Nematodes
- v) *C. nigrum* + Nematodes
- vi) *C. nigrum* - nematodes
- vii) *T. asperellum* + Nematodes
- viii) *T. asperellum* - Nematodes
- ix) Velum[®] + Nematodes
- x) Velum[®] - Nematodes
- xi) Nematodes alone
- xii) Untreated and un-inoculated

Comment [s11]:

The experiments were terminated 90 days after transplanting.

2.4.6 Evaluation of root-knot nematode disease parameters

The J2s of RKN were extracted from 200 cm³ of soil or 5 g of roots using modified Baermann technique (Hooper *et al.*, 2005; Coyne *et al.*, 2007). Five grams of roots were washed and chopped into 1 cm pieces and macerated in a blender for one minute. Nematodes were extracted using the above technique. The set-ups were incubated for 48 hours for nematode recovery. Then the nematode suspension was poured into 20µm sieve, backwashed gently and collected into a beaker and concentrated to 10 ml using a test sieve. Then 2 ml suspension of nematodes was pipetted into a nematode counting dish and nematodes counted at ×10 under dissecting microscope and enumerated from the original concentrated suspension for each sample. The nematodes were expressed per 200 cm³ of dry soil or 5 g of roots.

Galling and egg mass indices were determined using a scale of 0-5 (Quesenberry *et al.*, 1989) as shown in Table 1 below. Individual root systems were washed, and galling/egg mass indices were scored.

Table 1: Scoring scale for root-knot nematode galling and egg mass indices

Score	Description
0	No galls or No egg masses per root system
1	1-2 galls or egg masses per root system
2	3-10 galls or egg masses per root system
3	11-30 galls or egg masses per root system
4	31-100 galls or egg masses per root system
5	>100 galls or egg masses per root system

Phloxine-B (Holbrook *et al.*, 1983) was used to stain root systems to visualize egg masses and RKN females. The roots were immersed in phloxine-B for 20 minutes (Daykin and Hussey, 1985), and then rinsed in tap water to remove excess stain before viewing under a dissecting microscope.

The nematode reproduction factor (Rf) was determined from final nematode populations (Pf) and initial nematode populations (Pi) expressed as a ratio of Pf: Pi.

2.4.7 Evaluation of plant growth parameters

A centimeter graduated ruler was used to measure plant shoot height from the soil baseline to the newest apical shoots at the conclusion of the experiment. At the end of the experiment (90 days after transplanting), the dry weights of root and shoot were measured in grams. Plants were gently uprooted, and shoots and roots were separated by cutting at the base. To remove adhering soil, the roots were washed in flowing water under the tap and dried with blotting paper. The shoots and roots were placed in separate paper bags with clear labels and oven-dried at 60°C for three days to achieve constant mass.

2.5 Data analysis

The data was organized in excel sheets and analyzed using SAS version 9.2 computer software's one way analysis of variance (ANOVA). Tukey's Honest Significant Differences (HSD where $P \leq 0.05$) was used to separate the significant means. Regression and correlation analysis were done to evaluate the relationship between RKN disease parameters

Comment [s12]: Which growth parameters? And what is the methodology used?

3.5. RESULTS

3.1 Compatibility of Velum[®] prime with endophytic *Colletotrichum nigrum* and *Trichoderma asperellum* against J2s of RKN

3.1.1 Effect of Velum[®] on colony growth of endophytic *Colletotrichum nigrum* isolate and commercial *Trichoderma asperellum* in vitro

There was no significant difference ($P \geq 0.05$) in the colony diameter of endophytic *C. nigrum* exposed to Velum[®] compared to the untreated control (*C. nigrum* alone) as shown in Table 2 (experiments I and II) and plate 2. Commercial *T. asperellum* colony diameter did not also differ significantly with the un-treated control (*T. asperellum* only) on exposure to Velum[®] in experiment I and II as shown in Table 2 and plate 2.

Table 2: Effect of Velum[®] on growth (colony diameter) of *Colletotricum nigrum* isolate and *Trichoderma asperellum*

Treatments	Expt. I	Expt. II
	% Inhibition	% Inhibition
Isolated <i>C. nigrum</i> + Velum [®]	1.00 ± 0.79a	1.02 ± 0.73a
Isolated <i>C. nigrum</i> alone (control)	0.00 ± 0.00a	0.00 ± 0.00a
Commercial <i>T. asperellum</i> + Velum [®]	1.01 ± 0.58a	0.99 ± 0.33a
Commercial <i>T. asperellum</i> alone (control)	0.00 ± 0.00a	0.00 ± 0.00a
P-value	0.29	0.27

Comment [s13]: No need to write commercial everywhere. Once it is explained that is sufficient.

Data are means ± SE of four replicates. Means followed by the same letter(s) on the same column are not significantly different ($P \geq 0.05$) according to Tukey's Honestly Significant Difference (HSD) test

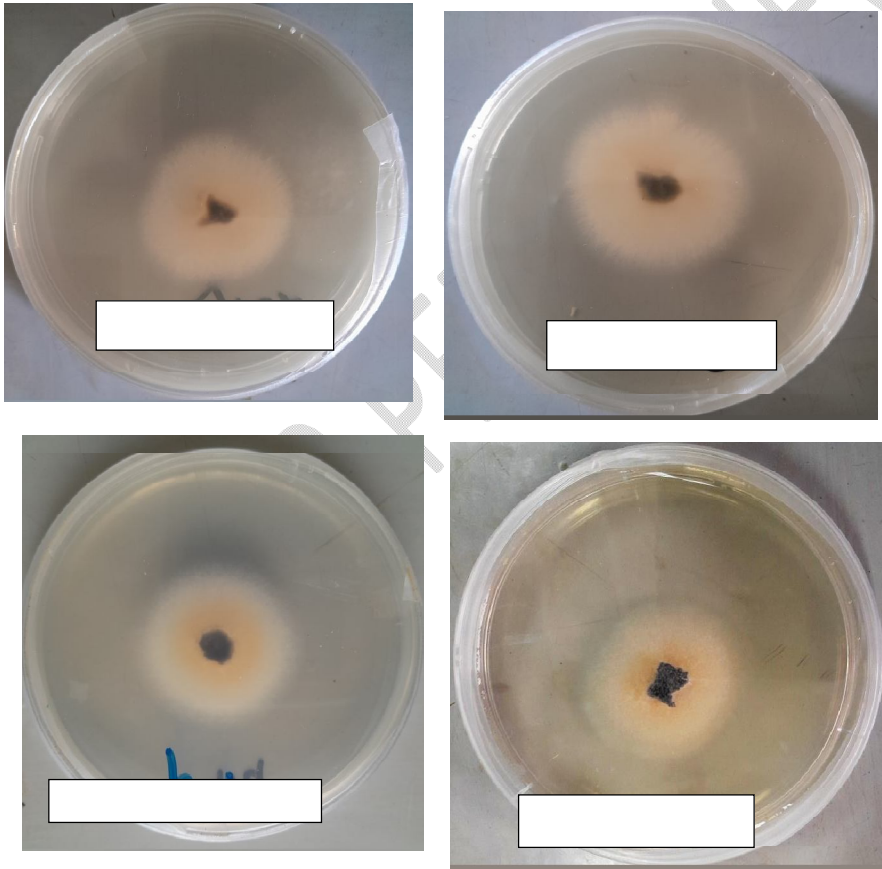


Plate 2: Effect of Velum[®] on colony diameter of endophytic *Colletotricum nigrum* and commercial *Trichoderma asperellum* in vitro

3.1.2 Efficacy of combining *Colletotrichumnigrum* and *Trichodermaasperellum* with Velum® against RKNs on tree tomato; Greenhouse tests I and II

3.1.2.1 Effect on second stage juveniles of RKN population in soil and roots

The soil population of J2s differed significantly ($P \leq 0.05$) among the treatments (Table 3). Nematode-inoculated plants treated with *C. nigrum* + Velum® had the lowest population of J2s per 200 cc of dry soil followed by those treated with *T. asperellum* + Velum® and Velum® alone, respectively (Table 3). All treatments significantly reduced nematode populations in the rhizospheric soil compared to the positive control.

The second stage juvenile populations (J2s) in roots were statistically different ($P \leq 0.05$) between all treatments. Nematode-inoculated plants with *C. nigrum* + Velum® treatment had the lowest J2s per 5g of dry roots followed by those treated with *T. asperellum* + Velum® and Velum® alone, respectively (Tables 3). All treatments significantly reduced RKN J2 populations in roots as compared to the positive control.

3.1.2.2 Effect on RKN reproduction factor

The RKN reproduction factor was statistically different ($P \leq 0.05$) between different treatments as shown in Table 3. The *C. nigrum* + Velum® treatment significantly reduced RKN reproduction factor followed by *T. asperellum* + Velum® and Velum® alone relative to positive control.

Table 3: Efficacy of combining *Colletotrichum nigrum* and *Trichoderma asperellum* with Velum® on J2 populations and RKN reproduction factor on tree tomato in the greenhouse test I and II

RKN disease parameters						
Treatments	Greenhouse test I			Greenhouse test II		
	J2s in soil	J2s in roots	RF	J2s in soil	J2s in roots	RF
<i>C.nigrum</i> + J2s	549.17 ± 24.85b	186.25 ± 23.04b	0.28 ± 0.01b	542.50 ± 12.96b	182.50 ± 31.52b	0.27 ± 0.01b
<i>T.asperellum</i> + J2s	580.83 ± 18.00b	198.75 ± 12.14b	0.29 ± 0.01b	574.17 ± 31.56b	191.25 ± 4.73b	0.29 ± 0.02b
<i>C. nigrum</i> + Velum® + J2s	215.83 ± 9.26d	45.00 ± 28.50d	0.11 ± 0.01cd	208.33 ± 12.29d	41.25 ± 4.27d	0.10 ± 0.01cd
<i>T. asperellum</i> + Velum® + J2s	226.67 ± 5.27d	57.50 ± 12.50d	0.14 ± 0.02cd	219.17 ± 9.44d	51.23 ± 2.39d	0.11 ± 0.01cd
Velum® + J2s	303.27 ± 6.21c	111.35 ± 9.05c	0.17 ± 0.03c	312.00 ± 14.07c	107.25 ± 9.08c	0.15 ± 0.02c
Positive control (J2s alone)	3223.33 ± 30.46a	516.25 ± 30.44a	1.61 ± 0.02a	3211.67 ± 113.81a	498.75 ± 14.63a	1.61 ± 0.06a
Negative control	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00d	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00d
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Data are means ± SE of four replicates. Means followed by different letter(s) in the same column are significantly different according to Tukey's Honestly significant difference (HSD) test at $P \leq 0.05$. **J2s** = second stage juveniles of RKNs; **Rf** = Nematode reproduction factor calculated as a ratio of final nematode populations (Pf) to initial nematode populations (Pi). Negative control = untreated control without nematodes.

Regression analysis indicated a positive linear association between nematode reproduction factor (Rf) and RKN J2 populations (Figure 1). A further investigation showed that the Rf and soil J2 populations correlated positively ($r=1$, $P=0.05$).

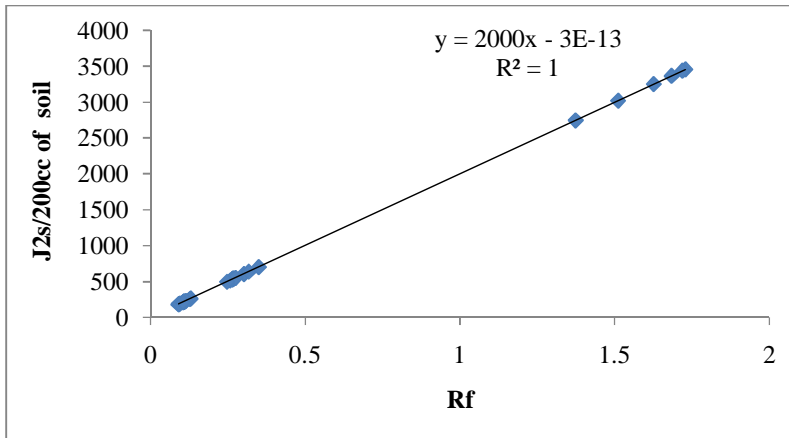


Figure 1: Relationship between nematode reproduction factor and J2 populations in the soil (Greenhouse test)

3.1.2.3 Effect on galling index and egg mass index

There was an existence of statistical difference ($P \leq 0.05$) in terms of indices on gall and egg masses among the treatments (Table 4). Plants treated with *C. nigrum* + Velum[®] had the lowest gall and egg mass indices followed by those treated with *T. asperellum* + Velum[®] and Velum[®] alone as shown in Table 4. The least gall and egg mass indices reduction was shown in *T. asperellum* standalone treatment.

Table 4: Efficacy of combining *Colletotrichum nigrum* and *Trichoderma asperellum* with Velum on indices of galling and egg masses on tree tomato in the greenhouse tests I and II

Treatments	Plant disease parameters			
	Greenhouse test I		Greenhouse test II	
	GI	EMI	GI	EMI
<i>Colletotrichumnigrum</i> + Nematodes	3.67 ± 0.21b	2.75 ± 0.25bc	3.83 ± 0.09b	3.00 ± 0.00b
<i>Trichodermaasperellum</i> + Nematodes	3.83 ± 0.17b	3.25 ± 0.25b	3.67 ± 0.56b	3.33 ± 0.50b
<i>C. nigrum</i> + Velum® + Nematodes	1.50 ± 0.43cd	1.50 ± 0.29cd	1.00 ± 0.45d	1.17 ± 0.31d
<i>T. asperellum</i> + Velum® + Nematodes	2.00 ± 0.43cd	2.00 ± 0.00c	1.50 ± 0.43d	1.33 ± 0.33d
Velum® + Nematodes	2.25 ± 0.51 c	2.05 ± 0.01c	2.00 ± 0.50c	2.00 ± 0.00c
Positive control (Nematodes alone)	5.00 ± 0.00a	5.00 ± 0.00a	5.00 ± 0.00a	5.00 ± 0.00a
Negative control (untreated/uninoculated)	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00e	0.00 ± 0.00e
P-value	<.0001	<.0001	<.0001	<.0001

Data are means ± SE of four replicates. Means followed by different letter(s) in the same column are significantly different according to Tukey's Honestly significant difference (HSD) test at $P \leq 0.05$. **J2s** = second stage juveniles of RKNs; **GI** = Galling index; **EMI** = Egg mass index; Negative control = untreated control without nematodes. GI and EMI were scored on a scale of 0-5, where 0 = no gall/egg masses; 1=1-2 galls/egg masses; 2=3-10 galls/egg masses; 3=11-30 galls/egg masses; 4=31-100 galls/egg masses and 5=>100 galls/egg masses (Quesenberry *et al.*, 1989).

3.1.2.4 Effect on shoot height

The heights of plants in various treatments significantly differed ($P \leq 0.05$) from one another. The heights of uninoculated (without nematodes) plants treated with *C. nigrum* and *Trichoderma asperellum* as standalone treatments had statistically higher ($P \leq 0.05$) shoots than those of the negative control (without nematodes and without treatments) as shown in Table 5. The heights of nematode-inoculated *C. nigrum* + Velum[®], *T. asperellum* + Velum[®], *C. nigrum* and *T. asperellum* treated plants were significantly higher than those of the positive control (nematodes alone). The results showed that all treatments increased the height of shoots of tree tomato relative to the positive control (Plate 3 and Table 5).

Comment [s14]: All the parameters should be explained in methodology.

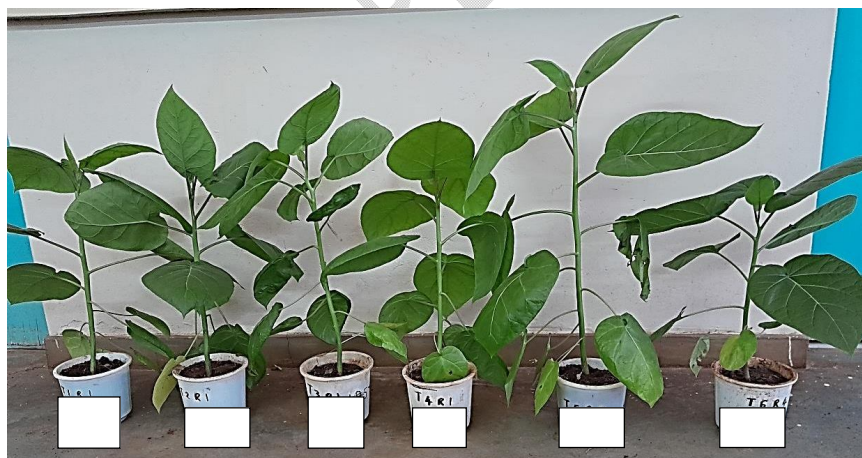




Plate 3: Effect of combining *Colletotrichum nigrum* and *Trichoderma asperellum* with Velum on shoot height of tree tomato in the greenhouse test.

a - h represents treatments where; **a** = inoculated + *Colletotrichumnigrum*; **b** = inoculated + *Trichodermaasperellum*; **c** = inoculated + *C. nigrum* + Velum®; **d** = inoculated + *T. asperellum* + Velum®; **e** = negative control (un-treated and un-inoculated); **f** = Positive control (un-treated and inoculated); **g** = *C. nigrum* alone; and **h** = *T. asperellum* alone

3.1.2.5 Effect on dry root weight

There was significant difference ($P \leq 0.05$) in dry root weight of plants among different treatments (Table 5). The highest dry root weight was recorded in un-inoculated (without nematodes) *C. nigrum* treated plants followed by un-inoculated plants treated with *T. asperellum* and negative control (untreated and un-inoculated). All un-inoculated treated plants had significantly higher dry root weights than those of inoculated treatments. The dry root weights of inoculated treatments had significantly higher dry root weights than those of the positive control (nematodes alone). *Colletotrichumnigrum* + Velum® had significantly higher dry root weight among nematode inoculated treatments compared to the positive control.

3.1.2.6 Effect on dry shoot weight

The dry shoot weights were significantly different ($P \leq 0.05$) among the treatments (Table 5). The dry shoot weights of un-inoculated and treated plants were significantly higher than those of the inoculated and the positive control. Un-inoculated plants treated with *C. nigrum* and Un-inoculated plants treated with *T. asperellum* had higher dry shoot weights as compared to the negative control (Table 5). The dry shoot weights of inoculated and treated plants were significantly higher than those of the positive control. Inoculated plants treated with *C. nigrum* + Velum® had the highest dry shoot weights compared to the positive control.

Table 5: Effect of combining *Colletotrichum nigrum* and *Trichoderma asperellum* with Velum on shoot height (cm) and dry weights (gms) of tree tomato in the greenhouse test I and II

Plant growth parameters						
Treatments	Greenhouse test I			Greenhouse test II		
	SH	DRW	DSW	SH	DRW	DSW
<i>C. nigrum</i> + Velum [®] + J2s	35.18 ± 0.64c	4.56 ± 0.07b	8.25 ± 1.5b	35.67 ± 2.57d	4.20 ± 0.40b	9.19 ± 1.32b
<i>C. nigrum</i> + Velum [®] - J2s	43.98 ± 1.49ab	6.84 ± 0.65 ab	10.25 ± 0.25ab	45.87 ± 1.11b	6.27 ± 0.58ab	11.34 ± 0.50a
<i>T. asperellum</i> + Velum [®] + J2s	34.37 ± 1.76c	3.99 ± 0.79c	8.08 ± 0.92b	35.05 ± 2.00d	3.85 ± 0.21b	9.01 ± 1.14b
<i>T. asperellum</i> + Velum [®] - J2s	43.81 ± 1.44ab	6.72 ± 0.69b	10.13 ± 0.96ab	45.03 ± 1.10b	6.23 ± 0.50ab	11.17 ± 0.53a
<i>C. nigrum</i> + J2s	33.22 ± 1.77c	3.11 ± 0.12c	7.87 ± 0.31c	34.92 ± 2.25d	3.09 ± 0.34bc	8.71 ± 1.06b
<i>C. nigrum</i> - J2s	46.87 ± 1.41a	7.64 ± 0.16a	11.65 ± 0.43a	48.67 ± 0.77a	7.15 ± 0.26a	12.14 ± 0.38a
<i>T. asperellum</i> + J2s	33.02 ± 0.59c	3.00 ± 0.66c	7.10 ± 0.53c	33.91 ± 1.15d	2.93 ± 0.24bc	8.64 ± 0.51b
<i>T. asperellum</i> - J2s	46.53 ± 0.50a	7.31 ± 0.48a	11.22 ± 0.27a	48.33 ± 0.98a	7.05 ± 0.39a	11.80 ± 0.56a
Velum [®] + J2s	34.01 ± 1.70c	4.14 ± 0.88b	6.99 ± 1.45c	34.47 ± 2.21d	4.03 ± 0.38b	6.09 ± 1.12c
Velum [®] - J2s	39.10 ± 0.57b	4.85 ± 0.81b	7.03 ± 0.41c	40.21 ± 0.99c	4.22 ± 0.40b	7.06 ± 1.09c
Positive control (J2s alone)	26.08 ± 0.64d	2.03 ± 0.33d	5.09 ± 0.20d	29.25 ± 1.91e	1.97 ± 0.20c	5.84 ± 0.43d
Negative control	42.63 ± 1.52ab	6.37 ± 0.64ab	10.02 ± 0.26ab	44.50 ± 1.68b	6.15 ± 0.49ab	10.47 ± 0.44ab
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Data are means ± SE of four replicates. Means followed by different letter(s) in the same column are significantly different according to Tukey's Honestly significant difference (HSD) test at P ≤ 0.05. **J2s** = second stage juveniles of RKNs; **SH** = shoot height; **DRW** = Dry root weight and **DSW** = Dry shoot weight. Negative control = untreated control without nematodes.

4.6. DISCUSSION

4.1 Compatibility of Velum prime with endophytic *Colletotrichum nigrum* and commercial *Trichoderma asperellum* against RKNs on tree tomato

Velum[®] (nematocide) did not inhibit the colony growths of *C. nigrum* and *T. asperellum* on PDA media. These results corroborate a research carried out by Kibunja. (2015) on the effect of selected endophytic fungi and resistant tomato cultivars on *Meloidogyne* spp. The author tested the compatibility of Mocap (active ingredient: ethoprophos= organophosphate) and *Trichoderma* spp. and reported that Mocap had no significant effect on *Trichoderma* isolates. In this study, this could be due to Velum[®] being a systemic non-fumigant is relatively less toxic to soil microflora (Wada and Toyota, 2008). Non-fumigants are said to have little effect on organisms that lack the nervous system since these nematocides act as cholinesterase inhibitors in the neurons (Bhattacharjee and Dey 2014).

Comment [s15]: The reference given is not clear and unable to explain the what finding are supportive???

The reduction in plant disease parameters could be due to production of nematotoxic secondary metabolites (flavonoids, terpenoids, trichodermin, colletonoic acid etc) and direct parasitism by the biocontrol fungi. Other studies have shown that biocontrol endophytic fungi produce toxic metabolites against RKNs (Kumar *et al.*, 2023; Yao *et al.*, 2023; Meyer *et al.*, 2020). Velum[®] is known to act against soil pathogens and enhance plant health (Chen *et al.*, 2020). The nematicidal effect of Velum[®] on nematodes has been variously described (Beeman and Tylka, 2018; Faske and Hurd, 2015; 2016; Roper, 2017). This could explain the reduced disease parameters by the combined application of biocontrol fungi with Velum[®]. Other studies have highlighted the nematicidal effect of Velum[®] (Dahlin, *et al.*, 2019). *C. colletotrichum nigrum* could have worked synergistically with Velum[®] against the RKN J2s resulting into decreased disease parameters and enhanced plant growth. These results are in agreement with Dahlin, *et al.* (2019) who noted that combining *Purpureicillium lilacinum* (strain PL251) with Velum[®] reduced J2 populations of *M. incognita* by 68% and gall index to 1.8 as compared to control (3.8). The same treatment also enhanced yield of tomatoes under greenhouse tests. Muthulakshmi *et al.* (2012) also found that combining carbofuran with biocontrol agents significantly increased the number and weight of potato tubers per plant and reduced potato cyst nematode populations in potatoes.

Comment [s16]: The sentence is not clear.

The biocontrol fungi increased the plant growth parameters of tree tomato when applied alone and in combination with Velum[®] in the greenhouse tests. As standalone applications, these biocontrol fungi (*C. nigrum* and *T. asperellum*) could be possessing plant growth promoting abilities. This could explain the increased shoot heights and dry weights of root and shoot of tree tomato plants. Some fungi such as *Trichoderma* spp. are known to act as biofertilizers which improves plant growth (Kubheka and Ziena, 2022). *Trichoderma* spp. have been used to produce volatile chemicals (alkaloids, colletonoic acid, esters, alcohols, ketones etc) against soil pathogens, solubilizes phosphates to make them available for plant absorption in acidic soils and also enhances uptake of micro and macro- nutrients by plants (Kubheka and Ziena, 2022). The endophytic fungi are also known to confer protection to plants against other pathogens (Silva Santos *et al.*, 2022) and this could explain the reduced plant disease parameters shown by their application in this study. Another research showed that endophytic *Colletotrichum tofieldiae* promoted plant growth of maize and tomato *in-vitro* resulting into higher shoot heights and weights (Conzalez-Diaz Sandra, *et al.*, 2020). Silva Santos *et al.* (2022) also found out that inoculating tomato plants with *Colletotrichum siamense* increased plant biomass. As combined treatments (Biocontrol fungi + Velum[®]) were able to enhance plant growth parameters (shoot

Comment [s17]: There is no link in the previous and next paragraph

height and dry weights) and also reduced plant disease parameters (nematode populations, egg masses and gall indices and reproduction factor).

Tree tomato farmers should utilize cost effective management options to maximize on their profit. This may involve use of biological control agents (BCAs) and/or nematicides. This could consist of combining compatible nematicides with biological agents for their additive effects against RKNs. The findings of this study could be used to promote integrated nematode management using less toxic nematicides and biocontrol agents especially under field conditions where efficacy of BCAs is limited (Koll et al, 2011).

5-7. CONCLUSION

Velum[®] was found compatible with endophytic *Colletotrichum nigrum* and commercial *T. asperellum*. This study demonstrated that their co-application significantly reduced root-knot nematode populations in the soil and roots of tree tomato and therefore have potential of being used in integrated nematode management especially under field conditions where efficacy of BCAs is erratic. The integration of chemical ~~nematocides~~ and biological approaches may become a critical component in the management of RKNs. Apart from Velum[®], other combinations should be tested for compatibility and applied as integrated approach rather than as standalone treatments in the management of RKNs. Integrated nematode management (INM) may not only be used for different categories of compatible RKN management but also embrace utilization of different components of BCAs in combination with favourable chemicals that have low toxicities.

6-8. RECOMMENDATION

Further research should be conducted to determine the mechanisms of action of the endophytic *Colletotrichum nigrum* on RKN J2s. Apart from Velum[®], other combinations should be tested for compatibility and applied as integrated approach rather than as standalone in the management of RKNs.

Data availability

All data supporting the findings of this study are presented in this paper.

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