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Compatibility of Microbial Bioagents Singly and in Combinations Against Plant-Parasitic Nematodes Infesting Sweet Orange Trees and Effects on Non-Parasitic Nematodes (Fungivorous and Free-Living Species)

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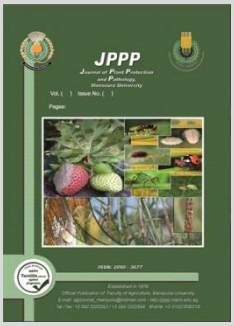

ABSTRACT

Four bioagents microbes were selected to assess effects singly and in combinations against plantparasitic nematodes (PPNs) and non-parasitic nematodes, in vitro and in vivo. Three of these microbes—*Trichoderma asperellum*, *T. harzianum*, and two strains of *Pseudomonas fluorescens* are widely utilized for nematode control, while the fourth, *Rhodosporidium paludigenum* as yeast, had not previously been tested against PPNs. The study evaluated the synergistic or antagonistic effects of these microbial mixtures. All combinations reduced PPN populations and increased other nematode species. Specifically, the mixture of *R. paludigenum* and *T. harzianum* was most effective against *Tylenchulus semipenetrans*, reducing its population by 68%, while *T. asperellum* combined with *P. fluorescens* strain 1 reduced *Helicotylenchus* spp. by 73.9%, and *P. fluorescens* strain 2 combined with *R. paludigenum* was most effective against *Xiphinema* spp., with a reduction of 67.2% after three months in the field. No reduction was observed in *Tylenchus* spp. and *Rhabditis* spp. These findings were corroborated by an *in vitro* experiment, where the mixture of *R. paludigenum* and *T. harzianum* deactivated the J2 stage of *T. semipenetrans* and reduced egg hatching by 63% and 20.6%, respectively, within four days of application. In conclusion, combining these tested microbes effectively reduces PPN populations in citrus without harming non-parasitic nematodes. It is recommended to incorporate these microbial mixtures with other biocontrol strategies to preserve soil health and reduce reliance on chemical nematicides in citrus groves.

Keywords: Citrus, Microbial mixtures, Plant parasitic nematodes, *Rhodosporidium paludigenum*, *Tylenchulus semipenetrans*.

INTRODUCTION

Plant parasitic nematodes (PPNs) have a damaging effect on citrus growing in Egypt and globally. Citrus trees are infested with many nematode species, including *Tylenchulus semipenetrans* (Cobb), *Helicotylenchus* spp. (Steiner), and *Xiphinema* spp. (Cobb), as the most distributed species, which can cause economic losses (Abd-Elgawad *et al.*, 2016; Ahuja and Somvanshi, 2021and Afzal *et al.,* 2021). Citrus nematode (*T. semipenetrans*) is the most dangerous species that infest citrus trees. The loss in crop production due to this nematode is estimated by 15-35% in crop yield losses (Afzal *et al.,* 2021) or 30-40% (Keshari and Mallikarjun, 2022). In Egypt, the economic losses of citrus production due to the infection by PPNs were estimated in 2016 at 10-30% (Abd-Elgawad *et al.,* 2016).

Citrus has economic importance in Egypt as a major exported crop for the European market. Egypt ranked 7th in the top 10 producing and exporting countries of those crops; about 1.3 million tons of oranges were exported in 2019, equal to 38 % of the world's exports; this percentage can be increased because of the expansion of the cultivation of these crops (Anonymous, 2020). This position was increased after one year to reach fifth. The fresh exported orange was 1.7 m. tons in 2022/2023 with a planted area of 172,200 ha. Most of this production is exported, primarily to Saudi Arabia, UAE, Russia, and the Netherlands; therefore, it ranked in the second category after South Africa in the continent of Africa (Anonymous, 2022).

The excessed use of the chemical nematicide is one of the biggest challenges facing citrus export for the global market due to the safety requirements set by the governments of these countries (Pretty and Bharucha, 2015). Therefore, in 2017 the Egyptian government passed many laws to rationalize the use of chemical pesticides on local farms, including encouraging farmers to use biopesticides, which raised the export efficiency of its crops, including citrus fruits (Anonymous, 2017).

On the other hand, regarding the cost-benefit of using biopesticides compared to chemicals, the bionematicides were effective as their low costs compared to the chemical nematicides; additionally, no environmental hazards were recorded with using these products (Abd-Elgawad and Askary, 2018). For example, the cost of the control PPNs on tomato plants in Egypt equals 187$ and 432$/ feddan using Oxamyl and Cadusafos, respectively. On the contrary, this cost decreased to 33$/feddan when using comparable bioproducts (Abd-Elgawad, 2020a).

These biopesticides varied; they could be bacteria, fungi, and sometimes other microorganisms like yeast. All these organisms shared with them their beneficial role in controlling soil-borne diseases, especially PPNs, without any environmental risk (Punja, 1997; Poveda *et al.*, 2020 and Lahlali *et al.,* 2022). This beneficial role extended to the non-parasitic nematode species by dramatically increasing their numbers and activity in the soil. These species play an essential antagonist role against soil-borne diseases; in addition, they decompose soil organic

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matter, which reflects plant health and growth (Yadav *et al.*, 2018 and Kekelis *et al.*, 2022).

The novelty of this research lies in its exploration of the synergistic effects of mixing various biocontrol agents, such as bacteria, fungi, and yeast, to manage PPNs in citrus cultivation. While previous studies have focused on the individual roles of these microbes, this study uniquely investigates how combining these biocontrol agents influences both PPNs and non-parasitic nematode species in citrus groves. By addressing the interaction between different microbial species and their collective impact on nematode communities, this research provides new insights into integrated pest management strategies. Additionally, it highlights the potential of microbial mixtures as a cost-effective, environmentally safe alternative to chemical nematicides, offering a sustainable solution to control nematode infestations while promoting soil health.

While the individual biological roles of these microbes in controlling PPNs have been well-documented, there is limited research on how mixing these microbes together may influence their combined effects on nematode species. Therefore, this study aims to investigate the biocontrol potential of mixing the tested microbes on the nematode community in citrus groves, with a particular focus on their impact on both plant-parasitic and non-parasitic nematode species.

MATERIALS AND METHODS

Preparation of the tested biocontrol agents and their binary forms for field and lab experiments

In this study, the tested microbes were determined for their efficacy on PPNs which were used individually in previous studies (Punja, 1997; Poveda *et al.,* 2020; Lahlali *et al.,* 2022 and Pires *et al.,* 2022). These microbes were *Rhodosporidium paludigenum* (Rp) as a yeast, *Trichoderma asperellum* (Ta), *T. harzianum* (Th) as a fungus, *Pseudomonas fluorescens* race1 (Pf1) and *P. fluorescens* race2 (Pf2) as a bacterium. The bioagent isolates *R. paludigenum* AUMC 7789, *T. harzianum* AUMC 5270, and *T. asperellum* AUMC 5570 were obtained from Assiut Mycological Center, Assiut University, Egypt.

The bacteria strains were provided by the Microbiological Resources Center (Mircen), Ain Shams University, Egypt, under their institutional accession protocols. Both isolates belong to the standard strain *P. fluorescens* ATCC 13525 Fig. (1). Race1 was confirmed through molecular analysis (GenBank accession: [AF207610] (https://www.ncbi.nlm.nih. gov/ nuccore/AF207610)), corresponding to its 16S rRNA sequence, while race 2 was verified as *P. fluorescens* (GenBank accession: [AY007258] (https://www.ncbi.nlm.nih.gov/ nuccore/AY007258).

To prevent contamination of the medium the strains were preserved in glycerol stocks at -80°C and routinely subcultured on nutrient agar to ensure purity. Species-specific PCR and sequencing were periodically performed to confirm identity and absence of contamination.

Therefore, the stock concentration of these microbes was prepared for each microbe as the following (2×10^6 CFU/ml) for Rp, Ta, and Th while Pf1 and Pf2 were (1×10^8 CFU/ml). These concentrations were attained from the Plant Pathology Department, Faculty of Agriculture, Zagazig University, Egypt.

Equal amounts (1000 ml) of each microbial concentration were mixed in a 3000 ml glass beaker for configuring the binary forms as follows (Rp+Ta), (Ta+Pf1), (Th+Pf2), (Rp+Th) and (Pf2+Rp). These microbial mixtures were applied later on marked trees as tested treatments and compared to the tested chemical nematicide.

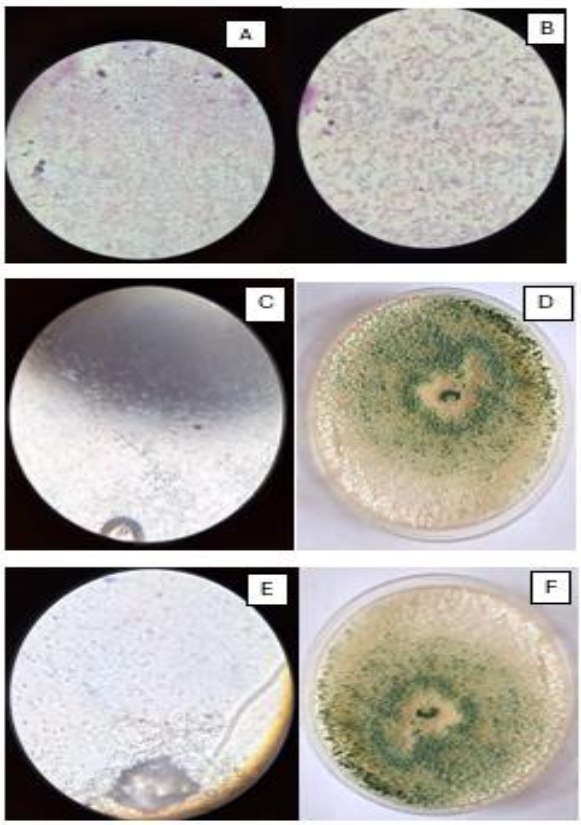

Fig. 1.The tested microbes tested aginst PPNs *Pseudomonas fluorescens* race1under light microscope (A), *P.fluorescens* race2 under light microscope (B), *Trichoderma harzianum* hyphates under light microscope (C) & in petri dish media (D),(E) *T. asperellum* hyhtes under light microscope & (F) in petri dish media .

Description of the experimental site

The field experiments were carried out along two successful fruiting seasons. They were conducted on a 4-ha farm cultivated with 13-year-old sweet orange (*Citrus sinensis*) grafted on sour orange rootstock *Citrus aurantium* cultivated in sandy clay soil (54% sand, 42% clay, and 4% silt). Trees are irrigated with a drip irrigation system and have the regular horticultural practices; no weeds grow between the trees. This farm is in Belbies distract, Al-Sharkia Governorate, Egypt. Five trees (replicates) were randomly marked inside a determined row to be one treatment; this process was done for all six treatments. Initial soil samples were collected from the marked trees from the canopy region at 25 cm depth and 1.5 m far from the trunk to determine the nematodes' initial population.

The collected samples were transferred in polyethylene bags to the nematology lab in the Faculty of Agriculture, Zagazig University, in an ice box, and stored in the refrigerator at 10 °C. The nematode was extracted on the second day using the (decanting method) a combination of sieves, and the Baermann trays technique (Van Bezooijen, 2006). After one week of the initial sampling, the experiment was conducted. First, the tested binary microbes were applied at 1500 ml of each mixture to the determined orange trees around it in the canopy region (1 to 2 m from the trunk) at 25 cm depth at the beginning of a not sunny or rainy day, and this mixture was added in the same quantity

weekly for three consecutive weeks and the first data had been recorded after a month of the end on the last week, this was repeated in the same way in the following season. The microbial density in the soil was measured one month after the treatment and at the end of the experiment (Martens, 1995). The weather conditions at the experiment site were observed to record any extreme changes. In contrast, the chemical nematicide was prepared to be compared with these bioagents. This nematicide was oxamyl (Vydate® 10% G), treated at 150g/ tree (59.5 kg/ha.) while the control treatment was without any applications.

The soil samples were collected after one, two, and three months at the nematode peak period (March, April, and May) in 2021 as the first season and the same months in 2022 as the second application season (El-Marzoky *et al.,* 2009 and Abd-Elgawad, 2020b). The soil samples were collected and nematodes were extracted, as explained above. After 24h. of extraction, the suspension was collected. The samples were examined in the Nematology Lab, Plant Protection Department, Zagazig University, Egypt (NLPPD) and the PPNs were morphologically identified using a fluorescence microscope (LEICA ICC50 HD) with a high-resolution digital camera at a 400x magnification power as in Fig. (2) (Mai and Lyon, 1975; Siddiqi, 1986 and Van den Berg *et al.,* 2017). Furthermore, *Tylenchus* spp. and *Rhabditis* spp. were recorded to attain population changes. All species were counted in one ml of the final extraction suspension, and the changes in the nematode numbers were calculated according to Equation (1) and (2)

$$\text{The reduction percentage (\%)} = \frac{Control - Treatment}{Control} \times 100 \quad (1)$$

$$\text{The percentage of increasing (\%)} = \frac{Treatment - Control}{Treatment} \times 100 \quad (2)$$

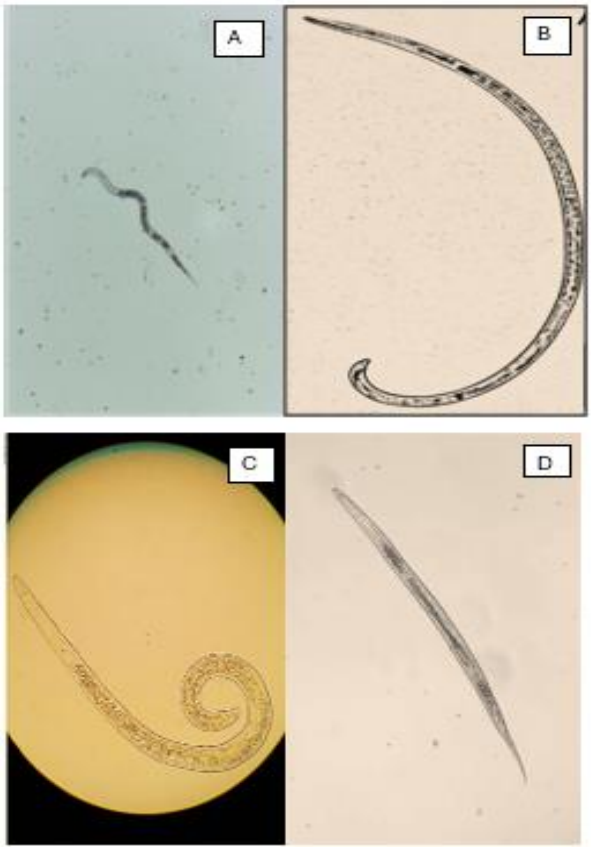

Fig. 2.The nematode species assoiated with citus trees (A) *Tylenchulus semipenetrans* J2 (B) *Xiphinema* spp. (C) *Helicotylenchus* spp. (D) *Tylenchus* spp.

Preparing second-stage juveniles (J2) and eggs of citrus nematode for lab experiments

A lab study was conducted to evaluate the suppressive effect of the binary mixtures of the tested microbes on citrus nematode stages (J2 and eggs). Therefore, five kg of the heavily infested soil was collected; this composite sample was collected from ten citrus seedlings of one-year-old sour orange (*C. aurantium*) cultivated in 50 cm pots containing light soil (70% sand + 30% loam) and artificially infected with a citrus nematode (J2+eggmasses) six months ago. The J2 was extracted from the soil sample using the same method above.

The final extraction solution was shaken well, and one ml was pipetted into a counting dish to count the J2. Each ml of the suspension was estimated to contain about 1100 J2. A 15 cm diameter sterilized Petri dish was used in the experiment. Initially, one ml of the nematode suspension was put in each Petri dish, and then five ml of the mixtures were added individually to each dish and replicated five times. On the other hand, the nematicide treatment was prepared using oxamyl 24% SL (soluble liquid) at 1000 ppm concentration (50 ml nematicide + 950 distilled water) and adding five ml to the dishes.

On the same side, the control treatment had no application; it contained one ml of nematode suspension + five ml of distilled water. All the dishes were arranged randomly and placed in a vertical incubator at 25 ±2°C and humidity at 75%. The immobile and straight J2 were counted, which were assumed inactive, not dead, because no chemical analysis was done on the J2 to confirm their death. The inactive J2s were recorded after 24,48, and 72 hours. The non-active J2 percentage was calculated according to equation (3).

$$\text{The non-active J2 percentage (\%)} = \frac{Numbers\ of\ non\ active\ J2}{Initial\ numbers\ of\ the\ J2} \times 100 \quad (3)$$

Regarding to egg preparation for the lab experiments, about ten g of the citrus root was collected from the infested seedlings mentioned above. These roots were soaked in the cleaned dish containing tap water for about ten minutes then carefully cut into pieces, each about 2 cm. These pieces were mixed with 200 ml of sodium hypochlorite solution (Naocl 0.5%) to dissolve the gelatinous matrix from the egg masses and separate the eggs. The solution was prepared by adding 20 ml of sodium hypochlorite 5% (commercial Clorox®) to 180 ml of distilled water. The root pieces and the solution were mixed well and shaken gently for 3 min.

Finally, the suspension was decanted through a 200-mesh sieve nestled upon a 500-mesh one. The impurities above the two sieves were immediately washed with light tap water to eliminate the Naocl and maintain egg vitality. Next, the collected eggs on the 500-mesh sieve were transferred with a small quantity of water to a 100 ml beaker. Then the number of eggs was counted using a counting dish on the one ml of the extraction suspension and stored in the refrigerator at 9 °C until using (Hussey and Barker, 1973 and Van Bezooijen, 2006). Next, one ml of the egg's extraction suspension containing about 500 eggs was pipetted in a 15 cm sterilized Petri dish, followed by adding five ml of the previously microbial mixtures individually. Each treatment was replicated five times. Finally, oxamyl was applied at the same concentration (1000 ppm).

Conversely, the control treatment contained eggs and distilled water only. The dishes were arranged randomly in an incubator at 25 ±2°C and humidity at 75%, and the number of non-hatched eggs was recorded after 24,48,72 h of application. The egg-hatching inhibition was calculated according to equation (4):

$$\text{The egg-hatching inhibition (\%)} = \frac{\text{Number of non-hatched eggs}}{\text{The initial number of the eggs}} \times 100 \quad (4)$$

Statistical analysis

The field experiments were implemented in a randomized complete block design. It was conducted in two successful fruiting seasons. Data were statistically analyzed using compare means analysis and calculating Duncan's multiple range test at probability level ($P \leq 0.05$) by SPSS (version 16) software (Anonymous, 2007).

RESULTS AND DISCUSSION

The effect of microbial mixtures on nematode community associated with sweet orange trees under field conditions

Three species of PPNs were recorded with sweet orange trees. These species were citrus nematode *T. semipenetrans*, spiral nematode *Helicotylenchus* spp., and sting nematode *Xiphinema* spp. Furthermore, *Tylenchus* spp. (fungivorus nematodes) and *Rhabditis* spp. (free-living nematodes) were identified as non-plant parasitic nematode species associated with the trees. Data in Table (1) showed the effect of the tested mixtures on PPNs and non-plant parasitic nematodes after one month of soil application during two sequenced fruiting seasons. In the first season, *T. semipenetrans* decreased by 53.3% in (Rp+Th) compared to 54.7 % in the nematicide treatment. While the least effective treatment was (Ta+Pf1) which decreased the population by 17.7%. On the other hand, the (Ta+Pf1) was the most effective mixture on *Helicotylenchus* spp. the reduction percentage was 40.3% compared to the nematicide treatment which was 52.2%. (Rp+Th) was the least effective in their effect on this species it was reduced number by 18.7%.

A different effect was recorded on *Xiphinema* spp., the (Pf2+Rp) was the most effective mixture compared to nematicide reducing the numbers by 29.8 and 53.8% respectively. In contrast, the (Rp+Ta) reduced the nematode number by 15.7%, the lowest reduction percentage.

Table 1. The effect of the combined biocontrol agents on nematodes community associated with sweet orange trees after one month of application.

Treatments	Nematode numbers in 250g soil									
	T. semipenetrans		*Helicotylenchus* spp.		*Xiphinema* spp.		*Tylenchus* spp.		*Rhabditis* spp.	
Season	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**
Negative control	3800.0a (0)	4153.3a (0)	89.3a (0)	97.7a (0)	99.6a (0)	109.0a (0)	97.0c (0)	106.0c (0)	93.3e (0)	102.3e (0)
Positive control (oxamyl)	1722.0f (-54.7)	1985.3f (-52.2)	42.7f (-52.24)	49.3f (-49.5)	46.0f (-53.8)	53.0f (-51.4)	89.0d (-8.2)	102.7c (-3.1)	47.7f (-48.9)	55.0f (-46.3)
(Rp+Ta)	1963.3d (-48.3)	2263.6d (-45.5)	58.3e (-34.7)	67.3d (-31.1)	84.0b (-15.7)	97.0b (-11.0)	171.0b (+43.3)	197.3b (+46.3)	120.3d (+22.5)	138.7d (+26.2)
(Ta+Pf1)	3126.3b (-17.7)	3604.6b (-13.2)	53.3d (-40.3)	61.3e (-37.2)	79.6c (-20.1)	91.3c (-16.2)	73.6e (-24.1)	85.0de (-19.8)	186.0a (+49.8)	214.3a (+52.3)
(Th+Pf2)	2670.0c (-29.7)	3078.6c (-25.9)	59.0e (-33.9)	68.0d (-30.4)	84.3b (-15.4)	97.3b (-10.7)	240.0a (+59.6)	276.7a (+61.7)	174.3c (+46.5)	201.0c (+49.1)
(Rp+Th)	1780.0f (-53.3)	2052.3f (-50.6)	72.6b (-18.7)	83.7b (-14.3)	73.3d (-26.4)	84.7d (-22.3)	75.0e (-22.7)	86.3d (-18.6)	180.3b (+48.2)	208.0b (+50.5)
(Pf2+Rp)	1899.3e (-50.0)	2190.0de (-47.3)	62.6e (-29.9)	72.3c (-25.9)	70.0e (-29.8)	81.0e (-25.7)	73.3e (-24.4)	84.7de (-20.1)	179.3c (+47.9)	206.6b (+50.5)

***Means in each separate column followed by the same letter(s) are not significantly different at P ≤0.05 according to Duncan's multiple range test.**
***Values between parentheses refer to percentages of the reduction (-) and the increasing (+) % according to the equations (1 and 2).**
*** *R. paludigenum* (Rp), *T. asperellum* (Ta), *T. harzianum* (Th), *P. fluorescens* race1 (Pf1), and *P. fluorescens* race2 (Pf2).**

Regarding the effect of the tested mixtures on non-target nematodes, it was monitored that (Rp+Ta) increased the nematode numbers by 43.3 and 22.5% for *Tylenchus* spp. and *Rhabditis* spp. respectively. in the same trend (Th+Pf2) increased the nematode numbers by 59.6 and 46.5% for the above-mentioned species, respectively. while, the exception of this trend was recorded in the treatment (Pf2+Rp), (Ta+Pf1), and (Rp+Th) it decreased *Tylenchus* spp. by 24.4, 24.1 and 22.7% successively, with no reduction effect on *Rhabditis* spp.

The same trend was recorded in the second year. The tested mixtures were arranged descendingly on their effect on *T. semipenetrans* as follows (Rp+Th), (Pf2+Rp), (Rp+Ta), (Th+Pf2), and finally (Ta+Pf1), with reduction percentages 50.6, 47.3,45.5, 25.9 and 13.2% respectively compared to the nematicide 52.2%.

The treatment (Ta+Pf1) was the most effective mixture that reduced *Helicotylenchus* spp. by 37.2% while the treatment (Rp+Th) was the least 14.3% in comparison with the nematicide treatment (49.5%). *Xiphinema* spp. was reduced by 25.7% in (Pf2+Rp) and this percentage was the lowest in (Th+Pf2) at 10.7% while this percentage was recorded (51.4%) in the nematicide treatment.

Regarding the effect of the tested mixtures on non-target nematodes, the treatment (Th+Pf2) increased the numbers of *Tylenchus* spp. by 61.7%. In comparison, the treatment (Ta+Pf1) increased the number of *Rhabditis spp.* by 52.3%.

After two months the results were clearer, this is described in Table (2). In the first season, *T. semipenetrans* decreased dramatically from 3895.0 J2/ 250 g soil in the control treatment to 1577.0 J2/ 250 g soil in (Rp+Th) treatment by 59.5%, and there is no significant difference between the last treatment and the nematicide treatment. The least effective treatment was (Ta+Pf1) which decreased the nematode number by 24.9%.

The treatment (Ta+Pf1) was the most effective no-chemical treatment on *Helicotylenchus* spp. it reduced nematode number by 58.4% and there are no significant differences between this treatment and (Rp+Ta), and

(Th+Pf2) which reduced numbers by 53.5 and 52.9% respectively while this percentage was 69.7% in the nematicide treatment.

The tested mixtures recorded varying effects on *Xiphinema* spp. no significant differences were recorded between (Th+Pf2) and (Rp+Ta) 73.0 and 72.7 individual/ in 250 g soil from one side and between (Ta+Pf1) and (Rp+Th) 68.3 and 62.0 individual/ in 250 g soil from the other side compared to the control treatment 109.7 individual/ in 250 g soil, the most effective treatment was gained by (Pf2+Rp) which recorded 46.5% compared to the nematicide treatment 68.4%.

Table 2. The effect of the combined biocontrol agents on nematodes community associated with sweet orange trees after two months of application.

Treatment	Nematode numbers in 250g soil									
	T. semipenetrans		*Helicotylenchus* spp.		*Xiphinema* spp.		*Tylenchus* spp.		*Rhabditis* spp.	
Season	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**
Negative control	3895.0a (0)	4257.3a (0)	103.3a (0)	113.0a (0)	109.7a (0)	120.0a (0)	121.3d (0)	132.7e (0)	107.7e (0)	117.7e (0)
Positive control (oxamyl)	1519.0f (-61.0)	1751.3g (-58.9)	31.3e (-69.7)	36.0f (-68.1)	34.7e (-68.4)	40.0f (-66.7)	66.0e (-45.6)	76.0f (-42.7)	33.7f (-68.7)	39.0f (-66.85)
(Rp+Ta)	1760.3d (-54.8)	2029.7d (-52.3)	48.0d (-53.5)	55.3d (-51.0)	72.7b (-33.7)	83.7b (-30.3)	254.0b (+52.3)	293.0b (+54.7)	193.3d (+44.3)	223.0d (+47.2)
(Ta+Pf1)	2923.3b (-24.9)	3370.7b (-20.8)	43.0d (-58.4)	49.6e (-56.0)	68.3c (-37.7)	79.0c (-34.2)	156.7c (+22.6)	180.7c (+26.6)	260.0a (+58.6)	300.0a (+60.8)
(Th+Pf2)	2467.0c (-36.7)	2844.3c (-33.2)	48.7d (-52.9)	56.0d (-50.4)	73.0b (-33.4)	84.0b (-30.0)	323.3a (+62.5)	373.0a (+64.4)	247.3c (+56.5)	285.0c (+58.7)
(Rp+Th)	1577.0f (-59.5)	1818.3f (-57.3)	62.3b (-39.7)	72.0b (-36.3)	62.0c (-43.5)	71.3d (-40.6)	158.0c (+23.2)	182.0c (+27.1)	253.3ab (+57.5)	292.0b (+59.7)
(Pf2+Rp)	1695.3e (-56.5)	1954.7e (-54.1)	52.3c (-49.4)	60.3c (-46.6)	58.7d (-46.5)	67.7e (-43.6)	153.0c (+20.7)	176.3d (+27.8)	252.3ab (+57.3)	291.0b (+59.6)

***Means in each separate column followed by the same letter(s) are not significantly different at P ≤0.05 according to Duncan's multiple range test.**
***Values between parentheses refer to percentages of the reduction (-) and the increasing (+) % according to the equations (1 and 2).**
*** *R. paludigenum* (Rp), *T. asperellum* (Ta), *T. harzianum* (Th), *P. fluorescens* race1 (Pf1), and *P. fluorescens* race2 (Pf2).**

Regarding the effect of the tested mixture on no-targeted nematodes, the tested mixtures were arranged for their increasing effect on *Tylenchus* spp. as follows: (Th+Pf2), (Rp+Ta), (Rp+Th), (Ta+Pf1) and (Pf2+Rp). Whereas the effect of the tested mixtures on the *Rhabditis* spp. was arranged descending by (Ta+Pf1), (Rp+Th), (Pf2+Rp), (Th+Pf2) and (Rp+Ta).

In the second season, no differences were recorded compared to the first season. The (Rp+Th) was the most effective mixture on *T. semipenetrans* which decreased the juvenile number by 57.3% while the (Ta+Pf1) was more effective on *Helicotylenchus* spp. the decreasing percentage recorded at 56.0%. Finally, the (Pf2+Rp) decreased *Xiphinema* spp. by 43.6%. Oppositely the (Th+Pf2) increased *Tylenchus* spp. by 64.4% and (Ta+Pf1) increased *Rhabditis* spp. by 60.8%.

After three months of application, the vision became clearer, and these appeared in Table (3). In the first season, *T. semipenetrans* numbers decreased by a percentage close to or exceeded fifty percent in all applications. The nematicide was the most effective treatment followed by (Rp+Th), (Rp+Ta), (Pf2+Rp), (Th+Pf2), and finally (Ta+Pf1) with reduction percentages of 71.1, 69.7, 65.1, 47.7 and 36.4% respectively.

Table 3. The effect of the combined biocontrol agents on nematodes community associated with sweet orange trees after three months of application

Treatment	Nematode numbers in 250g soil									
	T. semipenetrans		*Helicotylenchus* spp.		*Xiphinema* spp.		*Tylenchus* spp.		*Rhabditis* spp.	
Season	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**
Negative control	4045.0a (0)	4421.0a (0)	128.3a (0)	140.3a (0)	125.7a (0)	137.3a (0)	132.3e (0)	144.7e (0)	147.7c (0)	161.3e (0)
Positive control (oxamyl)	1169.3g (-71.1)	1348.3g (-69.5)	20.3f (-84.2)	23.3f (-83.4)	18.7f (-85.2)	21.7f (-84.2)	53.3f (-59.7)	62.0f (-57.1)	32.7d (-77.9)	37.7f (-76.7)
(Rp+Ta)	1410.3e (-65.1)	1626.0de (-63.2)	37.0d (-71.2)	42.7d (-69.6)	56.7b (-54.9)	65.3b (-52.4)	339.0b (+60.9)	391.0b (+63.0)	370.3b (+60.1)	427.0d (+62.2)
(Ta+Pf1)	2573.3b (-36.4)	2967.0b (-32.9)	32.0e (-75.1)	36.7e (-73.9)	52.7c (-58.1)	60.7c (-55.8)	241.7c (+45.2)	278.7c (+48.1)	437.0a (+66.2)	503.7a (+67.9)
(Th+Pf2)	2117.0c (-47.7)	2441.0c (-44.8)	37.7d (-70.7)	43.3d (-69.1)	57.0b (-54.6)	65.7b (-52.2)	408.3a (+67.6)	471.0a (+69.3)	424.3a (+65.2)	489.3b (+67.0)
(Rp+Th)	1227.0f (-69.7)	1414.7f (-68.0)	51.3b (-60.0)	59.3b (-57.7)	46.0d (-63.4)	53.0d (-61.4)	243.0c (+45.5)	280.0c (+48.3)	430.3a (+65.7)	496.0a (+67.5)
(Pf2+Rp)	1545.0d (-61.8)	1781.3d (-59.7)	45.3c (-64.7)	52.3c (-62.7)	42.3e (-66.1)	45.0e (-67.2)	188.3d (+29.7)	203.7d (+28.9)	399.7ab (+63.1)	460.7bc (+64.9)

***Means in each separate column followed by the same letter(s) are not significantly different at P ≤0.05 according to Duncan's multiple range test.**
***Values between parentheses refer to percentages of the reduction (-) and the increasing (+) % according to the equations (1 and 2).**
*** *R. paludigenum* (Rp), *T. asperellum* (Ta), *T. harzianum* (Th), *P. fluorescens* race1 (Pf1), and *P. fluorescens* race2 (Pf2).**

All treatments decreased *Helicotylenchus* spp. and *Xiphinema* spp. numbers by exceeding fifty percent as follows: the nematicide as (84.2& 85.2%), (Rp+Ta) as (71.2&54.9%), (Ta+Pf1) as (75.1&58.1%), (Th+Pf2) as (70.7&54.6%), (Rp+Th) as (60.0 &633.4%) and (Pf2+Rp) as (64.7 & 66.1%) for abovementioned species successively.

Regarding the effect of the tested treatments on *Tylenchus* spp. and *Rhabditis* spp., it was found that the individuals increased by (60.9 & 60.1%) in (Rp+Ta), (45.2 & 66.2%) in (Ta+Pf1), (67.6 & 65.2 %) in (Th+Pf2), (45.5& 65.7%) in (Rp+Th) and (29.7 & 63.1%) in (Pf2+Rp) sequentially for abovementioned species.

Data was confirmed in the second year, the most effective mixture on *T. semipenetrans* was (Rp+Th) which reduced it by 68.0% while the least effective was (Ta+Pf1) by 32.9%. Conversely, *Helicotylenchus* spp. reduced by 73.9% in (Ta+Pf1) and 57.7% in (Rp+Th). Ultimately, *Xiphinema* spp. reduced by 67.2% in (Pf2+Rp) and the least percentage gained was in (Th+Pf2) by 52.2%.

Considering the effect of the tested mixtures on *Tylenchus* spp. it increased by percentages varied between 69.3% in (Th+Pf2) and 28.9% in (Pf2+Rp) while in *Rhabditis* spp. increased by percentages ranged between 67.9% in (Ta+Pf1) and 62.2% in (Rp+Ta).

The effect of the tested microbial mixtures on *T. semipenetrans* under laboratory conditions

To confirm the obtained results of field experiments, a lab experiment was conducted to show the effect of the tested microbial mixtures on J2 activity and egg hatching of *T. semipenetrans* as a major PPN on citrus, data were recorded after two, four, and seven days after application, as well as numbers of immotile J2 and non-hatched eggs were recorded for each period separately. Data in Fig.3 show the effect of the tested mixtures on J2 mobility and egg hatching percentages by the time elapsed, after two days, significant differences existed between all treatments on J2 motility. The most effective treatment that reduced J2 motility was (Rp+Th) 16.7 % which was more than the nematicide treatment (15.2%) followed by (Pf2+Rp), (Rp+Ta), (Th+Pf2), and (Ta+Pf1) by 6.8, 5.8, 4.9 and 3.9% respectively compared to the control treatment (1.7%).

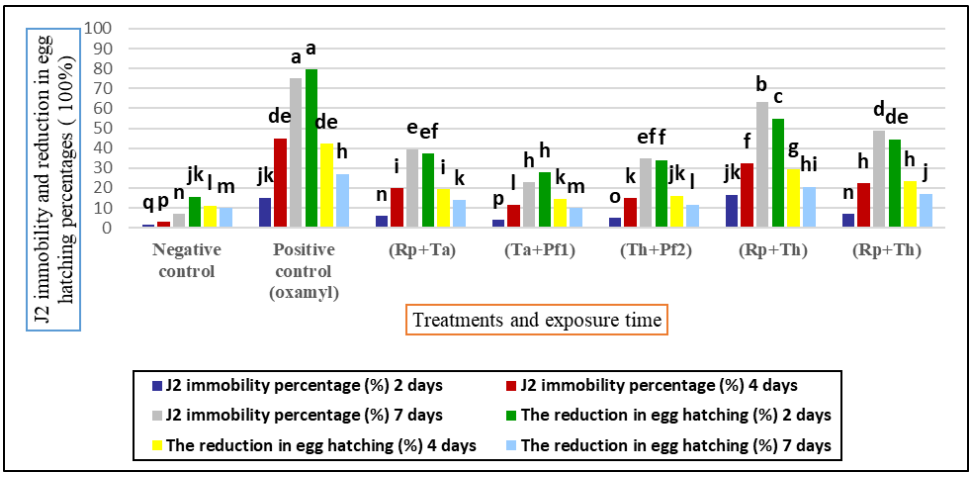

Fig. 3. The effect of exposure time on *T. semipenetrans* J2 and egg hatching for the tested mixtures after two, four, and seven days of application. The columns followed by the same letter are not significantly different according to the LSD test (P≤0.05).

The effect on the egg-hatching was described by counting the unhatched eggs (the reduction in the egg hatching%), these percentages recorded 54.9, 44.5, 37.2, 34.0 and 27.7% in (Rp+Th), (Pf2+Rp), (Rp+Ta), (Th+Pf2) and (Ta+Pf1) respectively while the percentage was 79.6% in the nematicide treatment and 15.7% in the control treatment.

After four days the number of nonmotile J2 and unhatched eggs increased. The J2 immobility percentage increased to 32.3% in (Rp+Th), 22.5% in (Pf2+Rp), 19.8% in (Rp+Ta), 14.8% in (Th+Pf2), and 11.3% in (Ta+Pf1) in comparison with the nematicide treatment 45.0%.

Regarding the effect of the tested mixtures on egg-hatching, the highest effect was the mine of (Rp+Th) 29.5% followed by (Pf2+Rp) 23.4%, (Rp+Ta) 19.4%, (Th+Pf2) 16.1%, while (Ta+Pf1) was the least effective treatment (14.5%). On the other hand, the nematicide treatment decreased egg hatching by 42.1%.

After seven days more accepted effects were recorded. It was found the tested mixture reduced J2 motility by 63.0, 48.7, 39.5, 34.7 and 22.8% in (Rp+Th), (Rp+Th), (Rp+Ta), (Th+Pf2) and (Ta+Pf1) respectively, in comparison with the value of the nematicide which was 75.0%. On the same trend, the effect on the egg hatching was in a similar arrangement, recorded at 20.6, 17.2, 13.8, 11.6, and 9.8% for the above-mentioned mixtures sequentially.

As mentioned above, some of the tested mixtures had reduced PPN numbers associated with citrus trees with an efficiency close to that of the chemical nematicide; as a result, it can use these microbial mixtures as an alternative eco-friendly and economical method for controlling PPNs in citrus orchards. Many authors tested the effect of microorganisms against PPNs, but there is no evidence data available about the effect of mixing those microbes.

The effect of the marine red yeast *Rhodosporidium paludigenum* on PPNs was not studied before, therefore for discussing the results, it was supported by the other yeast species studied before e.g., Hashem *et al.* (2008) screened the efficacy of twenty-two yeast strains in suppression of root-knot nematode *M. incognita* on flame seedless grapevines The most effective strains were *Pichia gluilliermondii* Moh10, *Pachytrichospora transvaalensis* Y 1240, *Candida albicans* Moh Y-5 and *Geotichum terrestre* Y 2162. The reduction effect of the yeast may be explained by increasing plant resistance to PPNs by supporting the production of the phenolics in the roots and cytokine hormone (Karajeh,2013).

Hamouda *et al.* (2019) studied the effect of the yeast *Saccharomyces cerevisiae* against root-knot nematode *Meloidogyne incognita* infesting banana plants with two concentrations (2 & 4 g/ 4kg soil). They reported the highest concentration was more effective than the lowest, it reduced

the galls, egg masses, and juveniles by 382.37, 333.48, and 1227.5 compared to 912.38, 897.3, and 6895 in the control treatment respectively.

Abokorah and Fathalla (2022) tested the yeast *Saccharomyces cerevisiae* and its effect on banana plant growth and PPNs associated with these plants, especially *Meloidogyne* spp. they found the number of J2 reduced from 1724 to 596 J2/ 100 cm^3 soil after 12 m. of application while in the oxamyl treatment, the number reduced from 1761 to 310 J2 / 100 cm^3 soil, and they decided that mixing yeast with fulvic acid and tryptophan have great benefits on plant growth and nematode reduction. D'Addabbo *et al.* (2024) suggested using the yeast *Papiliotrema terrestris* strain PT22AV at a dose of 1.0 kg/ha can significantly decrease the nematode population in soil and gall formation on tomato roots infesting with *M. incognita* under greenhouse conditions, and they reported that *P. terrestris* could represent an additional tool for non-chemical RKN management.

Rhodosporidium paludigenum NCYC 2663 and 2664 could produce carotenoids and lipids when cultivated on different sugar sources. This high content of carotenoids, furthermore lipids rich in oleic acid, and linoleic acid may offer antimicrobial activity against some microorganisms (Warjoto and Jennifer, 2020 and Sereti *et al.,* 2024).

The effect of the fungus *Trichoderma* on PPNs was more supported by studies than the yeast. Conversely, the mixing effect of this fungus with the other microbial agents has not been studied well. Siddiqui and Shaukat (2004) reported that the fungus *Trichoderma harzianum* had a synergistic effect on *P. fluorescens* when added to the soil infected with *M. javanica* and may improve the efficacy of bacteria against PPNs.

Lafta and Kasim (2019) referred to the efficacy of mixing *T. harzianum* and *Pseudomonas fluorescens* in controlling *Meloidogyne* spp. when tested individually and in combination form. They found that The combined treatment of *P. fluorescens* and *T. harzianum* resulted in low egg hatching and increased J2 mortality percentages (40.74% and 60.18%) respectively. Filamentous fungi especially *Trichoderma* can be an alternative biocontrol method for PPNs, this strategy is one to overcome the highly toxic chemical nematicide. These fungi could reduce the damage caused by PPNs directly by parasitism, antibiosis, and paralysis caused by the production of lytic enzymes. Moreover, its ability to induce resistance against nematodes by activating hormone-mediated like salicylic and jasmonic acid, which form a plant-defense mechanism, as stimulating the synthesis of plant secondary metabolites and different enzymes can also contribute to enhancing plant defenses against PPNs (Poveda *et al.*, 2020).

Almeida *et al.* (2022) evaluated two *T. harzianum* isolates (ALL42 and IBLF006) and the *T. asperellum* T00 strain as antagonists of *M. javanica* associated with banana plants. It has resulted in *T. harzianum* ALL42 and *T. harzianum* IBFL006 reducing the *M. javanica* population on banana roots by up to 55.2 % and 67.9 %, respectively.

Hashem and Abo-Elyousr (2011) mentioned to the importance of mixing *P. fluorescens* and *Paecilomyces lilacinus* in controlling *M. incognita* rather than using it individually. The J2 population in 250 g soil reduced from 3800 in the control treatment to 2011 in the combined treatment with improvement in plant health and production.

An unexpected increase in non-parasitic nematode numbers has been recorded. This increase may be due to most of these species feeding in bacteria and fungi and because of the competition between non-parasitic and parasitic species, this may be decreasing the number of PPNs (Timper *et al.,* 2021 and Hodda, 2022).

Moreover, most of the microorganisms could modify the soil to be suitable for fungivorous and free-living nematode species e.g., accelerating the process of the organic matter decomposition and making soil pH more suitable for free-living nematodes (Nisa *et al.,* 2021 and Jiajia *et al.,* 2022). These microbes are naturally available in the Egyptian environment, in addition, it can be multiplied greatly in these environments, especially in citrus orchards which are usually fertilized with decomposed materials. In the market, there are some of these products from other isolates which usually have a long shelf life, and low cost compared to the chemical nematicides. These results reinforce the use of these microbes in controlling PPNs in citrus orchards and reduce the use of traditional chemical nematicides this is to enhance the ecological balance.

CONCLUSION

It can be concluded that the combination of the tested microbial species exhibited a synergistic potential effect in controlling the plant-parasitic nematodes associated with citrus trees. Notably, the yeast species, which had not previously been used in the control of PPNs, demonstrated a clear positive effect when included in the microbial mixture. The integration of these biocontrol agents, when combined, enhanced their collective efficacy against the targeted nematodes. On the other hand, the populations of *Tylenchus* spp. *and Rhabditis* spp., two non-plant parasitic nematode species, showed an increase in number. These species are crucial to the soil ecosystem as they contribute to nutrient cycling and overall soil health. The results highlight the potential benefits of integrating different microbial species for a more holistic approach to PPN control, reinforcing the efficacy of mixed microbial strategies in nematode management.

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توافق بعض المواد البيولوجية فرديا او خلطا ضد النيماتودا المتطفلة التي تصيب أشجار البرتقال السكري وتأثيرها الجانبى على النيماتودا غير المتطفلة (الآكلة للفطريات والحرة المعيشة)

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الملخص

تم اختيار أربعة كائنات دقيقة لتقييم فعاليتها، في صورة فردية وفي مخاليط مزدوجة، ضد النيماتودا المتطفلة على النبات (PPNs) والنيماتودا غير المتطفلة، وذلك من خلال تجارب في المختبر وفي الحقل. شملت هذه الكائنات ثلاث ميكروبات معروفة بتأثيرها في مكافحة النيماتودا، وهي: *Trichoderma asperellum* و*T. harzianum*، وسلالتان من *Pseudomonas fluorescens* أما الكائن الرابع، وهو *Rhodosporidium paludigenum* (الخميرة البحرية الحمراء)، والأخير لم يتم اختباره سابقًا ضد النيماتودا المتطفلة. هدفت الدراسة إلى تحليل التأثيرات التآزرية أو التضادية لهذه المُخاليطالميكروبية. أظهرت جميع المُخاليطانخفاضًا ملحوظًا في أعداد النيماتودا المتطفلة، إلى جانب زيادة في أعداد الأنواع الاخرى وكان مخلوط *R. paludigenum* مع *T. harzianum* الأكثر فاعلية ضد *Tylenchulus semipenetrans*، حيث خفضت تعدادها بنسبة 68%. كما قلل المخلوط *T. asperellum* مع *P. fluorescens* (السلالة 1) أعداد *Helicotylenchus* spp. بنسبة 73.9%، بينما كانت مخلوط *P. fluorescens* (السلالة 2) مع *R. paludigenum* الأكثر تأثيرًا على *Xiphinema* spp.، حيث خفضت أعدادها بنسبة 67.2% بعد ثلاثة أشهر من المعاملة الحقلية. لم يُسجَّل أي انخفاض أعداد *Tylenchus* spp. و*Rhabditis* spp. وقد دعمت نتائج التجارب في المختبر هذه النتائج الحقلية، إذ تسبب مخلوط *R. paludigenum* و *T. harzianum* في تثبيط الطور الثاني اليرقي (J2) لنيماتودا *T. semipenetrans* وتقليل نسبة فقس البيض بنسبة 63% و20.6% على التوالي خلال أربعة أيام من التطبيق. تشير النتائج إلى أن استخدام هذه المُخاليطالميكروبية يُعد وسيلة فعّالة في خفض أعداد النيماتودا المتطفلة في بساتين الموالح دون التأثير السلبي على النيماتودا غير المتطفلة. ويوصى باعتماد هذه المُخاليطضمن برامج المكافحة الحيوية المتكاملة للنيماتودا المصاحبة لأشجار الموالح مع الحفاظ على التنوع الحيوي في التربة والحد من الاعتماد على المبيدات الكيميائية في زراعة الموالح.

الكلمات الدالة: الموالح والمُخاليط الميكروبية والنيماتودا المتطفلة على النبات والخميرة البحرية الحمراء و نيماتودا الموالح.