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Antagonistic potential of *Verticillium leptobactrum* against *Pratylenchus vulnus* associated with apple rootstock

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Abstract
In Tunisia, apples have an important place in the fruit sector. The root-lesion nematode is a major pest of pome due to the significant root damage it causes. In this study, we investigated under laboratory and greenhouse conditions the effect of *Verticillium leptobactrum* isolate (HR1) against *Pratylenchus vulnus* associated to three-apple rootstock (MM106, MM111 and Ba29). *In vitro* study, five concentration of *V. leptobactrum* were tested for nematicidal activity against *P.vulnus.* The microscopic observation of treated nematodes showed serious alterations caused by the fungal filtrate. The greatest activity was observed at 10 to 75% of filtrate dilutions. In greenhouse experiment, the reproduction of the root-lesion nematode and the parameters of growth of three-apple rootstock were evaluated. For the plants of Ba29, *V. leptobactrum* increased total weight and diameter but had no significant effect on the growth parameters of other rootstock (MM106 and MM111). Plants inoculated with the nematophagous fungi generally showed important reduction in the population of *P. vulnus* either in the soil or in roots than plants inoculated with chemical treatment (Mocap) and non-treated plants. The nematode multiplication rate differs significantly only for the MM106 rootstock.

Keywords: Biocontrol, *Pratylenchus vulnus*, *Verticillium leptobactrum*, apple rootstock

1. Introduction
Apple is widely produced in many parts of the world including Tunisia. Nevertheless, several constraints then whose principal ones are Root-lesion nematodes [1]. This genus is considered the most important plant-parasitic nematodes in terms of the economic losses caused in fruit tree throughout the temperate climate in the world [2]. *Pratylenchus vulnus* Allen and Jensen (1951) presented a major pathogen involved a reduction in the production of fruit tree in nurseries and orchard in the Mediterranean area [3, 4]. Within the genus *Pratylenchus*, 12 species have been reported as potential pathogens on apple [5]. In past, diseases caused by nematodes has managed by Nematicides [6]. The evaluated cost and harmful effects in environment and human has brought attention to other alternatives such as biological control [7]. Biological control agents represent an innovative alternative to synthetic pesticides and have potentials for control of plant parasitic nematodes [8, 9]. The biopesticides based on bacteria, filamentous fungi are generally eco-friendly, and less cost [10]. *Verticillium leptobactrum*, is Tunisian fungal isolates, was proved advantageous for suppression of plant parasitic nematodes such as Meloidogyne spp. on various crops [11].
The main objective of this work was to investigate the efficacy to use *V. leptobactrum* as protective agent against *P. vulnus* on apple rootstock. We have studied the effects of the nematophagous fungi on rootstock growth and on pathogen development. Also we tried to understand the mode of action of this nematophagous fungi on *P.vulnus* individuals.

2. Material and Methods
2.1 *In vitro* test of effect of *V.leptobactrum* against *P.vulnus*
Observation of mechanical parasitism
V.leptobactrum strain was kept in test tubes containing 2% potatoes dextrose agar (PDA), in the dark, at 4°C for 10 days. Culture disks, 4 mm in diameter, were extracted from fungi kept in the test tubes and plated into 9 cm diameter Petri dishes containing 20 ml of 2% potato dextrose agar, and then stored in the dark, at 25°C for 10 days. After the growth of the fungus,

new culture disks, 4 mm in diameter, were transferred to 9 cm diameter Petri dishes containing 20 ml of 2% water agar (2% WA) for 10 days. Ten individuals of *P.vulnus* previously surface sterilized were placed at the edge of the colonies. Periodic observations were made under a stereoscopic microscope.

Filtrate preparation

V.leptobactrum strain was cultured in Czapek-Dox broth (35 g l-1), in flasks with 50 ml of liquid medium, each inoculated with a 1 cm2 agar block chopped from a 2-week-old fungal colony growing on potato dextrose agar. The fungus was grown in stationary culture for 1 week at 25°C. The fungal biomass was removed by filtering through a Whatman filter paper N°1 followed by a 0.45 µm Millipore filter. The collected filtrate was used neat (100%) or diluted in distilled water (10%, 25%, 50%, 75%; v/v).

Effect of culture filtrate on individuals of P. vulnus motility

Individuals of *P. vulnus* were suspended in Sterile distilled water (100 individuals ml^{-1}). *P. vulnus* suspensions (0.01 ml) were placed into wells of a 96-well tissue culture plate (ca. 10 individual/well) with 0.9 ml of diluted fungal culture filtrates prepared as described, or a Czapek-Dox broth without fungal inoculate as control, in eight replications. The plates were maintained at 25°C, checking the effects on P. vulnus viability at 1, 24 and 72 h. The individual response was observed, under an inverted microscope, adding a drop of 1 M NaOH to each well [12]. The nematodes that responded by moving during 5 min were considered alive, whereas those not responding were considered dead. The assay was repeated twice.

2.2 Greenhouse experiment

Rootstock material

MM106, MM111 and Ba29 rootstock were obtained from an apple nursery located in the region of Oued Melliz, governorate of Jendouba Tunisia.

Rootstock with uniform growth were transplanted in a 4 liters of soil pots filled with a pasteurized Peat sandy soil (1:1:1).

Preparation of nematode inoculum

The *P.vulnus* population used was isolated from apple rootstock MM106 originated from an apple nursery located in the region of Oued Melliz, governorate of Jendouba Tunisia.

Pratylenchus population was cultured monoxenically on carrot disks according to method of [13]. Species identification was made by Chihani at Ghent University, Department of Biology, Nematology Unit [14]. *P. vulnus* isolate used in this study have proven to be pathogenic on Malus rootstocks**.** All inoculum used obtained by adding sterile water to carrots disks and collecting nematodes with a pipette.

Antagonist preparation

The antagonistic strain was cultivated in 1litre flasks containing 300g of wheat in 320 ml of deionised water, autoclaved at 120°C for 30 min and was incubated for 20 days at 20°C.

2.3 Statistical analysis

The data were subjected to analyses of variance (ANOVA) using SPSS 18.0 for Windows. The treatment means were compared by the Duncan's multiple range tests when the F-tests were statistically significant at $P < 0.05$.

3. Results and Discussion

3.1 Pathogenicity test on *P.vulnus* individuals *in vitro*

Given the movement of nematodes on agar surface, the confrontation test showed the paralysis effect of the fungus on the pathogen (Fig.1 A and B).

The fungus was observed as developing in the nematode. The mycelium was proliferating within nematode individuals 72 h after first contact digesting the nematode content, during the early stages of infection. Morphological alteration of nematodes was observed with hyphal penetration (Fig.2 A, B and C) however, the *P.vulnus* individuals on control remained viable and mobile until the end of the observations.

These microscopic observations of parasitism phenomenon are the first steps in understanding the interaction between nematophagous fungi and their hosts that involves three stages: recognition by phenomena of attractions and contact, followed by production of adhesives or lytic enzymes and differentiation of structures of infections named appressoria and trapping organs and finally the penetration and digestion of nematodes [15].

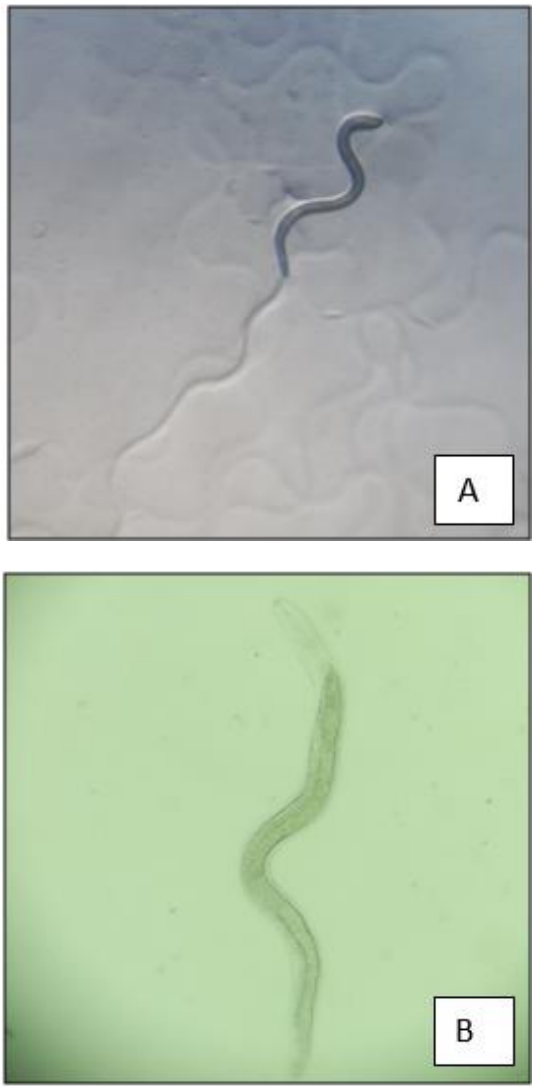

Fig 1: Microscopic observation of *P.vulnus* adults on agarose surface (A: *P.vulnus* adult moving and leaving traces on agar surface (control); B: adult of *P.vulnus* exposed to *V. leptobactrum*, paralysed on agar surface.

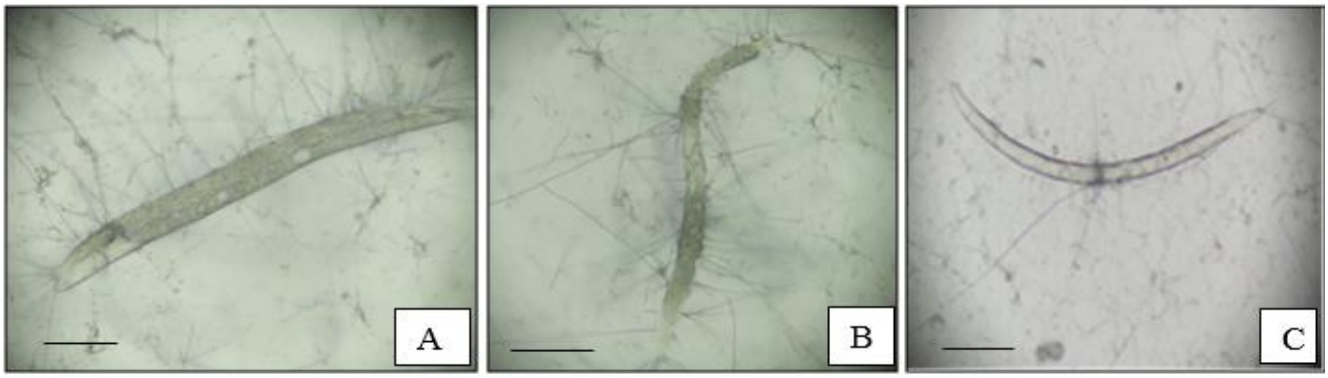

Fig 2 (A, B and C): Mobile stages of *P.vulnus* parasitized by the nematophagous fungi *V.leptobactrum* after 72 hours of exposure. Scale bars 5 µm.

3.2 Effect of fungal culture filtrate on mortality of *P.vulnus*

Nematode mortalities were proportional to the *V. leptobactrum* filtrate concentrations and the duration of exposure (Fig. 3). Filtrates showed a nematicidal activity toward *P.vulnus* at 25% dilutions, when the nematodes appeared paralysed after 24 h of exposure. Highest toxicity was observed at 75% dilutions and 72 h exposure. Mean mortalities in the fungal filtrates differed significantly from the control, which showed no nematostatic or nematicidal effects (Fig. 3).

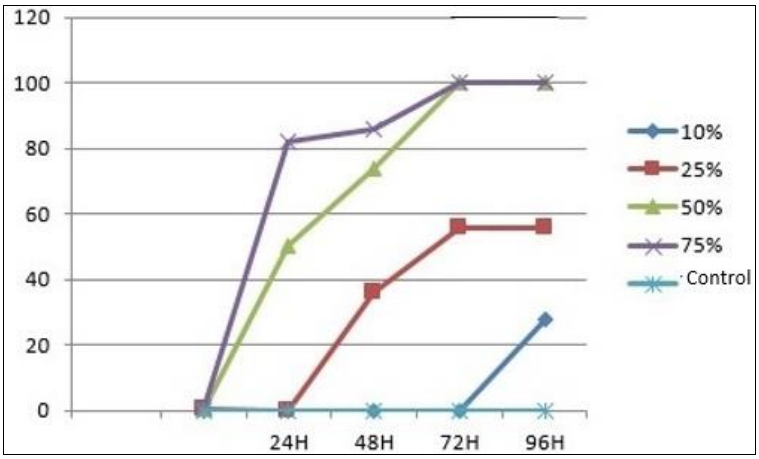

Fig 3: Effect of *V.leptobactrum* filtrates on Mortality of *P.vulnus* Individuals

After 4 days exposure to *V. leptobactrum* filtrate, the *P.vulnus* adults and larvae showed extensive damages and cuticle degradation, whereas untreated controls displayed and undisturbed cuticle (Fig. 4 A and B). In treated nematodes, the integrity of the external chitinous layers appeared compromised by the filtrate activity, and in some areas, their surface was interrupted or fractured.

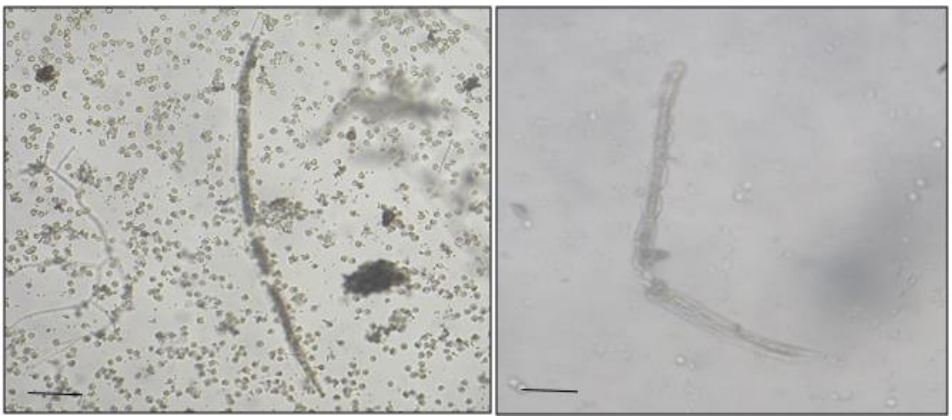

Fig 4: Effects of culture filtrate of *V. leptobactrum* at concentration of 50% on individuals of *P.vulnus* (A and B lysis of the cuticle and degradation within the nematodes).scale bars 5µm.

The culture filtrate of *V. leptobactrum* was lethal to larvae and adults of *P.vulnus*. The microscopic observations revealed serious alterations that depended on different dilutions and exposure time of nematode to the fungal filtrate. Regaeig *et al.* [11] showed the same effect of *V. leptobactrum* culture filtrate on *Meloidogyne* larvae and it was assumed that these changes depended on an enzymatic degradation process suggesting the ability of *V. leptobactrum* to produce one or

more enzymes capable of degrading nematode chitin layer. The same tendency was observed by [16] who tested *in vitro* the nematicidal effect of *Microsporum lysipagum* against *P. neglectus* resulted in 81% mortality of mobile stages of this nematode within 20 hours of exposure to the fungus.

In vivo effect of *V. leptobactrum* and Mocap on population of *P.vulnus*

Effect on parameters growth in different apple rootstock

Biological control of soil-borne pathogens by nematophagous fungi is notoriously susceptible to alterations in experimental conditions. Indeed, the addition of *V.leptobactrum* had no overall effect on plant growth or nematode multiplication for some of the rootstocks used. In contrary, to others studies, results showed in greenhouse experiment, *V. leptobactrum* isolate (HR1) an progress in plant growth of tomato founded previously by [11, 17].

No significant differences were observed in shoot length or shoot weight between untreated [control] and treated plants for all the rootstocks used. However, the application of the nematophagous fungi significantly enhanced Ba 29 rootstocks diameter compared to control and to the chemical treatment (table 1).

Table 1: Top weights, shoot lengths, shoot diameters, and root weights and length of MM106, MM111 and Ba 29 apple rootstocks evaluated under greenhouse conditions with 1000 P.vulnus per plant treated with V. leptobactrum and Mocap 3 months after nematode inoculation. The data are the means of 5 replicates. Means in the same column followed by the same letter do not differ according to Duncan's multiple range test (P_<0.05)

Rootstock	Treatment	Fresh top weight	Shoot diameter	Shoot length	Fresh root weight	Root length
MM106	Control	81,12a	0,736a	60,2a	25,22a	40,6a
	V.lep	131,88a	0,9a	78,6a	29,9a	43,2a
	Mocap	78,22a	0,78a	52,6a	23,06a	27,4a
MM111	Control	129,6a	0,846a	72,6a	43,92a	47a
	V.lep	124,4a	0,858a	77,8a	57,88a	42,6a
	Mocap	180,8a	0,872a	76a	37,84a	44,4a
Ba29	Control	112,6a	0,84a	49,2a	58,16a	56,8a
	V.lep	168,52a	1,18a	58,2a	49,02a	52,4a
	Mocap	120,52a	0,694b	52,6a	49,66a	53,6a

Effect on development of pathogen

Both chemical and biological treatment decreased *P.vulnus* multiplication rate compared to untreated plants, but this efficiency is only significant for the MM106 rootstock (Table. 2). These results suggest that the ability of *P. vulnus* to colonize root tissue in an area where the nematophagous fungi was already established depends on the suitability of the host for the nematode. Several similar studies have shown that *Paecilomyces lilacinus* reduced the populations of *Pratylenchus* in maize and *P. coffeae* in chickpeas, and the increase in the application rate of the fungus increased the percentage of reduction in the nematode population [18, 19].

Table 2: The reproduction of *Pratylenchus vulnus* on MM106, MM111 and Ba29 apple rootstocks treated with *V. leptobactrum* and Mocap, 3 months after inoculation with 1000 nematodes per plant. The data are the means of 5 replicates. Means in the same column followed by the same letter do not differ according to Duncan's multiple range test (P_<0.05). Pf/ Pi, Final population/initial population (nematode multiplication rate)

Treatment	Final population per plant (soil and roots)			Nematode/g root			final population/initial population		
Rootstock	MM106	MM111	Ba29	MM106	MM111	Ba29	MM106	MM111	Ba29
Control	3549,45b	3228,7a	3613,23a	210a	74,04a	72,24a	3,55b	3,22a	3,61a
V.lep	1275,08a	2521,99a	2832,71a	50,68a	73,8a	40,68a	1,27a	2,52a	2,83a
Mocap	1063,09a	1644,29a	1273,8a	61,31a	50,84a	23,32a	1,06a	1,64a	1,27a

4. Conclusion

The present study showed that *V. leptobactrum* as a biocontrol agent was efficient to control the root lesion nematodes (*Pratylenchus vulnus*) on the apple rootstock. Nonetheless, further studies performed under various field conditions and other fruit crops are still required.

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