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EFFECT OF THE PSEUDOMONAS AERUGINOSA ON POTATO CYST NEMATODE GLOBODERA PALLIDA

Abdelaziz Hajjaji, Bahia Rached, Rajaa Ait M'hand, Naima Rhallabi, Fouad Mellouki *

Address(es): Professor Fouad Mellouki,
[1] Research Unit of Microbiology Biomolecules and Biotechnologies (MicroBioTech) Laboratory of Chemistry-Physics and Biotechnology of Biomolecules and Materials (LCP2BM) Faculty of Sciences and Techniques Mohammedia, Hassan II University of Casablanca BP 146 Mohammedia 20650, Morocco.

*Corresponding author: fouad.mellouki@univh2c.ma

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ABSTRACT

The purpose of this study was to evaluate the efficiency of *Pseudomonas aeruginosa* strain Dokkala (OK383444) isolate against cyst nematode *Globodera pallida* strain Dokkala (MZ959187) as a biocontrol agent on potato cyst nematode. The test was first conducted *in vitro* with bacterial suspensions at 10^5, 10^6, 10^7 and 10^8 CFU/mL against 100 eggs, 100 second-stage juveniles (J2), and 10 cysts of *G. pallida* and then *in vivo* on potato variety Desiree infested by cyst nematode in the greenhouse. Four plant growing parameters were evaluated in the treated potato plants by *P. aeruginosa* in the presence of *G. pallida;* plant height (cm), tuber weight (kg), wet and dry weight (kg) of the aerial part, and root length (cm). The application of *P. aeruginosa in vitro* reduced the rate of eggs of *G. pallida* by 42% and juveniles by 56%. However, the percentages of colonized females with bacteria were not significant (8% and 16% after 96 and 240 h, respectively), whereas normal J2 controls hatched at 100% after 48 h. Furthermore, the *in vivo* treatment showed an enhancement of 650 g in tuber weight and 19.2 cm in plant length compared to untreated *G. Pallida* infested plants and 33.34% colonized cysts. This research clearly indicates the ability of *P. aeruginosa* to reduce the density of potato cyst nematodes and has the potential for the biological control of *G. pallida.*

Keywords: Biological control, *Globodera pallida*, Potato, *Pseudomonas aeruginosa*

INTRODUCTION

The potato cyst nematode (PCN), *Globodera pallida* (Stone) (**Behrens, 1973**), is an important economic plant pest that affects the cultivation of *Solanum tuberosum* L. (Solanaceae) around the world (**Van Riel and Mulder, 1998**), causing up to 9% yield loss of world production (**Turner, 2011**). Once introduced into the field, the control of this nematode is difficult due to its high multiplication rate (**Desgarennes *et al*., 2006; Turner, 1996**). The main symptoms of infection are yellowing and water stress (**EPPO, 2009**). When the second-stage juveniles (J2) penetrate the root, they move through the cortex and dissolve the cell walls, then become sedentary, forming feeding sites called syncytia (**Sobczak and Golinowski, 2011**).
The control of PCN involves the application of chemical nematicides. However, this application presents many problems, including the high mobility of these chemicals in the soil that cause negative effects on the environment and potential contamination of groundwater (**Haydock *et al*., 2006**). Moreover, due to their residual effect, the chemical residue may remain in the potato tuber until harvest, which implies a risk not only to farmers but also to consumers (**Mendes *et al.*, 2005**). Investigations are conducted to use an integrated management approach that includes the control of *G. pallida* with agro-ecological methods such as biological control and crop rotation (**Lichtfouse *et al.*, 2009**). The integrated management can favor pest control due to the combination of various control methods, which improves the effectiveness of each method (**Gurr *et al.*, 2004**). **Hallmann *et al.* (2001)** have shown that endophytic bacteria are associated with beneficial effects such as promoting plant growth and having potential for biological control against phytoparasitic nematodes. Among these bacteria. *Pseudomonas* spp. which are able to locate the roots by dint of exudates such as carbohydrates or amino acids which would stimulate their chemotaxis (**Somers *et al.*, 2004**). These bacteria colonize the rhizosphere of the potato and often confer beneficial effects, such as growth stimulation and reduction of possible phytosanitary problems caused by PCN (**Rajkumar *et al.*, 2010**).
The goal of this research was to evaluate, for the first time, the potential effect of *P. aeruginosa* on the population density of *G. pallida*., especially on eggs, juveniles (J2) and cysts *in vitro* and the efficacity *in vivo* to control the nematode on the susceptible potato variety 'Desiree'.

MATERIAL AND METHODS

Nematode populations

Globodera pallida strain Dokkala (NCBI access number: MZ959187) used in the experiments was originally obtained from soil samples collected during 2020 from the potato-production region in the western-center of Morocco (N33.681600; W-7.327000) known to be infested with *Globodera pallida* (**Hajjaji *et al.*, 2021**). The nematode cysts were extracted from the dry soil samples by flotation and sieving using Fenwick's apparatus and then sorted visually by stereomicroscopy (**Fenwick, 1940**). Briefly, the apparatus is filled with water to the brim, the dried soil is dragged by a jet of water through the upper sieve with a 1 to 2 mm mesh and then to a funnel which plunges into the body of the apparatus. The cysts float and are dragged by overflow into the collection collar, under which a 250 µm sieve is placed. The water supply is maintained until the sample is exhausted and clear water overflows. The sample is placed in 0.5 mL tubes and stored at 4°C until use.
Species confirmation was achieved through morphological identification using several characters: color, size and Granek's ratio (the vulva – anus distance divided by vulval basin diameter) and count of cuticular ridges between fenestra and anus for cysts, stylet length, tail length, length of the hyaline terminal part of tail and stylet button shape for juveniles J2 that were heat-set before measurement and identification (**Skantar *et al.*, 2007**).

Isolation of *P. aeruginosa* strain

P. aeruginosa strain was directly isolated from *G. pallida* cysts. For this purpose, 10 *G. pallida* cysts were surface-sterilized in 0.5% sodium hypochlorite (NaOCl) for 2 minutes, rinsed three times in sterile distilled water, gently dried on absorbent paper and then incubated in a Petri dish on King's B Agar (KBA) medium (**King *et al.*, 1954**) containing penicillin (90 $\mu g.ml^{-1}$) and streptomycin (30 $\mu g.ml^{-1}$) and incubated at 24-26°C for 24-48h (Figure 2H).
Isolated bacterial colonies were examined under fluorescent light with an ultraviolet lamp at a wavelength of 366 nm used for strain identification. Further identification was based on the biochemical test using the Gram stain and the Galerie Api 20 NE tests.
DNA sequencing method was performed for confirmation of *P. aeruginosa* species and for phylogenetic tree analysis of 16S rRNA gene in local isolates of *P. aeruginosa* and compared with isolates of *P. aeruginosa* NCBI-Genbank and final

submission of current isolates to the NCBI-Genbank database. The 16S rRNA gene was purified from the agarose gel using the Bioneer AccuPrep-kit DNA gel extraction kit. The purified 16S rRNA gene was used for DNA sequencing using the AB DNA sequencing system. Phylogenetic tree analysis was performed based on NCBI-Blast alignment identification and neighbour distance phylogenetic tree analysis (Mega version 11).

In vitro tests

In vitro tests of the effect of isolated strains of *P. aeruginosa* were carried out on eggs, second-stage juveniles and cysts of *G. pallida* respectively. The cysts were surface sterilized by immersion in 0.5% sodium hypochlorite, rinsed twice in sterile distilled water and gently dried on absorbent paper. The eggs were released by bursting the cysts and were collected with a Pasteur pipette (**Terrell, 1995**). 200 µl of the bacterial strain at 10^5, 10^6, $10^7$and 10^8 CFU/ml was added to microtubes containing either 100 eggs or 100 juveniles (J2) or 10 cysts of *G. pallida.* 200 µL of distilled water was used as control containing the same number of individuals. After 24, 48, 96, and 240 hours of incubation at 25°C, the eggs, J2 and diseased and normal cysts were counted using an optical microscope (Olympus BX43). Reduction of the number of Eggs (RNE), of Juveniles (RNJ) and of Cysts (RNC) were determined based on the mobility of the juveniles, infection of the walls of the juveniles and eggs and the hatching of cysts and calculated to evaluate the effect of *P. aeruginosa* respectively on eggs, juveniles and cysts of *G. pallida,* using the following formula:
Reduction of the Number = (number of eggs or juveniles or cysts infected / total number of eggs or juveniles or cysts) x 100.
All tests were performed with 5 replicates on different days.

In vivo tests

For *in vivo* tests, we used a silty-sandy soil (sand 80%, clay 10% and silt 10%) autoclaved at 121°C for 20 min. The Desiree potato seeds, sterilized with 5% sodium hypochlorite for 30 minutes and rinsed in water, were sown in 15L pots containing 20 cysts per 1 kg of soil. The control lot did not contain cysts. The test was carried out in triplicate and in two different times (September to December and January to the end of April for the second experiment). After one week, the pots were treated by injection into the soil with irrigation water at a dose of 5 mL per kg of soil by *P. aeruginosa* bacterial suspension at 10^7 CFU/ml. The experiments were monitored during 120 days of incubation in a glasshouse at 25°C, 70% relative humidity and under photoperiodic lighting (16 hours of light:8 hours of dark) programs. The pots were watered once a week and mineral fertilizers were added according to the vegetative stages of the potato. A first addition containing 12% nitrogen (N) and 61% phosphorus (P2O5) one month after the sowing and a second addition a week before flowering with 10% N, 10% P2O5 and 25% K2O.
Throughout the experiment, plant parameters measured were: plant height (cm), tuber weight (kg), wet and dry weights (kg) of the aerial part and root length (cm). At the end of the four months after sowing, the cysts were extracted from the entire soil using Fenwick method. Furthermore, the walls of the cysts were observed using a light microscope (Olympus BX43) at x40 magnification and exploded manually to separate cysts that contain juveniles and empty cysts. The number of cysts that contain juveniles and empty cysts were calculated.
Reduction of the number of Cysts (RNC) was determined based on hatched cysts and calculated to evaluate the effect of *P. aeruginosa* respectively on cysts of *G. pallida,* using the following formula**:**
RNC = (number of cysts hatched / total number cysts) x 100.

Statistical analysis

The *in vivo* test data were analyzed by the Principal Component Analysis (PCA) to highlight all of the variables that influence the response in the same way and those that influence it differently. Data from the *in vitro* experiments were subjected to a non-parametric ANOVA test using a Friedman test followed by a multiple comparison in reducing the number obtained for eggs, juveniles and cysts with significance level at P < 0.05.

RESULTS ET DISCUSSION

Identification of *P. aeruginosa*

P. aeruginosa isolates from cysts were identified by biochemical tests. The colonies were whitish, grey, and enhanced with a diffusible yellowish green pigment which fluoresces blue under ultraviolet light (366 nm). The Gram staining test was Gram-negative with confirmation by the API 20 NE gallery (ADH+, CIT+, URE+, GEL+, GLU+, ARA+, OX+). Molecular identification by sequencing of 16S rRNA gene *P. aeruginosa* isolated from *G. pallida* cysts gave a single population of *P. aeruginosa* strain Dokkala (OK383444.1) at 1374 bp (Figure 1).

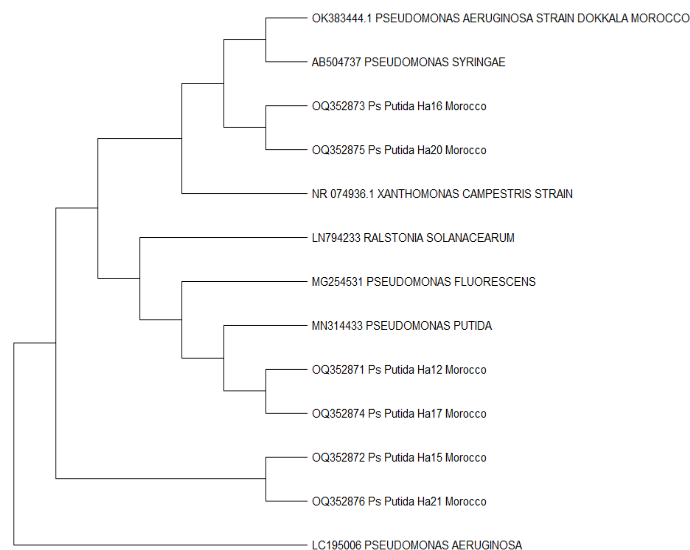

Figure 1 Phylogenetic tree of Moroccan *Pseudomonas aeruginosa* strain Dokkala (OK383444.1) isolated from *Globodera pallida* cysts

In vitro effect of P. *aeruginosa* strain on *G. pallida*

The reduction of the number of eggs and juveniles J2 were high significantly different compared to uninoculated control, and there were strong effects in measured infection dependently on bacteria inoculum density (P < 0.05) (Tab 1). The effect of *P. aeruginosa* at a concentration of 10^8 CFU/ml on the eggs of *G. pallida* (Figure 2F) and J2 juveniles (Figure 2A, B; E) was clear and allowed to eliminate 42% of eggs and 56 to 58% of juveniles. The concentration at 10^5 CFU/ml has no effect on the reduction of the number of eggs and cysts, but on juveniles, this concentration killed 22% after 10 days of inoculation. The suspension bacteria at 10^6 CFU/ml showed weak effects on eggs and on cysts with rate values of 6% and 2%, respectively, while juveniles mortality reached 30%.
Furthermore, the percentages of colonized cysts were fairly insignificant, with RNC values of 8%, 16% after 96h and 240h respectively (Figure 2D); giving 84% normal hatching (Figure 2C) compared to control eggs which hatched at 100% after 48h. J2 inside the eggs remained alive and were observed at time intervals of 12h, 24h, 48h, 96h, 240h, and 480h. The RNJ for the untreated control revealed a mortality rate of 15% after 10 days.

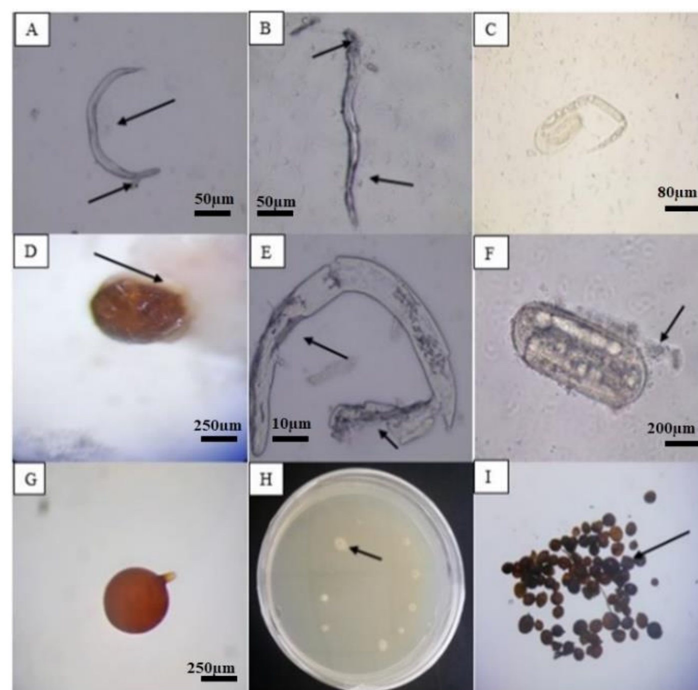

Figure 2 Effect of the *Pseudomonas aeruginosa* on *Globodera pallida.*
Legend: Juvenile colonized (A, B). Normal juvenile hatch (C). Colonized cyst (D, I). Infection level after 480h (E). Colonized egg (F). Normal cyst (G), Isolation of *P. aeruginosa* strain on King B Agar medium (H).

In vivo effect of *P. aeruginosa* strain on *G. pallida*

Significantly differences were observed between data from the experiments of *G. pallida* treated with *P. aeruginosa* (Trt) compared to the untreated control (C^+). The RNC in the treated plants with *G. pallida* in the presence of *P. aeruginosa* isolates was 33.34% (Tab 2). The height of the treated plant with *P. aeruginosa* increased by 19.2 cm (+31%) compared to the control. The root length, the weight of tubers and the wet and dry weights significantly increased respectively by 4.46 cm (19.5%), 650 g (54%), 185 g (21%) and 34.5 g (9%) (Tab 2).
Principal Component Analysis Biplot (Figure 3) showed that all growth parameters measured for the plants (height, root length, wet weight, dry weight and weight of tubers) were ranged into the same group for treated plants (Trt) with *P. aeruginosa* and for untreated plants (C^-) and separately from data related to positive control consisting in plant infected by *G. pallida* and not treated by *Pseudomonas*.

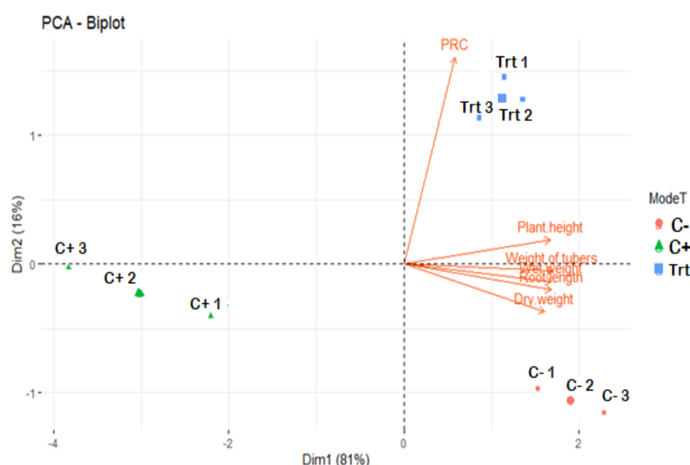

Figure 3 Biplot of Principal Component Analysis (PCA) for the in-vivo test.
Legend: C-: Potato without *G. pallida* cyst - C+: Potato + *G. pallida* cyst - Trt: Potato + *G. pallida* cyst + Treatment (*P. aeruginosa*) – 1, 2, 3: number of replicate - PRC: Parasitism rate of cysts.

Table 1 Percentage of eggs, juveniles and cysts of *G. pallida* infected by various concentrations of treatment with *P. aeruginosa.*

			RNE%				RNJ %				RNC %			
	Serial dilution CFU.mL-1	N	24h	48h	96h	240h	24h	48h	96h	240h	24h	48h	96h	240h
C	-	5	0	0	0	0	0	0	10	15	0	0	0	0
Trt	10^5	5	0	0	0	0^a	0	4	14	22^a	0	0	0	0^a
	10^6	5	0	0	2	6^b	4	16	26	30^b	0	0	0	2^a
	10^7	5	4^c	12^c	26	40^c	20	32^c	42^c	58^c	0	0	6	16^b
	10^8	5	6^c	16^c	28	42^c	40	36^c	42^c	56^c	0	0	8	16^b

Legend: Control (egg, juvenile and cyst without treatment) (C). Egg, juvenile and cyst with treatment (*P. aeruginosa*) (Trt). Number of tests carried out(N). Reduction of the Number of Eggs (RNE). Reduction of the Number of Juveniles (RNJ). Reduction of the Number of Cysts (RNC). *abc the same letter are not significantly different according to Friedman test at P<0.05.

Table 2 Effects of treatment of potato with *Pseudomonas aeruginosa* on plant growth and cysts infection.

	N	PRC %	Plant height (cm)	Root length (cm)	Wet weight (kg)	Dry weight (kg)	Weight of tubers (kg)
C^-	3	0	62.85	28.975	1.11	0.4	2
C^+	3	0.83	42.5	22.78	0.865	0.356	1.2
Trt	3	33.34	61.7	27.24	1.05	0.3905	1.85

Legend: Potato without *G. pallida* cyst (C^-). Potato + *G. pallida* cyst (C^+). Potato + *G. pallida* cyst + Treatment (*Pseudomonas aeruginosa)* (Trt). number of tests performed(N). Reduction of the Number of Cysts (RNC).

In this study, we evaluated the effect of *Pseudomonas aeruginosa* strain Dokkala (OK383444) isolated from a cyst of *Globodera pallida* strain Dokkala (MZ959187). The *in vitro* test showed that the reduction of the number of eggs (RNE), J2 (RNJ) and cysts (RNC) of *G. pallida* were respectively 42%, 56% and 16% after 240h of treatment with *P. aeruginosa* at 10^8 CFU/ml. These results indicate that *P. aeruginosa* isolate was capable of decreasing the density of *G. pallida.*
P. aeruginosa has been reported by several researchers as a biocontrol for plant parasitic nematodes but note on potato cyst nematodes. Our research targets the *G. pallida* for the first time. **Siddiqui *et a1.* (2000)** found that when applied in soil, *P. aeruginosa* reduced root-knot development and nematode population density. **Trifonova *et al.* (2014)** have also shown that the *Pseudomonas putida* and *Pseudomonas aurantiacea* strains significantly reduced *Globodera rostochiensis* populations by 40.7% - 42.2 % compared to the control. **Seenivasan (2020)** reported that *Pseudomonas fluorescens* have reduced the density of *G. pallida* and *G. rostochiensis* eggs up to 86.5% and have diminished root penetration by second-stage juveniles to 67.1 to 68.1%. The number of cysts has decreased to a percentage of 36%. Further research has been evaluated the biological control of *G. pallida* using *Trichoderma harzianium* and 49% reduction in the population of *G. pallida* has been obtained (**Contina *et al.*, 2017**). Dandurand and Knudsen, (2016) evaluated the effect of the trap plant *Solanum sisymbriifolium* alone or in combination with two fungi *T. harzianum* or *Plectosphaerella cucumerina* and have found a reduction in the population of *G. pallida* reached 47%.
Our results showed a weak effect of *P. aeruginosa* on the cysts due to the protective form of the females and the bursting of the cysts. However, the J2 contained in the cysts may perceive the root exudates emitted by the host plant then they leave the cyst and disperse in the soil containing *P. aeruginosa* which thereafter hinders their movement towards the elongation zones of the host root. Effectively, in the *in vivo* test, we noticed remarkable growth of the treated potato plants with respectively plant height and root length and difference in tuber weight compared to untreated plants. These results can be explained by the probable effects of *P. aeruginosa* bacteria on the juveniles just after hatching by reducing roots penetration by larvae and causing damage which disrupted the life cycle of the nematode, including the decrease in the density of cysts in the soil.

CONCLUSION

In summary, in this study we evaluated the efficiency of bacteria *P. aeruginosa* on the reduction of populations of *G. pallida* and on the growth parameters of potato. Our results clearly indicate that *P. aeruginosa* could be useful for the biological control of *G. pallida*. Data from greenhouse experiments showed that the percentage of infection of J2 is greater than that of eggs and cysts, which explains the importance of the contribution of the treatment by the *P. aeruginosa* at the time of the root growth stage. The roots favor the release of J2 from cysts under the effect of their exudates, and at this stage, the bacteria reduces the density of nematodes by dint of its toxic secretions. However, further investigations are needed by extending the bacteria tested to other *Pseudomonas* species from the rhizosphere to find more effective antagonistic strains against PCN and examine their mechanisms of action behind the reduction of the nematode population. The application of such biological control of potato cyst nematodes should be done in integrated strategies by combination with rotation of non-hosts culture, with nematophagous fungi as well as by using of trap cultures.

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