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Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse.

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Running title: Effect of plant resistance and BioAct WG on RKN in greenhouse.

Abstract

BACKGROUND: The effectiveness of combining resistant tomato with BioAct WG (*Purpureocillium lilacinum* strain 251; Pl251) against *Meloidogyne incognita* was assessed in a tomato-cucumber rotation in greenhouse over two years. Additionally, the enzymatic activity of the fungus, the percentage of fungal egg and juvenile parasitism, cardinal temperatures and the effect of water potential on mycelia growth and the soil receptivity to Pl251 were determined *in vitro*.

RESULTS: Plant resistance was the only factor that suppressed nematode and crop yield losses. Percentage of egg parasitism in plots treated with BioAct WG was less than 2.6 %. However under *in vitro* conditions, Pl251 showed protease, lipase, and chitinase activities, and parasitized 94.5 % of eggs, but no juveniles. Cardinal temperatures were 14.2, 24-26, and 35.4 ^{o}C. The maximum Pl251 mycelial growth was at -0.25 MPa and 25 ^{o}C. Soil temperatures and water potential in the greenhouse were in

the range of the fungus. However, soil receptivity was less in greenhouse soil, irrespective of sterilization, than in sterilized sand.

CONCLUSIONS: Plant resistance was the only factor able to suppress nematode densities, disease severity and yield losses, and to protect the following cucumber crop. Environmental factors involved in soil receptivity could have negatively affected fungus effectiveness.

Key words

Biological control, *Cucumis sativus*, doble-cropping system, integrated management, root-knot nematodes, *Solanum lycopersicum*.

1 INTRODUCTION

Spain is the main producer of vegetables under protected cultivation in the Mediterranean area, with 71.003 ha.[1] The major crops are tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.), frequently cultivated in a double cropping system.[2] Greenhouse tomato is grown on 18.501 ha with an annual production of 1.881.922 tonnes, which represents 38 % of the tomato production area and 47 % of the total yield. Greenhouse cucumber is grown on 7.768 ha with an annual yield of 717.693 tonnes, representing 88 % of the total production area and 95 % of the total yield.[1]

Root-knot nematodes (RKN), *Meloidogyne* spp., are one of the most important soil pathogens limiting horticulture production worldwide, especially under protected cultivation.[3] *Meloidogyne* spp. are widely spread in all vegetable production areas in Spain. Yield losses caused by RKN can reach 60 % in tomato and 88 % in cucumber.[4,5] RKN is mainly managed by fumigant and non-fumigant nematicides.[6] However, the

limitations imposed by the Directive 2009/128/EC have encouraged research finding alternatives to chemical pesticides as well as to design effective and durable strategies to manage RKN.

There are a large number of non-chemical alternatives to control RKN,[3] including crop rotation with resistant cultivars and biological control. In nematology, resistance is defined as the ability of a plant to suppress infection, development and/or reproduction of plant-parasitic nematodes.[7] Therefore, resistant tomato cultivars or rootstocks carrying the *Mi*-gene are widely used because of their effectiveness in suppressing *M. arenaria, M. incognita* and *M. javanica*.[4,7] However, its expression can be limited by i) constant soil temperatures above 28 ^{o}C at transplanting,[8] ii) the genetic background of the tomato cultivar or rootstock,[9] iii) RKN species: not effective against *M. hapla*, *M. chitwoodi* race 3,[10] *M. enterolobi*,[11] or *M. exigua*;[12], and iv) virulent populations, which can occur suddenly or be selected by repeated cultivation of resistant cultivars.[13,14] Inclusion of resistant tomato cultivars in a cropping sequence helps to suppress the reproduction of RKN and to reduce yield losses of the following susceptible crop,[15] such as cucumber,[2] because yield losses are related to nematode densities in soil at transplanting.[16]

Several microorganisms have been evaluated for biological control of plant-parasitic nematodes.[17] The use of biological control agents able to suppress the buildup of RKN can be of interest to reduce the pressure on R genes avoiding the selection of virulent populations and contribute to maintain nematode densities below the economic threshold level. Out of those, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) strain 251 (Pl251) is the only biological nematicide listed in the annex 1 of the European register of active substances.[18] *Purpureocillium lilacinum* is a common soil hyphomycete able to parasitize RKN sedentary stages by direct hyphal penetration and

by using hydrolytic enzymes.[19-21] Besides, the effectiveness of Pl251 to control RKN has been widely reported under controlled conditions and in pot tests,[22-24] although few reports are available on its effectiveness under field conditions.[25,26]

The aim of the present study was to evaluate the effectiveness of combining the resistant tomato cv. Monika with *P. lilacinum* strain 251 (BioAct WG®, Belchim Crop Protection, Londerzeel, Belgium) over two consecutive growing seasons in a greenhouse to manage RKN in a tomato-cucumber rotation. Additionally, *in vitro* experiments were carried out to determine extracellular enzymes produced by Pl251 and its capability to parasitize eggs and second-stage juveniles of RKN, as well as to know cardinal temperatures and the effect of temperature and water potential on mycelia growth, and soil receptivity to Pl251.

2 MATERIAL AND METHODS

2.1 Greenhouse trial

The field trial was conducted in a 700 m^2 greenhouse infested with *M. incognita* in Viladecans (41º 17' 18''N; 2º 2' 39''E, Barcelona, Spain) during, 2011 and 2012. The soil was a sandy loam with 83.8 % sand, 6.7 % silt and 9.5 % clay; pH 8.7; 1.8 % of organic matter (w/w) and 0.5 dS/m of electric conductivity. Soil was infested with *M. incognita* in 2007 and rotations with resistant or susceptible tomato cultivars and cucumber or black fallow were carried out from 2008 to 2010.

The rotation sequence consisted of resistant tomato cv. Monika (bearing the *Mi* gene) or the susceptible cv. Durinta from March to July, followed by cucumber cv. Dasher II cultivated from July to October-November. Individual plots of 9.6 m^2 comprising of 4 rows with 6 plants per row. Plant spacing was 50 cm between rows and 55 cm within

rows. The distance between individual plots was 110 cm between rows and 100 cm along rows.

In 2011, combinations of resistant (TR) or susceptible tomato (TS) with or without BioAct WG preceding cucumber with or without BioAct WG were assessed. Each combination was replicated 10 times according to a stratified randomized block design. In 2012, the combinations were located on the same plots as in 2011 with slight modifications. Plots cultivated with TR did not receive any treatment with BioAct WG because of results obtained in 2011. Then, combinations including TR or TS without BioAct WG were replicated 10 times, and 20 for the combination TS with BioAct WG. Cucumber crop cultivated after TS with application or not application of BioAct WG was replicated 15 times, and five times each combination after TR.

Dates and rates of application of BioAct WG to soil and seedlings were as recommended by the manufacturer. Briefly, the first soil application of the commercial product BioAct WG (1×10^{10} viable spores g^{-1} of *P. lilacinus* strain 251 dried on glucose) was carried out 14 days before transplanting by drip irrigation at a rate of 0.4 g m^{-1} linear and 10 cm width. The following fungal applications to soil were repeated at the same rate at six weeks interval; two applications during the tomato and cucumber crops in 2011 and cucumber in 2012, and three during the tomato crop in 2012. Seedlings were watered with a suspension of 0.l g BioAct WG 1 L^{-1} just before transplanting.

Tomato was cultivated from 31st March to 6th July (98 days), and cucumber from 29th July to 26th October (90 days) in 2011. In 2012, tomato was cultivated from 5th March to 17th July (135 days) and cucumber from 31st July to 5th November (98 days).

The plants were irrigated by drip irrigation and fertilized weekly with a NPK solution (15–5–30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Crops were

vertically trellised. Weeds were removed manually during and between cropping cycles. Tomato yield was assessed from the first six fruit sets produced from the eight central plants in each plot. Fruits were harvested according to commercial standards as they reached maturity. Similar, cucumber fruits were harvested from the eight central plants of each plot when they reached the standard commercial size. Total yield per crop cycle was expressed as kilograms plant^{-1}.

Soil temperatures were recorded at 30 min intervals in order to estimate the number of the nematode generations, and the effect of soil temperature on the fungus growth. In 2011 were recorded with soil probes 107 (Campbell Scientific, Logan, USA) placed at a depth of 15 cm. In 2012, soil temperatures and water potential were recorded at the same interval and soil depth with 5TM and MPS-1 probes (Decagon devices, Inc, Pullman, USA).

Composite soil samples were collected from each plot at the beginning and at the end of each crop to estimate initial (Pi) and final (Pf) nematode population densities, respectively. Each soil sample consisted of eight subsamples from the top 30 cm taken with an auger (2.5 cm diameter). Soil samples were sieved through a 4 mm aperture screen to remove stones, and carefully homogenized to extract nematodes from 500 cm^3 by Baermann trays.[27] Second-stage juveniles (J2) that migrated to the water were collected one week later, concentrated on a 75 µm sieve, counted, and then expressed as number of J2 per 250 cm^3 of soil. At the end of each crop, plants were removed with a pitchfork, cut at ground level, and the disease severity was assessed using the Zeck's galling[28] index on a scale of 0 to 10, where 0 means complete and healthy root system (no galls observed) and 10 means plants and roots dead. Afterwards, the roots were weighted and chopped into 2 cm long segments and two 10 g subsamples were used to extract eggs by blender maceration in a 1 % NaOCl solution.[29] After 10 minutes of

maceration, the egg suspension was passed through a 75 µm sieve, to retain the plant material, and a 25 µm sieve to retain the eggs. The number of eggs was counted and expressed per gram of fresh root weight.

To assess fungal egg parasitism, the procedure described in Giné *et al.*[30] was used. In brief, 20 egg masses per individual plot were handpicked from the remaining roots and placed in a watchglass containing sterile demineralised water. The outer part of the gelatinous matrix was removed, and eggs were dispersed in an Eppendorf microcentrifuge tube containing 1 mL of sterile demineralised water with a pestle. A 333 µL aliquots of egg suspension were spread onto each of three replicated Petri plates containing a growth restricting medium,[31] and incubated at 25 ºC ± 0.5 ºC in the dark. Number of parasitized eggs was recorded after 24 and 48 hours under a dissecting microscope and percentage of egg parasitism was then calculated as the number of parasitized eggs per plate per total eggs per plate. Eggs were considered parasitized if fungal hyphae grew from inside of unhatched eggs. Parasitized eggs were individually transferred to the growth restricting medium to establish pure cultures and fungal species were identified by cultural and morphological characteristics.[32]

2.2 Extracellular enzymes production

Extracellular enzyme production by Pl251 was evaluated using a semiquantitative API ZYM ® (BioMérieux, Marcy l'Etoile, France) system which identifies 19 cellular enzymes, i.e. alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtol-AS-BI phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, nacetyl-β-glucosaminidase, α-mannosidase and α- fucosidase.

Conidia of Pl251 were collected from 3-week-old cultures of the fungus growing on potato dextrose agar (PDA; 39 g L^{-1}) at 25ºC in the dark. The colony was washed with 5 mL of sterile distilled water. The number of conidia was then counted with a haemocytometer and adjusted to 1 x 10^6 conidia mL^{-1}. The API ZYM system was used according to the manufacturer's instructions. Briefly, 65 µL of the conidia suspension was placed into each cupule, and incubated at 25 °C for 6 h in the dark.[33] Enzyme activity was observed after addition of a drop of each ZYM A and ZYM B reagents and exposed to sunlight for an hour to eliminate the yellowing from the reagents. The enzyme production was assessed according to the scale from 0 (no enzyme production) to 5 (maximum enzyme production) according the color chart provided by the manufacturer. The experiment was carried out once.

2.3 Capability of Pl251 to parasitize *Meloidogyne incognita* eggs and J2 *in vitro*

Individual plugs of 9 mm-diameter from the edge of a Pl251 colony growing on PDA were placed in the centre of a total of six Petri dishes (90 mm) containing water agar (agar 12 g L^{-1}), and incubated at 25 ºC ± 0.5 ºC in the dark for 3 days.

Surface sterilized eggs or J2 of the same *M. incognita* population used to inoculate the soil of the greenhouse in 2007 and maintained in tomato in pots were used to determine the parasitic ability of Pl251. *Meloidogyne incognita* was identified by morphology of perineal pattern, esterase pattern, and SCAR marker. Eggs from 30 egg masses handpicked from tomato roots were surface sterilized following the protocol of Verdejo *et al.*[34] with some modifications. Egg masses were placed into a conical sterile tube with 1 mL of a 0.5 % NaOCl solution for four min. and were shaken at 30 s intervals for 10 seconds. After that, the solution was diluted 10 times with sterile distilled water and left undisturbed for 30 min. to allow the eggs to settle at the bottom of the tube. Then, eggs were taken and spread into three Petri dishes at 1 cm of the edge

of the colony of the Pl251 using a sterile Pasteur pipette, and incubated at 25 ºC ± 0.5 ºC in the dark for one week. The assessment of egg parasitism was carried out as described previously.

Second-stage juveniles of *M. incognita* were obtained by extraction of eggs from tomato roots by the Hussey and Barker method[29] and left to emerge on Baermann trays.[27] Then, J2 were surface sterilized following the Mountain[34] procedure with some modifications. Briefly, a suspension of 100 J2 in 0.5 mL water was placed in a conical sterile tube containing streptomycin 0.1 % for 4 hours. The suspension was shaken at one hour interval for 10 seconds. The solution was then diluted 20 times with sterile distilled water and left undisturbed for 30 min. for J2 to settle at the bottom of the tube. Then, J2 were taken and placed in three Petri dishes at 1 cm of the edge of the Pl251 colony using a sterile Pasteur pipette, and incubated at 25 ºC ± 0.5 ºC in the dark for one week. The assessment of J2 parasitism was carried out as described previously for egg parasitism.

Percentage of parasitism was then calculated as the number of unhatched parasitized eggs or J2 per Petri dish divided by the number of unhatched eggs or J2 per Petri dish.

Experiments were conducted once.

2.4 Cardinal temperatures and the effect of temperature and water potential on Pl251 mycelia growth *in vitro*

Cardinal temperatures of Pl251 were determined placing individual 9 mm-diameter plugs of Pl251 in the center of each of 24 Petri dishes containing water agar (WA; 12 g L^{-1}). Petri dishes were incubated at 4, 10, 15, 20, 25, 30, 35 or 40ºC in the dark (three dishes per temperature). Minimum and maximum diameters (mm) of fungal growth were measured every 24 h until the colonies occupied 80 % of the surface of the Petri

plate. The mycelia growth rate (mm day $^{-1}$) was calculated as the relation between mean colony diameter (mm) and growth time (day).

Concurrently, the effect of water potential (Ψ) and temperature on Pl251 growth was assessed. Water agar media with different concentrations of Polyethylene Glycol 8000 (PEG8000) was prepared according to the Michel's equation[36] to achieve Ψ between 1.25, -1, -0.75, -0.5 and -0.25 MPa. Mycelia plugs of 9 mm diameter from the edge of a fungal the colony were placed in the centre of the Petri plates and then incubated at 15, 20, 25, and 30 ºC in the dark. Each combination of temperature-PEG8000 concentration was repeated three times. Minimum and maximum diameters of fungal growth were measured daily until the colonies occupied 80 % of the surface of the Petri plate. For each water potential, a linear regression was calculated between the mean diameter of the colony per day and the temperature, and the slopes were used to construct the regressions to determine the effect of temperature and water potential on mycelia growth. Experiments were repeated once.

2.5 Soil receptivity

Soil collected from BioAct WG non-treated plots of the greenhouse trial was assessed for receptivity to the fungal isolate. A part of soil was two times sterilized at 121 ^{o}C for 1 h within 24 h. The other part remained non-sterilized. The experiment was carried out following the procedure described by Monfort *et al.*[37] Briefly, 40 g of sterilized or non-sterilized air-dried soil was placed in Petri dishes and saturated with sterile distilled water. Soils included in the experiment were: (i) sterilized greenhouse soil; (ii) non-sterilized greenhouse soil and (iii) sterilized sand. A polyvinylidene difluoride (PVDF) membrane (0.22-µm-pore-size and 45 mm-diameter) was sterilized at 121 ^{o}C for 20 min and placed on top of the soil ensuring full contact. A 4 mm-diameter plug of Pl251 was placed in the middle of each membrane. Petri dishes were

then sealed and incubated at 25 °C ± 0.5 ºC in the dark. After three weeks, the membranes were washed with sterile distilled water and then dried in a laminar air flow cabinet. Then, the membranes where incubated in a solution of 1 % trypan blue in lactic acid for 12 h at room temperature to stain the mycelia . After that, the excess of the stain was removed with sterile distilled water, and minimum and maximum colony diameters were measured. The experiment was repeated once.

2.6 Statistical analysis

Statistical analyses were done using SAS v. 9 (SAS Institute Inc.). Data from field experiments were transformed when required to log10 (x + 1) or square-root (x+0.5) to normalize the data. The greenhouse trial was analyzed by analysis of variance by the general lineal model (PROC GLM) according to a factorial design to compare the effect of the tomato cultivar, the application of BioAct WG, and the interaction (except for tomato 2012), on nematode densities in soil, eggs in roots, disease severity, and crop yield per cropping season. Analysis of variance was also carried out to compare the effect of the temperature and kind of soil on the growth of Pl251.

3 RESULTS

3.1 Greenhouse trial

Minimum, maximum and average soil temperatures during the cultivation of each crop and year are provided in Table 1. Accumulated soil temperatures during the tomato and cucumber crops in 2011 were 1504 DD (degree days; base temperature (Tb) = 10 ºC) and 1473 DD (Tb = 11.4 ºC), respectively. According to thermal requirements of tomato and cucumber, [5,38] *M. incognita* completed two generations in tomato (thermal constant (S) = 600-700 DD over Tb=10 ºC),[38] and in cucumber (S = 500 DD over Tb = 11.4 ºC).[5]

In 2011, the tomato cultivar was the only factor that explained differences ($P < 0.05$) in nematode densities in the soil and roots, galling index and crop yield, both in tomato and cucumber (Tables 2 and 3). Nematode densities in the soil, roots and the galling index at the end of the resistant tomato crop were 8, 6 and 18 % those recorded at the end of the susceptible cultivar, which yielded 78 % less than the resistant one. The percentage of fungal egg parasitism at the end of the crop was less than 0.1 % (Table 2). At cucumber transplanting, higher ($P < 0.05$) soil nematode densities occurred in plots preceded by a susceptible than a resistant tomato cultivar. Galling indices were lower ($P < 0.05$) after a resistant than a susceptible tomato cultivar. Eggs from cucumber plants were only recovered from plants preceded by a resistant tomato because cucumber following susceptible tomato died (data not shown). Egg parasitism by the fungus was less than 0.5 % (Table 3).

In 2012, accumulated temperatures during the tomato and cucumber crops were 1959 (Tb = 10 ºC) and 1524 DD (Tb = 11.4 ºC), respectively. Hence, *M. incognita* completed three generations in both crops according to its thermal requirements. In this cropping season, the tomato cultivar was also the factor responsible for the differences ($P < 0.05$) in nematode densities in the soil and roots, disease severity, as well as crop yield in both tomato and cucumber crops (Tables 4 and 5). As in the previous season, cucumber following susceptible tomato had higher ($P < 0.05$) nematode levels at transplanting, and all plants died at the end of the experiment. The percentage of fungal egg parasitism at the end of tomato and cucumber crops was 2.4 and 2.6 %, respectively (Table 5).

3.2 Extracellular enzymes production

Six extracellular enzymes were produced by Pl251. The enzyme produced in highest amounts by the fungus was leucine arylamidase (value of the color scale: 5) followed by esterase and acid phosphatase (value of the color scale: 4), esterase-lipase (value of the

color scale: 3) and the lowest were naphtol-AS-BI- phosphohydrolase and N-acetyl-β-glucosaminidase (value of the color scale: 2).

3.3 Capability of Pl251 to parasitize RKN eggs and J2 *in vitro*

Pl251 parasitized 94.91 % ± 2.88 (mean ± standard error) of *M. incognita* unhatched eggs. However, no J2 were parasitized.

3.4 Cardinal temperatures and the effect of temperature and water potential on Pl251 mycelia growth *in vitro*

Mycelia growth of Pl251 occurred between 14.2 ºC (minimum) and 35.4 ºC (maximum), with 24-26 ºC as the optimal growth temperatures range (Figure 1). No growth was detected at 4, 10 and 40 ºC. The water potential (-1.25 to -0.25 MPa) directly influenced the mycelia growth of Pl251. The mycelia growth was higher at optimal temperatures (25 ºC) and highest water potential (-0.25 MPa), followed by 30 ºC, 20 ºC and 15 ºC (Figure 2).

3.5 Soil receptivity

The mycelia growth of Pl251 in sterile sand (2.55cm ± 0.06; mean ± standard error) was 91.83 % higher ($P < 0.05$) compared with non-sterile (0.18cm ± 0.06; mean ± standard error) or sterile greenhouse soil (0.23cm ± 0.07; mean ± standard error). However, no differences ($P > 0.05$) were found between sterile and non-sterile greenhouse soil.

4. DISCUSSION

The effectiveness of combining a tomato cultivar carrying the *Mi* gene for resistance to *M. incognita* and BioAct WG based on the nematode antagonist *P. lilacinum* strain 251 against RKN has been assessed in a tomato – cucumber rotation under greenhouse conditions. The initial hypothesis considered a synergistic effect of both methods to suppress nematode densities. The first one, mediated by plant resistance, should

suppress nematode infection, development and reproduction. The second one, due to Pl251, should parasitize eggs produced by nematodes that escaped plant resistance. In the following cucumber crop, fewer RKN at transplanting after resistant tomato should mean more percentage of egg parasitism, because most egg masses should be on the root surface favoring egg infection, as well as reduce yield losses due to fewer J2 at transplanting. However, the hypothesis was not confirmed in our trial, with plant resistance being the only factor that consistently suppressed RKN. The effectiveness of tomato cultivars or rootstocks carrying the *Mi* gene against RKN in greenhouses in Spain was consistent with previous reports.[4,9,14,15] In this study, resistant tomato suppressed disease severity and reproduction by 82 – 91 % and 87 – 95 % compared to the susceptible cultivar, each year, respectively. The effect of intermittent peaks of soil temperatures over 28 ^{o}C did not affect the effectiveness of the *Mi* gene as previously reported.[39] During the tomato crop in 2011, the numbers of days with maximum soil temperatures over 28 ºC were 23, after 35 days of transplanting, and 38 days, after 62 days of transplanting in 2012. In addition, the benefit of cropping a resistant tomato cultivar on yield losses of the following susceptible crop was also observed as previously stated.[2,15]

Unlike to plant resistance, there are few reports about the effectiveness of BioAct WG alone and/or in combination with other control methods against RKN under Mediterranean conditions.[25,26] In studies conducted in Greece and Turkey, BioAct WG did not provide satisfactory RKN control. However, in several *in vitro* and pot tests the antagonistic capability of Pl251 against several plant-parasitic nematode species was reported .[11,20-24,40-42] The ability of Pl251 to penetrate eggs and cuticles of sedentary stages of RKN by mechanical and chemical mechanisms has been reported.[20,21] The results obtained by the API ZYM method showed high protease and lipase activity and

low chitinase activity able to degrade the main components of egg shell and nematode cuticle.[43] Therefore, a high proportion of egg parasitism was expected, as it was in our *in vitro* experiment (94.9 %). In addition, Kahn *et al.*[21] pointed out the parasitic ability of Pl251 on all stationary stages of *M. javanica*, that is: eggs, juvenile contained in eggs, post-infective juvenile stages: from swollen J2 to J4, and females, but they did not assess the effect on the mobile infective J2. The results of this study showed that Pl251 was not able to parasitize the infective J2 stage of *M. incognita* neither sedentary stages of RKN because Pl251 is not a root endophyte of tomato or cucumber plants.[44,45] Thus, Pl251 could exert its parasitic potential only on eggs and juveniles contained in eggs that remained in soil at the end of the crop, or on those produced on roots and exposed to the soil. In fact, in greenhouse conditions, the percentage of egg parasitism was less than 2.6 % in both crops and years. Moreover, microorganisms associated to the gelatinous matrix of the egg masses can inhibit fungal egg parasites as *Pochonia chlamydosporia.*[46] Thus, fungal application did not affect nematode development even though four applications per-crop and year.

Environmental factors can play an important role in nematode biocontrol.[47] Rumbos *et al.*[42] reported a negative correlation between the persistence of Pl251 in soil and the sand content of soil. Thus, sandy soils, as in this study (83.8 % sand) would not be suitable for the fungus. However, the test of soil receptivity showed that mycelia growth was better in sterilized sand than in the sterilized sandy loam soil from the greenhouse experiments. This indicates that other factors different to microbial communities or thermo-sensitive chemicals in soil could limit the effectiveness of Pl251 because mycelia growth was equally poor in sterile and non-sterile sandy loam soil from the greenhouse.

Soil temperatures during the cropping period or time of application could also affect BioAct WG effectiveness. In this study, cardinal temperatures of mycelia growth were determined. Maximum temperature of Pl251 mycelia growth in water agar was similar to that reported by Kiewnick,[41] but not the optimal range, which was less wide (24-26 *vs* 24-30 ^{o}C). Moreover, in this study, minimum temperature was also estimated (14.2 ^{o}C) because low soil temperatures at transplanting tomato in spring (17 – 19 ^{o}C) could affect fungal and thus its effectiveness. Soil temperatures during the cultivation of tomato and cucumber were in the range of the fungus development according to cardinal temperatures, but 37 out of 98 days and 60 out of 90 days during each tomato crop were over optimal temperatures, and 63 out of 135 and 59 out of 98 days during each cucumber crop. At soil temperatures between the optimal for fungal growth (24-26 ^{o}C) and the optimal for *M incognita* development (30 ^{o}C),[48] the nematode could take advantage over Pl251 that can reduce its effectiveness. In fact, at temperatures of 28 ± 1.5 ^{o}C no dose-response relationship was observed but it did at 25 ± 1 ^{o}C.[24]

The soil water potential recorded during the cropping period should not affect the fungal growth because it was near field capacity (-0.033MPa), and according to the results in *in vitro* test, Pl251 mycelia grow more at higher water potential.

Another putative explanation for the lack of efficacy of BioAct WG in the greenhouse trial could be the content of glucose in the formulation, which inhibits the protease activity and consequently the capability to parasitize RKN eggs.[49]

The present study aimed to demonstrate the usefulness of combining plant resistance with BioAct WG to manage RKN. However, Pl251 was not able to parasitize eggs in greenhouse due to suboptimal soil temperatures for several days during the cropping period and/or inhibition of enzymes produced by the fungus by the components of the formulation and/or non-thermo-sensitive chemical factors in soil. Therefore, no

synergistic effect was observed. Resistant tomato suppressed nematode reproduction and yield losses of tomato as well as yield losses of the following cucumber crop, but nematode populations increased at the end of the crop. *P. lilacinum* is found naturally worldwide,[47] and it has been isolated from RKN in Spain.[30] Pl251 was isolated from Philippines,[46] and despite its effectiveness to parasitize eggs *in vitro,* well is known that native isolates can be more suitable and can performance better than the foreign ones in field conditions.[50] More studies are needed to optimize BioAct WG usage and to improve knowledge on optimal environmental conditions to improve its effectiveness.

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Table 1. Cropping dates of the rotation sequence tomato-cucumber and soil temperatures in the greenhouse during two consecutive growing seasons.

Year	Crop	Cropping dates	Average soil temperature (ºC) [a]	Minimum and maximum temperatures (ºC) [b]
2011	Tomato	31 March-6 July	25.4	19.8-34.3
	Cucumber	29 July-26 October	27.8	20.2-32.9
2012	Tomato	5 March-17 July	24.4	17.0-31.4
	Cucumber	31 July-5 November	27.0	17.5-31.2

[a] Average soil temperature at 15cm depth: mean of daily mean temperatures during the cropping period.

[b] Absolute minimum or maximum soil temperature at 15cm depth during the cropping period.

Table 2. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the resistant tomato cv. Monika (TR) and susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG cultivated from 31st March to 6th July of 2011.

BioAct WG [a]	Tomato cultivar	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	663±241 a	334±113 b	1.2±0.2 b	421±1100 b	0±0	1.5±0.2 a
	TS	612±182 a	2347±331 a	6.8±0.2 a	7499±347 a	0±0	0.3±0.2 b
Application	TR	579±210 a	100±24 b	1.3±0.2 b	482±110 b	0.04±0.02	1.3±0.1 a
	TS	576±161 a	3300±649 a	7.4±0.2 a	6957±441 a	0.02±0.01	0.3±0.1 b

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 3. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 29th July to 26th October of 2011.

BioAct WG [a]	Previous Crop	Pi (J2 250 cm^{-3}soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	241±99 b	357.85±91 a	6.6 ±0.6 b	1352±561 a	0±0	0
	TS	3202±700 a	234.60±99 b	9.7±0.3 a	179±121 b	0±0	0
Application	TR	193±80 b	684.30±183 a	6.9±0.7 b	3094±956 a	0.44±0.27	0
	TS	2446±243 a	185.05±64 b	10.0±0 a	0±0 b	0.09±0.09	0

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 4. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG, and the resistant tomato cv. Monika (TR) alone, cultivated from 5th March to 17th July of 2012.

BioAct WG [a]	Tomato cultivar	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	358±91 a	1009±232 b	2.9±0.3 b	811±250 b	0	2.2±0.1 a
	TS	185±80 b	4498±705 a	7.2±0.3 a	6406±1695 a	0	0.9±0.2 b
Application	TS	363±106 a	4010±513 a	7.7±0.3 a	8586±989 a	2.39±1.23	0.8±0.1 b

Data are mean ± standard error of 10 replicates of the combination of "no BioAct application with TR or TS" and 20 replications of the combination of "BioAct application with TS".

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 5. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 31st July to 5th November of 2012.

BioAct WG [a]	Previous Crop	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	1187±400 b	1379±253 a	7.6±1.1 b	1083±381	0±0	0.2±0.1 a
	TS	4319±464 a	659±162 ab	10.0±0 a	na	na	0.03±0.002 b
Application	TR	801±199 b	768±184 ab	8.7±0.5 b	3646±1482	2.60±1.01	0.10±0.04 a
	TS	3968±695 a	522±217 b	10.0±0 a	na	na	0.02±0.002 b

Data are mean ± standard error of 15 replicates of the combination each combination with TS and 5 replicates of each combination with TR.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Na: no available

Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251) ~~against~~ on *Meloidogyne incognita* in a tomato–cucumber rotation in ~~plastic~~ a greenhouse.

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Running title: Effect of plant resistance and BioAct WG ~~(*P. lilacinus* strain 251)~~ ~~against~~ on RKN in greenhouse.

Abstract

BACKGROUND: The effectiveness of combining resistant ~~or susceptible~~ tomato ~~cultivars~~ with BioAct WG (*Purpureocillium lilacinum* strain 251; Pl251) against *Meloidogyne incognita* was assessed in ~~into~~ a tomato-cucumber rotation in ~~a plastic~~ greenhouse over ~~during~~ two years. Additionally, ~~In addition~~ enzymatic activity of the fungus, the percentage of fungal eggs ~~or~~ and juveniles parasitism, cardinal temperatures and the effect of water potential on mycelia growth and the soil receptivity to Pl251, were determined *in vitro*. ~~experiments were conducted to determine the~~

RESULTS: Plant resistance was the only factor that suppressed nematode reproduction~~, and tomato and the following cucumber~~ crop yield losses. Percentage of ~~fungal~~ egg parasitism in plots treated with BioAct WG was less than 2.6%. However ~~in~~ under *in vitro* conditions, Pl251 showed protease, lipase, and chitinase activities, and

parasitized 94.5% of ~~*M. incognita*~~ eggs, but no juveniles. Cardinal temperatures were 14.2, 24-26, and 35.4 °C. The maximum Pl251 mycelial growth was at -0.25 MPa and 25 °C. Soil temperatures and water potential in the greenhouse ~~field conditions~~ were in the range of the fungus ~~development~~. However, soil receptivity was less in ~~the plastic~~ greenhouse soil, irrespective of sterilization, than in sterilized sand.

CONCLUSIONS: ~~No synergistic effect of combining resistant cultivars and BioAct WG was observed, being with~~ Plant resistance was the only factor able to suppress nematode densities, disease severity and yield losses, and to protect the following ~~susceptible~~ cucumber crop. ~~Soil temperatures in field for fungal growth and~~ Environmental factors involved in soil receptivity could have negatively affected ~~its~~ fungus effectiveness.

Key~~s~~ words

Biological control, *Cucumis sativus*, doble-cropping system, integrated management, root-knot nematodes, *Solanum lycopersicum*.

1 INTRODUCTION

Spain is the main producer of vegetables under protected cultivation in the Mediterranean area, ~~dedicating~~ with 71.003 ha.[1] The major crops ~~belong to Solanaceae and Cucurbitaceae families. The most important~~ are tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.), ~~two of the most cultivated crops, which are~~ frequently cultivated in a double cropping system.[2] ~~In g~~Greenhouse~~,~~ tomato is ~~cultivated in~~ grown on 18.501 ha with an annual production of 1.881.922 tonnes, which represents 38% of the tomato production area and 47% of the total ~~tomato surface and~~ yield~~,~~ ~~respectively~~. ~~Concerning~~ Greenhouse cucumber~~, it~~ is grown on ~~in~~ 7.768 ha with an

annual yield of 717.693 tonnes, ~~which~~ representing ~~an~~ 88% of the total production area and 95 % of the total yield ~~cucumber surface and production, respectively.~~[1]

Root-knot nematodes (RKN), *Meloidogyne* spp., are one of the most important soil pathogens limiting horticulture production worldwide, especially under protected cultivation.[3] *Meloidogyne* spp. ~~is~~are widely spread in all vegetable production areas in Spain. Yield losses caused by RKN can reach 60% in tomato and 88% in cucumber.[4,5] RKN is mainly managed by fumigant and non-fumigant nematicides.[6] However, the limitations imposed by the Directive 2009/128/EC ~~into the EU countries~~ have encouraged research finding alternatives to chemical pesticides as well as to design effective and durable strategies to manage ~~plant damaging organisms such as~~ RKN.

There are a large number of non-chemical ~~available~~ alternatives ~~to chemicals~~ to control RKN,[3] including crop rotation with resistant cultivars ~~plants~~ and biological control. In ~~N~~nematology, ~~plant~~ resistance is defined as the ability of a plant to suppress infection, development and/or reproduction of plant-parasitic nematodes.[7] Therefore, resistant tomato cultivars or rootstocks carrying the *Mi*-gene are widely used because of their effectiveness in suppressing *M. arenaria, M. incognita* and *M. javanica* ~~RKN~~.[4,7] However, its expression can be ~~conditioned~~ limited by i) ~~by~~ constant soil temperatures above 28 ^{o}C at transplanting,[8] ii) ~~by~~ the genetic background of the tomato cultivar or rootstock,[9] ~~and~~ iii) ~~by~~ RKN species: not effective against *M. hapla*, ~~race 3 of~~ *M. chitwoodi* race 3,[10] *M. enterolobi*,[11] or *M. exigua*;[12], ~~as well as~~ and iv) virulent populations, which can occur suddenly or be selected by repeated cultivation of resistant cultivars ~~tomatoes~~.[13,14] Inclusion of resistant tomato cultivars in a cropping sequence ~~contributes~~ helps to suppress the ~~multiplication~~ reproduction of RKN and to reduce yield losses of the following susceptible crop,[15] such as cucumber,[2] because yield losses are related to nematode densities in soil at transplanting.[16]

Several microorganisms have been evaluated ~~as~~ for biological control of plant-parasitic nematodes~~.~~.[17] The use of biological control agents able to suppress the buildup of RKN can be of interest to reduce the pressure on R genes avoiding the selection of virulent populations and contribute to maintain nematode densities below the economic threshold level. ~~however~~Out of those, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) strain 251 (Pl251) is the only biological ~~control agent included as~~ nematicide listed in the annex 1 of the European register of active substances.[18] *Purpureocillium lilacinum* is a common soil hyphomycete able to parasitize RKN sedentary stages by direct hyphal penetration and by using hydrolytic enzymes.[19-21] Besides, the effectiveness of Pl251 to ~~manage~~ control RKN has been widely reported ~~in~~ under controlled conditions and in pot tests,[22-24] although few reports are available on its effectiveness ~~in~~ under field conditions ~~are available~~.[25,26]

The aim of the present study was to evaluate the effectiveness of combining the resistant tomato cv. Monika with ~~and~~ *P. lilacinum* strain 251 (BioAct WG®, Belchim Crop Protection, Londerzeel, Belgium) over two consecutive growing seasons in a greenhouse to manage RKN in a tomato-cucumber rotation ~~sequence~~. Additionally, ~~several~~ *in vitro* experiments were carried out to determine extracellular enzymes produced by Pl251 and its capability to parasitize eggs and second-stage juveniles of RKN, as well as to know cardinal temperatures and the effect of temperature and water potential on mycelia growth, and soil receptivity to Pl251~~to the fungus isolate~~.

2 MATERIAL AND METHODS

2.1 ~~Field~~ Greenhouse trial

The field trial was conducted in a 700 m^2 ~~plastic~~ greenhouse infested with *M. incognita* in Viladecans (41º 17’ 18’’N; 2º 2’ 39’’E, Barcelona, Spain) during ~~two~~

~~consecutive years,~~ 2011 and 2012. The soil ~~texture~~ was a sandy loam with 83.8% sand, 6.7% silt and 9.5% clay; pH 8.7; 1.8% of organic matter (w/w) and 0.5 dS/m of electric conductivity. Soil was infested with *M. incognita* in 2007 and rotations with resistant or susceptible tomato cultivars and cucumber or black fallow were carried out from 2008 to 2010.

The rotation sequence consisted of ~~was integrated by~~ resistant tomato cv. Monika (bearing the *Mi* gene) or the susceptible cv. Durinta ~~cultivated~~ from March to July, followed by cucumber cv. Dasher II cultivated from July to October-November. Individual plots of 9.6 m^2 comprising of 4 rows with 6 plants per row. Plant spacing was 50 cm between rows and 55 cm within rows. The distance between individual plots was 110 cm between rows and 100 cm along rows.

In 2011, combinations of resistant (TR) or susceptible tomato (TS) with or without BioAct WG preceding cucumber with or without BioAct WG were assessed. ~~In 2011, eight combinations were included in the experiment: (i) TR-BioAct-C-BioAct; (ii) TR-BioAct-C; (iii) TR-C-BioAct; (iv) TR-C; (v) TS-BioAct-C-BioAct; (vi) TS-BioAct-C; (vii) TS-C-BioAct; (viii) TS-C.~~ Each combination was replicated ~~five~~ 10 times according to a stratified randomized block design. In 2012, the combinations were located ~~at~~ on the same plots ~~than~~ as in 2011 with slight modifications. Plots cultivated with ~~resistant tomato~~ TR did not receive any treatment with BioAct WG because of results obtained in 2011~~, but did when cucumber was cultivated~~. Then, combinations ~~of susceptible tomato with or without BioAct WG and resistant tomato without BioAct WG preceding cucumber with or without BioAct WG were assessed.~~ including TR or TS without BioAct WG were replicated 10 times, and 20 for the combination TS with BioAct WG. Cucumber crop cultivated after TS with application or not application of BioAct WG was replicated 15 times, and five times each combination after TR. ~~six~~

~~combinations were assessed: (i) TR-C BioAct; (ii) TR-C; (iii) TS BioAct-C BioAct; (iv) TS BioAct-C; (v) TS-C BioAct; (vi) TS-C.~~

Dates and rates of application of BioAct WG to soil and seedlings were ~~those~~ as recommended by the manufacturer. Briefly, the first soil application of the commercial product BioAct WG (1 x 10^{10} viable spores g^{-1} of *P. lilacinus* strain 251 dried on glucose) was carried out 14 days before transplanting by drip irrigation at a rate of 0.4 g m^{-1} linear and 10 cm width. The following fungal applications to soil were repeated at the same rate at six weeks interval; two applications during the tomato and cucumber crops in 2011 and cucumber in 2012, and three during the tomato crop in 2012. Seedlings were watered with a suspension of 0.1 g BioAct WG 1 L^{-1} just before transplanting.

Tomato was cultivated from 31st March to 6th July (98 days), and cucumber from 29th July to 26th October (90 days) in 2011. In 2012, tomato was cultivated from 5th March to 17th July (135 days) and cucumber from 31st July to 5th November (98 days).

The plants were irrigated by drip irrigation and fertilized weekly with a NPK solution (15–5–30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1}. Crops were vertically trellised. Weeds were removed manually during and between cropping cycles. Tomato yield was assessed from the first six fruit sets produced from the eight central plants in each plot. ~~Commercial standard~~ Fruits were harvested according to commercial standards as they reached maturity. Similar, cucumber fruits were harvested from the eight central plants of each plot ~~were harvested~~ when they reached the standard commercial size. Total yield per crop cycle was expressed as kilograms $plant^{-1}$.

Soil temperatures were recorded at 30 min intervals in order to estimate the number of the nematode generations, and the effect of soil temperature on the fungus growth. In 2011 were recorded with soil probes 107 (Campbell Scientific, Logan, USA) placed at a

depth of 15 cm. In 2012, soil temperatures and water potential were recorded at the same interval and soil depth with 5TM and MPS-1 probes (Decagon devices, Inc, Pullman, USA).

Composite soil samples were collected from each plot at the beginning and at the end of each crop to estimate initial (Pi) and final (Pf) nematode population densities, respectively. Each soil sample consisted ~~in~~ of eight subsamples from the ~~firsts~~ top 30 cm ~~of depth tacked~~ taken with an auger ~~sampling tube~~ (2.5 cm diameter). Soil~~s~~ samples were sieved through a 4 mm aperture screen to remove stones ~~from~~ ~~soil~~, and carefully homogenized to extract nematodes from 500 cm^3 by Baermann trays.[27] Second-stage juveniles (J2) that migrated to the water were collected one week later, concentrated on a 75 µm sieve, counted, and then expressed as number of J2 per 250 cm^3 of soil. At the end of each crop, plants were removed with a pitchfork, cut at ground level, and the disease severity was assessed using the Zeck's galling[28] index on a scale of 0 to 10, where 0 means complete and healthy root system (no galls observed) and 10 means plants and roots dead. Afterwards, the roots were weighted ~~bulked~~ and chopped into 2 cm long segments and two 10 g subsamples were used to extract eggs by blender maceration in a 1% NaOCl solution.[29] After 10 minutes of maceration, the egg suspension was passed through a 75 µm sieve, to retain the plant material, and ~~through~~ a 25 µm sieve to retain the eggs. The number of eggs was counted and expressed per gram of fresh root weight.

To assess fungal egg parasitism, the procedure described in Giné *et al.*[30] was used. In brief, 20 egg masses per individual plot were handpicked from the remaining roots and placed in a watchglass containing sterile demineralised water. The outer part of the gelatinous matrix was removed, and eggs were dispersed in an Eppendorf microcentrifuge tube containing 1 mL of sterile demineralised water with a pestle. A

333 µL aliquots of eggs suspension were was spread onto each of three replicated Petri plates containing a growth restricting medium,[31] and incubated at 25 ºC ± 0.5 ºC at in the dark. Number of parasitized eggs was recorded after 24 and 48 hours under a dissecting microscope and percentage of egg parasitism was then calculated as the number of parasitized eggs per plate per total eggs per plate. Eggs were considered parasitized if fungal hyphae grew from inside of unhatched eggs. Parasitized eggs were individually transferred to the growth restricting medium to establish pure cultures and fungal species were identified by cultural and morphological characteristics.[32]

2.2 Extracellular enzymes production

Extracellular enzyme production by Pl251 was evaluated using a semiquantitative API ZYM ® (BioMérieux, Marcy l'Etoile, France) system which identifies 19 cellular enzymes, i.e. alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtol-AS-BI phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, nacetyl-β-glucosaminidase, α-mannosidase and α- fucosidase.

Conidia of Pl251 were collected from 3-week-old cultures of the fungus growing on potato dextrose agar (PDA; 39 g L^{-1}) at 25ºC at in the dark. The colony was washed with 5 mL of sterile distilled water. The number of conidia was then counted with a haemocytometer and adjusted to 1 x 10^6 conidia mL^{-1}. The API ZYM system was used according to the manufacturer's instructions. In brief Briefly, 65 µL of the conidia suspension was dispended placed into each cupule, and incubated at 25 °C for 6 h at in the dark.[33] Enzyme activity was observed after addition of a drop of each ZYM A and ZYM B reagents and exposed to sunlight for an hour to eliminate the yellowing from the reagents. The enzyme production was assessed according to the scale from 0 (no

enzyme production) to 5 (maximum enzyme production) according the color chart provided by the manufacturer. The experiment was carried out once.

2.3 Capability of Pl251 to parasitize ~~RKN~~ *Meloidogyne incognita* eggs and J2 ~~in~~ *in vitro* ~~test~~

Individual plugs of 9 mm-diameter from the edge of a Pl251 colony growing ~~in~~ on PDA ~~was~~ were placed ~~at~~ in the centre of a total of six Petri dishes (90 mm) containing water agar (~~bacteriological~~ agar 12 g L^{-1}), and incubated at 25 ºC ± 0.5 ºC ~~at~~ in the dark for 3 days ~~weeks~~.

Surface sterilized eggs or J2 of the same *M. incognita* population used to inoculate the soil of the greenhouse in 2007 and maintained in tomato in pots were used to determine the parasitic ability of Pl251. *Meloidogyne incognita* was identified by morphology of perineal pattern, esterase pattern, and SCAR marker. Eggs from 30 egg masses handpicked from tomato roots were surface sterilized following the protocol of Verdejo *et al.*[34] with some modifications. Egg masses were placed into a conical sterile tube with 1 mL of a 0.5% NaOCl solution for four min. and were shaken at 30 s intervals for 10 seconds. After that, the solution was diluted 10 times with sterile distilled water and left undisturbed for 30 min. to allow the eggs to settle at the bottom of the tube. Then, eggs were taken and ~~were~~ spread into three Petri dishes at 1 cm of the edge of the colony of the Pl251 using a sterile Pasteur pipette, and incubated at 25 ºC ± 0.5 ºC ~~at~~ in the dark for one week ~~3 weeks~~. The assessment of egg parasitism was carried out as described previously.

Second-stage juveniles of *M. incognita* were obtained by extraction of eggs from tomato roots by the Hussey and Barker method[29] and left to emerge ~~in~~ on Baermann trays.[27] Then, J2 were surface sterilized following the Mountain[34] procedure with some modifications. Briefly, a suspension of 100 J2 in 0.5 mL water was placed in a conical

sterile tube containing streptomycin 0.1% for 4 hours. The suspension was shaken at one hour interval for 10 seconds. The solution was then diluted 20 times with sterile distilled water and left undisturbed for 30 min. for J2 to settle at the bottom of the tube ~~deposition~~. Then, J2 were taken and placed in three Petri dishes at 1 cm of the edge of the Pl251 colony using a sterile Pasteur pipette, and incubated at 25 ºC ± 0.5 ºC ~~at~~ in the dark for one week ~~3 weeks~~. The assessment of J2 parasitism was carried out as described previously for egg parasitism.

Percentage of parasitism was then calculated as the number of unhatched parasitized eggs or ~~juveniles~~ J2 per Petri dish divided by the number of unhatched eggs or ~~juveniles~~ J2 per Petri dish.

Experiments were conducted once.

2.4 Cardinal temperatures and the effect of temperature and water potential on Pl251 mycelia growth ~~in~~ *in vitro* ~~tests~~

Cardinal temperatures of Pl251 were determined placing individual 9 mm-diameter plugs of Pl251 in ~~at~~ the center of each of 24 Petri dishes containing water agar (WA; 12 g L^{-1}). ~~Three out 24~~ Petri dishes were incubated at 4, 10, 15, 20, 25, 30, 35 ~~and~~ or 40ºC ~~at~~ in the dark (three dishes per temperature). Minimum and maximum diameters (mm) of fungal growth were measured every 24 h until the ~~fungi~~ colonies occupied ~~reach the~~ 80% of the surface of the Petri plate. The mycelia growth rate (mm day^{-1}) was calculated as the relation between mean colony diameter (mm) and growth time (day).

Concurrently, the effect of water potential (Ψ) and temperature on Pl251 growth was assessed. Water agar media with different concentrations of Polyethylene Glycol 8000 (PEG8000) was prepared according to the Michel's equation[36] to achieve Ψ between 1.25, -1, -0.75, -0.5 and -0.25 MPa. Mycelia plugs of 9 mm diameter from the edge of a fungal the colony were placed in ~~at~~ the centre of the Petri plates and then incubated at

15, 20, 25, and 30 ºC ~~at~~ in the dark. Each combination of temperature-PEG8000 concentration was repeated three times. Minimum and maximum diameters of fungal growth were measured daily until the colonies occupied 80% of the surface of the Petri plate. ~~Per~~ For each water potential, a linear regression~~s~~ was calculated between the mean diameter of the colony per ~~each~~ day ~~of assessment~~ and the temperature ~~were constructed~~, and the slopes were used to construct ~~other~~ the regressions to determine the effect of temperature and water potential on mycelia growth. Experiments were repeated once.

2.5 Soil receptivity

Soil ~~coming~~ collected from BioAct WG non-treated plots ~~in of which~~ the greenhouse~~field~~ trial ~~was carried out~~ was assessed for receptivity to the fungal isolate. A part of soil was two times sterilized at 121 ^{o}C for 1 h ~~and the procedure was repeated after~~ within 24 h. The other part remained non-sterilized. The experiment was carried out following the procedure described by Monfort *et al.*[37] ~~procedure~~. ~~In brief~~ Briefly, 40 g of sterilized or non-sterilized air-dried soil was placed in Petri dishes and saturated with sterile distilled water. Soils included in the experiment were: (i) sterilized ~~plastic~~ greenhouse soil; (ii) non-sterilized ~~plastic~~ greenhouse soil and (iii) sterilized sand. A polyvinylidene difluoride (PVDF) membrane (0.22-µm-pore-size and 45 mm-diameter) was sterilized at 121 ^{o}C for 20 min and placed ~~at the~~ on top of the soil~~s~~ ensuring full contact. A 4 mm-diameter plug of Pl251 was placed in the middle of each membrane. Petri dishes were then sealed and incubated at 25 °C ± 0.5 ºC ~~at~~ in the dark. After three weeks, the membranes were ~~cleaned~~ washed with sterile distilled water and then dried in a laminar air flow cabinet. Then, the membranes where incubated in a solution of 1% trypan blue in lactic acid for 12 h at room temperature to stain the mycelia ~~in blue~~. After

that, the excess of the stain was removed with sterile distilled water, and minimum and maximum colony diameters were measured. The experiment was repeated once.

2.6 Statistical analysis

Statistical analyses were done using SAS v. 9 (SAS Institute Inc.). Data from field experiments were transformed when required to log10 (x + 1) or ~~arcsine~~ square-root (x+0.5) to normalize the data. The greenhouse trial was analyzed by analysis of variance by the general lineal model (PROC GLM) according to a factorial design to compare the effect of ~~i)~~ the tomato cultivar, the application of BioAct WG, and the interaction (except for tomato 2012), on nematode densities in soil, eggs in roots, disease severity, and crop yield per ~~every~~ cropping season. Analysis of variance was also carried out to compare the effect of the temperature and kind of soil on the ~~mycelia~~ growth of Pl251. ~~; and ii)~~

3 RESULTS

3.1 ~~Field~~ Greenhouse trial

Minimum, maximum and average soil temperatures during the cultivation of each crop and year are provided in Table 1. Accumulated soil temperatures during the tomato and cucumber crops in 2011 were 1504 DD (degree days; base temperature (Tb) =10 ºC) and 1473 DD (Tb=11.4 ºC), respectively. According to thermal requirements of tomato and cucumber, [5,38] *M. incognita* completed two generations in tomato (thermal constant (S) = 600-700 DD over Tb=10 ºC),[38] and in cucumber (S = 500 DD over Tb = 11.4 ºC).[5]

In 2011, the tomato cultivar was the only factor that explained ~~variability~~ differences ($P < 0.05$) ~~on~~ in nematode densities in the soil and roots, galling index and crop yield, both ~~than~~ in tomato ~~as~~ and cucumber ~~crops~~ (Tables 2 and 3). Nematode densities in the

soil, roots and the galling index ~~registered~~ at the end of the resistant tomato crop were 8, 6 and 18% those ~~registered~~ recorded at the end of the susceptible ~~one~~ cultivar, which ~~in turn~~ yielded 78% less than the resistant one. The percentage of fungal egg parasitism at the end of the crop was less than 0.1% (Table 2). At cucumber transplanting, higher ($P < 0.05$) soil nematode densities ~~in soil were registered~~ occurred in plots preceded by a susceptible than a resistant tomato cultivar~~s~~. Galling indices were lower ($P < 0.05$) after a resistant than a susceptible tomato cultivar. ~~The~~ Eggs from cucumber plants were only recovered from plants preceded by a resistant tomato because ~~high percent of plants~~ cucumber following susceptible tomato died (data not shown). Egg parasitism by the fungus was less than 0.5~~1~~% (Table 3).

In 2012, accumulated temperatures during the tomato and cucumber crops were 1959 (Tb = 10 ºC) and 1524 DD (Tb = 11.4 ºC), respectively. Hence, *M. incognita* completed three generations in both crops according to its thermal requirements. In this cropping season, the tomato cultivar was also the factor responsible ~~to~~ for the differences ($P < 0.05$) ~~on~~ in nematode densities in the soil and roots, disease severity, as well as crop yield in both tomato and cucumber crops (Tables 4 and 5). As in the previous season, cucumber ~~preceded by~~ following susceptible tomato ~~was exposed to~~ had higher ($P < 0.05$) nematode ~~density~~ levels at transplanting, and all plants died~~were dead~~ at the end of the experiment. The percentage of fungal egg parasitism at the end of tomato and cucumber crops was 2.4 and 2.6 %, respectively (Table 5).

3.2 Extracellular enzymes production

Six extracellular enzymes were produced by Pl251. The ~~highest~~ enzyme produced in highest amounts by the fungus ~~isolate~~ was leucine arylamidase (value of the color scale: 5) followed by esterase and acid phosphatase (value of the color scale: 4), esterase-

lipase (value of the color scale: 3) and the lowest were naphtol-AS-BI-phosphohydrolase and N-acetyl-*β*-glucosaminidase (value of the color scale: 2).

3.3 Capability of Pl251 to parasitize RKN eggs and J2 ~~in~~ *in vitro* ~~test~~

Pl251 parasitized 94.91% ± 2.88 (mean ± standard error) of *M. incognita* unhatched eggs. However, no ~~juveniles~~ J2 were parasitized ~~by the fungal isolate~~.

3.4 Cardinal temperatures and the effect of ~~the relationship~~ temperature and water potential on Pl251 mycelia growth ~~in~~ *in vitro* ~~tests~~

Mycelia growth of Pl251 occurred between 14.2 ºC (minimum) and 35.4 ºC (maximum), with ~~being~~ 24-26 ºC as the optimal growth temperatures range (Figure 1). No ~~fungal~~ growth was detected at 4, 10 and 40 ºC. The ~~values of~~ water potential ~~assessed~~ (-1.25 to -0.25 MPa) directly influenced the mycelia growth of Pl251. The mycelia growth was higher at optimal temperatures (25 ºC) and highest water potential (-0.25 MPa), followed by 30 ºC, 20 ºC and 15 ºC (Figure 2).

3.5 Soil receptivity

The mycelia growth of Pl251 in sterile sand (2.55cm ± 0.06; mean ± standard error) was 91.83% higher ($P < 0.05$) compared with non-sterile (0.18cm ± 0.06; mean ± standard error) or sterile ~~plastic~~ greenhouse soil (0.23cm ± 0.07; mean ± standard error). However, no differences ($P > 0.05$) were found between sterile and non-sterile greenhouse soil.

4. DISCUSSION

The effectiveness of combining a tomato cultivar carrying the *Mi* gene ~~of~~ for resistance to *M. incognita* and BioAct WG based on the nematode antagonist *P. lilacinum* strain 251 against RKN has been assessed in ~~the~~ a tomato – cucumber rotation ~~sequence cropped in a plastic~~ under greenhouse conditions. The initial hypothesis considered a synergistic effect of ~~between~~ both ~~control~~ methods to suppress nematode

densities ~~across the rotation sequence in two ways~~. The first one, mediated by plant resistance, should ~~act~~ suppress~~ing~~ nematode infection, development and reproduction. The second one, due to Pl251, should ~~act~~ parasitize eggs produced by nematodes that escaped ~~the action of~~ plant resistance, ~~and then further increase the level of suppression against RKN~~. In the following cucumber crop, ~~less number of~~ fewer RKN at transplanting after resistant tomato should mean more percentage of egg parasitism, because most egg masses should be ~~outside the root~~ on the root surface favoring egg infection, as well as ~~less~~ reduce yield losses due to ~~less number of~~ fewer J2 at transplanting. However, the hypothesis was not confirmed in our ~~conditions~~ trial, ~~being~~ with plant resistance being the only ~~control methods~~ factor that consistently suppressed RKN~~nematode built up in every rotation sequence~~. The effectiveness of tomato cultivars or rootstocks carrying the *Mi* gene against RKN ~~cropped~~ in ~~plastic~~ greenhouses in Spain was consistent with previous reports.[4,9,14,15] In this study, ~~cropping~~ resistant tomato suppressed disease severity and reproduction ~~in~~ by 82 - 91% and 87 - 95% ~~than which were registered~~ compared to the susceptible ~~one~~ cultivar, each year, respectively. The effect of intermittent peaks of soil temperatures over 28 ºC did not affect the effectiveness of the *Mi* gene as previously reported.[39] During the tomato crop in 2011, the numbers of days with maximum soil temperatures over 28 ºC were 23, after 35 days of transplanting, and 38 days, after 62 days of transplanting in 2012. In addition, the benefit of cropping a resistant tomato cultivar on yield losses of the following susceptible crop was also observed as previously stated.[2,15]

~~Conversely~~ Unlike to plant resistance, there are few reports about the effectiveness of ~~*Purpureocillium lilacinus* strain 251~~ BioAct WG alone and/or in combination with other control methods against RKN ~~in~~ under Mediterranean conditions.[25,26] In studies conducted in Greece and Turkey, BioAct WG did not provide~~d~~ satisfactory RKN

~~management~~ control. However, in several *in vitro* and pot tests the antagonistic capability of Pl251 against several plant-parasitic nematode species was reported .[11,20-24,40-42] The ability of Pl251 to penetrate eggs and cuticles of sedentary stages of RKN by mechanical and chemical mechanisms has been reported.[20,21] The results obtained by the API ZYM method showed high protease and lipase activity and low chitinase activity able to degrade the main components of egg shell and nematode cuticle.[43] Therefore, ~~the~~ a high proportion of egg parasitism was expected, as it was in our *in vitro* experiment (94.9 %). ~~because protease alone or in combination with chitinase are more important for nematode parasitism than chitinase alone.~~[37] In addition, Kahn *et al.*[21] pointed out the parasitic ability of Pl251 on all stationary ~~developing~~ stages of *M. javanica*, that is: eggs, juvenile contained in eggs, post-infective juvenile stages: from swollen J2 to J4, and females, but they did not assess the effect on the mobile infective J2. The results of this study showed that Pl251 was not able to parasitize the infective J2 stage of *M. incognita* neither sedentary stages of RKN because Pl251 is not a root endophyte of tomato or cucumber plants.[44,45] Thus, Pl251 could exert its parasitic potential only on eggs and juveniles contained in eggs that remained in soil at the end of the crop, or on those produced on roots and exposed to the soil ~~conditions~~. ~~Otherwise, the fungus could not be able to parasitize the~~ In fact, in ~~plastic~~ greenhouse conditions, the percentage of egg parasitism was less than 2.6% in both crops and years. Moreover, microorganisms associated to the gelatinous matrix of the egg masses can inhibit fungal egg parasites as *Pochonia chlamydosporia*.[46] Thus, fungal application did not affect nematode development ~~despite~~ even though four ~~treatments~~ applications per ~~were applied during each~~ crop and year.

Environmental factors can play an important role ~~on~~ in nematode biocontrol. [47] Rumbos *et al.*[42] reported a negative correlation between the persistence of Pl251 in soil

and the sand content of soil. Thus, sandy soils, ~~such that~~ as in this study (83.8% sand) ~~could~~ would not be suitable for the fungus. However, the test of soil receptivity showed ~~more fungal~~ that mycelia growth was better in sterilized sand than in the sterilized sandy loam soil ~~in which~~ from the ~~field~~ greenhouse experiments ~~were carried out~~. ~~Then it seems~~ This indicates that other factors ~~than sand content~~ different to microbial communities or thermo-sensitive chemicals in soil could ~~influence~~ limit the effectiveness of Pl251 because ~~similar~~ mycelia growth was equally poor in sterile and non-sterile sandy loam soil from the ~~plastic~~ greenhouse ~~were recorded in the test of soil receptivity~~.

Soil temperatures during the cropping period or time of application could also affect ~~Pl251~~ BioAct WG effectiveness. In this study, cardinal temperatures of mycelia growth were determined. Maximum temperature of Pl251 mycelia growth in water agar was similar to that reported by Kiewnick[41], but not the optimal range, which was less wide (24-26 *vs* 24-30 ^{o}C). Moreover, in this study, ~~minimal~~ minimum temperature was also estimated (14.2 ^{o}C) because low soil temperatures at transplanting tomato in ~~the~~ spring ~~crop~~ (17 – 19 ^{o}C) could affect fungal ~~development~~ and thus its effectiveness. Soil temperatures during the cultivation of tomato and cucumber were in the range of the fungus development according to cardinal temperatures, but 37 out of 98 days and 60 out of 90 days during each tomato crop were over ~~the range of~~ optimal temperatures, and 63 out of 135 and 59 out of 98 days during each cucumber crop. At soil temperatures ~~from~~ between the optimal for fungal growth (24-26 ^{o}C) ~~to~~ and the optimal for *M incognita* development (30 ^{o}C),[48] the nematode could take advantage over Pl251 that can reduce its effectiveness. In fact, at temperatures of 28 ± 1.5 ^{o}C no dose-response relationship was observed but it did at 25 ± 1 ^{o}C.[24] ~~In addition, Pl251 was~~

~~more effective against *Belonolaimus longicaudatus* when applied during cooler winter and spring than in hot summer in Florida.[67] Conversely, t~~

The soil water potential recorded during the cropping period should not affect the fungal growth because it was near field capacity (-0.033MPa), and according to the results in *in vitro* test, Pl251 mycelia grow more at higher water potential.

Another putative explanation ~~of~~ for the lack of efficacy of ~~Pl251~~ BioAct WG in the ~~plastic~~ greenhouse trial ~~conditions~~ could be the content of glucose in the formulation, which inhibits the protease activity and consequently the capability to parasitize RKN eggs.[49]

The present ~~work tried to point out~~ study aimed to demonstrate the usefulness of combining plant resistance with ~~biological control~~ BioAct WG to manage RKN. However, Pl251 was not able to parasitize eggs in ~~field~~ greenhouse ~~conditions~~ due to suboptimal soil temperatures ~~during~~ for several days during the cropping period~~s~~ and/or inhibition of enzymes produced by the fungus by the components of the formulation and/or non-thermo-sensitive chemical factors in soil. ~~Definitively~~Therefore, no synergistic effect was observed. Resistant tomato suppressed nematode reproduction and yield losses of tomato as well as yield losses of the following cucumber crop, but nematode populations increased at the end of the crop. ~~The use of biological control agents able to suppress the buildup of RKN can be of interest to reduce the pressure on R genes avoiding the selection of virulent populations and contribute to maintain nematode densities above below the economic threshold level~~. *P. lilacinum* is found naturally worldwide,[47] and it has been isolated from ~~cyst nematodes and~~ RKN in Spain.[30] ~~*P. lilacinum* strain 251~~ Pl251 was isolated from Philippines,[46] and despite its effectiveness to parasitize eggs *in vitro* ~~test~~, well is known that native isolates ~~are~~ can be more suitable and can performance better than the foreign ones in field conditions.[50]

More studies ~~must be done~~ are needed to optimize ~~Pl251~~ BioAct WG usage and to improve knowledge on optimal environmental conditions to improve its effectiveness.

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Table 1. Cropping dates of the rotation sequence tomato-cucumber and soil temperatures in the greenhouse during two consecutive growing seasons.

Year	Crop	Cropping dates	Average soil temperature (ºC) [a]	Minimum and maximum temperatures (ºC) [b]
2011	Tomato	31 March-6 July	25.4	19.8-34.3
	Cucumber	29 July-26 October	27.8	20.2-32.9
2012	Tomato	5 March-17 July	24.4	17.0-31.4
	Cucumber	31 July-5 November	27.0	17.5-31.2

[a] Average soil temperature at 15cm depth: mean of daily mean temperatures during the cropping period.

[b] Absolute minimum or maximum soil temperature at 15cm depth during the cropping period.

Table 2. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the resistant tomato cv. Monika (TR) and susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG cultivated from 31st March to 6th July of 2011.

BioAct WG [a]	Tomato cultivar	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	663±241 a	334±113 b	1.2±0.2 b	421±1100 b	0±0	1.5±0.2 a
	TS	612±182 a	2347±331 a	6.8±0.2 a	7499±347 a	0±0	0.3±0.2 b
Application	TR	579±210 a	100±24 b	1.3±0.2 b	482±110 b	0.04±0.02	1.3±0.1 a
	TS	576±161 a	3300±649 a	7.4±0.2 a	6957±441 a	0.02±0.01	0.3±0.1 b
~~BioAct [c] *vs* no BioAct~~		~~NS~~	~~NS~~	~~NS~~	~~NS~~		~~NS~~
~~TR *vs* TS~~		~~NS~~	~~S~~	~~S~~	~~S~~		~~S~~
~~T x BioAct~~		~~NS~~	~~NS~~	~~NS~~	~~NS~~		~~NS~~

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

~~[c] S and NS indicate statistically significant or non-significant differences between factors, respectively, according to Tukey's test ($P < 0.05$).~~ Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 3. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 29th July to 26th October of 2011.

BioAct WG [a]	Previous Crop	Pi (J2 250 cm^{-3}soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	241±99 b	357.85±91 a	6.6 ±0.6 b	1352±561 a	0±0	0
	TS	3202±700 a	234.60±99 b	9.7±0.3 a	179±121 b	0±0	0
Application	TR	193±80 b	684.30±183 a	6.9±0.7 b	3094±956 a	0.44±0.27	0
	TS	2446±243 a	185.05±64 b	10.0±0 a	0±0 b	0.09±0.09	0
~~BioAct [e] *vs* no BioAct~~		~~NS~~	~~NS~~	~~NS~~	~~NS~~		
~~TR *vs* TS~~		~~S~~	~~S~~	~~S~~	~~S~~		
~~T x BioAct~~		~~NS~~	~~NS~~	~~NS~~	~~NS~~		

Data are mean ± standard error of 10 replicates.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

~~[e] S and NS indicate statistically significant or non-significant differences between factors, respectively, according to Tukey's test ($P < 0.05$).~~ Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 4. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG, and the resistant tomato cv. Monika (TR) alone, cultivated from 5th March to 17th July of 2012.

BioAct WG [a]	Tomato cultivar	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	358±91 a	1009±232 b	2.9±0.3 b	811±250 b	0	2.2±0.1 a
	TS	185±80 b	4498±705 a	7.2±0.3 a	6406±1695 a	0	0.9±0.2 b
Application	TS	363±106 a	4010±513 a	7.7±0.3 a	8586±989 a	2.39±1.23	0.8±0.1 b
~~TR[e] *vs* TS~~		~~NS~~	~~NS~~	~~NS~~	~~NS~~		~~NS~~
~~T x BioAct~~		~~NS~~	~~S~~	~~S~~	~~S~~		~~S~~

Data are mean ± standard error of 10 replicates of the combination of "no BioAct application with TR or TS" and 20 replications of the combination of "BioAct application with TS".

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

~~[e] S and NS indicate statistically significant or non-significant differences between factors, respectively, according to Tukey's test ($P < 0.05$).~~ Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Table 5. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato cv. Monika (TR) or susceptible cv. Durinta (TS) from 31st July to 5th November of 2012.

BioAct WG [a]	Previous Crop	Pi (J2 250 cm^{-3} soil)	Pf (J2 250 cm^{-3} soil)	Galling index [b]	Eggs g^{-1} root	Egg parasitism (%)	Yield (kg $plant^{-1}$)
No application	TR	1187±400 b	1379±253 a	7.6±1.1 b	1083±381	0±0	0.2±0.1 a
	TS	4319±464 a	659±162 ab	10.0±0 a	na	na	0.03±0.002 b
Application	TR	801±199 b	768±184 ab	8.7±0.5 b	3646±1482	2.60±1.01	0.10±0.04 a
	TS	3968±695 a	522±217 b	10.0±0 a	na	na	0.02±0.002 b
~~BioAct [e] *vs* no BioAct~~		~~NS~~	~~NS~~	~~NS~~			~~NS~~
~~TR *vs* TS~~		~~S~~	~~S~~	~~S~~			~~S~~
~~T x BioAct~~		~~NS~~	~~NS~~	~~NS~~			~~NS~~

Data are mean ± standard error of 15 replicates of the combination each combination with TS and 5 replicates of each combination with TR.

[a] BioAct WG (1 x 10^{10} viable spores g^{-1}) Treatments: At soil, 14 days before transplanting and every six weeks after transplanting at rate of 0.4 g m^{-1} linear and 10 cm width: , in seedling before transplanting at 0.1g L^{-1} rate.

[b] Galling index based on the Zeck'[45] scale; from 0 (healthy plants) to 10 (dead plants).

[e] ~~S and NS indicate statistically significant or non-significant differences between factors, respectively, according to Tukey's test ($P < 0.05$).~~ Different letters in the same column indicate differences ($P < 0.05$) according to Tukey's test.

Na: no available

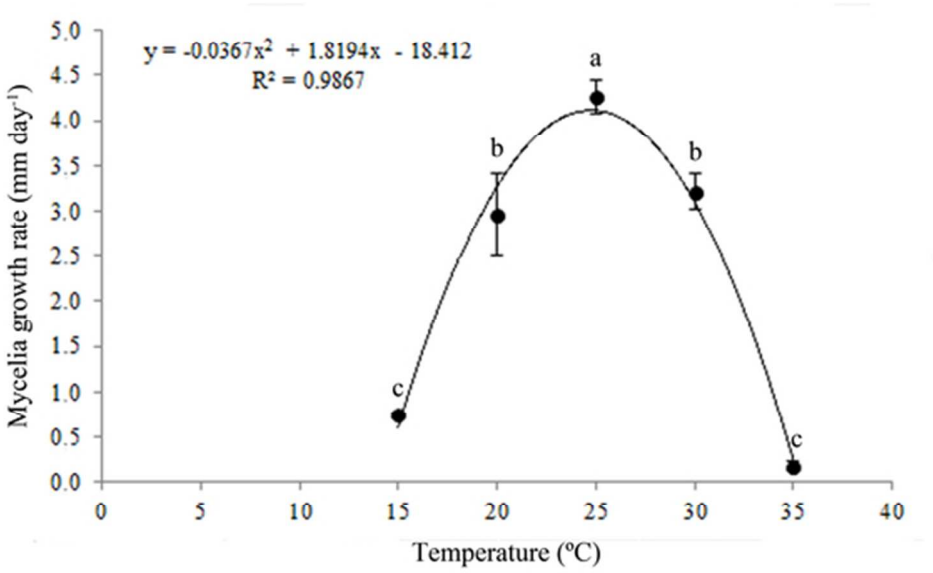

Figure 1. Mycelia growth rate (mm day^{-1}) of *Purpureocillium lilacinum* strain 251 in water agar (12 g L^{-1}) at 15, 20, 25, 30 and 35ºC. Bars represent the standard error (n=6) Different letters indicate differences at $P < 0{\cdot}05$ according to LSD's test.

Figure 1
25x16mm (600 x 600 DPI)

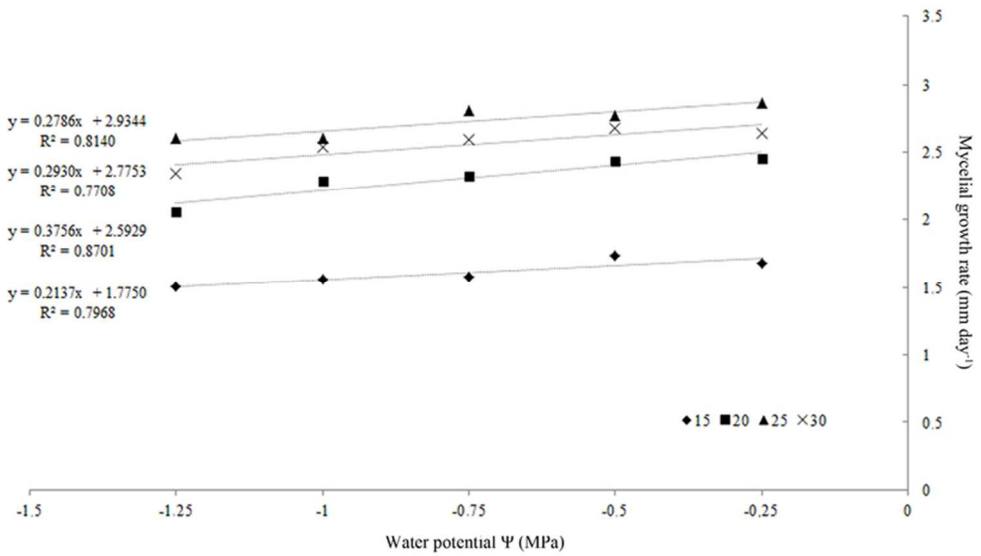

Figure 2. Mycelial growth rate (mm dia^{-1}) of *Purpureocillium lilacinum* strain 251 at 15, 20, 25, 30 ºC and water potentials from -1,25 to -0,25 MPa.
Figura 2
35x19mm (600 x 600 DPI)