



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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3 1 **Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251)**
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5 2 **on *Meloidogyne incognita* in a tomato–cucumber rotation in a greenhouse.**

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23 10 **Running title:** Effect of plant resistance and BioAct WG on RKN in greenhouse.
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26
27 12 **Abstract**

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29 13 **BACKGROUND:** The effectiveness of combining resistant tomato with BioAct WG
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31 14 (*Purpureocillium lilacinum* strain 251; PI251) against *Meloidogyne incognita* was
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33 15 assessed in a tomato-cucumber rotation in greenhouse over two years. Additionally, the
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35 16 enzymatic activity of the fungus, the percentage of fungal egg and juvenile parasitism,
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37 17 cardinal temperatures and the effect of water potential on mycelia growth and the soil
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39 18 receptivity to PI251 were determined *in vitro*.

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43 19 **RESULTS:** Plant resistance was the only factor that suppressed nematode and crop
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45 20 yield losses. Percentage of egg parasitism in plots treated with BioAct WG was less
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47 21 than 2.6 %. However under *in vitro* conditions, PI251 showed protease, lipase, and
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49 22 chitinase activities, and parasitized 94.5 % of eggs, but no juveniles. Cardinal
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51 23 temperatures were 14.2, 24-26, and 35.4 °C. The maximum PI251 mycelial growth was
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53 24 at -0.25 MPa and 25 °C. Soil temperatures and water potential in the greenhouse were in
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1 the range of the fungus. However, soil receptivity was less in greenhouse soil,
2 irrespective of sterilization, than in sterilized sand.

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

7 8 **Key words**

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

11 12 **1 INTRODUCTION**

13 Spain is the main producer of vegetables under protected cultivation in the
14 Mediterranean area, with 71.003 ha.¹ The major crops are tomato (*Solanum*
15 *lycopersicum* L.) and cucumber (*Cucumis sativus* L.), frequently cultivated in a double
16 cropping system.² Greenhouse tomato is grown on 18.501 ha with an annual production
17 of 1.881.922 tonnes, which represents 38 % of the tomato production area and 47 % of
18 the total yield. Greenhouse cucumber is grown on 7.768 ha with an annual yield of
19 717.693 tonnes, representing 88 % of the total production area and 95 % of the total
20 yield.¹

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

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3 1 limitations imposed by the Directive 2009/128/EC have encouraged research finding
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5 2 alternatives to chemical pesticides as well as to design effective and durable strategies
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7 3 to manage RKN.
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10 4 There are a large number of non-chemical alternatives to control RKN,³ including
11
12 5 crop rotation with resistant cultivars and biological control. In nematology, resistance is
13
14 6 defined as the ability of a plant to suppress infection, development and/or reproduction
15
16 7 of plant-parasitic nematodes.⁷ Therefore, resistant tomato cultivars or rootstocks
17
18 8 carrying the *Mi*-gene are widely used because of their effectiveness in suppressing *M.*
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20 9 *arenaria*, *M. incognita* and *M. javanica*.^{4,7} However, its expression can be limited by i)
21
22 10 constant soil temperatures above 28 °C at transplanting,⁸ ii) the genetic background of
23
24 11 the tomato cultivar or rootstock,⁹ iii) RKN species: not effective against *M. hapla*, *M.*
25
26 12 *chitwoodi* race 3,¹⁰ *M. enterolobi*,¹¹ or *M. exigua*,¹² and iv) virulent populations, which
27
28 13 can occur suddenly or be selected by repeated cultivation of resistant cultivars.^{13,14}
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30 14 Inclusion of resistant tomato cultivars in a cropping sequence helps to suppress the
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32 15 reproduction of RKN and to reduce yield losses of the following susceptible crop,¹⁵ such
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34 16 as cucumber,² because yield losses are related to nematode densities in soil at
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36 17 transplanting.¹⁶
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41 18 Several microorganisms have been evaluated for biological control of plant-parasitic
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43 19 nematodes.¹⁷ The use of biological control agents able to suppress the buildup of RKN
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45 20 can be of interest to reduce the pressure on R genes avoiding the selection of virulent
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47 21 populations and contribute to maintain nematode densities below the economic
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49 22 threshold level. Out of those, *Purpureocillium lilacinum* (formerly *Paecilomyces*
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51 23 *lilacinus*) strain 251 (PI251) is the only biological nematicide listed in the annex 1 of the
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53 24 European register of active substances.¹⁸ *Purpureocillium lilacinum* is a common soil
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55 25 hyphomycete able to parasitize RKN sedentary stages by direct hyphal penetration and
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1 by using hydrolytic enzymes.¹⁹⁻²¹ Besides, the effectiveness of PI251 to control RKN
2 has been widely reported under controlled conditions and in pot tests,²²⁻²⁴ although few
3 reports are available on its effectiveness under field conditions.^{25,26}

4 The aim of the present study was to evaluate the effectiveness of combining the
5 resistant tomato cv. Monika with *P. lilacinum* strain 251 (BioAct WG[®], Belchim Crop
6 Protection, Londerzeel, Belgium) over two consecutive growing seasons in a
7 greenhouse to manage RKN in a tomato-cucumber rotation. Additionally, *in vitro*
8 experiments were carried out to determine extracellular enzymes produced by PI251 and
9 its capability to parasitize eggs and second-stage juveniles of RKN, as well as to know
10 cardinal temperatures and the effect of temperature and water potential on mycelia
11 growth, and soil receptivity to PI251.

12

13 2 MATERIAL AND METHODS

14 2.1 Greenhouse trial

15 The field trial was conducted in a 700 m² greenhouse infested with *M. incognita* in
16 Viladecans (41° 17' 18''N; 2° 2' 39''E, Barcelona, Spain) during, 2011 and 2012. The
17 soil was a sandy loam with 83.8 % sand, 6.7 % silt and 9.5 % clay; pH 8.7; 1.8 % of
18 organic matter (w/w) and 0.5 dS/m of electric conductivity. Soil was infested with *M.*
19 *incognita* in 2007 and rotations with resistant or susceptible tomato cultivars and
20 cucumber or black fallow were carried out from 2008 to 2010.

21 The rotation sequence consisted of resistant tomato cv. Monika (bearing the *Mi* gene)
22 or the susceptible cv. Durinta from March to July, followed by cucumber cv. Dasher II
23 cultivated from July to October-November. Individual plots of 9.6 m² comprising of 4
24 rows with 6 plants per row. Plant spacing was 50 cm between rows and 55 cm within

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

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

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

21 Tomato was cultivated from 31st March to 6th July (98 days), and cucumber from 29th
22 July to 26th October (90 days) in 2011. In 2012, tomato was cultivated from 5th March to
23 17th July (135 days) and cucumber from 31st July to 5th November (98 days).

24 The plants were irrigated by drip irrigation and fertilized weekly with a NPK solution
25 (15–5–30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1} . Crops were

1 vertically trellised. Weeds were removed manually during and between cropping cycles.
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3 Tomato yield was assessed from the first six fruit sets produced from the eight central
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5 plants in each plot. Fruits were harvested according to commercial standards as they
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7 reached maturity. Similar, cucumber fruits were harvested from the eight central plants
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9 of each plot when they reached the standard commercial size. Total yield per crop cycle
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11 was expressed as kilograms plant⁻¹.
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16 Soil temperatures were recorded at 30 min intervals in order to estimate the number
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18 of the nematode generations, and the effect of soil temperature on the fungus growth. In
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20 2011 were recorded with soil probes 107 (Campbell Scientific, Logan, USA) placed at a
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22 depth of 15 cm. In 2012, soil temperatures and water potential were recorded at the
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24 same interval and soil depth with 5TM and MPS-1 probes (Decagon devices, Inc,
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26 Pullman, USA).
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29 Composite soil samples were collected from each plot at the beginning and at the end
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31 of each crop to estimate initial (Pi) and final (Pf) nematode population densities,
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33 respectively. Each soil sample consisted of eight subsamples from the top 30 cm taken
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35 with an auger (2.5 cm diameter). Soil samples were sieved through a 4 mm aperture
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37 screen to remove stones, and carefully homogenized to extract nematodes from 500 cm³
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39 by Baermann trays.²⁷ Second-stage juveniles (J2) that migrated to the water were
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41 collected one week later, concentrated on a 75 µm sieve, counted, and then expressed as
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43 number of J2 per 250 cm³ of soil. At the end of each crop, plants were removed with a
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45 pitchfork, cut at ground level, and the disease severity was assessed using the Zeck's
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47 galling²⁸ index on a scale of 0 to 10, where 0 means complete and healthy root system
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49 (no galls observed) and 10 means plants and roots dead. Afterwards, the roots were
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51 weighted and chopped into 2 cm long segments and two 10 g subsamples were used to
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53 extract eggs by blender maceration in a 1 % NaOCl solution.²⁹ After 10 minutes of
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3 1 maceration, the egg suspension was passed through a 75 μm sieve, to retain the plant
4 material, and a 25 μm sieve to retain the eggs. The number of eggs was counted and
5 expressed per gram of fresh root weight.
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10 4 To assess fungal egg parasitism, the procedure described in Giné *et al.*³⁰ was used. In
11 brief, 20 egg masses per individual plot were handpicked from the remaining roots and
12 placed in a watchglass containing sterile demineralised water. The outer part of the
13 gelatinous matrix was removed, and eggs were dispersed in an Eppendorf
14 microcentrifuge tube containing 1 mL of sterile demineralised water with a pestle. A
15 333 μL aliquots of egg suspension were spread onto each of three replicated Petri plates
16 containing a growth restricting medium,³¹ and incubated at 25 $^{\circ}\text{C} \pm 0.5$ $^{\circ}\text{C}$ in the dark.
17
18 Number of parasitized eggs was recorded after 24 and 48 hours under a dissecting
19 microscope and percentage of egg parasitism was then calculated as the number of
20 parasitized eggs per plate per total eggs per plate. Eggs were considered parasitized if
21 fungal hyphae grew from inside of unhatched eggs. Parasitized eggs were individually
22 transferred to the growth restricting medium to establish pure cultures and fungal
23 species were identified by cultural and morphological characteristics.³²
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38 2.2 Extracellular enzymes production

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40 18 Extracellular enzyme production by PI251 was evaluated using a semiquantitative API
41 ZYM $\text{\textcircled{R}}$ (BioMérieux, Marcy l'Etoile, France) system which identifies 19 cellular
42 enzymes, i.e. alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14),
43 leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin,
44 acid phosphatase, naphthol-AS-BI phosphohydrolase, α -galactosidase, β -galactosidase,
45 β -glucuronidase, α -glucosidase, β -glucosidase, n-acetyl- β -glucosaminidase, α -
46 mannosidase and α - fucosidase.
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3 1 Conidia of PI251 were collected from 3-week-old cultures of the fungus growing on
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5 2 potato dextrose agar (PDA; 39 g L⁻¹) at 25°C in the dark. The colony was washed with 5
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7 3 mL of sterile distilled water. The number of conidia was then counted with a
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9 4 haemocytometer and adjusted to 1 x 10⁶ conidia mL⁻¹. The API ZYM system was used
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11 5 according to the manufacturer's instructions. Briefly, 65 µL of the conidia suspension
12
13 6 was placed into each cupule, and incubated at 25 °C for 6 h in the dark.³³ Enzyme
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15 7 activity was observed after addition of a drop of each ZYM A and ZYM B reagents
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17 8 and exposed to sunlight for an hour to eliminate the yellowing from the reagents. The
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19 9 enzyme production was assessed according to the scale from 0 (no enzyme production)
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21 10 to 5 (maximum enzyme production) according the color chart provided by the
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23 11 manufacturer. The experiment was carried out once.

12 2.3 Capability of PI251 to parasitize *Meloidogyne incognita* eggs and J2 *in vitro*

13 Individual plugs of 9 mm-diameter from the edge of a PI251 colony growing on PDA
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15 14 were placed in the centre of a total of six Petri dishes (90 mm) containing water agar
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17 15 (agar 12 g L⁻¹), and incubated at 25 °C ± 0.5 °C in the dark for 3 days.

16 Surface sterilized eggs or J2 of the same *M. incognita* population used to inoculate
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18 17 the soil of the greenhouse in 2007 and maintained in tomato in pots were used to
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20 18 determine the parasitic ability of PI251. *Meloidogyne incognita* was identified by
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22 19 morphology of perineal pattern, esterase pattern, and SCAR marker. Eggs from 30 egg
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24 20 masses handpicked from tomato roots were surface sterilized following the protocol of
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26 21 Verdejo *et al.*³⁴ with some modifications. Egg masses were placed into a conical sterile
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28 22 tube with 1 mL of a 0.5 % NaOCl solution for four min. and were shaken at 30 s
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30 23 intervals for 10 seconds. After that, the solution was diluted 10 times with sterile
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32 24 distilled water and left undisturbed for 30 min. to allow the eggs to settle at the bottom
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34 25 of the tube. Then, eggs were taken and spread into three Petri dishes at 1 cm of the edge
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1 of the colony of the PI251 using a sterile Pasteur pipette, and incubated at 25 °C ± 0.5
2 °C in the dark for one week. The assessment of egg parasitism was carried out as
3 described previously.

4 Second-stage juveniles of *M. incognita* were obtained by extraction of eggs from
5 tomato roots by the Hussey and Barker method²⁹ and left to emerge on Baermann
6 trays.²⁷ Then, J2 were surface sterilized following the Mountain³⁴ procedure with some
7 modifications. Briefly, a suspension of 100 J2 in 0.5 mL water was placed in a conical
8 sterile tube containing streptomycin 0.1 % for 4 hours. The suspension was shaken at
9 one hour interval for 10 seconds. The solution was then diluted 20 times with sterile
10 distilled water and left undisturbed for 30 min. for J2 to settle at the bottom of the tube.
11 Then, J2 were taken and placed in three Petri dishes at 1 cm of the edge of the PI251
12 colony using a sterile Pasteur pipette, and incubated at 25 °C ± 0.5 °C in the dark for one
13 week. The assessment of J2 parasitism was carried out as described previously for egg
14 parasitism.

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

17 Experiments were conducted once.

18 **2.4 Cardinal temperatures and the effect of temperature and water potential on** 19 **PI251 mycelia growth *in vitro***

20 Cardinal temperatures of PI251 were determined placing individual 9 mm-diameter
21 plugs of PI251 in the center of each of 24 Petri dishes containing water agar (WA; 12 g
22 L⁻¹). Petri dishes were incubated at 4, 10, 15, 20, 25, 30, 35 or 40°C in the dark (three
23 dishes per temperature). Minimum and maximum diameters (mm) of fungal growth
24 were measured every 24 h until the colonies occupied 80 % of the surface of the Petri

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

3 Concurrently, the effect of water potential (Ψ) and temperature on PI251 growth was
4 assessed. Water agar media with different concentrations of Polyethylene Glycol 8000
5 (PEG8000) was prepared according to the Michel's equation³⁶ to achieve Ψ between
6 1.25, -1, -0.75, -0.5 and -0.25 MPa. Mycelia plugs of 9 mm diameter from the edge of a
7 fungal the colony were placed in the centre of the Petri plates and then incubated at 15,
8 20, 25, and 30 °C in the dark. Each combination of temperature-PEG8000 concentration
9 was repeated three times. Minimum and maximum diameters of fungal growth were
10 measured daily until the colonies occupied 80 % of the surface of the Petri plate. For
11 each water potential, a linear regression was calculated between the mean diameter of
12 the colony per day and the temperature, and the slopes were used to construct the
13 regressions to determine the effect of temperature and water potential on mycelia
14 growth. Experiments were repeated once.

15 **2.5 Soil receptivity**

16 Soil collected from BioAct WG non-treated plots of the greenhouse trial was
17 assessed for receptivity to the fungal isolate. A part of soil was two times sterilized at
18 121 °C for 1 h within 24 h. The other part remained non-sterilized. The experiment was
19 carried out following the procedure described by Monfort *et al.*³⁷ Briefly, 40 g of
20 sterilized or non-sterilized air-dried soil was placed in Petri dishes and saturated with
21 sterile distilled water. Soils included in the experiment were: (i) sterilized greenhouse
22 soil; (ii) non-sterilized greenhouse soil and (iii) sterilized sand. A polyvinylidene
23 difluoride (PVDF) membrane (0.22- μm -pore-size and 45 mm-diameter) was sterilized
24 at 121 °C for 20 min and placed on top of the soil ensuring full contact. A 4 mm-
25 diameter plug of PI251 was placed in the middle of each membrane. Petri dishes were

1 then sealed and incubated at $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ in the dark. After three weeks, the
2 membranes were washed with sterile distilled water and then dried in a laminar air flow
3 cabinet. Then, the membranes were incubated in a solution of 1 % trypan blue in lactic
4 acid for 12 h at room temperature to stain the mycelia. After that, the excess of the stain
5 was removed with sterile distilled water, and minimum and maximum colony diameters
6 were measured. The experiment was repeated once.

7 **2.6 Statistical analysis**

8 Statistical analyses were done using SAS v. 9 (SAS Institute Inc.). Data from field
9 experiments were transformed when required to $\log_{10}(x + 1)$ or square-root ($x+0.5$) to
10 normalize the data. The greenhouse trial was analyzed by analysis of variance by the
11 general linear model (PROC GLM) according to a factorial design to compare the effect
12 of the tomato cultivar, the application of BioAct WG, and the interaction (except for
13 tomato 2012), on nematode densities in soil, eggs in roots, disease severity, and crop
14 yield per cropping season. Analysis of variance was also carried out to compare the
15 effect of the temperature and kind of soil on the growth of PI251.

17 **3 RESULTS**

18 **3.1 Greenhouse trial**

19 Minimum, maximum and average soil temperatures during the cultivation of each
20 crop and year are provided in Table 1. Accumulated soil temperatures during the tomato
21 and cucumber crops in 2011 were 1504 DD (degree days; base temperature (T_b) = 10
22 $^{\circ}\text{C}$) and 1473 DD ($T_b = 11.4\text{ }^{\circ}\text{C}$), respectively. According to thermal requirements of
23 tomato and cucumber,^{5,38} *M. incognita* completed two generations in tomato (thermal
24 constant (S) = 600-700 DD over $T_b=10\text{ }^{\circ}\text{C}$),³⁸ and in cucumber ($S = 500$ DD over $T_b =$
25 $11.4\text{ }^{\circ}\text{C}$).⁵

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3 1 In 2011, the tomato cultivar was the only factor that explained differences ($P < 0.05$)
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5 2 in nematode densities in the soil and roots, galling index and crop yield, both in tomato
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7 3 and cucumber (Tables 2 and 3). Nematode densities in the soil, roots and the galling
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9 4 index at the end of the resistant tomato crop were 8, 6 and 18 % those recorded at the
10
11 5 end of the susceptible cultivar, which yielded 78 % less than the resistant one. The
12
13 6 percentage of fungal egg parasitism at the end of the crop was less than 0.1 % (Table 2).
14
15 7 At cucumber transplanting, higher ($P < 0.05$) soil nematode densities occurred in plots
16
17 8 preceded by a susceptible than a resistant tomato cultivar. Galling indices were lower (P
18
19 9 < 0.05) after a resistant than a susceptible tomato cultivar. Eggs from cucumber plants
20
21 10 were only recovered from plants preceded by a resistant tomato because cucumber
22
23 11 following susceptible tomato died (data not shown). Egg parasitism by the fungus was
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25 12 less than 0.5 % (Table 3).
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30 13 In 2012, accumulated temperatures during the tomato and cucumber crops were 1959
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32 14 ($T_b = 10\text{ }^\circ\text{C}$) and 1524 DD ($T_b = 11.4\text{ }^\circ\text{C}$), respectively. Hence, *M. incognita* completed
33
34 15 three generations in both crops according to its thermal requirements. In this cropping
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36 16 season, the tomato cultivar was also the factor responsible for the differences ($P < 0.05$)
37
38 17 in nematode densities in the soil and roots, disease severity, as well as crop yield in both
39
40 18 tomato and cucumber crops (Tables 4 and 5). As in the previous season, cucumber
41
42 19 following susceptible tomato had higher ($P < 0.05$) nematode levels at transplanting,
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44 20 and all plants died at the end of the experiment. The percentage of fungal egg parasitism
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46 21 at the end of tomato and cucumber crops was 2.4 and 2.6 %, respectively (Table 5).
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50 22 **3.2 Extracellular enzymes production**

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52 23 Six extracellular enzymes were produced by Pl251. The enzyme produced in highest
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54 24 amounts by the fungus was leucine arylamidase (value of the color scale: 5) followed by
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56 25 esterase and acid phosphatase (value of the color scale: 4), esterase-lipase (value of the
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3 1 color scale: 3) and the lowest were naphthol-AS-BI- phosphohydrolase and N-acetyl- β -
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5 2 glucosaminidase (value of the color scale: 2).
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7 **3.3 Capability of PI251 to parasitize RKN eggs and J2 *in vitro***

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9 4 PI251 parasitized 94.91 % \pm 2.88 (mean \pm standard error) of *M. incognita* unhatched
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11
12 5 eggs. However, no J2 were parasitized.
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14 **3.4 Cardinal temperatures and the effect of temperature and water potential on** 15 16 **PI251 mycelia growth *in vitro***

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18 8 Mycelia growth of PI251 occurred between 14.2 °C (minimum) and 35.4 °C
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20 9 (maximum), with 24-26 °C as the optimal growth temperatures range (Figure 1). No
21
22 10 growth was detected at 4, 10 and 40 °C. The water potential (-1.25 to -0.25 MPa)
23
24 11 directly influenced the mycelia growth of PI251. The mycelia growth was higher at
25
26 12 optimal temperatures (25 °C) and highest water potential (-0.25 MPa), followed by 30
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28 13 °C, 20 °C and 15 °C (Figure 2).
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31 **3.5 Soil receptivity**

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34 15 The mycelia growth of PI251 in sterile sand (2.55cm \pm 0.06; mean \pm standard error)
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36 16 was 91.83 % higher ($P < 0.05$) compared with non-sterile (0.18cm \pm 0.06; mean \pm
37
38 17 standard error) or sterile greenhouse soil (0.23cm \pm 0.07; mean \pm standard error).
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40 18 However, no differences ($P > 0.05$) were found between sterile and non-sterile
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42 19 greenhouse soil.
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45 **4. DISCUSSION**

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47 21 The effectiveness of combining a tomato cultivar carrying the *Mi* gene for resistance
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49 22 to *M. incognita* and BioAct WG based on the nematode antagonist *P. lilacinum* strain
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51 23 251 against RKN has been assessed in a tomato – cucumber rotation under greenhouse
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53 24 conditions. The initial hypothesis considered a synergistic effect of both methods to
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55 25 suppress nematode densities. The first one, mediated by plant resistance, should
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1 suppress nematode infection, development and reproduction. The second one, due to
2 PI251, should parasitize eggs produced by nematodes that escaped plant resistance. In
3 the following cucumber crop, fewer RKN at transplanting after resistant tomato should
4 mean more percentage of egg parasitism, because most egg masses should be on the
5 root surface favoring egg infection, as well as reduce yield losses due to fewer J2 at
6 transplanting. However, the hypothesis was not confirmed in our trial, with plant
7 resistance being the only factor that consistently suppressed RKN. The effectiveness of
8 tomato cultivars or rootstocks carrying the *Mi* gene against RKN in greenhouses in
9 Spain was consistent with previous reports.^{4,9,14,15} In this study, resistant tomato
10 suppressed disease severity and reproduction by 82 – 91 % and 87 – 95 % compared to
11 the susceptible cultivar, each year, respectively. The effect of intermittent peaks of soil
12 temperatures over 28 °C did not affect the effectiveness of the *Mi* gene as previously
13 reported.³⁹ During the tomato crop in 2011, the numbers of days with maximum soil
14 temperatures over 28 °C were 23, after 35 days of transplanting, and 38 days, after 62
15 days of transplanting in 2012. In addition, the benefit of cropping a resistant tomato
16 cultivar on yield losses of the following susceptible crop was also observed as
17 previously stated.^{2,15}

18 Unlike to plant resistance, there are few reports about the effectiveness of BioAct
19 WG alone and/or in combination with other control methods against RKN under
20 Mediterranean conditions.^{25,26} In studies conducted in Greece and Turkey, BioAct WG
21 did not provide satisfactory RKN control. However, in several *in vitro* and pot tests the
22 antagonistic capability of PI251 against several plant-parasitic nematode species was
23 reported.^{11,20-24,40-42} The ability of PI251 to penetrate eggs and cuticles of sedentary
24 stages of RKN by mechanical and chemical mechanisms has been reported.^{20,21} The
25 results obtained by the API ZYM method showed high protease and lipase activity and

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3 1 low chitinase activity able to degrade the main components of egg shell and nematode
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5 2 cuticle.⁴³ Therefore, a high proportion of egg parasitism was expected, as it was in our
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7 3 *in vitro* experiment (94.9 %). In addition, Kahn *et al.*²¹ pointed out the parasitic ability
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9 4 of PI251 on all stationary stages of *M. javanica*, that is: eggs, juvenile contained in eggs,
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11 5 post-infective juvenile stages: from swollen J2 to J4, and females, but they did not
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13 6 assess the effect on the mobile infective J2. The results of this study showed that PI251
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15 7 was not able to parasitize the infective J2 stage of *M. incognita* neither sedentary stages
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17 8 of RKN because PI251 is not a root endophyte of tomato or cucumber plants.^{44,45} Thus,
18
19 9 PI251 could exert its parasitic potential only on eggs and juveniles contained in eggs
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21 10 that remained in soil at the end of the crop, or on those produced on roots and exposed
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23 11 to the soil. In fact, in greenhouse conditions, the percentage of egg parasitism was less
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25 12 than 2.6 % in both crops and years. Moreover, microorganisms associated to the
26
27 13 gelatinous matrix of the egg masses can inhibit fungal egg parasites as *Pochonia*
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29 14 *chlamydosporia*.⁴⁶ Thus, fungal application did not affect nematode development even
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31 15 though four applications per-crop and year.

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36 16 Environmental factors can play an important role in nematode biocontrol.⁴⁷ Rumbos
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38 17 *et al.*⁴² reported a negative correlation between the persistence of PI251 in soil and the
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40 18 sand content of soil. Thus, sandy soils, as in this study (83.8 % sand) would not be
41
42 19 suitable for the fungus. However, the test of soil receptivity showed that mycelia growth
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44 20 was better in sterilized sand than in the sterilized sandy loam soil from the greenhouse
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46 21 experiments. This indicates that other factors different to microbial communities or
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48 22 thermo-sensitive chemicals in soil could limit the effectiveness of PI251 because
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50 23 mycelia growth was equally poor in sterile and non-sterile sandy loam soil from the
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52 24 greenhouse.
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3 1 Soil temperatures during the cropping period or time of application could also affect
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5 2 BioAct WG effectiveness. In this study, cardinal temperatures of mycelia growth were
6
7 3 determined. Maximum temperature of PI251 mycelia growth in water agar was similar
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9 4 to that reported by Kiewnick,⁴¹ but not the optimal range, which was less wide (24-26 vs
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11 5 24-30 °C). Moreover, in this study, minimum temperature was also estimated (14.2 °C)
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13 6 because low soil temperatures at transplanting tomato in spring (17 – 19 °C) could
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15 7 affect fungal and thus its effectiveness. Soil temperatures during the cultivation of
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17 8 tomato and cucumber were in the range of the fungus development according to
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19 9 cardinal temperatures, but 37 out of 98 days and 60 out of 90 days during each tomato
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21 10 crop were over optimal temperatures, and 63 out of 135 and 59 out of 98 days during
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23 11 each cucumber crop. At soil temperatures between the optimal for fungal growth (24-26
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25 12 °C) and the optimal for *M incognita* development (30 °C),⁴⁸ the nematode could take
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27 13 advantage over PI251 that can reduce its effectiveness. In fact, at temperatures of 28 ±
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29 14 1.5 °C no dose-response relationship was observed but it did at 25 ± 1 °C.²⁴
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34 15 The soil water potential recorded during the cropping period should not affect the
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36 16 fungal growth because it was near field capacity (-0.033MPa), and according to the
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38 17 results in *in vitro* test, PI251 mycelia grow more at higher water potential.

39 18 Another putative explanation for the lack of efficacy of BioAct WG in the
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41 19 greenhouse trial could be the content of glucose in the formulation, which inhibits the
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43 20 protease activity and consequently the capability to parasitize RKN eggs.⁴⁹
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47 21 The present study aimed to demonstrate the usefulness of combining plant resistance
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49 22 with BioAct WG to manage RKN. However, PI251 was not able to parasitize eggs in
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51 23 greenhouse due to suboptimal soil temperatures for several days during the cropping
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53 24 period and/or inhibition of enzymes produced by the fungus by the components of the
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55 25 formulation and/or non-thermo-sensitive chemical factors in soil. Therefore, no
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1 synergistic effect was observed. Resistant tomato suppressed nematode reproduction
2 and yield losses of tomato as well as yield losses of the following cucumber crop, but
3 nematode populations increased at the end of the crop. *P. lilacinum* is found naturally
4 worldwide,⁴⁷ and it has been isolated from RKN in Spain.³⁰ PI251 was isolated from
5 Philippines,⁴⁶ and despite its effectiveness to parasitize eggs *in vitro*, well is known that
6 native isolates can be more suitable and can performance better than the foreign ones in
7 field conditions.⁵⁰ More studies are needed to optimize BioAct WG usage and to
8 improve knowledge on optimal environmental conditions to improve its effectiveness.

9 ACKNOWLEDGMENTS

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16 REFERENCES

- 17 1 Anuario de estadística agraria. ed. by Ministerio de Agricultura, Alimentación y
18 Medio Ambiente, Madrid, España (2013).
- 19 2 Ornat C, Verdejo-Lucas S and Sorribas FJ, Effect of the previous crop on population
20 densities of *Meloidogyne javanica* and yield of cucumber. *Nematropica* **27**:85-
21 90 (1997).
- 22 3 Sikora R and Fernandez E, Nematode parasites of vegetables, in *Plant Parasitic*
23 *Nematodes in Subtropical and Tropical Agriculture*, eds. by Luc M, Sikora RA
24 and Bridge J, CAB International Wallingford, UK, pp. 319–392 (2005).

- 1
2
3 1 4 Sorribas FJ, Ornat C, Verdejo-Lucas S, Galeano, M and Valero J, Effectiveness and
4
5 2 profitability of the Mi-resistant tomatoes to control root-knot nematodes. *Eur J*
6
7 3 *Plant Pathol* **111**(1):29-38 (2005).
8
9
10 4 5 Giné A, López-Gómez M, Vela MD, Ornat C, Talavera M, Verdejo-Lucas S and
11
12 5 Sorribas FJ Thermal requirements and population dynamics of root-knot
13
14 6 nematodes on cucumber and yield losses under protected cultivation. *Plant*
15
16 7 *Pathol* **63**(6):1446-1453 (2014).
17
18 8 6 Talavera M, Sayadi S, Chiroso-Rios M, Salmeron T, Flor-Peregrin E and Verdejo-
19
20 9 Lucas S, Perception of the impact of root-knot nematode-induced diseases in
21
22 10 horticultural protected crops of south-eastern Spain. *Nematology* **14**(5):517–527
23
24 11 (2012).
25
26 12 7 Roberts PA, Concepts and consequences of resistance, in *Plant Resistance to*
27
28 13 *Parasitic Nematodes*, eds. by Starr JL, Cook R and Bridge J, CABI
29
30 14 International, Wallingford, pp. 23-41 (2002).
31
32 15 8 Dropkin VH, The necrotic reaction of tomatoes and other hosts resistant to
33
34 16 *Meloidogyne*: Reversal by temperature. *Phytopathology* **59**:1632-1637 (1969).
35
36 17 9 Cortada L, Sorribas F J, Ornat C, Andrés M F and Verdejo-Lucas S, Response of
37
38 18 tomato rootstocks carrying the *Mi*-resistance gene to populations of *Meloidogyne*
39
40 19 *arenaria*, *M. incognita* and *M. javanica*. *Eur J Plant Pathol* **124**(2):337-343
41
42 20 (2009).
43
44 21 10 Brown CR, Mojtahedi H, Santo GS and Williamson VM, Effect of the *Mi* gene in
45
46 22 tomato on reproductive factors of *Meloidogyne chitwoodi* and *M. hapla*, *J*
47
48 23 *Nematol* **29**(3):416-419(1997).
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 11 Kiewnick S, Dessimoz M and Franck L, Effects of the *Mi-1* and N root-knot
4
5 2 nematode-resistance gene on infection and reproduction of *Meloidogyne*
6
7 3 *enterolobii* on tomato and pepper cultivars. *J Nematol* **41**(2):134-139 (2009).
8
9
10 4 12 Silva RV, Oliviera RDL, Ferreira PS and Castro DB, Efeito do gene *Mi* na
11
12 5 reprodução de populações de *Meloidogyne exigua* em tomateiro. *Nematol Bras*
13
14 6 **32**:150-153 (2008).
15
16 7 13 Ornat C, Verdejo-Lucas S and Sorribas FJ, A population of *Meloidogyne javanica* in
17
18 8 Spain virulent to the *Mi* resistance gene in tomato. *Plant Dis* **85**:271-276 (2001).
19
20
21 9 14 Verdejo-Lucas S, Cortada L, Sorribas FJ and Ornat C, Selection of virulent
22
23 10 populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance
24
25 11 gene tomato rootstocks under field conditions. *Plant Pathol* **58**(5):990-998
26
27 12 (2009).
28
29
30 13 15 Talavera M, Verdejo-Lucas S, Ornat C, Torres J, Vela MD, Macias FJ, Cortada L,
31
32 14 Arias DJ, Valero J and Sorribas FJ, Crop rotations with *Mi* gene resistant and
33
34 15 susceptible tomato cultivars for management of root-knot nematodes in plastic-
35
36 16 houses. *Crop Prot* **28**(8):662-667 (2009).
37
38
39 17 16 Seinhorst JW, The relation between nematode density and damage to plants.
40
41 18 *Nematologica* **11**(1):137-154 (1965).
42
43 19 17 Sikora RA, Management of the antagonistic potential in agricultural ecosystems for
44
45 20 the biological control of plant parasitic nematodes. *Annu Rev Phytopathol*
46
47 21 **30**(1):245-270 (1992).
48
49
50 22 18 EUROPEAN UNION (2009). Regulation (EC) No 1107/2009 of the European
51
52 23 Parliament and of the Council of 21 October 2009 concerning the placing of
53
54 24 plant protection products on the market and repealing Council Directives
55
56
57
58
59
60

- 1
2
3 1 79/117/EEC and 91/414/EEC. *Official Journal of the European Union* L309, pp.
4
5 2 50.
6
7 3 19 Holland RJ, Williams KL and Khan A, Infection of *Meloidogyne javanica* by
8
9 4 *Paecilomyces lilacinus*. *Nematology* **1**(2):131-139 (1999).
10
11 5 20 Khan A, Williams KL and Nevalainen HK, Effects of *Paecilomyces lilacinus*
12
13 6 protease and chitinase on the eggshell structures and hatching of *Meloidogyne*
14
15 7 *javanica* juveniles. *Biol Control* **31**(3):346-352 (2004).
16
17 8 21 Khan A, Williams KL and Nevalainen HK, Infection of plant-parasitic nematodes by
18
19 9 *Paecilomyces lilacinus* and *Monacrosporium lysipagum*. *Biocontrol* **51**(5):659-
20
21 10 678 (2006).
22
23 11 22 Kiewnick S and Sikora RA, Biological control of the root-knot nematode
24
25 12 *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. *Biol Control*
26
27 13 **38**(2):179-187 (2006).
28
29 14 23 Kiewnick S and Sikora RA, Evaluation of *Paecilomyces lilacinus* strain 251 for the
30
31 15 biological control of the northern root-knot nematode *Meloidogyne hapla*
32
33 16 Chitwood. *Nematology* **8**(1):69-78(2006).
34
35 17 24 Kiewnick S, Neumann S, Sikora RA, and Frey JE, Effect of *Meloidogyne incognita*
36
37 18 inoculum density and application rate of *Paecilomyces lilacinus* strain 251 on
38
39 19 biocontrol efficacy and colonization of egg masses analyzed by real-time
40
41 20 quantitative PCR. *Phytopathology* **101** (1):105-112 (2011).
42
43 21 25 Anastasiadis IA, Giannakou IO, Prophetou-Athanasiadou DA and Gowen SR, The
44
45 22 combined effect of the application of a biocontrol agent *Paecilomyces lilacinus*,
46
47 23 with various practices for the control of root-knot nematodes. *Crop Prot*
48
49 24 **27**(3):352-361 (2008).
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 1 26 Kaşkavalci G, Tüzel Y, Dura O and Öztekin GB, Effects of alternative control
4
5 2 methods against *Meloidogyne incognita* in organic tomato production. *Ekoloji*
6
7 3 **18**(72):23-31 (2009).
8
9
10 4 27 Whitehead AG and Hemming JR, A comparison of some quantitative methods of
11
12 5 extracting small vermiform nematodes from soil. *Ann Appl Biol* **55**(1):25–38
13
14 6 (1965).
15
16 7 28 Zeck WM, A rating scheme for field evaluation of root-knot nematode infection.
17
18 8 *Pflanzenschutz-Nachrichten* **24**:141-144 (1971).
19
20
21 9 29 Hussey RS and Barker KR, A comparison of methods of collecting inocula of
22
23 10 *Meloidogyne* spp. including a new technique. *Plant Dis Rep* **57**:1025-1028
24
25 11 (1973).
26
27
28 12 30 Giné A, Bonmatí M, Sarro A, Stchiegel A, Valero J, Ornat C, Fernández C and
29
30 13 Sorribas FJ, Natural occurrence of fungal egg parasites of root-knot nematodes,
31
32 14 *Meloidogyne* spp. in organic and integrated vegetable production systems in
33
34 15 Spain. *BioControl* **58**(3):407-416 (2013).
35
36
37 16 31 Lopez-Llorca LV and Duncan JM, New media for the estimation of fungal infection
38
39 17 in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica*
40
41 18 **32**(4):486-489 (1986).
42
43
44 19 32 Samson RA, Paecilomyces and some allied Hyphomycetes. Studies in Mycology,
45
46 20 No. 6. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. (1974).
47
48 21 33 Castellá G, Bragulat MR and Cabañes FJ, Extracellular enzymatic activity of
49
50 22 *Fusarium* section *Liseola* isolates. *Mycopathologia* **144**(1):55-60 (1999).
51
52 23 34 Verdejo S, Jaffee BA and Mankau R, Reproduction of *Meloidogyne javanica* in plant
53
54 24 roots genetically transformed by *Agrobacterium rhizogenes*. *J Nematol*
55
56 25 **20**(4):599-604 (1988).
57
58
59
60

- 1
2
3 1 35 Mountain WB, A method of culturing plant parasitic nematodes under sterile
4
5 2 conditions. *Proc Helminthol Soc Wash* **22**(1):49-52 (1955).
6
7 3 36 Michel BE, Evaluation of the water potential of solutions of polyethylene glycol
8
9 4 8000 both in the absence and presence of other solutes. *Plant Physiol* **72**(1):66-
10
11 5 70 (1983).
12
13 6 37 Monfort E, Lopez-Llorca LV, Jansson HB and Salinas J, *In vitro* soil receptivity
14
15 7 assays to egg-parasitic nematophagous fungi. *Mycological Progress* **5**(1):18-23
16
17 8 (2006).
18
19 9 38 Ferris H, Roberts PA and Thomason IJ, Nematodes, in *Integrated Pest Management*
20
21 10 *for Tomatoes*, ed.; Statewide Integrated Pest Management Project; Division of
22
23 11 Agriculture and Natural Resources, University of California, pp 60–65. (1985).
24
25 12 39 Verdejo-Lucas S, Blanco M, Cortada L and Sorribas F J, Resistance of tomato
26
27 13 rootstocks to *Meloidogyne arenaria* and *Meloidogyne javanica* under
28
29 14 intermittent elevated soil temperatures above 28 °C. *Crop Prot* **46**:57-62 (2013).
30
31 15 40 Khan A, Williams KL and Nevalainen HK, Control of plant-parasitic nematodes by
32
33 16 *Paecilomyces lilacinus* and *Monacrosporium lysipagum* in pot trials. *Biocontrol*
34
35 17 **51**(5):643-658 (2006).
36
37 18 41 Kiewnick S, Effect of temperature on growth, germination, germ-tube extension and
38
39 19 survival of *Paecilomyces lilacinus* strain 251. *Biocontrol Sci Techn* **16**(5):535-
40
41 20 546 (2006).
42
43 21 42 Rumbos C, Mendoza A, Sikora R and Kiewnick S, Persistence of the
44
45 22 nematophagous fungus *Paecilomyces lilacinus* strain 251 in soil under controlled
46
47 23 conditions. *Biocontrol Sci Techn* **18**(10):1041-1050 (2008).
48
49 24 43 Bird AF and Bird J, The structure of nematodes. Academic Press, San Diego. (1991).
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 1 44 Holland RJ, Williams KL and Nevalainen KH, *Paecilomyces lilacinus* strain
4
5 2 Bioact251 is not a plant endophyte. *Australas Plant Path* **32**(4):473-478 (2003).
6
7 3 45 Rumbos CI and Kiewnick S, Effect of plant species on persistence of *Paecilomyces*
8
9 4 *lilacinus* strain 251 in soil and on root colonization by the fungus. *Plant Soil*
10
11 5 **283**(1-2):25-31 (2006).
12
13 6 46 Kok CH and Papert A, Microflora of *Meloidogyne* egg masses: species composition,
14
15 7 population density and effect on the biocontrol agent *Verticillium*
16
17 8 *chlamydosporium* (Goddard). *Nematology* **3**(8): 729-734 (2001).
18
19 9 47 Stirling GR. *Biological control of plant-parasitic nematodes: soil ecosystem*
20
21 10 *management in sustainable agriculture*. CAB International, Wallingford, UK
22
23 11 (2014).
24
25 12 48 Ploeg AT and Maris PC, Effects of temperature on the duration of the life cycle of a
26
27 13 *Meloidogyne incognita* population. *Nematology* **1**(4):389-393 (1999).
28
29 14 49 Bonants PJM, Flitters PFL, Thijs H, den Belder E, Waalwijk C and Henfling JWDM,
30
31 15 A basic serine protease from *Paecilomyces lilacinus* with biological activity
32
33 16 against *Meloidogyne hapla* eggs. *Microbiology* **141**(4):775-784 (1995).
34
35 17 50 Sorribas FJ, Ornat C, Galeano M and Verdejo-Lucas S, Evaluation of a native and
36
37 18 introduced isolate of *Pochonia chlamydosporia* against *Meloidogyne*
38
39 19 *javanica*. *Biocontrol Sci Techn* **13**(8): 707-714 (2003).
40
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1 Table 1. Cropping dates of the rotation sequence tomato-cucumber and soil
2 temperatures in the greenhouse during two consecutive growing seasons.

Year	Crop	Cropping dates	Average soil temperature (°C) ^a	Minimum and maximum temperatures (°C) ^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

3 ^a Average soil temperature at 15cm depth: mean of daily mean temperatures during the
4 cropping period.

5 ^b Absolute minimum or maximum soil temperature at 15cm depth during the cropping
6 period.

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1 Table 2. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the resistant tomato cv. Monika (TR) and susceptible cv. Durinta (TS) alone or combined with the application of
 3 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

4 Data are mean ± standard error of 10 replicates.

5 ^a BioAct WG (1 x 10¹⁰ 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; ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Gallings index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

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

1 Table 3. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato
 3 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

4 Data are mean ± standard error of 10 replicates.

5 ^a BioAct WG (1 x 10¹⁰ 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: ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Gallings index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

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

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6 1 Table 4. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
7 egg parasitism, and yield of the susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG, and the resistant tomato cv.
8 Monika (TR) alone, cultivated from 5th March to 17th July of 2012.
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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

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22 4 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
23 with TS”.

24 6 ^a BioAct WG (1 × 10¹⁰ 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: ,
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26 7 in seedling before transplanting at 0.1g L⁻¹ rate.

27 8 ^b Gallings index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

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29 9 Different letters in the same column indicate differences ($P < 0.05$) according to Tukey’s test.
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1 Table 5. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato
 3 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

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

5 ^a BioAct WG (1 x 10¹⁰ 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: ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Gallings index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

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

9 Na: no available

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2
3 1 Effect of plant resistance and BioAct WG (*Purpureocillium lilacinum* strain 251)
4
5 2 ~~against-on~~ *Meloidogyne incognita* in a tomato–cucumber rotation in ~~plastic-a~~
6
7 3 ~~greenhouse.~~
8

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10 4 Ariadna Giné and Francisco J Sorribas*

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24
25 11 **Running title:** Effect of plant resistance and BioAct WG (~~*P. lilacinus* strain 251~~)
26
27 12 ~~against-on~~ RKN in greenhouse.
28

29 13

30
31
32 14 **Abstract**

33
34 15 BACKGROUND: The effectiveness of combining resistant ~~or-susceptible~~ tomato
35
36 16 ~~cultivars~~ with BioAct WG (*Purpureocillium lilacinum* strain 251; PI251) against
37
38 17 *Meloidogyne incognita* was assessed ~~in into~~ a tomato-cucumber rotation in ~~a-plastic~~
39
40 18 greenhouse ~~over during~~ two years. ~~Additionally, In-addition~~ enzymatic activity of the
41
42 19 ~~fungus, the percentage of fungal eggs or~~ and juveniles parasitism, cardinal temperatures
43
44 20 ~~and the effect of water potential on mycelia growth and the soil receptivity to PI251,~~
45
46 21 ~~were determined in vitro. experiments were conducted to determine the~~
47

48
49 22 RESULTS: Plant resistance was the only factor that suppressed nematode
50
51 23 reproduction, ~~and tomato and the following cucumber crop yield losses.~~ Percentage of
52
53 24 ~~fungal~~ egg parasitism in plots treated with BioAct WG was less than 2.6%. However ~~in~~
54
55 25 ~~under~~ *in vitro* conditions, PI251 showed protease, lipase, and chitinase activities, and
56
57
58
59
60

1 parasitized 94.5% of *M. incognita* eggs, but no juveniles. Cardinal temperatures were
2 14.2, 24-26, and 35.4 °C. The maximum PI251 mycelial growth was at -0.25 MPa and
3 25 °C. Soil temperatures and water potential in the greenhouse field conditions were in
4 the range of the fungus development. However, soil receptivity was less in the plastic
5 greenhouse soil, irrespective of sterilization, than in sterilized sand.

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

12

13 **Keys words**

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

16

17 **1 INTRODUCTION**

18 Spain is the main producer of vegetables under protected cultivation in the
19 Mediterranean area, dedicating with 71.003 ha.¹ The major crops belong to Solanaceae
20 and Cucurbitaceae families. The most important are tomato (*Solanum lycopersicum* L.)
21 and cucumber (*Cucumis sativus* L.), two of the most cultivated crops, which are
22 frequently cultivated in a double cropping system.² In Greenhouse, tomato is cultivated
23 in grown on 18.501 ha with an annual production of 1.881.922 tonnes, which represents
24 38% of the tomato production area and 47% of the total tomato surface and yield,
25 respectively. Concerning Greenhouse cucumber, it is grown on in 7.768 ha with an

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

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

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

1 Several microorganisms have been evaluated ~~as for~~ biological control of plant-
2 parasitic nematodes.¹⁷ ~~The use of biological control agents able to suppress the buildup~~
3 ~~of RKN can be of interest to reduce the pressure on R genes avoiding the selection of~~
4 ~~virulent populations and contribute to maintain nematode densities below the economic~~
5 ~~threshold level. however~~ Out of those, *Purpureocillium lilacinum* (formerly
6 *Paecilomyces lilacinus*) strain 251 (PI251) is the only biological ~~control agent included~~
7 ~~as~~ nematicide ~~listed~~ in the annex 1 of the European register of active substances.¹⁸
8 *Purpureocillium lilacinum* is a common soil hyphomycete able to parasitize RKN
9 sedentary stages by direct hyphal penetration and by using hydrolytic enzymes.¹⁹⁻²¹
10 Besides, the effectiveness of PI251 to ~~manage control~~ RKN has been widely reported ~~in~~
11 ~~under~~ controlled conditions and ~~in~~ pot tests,²²⁻²⁴ ~~although~~ few reports ~~are available on its~~
12 effectiveness ~~in under~~ field conditions ~~are available~~.^{25,26}

13 The aim of the present study was to evaluate the effectiveness of combining the
14 resistant tomato cv. Monika ~~with and~~ *P. lilacinum* strain 251 (BioAct WG[®], Belchim
15 Crop Protection, ~~Londerzeel, Belgium~~) over two consecutive growing seasons in a
16 greenhouse to manage RKN in a tomato-cucumber rotation ~~sequence~~. Additionally,
17 ~~several~~ *in vitro* experiments were carried out to determine extracellular enzymes
18 produced by PI251 and its capability to parasitize eggs and second-stage juveniles of
19 RKN, as well as to know cardinal temperatures and the effect of temperature and water
20 potential on mycelia growth, and soil receptivity ~~to PI251 to the fungus isolate~~.

21

22 2 MATERIAL AND METHODS

23 2.1 Field Greenhouse trial

24 The field trial was conducted in a 700 m² ~~plastic~~ greenhouse infested with *M.*
25 *incognita* in Viladecans (41° 17' 18''N; 2° 2' 39''E, Barcelona, Spain) during ~~two~~

1
2
3 1 ~~consecutive years~~, 2011 and 2012. The soil ~~texture~~ was a sandy loam with 83.8% sand,
4
5 2 6.7% silt and 9.5% clay; pH 8.7; 1.8% of organic matter (w/w) and 0.5 dS/m of electric
6
7 3 conductivity. Soil was infested with *M. incognita* in 2007 and rotations with resistant or
8
9 4 susceptible tomato cultivars and cucumber or black fallow were carried out from 2008
10
11 5 to 2010.

12
13
14 6 The rotation sequence ~~consisted of~~ ~~was integrated by~~ resistant tomato cv. Monika
15
16 7 (bearing the *Mi* gene) or the susceptible cv. Durinta ~~cultivated~~ from March to July,
17
18 8 followed by cucumber cv. Dasher II cultivated from July to October-November.

19
20 9 Individual plots of 9.6 m² comprising of 4 rows with 6 plants per row. Plant spacing was
21
22 10 50 cm between rows and 55 cm within rows. The distance between individual plots was
23
24 11 110 cm between rows and 100 cm along rows.

25
26
27 12 In 2011, combinations of resistant (TR) or susceptible tomato (TS) with or without
28
29 13 BioAct WG preceding cucumber with or without BioAct WG were assessed. ~~In 2011,~~
30
31 14 ~~eight combinations were included in the experiment: (i) TR-BioAct-C-BioAct; (ii) TR~~
32
33 15 ~~BioAct-C; (iii) TR-C-BioAct; (iv) TR-C; (v) TS-BioAct-C-BioAct; (vi) TS-BioAct-C;~~
34
35 16 ~~(vii) TS-C-BioAct; (viii) TS-C.~~ Each combination was replicated ~~five~~ 10 times
36
37 17 according to a stratified randomized block design. In 2012, the combinations were
38
39 18 located ~~at-on~~ the same plots ~~than-as~~ in 2011 with slight modifications. Plots cultivated
40
41 19 with ~~resistant tomato~~ TR did not receive any treatment with BioAct WG because of
42
43 20 results obtained in 2011, ~~but did when cucumber was cultivated.~~ Then, combinations of
44
45 21 ~~susceptible tomato with or without BioAct WG and resistant tomato without BioAct~~
46
47 22 ~~WG preceding cucumber with or without BioAct WG were assessed.~~ including TR or
48
49 23 TS without BioAct WG were replicated 10 times, and 20 for the combination TS with
50
51 24 BioAct WG. Cucumber crop cultivated after TS with application or not application of
52
53 25 BioAct WG was replicated 15 times, and five times each combination after TR. ~~six~~

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

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

10 Seedlings were watered with a suspension of 0.1 g BioAct WG 1 L^{-1} just before
11 transplanting.

12 Tomato was cultivated from 31st March to 6th July (98 days), and cucumber from 29th
13 July to 26th October (90 days) in 2011. In 2012, tomato was cultivated from 5th March to
14 17th July (135 days) and cucumber from 31st July to 5th November (98 days).

15 The plants were irrigated by drip irrigation and fertilized weekly with a NPK solution
16 (15–5–30) at 31 kg ha^{-1} and iron chelate and micronutrients at 0.9 kg ha^{-1} . Crops were
17 vertically trellised. Weeds were removed manually during and between cropping cycles.
18 Tomato yield was assessed from the first six fruit sets produced from the eight central
19 plants in each plot. Commercial standard Fruits were harvested according to commercial
20 standards as they reached maturity. Similar, cucumber fruits were harvested from the
21 eight central plants of each plot were harvested when they reached the standard
22 commercial size. Total yield per crop cycle was expressed as kilograms plant^{-1} .

23 Soil temperatures were recorded at 30 min intervals in order to estimate the number
24 of the nematode generations, and the effect of soil temperature on the fungus growth. In
25 2011 were recorded with soil probes 107 (Campbell Scientific, Logan, USA) placed at a

1
2
3 1 depth of 15 cm. In 2012, soil temperatures and water potential were recorded at the
4
5 2 same interval and soil depth with 5TM and MPS-1 probes (Decagon devices, Inc,
6
7 3 Pullman, USA).

9
10 4 Composite soil samples were collected from each plot at the beginning and at the end
11
12 5 of each crop to estimate initial (Pi) and final (Pf) nematode population densities,
13
14 6 respectively. Each soil sample consisted ~~in~~ of eight subsamples from the ~~firsts-top~~ 30
15
16 7 cm ~~of depth-tacked-taken~~ with an ~~auger sampling-tube~~ (2.5 cm diameter). Soils samples
17
18 8 were sieved through a 4 mm aperture screen to remove stones ~~from soil~~, and carefully
19
20 9 homogenized to extract nematodes from 500 cm³ by Baermann trays.²⁷ Second-stage
21
22 10 juveniles (J2) that migrated to the water were collected one week later, concentrated on
23
24 11 a 75 µm sieve, counted, and then expressed as number of J2 per 250 cm³ of soil. At the
25
26 12 end of each crop, plants were removed with a pitchfork, cut at ground level, and the
27
28 13 disease severity was assessed using the Zeck's galling²⁸ index on a scale of 0 to 10,
29
30 14 where 0 means complete and healthy root system (no galls observed) and 10 ~~means~~
31
32 15 plants and roots dead. Afterwards, the roots were ~~weighted bulked~~ and chopped into 2
33
34 16 cm long segments and two 10 g subsamples were used to extract eggs by blender
35
36 17 maceration in a 1% NaOCl solution.²⁹ After 10 minutes ~~of maceration~~, the egg
37
38 18 suspension was passed through a 75 µm sieve, to retain the plant material, and ~~through a~~
39
40 19 25 µm sieve to retain the eggs. The number of eggs was counted and expressed per
41
42 20 gram of fresh root weight.

43
44
45
46
47 21 To assess fungal egg parasitism, the procedure described in Giné *et al.*³⁰ was used. In
48
49 22 brief, 20 egg masses per individual plot were handpicked from the remaining roots and
50
51 23 placed in a watchglass containing sterile demineralised water. The outer part of the
52
53 24 gelatinous matrix was removed, and eggs were dispersed in an Eppendorf
54
55 25 microcentrifuge tube containing 1 mL of sterile demineralised water with a pestle. A
56
57
58
59
60

1 333 μL aliquots of eggs suspension ~~were was~~ spread onto each of three replicated Petri
2 plates containing a growth ~~restricting medium~~,³¹ and incubated at $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ ~~at-in~~
3 ~~the dark~~. Number of parasitized eggs was recorded after 24 and 48 hours under a
4 dissecting microscope and percentage of egg parasitism was then calculated as the
5 number of parasitized eggs per plate per total eggs per plate. Eggs were considered
6 parasitized if fungal hyphae grew from inside ~~of unhatched eggs~~. Parasitized eggs were
7 individually transferred to the growth ~~restricting medium~~ to establish pure cultures and
8 fungal species were identified by cultural and morphological characteristics.³²

9 2.2 Extracellular enzymes production

10 Extracellular enzyme production by PI251 was evaluated using a semiquantitative API
11 ZYM ® (BioMérieux, ~~Marcy l'Etoile, France~~) system which identifies 19 cellular
12 enzymes, ~~i.e.~~ alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14),
13 leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin,
14 acid phosphatase, naphthol-AS-BI phosphohydrolase, α -galactosidase, β -galactosidase,
15 β -glucuronidase, α -glucosidase, β -glucosidase, n-acetyl- β -glucosaminidase, α -
16 mannosidase and α - fucosidase.

17 Conidia of PI251 were collected from 3-week-old cultures of the fungus growing on
18 potato dextrose agar (PDA; 39 g L^{-1}) at 25°C ~~at in the~~ dark. The colony was washed
19 with 5 mL of sterile distilled water. The number of conidia was then counted with a
20 haemocytometer and adjusted to 1×10^6 conidia mL^{-1} . The API ZYM system was used
21 according to the manufacturer's instructions. ~~In-brief~~ Briefly, 65 μL of the conidia
22 suspension was ~~dispended-placed~~ into each cupule, and incubated at $25\text{ }^{\circ}\text{C}$ for 6 h ~~at in~~
23 ~~the dark~~.³³ Enzyme activity was observed after addition of a drop of each ZYM A and
24 ZYM B reagents and exposed to sunlight for an hour to eliminate the yellowing from
25 the reagents. The enzyme production was assessed according to the scale from 0 (no

1
2
3 1 enzyme production) to 5 (maximum enzyme production) according the color chart
4
5 2 provided by the manufacturer. The experiment was carried out once.

6
7 3 **2.3 Capability of PI251 to parasitize *Meloidogyne incognita* eggs and J2 in**
8
9 4 ***in vitro* test**

10 5 Individual plugs of 9 mm-diameter from the edge of a PI251 colony growing ~~in~~ on
11
12 6 PDA ~~was-were~~ placed ~~at~~ in the centre of a total of six Petri dishes (90 mm) containing
13
14 7 water agar (~~bacteriological~~ agar 12 g L⁻¹), and incubated at 25 °C ± 0.5 °C ~~at-in~~ the dark
15
16 8 for 3 ~~days~~ weeks.

17
18
19 9 Surface sterilized eggs or J2 of the same *M. incognita* population used to inoculate
20
21 10 the soil of the greenhouse in 2007 and maintained in tomato in pots were used to
22
23 11 determine the parasitic ability of PI251. *Meloidogyne incognita* was identified by
24
25 12 morphology of perineal pattern, esterase pattern, and SCAR marker. Eggs from 30 egg
26
27 13 masses handpicked from tomato roots were surface sterilized following the protocol of
28
29 14 Verdejo *et al.*³⁴ with some modifications. Egg masses were placed into a conical sterile
30
31 15 tube with 1 mL of a 0.5% NaOCl solution for four min. and were shaken at 30 s
32
33 16 intervals for 10 seconds. After that, the solution was diluted 10 times with sterile
34
35 17 distilled water and left undisturbed for 30 min. to allow the eggs to settle at the bottom
36
37 18 of the tube. Then, eggs were taken and ~~were~~ spread into three Petri dishes at 1 cm of the
38
39 19 edge of the colony of the PI251 using a sterile Pasteur pipette, and incubated at 25 °C ±
40
41 20 0.5 °C ~~at~~ in the dark for one week ~~3-weeks~~. The assessment of egg parasitism was
42
43 21 carried out as described previously.

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45
46
47
48
49 22 Second-stage juveniles of *M. incognita* were obtained by extraction of eggs from
50
51 23 tomato roots by the Hussey and Barker method²⁹ and left to emerge ~~in~~ on Baermann
52
53 24 trays.²⁷ Then, J2 were surface sterilized following the Mountain³⁴ procedure with some
54
55 25 modifications. Briefly, a suspension of 100 J2 in 0.5 mL water was placed in a conical
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1 sterile tube containing streptomycin 0.1% for 4 hours. The suspension was shaken at
2 one hour interval for 10 seconds. The solution was then diluted 20 times with sterile
3 distilled water and left undisturbed for 30 min. for J2 to settle at the bottom of the tube
4 deposition. Then, J2 were taken and placed in three Petri dishes at 1 cm of the edge of
5 the PI251 colony using a sterile Pasteur pipette, and incubated at $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ at-in the
6 dark for one week 3-weeks. The assessment of J2 parasitism was carried out as
7 described previously for egg parasitism.

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

11 Experiments were conducted once.

12 **2.4 Cardinal temperatures and the effect of temperature and water potential on** 13 **PI251 mycelia growth in in vitro tests**

14 Cardinal temperatures of PI251 were determined placing individual 9 mm-diameter
15 plugs of PI251 in at the center of each of 24 Petri dishes containing water agar (WA; 12
16 g L⁻¹). Three-out-24-Petri dishes were incubated at 4, 10, 15, 20, 25, 30, 35 and or 40°C
17 at in the dark (three dishes per temperature). Minimum and maximum diameters (mm)
18 of fungal growth were measured every 24 h until the fungi-colonies occupied reach-the
19 80% of the surface of the Petri plate. The mycelia growth rate (mm day⁻¹) was
20 calculated as the relation between mean colony diameter (mm) and growth time (day).

21 Concurrently, the effect of water potential (Ψ) and temperature on PI251 growth was
22 assessed. Water agar media with different concentrations of Polyethylene Glycol 8000
23 (PEG8000) was prepared according to the Michel's equation³⁶ to achieve Ψ between
24 1.25, -1, -0.75, -0.5 and -0.25 MPa. Mycelia plugs of 9 mm diameter from the edge of a
25 fungal the colony were placed in at the centre of the Petri plates and then incubated at

1 15, 20, 25, and 30 °C ~~at in the~~ dark. Each combination of temperature-PEG8000
2 concentration was repeated three times. Minimum and maximum diameters of fungal
3 growth were measured daily until the colonies occupied 80% of the surface of the Petri
4 plate. ~~Per-For~~ For each water potential, a linear regressions was calculated between the
5 mean diameter of the colony per each day of assessment and the temperature were
6 ~~constructed~~, and the slopes were used to construct ~~other the~~ regressions to determine the
7 effect of temperature and water potential on mycelia growth. Experiments were repeated
8 once.

2.5 Soil receptivity

9 Soil ~~coming-collected~~ from BioAct WG non-treated plots ~~in-of-which~~ the
10 greenhousefield trial was carried-out was assessed for receptivity to the fungal isolate. A
11 part of soil was two times sterilized at 121 °C for 1 h and the procedure was repeated
12 after within 24 h. The other part remained non-sterilized. The experiment was carried
13 out following the procedure described by Monfort *et al.*³⁷ procedure. ~~In-brief~~ Briefly, 40
14 g of sterilized or non-sterilized air-dried soil was placed in Petri dishes and saturated
15 with sterile distilled water. Soils included in the experiment were: (i) sterilized ~~plastic~~
16 greenhouse soil; (ii) non-sterilized ~~plastic~~-greenhouse soil and (iii) sterilized sand. A
17 polyvinylidene difluoride (PVDF) membrane (0.22- μ m-pore-size and 45 mm-diameter)
18 was sterilized at 121 °C for 20 min and placed ~~at-the-on~~ top of the soils ensuring full
19 contact. A 4 mm-diameter plug of P1251 was placed in the middle of each membrane.
20 Petri dishes were then sealed and incubated at 25 °C \pm 0.5 °C ~~at-in the~~ dark. After three
21 weeks, the membranes were ~~cleaned washed~~ with sterile distilled water and then dried
22 in a laminar air flow cabinet. Then, the membranes where incubated in a solution of 1%
23 trypan blue in lactic acid for 12 h at room temperature to stain the mycelia ~~in-blue~~. After

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

3 2.6 Statistical analysis

4 Statistical analyses were done using SAS v. 9 (SAS Institute Inc.). Data from field
5 experiments were transformed when required to $\log_{10}(x + 1)$ or ~~arcsine~~ square-root
6 $(x+0.5)$ to normalize the data. ~~The greenhouse trial was analyzed by~~ analysis of variance
7 by the general lineal model (PROC GLM) ~~according to a factorial design~~ to compare the
8 effect of ~~i)~~ the tomato cultivar, the application of BioAct WG, and the interaction
9 (except for tomato 2012), on nematode densities in soil, eggs in roots, disease severity,
10 and crop yield per ~~every~~ cropping season. ~~Analysis of variance was also carried out to~~
11 ~~compare the effect of~~ the temperature and kind of soil on the ~~mycelia~~ growth of PI251. ~~;~~
12 ~~and ii)~~

13 3 RESULTS

14 3.1 Field Greenhouse trial

15 Minimum, maximum and average soil temperatures during the cultivation of each
16 crop and year are provided in Table 1. Accumulated soil temperatures during the tomato
17 and cucumber crops in 2011 were 1504 DD (degree days; ~~base temperature (Tb) =10~~
18 °C) and 1473 DD (~~Tb=11.4 °C~~), respectively. ~~According to thermal requirements of~~
19 ~~tomato and cucumber,~~^{5,38} *M. incognita* completed two generations in tomato (thermal
20 constant (S) = 600-700 DD over ~~Tb=10 °C~~),³⁸ and in cucumber (S = 500 DD over ~~Tb =~~
21 11.4 °C).⁵

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

1 soil, roots and the galling index ~~registered~~ at the end of the resistant tomato crop were 8,
2 6 and 18% those ~~registered recorded~~ at the end of the susceptible ~~one cultivar~~, which ~~in~~
3 ~~turn~~-yielded 78% less than the resistant one. The percentage of fungal egg parasitism at
4 the end of the crop was less than 0.1% (Table 2). At ~~cucumber transplanting~~, higher (P
5 < 0.05) soil nematode densities ~~in soil were registered~~ occurred in plots preceded by a
6 susceptible than a resistant tomato cultivars. Galling indices were lower ($P < 0.05$) after
7 a resistant than a susceptible tomato cultivar. ~~The Eggs from cucumber plants~~ were only
8 recovered from plants preceded by a resistant tomato because ~~high percent of plants~~
9 ~~cucumber following susceptible tomato~~ died (data not shown). Egg parasitism by the
10 fungus was less than 0.51% (Table 3).

11 In 2012, accumulated temperatures during the tomato and cucumber crops were 1959
12 ($T_b = 10\text{ }^\circ\text{C}$) and 1524 DD ($T_b = 11.4\text{ }^\circ\text{C}$), respectively. Hence, *M. incognita* completed
13 three generations in both crops according to its thermal requirements. In this cropping
14 season, the tomato cultivar was also the factor responsible ~~to~~ for the differences ($P <$
15 0.05) ~~on~~ in nematode densities in the soil and roots, disease severity, as well as crop
16 yield in both tomato and cucumber crops (Tables 4 and 5). As in the previous season,
17 cucumber ~~preceded by~~ following susceptible tomato ~~was exposed to had~~ higher ($P <$
18 0.05) nematode ~~density levels~~ at transplanting, and all plants ~~died were dead~~ at the end
19 of the experiment. The percentage of fungal egg parasitism at the end of tomato and
20 cucumber crops was 2.4 and 2.6 %, respectively (Table 5).

21 3.2 Extracellular enzymes production

22 Six extracellular enzymes were produced by P1251. The ~~highest~~ enzyme produced in
23 ~~highest amounts~~ by the fungus ~~isolate~~ was leucine arylamidase (~~value of the color scale:~~
24 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 naphthol-AS-BI-phosphohydrolase and N-acetyl- β -glucosaminidase (value of the color scale: 2).

3.3 Capability of PI251 to parasitize RKN eggs and J2 ~~in in vitro test~~

PI251 parasitized $94.91\% \pm 2.88$ (mean \pm 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 PI251 mycelia growth ~~in in vitro tests~~

Mycelia growth of PI251 occurred between 14.2 °C (minimum) and 35.4 °C (maximum), ~~with being~~ 24-26 °C as the optimal ~~growth~~ temperatures range (Figure 1).

No ~~fungal~~ growth was detected at 4, 10 and 40 °C. The ~~values of~~ water potential ~~assessed~~ (-1.25 to -0.25 MPa) directly influenced the mycelia growth of PI251. The mycelia growth was higher at optimal temperatures (25 °C) and highest water potential ~~(-0.25 MPa)~~, followed by 30 °C, 20 °C and 15 °C (Figure 2).

3.5 Soil receptivity

The mycelia growth of PI251 in sterile sand ($2.55\text{cm} \pm 0.06$; mean \pm standard error) was 91.83% higher ($P < 0.05$) compared with non-sterile ($0.18\text{cm} \pm 0.06$; mean \pm standard error) or sterile ~~plastic~~-greenhouse soil ($0.23\text{cm} \pm 0.07$; mean \pm 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

1 densities ~~across the rotation sequence in two ways~~. The first one, mediated by plant
2 resistance, should ~~aet~~ suppressing nematode infection, development and reproduction.
3 The second one, due to PI251, should ~~aet-parasitize~~ eggs produced by nematodes that
4 escaped ~~the action of~~ plant resistance, ~~and then further increase the level of suppression~~
5 ~~against RKN~~. In the following cucumber crop, ~~less number of fewer~~ RKN at
6 transplanting after resistant tomato should mean more percentage of egg parasitism,
7 because most egg masses should be ~~outside the root on the root surface~~ favoring egg
8 infection, as well as ~~less reduce~~ yield losses due to ~~less number of fewer~~ J2 at
9 transplanting. However, the hypothesis was not confirmed in our ~~conditions-trial~~, ~~being~~
10 ~~with~~ plant resistance ~~being~~ the only ~~control-methods~~ factor that consistently suppressed
11 ~~RKNnematode built up in every rotation sequence~~. The effectiveness of tomato cultivars
12 or rootstocks carrying the *Mi* gene against RKN ~~eropped~~ in ~~plastic~~ greenhouses in Spain
13 was consistent with previous reports.^{4,9,14,15} In this study, ~~eropping~~ resistant tomato
14 suppressed disease severity and reproduction ~~in by~~ 82 - 91% and 87 - 95% ~~than which~~
15 ~~were registered compared to~~ the susceptible ~~one~~ cultivar, each year, respectively. The
16 effect of intermittent peaks of soil temperatures over 28 °C did not affect the
17 effectiveness of the *Mi* gene as previously reported.³⁹ During the tomato crop in 2011,
18 the numbers of days with maximum soil temperatures over 28 °C were 23, after 35 days
19 of transplanting, and 38 days, after 62 days of transplanting in 2012. In addition, the
20 benefit of cropping a resistant tomato cultivar on yield losses of the following
21 susceptible crop was also observed as previously stated.^{2,15}

22 ~~Conversely~~ Unlike to plant resistance, there are few reports about the effectiveness of
23 ~~Purpureocillium lilacinus strain 251~~ BioAct WG alone and/or in combination with other
24 control methods against RKN ~~in~~ under Mediterranean conditions.^{25,26} In studies
25 conducted in Greece and Turkey, BioAct WG did not provided satisfactory RKN

1 ~~management control~~. However, in several *in vitro* and pot tests the antagonistic
2 capability of PI251 against several plant-parasitic nematode species was reported.^{11,20-}
3 ^{24,40-42} The ability of PI251 to penetrate eggs and cuticles of sedentary stages of RKN by
4 mechanical and chemical mechanisms has been reported.^{20,21} The results obtained by the
5 API ZYM method showed high protease and lipase activity and low chitinase activity
6 able to degrade the main components of egg shell and nematode cuticle.⁴³ Therefore, ~~the~~
7 ~~a high proportion of egg parasitism was expected, as it was in our *in vitro* experiment~~
8 ~~(94.9 %). because protease alone or in combination with chitinase are more important~~
9 ~~for nematode parasitism than chitinase alone.~~³⁷ In addition, Kahn *et al.*²¹ pointed out the
10 parasitic ability of PI251 on all stationary ~~developing~~ stages of *M. javanica*, that is:
11 eggs, juvenile contained in eggs, post-infective juvenile stages: from swollen J2 to J4,
12 and females, but they did not assess the effect on the ~~mobile~~ infective J2. The results of
13 this study showed that PI251 was not able to parasitize the infective J2 stage of *M.*
14 *incognita* ~~neither sedentary stages of RKN because PI251 is not a root endophyte of~~
15 ~~tomato or cucumber plants.~~^{44,45} Thus, PI251 could exert its parasitic potential only on
16 eggs and juveniles contained in eggs that remained in soil at the end of the crop, or on
17 those produced on roots and exposed to ~~the soil conditions. Otherwise, the fungus could~~
18 ~~not be able to parasitize the~~ In fact, in ~~plastic~~ greenhouse conditions, the percentage of
19 egg parasitism was less than 2.6% in both crops and years. ~~Moreover, microorganisms~~
20 ~~associated to the gelatinous matrix of the egg masses can inhibit fungal egg parasites as~~
21 *Pochonia chlamydosporia*.⁴⁶ Thus, fungal application did not affect nematode
22 development ~~despite even though~~ four ~~treatments applications per were applied during~~
23 ~~each~~ crop and year.

24 Environmental factors can play an important role ~~on-in~~ nematode biocontrol.⁴⁷
25 Rumbos *et al.*⁴² reported a negative ~~correlation~~ between the persistence of PI251 in soil

1 and the sand content of soil. Thus, sandy soils, ~~such that as~~ in this study (83.8% sand)
2 ~~could would~~ not be suitable for the fungus. However, the test of soil receptivity showed
3 ~~more fungal that~~ mycelia growth ~~was better~~ in sterilized sand than in the sterilized
4 sandy loam soil ~~in which from the field greenhouse experiments were carried out. Then~~
5 ~~it seems~~ This indicates that other factors ~~than sand content~~ different to microbial
6 communities or thermo-sensitive chemicals in soil could ~~influence limit~~ the
7 effectiveness of PI251 because ~~similar~~ mycelia growth ~~was equally poor~~ in sterile and
8 non-sterile sandy loam soil from the ~~plastic greenhouse were recorded in the test of soil~~
9 ~~receptivity.~~

10 Soil temperatures during the ~~cropping period~~ or time of application could also affect
11 ~~PI251 BioAct WG~~ effectiveness. In this study, cardinal temperatures of mycelia growth
12 were determined. Maximum temperature of PI251 mycelia growth in water agar was
13 similar to that reported by Kiewnick⁴¹, but not the optimal range, which was less wide
14 (24-26 vs 24-30 °C). Moreover, in this study, ~~minimal minimum~~ temperature was also
15 estimated (14.2 °C) because low soil temperatures at transplanting ~~tomato in the~~ spring
16 ~~crop~~ (17 – 19 °C) could affect fungal ~~development~~ and thus its effectiveness. Soil
17 temperatures during the cultivation of tomato and cucumber were in the range of the
18 fungus development according to cardinal temperatures, but 37 out of 98 days and 60
19 out of 90 days during each tomato crop were over ~~the range of~~ optimal temperatures,
20 and 63 out of 135 and 59 out of 98 days during each cucumber crop. At soil
21 temperatures ~~from between~~ the optimal for fungal growth (24-26 °C) ~~to and~~ the optimal
22 for *M incognita* development (30 °C),⁴⁸ the nematode could take advantage over PI251
23 that can reduce its effectiveness. In fact, at temperatures of 28 ± 1.5 °C no dose-
24 response relationship was observed but it did at 25 ± 1 °C.²⁴ ~~In addition, PI251 was~~

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

3 The soil water potential recorded during the cropping period should not affect the
4 fungal growth because it was near field capacity (-0.033MPa), and according to the
5 results in *in vitro* test, PI251 mycelia grow more at higher water potential.

6 Another putative explanation ~~of for~~ the lack of efficacy of ~~PI251 BioAct WG~~ in the
7 ~~plastic~~ greenhouse ~~trial conditions~~ could be the content of glucose in the formulation,
8 which inhibits the protease activity and consequently the capability to parasitize RKN
9 eggs.⁴⁹

10 The present ~~work tried to point out study~~ aimed to demonstrate the usefulness of
11 combining plant resistance with ~~biological control~~ BioAct WG to manage RKN.
12 However, PI251 was not able to parasitize eggs in ~~field greenhouse conditions~~ due to
13 suboptimal soil temperatures ~~during for~~ several days during the cropping periods and/or
14 inhibition of enzymes produced by the fungus by the components of the formulation
15 ~~and/or non-thermo-sensitive chemical factors in soil.~~ ~~Definitively~~Therefore, no
16 synergistic effect was observed. Resistant tomato suppressed nematode reproduction
17 and ~~yield losses of tomato as well as yield losses of the~~ following cucumber crop, but
18 nematode populations increased at the end of the crop. ~~The use of biological control~~
19 ~~agents able to suppress the buildup of RKN can be of interest to reduce the pressure on~~
20 ~~R-genes avoiding the selection of virulent populations and contribute to maintain~~
21 ~~nematode densities above below the economic threshold level.~~ *P. lilacinum* is found
22 naturally worldwide,⁴⁷ and it has been isolated from ~~cyst nematodes and~~ RKN in
23 Spain.³⁰ ~~*P. lilacinum* strain 251 PI251~~ was isolated from Philippines,⁴⁶ and despite its
24 effectiveness to parasitize eggs *in vitro* ~~test~~, well is known that native isolates ~~are~~ can be
25 more suitable and can performance better than the foreign ones ~~in field conditions.~~⁵⁰

1
2
3 1 More studies ~~must be done~~ are needed to optimize ~~P1251~~ BioAct WG usage and to
4
5 2 improve knowledge on optimal environmental conditions to improve its effectiveness.
6

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20 REFERENCES

- 21
22
23 10 REFERENCES
24
25 11 1 Anuario de estadística agraria. ed. by Ministerio de Agricultura, Alimentación y
26
27 12 Medio Ambiente, Madrid, España (2013).
28
29 13 2 Ornat C, Verdejo-Lucas S and Sorribas FJ, Effect of the previous crop on population
30
31 14 densities of *Meloidogyne javanica* ~~gene~~ and yield of cucumber. *Nematropica*
32
33 15 27:85-90 (1997).
34
35 16 3 Sikora R and Fernandez E, Nematode parasites of vegetables, in *Plant Parasitic*
36
37 17 *Nematodes in Subtropical and Tropical Agriculture*, eds. by Luc M, Sikora RA
38
39 18 and Bridge J, CAB International Wallingford, UK, pp. 319–392 (2005).
40
41 19 4 Sorribas FJ, Ornat C, Verdejo-Lucas S, Galeano, M and Valero J, Effectiveness and
42
43 20 profitability of the Mi-resistant tomatoes to control root-knot nematodes. *Eur J*
44
45 21 *Plant Pathol* 111(1):29-38 (2005).
46
47 22 5 Giné A, López-Gómez M, Vela MD, Ornat C, Talavera M, Verdejo-Lucas S and
48
49 23 Sorribas FJ Thermal requirements and population dynamics of root-knot
50
51 24 nematodes on cucumber and yield losses under protected cultivation. *Plant*
52
53 25 *Pathol* 63(6):1446-1453 (2014).
54
55
56
57
58
59
60

- 1
2
3 1 6 Talavera M, Sayadi S, Chiroso-Rios M, Salmeron T, Flor-Peregrin E and Verdejo-
4
5 2 Lucas S, Perception of the impact of root-knot nematode-induced diseases in
6
7 3 horticultural protected crops of south-eastern Spain. *Nematology* **14**(5):517–527
8
9 4 (2012).
- 10
11 5 7 Roberts PA, Concepts and consequences of resistance, in *Plant Resistance to*
12
13 6 *Parasitic Nematodes*, eds. by Starr JL, Cook R and Bridge J, CABI
14
15 7 International, Wallingford, pp. 23-41 (2002).
- 16
17 8 8 Dropkin VH, The necrotic reaction of tomatoes and other hosts resistant to
18
19 9 *Meloidogyne*: Reversal by temperature. *Phytopathology* **59**:1632-1637 (1969).
- 20
21 10 9 Cortada L, Sorribas F J, Ornat C, Andrés M F and Verdejo-Lucas S, Response of
22
23 11 tomato rootstocks carrying the *Mi*-resistance gene to populations of *Meloidogyne*
24
25 12 *arenaria*, *M. incognita* and *M. javanica*. *Eur J Plant Pathol* **124**(2):337-343
26
27 13 (2009).
- 28
29 14 10 Brown CR, Mojtahedi H, Santo GS and Williamson VM, Effect of the *Mi* gene in
30
31 15 tomato on reproductive factors of *Meloidogyne chitwoodi* and *M. hapla*, *J*
32
33 16 *Nematol* **29**(3):416-419(1997).
- 34
35 17 11 Kiewnick S, Dessimoz M and Franck L, Effects of the *Mi-I* and N root-knot
36
37 18 nematode-resistance gene on infection and reproduction of *Meloidogyne*
38
39 19 *enterolobii* on tomato and pepper cultivars. *J Nematol* **41**(2):134-139 (2009).
- 40
41 20 12 Silva RV, Oliviera RDL, Ferreira PS and Castro DB, Efeito do gene *Mi* na
42
43 21 reprodução de populações de *Meloidogyne exigua* em tomateiro. *Nematol Bras*
44
45 22 **32**:150-153 (2008).
- 46
47 23 13 Ornat C, Verdejo-Lucas S and Sorribas FJ, A population of *Meloidogyne javanica* in
48
49 24 Spain virulent to the *Mi* resistance gene in tomato. *Plant Dis* **85**:271-276 (2001).
- 50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 1 14 Verdejo-Lucas S, Cortada L, Sorribas FJ and Ornat C, Selection of virulent
4
5 2 populations of *Meloidogyne javanica* by repeated cultivation of *Mi* resistance
6
7 3 gene tomato rootstocks under field conditions. *Plant Pathol* **58**(5):990-998
8
9 4 (2009).
- 10
11 5 15 Talavera M, Verdejo-Lucas S, Ornat C, Torres J, Vela MD, Macias FJ, Cortada L,
12
13 6 Arias DJ, Valero J and Sorribas FJ, Crop rotations with *Mi* gene resistant and
14
15 7 susceptible tomato cultivars for management of root-knot nematodes in plastic-
16
17 8 houses. *Crop Prot* **28**(8):662–667 (2009).
- 18
19
20 9 16 Seinhorst JW, The relation between nematode density and damage to plants.
21
22 *Nematologica* **11**(1):137-154 (1965).
- 23
24
25 11 17 Sikora RA, Management of the antagonistic potential in agricultural ecosystems for
26
27 12 the biological control of plant parasitic nematodes. *Annu Rev Phytopathol*
28
29 13 **30**(1):245-270 (1992).
- 30
31
32 14 18 EUROPEAN UNION (2009). Regulation (EC) No 1107/2009 of the European
33
34 15 Parliament and of the Council of 21 October 2009 concerning the placing of
35
36 16 plant protection products on the market and repealing Council Directives
37
38 17 79/117/EEC and 91/414/EEC. *Official Journal of the European Union* L309, pp.
39
40 18 50.
- 41
42
43 19 19 Holland RJ, Williams KL and Khan A, Infection of *Meloidogyne javanica* by
44
45 20 *Paecilomyces lilacinus*. *Nematology* **1**(2):131-139 (1999).
- 46
47 21 20 Khan A, Williams KL and Nevalainen HK, Effects of *Paecilomyces lilacinus*
48
49 22 protease and chitinase on the eggshell structures and hatching of *Meloidogyne*
50
51 23 *javanica* juveniles. *Biol Control* **31**(3):346-352 (2004).
- 52
53
54
55
56
57
58
59
60

- 1
2
3 1 21 Khan A, Williams KL and Nevalainen HK, Infection of plant-parasitic nematodes by
4
5 2 *Paecilomyces lilacinus* and *Monacrosporium lysipagum*. *Biocontrol* **51**(5):659-
6
7 3 678 (2006).
8
9
10 4 22 Kiewnick S and Sikora RA, Biological control of the root-knot nematode
11
12 5 *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. *Biol Control*
13
14 6 **38**(2):179-187 (2006).
15
16 7 23 Kiewnick S and Sikora RA, Evaluation of *Paecilomyces lilacinus* strain 251 for the
17
18 8 biological control of the northern root-knot nematode *Meloidogyne hapla*
19
20 9 Chitwood. *Nematology* **8**(1):69-78(2006).
21
22
23 10 24 Kiewnick S, Neumann S, Sikora RA, and Frey JE, Effect of *Meloidogyne incognita*
24
25 11 inoculum density and application rate of *Paecilomyces lilacinus* strain 251 on
26
27 12 biocontrol efficacy and colonization of egg masses analyzed by real-time
28
29 13 quantitative PCR. *Phytopathology* **101** (1):105-112 (2011).
30
31
32 14 25 Anastasiadis IA, Giannakou IO, Prophetou-Athanasiadou DA and Gowen SR, The
33
34 15 combined effect of the application of a biocontrol agent *Paecilomyces lilacinus*,
35
36 16 with various practices for the control of root-knot nematodes. *Crop Prot*
37
38 17 **27**(3):352-361 (2008).
39
40
41 18 26 Kaşkavalci G, Tüzel Y, Dura O and Öztekin GB, Effects of alternative control
42
43 19 methods against *Meloidogyne incognita* in organic tomato production. *Ekoloji*
44
45 20 **18**(72):23-31 (2009).
46
47
48 21 27 Whitehead AG and Hemming JR, A comparison of some quantitative methods of
49
50 22 extracting small vermiform nematodes from soil. *Ann Appl Biol* **55**(1):25-38
51
52 23 (1965).
53
54 24 28 Zeck WM, A rating scheme for field evaluation of root-knot nematode infection.
55
56 25 *Pflanzenschutz-Nachrichten* **24**:141-144 (1971).
57
58
59
60

- 1
2
3 1 29 Hussey RS and Barker KR, A comparison of methods of collecting inocula of
4
5 2 *Meloidogyne* spp. including a new technique. *Plant Dis Rep* **57**:1025-1028
6
7 3 (1973).
8
9
10 4 30 Giné A, Bonmatí M, Sarro A, Stchiegel A, Valero J, Ornat C, Fernández C and
11
12 5 Sorribas FJ, Natural occurrence of fungal egg parasites of root-knot nematodes,
13
14 6 *Meloidogyne* spp. in organic and integrated vegetable production systems in
15
16 7 Spain. *BioControl* **58**(3):407-416 (2013).
17
18 8 31 Lopez-Llorca LV and Duncan JM, New media for the estimation of fungal infection
19
20 9 in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica*
21
22 10 **32**(4):486-489 (1986).
23
24 11 **32 Samson RA, Paecilomyces and some allied Hyphomycetes. Studies in Mycology,**
25
26 12 **No. 6. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. (1974).**
27
28
29 13 33 Castellá G, Bragulat MR and Cabañes FJ, Extracellular enzymatic activity of
30
31 14 *Fusarium* section *Liseola* isolates. *Mycopathologia* **144**(1):55-60 (1999).
32
33
34 15 34 Verdejo S, Jaffee BA and Mankau R, Reproduction of *Meloidogyne javanica* in plant
35
36 16 roots genetically transformed by *Agrobacterium rhizogenes*. *J Nematol*
37
38 17 **20**(4):599-604 (1988).
39
40 18 35 Mountain WB, A method of culturing plant parasitic nematodes under sterile
41
42 19 conditions. *Proc Helminthol Soc Wash* **22**(1):49-52 (1955).
43
44
45 20 36 Michel BE, Evaluation of the water potential of solutions of polyethylene glycol
46
47 21 8000 both in the absence and presence of other solutes. *Plant Physiol* **72**(1):66-
48
49 22 70 (1983).
50
51
52 23 37 Monfort E, Lopez-Llorca LV, Jansson HB and Salinas J, *In vitro* soil receptivity
53
54 24 assays to egg-parasitic nematophagous fungi. *Mycological Progress* **5**(1):18-23
55
56 25 (2006).
57
58
59
60

- 1
2
3 1 38 Ferris H, Roberts PA and Thomason IJ, Nematodes, in *Integrated Pest Management*
4 *for Tomatoes*, ed.; Statewide Integrated Pest Management Project; Division of
5 2 *Agriculture and Natural Resources*, University of California, pp 60–65. (1985).
6
7 3
8
9
10 4 39 Verdejo-Lucas S, Blanco M, Cortada L and Sorribas F J, Resistance of tomato
11 5 rootstocks to *Meloidogyne arenaria* and *Meloidogyne javanica* under
12 6 intermittent elevated soil temperatures above 28 °C. *Crop Prot* **46**:57-62 (2013).
13
14
15
16 7 40 Khan A, Williams KL and Nevalainen HK, Control of plant-parasitic nematodes by
17 8 *Paecilomyces lilacinus* and *Monacrosporium lysipagum* in pot trials. *Biocontrol*
18 9 **51**(5):643-658 (2006).
19
20
21
22
23 10 41 Kiewnick S, Effect of temperature on growth, germination, germ-tube extension and
24 11 survival of *Paecilomyces lilacinus* strain 251. *Biocontrol Sci Techn* **16**(5):535-
25 12 546 (2006).
26
27
28
29
30 13 42 Rumbos C, Mendoza A, Sikora R and Kiewnick S, Persistence of the
31 14 nematophagous fungus *Paecilomyces lilacinus* strain 251 in soil under controlled
32 15 conditions. *Biocontrol Sci Techn* **18**(10):1041-1050 (2008).
33
34
35
36 16 43 Bird AF and Bird J, The structure of nematodes. Academic Press, San Diego. (1991).
37
38
39 17 44 Holland RJ, Williams KL and Nevalainen KH, *Paecilomyces lilacinus* strain
40 18 Bioact251 is not a plant endophyte. *Australas Plant Path* **32**(4):473-478 (2003).
41
42
43 19 45 Rumbos CI and Kiewnick S, Effect of plant species on persistence of *Paecilomyces*
44 20 *lilacinus* strain 251 in soil and on root colonization by the fungus. *Plant Soil*
45 21 **283**(1-2):25-31 (2006).
46
47
48
49 22 46 Kok CH and Papert A, Microflora of *Meloidogyne* egg masses: species composition,
50 23 population density and effect on the biocontrol agent *Verticillium*
51 24 *chlamydosporium* (Goddard). *Nematology* **3**(8): 729-734 (2001).
52
53
54
55
56
57
58
59
60

- 1
2
3 1 47 Stirling GR. *Biological control of plant-parasitic nematodes: soil ecosystem*
4
5 2 *management in sustainable agriculture*. CAB International, Wallingford, UK
6
7 3 (2014).
8
9
10 4 48 Ploeg AT and Maris PC, Effects of temperature on the duration of the life cycle of a
11
12 5 *Meloidogyne incognita* population. *Nematology* **1**(4):389-393 (1999).
13
14 6 49 Bonants PJM, Flitters PFL, Thijs H, den Belder E, Waalwijk C and Henfling JWDM,
15
16 7 A basic serine protease from *Paecilomyces lilacinus* with biological activity
17
18 8 against *Meloidogyne hapla* eggs. *Microbiology* **141**(4):775-784 (1995).
19
20 9 50 Sorribas FJ, Ornat C, Galeano M and Verdejo-Lucas S, Evaluation of a native and
21
22 10 introduced isolate of *Pochonia chlamydosporia* against *Meloidogyne*
23
24 11 *javanica*. *Biocontrol Sci Techn* **13**(8): 707-714 (2003).
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
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1 Table 1. Cropping dates of the rotation sequence tomato-cucumber and soil
 2 temperatures in the greenhouse during two consecutive growing seasons.

Year	Crop	Cropping dates	Average soil temperature (°C) ^a	Minimum and maximum temperatures (°C) ^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

3 ^a Average soil temperature at 15cm depth: mean of daily mean temperatures during the
 4 cropping period.

5 ^b Absolute minimum or maximum soil temperature at 15cm depth during the cropping
 6 period.

7

1 Table 2. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the resistant tomato cv. Monika (TR) and susceptible cv. Durinta (TS) alone or combined with the application of
 3 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^e vs no BioAct		NS	NS	NS	NS		NS
TR vs TS		NS	S	S	S		S
T x BioAct		NS	NS	NS	NS		NS

4 Data are mean ± standard error of 10 replicates.

5 ^a BioAct WG (1 × 10¹⁰ 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: ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Galling index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

8 ^eS 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
 9 column indicate differences ($P < 0.05$) according to Tukey's test.

1 Table 3. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato
 3 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		

4 Data are mean ± standard error of 10 replicates.

5 ^a BioAct WG (1 x 10¹⁰ 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; ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Galling index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

8 ^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
 9 column indicate differences ($P < 0.05$) according to Tukey's test.

1 Table 4. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the susceptible cv. Durinta (TS) alone or combined with the application of BioAct WG, and the resistant tomato cv.
 3 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^c vs TS		NS	NS	NS	NS		NS
T x BioAct		NS	S	S	S		S

4 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
 5 with TS”.

6 ^a BioAct WG (1 x 10¹⁰ 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: ,
 7 in seedling before transplanting at 0.1g L⁻¹ rate.

8 ^b Gallings index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

9 ^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
 10 column indicate differences (P < 0.05) according to Tukey's test.

1 Table 5. Initial (Pi) and final (Pf) population densities of *Meloidogyne incognita* in soil, galling index, eggs per gram of root, percentage of fungal
 2 egg parasitism, and yield of the cucumber cv. Dasher II alone or combined with the application of BioAct WG cultivated after resistant tomato
 3 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

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

5 ^a BioAct WG (1 x 10¹⁰ 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: ,
 6 in seedling before transplanting at 0.1g L⁻¹ rate.

7 ^b Galling index based on the Zeck⁴⁵ scale; from 0 (healthy plants) to 10 (dead plants).

8 ~~^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
 9 column indicate differences ($P < 0.05$) according to Tukey's test.

10 Na: no available

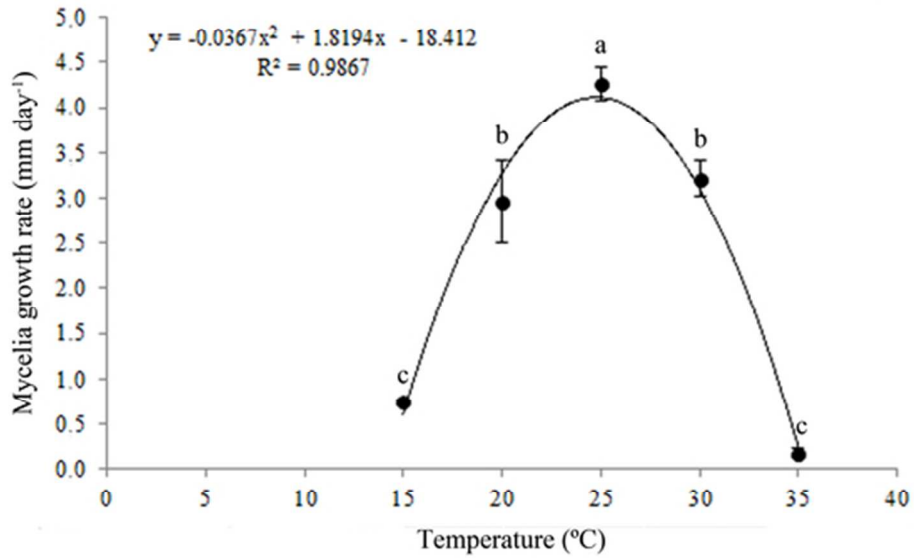


Figure 1. Mycelia growth rate (mm day⁻¹) of *Purpureocillium lilacinum* strain 251 in water agar (12 g L⁻¹) at 15, 20, 25, 30 and 35°C. Bars represent the standard error (n=6) Different letters indicate differences at $P < 0.05$ according to LSD's test.

Figure 1

25x16mm (600 x 600 DPI)

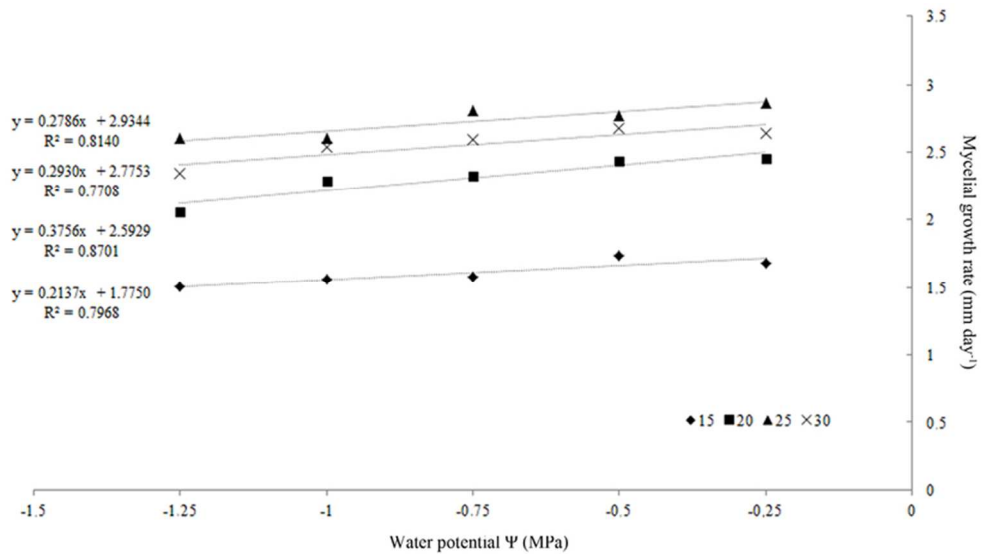


Figure 2. Mycelial growth rate (mm dia⁻¹) of *Purpureocillium lilacinum* strain 251 at 15, 20, 25, 30 °C and water potentials from -1,25 to -0,25 MPa.

Figura 2
35x19mm (600 x 600 DPI)