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ORIGINAL PAPER

In vitro compatibility of *Brassicaceae* extracts with nematophagous fungi and their effects against *Nacobbus celatus*

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Abstract

Nacobbus celatus sp. n. is one of the main root-knot nematodes in the field destined for horticultural production of the central region of Argentine due to its ability to infect several host plants. The lack of new and safe active ingredients against this nematode has restricted control alternatives for growers. Egg-parasitic fungi and biofumigation with brassicaceae have been considered as potential candidates for the development of bionematicides. Nematicidal effects of *Brassica oleracea* var. *italica* (broccoli) and *Brassica oleracea* var. *capitata* (cabbage) aqueous extracts (AEs) against second-stage juveniles (J2) of *N. celatus* were evaluated in vitro. Fisher LSD tests evidenced significant nematicidal ($\alpha = 0.05$) effects of the two AEs tested, with LD_{100} of 250 and 500 µL mL^{-1} for broccoli and cabbage, respectively. Compatibility assays between AEs and five nematophagous fungi were performed on soil extract medium conditioned at 0.99 water activity and incubated at 30, 25 and 20 °C. *Purpureocillium lilacinum* SR14 was the fungal strain that showed compatibility at levels of spore viability, growth rate and conidia productions at LD_{50} (125 µL mL^{-1}) and LD_{25} (60 µL mL^{-1}) of broccoli aqueous extract (BAE) and enhanced the nematophagous effect. Moreover, phytotoxic studies revealed that 125 µL mL^{-1} of BAE applied at the transplantation time could be safely used without affecting tomato culture. In conclusion, the integrated application of BAE with *P. lilacinum* SR14, which combines two action mechanisms, represents a promising integrated strategy to management phytoparasitic nematodes.

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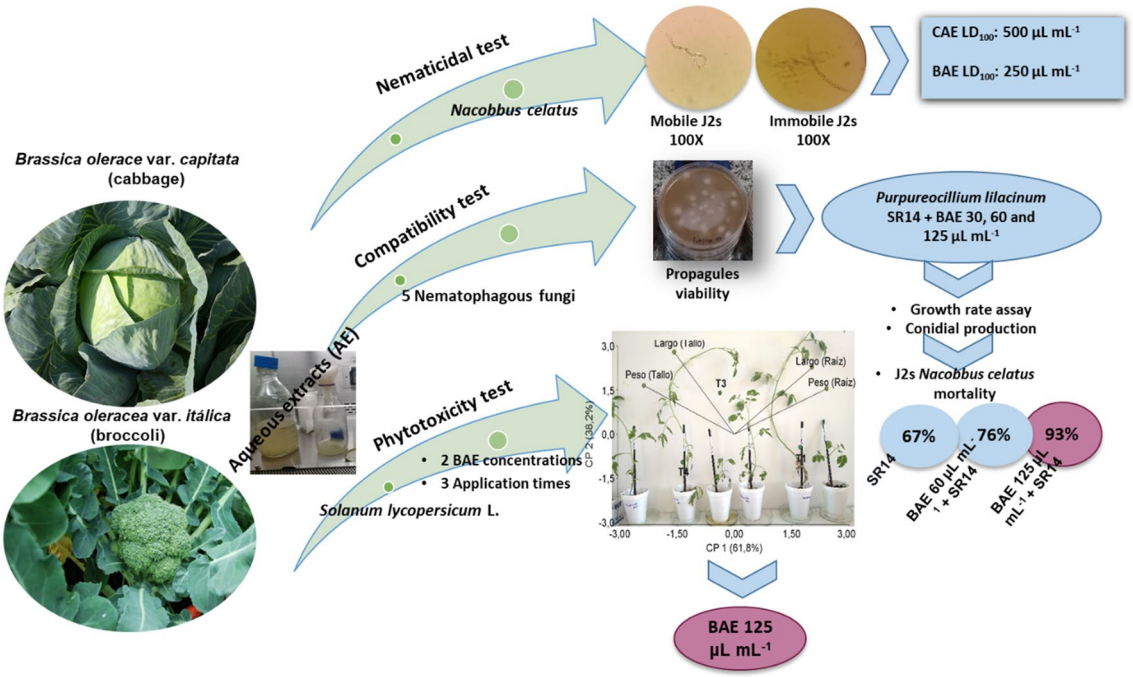
Graphical abstract



Keyword Plant-parasitic nematodes · Biological control · Botanical extracts · Horticultural agrosystem

Abbreviations

AEs	Aqueous extracts
BAE	Broccoli aqueous extract
CAE	Cabbage aqueous extract
NF	Nematophagous fungi
LD	Lethal doses
t	Time
a_W	Water activity
T	Temperature
SEM	Soil-extract medium
PPN	Plant-parasitic nematodes
Purpureocillium lilacinum	SR7, SR14, SR38
Metarhizium robertsii	SR51
Plectosphaerella plurivora	SRA14

Introduction

Agricultural damage caused by plant-parasitic nematodes (PPN) is estimated to be 80 billion dollars annually worldwide (Degenkolb and Vilcinskas 2016; Khan et al. 2020). The genus *Nacobbus*, the false root-knot nematode, is endemic to Argentina, Bolivia, Chile, Ecuador, México, Peru, and the USA (EPPO, 2020). *Nacobbus aberrans* s.l. (Thorne 1935) Thorne and Allen (1944) is an agricultural pest of quarantine importance (Singh et al. 2013) and has been listed as one of the top 10 most relevant nematodes worldwide in plant pathology (Jones et al. 2013). This is a polyphagous pest, with 18 botanical families and 93 species as hosts known so far (Jeger et al. 2018, Manzanillas-Lopez 2010). Costilla et al. (1977) were the first that reported the presence of *Nacobbus celatus* sp. n. (Lax et al. 2021), previously named *Nacobbus aberrans*, in Argentina in potato crops at 2000 m above sea level. In our region, *N. celatus* constitutes one of the recurrent biotic adversities in the crops both at the field and undercover of the Riocuartense Horticultural Belt (Doucet 1989; Doucet and Lax 2005; Lax et al. 2011; Sosa et al. 2018).

Numerous strategies to effectively reach pests management have been recently reported (Cortez Hernandez et al. 2019; Prajapati et al. 2020; Sosa et al. 2020; Vázquez-Sánchez et al., 2018), however, chemical control is the principal practice adopted by producers in Argentina (Tierra Viva 2021). In this country, the only pesticide registered for the chemical control of soil pests in vegetables is chlorpyrifos (Cichón et al. 2017). This chemical product is a broad-spectrum, chlorinated organophosphate insecticide, acaricide and nematicide. Chlorpyrifos affects the nervous system by inhibiting the breakdown of acetylcholine, a neurotransmitter, whose use is restricted. Moreover, fumigation with methyl bromide (MB) was a widespread practice adopted by Argentinean horticulturists (Parrilla 2018), even

though, Argentina adhered in 2006 to the Montreal Protocol that prohibits the use of MB (SENASA 2006; MBTOC 2010; Parrilla, 2018). Different alternatives to control PPN were evaluated due to the growing concern about the effects of chemical pesticide applications on the environment and human health (Akhtar and Siddiqu 2008).

Among the practices of low environmental impact used for endoparasitic nematodes management are the exploitation of biological agents (Mondino et al. 2019) and the application of botanical extracts (Sosa et al. 2020). Considering the first alternative, in a previous study carried out in our laboratory, 66 fungal strains isolated from rhizospheric and no rhizospheric soils of chard and beet were tested for their nematophagous potentiality (Sosa et al. 2018). This study allowed us to select five fungal strains that showed high infection percentages in the order of 80 and 63% for J1s and J2s, respectively. On the other hand, in recent years, biofumigation has emerged as an effective nonchemical alternative, the suppression of soilborne pests and pathogens, including PPN, by incorporation of brassica green manures into various cropping systems (Rahman and Somers 2005; Lazzeri et al. 2009). The combined application of aqueous extracts of *Brassicaceae* compatible with biological control agents is another complementary option for management of the phytoparasitic nematode, *N. celatus*.

Ecophysiological factors such as temperature and water potential primarily influence fungal colonization and enzymatic production (Vieira do Santos et al. 2012). Slight variations in microclimatic conditions have a significant effect on biocontrol activity (Hannusch and Boland 1996). Hence, it is important to know if the stress caused by environmental factors could affect the compatibility between the NF and the botanical extract under study.

Therefore, the following objectives are proposed: (i) to determine through in vitro studies the nematicidal activity of the AEs of *Brassica oleracea* var. *italica* (broccoli) and *Brassica oleracea* var. *capitata* (cabbage) on the infective stage J2 of *N. celatus*; (ii) to evaluate the compatibility of NF with AEs at different water activities (a_w) and temperatures; (iii) to elucidate the combined impact of AEs and NF on J2 of *N. celatus*; and (iv) to determine the phytotoxic effects of AEs on tomato seedlings.

Materials and methods

Nematode population

The population of *N. celatus* sp. n. (MH000315), isolated from a horticultural field of Río Cuarto, Córdoba, Argentina (Sosa et al. 2018) was kept in tomato plants (*Solanum lycopersicum* L.) of cv. Valouro under greenhouse conditions. Egg masses were directly extracted from infected root galls and disinfected using 1% sodium hypochlorite (NaClO). The separated eggs were gently washed with sterile water to remove the NaClO (Cortez-Hernández et al. 2019). Egg masses were kept in distilled water in the dark at 25 °C. After 48 h, mobile J2 were recovered to conduct the nematicidal assays.

Nematophagous fungi

Five strains [*Purpureocillium lilacinum* (SR7, SR14, and SR38), *Metarhizium robertsii* (SR51), and *Plectosphaerella plurivora* (SRA14)] were used in these experiments. These fungi were isolated from rhizospheric soil samples of chard (*B. vulgaris* L. var. *cicla*), and beet (*B. vulgaris* var. *conditiva*) collected from a horticultural region located in Río Cuarto, Córdoba, Argentina, and were identified and deposited in GenBank with the following accession number: MF996811, MF996818, MF996813, MF996819, and MF996823, respectively. All strains showed virulence against J1 and J2 of *N. celatus* (Sosa et al. 2018). These strains are stored in the Microbiology and Immunology Department Collection of the National University of Río Cuarto, Córdoba (Argentina). Fungal strains were cultivated on potato dextrose agar for 7 days at 25 °C to obtain strongly sporulated cultures to perform the compatibility and biological control assays.

Vegetable extracts

The AEs were obtained following the methodology proposed by Curimilma Campos (2015) with modifications. Fresh leaf samples of cabbage and broccoli were collected. A portion of 100 g was crushed in a blender with 150 mL of sterile distilled water for 30 s. The mixed solution was filtered through a wire mesh screen, allowing the solid residue to be removed, which provided the emulsified filtrate that was then heated at 60 °C for 20 min. The extract was filtered through a Whatman No. 4 filter paper and centrifuged at 10,000 rpm for 10 min. Finally, the supernatant was recovered and sterilized by filtration (0.2 µm). The broccoli and cabbage extract solutions were considered as standard (100%) and different aqueous dilutions were obtained.

Nematicidal assay in vitro

Twenty microlitres of water containing 20 J2 of *N. celatus* were placed into vials with 980 µL of the AEs solutions. The doses assayed for BAE and CAE were 1000, 500, 250, 200, 170, 120, and 60 µL mL^{-1}. A control treatment with sterile distilled water was performed. The vials were incubated for 24 h at 25 °C. The number of immobile J2 was determined 2, 4, and 24 h after the treatment. Then, the immobile J2 were transferred to vials with sterile distilled water for 24 h

to assess whether they regained mobility. Eight replicates were performed for each treatment and the test was repeated. In order to determine the LD_{50} for the AEs, the results of J2 mortality percentages were analyzed in relation to the different doses tested at the final exposure time (24 h).

Compatibility assays

The compatibility of the five fungal strains with the BAE and CAE was evaluated following the methodology described by Barra et al. (2013). The fungal count was determined using decimal dilutions from a spore suspension of 10^7 spores mL^{-1} in 0.1% peptone water. An aliquot of 0.1 mL of 10^{-5} and 10^{-6} dilutions was spread on the soil extract medium (SEM) (James, 1958), conditioned to 0.99 a_W by adding glycerol (2.76 g/100 mL) (Dallyn and Fox 1980) and enriched with AEs. Different doses of AEs (900, 500, 250, 200, 170, 125, 60, and 30 µL mL^{-1} for broccoli and 900, 500, 250, 125, 60, and 30 µL mL^{-1} for cabbage) were added to the SEM after cooling to 50 °C, homogenized, and poured into sterile Petri dishes. The equivalent amount of sterile distilled water was added to the control treatments. Petri dishes were sealed in polyethylene bags and incubated at 25 °C for 15 days. The viability of the fungal propagules (CFU mL^{-1}) was determined in each treatment. The data collected were compared with controls to determine the effect of the AEs on propagules viability. The treatments were grouped as compatible or non-compatible (Ganga Visalakshy et al. 2006).

Growth rate assay

This assay was performed with the higher compatible doses of BAE (LD_{50} and LD_{25}) and *P. lilacinum* SR14 isolated. SEM plates (0.99 aw) containing BAE (125 and 60 µL mL^{-1}) were inoculated with a drop (5 µL) of *P. lilacinum* SR14 conidial suspension (10^6 spores mL^{-1}). Culture medium without AEs was used as a control. Petri plates were sealed in polyethylene bags to maintain the a_W value and incubated for 15 days at 20, 25 and 30 °C. The tests were carried out in triplicate. The fungal growth was examined daily and the growth rate was calculated (Passone et al. 2005).

Conidial production

To assess conidial production, fungal colonies were harvested from growth rate assay plates treated with BAE at 25 °C. The fungal mycelium was harvested by adding 10 mL of water with Tween 20 (0.05%), and the obtained material was transferred to sterile test tubes. The suspensions were vigorously shaken. Subsequently, two aliquots of each suspension were taken, and the spore count was performed in the Neubauer chamber under optical microscopy (400×). The count results allowed estimating the mean concentration of the spores produced by *P. lilacinum* SR14 in each treatment.

Inhibitory activity of BAE and *P. lilacinum* SR14 against *N. celatus*

Petri dishes containing SEM enriched with BAE (125 and 60 µL mL^{-1}) were inoculated with 5 µL of *P. lilacinum* SR14 conidial suspension (10^6 spores mL^{-1}) at the centre of the plate and incubated at 25 °C for 7 days (colony diameter approximately 2 cm). Then, 50 J2 were inoculated near the edge of the fungal colony. The plates were incubated at 25 °C for 7 days. The treatments evaluated were: *P. lilacinum* SR14, BAE, and *P. lilacinum* SR14+BAE. Plates with SEM without treatments and inoculated with J2 were used as control. Parasitized J2 were determined by microscopy (Peraza Padilla et al. 2014). The test was carried out in quadruplicate and repeated in time.

Phytotoxic effect of BAE on tomato plants

To evaluate the effect of the extract on tomato plants, the methodology proposed by Mendez Navarrete (2019) was followed with some modifications. A volume of 6 and 12.5 mL of the BAE stock solution was added to a sterile substrate (100 g). The treatment was applied at different times 0, 1, 3 and 7 days before transplantation. Three-week-old tomato seedlings were transplanted into the pots containing the treated substrate. The equivalent of water was applied in the control pots. All treatments were incubated in a chamber conditioned at 25 °C; 80% RH, photoperiod light cycle 16 h: 8 h (light: dark) for 30 days and irrigated daily. The test was repeated in time with four replicates for each treatment. After the incubation time, the following variables were evaluated: length and weight of root and stem and the number of stained leaves.

Data analysis

Statistical analyses were performed through the program InfoStat version 2017. InfoStat Group, FCA, National University of Cordoba, Argentina. http://www.infostat.com.ar URL (Di Rienzo et al. 2012). Means data of fungal count, fungal growth, and parasitized larvae were determined by the analyses of variance (ANOVA). Fisher's Least Significant Difference test (LSD) ($\alpha = 0.05$) was performed to establish significant differences between control and treatments. Lethal doses (LD_{50}) of AEs on *N. celatus* J2 were calculated by Probit Analysis using the Statgraphics® Centurion version XVII program (Manugistics, Inc., Maryland, USA).

Results

Nematicidal effects

Broccoli

The effect of BAE on *N. celatus* J2 was determined by testing seven different concentrations (60–1000 µL mL^{-1}). The ANOVA test showed that both exposure time (T) ($F = 16.37$; $p < 0.0001$) and BAE concentrations (C) ($F = 234.74$; $p < 0.0001$) and their interactions (T × C) ($F = 2.27$; $p = 0.0067$) significantly affected the viability of *N. celatus* J2. All concentrations of the BAE tested significantly reduced the larvae viability compared to the control ($\alpha < 0.05$) (Table 1). Although, it is noteworthy that the nematicidal effect of BAE was dependent on the dose tested and the exposure time. The highest doses (500–1000 µL mL^{-1}) showed the highest nematicidal effect, with J2 mortality of 100% after 2 h of exposure. Low mortality rates (7.8–26%) were observed 2 h after larvae exposition at the lowest BAE concentrations (60–130 µL mL^{-1}). Moreover, the viability of the larvae decreased as the exposure time increased, observing mortality rates between 18.5 and 50% at the above concentrations. The mobility of J2 in presence of water (control) was maintained until the end of the exposure period (24 h).

Table 1 Nematicidal effect of BAE and CAE on *N. celatus* J2 evaluated at 2, 4 and 24 h

Aqueous extracts	Concentrations (µL mL^{-1})	Time of exposition		
		2	4	24
Broccoli	0	0^{I}	0^{I}	0^{I}
	60	7.8^{HI}	13.8^{GH}	18.5^{GH}
	120	26.0^{G}	40.7^{F}	50.3^{EF}
	170	53.9^{DE}	65.0^{CD}	80.8^{BC}
	200	46.5^{EF}	81.1^{BC}	90.1^{AB}
	250	89.4^{AB}	91.1^{AB}	99.4^{A}
	500	100^{A}	100^{A}	100^{A}
	1000	100^{A}	100^{A}	100^{A}
Cabbage	0	0^{H}	0^{H}	0^{H}
	60	4.7^{GH}	14.0^{EFGH}	60.8^{BC}
	120	12.2^{FGH}	24.7^{EF}	51.2^{CD}
	200	17.9^{EFG}	30.6^{DE}	58.3^{BC}
	500	22.6^{EFG}	51.9^{CD}	99^{A}
	1000	46.3^{CD}	72.6^{B}	98.4^{A}

The data represent the average percentage of dead J2 for each concentration. Different letters for each AE, at each time, indicate significant differences concerning the control according to the LSD Fisher test ($\alpha = 0.05$)

Probit analysis revealed that BEA was highly toxic on *N. celatus* J2 with a LD_{50} value of 125 µL mL^{-1} at 24 h of exposure.

Cabbage

The nematicidal effect of cabbage aqueous extract (CAE) was determined by testing a total of five different concentrations (60–1000 µL mL^{-1}). The ANOVA test showed that both the variables T ($F = 57.31$; $p < 0.0001$), C ($F = 47.82$; $p < 0.0001$), as well as their interaction T × C ($F = 4.42$; $p < 0.0001$) significantly affected the viability of *N. celatus* J2.

Considering the total of CAE concentrations (60–1000 µL mL^{-1}) evaluated, the nematicidal effect after 24 h of exposure ranged between 51.3 and 100% (Table 1). Moreover, the nematicidal capacity of the five concentrations evaluated were statistically significant ($\alpha < 0.05$) after 2 h of exposure, where the mortality percentages ranged between 4.7 and 46.3%. Only the highest concentrations tested (500–1000 µL mL^{-1}) were able to immobilize almost all of the larvae after 24 h of exposure, with percentages of 99.4 and 98.4%, respectively. It was also observed that the effectiveness of the lowest doses tested (60–200 µL mL^{-1}) increased significantly ($\alpha < 0.05$) as the exposure time of J2 to CAE was extended. In the control samples (water), the mobility of the J2 was maintained until the end of the exposure period (24 h).

Probit analysis revealed that the CAE was highly toxic on *N. celatus* J2 with a LD_{50} value of 110 µL mL^{-1} at 24 h of exposure.

Compatibility assays

Effect on the viability of fungal propagules

Broccoli

According to the ANOVA test the concentration of BAE significantly affected ($F = 544.38$; $p < 0.0001$) the viability of NF propagules. The count levels of the five NF in the control cultures (mean count: 8.4×10^6 cfu mL^{-1}) were similar to the counts obtained in the treatments that contained the lowest doses of BAE (mean counts at 30 µL $mL^{-1} = 7.9 \times 10^6$ cfu mL^{-1}; 60 µL $mL^{-1} = 1.4 \times 10^6$ cfu mL^{-1}; 125 µL $mL^{-1} = 7.0 \times 10^6$ cfu mL^{-1}) (Fig. 1). Meanwhile, statistically significant reductions were observed at the remaining concentrations. *Metarhizium robertsii* SR51 was the only strain able to grow in the presence of the highest concentrations of the BAE (170 and 200 µL mL^{-1}), although significant reductions ($\alpha < 0.05$) in the number of propagules were estimated in the order of 11 and 28%, respectively.

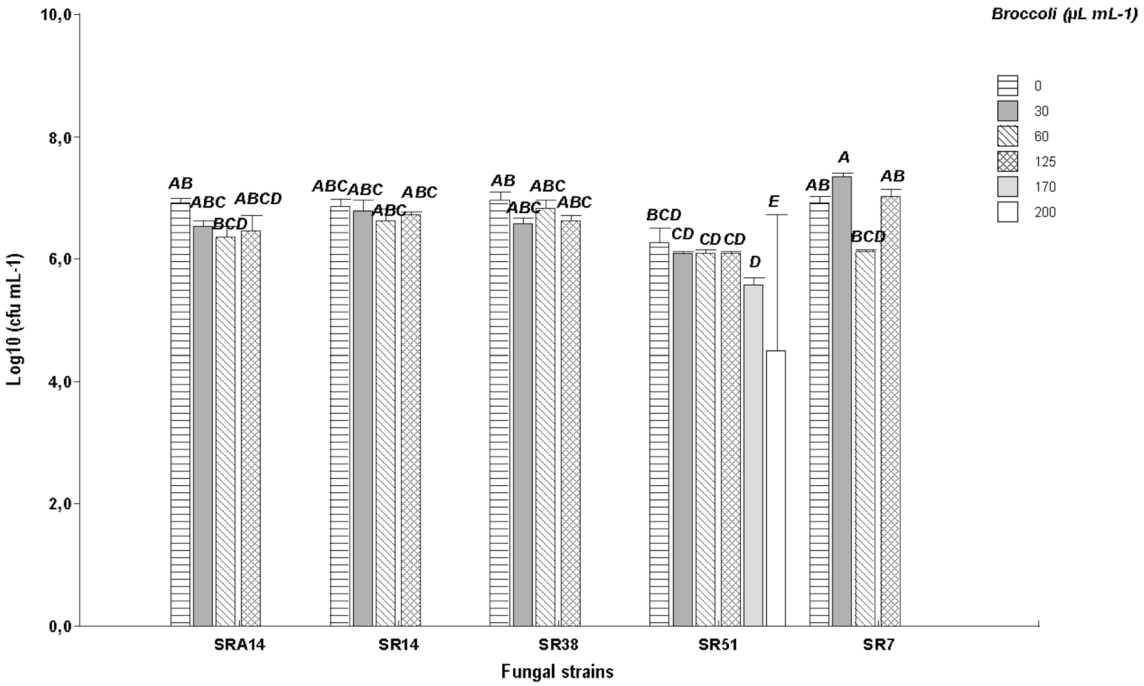


Fig. 1 Effect of BAE concentrations on the viability of the spores of five fungal strains on SEM conditioned at 0.99 a_W and incubated at 25 °C, after 25 days of incubation. The bars represent the means and the standard error of the different treatments. Different letters on each bar indicate significant differences between treatments and controls based on the LSD Fisher test ($\alpha < 0.05$)

It is important to highlight that *P. lilacinum* SR14 demonstrated compatibility with the three lowest BAE concentrations (30, 60 and 125 µL mL^{-1}), with a mean count of 6.5×10^6 cfu mL^{-1}. Moreover, the fungal development of *P. lilacinum* SR7 increased above 6% in presence of the lowest concentration of BEA. Meanwhile, no significant difference with respect to the control was observed when this fungus grew in contact with 125 µL mL^{-1} of AE. Therefore it was also shown to be compatible with the botanical extract under study. The higher doses 250, 500 and 900 µL mL^{-1} completely inhibited the development of the five NF.

Cabbage

The results regarding the effect of different doses of CAE revealed that no fungal compatibility with any of the fungal strains was observed at any of the six concentrations evaluated. The results were evaluated after 15 and 25 days of incubation, confirming a fungicidal effect, and not a delay in fungal development (fungistatic).

Effect on fungal growth rate

When the results obtained were analyzed, the ANOVA test showed that both the different concentrations of the BAE (60 and 125 µL mL^{-1}) ($F = 3.51$; $p = 0.0422$) and the temperature variations (20, 25 and 30 °C) ($F = 87.50$; $p < 0.0001$) significantly influenced the growth rate of *P. lilacinum* SR14. Reductions of the order of 25 and 43% were observed at 20 and 30 °C, respectively, being 25 °C the optimum temperature for its development (Table 2). Also, fungal development was significantly affected ($F = 4.68$; $p = 0.0045$)

Table 2 Effect of different BAE concentrations and temperatures on the growth rate and conidial production of *P. lilacinum* SR14

Concentrations (µL mL^{-1})	Growth rate of *P. lilacinum* SR14			Production of conidia (conidia mL^{-1})
	Temperature (°C)			
	20	25	30	
0	0.09 ± 0.01^{D}	0.13 ± 0.01^{AB}	0.12 ± 0.01^{BC}	$5.1 \times 10^7 \pm 3.8 \times 10^{6A}$
60	0.09 ± 0.01^{D}	0.14 ± 0.01^{A}	0.12 ± 0.01^{BC}	$3.4 \times 10^7 \pm 4.3 \times 10^{6B}$
125	0.06 ± 0.01^{E}	0.14 ± 0.004^{A}	0.11 ± 0.01^{C}	$1.1 \times 10^6 \pm 3.8 \times 10^{6C}$

The different letters for each EA concentration, at each temperature, indicate significant differences in the parameter evaluated according to the LSD Fisher test ($\alpha = 0.05$)

by the interaction of the understudy variables (BAE concentration × temperature). The optimal conditions for the development of SR14 were at 25 °C and when the SEM was enriched with BAE. Under these conditions, stimulation of fungal growth (8%) was registered concerning the control.

Effect on the production of fungal conidia

The ANOVA test showed that the applied treatments (BAE 60 and 125 µL mL^{-1}) significantly affected ($F = 42.41$; $p < 0.0001$) the production of conidia by *P. lilacinum* SR14 at 25 °C. The supplementation of AES with BAE increased the production of conidia concerning the control treatment. When the fungus developed in the presence of 60 and 125 µL mL^{-1}, the increase of conidial production was estimated in the order of 25 and 28%, respectively (Table 2).

In vitro biocontrol assay

According to the MLGM test with a binomial distribution, the viability of *N. celatus* J2 was significantly affected ($F = 6.46$; $p = 0.0064$) by the treatments evaluated. Infection capacity on J2 was variable, the highest proportion (0.93) was recorded in the treatment with the combined application of BAE 125 µL mL^{-1}+SR14. However, it is noteworthy that the nematophagous effect was lower in the presence of SR14 and BAE 60 µL L^{-1}+SR14, being the proportion of infected J2 around 0.67 and 0.76, respectively. Therefore, the results showed that *P. lilacinum* SR14 maintained and incremented its infective capacity on J2 in presence of BAE.

Phytotoxicity test

The results of the variance analysis according to the MLMix showed that the concentration (60 and 125 µL mL^{-1}) and the application time before transplantation (0, 1, 3 and 7 days) of the BAE did not significantly influence the vigour variables analyzed as shown in the table (Online Resource 1), so the application time was considered as covariance in the MLMix. The tomato plants developed in the presence of the highest concentration of BAE (125 µL mL^{-1}) showed slight increases in the weight and length of the aerial part of the order of 12 and 8%, respectively, while no differences were observed in the root parameters. However, at the lowest concentration of BAE, slight reductions in the vigour characteristics were recorded, estimated at 12 and 13% for the aerial and root parameters, respectively (Online resource 2).

According to the analysis of variance by the MLGM, both the concentration ($F = 168.8$; $p = 0.0003$) and the time of application ($F = 157.8$; $p = 0.0125$) of the BAE significantly affected the chlorosis of leaf tissue in tomato plants.

The highest proportion of chlorotic leaves (0.20) was recorded when the substrate was treated with the highest dose of BAE (125 µL mL^{-1}), 1 and 3 days before transplantation with statistically significant differences ($\alpha < 0.05$) compared to the control (Fig. 2). However, the above treatment did not have a negative impact when it was applied at the same time of transplantation (0 day), with the percentage of chlorotic leaves similar to the control, around 10%. Similarly, the scope of foliar chlorosis in the plants treated with the lowest dose of BAE (60 µL mL^{-1}) was comparable with the control, regardless of the application time.

When the vigour and chlorosis results were descriptively analyzed by the Principal Component test, the statistical analysis showed that the sum of two main components (CP1 and CP2) explained 79% of the total variability of the data (Fig. 3). The six evaluated variables (length and weight of root and stem, number and chlorotic leaves) contributed significantly to the construction of CP1. Three different groups resulted in the biplot: (i) a group composed by plants

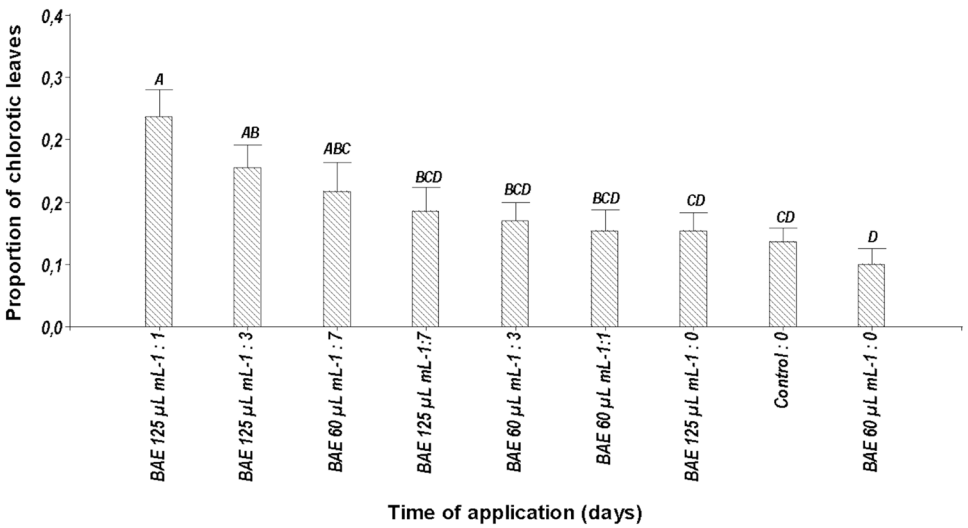


Fig. 2 Effect of BAE concentrations (60 and 125 µL mL^{-1}) at different application times (0, 1, 3 and 7 days before transplantation) on the proportion of chlorotic leaves of tomato plants. Different letters on each bar indicate significant differences in the evaluated parameter according to the LSD Fisher test ($\alpha = 0.05$)

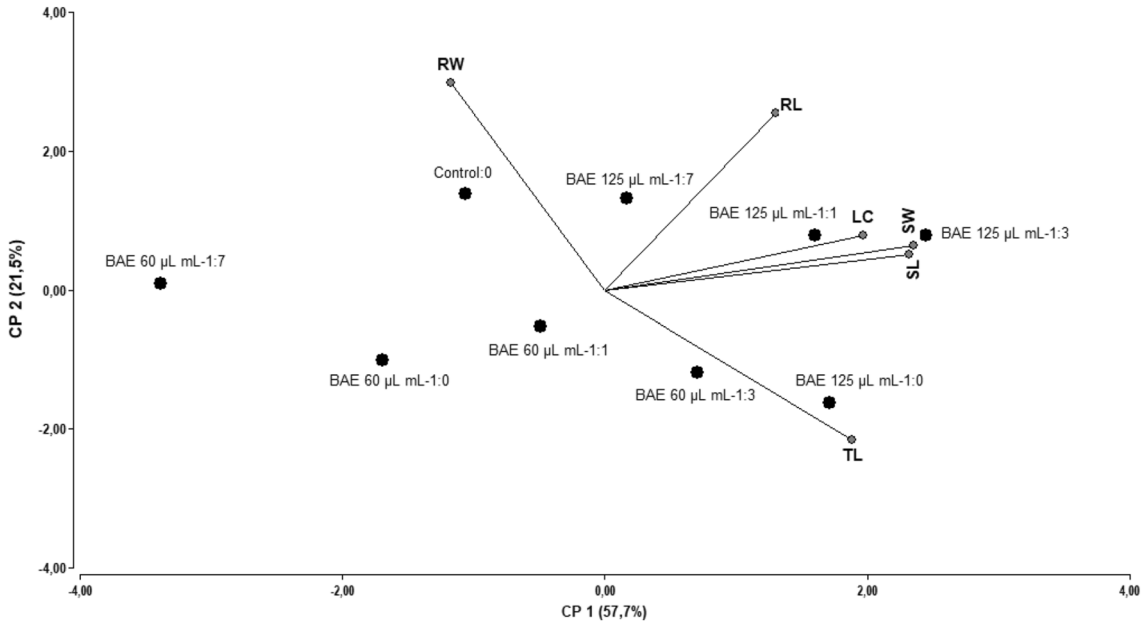


Fig. 3 Analysis of main components of different combinations of BAE concentrations (60 and 125 µL mL^{-1}) at interval times (0, 1, 3 and 7 days before transplantation) on the vigour and chlorosis of the tomato plants. Variables analyzed: *SW* stem weight, *SL* stem length, *RW* root weight, *RL* root length, *TL* total leaves, and *LC* leaf chlorosis

treated with 60 µL mL^{-1} applied 7 days before transplantation, characterized by low vigour plants, this group showed a significant reduction (angle between vectors greater than 90°) of the understudy variables. (ii) Another group formed by the plants treated with 60 µL mL^{-1} applied 0, 1 and 3 days before transplantation and with 125 µL mL^{-1} applied 7 days previous, which presented similar characteristics to the control plants; and (iii) a third group conformed by the plants treated with 125 µL mL^{-1} applied 0, 1 and 3 days before transplantation that presented vigour characteristics higher to the control plants. In addition, it is noteworthy that the plants in pots treated with 125 µL L^{-1} of BAE applied at the time of transplantation presented the highest number of leaves and the lower proportion of chlorotic leaves.

Discussion

Brassicaceae have a sharp and potent flavour, attributable to their principal metabolites, glucosinolates (GLS), which contains sulfur (Mithen et al. 2010; Björkman et al. 2011). In addition to beneficial health properties, GLS and their hydrolysis products, among which stand out, isothiocyanates, sulfurans, nitriles, and thiocyanates, also include a broad spectrum of biocidal activities such as insecticides, fungicides, nematicides, and phytotoxic effects (Björkman et al. 2011; Ntalli and Menkissoglu-Spiroudi 2011; Ntalli and Caboni 2012). In the present study, the first results of the nematicidal effect of the AEs of *Brassica oleracea* var. *capitata* (cabbage) and *Brassica oleracea* var. *italica* (broccoli) on *N. celatus* J2, showing high nematicidal activity in vitro with LD_{50} of 110 and 125 µL mL^{-1}, respectively. It could also establish that all doses tested of AEs decreased the mobility of the *N. celatus* J2 concerning the controls, being even more effective in the higher doses and with a longer exposure time. Mortality could be confirmed when specimens were transferred to vials containing sterile water, and they did not regain mobility. In an *in-plant* study conducted by Garita (2019), finely cut cabbage heads were incorporated into pots containing tomato plants and inoculated with 5000 eggs and larvae of *N. aberrans*. The reproduction factor values were 12.8, 7.09 and 2.49 for the control without biofumigation and the treatments with 140 and 280 g kg^{-1} of cabbage, respectively. The authors observed the same statistical differences in the number of eggs according to Fisher's LSD test ($p < 0.01$). In general, fumigant nematicides are liquid formulations with the characteristic that they can vaporize in contact with the air (Galbieri and Belot 2016). With the detachment of their molecules in the vapour phase, they move in the soil typically in-depth. When these compounds are exposed to the water available in the ground, they decompose into products that penetrate directly into the cuticle of the nematode, reacting rapidly with amino acids, oxidases, and proteins, causing metabolic dysfunctions (Galbieri and Belot 2016). Broad-spectrum fumigant nematicides penetrate directly into the body wall of the nematode and do not need to ingest to be effective. Once

inside the cavity of the nematode body, they affect different internal organs (Nowling 1997). Isocyanate acts in various ways in the cell, the main ones being the inhibition of electron transport in the respiratory chain, enzymatic inactivation, and signalling for the induction of cell apoptosis (Aguiar 2012). The mechanism of the nematicides belonging to the halogenated aliphatic compounds, like MB, are similar to the isocyanates, serving as alkylating agents of proteins and, also, oxidizing the Fe^{2+} centres in the cytochrome, blocking respiration (Chitwood 2002).

The fact that brassicas are susceptible to *N. celatus* indicates that the sole presence of this type of compound is not what exerts control but rather that other mechanisms and interactions are involved (Winde and Wittstock 2011). Therefore, to evaluate a combined control strategy involving different mechanisms of action on the phytoparasitic nematode, *N. celatus*, several compatibility tests between AEs and NFs were carried out. For this, in vitro effect of AEs on the spore viability of the five fungal strains with nematophagous activity was determined. The result recorded in this investigation demonstrated that the highest concentrations of BAE (250, 500 and 1000 µL mL^{-1}) completely inhibited fungal development. All strains were able to grow when exposed to the lower doses (30, 60 and 125 µL mL^{-1}) of BAE, but only *P. lilacinum* SR14 and SR7 were able to present statistically similar counts to those obtained in the control treatment, so they turned out to be compatible with the botanical agent. While the results obtained in the test with CAE demonstrated incompatibility with all fungal strains at all the concentrations evaluated. Similar results were obtained by Smolinska et al. (2003), reported incompatibilities between the incorporation of brassicas with *Fusarium oxysporum*. Meanwhile, another study carried out by Pérez-Rodríguez et al. (2011) evaluated the combined effect of cabbage fragments+*Pochonia chlamydosporia* for controlling *N. aberrans* in the *Capsicum annuum* L. crop. In addition, the authors determined the levels of the biological control agent in root and soil 50 days after transplantation, reporting counts of 3.2×10^3 and 7.0×10^2 CFU g^{-1}, respectively. Specific GLS can act as deterrents or stimulants for feeding, depending on the species of microorganism. Therefore, natural selection could favour the presence of various profiles of GLS within a given species (Donkin et al. 1995; Li et al. 2000; Sellam et al. 2007; Tierens et al. 2001). Buxdorf et al. (2013) evaluated *Arabidopsis thaliana* mutants containing different amounts of GLS and its decomposition products to study the effects of these phytochemicals on phytopathogenic fungi. They observed that the isolates of *Botrytis cinerea* showed variable sensitivity depending on the composition of GLS and its hydrolysis products, while *Alternaria brassicicola* was more strongly affected by the decomposition products of GLS such as aliphatic glucosinolates and isothiocyanates.

In the present study, the application of compatible treatment (BAE 125 µL mL^{-1}+*P. lilacinum* SR14) enhanced the effectiveness of individual ones on *N. celatus* J2 in the order of 30%. Similarly, Pérez-Rodríguez et al. (2011) reported significant reductions ($p = 0.05$) in the gall index (26.5%), juveniles (76.0%) and mature females (41.7%) of *N. aberrans* population by the application of the combined treatment (cabbage fragments+*P. chlamydosporia*). In addition to the nematicidal effect attributed to GLS and their hydrolysis products present in BAE, as previously explained, the nematophagous activity of the *P. lilacinum* SR14 tested in previous works was combined in this study. In previous investigations, high infection capacity of eggs and J2 of *N. celatus* of 73 and 64%, respectively were attributed to this strain (Sosa et al. 2018). Moreover, the nematophagous activity could be attributed to high levels of chitinases (0.14 ± 0.02 U h^{-1} mL^{-1}) and proteases (0.15 ± 0.06 U min^{-1} ml^{-1}) and the production of paecylotoxins (1850 ± 1322.17 and 532.99 ± 328.67 ng μl^{-1} for leucinostatins A and B, respectively) (Girardi et al. 2022), that confer nematicidal properties according to Park et al. (2004).

Finally, the potential phytotoxic effects of BAE on tomato plants were also evaluated in the present work. Those plants treated with the concentration of 125 µL mL^{-1} BAE on days 0, 1 and 3 before transplantation presented vigour characteristics (height and weight of root and stem) superior to the control plants. In addition, these plants had the highest number of leaves and the lowest proportion of chlorotic leaves. Garita (2019) analyzed two variables related to stress in tomato plants treated with cabbage pieces. The author observed a significant increase in the concentration of proline and malondialdehyde in the root tissue of biofumigated plants. Although the plants where cabbage was incorporated had high growth, these results could indicate some phytotoxicity type. Mojtahedi et al. (1991) observed a phytotoxic effect in tomato plants that were placed in pots where green rapeseed material had previously been incorporated. This work reported plant death and lower root growth, attributing these results to some substance produced during the brassica decomposition. Mian and Rodríguez-Kábana (1982) indicate a direct relationship between the nitrogen content of the amendment and its nematicidal activity exerted by the release of ammonia. These authors suggest that, although high N contents are desirable in terms of nematological control, excesses of ammonia can have phytotoxic effects.

As conclusions of this work, even though both AEs showed high nematicidal activity in 24 h, with LD_{50} of 110 and 125 µL mL^{-1} for cabbage and broccoli, respectively, BAE showed compatibility with *P. lilacinum* SR14 and SR7 at the lowest doses assayed (30, 60 and 125 µL mL^{-1}). *Purpureocillium lilacinum* SR14 developed under all evaluated conditions, but the compatibility with 60 and 125 µL mL^{-1} could only establish at 25 °C, where stimulations

of the studied parameters about 8 and 25% for growth rate and conidiation, respectively, could be registered. The nematophagous activity of *P. lilacinum* SR14 increased significantly in the presence of the highest concentration of BAE (125 µL mL^{-1}). Consequently, the highest compatible doses of BAE (60 and 125 µL mL^{-1}) were selected to continue the *in-plant* studies. Concerning the phytotoxicity test, the results showed that the BAE concentration and the time of application did not significantly affect the vigour characteristics of the tomato plants and those vegetables that developed in the substrate treated with the dose of 125 µL mL^{-1} at the time of transplantation presented greater vigour. Therefore, the results of this study revealed that the dose of 125 µL mL^{-1} BAE applied at the time of tomato transplantation in combination with the compatible strain *P. lilacinum* SR14 could be an effective strategy for the management of the phytonematode, *N. celatus*, without producing any baneful effect on host culture. *In-plant* studies to evaluate the effectiveness of the combined management strategy on *N. celatus* population are actually conducted on tomato under chamber and greenhouse conditions.

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Declarations

Conflict of interest The authors have declared that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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