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Nematicidal effect of chestnut tannin solutions on the potato cyst nematode *Globodera rostochiensis* (Woll.) Barhens

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Summary

Recently, tannins have been reported for their nematicidal activity against the root-knot nematode *Meloidogyne javanica* both *in vitro* and in pot experiments in addition to a biocidal effect on a wide range of fungi, bacteria and yeasts. However, no information is available on the effect of these polyphenols on plant parasitic cyst nematodes. Therefore, an *in vitro* and a pot experiments on potato were undertaken to investigate the nematicidal activity of tannin aqueous solutions at different concentrations on the potato cyst nematode *Globodera rostochiensis*. In the *in vitro* experiment different tannin concentrations in a geometric scale (from 0.32 to 20.48 g/l) were tested for their effect on the egg hatch of the nematode. All tested tannin concentrations were effective to reduce egg viability from 56 to 87%, in comparison to the untreated control. In the pot experiment, tannins, as aqueous solutions at rates of 100, 250 and 450 g/m^2, were applied to soil at two different application times (at sowing and at sowing and two weeks later). All tested doses were effective to reduce the number of cyst/100 g soil, eggs and juveniles/g soil and reproduction rate in comparison to untreated control. The number of eggs and juveniles/cyst was not influenced by the different applied rates of tannins.

Keywords: golden nematode; nematode control; polyphenols; *Castanea sativa* L.

Introduction

The golden nematode, *Globodera rostochiensis* (Woll.) Barhens causes severe yield losses in many potato growing areas of the world (Anand *et al.*, 1998). The extent of yield loss is related to the nematode population density in the soil, which should be always maintained below the estimated damage threshold (1.9 eggs and juveniles/g soil) (Greco *et al*., 1982; Sasanelli, 1994). *G. rostochiensis* is a quarantine pest included in the part A of the section II of the Annex 1 of the EU Directive 29/2000 (harmful organisms known to occur in the Community and relevant for the entire Community) and in the quarantine list of phytoparasitic nematodes in Europe, A1 list, of the European and Mediterranean Plant Protection Organization (EPPO). Soil treatments with nematicides in different formulations (fumigants, granulars or liquids) can successfully and economically control *G. rostochiensis* (Greco *et al*., 1984; Been & Schomaker, 1999). However, the recent European Legislation (Reg. CE 396/2005; 1095/2007; 33/2008; 299/2008 and 1107/2009) has deeply restricted and revised the use of pesticides on agricultural crops focusing the attention on enviromental safety, human and animal health. Therfore, research on low environmental impact alternatives to chemicals has received a stronge impulse with a wide range of options. Among the alternatives are soil solarization (McSorley & McGovern, 2000), organic amendments (Renčo *et al*., 2007, 2011; Hu & Qi, 2010; D'Addabbo *et al*., 2011; Toden *et al*., 2011), biofumigation (Aires *et al*., 2009), mycorrhization (Ryan & Jones, 2004; Sasanelli *et al*., 2007, 2009) or, more recently, natural plant-derived compounds (Céspedes *et al*., 2006). In particular, plants may represent a source of natural nematicide, as a higher number of nematicidal compounds are already reported in many species (Chitwood, 2002). Commercial formulations based on plant-derived extracts of (*Quillaja saponaria* Molina), marigold (*Tagetes erecta* L.) and neem (*Azadirachta indica* Juss) are already available. Tannins, natural products extracted from many plants, have been reported to possess antihelmintic activity especially for gastrointestinal nematodes in ruminants (Hoste *et al*., 2006). They are secondary plant polyphenols whose chemical and physical properties can change according to the plants (i.e. *Castanea sativa* Miller or *Schinopsis lorentzii* (Schldl.) Barkley & Meyer, etc.), parts of the plants and the season in which they are produced (Waterman, 1999; Waghorn & McNabb, 2003). Moreover, tannins protect several plants against

herbivores (Feeny, 1976) and are toxic to a wide range of fungi, bacteria and yeasts (Scalbert, 1991).
Recently, tannins have been reported for their nematicidal activity against the root-knot nematode *Meloidogyne javanica* (Treub) Chitw. both *in vitro* and in pot experiments (Maistrello *et al*., 2010). However, there is no information on the effect of these polyphenols on plant parasitic cyst nematodes. Therefore, an *in vitro* and a pot experiments were undertaken to investigate the nematicidal activity of tannin aqueous solutions at different concentrations on the potato cyst nematode *G. rostochiensis.*

Materials and methods

Hatching test
The population of *Globodera rostochiensis*, previously identified as pathotype Ro1, was obtained from an infested soil at Avezzano (province of L'Aquila, Italy). The cysts were collected by Fenwick can from dried soil. Batches of 50 cysts of similar size (about 115 eggs and juveniles/cyst) were placed in 2 cm diam sieves (215 µm aperture). Each sieve was put in a 3.2 cm diam Petri dish (Shepherd, 1986) and all dishes were arranged according to a complete randomised block design in a growth cabinet at 20 $\pm$ 2 °C (Ekanayake & Di Vito, 1984) with four replicates for each treatment.
The tannins were provided by Agrostar s.r.l. (Cavriago, province of Reggio Emilia, Italy) and they were extracted by vapour from chestnut wood, without chemical solvents, in powder form after dehydration. Different concentrations from 0.32 to 20.48 g/l, in a geometric series, were obtained by dissolving the largest rate of tannin in distilled water (Table 1). Each tested aqueous tannin solution was prepared considering a double concentration, because each of them was adjusted with an equal amount of potato root leachate used as natural hatching agent.
Four ml of each test solution, sufficient to cover cysts, were then added to four batches of cysts. Potato root leachate, which was used as control, was collected from three month-old actively growing potato plants, cultivated in fifteen 2,500 cm^3 clay pots, by drenching the soil with excess tap water. The leachate was then centrifuged at 1,300 rpm for 15 minutes, stored in plastic bottles, and kept in a freezer until required. Only small amounts were kept in a refrigerator at 5 °C for immediate use.
Juveniles (J_2) emerging from cysts were removed and counted every week over a 12 week period. The solutions were renewed weekly but after four weeks the test solutions were removed and for the remaining eight more weeks the incubation continued only in potato root leachate, according to an already described methodology (Sasanelli & Di Vito, 1991; Sasanelli & D'Addabbo, 1992) (Table 1). At the end of the experiment cysts were crushed according to Seinhorst and Den Ouden (1966), and unhatched eggs and juveniles were counted. Numbers of second stage juveniles emerging weekly were expressed as cumulative percentages of the total egg content of the cysts (hatched + unhatched eggs).
Data were subjected to analysis of variance (ANOVA), after arc sin square root transformation (Bliss' Tables), and means were compared by Least Significant Difference's Test. All statistical analyses were performed using the PlotIT program. The Abbott's formula (Abbott, 1925) was used to calculate the mortality of eggs in tannin treated cysts at the different tested concentrations.

Glasshouse experiment
The same population of *G. rostochiensis* used in the *in vitro* experiment was also used for the pot experiment carried out in glasshouse. The cysts were extracted from an infested field (Avezzano, AQ) and thoroughly mixed into 2.9 kg steam sterilised sandy soil. Ten 10 g soil samples were taken from this inoculum and the cysts were extracted, counted and their egg content estimated. Appropriate amounts (100 g) of this inoculum were then thoroughly mixed with the steam sterilised soil in each clay pot containing 3,900 g soil to give an initial nematode population density of 5 eggs and juveniles/g soil. The pots were arranged on benches in a glasshouse at 23 $\pm$ 2 °C according to a randomized block design with four replicates for each treatment. Three tannin concentrations were considered 1) 100 g/m^2; 2) 250 g/m^2 and 3) 450 g/m^2, applied only at sowing or at sowing and two weeks later, for a total of six tannin treatments (Table 2). Nematode infested untreated soil was used as control. Tannins were applied as aqueous solutions (1,400 ml/pot), calibrated on the holding capacity of the soil.
After inoculation, to all clay pot one potato tuber (cv. Désirée) was planted. During the experiment potato plants were maintained in the glasshouse randomizing the position of the blocks and at the same time repositioning each plant within a block every week, to avoid a block position effect and at the same time the factor position of the plant within the block. Plants received all the necessary maintenance (irrigation, fertilization, etc.).
Three months later, at the end of the experiment, potato plants were uprooted and fresh and dry top and root weight and height were recorded. Soil from each pot was mixed thoroughly and then a soil sample was collected and air dried. Cysts were extracted by Fenwick can from 200 g sub-sample, crushed (Seinhorst & Den Ouden, 1966) and their egg content estimated.
Data from the experiment were subjected to analysis of variance (ANOVA) and means compared by Least Significant Difference's Test. Statistical analysis were performed using the PlotIT program. TableCurve program was used to analyze the relationships between different tannin doses and nematological parameters of the potato cyst nematode.

Results

Hatching test
During the first week, nematode hatch in the untreated control was significantly higher than all other treatments with tannin aqueous solutions at different concentration

Table 1. Effect of different aqueous concentrations of tannins on hatching of the potato cyst nematode *Globodera rostochiensis*

Treatment	Cumulative percentage of juveniles emerging weekly																								Mortality (%)[3]
	Incubation period (weeks)																								
	In test solutions								In potato root leachates																
	1		2		3		4		5		6		7		8		9		10		11		12		
Control	4.0[1]	A[2]	8.9	A	14.6	A	21.3	A	26.4	A	30.8	A	35.9	A	39.9	A	43.9	A	46.6	A	48.5	A	49.7	A	---
0.32 g/l	1.7	B	3.0	B	5.3	B	7.2	B	9.9	B	13.1	B	15.8	BC	17.8	BC	19.1	BC	19.8	BC	20.2	BC	20.3	BC	59.1
0.64 g/l	1.7	B	3.6	B	5.0	B	6.9	B	10.6	B	13.5	B	16.6	B	18.4	B	20.1	B	20.9	B	21.4	B	21.9	B	55.9
1.28 g/l	1.1	BC	2.8	B	4.2	B	5.5	B	9.7	B	13.1	B	15.9	B	17.5	BC	18.5	BC	19.3	BC	19.8	BC	19.9	BCD	60.0
2.56 g/l	0.6	CD	1.3	C	2.2	C	2.8	C	5.9	C	8.9	C	12.0	CD	13.6	CD	14.7	CD	15.1	CD	15.7	CD	15.9	CD	68.0
5.12 g/l	0.6	DE	1.1	CD	1.7	CD	2.2	CD	4.6	C	7.4	C	9.6	D	11.4	D	13.3	D	13.8	D	14.3	D	14.7	D	70.4
10.24 g/l	0.2	EF	0.7	D	1.1	D	1.3	D	2.8	D	4.2	D	5.7	E	6.9	E	8.2	E	8.9	E	9.6	E	9.7	E	80.5
20.48 g/l	0.1	F	0.3	E	0.5	E	0.6	E	1.8	D	2.7	D	3.9	E	4.4	E	5.2	E	5.9	E	6.4	E	6.4	E	87.1

[1] Each value is an average of four replications;
[2] Data flanked in each column by the same letters are not statistically different according to Least Significant Difference's Test (P=0.01);
[3] Values were calculated according to Abbott's formula.

Table 2. Morphological parameters of potato plants (cv. Désirée) grown in pots (4 l) in *Globodera rostochiensis* (Ro1) infested soil and treated with tannin at different doses and application times

Treatment	Dose (g/m^2)	Application time	Stem – Top weight (g)				Stem – height (cm)		Root weight (g)[3]	
			fresh		dry					
Untreated control	0		238[1]	A[2]	24.4	A	62	A	20.4	A
Tannin	100	At sowing	225	A	20.6	A	65	A	25.6	A
Tannin	100	At sowing + 2 weeks later	205	A	20.0	A	63	A	19.2	A
Tannin	250	At sowing	239	A	23.6	A	60	A	22.3	A
Tannin	250	At sowing + 2 weeks later	235	A	22.9	A	64	A	23.9	A
Tannin	450	At sowing	180	B	17.2	B	31	B	16.6	B
Tannin	450	At sowing + 2 weeks later	172	B	18.0	B	41	B	17.5	B

[1] Each value i san average of 4 replications;
[2] Data flanked in each column by the same letter are not statistically different according to Least Significant Difference's Test (P=0.01);
[3] Without tubers

(Table 1). The highest per cent hatch reduction (0.1%) was observed at the concentration of 20.48 g/l tannin solution, although this percentage was no statistically different from the one recorded at 10.24 g/l tannin concentration (Table 1). During the first four weeks, emergence of juveniles from *G. rostochiensis* cysts was suppressed in all tannin aqueous solutions. All treatments with tannins in the range 0.32 – 1.28 g/l did not significantly differ among each other (Table 1). Moreover, no significant difference was observed between tannin treatments at 2.56 and 5.12 g/l. From the second week until the fourth week the percent hatch of juveniles from cysts treated with tannin at the highest concentration (20.48 g/l) was significantly lower than that observed at 10.24 g/l (Table 1).

When tannin aqueous solutions were removed and the incubation continued in potato root leachate, the emergence of juveniles increased in all treatments, although it remained significantly lower than in the control until the end of the experiment (Table 1). In general, in each week between the fifth and the seventh week, no significant statistical differences were observed in the percentage of hatch in cysts treated in aqueous tannin solutions in the ranges of 0.32 – 1.28, 2.56 – 5.12 and 10.24 – 20.48 g/l concentrations (Table 1). Statistical differences were significantly more evident in the incubation period between the eighth and the twelfth week. At the end of the experiment, percent mortality of eggs from cysts treated with tannin solutions ranged between 55.9 and 87.1 (Table 1). The lowest and the highest percentage of egg mortality were detected at 0.64 and 20.48 g/l tannin concentration, respectively.

Glasshouse experiment

All treatments with tannin did not significantly (P=0.01) increase potato plants growth variables (top fresh and dry stem and root weights and stem height) compared with the untreated control (Table 2). However, treatments with the highest dose of tannin (450 g/m^2) applied at sowing and again 2 weeks later, significantly reduced plant growth in comparison to the lower applied doses and to the inoculated and untreated control (Table 2).

The number of *G. rostochiensis* cysts/100 g soil, the number of eggs and second stage juveniles/g soil and the reproduction rate (r = *Pf*/*Pi*) were significantly reduced by all tannin treatments in comparison to the untreated control (Table 3). The highest reduction of these parameters was recorded in tannin treatment at dose of 450 g/m^2 applied at sowing and at sowing and 2 weeks later, although this treatment was not significantly different from that at the same dose applied only at sowing and that at 250 g/m^2 applied two times (Table 3). The tannin treatments at different doses did not significantly affect the number of eggs/cyst (Table 3). No significant differences, in all nematological parameters, were found between the tannin applications at sowing and at sowing and 2 weeks later for all the three tested concentrations (100, 250 and 450 g/m^2) (Table 3).

Table 3. Nematological parameters of potato plants (cv. Désirée) grown in pots (4 l) in *Globodera rostochiensis* (Ro1) infested soil and treated with tannin at different doses and application times.

Treatment	Dose (g/m2)	Application time	Number of cysts/100 g soil		Eggs and J_2/cyst		Eggs and J_2/g soil		r = *Pf*/*Pi*	
Untreated control	0		78.8[(1)]	A[(2)]	150	A	118.0	A	23.6	A
Tannin	100	At sowing	23.5	BC	172	A	40.6	B	8.1	B
Tannin	100	At sowing + 2 weeks later	30.5	B	138	A	41.0	BC	8.2	BC
Tannin	250	At sowing	20.5	BC	174	A	36.4	BC	7.3	BC
Tannin	250	At sowing + 2 weeks later	17.5	CD	141	A	24.4	CD	4.9	CD
Tannin	450	At sowing	12.5	CD	130	A	16.2	D	3.2	D
Tannin	450	At sowing + 2 weeks later	8.0	D	141	A	11.0	D	2.2	D

[(1)] Each value is an average of 4 replications;

[(2)] Data flanked in each column by the same letter are not statistically different according to Least Significant Difference's Test (P=0.01);

Based on the results, significant negative correlations were found in the relationship between the four different applied doses, including the control, and the values of the number of cysts/100 g soil, eggs and juveniles/g soil and "r" (Fig. 1). The equations reasonably explain the above relationships, as indicated by the high values of the correlation coefficients (r^2) (Fig. 1).

Discussion and Conclusions

Studies on the effect of tannins on plant parasitic nematodes are few, whereas the studies on tannins as suppressant of gastrointestinal nematodes, bacteria and yeasts are numerous (Scalbert, 1991; O´Donovan & Brooker, 2001; Paolini *et al.,* 2003; Nguyen *et al.,* 2005; Elizondo *et al.*, 2010). In our *in vitro* experiment, chestnut tannin solutions significantly reduced *G. rostochiensis* egg hatch during the first fourth weeks in comparison to untreated control. Also, when tannin aqueous solutions were removed and the incubation continued in potato root leachate, the emergence of juveniles remained significantly lower compared to the untreated control until the end of the experiment demonstrating a nematicidal effect of these solutions. Similar results, using the same chestnut tannin at the same concentrations in an *in vitro* experiment, were found on the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood by Maistrello *et al*. (2010). Also Chen *et al.* (1997) found a suppressive effect on egg hatch of the soybean cyst nematode *Heterodera glycines* in *in vitro* experiment with tannic acid extracted from tara *Caesalpinia spinosa* (Molina) Kuntze applied at concentrations between 0.156 and 10g/l, whereas the lower tannic acid concentration (0.040 g/l) increased nematode egg hatch. In all *in vitro* experi-

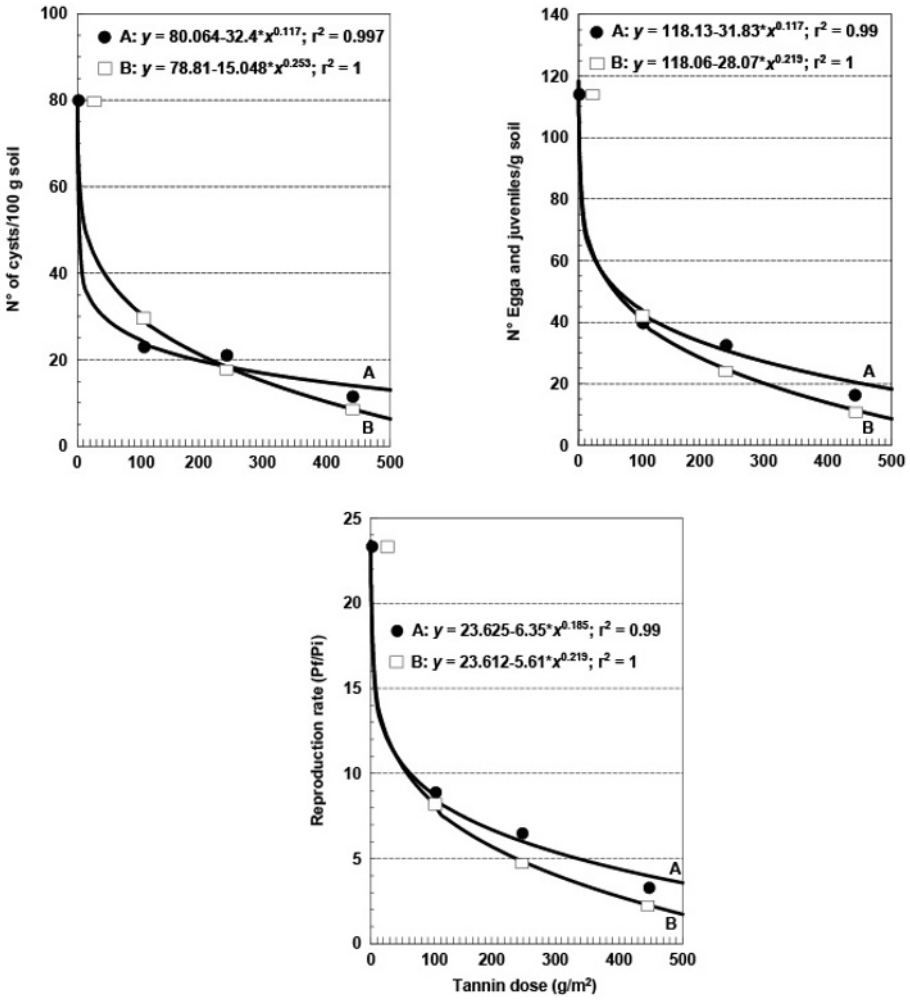

Fig. 1. Relationship between doses of soil applied tannin aqueous solution and number of cysts/100 g soil, eggs and juveniles/g soil and reproduction rate of *G. rostochiensis* (Ro1).
Each experimental point represents the average of four replicates. Lines A and B represent the predicted function calculated by fitting experimental data to different equations and relative to tannin application at transplant and to tannin applications at transplant and two weeks later, respectively.

ments, including also this experiment, percent egg hatch significantly decreased with the increase of tannin concentrations and with longer exposure times, which are probably the most important factors involved in nematode suppression.

Results from glasshouse experiment showed no effect on stimulation of potato plant growth parameters (top fresh and dry stem and root weights and stem height) compared with the untreated control, and treatments with the highest dose of tannin (450 g/m^2) applied at sowing and at sowing and 2 weeks later showed a considerable phytotoxicity on potato plants. These findings are in agreement with that found by Mian and Rodríguez-Kábana (1982) or Hewlett *et al.* (1997), on other crops, where the use of higher amount of tannins showed phytotoxic effect on growth parameters of squash and tomato plants, respectively. Some residues of plants contain volatile fatty acids (VFAs) and phenolic compounds (PCs) which are phytotoxic on other plant species growth as found by Wanniarachchi and Voroney (1997). They tested the release of VFAs and PCs from root, stem and leaves residues of canola plants (rapeseed) (*Brassica napus* L.) and their inhibiting (P≤0.01) effect on seedling growth (coleoptile and radicle lengths) of corn (*Zea mays* L.), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) plants. Toxicity of residue extracts was not related to the amounts of VFAs and PCs found. However, toxicity appeared to be most related to the presence of total phenolic compounds in the residue extracts.

In contrast, on tomato plants grown in soil infested with root-knot nematode *Meloidogyne javanica*, Maistrello *et al*. (2010) found no phytotoxic effect on plant growth of chestnut tannin at rate of 450 g/m^2 as used in our experiment on potato.

In the pot experiment all doses of chestnut tannin (100, 250 and 450 g/m^2) significantly reduced the number of cyst/100 g soil, number of eggs and J_2/g soil and the reproduction rate "*r*" in comparison to untreated soil (control). This agrees with results from Maistrello *et al*. (2010) where the same chestnut tannin, applied at the same doses, significantly reduced the number of eggs and J_2/g root, the final nematode population density/cm^3 soil and the "*r*" of

Meloidogyne javanica on tomato plants. Similarly, in a field experiment on squash, also Mian and Rodríguez-Kábana (1982) recorded *Meloidogyne arenaria* suppression after tannic acid treatments. Taylor and Murant (1966) found a reduction of number of *Longidorus elongatus* (de Man) in soil treated with two powdered tannin extracts from mimosa (*Acacia mollisima* Willd.) and quebracho (*Schinopsis lorentzii* (Griseb.) Engler). Moreover, *L. elongatus* is a virus vector of plant soil borne viruses which were previously suppressed by adding tannins to the soil (Cadman & Harrison, 1960). Considering that these viruses are not free in the soil ut they are transmitted by *L. elongatus*, Tylor and Murante (1966) hypothesized that viruses plant protection was obtained by the control of the vector virus and not by the direct effect of tannin on the virus.

Results from the pot experiment might be due to a double effect of tannin solutions: a) the nematicidal effect as confirmed in the hatching test and b) to the repellent action towards to *G. rosatochiensis* juveniles.

Application of repellents at sowing, planting or before transplant, may serve to disorientate plant parasitic nematodes causing them difficulties in locating the root systems and potentially reducing nematode attack as previously found by Hewlett *et al.* (1997) for *Radopholus similis* and Maistrello *et al*. for *M. javanica* (2010).

In the pot experiment, a significant reduction of the nematode population was obtained by the use of all tannin doses and no significant differences were observed between the two application times, at sowing and at sowing and 2 weeks later, at the same rate (100, 250 and 450 g/m^2), demonstrating the positive effect of only one application of the tannin solution. Based on our results it seems that the best application rate is 250 g/m^2 applied at sowing because of at this dose of chestnut tannin it is possible to obtain on potato the highest nematicidal effect without phytotoxicity. These results are in agreement with those found by Maistrello *et al*. (2010) which reported that the best suppression of *M. javanica* nematode population was obtained at the dose of 250 g/m^2 of tannin applied at transplant and no phytotoxicity was shown at all doses tested (100, 250 and 450g/m^2).

Data from glasshouse experiment on the same population of the potato cyst nematode *G. rostochiensis*, patotype Ro1, confirm results obtained in the *in vitro* experiment.

In conclusion, the use of tannins from chestnut seems to be a promising control method of potato cyst nematodes in sustainable agriculture. However, further studies are suggested to investigate the effect of tannins derived from different plants (chestnut, quebracho, mimosa etc.), in different types of soils, on different nematode species and on beneficial soil free living nematodes.

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