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Effect of coffee silver skin and brewers' spent grain in the control of root-knot nematodes

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Summary

Plant parasitic nematodes (PPN) are important pests of numerous agricultural crops especially vegetables, able to cause remarkable yield losses correlated to soil nematode population densities at sowing or transplant. The concern on environmental risks, stemming from the use of chemical pesticides acting as nematicides, compels to their replacement with more sustainable pest control strategies. To verify the effect of aqueous extracts of the agro-industry waste coffee silverskin (CS) and brewers' spent grain (BSG) on the widespread root-knot nematode *Meloidogyne incognita*, and on the physiology of tomato plants, a pot experiment was carried out in a glasshouse at 25 ± 2 °C. The possible phytotoxicity of CS and BSG extracts was assessed on garden cress seeds. Tomato plants (landrace of Apulia Region) were transplanted in an artificial nematode infested soil with an initial population density of 3.17 eggs and juveniles/mL soil. CS and BSG were applied at rates of 50 and 100 % (1L/pot). Untreated and Fenamiphos EC 240 (nematicide) (0.01 µL a.i./mL soil) treated plants were used as controls. Reactive oxygen species (ROS) and chlorophyll content of tomato plants were estimated during the experiment. CS extract, at both doses, significantly reduced nematode population in comparison to the untreated control, although it was less effective than Fenamiphos. BSG extract did not reduce final nematode population compared to the control. Ten days after the first treatment, CS 100 %, BSG 50 % and BSG 100% elicited the highest ROS values, which considerably affected the growth of tomato plants in comparison to the untreated plants. The control of these pests is meeting with difficulties because of the current national and international regulations in force, which are limiting the use of synthetic nematicides. Therefore, CS extracts could assume economic relevance, as alternative products to be used in sustainable strategies for nematode management.

Keywords: *Meloidogyne incognita*; phytochemicals; sustainable nematode control; tomato; by-products valorization

Introduction

Plant parasitic nematodes (PPN) are important pests of numerous agricultural crops especially vegetables, able to cause remarkable yield losses correlated to soil nematode population densities at sowing or transplant (Sasanelli, 1994; Perry & Moens, 2011). They can also cause indirect damages by opening penetration ways to soil pathogens (*Fusarium* spp., *Verticillium* spp., *Pyreno-*

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chaeta lycopersici etc.) and/or to viruses (Brown *et al*., 1988) because of the mechanical action of their stylet on the root surface (Ciccarese *et al*., 2008; Sasanelli *et al*., 2008).
In particular, the widespread root-knot nematodes (*Meloidogyne* spp.) are of remarkable importance due to their polyphagy. Some of these species are included in the quarantine pest list either of the European Union (EU, Directive 2000/29) and of the European and Mediterranean Plant Protection Organization (EPPO) (Wesemael *et al*., 2010). Concerns for the environmental risks, stemming from the use of chemical pesticides acting as nematicides, recently ended in restrictions provided by the European legislation (EU Reg. 396/2005, 1095/2007, 33/2008, 299/2008, 1107/2009, 459/2010 and 293/2013), that impel their replacement with more sustainable pest control strategies (Renčo, 2013; Abdel-Daym *et al*., 2014). The use of eco-friendly agro-industrial by-products in pest control is nowadays regarded with increasing interest (Abdel-Dayem *et al*., 2012; Luque & Clark, 2013). In particular, those with high polyphenols content seems to be particularly effective in controlling plant parasitic nematodes (Chitwood, 2002; Oka, 2010). From this point of view, coffee silverskin (CS) and brewer's spent grain (BSG) are among the most interesting readily available, high volume and low cost agro-industry by products with high polyphenols content. These by-products, rich in polyphenols content (Regazzoni *et al*., 2016; Santi Stefanello *et al*., 2018), are produced in large amount throughout the year (Mussatto & Teixeira, 2010; Lynch *et al*., 2016).
Coffee silverskin, the only by-product generated during the coffee roasting process (dos Santos Polidoro *et al*., 2017), is a thin tegument of the outer layer of coffee beans and represents about 4.2 % (w/w) of the entire seed weight (Janissen & Huynh, 2018). The average basic chemical composition of CS is 16 – 18 % of proteins, 2 % of lipids and 4 – 7 % of ash (Borrelli *et al*., 2004; Carneiro *et al*., 2009). This by-product is also rich in specific bioactive compounds such as chlorogenic acids (1 – 6 %), caffeine (0.8 – 1.3 %), and melanoidins (17 – 23 %) (Mesías *et al*., 2014; Behrouzian *et al*., 2016). CS is used as biofuel (Woldesenbet *et al*., 2016), fertilizer (Hachicha *et al*., 2012) and as mushroom cultivation substrate (Fan *et al*., 2003).
Brewer's spent grain is the by-product of the beer fermentation process and consists of the husk-pericarp-seed coat layers covering the barley grain. The husk contains considerable amounts of silica and polyphenolic components of the barley grain (Macleod, 1979). The chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions (Huige, 1994; Santos *et al*., 2003). In general, BSG is considered as a lignocellulosic material rich in protein and fibers, containing 15 – 24 % of proteins, 10 % lipids and 2 – 4 % of ash (Kanauchi *et al*. 2001; Mussatto & Roberto, 2005) and remarkable quantity of bioactive phytochemicals, such as phenolic compounds (Connolly *et al*., 2015). Among its different uses, it is employed to increase the protein and dietary fibre content of food, in animal feeding (Öztürk *et al*., 2002) and in industrial processes (Tsaousi *et al*., 2011; Aggelopoulos *et al*., 2013).
This work was aimed at studying, in a tomato plant pot experiment, the effect of the aqueous extract of CS and BSG on the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitw. and on the physiology of tomato plants.

Materials and Methods

The pot experiment was carried out at the Institute of Sustainable Plant Protection (IPSP) of the Italian National Research Council (CNR) in Bari (Italy) (40°16'22"N, 16°88'16"East Greenwhich) in a glasshouse, the temperature of which was set at 25 ± 2 °C.

Extracts preparation and characterization
CS and BSG were crushed and suspended in deionized water (1:10 w/vol) in a blender at 8,000 rpm for 5 min, shaken for 1 hour and filtered using a Whatman n.1 filter. The pH of extracts was measured using the pHmeter Basic 20 Crison and the electrical conductivity (EC) by a Sension+ EC7 (Hach) conductivity meter. Total nitrogen and total polyphenols were determined according to Bremner (1996) and Waterhouse's (2002) methods, respectively. UHPLC Dionex Ultimate 3000 RS system coupled by the HESI–II probe and TSQ Quantum Access Max triple quad mass spectrometer (Thermo Fischer Scientific) was used for the qualitative assessment of polyphenols in CS and BSG aqueous extracts. The separation of compounds was performed at 30 °C on Hipersyl Gold C18 column, 3 µm particle size, i.d. 2.1 mm, 100 mm length (Thermo Fischer Scientific). A binary mobile phase made of a) formic

Table 1. Physical and chemical main characteristics of coffee silverskin (CS) and brewer's spent grain (BSG) extracts.

Parameters	Unit	BSG	CS	LSD	
				0.05	0.01
pH	$[H^+]$	6.9* ± 0.1	5.6 ± 0.1	0.13	0.22
Electrical Conductivity	mS/cm	4.9 ± 0.3	4.6 ± 0.2	0.57	0.95
Total Nitrogen	g/L	1.2 ± 0.1	0.7 ± 0.2	0.35	0.57
Total Polyphenols	mg/L	353 ± 15	403 ± 22	44.0	73.0

*Each value is an average of three replications ± SE

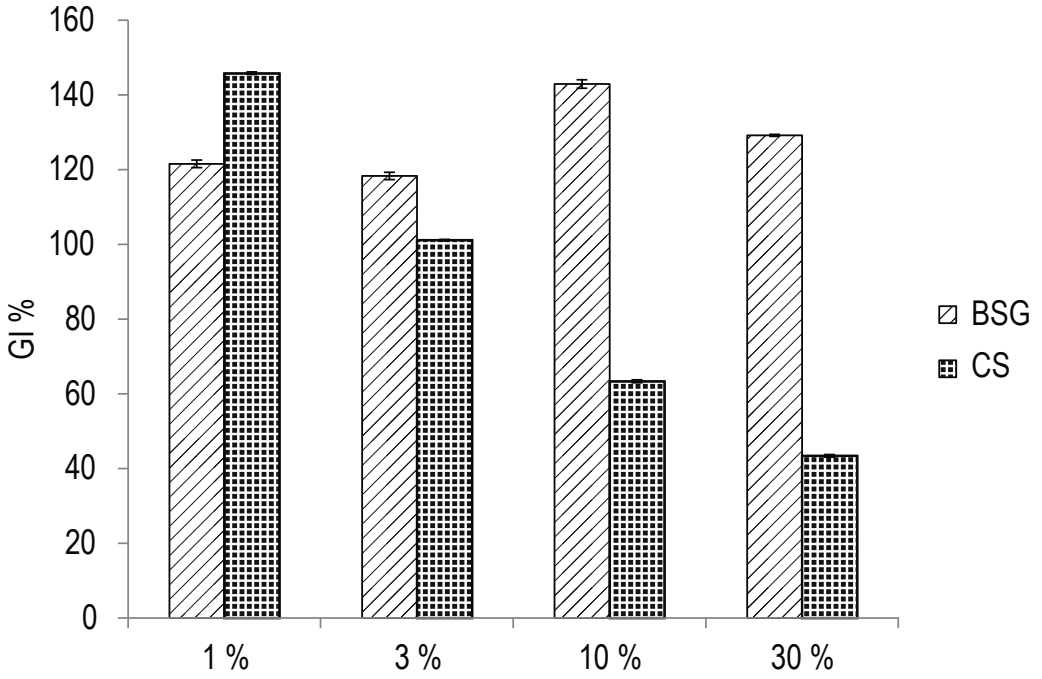

Fig. 1. Phytotoxicity test. Effect of different BSG and CS aqueous extract concentrations on germination index (GI) of garden cress seeds (*Lepidium sativum* L.).

acid aqueous solution at 0.1 % and b) formic acid in acetonitrile solution at 0.1 %, at a constant flow rate of 0.2 mL/min was used. The gradient program of solvent b was set to increase from 10 to 70 % in 20 min. The conditions of the MS system were the following: 320 °C for capillary temperature, 280 °C for source heater temperature, nebulizer gas N_2, collision gas Ar, sheath gas flow 35 psi, auxiliary gas flow 10 units, capillary voltage -2.8 kV, tube lens offset 78, 111 and 160 for Q1, Q2 and Q3, respectively. Calibration curves were performed using pure standard phenols solutions of chlorogenic and ferulic acid at concentration ranging from 2.5 mg/L to 20 mg/L. These calibrations, based on ion extracted chromatogram at m/z = $[M\text{-}H]^-$ from the total ion chromatogram, were used to obtain semi-quantitative data of the caffeoyl quinic and feruloyl quinic derivates compounds identified in the extracts.

Phytotoxicity tests

Phytotoxicity of the CS and BSG aqueous extracts was evaluated measuring the Germination Index (GI) of the garden cress seeds (*Lepidium sativum* L.) (Zucconi *et al*., 1981). *L. sativum* was exposed to the extracts diluted at 30 %, 10 %, 3 % and 1 %. GI was calculated according to the following formula:

$$GI = \frac{N_S \times E_S}{N_W \times E_W} \times 100$$

where N_s is the number of germinated seeds, E_s the root elongation measured in mm and N_w and E_w are the same parameters measured in the control treatment.

Preparation of infested soil and pot experiment

An Italian population of *Meloidogyne incognita* race 1 (Hartman & Sasser, 1985) was reared for two months on tomato [*Lycopersicum esculentum* Mill. (L.)] plants (cv. Marmande) in a glasshouse at 25 ± 2 °C. When large mature egg masses were formed, tomato plants were uprooted and their roots gently washed, to free them of adhering soil particles, and finely chopped. To estimate the numbers of eggs and second stage juveniles (J2s) in the chopped roots, ten 5-g root samples were suspended in a 1 % aqueous solution of sodium hypochlorite (NaClO) in 150 mL jars for 3 minutes, after which the eggs and J2s released in the suspension were counted (Hussey & Barker,1973). The roots were then thoroughly mixed with 4 kg of steam sterilized sandy soil (pH 7.9; sand = 85.7 %; silt = 7.1 %; clay = 7.2 % and organic matter = 0.6 %) and used as inoculum. Appropriate amounts of this inoculum were then thoroughly mixed with steam sterilized silty clay loam soil (USDA) in a concrete mixer to obtain a uniformly infested soil. Nematodes, eggs and J2s, were extracted from 8 soil samples to determine the initial population density corresponding to 3.17 eggs and J2s/mL soil (*Pi*). This infested soil in an amount of 6.5 L was then used to fill plastic pots (V = 7.5 L).

One month old seedling of tomato (landrace of Apulia Region) was transplanted into each pot. There were five replications for each treatment and pots were arranged on benches, in a glasshouse at 25 ± 2 °C, according to a randomized block design. During the experiment tomato plants were maintained 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

Table 2. Identification and quantification of compounds obtained by LC-MS/MS analysis of silver skin coffee extract (in brackets the relative abundance of each signal).

RT	$[M-H]^-$	MS^2	mg/L	Name *
1.44	191	85(100) 127(50)	54.78	QA
2.23	353	135(100) 191(80) 179(10)	3.43	3-CQA
3.22	353	191(100) 161(5) 173(6)	4.54	5-CQA
3.39	353	136(100) 191(60) 94(40) 173(30)	7.44	4-CQA
3.56	367	135(100) 193(10) 179(5) 118(5) 94(5)	2.26	3-FQA
4.01	367	367(100) 269(95) 287(40) 148(20) 349(15)	2.85	FQA1
4.67	367	367(100) 287(40) 243(40) 349(30)	2.02	FQA2
6.11	367	173(100) 134(80) 94(60) 193(15)	2.53	4-FQA
6.27	367	191(100) 135(40) 94(35) 193(15)	7.71	5-FQA
6.53	559	351(100)	0.36	3Si-4CQA
9.87	381	358(100) 363(74) 257(48) 273(35) 319(27) 363(25) 336(23)	4.25	3-DQA
12.34	397	397(100) 325(20) 219(10)	0.37	SiQA

*Q=quinic, F=Feruloyl, C=Caffeoyl, Si=Sinapoyl, D=Dimethoxycinnamoyl, A=Acid

and at the same time the factor position of the plant within the block. The experiment was performed twice. Plants received all the necessary maintenance (irrigation, fertilization, etc.). Plants were irrigated when it was necessary before their wilting. Hoagland solution (1 L/pot) was used for fertilization (2 times during the experiment) to avoid macro and micro elements deficiency (Hoagland & Arnon, 1950).

The pots were treated with CS and BSG aqueous extracts, obtained as described in the paragraph "Extracts preparation and characterization", at concentrations of 50 and 100 %. Untreated and Fenamiphos EC 240 (0.01 µL a.i./mL soil) treated pots were used as controls. Each pot received 1 L of extract, or nematicide suspension. CS and BSG treatments were applied twice: at plant transplant and 20 days later.

At the end of the experiment (2 months) plants were uprooted and height, fresh and dry top and root weights were recorded. Root gall index (RGI) was estimated according to a 0 – 10 scale, where 0 = no galls; 1 – 4 = galling of secondary roots only, 5 – 10 = galling of primary laterals and tap root, with 5 equal to 50 % of roots galled and 10 the maximum nematode infestation possible (Bridge & Page, 1980).

Final soil nematode population density was determined in each pot processing 500 mL soil by the Coolen's method (Coolen, 1979). *M. incognita* density in roots was assessed by cutting up each root

Table 3. Identification and quantification of compounds obtained by LC-MS/MS analysis of brewer's spent grain extract (in brackets the relative abundance of each signal).

RT	**$[M-H]^-$**	**MS^2**	**Structural hypothesis**
7.58	394	289(100) 333(88) 394(88) 305(82) 351(78) 271(36) 297(26)	Ca (289)
7.69	329	82(100) 247(99) 96(43) 163(37) 125(36) 148(33) 81(30) 173(28)	Co(163) Q frg(173) Dq(329)
8.2	265	123(100) 86(59) 175(45) 153(19) 168(16) 114(12) 106(11)	P(153)
8.68	357	163(100) 233(50) 151(10)	Co(163)
8.99	331	249(100) 153(32) 207(15) 234(13) 150(11)	P(153)
9.01	271	146(100) 148(59) 136(46) 176(20) 120(17) 163(11) 191(11)	Co(163) Q(191)
9.36	373	212(100) 283(53) 248(46) 191(43) 209(39) 194(35)	F(194) Q(191)
10.73	375	312(100) 191(81) 246(52) 187(48) 176(35) 219(24)	Q(191)
12.27	538	180(100) 414(59) 283(48) 206(28) 383(16) 184(11)	C(180)
12.94	480	173(100) 262(73) 306(32) 231(22) 480(20) 188(16) 204(15)	Q frg(173)
13.54	331	157(100) 314(74) 144(39) 153(23) 155(19) 138(18) 171(15)	P (153)
13.95	329	211(100) 222(44) 173(38) 212(38) 203(37) 163(16)	Co(163) Q(173) Dq(329)
15.58	317	153(100) 233(30) 112(28) 163(23) 133(19) 215(17)	Co(163) P(153)
17.19	541	230(100) 117(20) 194(15) 212(10) 153(5)	F (194) P(153)

Ca=catechin; Co=coumaric; Q=quinic; Dq=dimethylquercetin; P=protocatechuic; F=ferulic; C=caffeic; Frg=fragment.

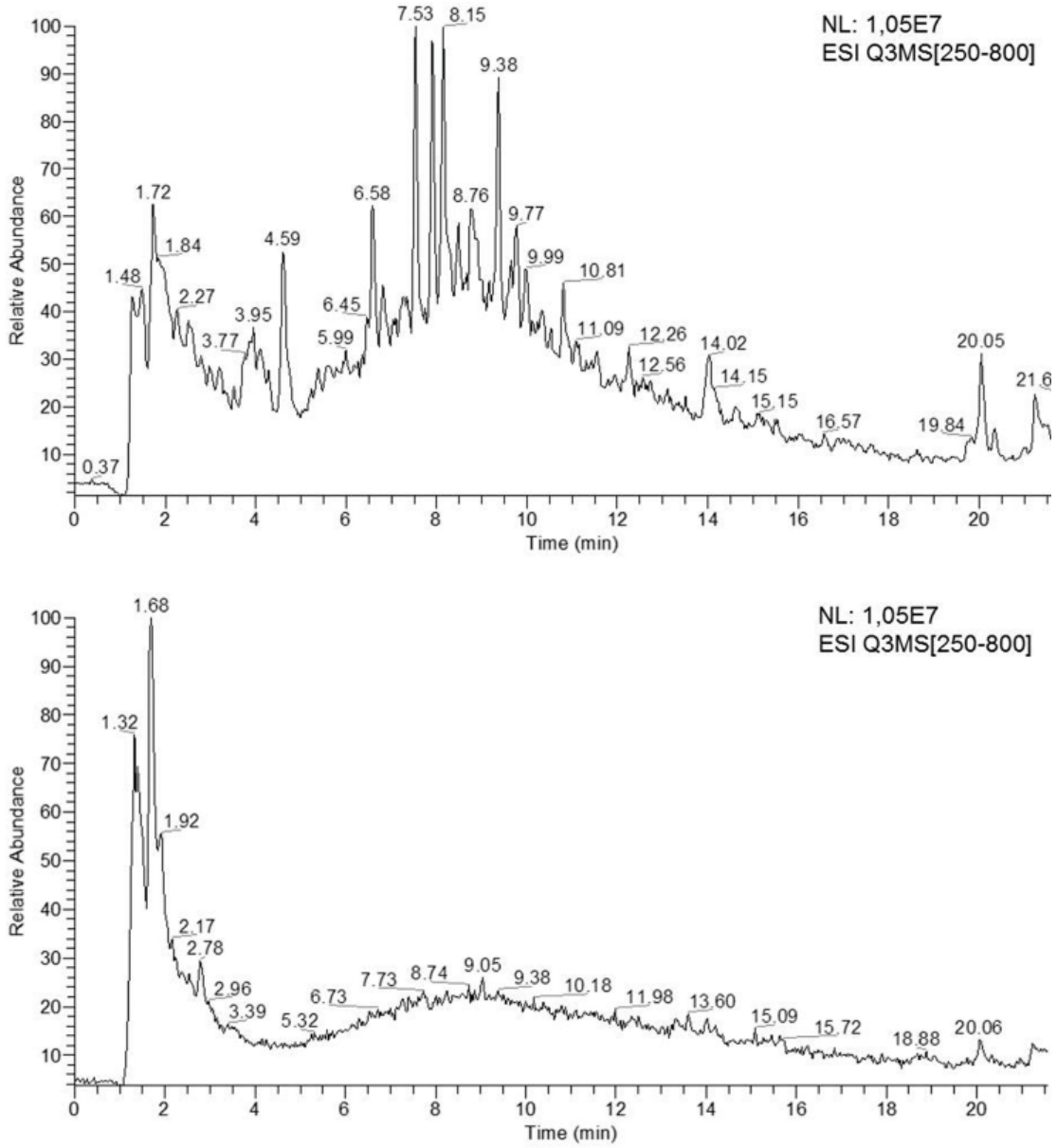

Fig. 2. Total ions chromatograms of silverskin coffee extract (top) and brewer's spent grain (bottom) obtained by LC-MS analysis.

system into small pieces and further comminuting them in a blender, containing 1 % aqueous solution of sodium hypochlorite for three periods of 20 sec (Marull & Pinochet, 1991). The water suspension was sieved on a 250 µm pore sieve over a 22 µm pore sieve. Nematodes and root debris gathered on the 22 µm pore sieve were separated by centrifugation (Beckman, Mod. Allegra X-12) at 2,000 rpm for five min in a magnesium sulfate solution of 1.16 specific gravity. Then eggs and juveniles in the water suspension were sieved again through the 22 µm pore sieve, sprayed with tap water to wash away the magnesium sulfate solution and collected in about 40-60 mL water. Eggs and juveniles in the water suspension were counted and final nematode population density (*Pf*) in each pot was determined by summing nematodes recovered from soil and roots. The nematode reproduction factor *r* was expressed as ratio between final and initial population density (*Pf*/*Pi*) of *M. incognita*.

Effects of CS and BSG treatments on Reactive Oxygen Species (ROS) and Chlorophyll levels

To test whether CS and BSG treatments at different concentration doses triggered an oxidative burst, the accumulation of ROS was quantified in tomato roots. ROS contents were determined following the method described in Melillo *et al*. (2006). Root portions were excised and pre-incubated for 30 min in potassium

Table 4. Effects of two different concentrations of aqueous solutions of coffee silverskin (CS) and brewer's spent grain (BSG) on the growth of tomato plants (landrace of Apulia Region) infested by the root-knot nematode *Meloidogyne incognita*.

Treatment	Dose (%)	Top weight (g) fresh			Top weight (g) dry			Height (cm)			Root weight (g)		
CS	50	76.1* ± 6.7	bc**	ABC	9.3 ± 0.8	bc	ABC	88.2 ± 4.4	ab	A	8.7 ± 0.7	b	AB
CS	100	80.7 ± 10.9	bcd	ABC	9.6 ± 1.2	bc	BC	91.4 ± 7	b	A	12.2 ± 0.7	c	BC
BSG	50	101.5 ± 6.9	d	C	12.4 ± 0.9	d	C	85.6 ± 2.6	ab	A	18.6 ± 1.5	d	D
BSG	100	94.4 ± 3.5	cd	BC	11.1 ± 0.6	cd	C	86.8 ± 5.6	ab	A	13.3 ± 1.4	c	C
Fenamiphos 240 EC (liquid formulation)	0.01 µL a.i./cm^3 soil	64.3 ± 11.1	ab	AB	7.2 ± 1.4	ab	AB	94.2 ± 7.5	b	A	4.7 ± 0.5	a	A
Untreated control	--	52.5 ± 4.5	a	A	5.8 ± 0.5	a	A	73.8 ± 5.9	a	A	12.3 ± 0.9	c	BC

* Each value is an average of 5 replications ± SE; **Data followed by the same letters in each column are not statistically different according to Least Significant Difference's Test (small letters for P= 0.05; capital letters for P= 0.01).

Table 5. Effects of different concentrations of aqueous solutions of coffee silverskin (CS) and brewer's spent grain (BSG) on the root-knot nematode *Meloidogyne incognita* infecting tomato plants (landrace Apulia Region).

Treatment	Dose (%)	Root gall index (0-10)			Eggs and juveniles/g root (x 1,000)			Eggs and juveniles/mL soil			Final population/mL soil (from roots and soil)			Reproduction rate *r = Pf/Pi*		
CS	50	3.8* ± 0.7	b**	B	19.8 ± 1.3	ab	A	14.8 ± 1.6	b	BC	42.9 ± 2.8	b	AB	13.5 ± 0.9	b	AB
CS	100	4 ± 0.3	bc	B	22.4 ± 1.5	b	AB	4.8 ± 0.7	a	A	52.9 ± 9.4	b	BC	16.7 ± 1.1	b	BC
BSG	50	4.6 ± 0.5	bc	B	35.2 ± 2.1	cd	CD	5.4 ± 1.3	a	A	107 ± 10.9	d	D	33.7 ± 3.4	d	D
BSG	100	4.2 ± 0.6	bc	B	29.3 ± 1.7	c	BC	16.8 ± 3.3	b	C	69.1 ± 11.4	bc	BC	21.8 ± 3.6	bc	BC
Fenamiphos 240 EC (liquid formulation)	0.01 µL/ cm^3 soil	1.4 ± 0.5	a	A	13.8 ± 2.9	a	A	5.8 ± 0.9	a	A	13.1 ± 1.2	a	A	4.1 ± 0.4	a	A
Untreated control	--	5.4 ± 0.2	c	B	40.3 ± 3.5	d	D	8.2 ± 1.8	a	AB	82.2 ± 13.6	cd	CD	25.9 ± 4.3	cd	CD

* Each value is an average of 5 replications ± SE; **Data followed by the same letters in each column are not statistically different according to Least Significant Difference's Test (small letters for P= 0.05; capital letters for P= 0.01).

phosphate buffer (20 mM, pH 6). Root tissues were homogenized (in a ratio of 1 mL/50 mg of tissue) inside a working solution containing 50 µM 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Sigma, St Louis, MO, USA) dissolved in a potassium phosphate buffer 20 mM pH 6 with 0.2 g/mL of porcine liver esterase (Sigma) and then incubated for 30 min at 25 °C on a shaker. Fluorescence (E_x 488 nm, E_m 525 nm) caused by the oxidation of DCFH to DCF was measured by a fluorometer (GloMax-Multi Jr, Promega, Madison, WI, USA). For statistical purposes, fluorometry experiments were performed on six samples.

To verify the effect of CS and BSG treatments on chlorophyll contents, the following methods were used: a) indirect measures of chlorophyll content were recorded with a quick method using the SPAD-502 chlorophyll meter (Konica Minolta, Japan). For each plant ten measurements were recorded between the base and the apex of each leaf lamina and their average calculated as single SPAD value; b) 3 leaves of each treated or untreated plant were sampled and three disks, for each of them, were collected and immediately placed in vials containing 10 mL Dimethyl sulfoxide (DMSO). Chlorophyll extraction was obtained following the Tait and Hik's method (2003). Total chlorophyll content and its concentration were determined by UV-Vis spectrophotometer (Mod. Lambda 25 - Perkin Elmer). Contents of total chlorophyll and chlorophyll *a* and *b*, were assessed by using the equations described by Barnes *et al*. (1992). ROS contents and chlorophyll contents were determined 5 and 10 days after treatments and 25 days later after a second CS and BSG treatments.

Statistical analysis

Data from pot experiment, chlorophyll and SPAD assessments were statistically analyzed by analysis of variance (ANOVA). The Least Significant Difference's Test (LSD's Test) was used for post-hoc analysis of physical and chemical main characteristics of CS and BSG extracts. Student's *t*-tests ($P \leq 0.05$ and $P \leq 0.01$) was used for experimental design of ROS analysis in which we wished to make pairwise comparisons between treatments and their respective controls. Statistical analysis was performed using the Plot IT program Ver. 3.2 (Scientific Programming Enterprises, Haslett, MI, USA).

Results and Discussion

Extracts physical and chemical characteristics

The BSG extract showed a pH value close to neutrality (6.9) whereas a sub acid pH (5.6) was recorded for the CS extract (Table 1). No significant difference was observed for electrical conductivity. Total nitrogen content was significantly higher in BSG than in CS (about 70 %). Total polyphenols were significantly lower in BSG extract in comparison to CS extract (about 15 %) ($P < 0.05$).

Both CS and BSG extracts exerted a bio stimulating effect at concentration of 1 and 3 %, whereas at concentration of 10 and 30 % BSG increased its bio stimulating effect and CS approached the GI toxicity threshold of 40 % (Zucconi *et al*., 1981), remaining, however, in the non-toxic range (Fig. 1). It is interesting to note that BSG extract, at a concentration of 10 %, showed a stimulating activity close to 140 % of the control.

As showed in Figure 2 (top), LC-MS/MS analysis of polyphenols of CS extracts allowed to detect 77 signals. Only 12 of them were identified as quinic derivate considering their MS^2 spectra as compared to those reported by Clifford *et al*. (2003; 2006) and Jaiswal *et al*. (2010). The 3 isomers of caffeoylquinic acid, 3-CQA, 4-CQA and 5-CQA, were identified using their $[M-H]^-$ at *m/z* 353 and the diagnostic signals with relative abundances at *m/z* 179, 191 and 173, respectively. Similarly, the feruloyl quinc isomers, 3-FQA, 4-FQA and 5-FQA, were identified using *m/z* 367 $[M-H]^-$ and the diagnostic signals at *m/z* 193, 173 and 191, respectively. The quinic acid (QA), feruloyl quinic acid-1 (FQA1), feruloyl quinic acid-2

Table 6. Effect of coffee silverskin (CS) and brewer's spent grain (BSG) extracts soil treatments, at two concentrations (50 and 100%), on plant growth and reactive oxygen species (ROS) accumulation in tomato roots (landrace Apulia Region).

Treatment	Dose (%)	5 days after 1st treatment		10 days after 1st treatment		5 days after 2nd treatment	
		ROS content	Plant weight (g)	ROS content	Plant weight (g)	ROS content	Plant weight (g)
BSG	**50**	3,482 ± 125	4.96 ± 1.38	3,738 ± 69**	6.45 ± 0.86*	3,682 ± 77	30.83 ± 2.63
BSG	**100**	3,725 ± 71	3.37 ± 0.79	3,840 ± 50**	5.36 ± 1.05*	2,999 ± 82	28.97 ± 1.31
CS	**50**	3,444 ± 127	4.57 ± 0.82	3,325 ± 129	7.91 ± 1.44	3,392 ± 157	26.89 ± 2.04
CS	**100**	3,777 ± 165	4.11 ± 1.08	3,764 ± 130**	6.42 ± 1.18*	4,681 ± 139**	29.93 ± 0.49
Control		3,372[1] ± 182	4.91 ± 0.94	3,131 ± 148	11.00 ± 0.93	3,360 ± 203	27.13 ± 3.06

[1]Each value is an average of fluorescence units (FSU 50 mg^{-1} root fresh weight) of two experiments each containing six replications ± SE; Asterisks indicate statistically significant difference in comparison to the untreated control according to Student's *t*-test (* for P≤0.05, ** for P≤0.01).

(FQA2), 3-sinapoyl-4-caffeoyl quinic acid (3Si-4CQA), 3-dimethoxycinnamoyl quinic acid (3-DQA) and sinapoyl quinic acid (SiQA) were identified by partial matching with the expected monoisotopic mass and the diagnostic signals and reported in Table 2.

In Figure 2 (bottom), 35 signals detected in LC-MS/MS analysis of BSG extract were reported. Unfortunately, no one of them matched with those described by Quifer-Rada *et al*. (2015) and Munekata *et al*. (2016) for beer polyphenols and residues. Therefore, Table 3 reports a structural hypothesis about the possible nature of the molecules found in BSG.

Pot experiment

In Table 4 the effects of the CS and BSG extracts on the growth of tomato plants infested by *M. incognita* are reported. The nematode caused a significant reduction in fresh and dry top weight of tomato plants in comparison to CS and BSG treatments. Fresh

Table 7. Effect of different concentrations of coffee silverskin (CS) and brewer's spent grain (BSG) aqueous extracts on chlorophyll content (Chl) of leaves of treated or untreated (control) tomato plants (landrace of Apulia Region) at 5, 10 and 25 days after treatments.

15/11/2017 (5 days – after the first treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	27.6* ± 1.7	a**	7 ± 1.6	a	34.6 ± 3.2	a
CS	**100**	26.7 ± 4	a	7.3 ± 1.1	a	34.1 ± 5.1	a
BSG	**50**	28.9 ± 2.7	a	8.1 ± 0.7	a	37 ± 3.4	a
BSG	**100**	26.7 ± 2.9	a	7.7 ± 1.2	a	34.3 ± 4	a
Control		28.1± 1.3	a	7.9 ± 0.4	a	36.1 ± 1.7	a
20/11/2017 (10 days - after the first treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	29.4 ± 2	a	7.4 ± 0.3	a	36.7 ± 2.1	a
CS	**100**	31.4 ± 0.6	a	8.3 ± 0.4	a	39.7 ± 0.9	a
BSG	**50**	28.8 ± 1.9	a	7.8 ± 0.6	a	36.5 ± 1.7	a
BSG	**100**	29.3 ± 2.1	a	7.8 ± 0.7	a	37.2 ± 2.5	a
Control		32 ± 0.7	a	8.4 ± 0.4	a	40.5 ± 1.1	a
05/12/2017 (5 days - after the second treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	33.6 ± 1.7	a	11.4 ± 2.7	a	45.1 ± 4.4	a
CS	**100**	29.7 ± 0.7	b	7.9 ± 0.1	a	37.7 ± 0.7	a
BSG	**50**	34.3 ± 1.9	a	8.9 ± 0.7	a	43.2 ± 2.6	a
BSG	**100**	33.3 ± 0.2	a	9.1 ± 0.2	a	42.4 ± 0.4	a
Control		33.3 ± 2.2	a	9.5 ± 1.2	a	42.8 ± 3.4	a

* Each value is an average of 3 replications ±SE;** Data followed in each column by the same letter are not significantly different according to Least Significant Difference's Test (LSD's Test) (P≤0.05).

and dry top weights ranged between 52.5 and 101.5 g and 5.8 and 12.4 g, respectively. No statistical difference was observed between the 2 controls (Fenamiphos treated and untreated). All CS and BSG treatments did not differ from each other for plant fresh and dry top weights (P=0.01). These morphological parameters were not different in CS treated plants and in Fenamiphos treated plants (P=0.01) either. On the contrary, a significant difference was observed between the 2 BSG treatments and the Fenamiphos one (P=0.05). Both CS and BSG treatments had a stimulating effect on tomato growth compared to the untreated plants. Plant heights across treatments ranged between 85.6 cm and 94.2 cm, being not significantly different from those of control pots (73.8) at P=0.01.

Root weights in plants treated with BSG at a concentration of 50 % (18.6 g/pot) and Fenamiphos (4.7 g/pot) were significantly higher and lower, respectively, than those in the untreated control (12.3 g/pot) (Table 4). The higher root weight of tomato plants in comparison to Fenamiphos treated pots was due to the presence of numerous galls increasing root weight as already reported by D'Addabbo and Sasanelli (2005). All the other treatments did not influence root weight.

The nematological analysis pointed out that both CS and BSG treatments, independently from the dose, did not reduce root gall index (RGI), if compared to the untreated control (Table 5) (P=0.01). On the contrary, Fenamiphos was able to reduce the RGI to 1.4, a value significantly lower than those of all other treatments, including the untreated control, that spanned from 3.8 to 5.4.

Nevertheless, CS treatments were effective to reduce eggs and juveniles/g root by 50.9 and 44.4 % respectively, compared to the untreated control (Table 5) (P=0.01), irrespective of the concentration used. In addition, the observed nematicidal effect was no significantly different from that observed in Fenamiphos treated pots (P=0.01). CS treatments were more effective than BSG treatments in reducing nematode population on the roots (P = 0.05).

Soil nematode population density was lowered by CS 100 % (4.8 eggs and juveniles/mL soil) and BSG 50 % (5.4 eggs and juveniles/mL soil) treatments and was no statistically different from Fenamiphos (5.8 eggs and juveniles/mL soil) and the untreated control (8.2 eggs and juveniles/mL soil).

The final nematode population density, calculated summing nematodes from roots and soil, was reduced in CS 50 % and 100 % treatments by 47.8 and 35.6 %, respectively if compared to the untreated control. Interestingly, this parameter, in the CS 50 % treatment, was not significantly different (P = 0.05) from that recorded in Fenamiphos treated pots. By contrast, BSG treatments, at both concentrations, were not effective in reducing the final nematode population in comparison to the control.

The same results were obtained for the nematode reproduction factor (*Pf*/*Pi*). The lowest and the highest reproduction factors were recorded in Fenamiphos and BSG 50 % treatments, respectively (Table 5).

It is well known that substances with a high polyphenols content display a substantial nematicidal effect, the intensity of which is also related to the species of nematode concerned. Considering that CS and BSG extracts have both a high content of polyphenols, their different performances in assuring nematode control can be explained with their different polyphenols composition. This hypothesis is in line with the results of D'Addabbo *et al*. (2013), who studied the nematicidal activity of pure chlorogenic and caffeic acids and the extract of *Artemisia annua* on the nematodes *M. incognita*, *Globodera rostochiensis* (Woll.) Behrens and *Xiphinema index* Thorne *et* Allen. They found a high effect of these compounds on *G. rostochiensis*, a partial response on *X. index* and a low activity on *M. incognita*. Chlorogenic acid was able to elicit 100 % of juvenile mortality in *X. index* at a concentration of 125 µg/mL after 8 h of exposure, whereas caffeic acid produced the same result after 4 hrs of exposure, at the same concentration. The authors found a very little effect of these compounds against *M. incognita*, with the maximum observed effect after more than 24 h of exposure at the maximum concentration (500 µg/mL). The plant extracts were not able to produce the same nematicidal activity observed for pure chlorogenic and caffeic acids. However, *A. annua* extract was able to reduce significantly (50 %) the hatching percentage of eggs of *M. incognita* and *G. rostochiensis* in comparison to the control (distilled water). Considering these results, it is plausible that caffeoyl and feruloyl quinic derivates, identified in the CS extract, could be responsible for the observed nematicidal effect on the *M. incognita* population in our pot experiment.

Effects of CS and BSG treatments on ROS and Chlorophyll levels

In plants ROS are implicated as key signaling molecules in the regulation of numerous biological processes such as growth, development and responses to biotic and/or abiotic stimuli (Baxter *et al.*, 2014). ROS production was used as a phytotoxicity index of the tomato roots exposed to both doses of CS and BSG extracts. No ROS accumulation was detected in roots 5 days after treatments at the two applied doses (50 and 100 %) (Table 6) in comparison to the untreated plants. A significant increase (P=0.01) in ROS content was detected 10 days after BSG treatments in roots at both concentrations. In roots treated with 100 % of CS a significant ROS increase was recorded (Table 6). Twenty days after the first treatment, plants were newly treated with both extracts and ROS amount was evaluated 5 days later. A significant ROS accumulation was evident only in roots treated with 100 % CS compared to untreated and BSG-treated plants.

The treatments CS 100 %, BSG 50 % and BSG 100 % elicited the highest ROS values, which considerably affected the growth of tomato plants (Table 6). Five days after the second treatment plants recovered the loss of weight, compared to control. Because cell membranes are one of the major sites of ROS activity under environmental stress (Mittler, 2002), higher levels of ROS in CS- and BSG-treated plants might induce cellular damage leading to apoptic-like programmed cell death. Moreover, as high levels of ROS serve as substrates for synthesis of secondary metabolites

or as components enforcing the physical barrier of the cell walls their accumulation in treated roots alert plants to limit nematode infection.

Chlorophyll content (Chl) in all treated tomato plants did not differ from that of the control at 5 and 10 days after the first treatment (Table 7). No difference, at both observation times, was found in total chlorophyll, Chl *a* and *b*. After the second treatment, a significant difference was observed in chlorophyll *a* content in plants treated with CS 100 % (29.7 μg/cm^2) in comparison to all other treatments included the control (33.3 μg/cm^2) (Table 7). It is well known that ROS enhancement can cause the degradation of photosynthetic pigments and damage to photosynthetic machinery (Mittler, 2002). High ROS levels found after treatments may have directly or indirectly contributed to the decline in the observed chlorophyll levels.

Conclusions

Yield losses caused worldwide by root-knot nematodes require to find ecofriendly control strategies with low environmental impact (Stirling, 2014) considering that the control of these pests is meeting with difficulties as current national and international regulations are limiting the use of synthetic nematicides which exert serious detrimental effects on the environment (Sánchez-Moreno *et al*., 2009). In our study both tested by-product extracts were not phytotoxic to tomato plants as shown by the morpho-physiological parameters of the treated plants. All this makes CS extracts of economic relevance, as alternative products to be used in sustainable strategies for nematode management. The observed nematicidal effect of CS extract against *M. incognita* is related to the release of polyphenols (caffeoyl and feruloyl quinic derivates) from the coffee epidermis as demonstrated for other agro-industrial by-products as grape pomace (D'Addabbo *et al*., 2000) or olive mill wastes (Sasanelli *et al*., 2002).

Future research needs consist of the setup of techniques able to produce commercial CS extracts with a standardized composition and known nematicidal efficacy. However, further studies are also needed to investigate the effect of CS extracts on different nematode species and types of soils.

Conflict of Interest

Authors state no conflict of interest

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