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Plant defense elicitors' purification in soybean and bean from pathogenic nematode

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Plant resistance induction against pathogens is an alternative disease control method, which involves the activation of the plant defense mechanisms, such as the phytoalexins induction. The eliciting molecules have the capacity of inducing and activating such responses and thus, techniques have searched to isolate and characterize fractions with eliciting aspect. This study aimed to purify, through ion exchange chromatography and gel filtration chromatography, eliciting molecules from phytopathogenic nematodes, and test them in the induction of phaseolin in bean hypocotyls and gliceolin in soybeans cotyledons. A Tris HCl 0.05 M (pH 6.8) buffer was used as control treatment and acibenzolar-S-methly (50 mg i.a. L^{-1}) and *Saccharomyces cerevisiae* (20 mg m L^{-1}) was used as induction standard treatment. Ion exchange chromatography (IEC) and gel filtration chromatography (GFC) were applied to separate power eliciting fractions from five hundred female root knot nematodes (*Meloidogyne javanica*). The purification of elicitors, through IEC, resulted in sixty glicidic fractions and six glycoprotein ones. They were classified according to their nature, being twenty-six glicidic fractions and thirty-seven glycoprotein ones, with molecular masses ranging from 29.19 to 2,989.25 kDa. From the purified fractions, eight of them presented phaseolin inducing effect potential, whereas fifteen fractions presented gliceolin inducing effect. Chromatography proved to be efficient in purifying the eliciting compounds. Compounds having gliceolin and phaseolin suppressing characteristics were verified in the bioassays. For those fractions obtained through IEC and posteriorly submitted to GFC that did not induce phytoalexin, it is suggested that the molecules need to act jointly so there is eliciting effect and thus induce a defense response in the plant.

Key words: gel filtration chromatography, ion exchange chromatography, *Meloidogyne javanica*, phytoalexins, resistance induction.

INTRODUCTION

In phytopathology, resistance is the rule and susceptibility to pathogenic agents is the exception. The flourishing and development of an illness is the result of the interaction between susceptible host, virulent pathogenic agent and favorable environment. The incompatibility of these three components results in the non-manifestation of the disease. In this sense, one of the primary events to occur is the recognition of the pathogenic agent by the hosting plant and the activation of its defense mechanisms (Pascholati, 2011). Such mechanisms are responsible for the plant resistance, so that it can be induced by the expression of a set of defense genes, which aim to restrain the pathogen growth and/or activity, as well as diminish the incidence of diseases (Métraux, 2001) of biotic origin in plants. Thus, the resistance induction in plants against illnesses has become a fundamental tool for research tuned at studies over phytosanitary issues. One of the main focus of agro-ecological agriculture is the alternative control of diseases by the induction of plant defense mechanisms. Bonaldo et al. (2005) suggested that the pathogenic agent control such as viruses, bacteria, fungi and nematodes could be established by resistance mechanism induction and that, according to Stangarlin et al. (1999), are activated by eliciting molecules, which they produce against phytopathogens.

The elicitors are molecules released by the pathogen or by the plant itself, by the pathogen action, and that are recognized by present receptors in the plant cell membrane (Durrant and Dong, 2004; Jalali et al., 2006). The pathogen elicitors belong to a signaling molecule class that participates in the signal swapping between plant and pathogenic agent (Kamoun, 2006) able to induce the phytoalexin synthesis (Hahn, 1996), which to Barros et al. (2010) and Mazaro et al. (2013) are biocide compounds that act on the plant biochemical defense against pathogens. The soybean isolated gliceolin (Paxton, 1995) and phaseolin in beans (Müller, 1958), are examples of phytoalexins being studied currently and that perform an important role as a plant-pathogen interaction defense response (Franzener et al., 2000). Currently, there are resistance inductors available in the market, whose purified and tested molecules presented an elicitor aspect able to activate and/or induce defense responses in plants, such as Messenger™, a formulation obtained from the harpin protein from the bacteria *Erwinia amylovora* (Sobrinho et al., 2005). The elicitor characterization present in nematode females has as its objective to optimize the advance in studies of elicitor molecule products and develop viable sustainable alternatives for disease control. The elicitor molecule capacity originating from nematode females in the phytoalexin induction is still unknown.

This study seeks to purify, through ion exchange chromatography and gel-filtration chromatography, protein and glicidic elicitor molecules, from the root knot nematode (*Meloidogyne javanica*) and characterize them as being molecules with phaseolin phytoalexin inductor potential in bean hypocotyl and gliceolin in soybean cotyledons.

MATERIALS AND METHODS

The *Meloidogyne javanica* population used was obtained from "Santa Clara" tomato cv. plants and identified based on the perineal configuration (Hartman and Sasser, 1985). With the aid of a needle, 500 females were detached from their galls and were macerated in a 200 µl Tris HCl buffer at 0.5 M, pH 6.8 in a microcentrifuge tube with the aid of a glass rod. The sample volume was completed to 1 ml using the same buffer and subsequently filtered in syringe filters (Millipore) with 0.45 µl in diameter, being the sample final volume adjusted to 2 ml with the same buffer.

Firstly, an ion exchange chromatography (IEC) was conducted. A volume of 1.5 ml from the prepared sample was applied and eluted with a buffer (Tris-HCl 0.025 M (pH7.5)), in a 1.5 ml min^{-1} flux being collected in 6 ml fractions. After the non-absorbed material removal, the material retained was displaced by NaCl linear gradient on the buffer in sub sequential concentrations from 0 to 100%, and was determined by electrical conductivity. The fractions were eluted in NaCl and only those on the Tris-HCl buffer were dialyzed in 12 to 16 KDa molecule exclusion limit membranes against polyethylene glycol (PEG) 20,000, according to Franzener (2011). The aliquot of fractions obtained were adjusted to a final volume of 3 ml with a Tris-HCl 0.05 M (pH 6.8). From this elution pattern, 12 fractions were obtained, which represent the treatments.

Next, purification was performed by the gel-filtration chromatography (GFC). From the fractions compiled through IEC, 1.5 ml of sample was applied over the column bed, eluting the sample with the buffer in a flux of 0.5 ml min^{-1}, with 1 ml of the GFC elution pattern fraction collected which resulted in 63 fractions, and this represent the treatments. The protein relative molecular masses of each fraction, obtained by GFC, were calculated by the equation:

$$y = 8867.4e^{-3.231x}$$

The standard curve for the dosage of carbohydrate reducers was performed through the Lever method (1972):

$$y = 0.0414x - 0.0297$$

The total content quantification of proteins was calculated by a standard curve, the Bradford method (1976):

$$y = 0.0434x + 0.0431$$

The IEC and GFC protein and glicidic fractions were collected according to their distribution patterns and monitored via

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spectrophotometer at 280 and 410 nm, respectively. They were then tested for their capacity of inducing gliceolin phytoalexins in soybean cotyledons and bean hypocotyls.

Bioassay for the production of phytoalexins in soybean cotyledons

Soybean seeds (*Glycine max* L.), Cultivar VMAX, were sanitized in sodium hypochlorite 1% (5 min), washed with distilled water, and sowed in plastic trays containing autoclaved sand (three times for 1 h at 121°C and 1 atm). After 7 days, the recently-opened cotyledons were detached from the seedlings for the bioassay execution. The cotyledons were placed on a petri dish (five cotyledons/dish) containing two sheets of paper filter sterilized and moistened with 1 ml of sterilized distilled water. A “wedge” was made on each cotyledon, on the adaxial surface, with the aid of a stiletto and, on each one of them was added an aliquot of 20 µl of the treatments (fractions). The dishes were incubated in a biochemical oxygen demand (BOD) incubator at 25°C, in the dark, for 20 h. After this period, the cotyledons were transferred to vials containing 15 ml of sterilized distilled water and left for shaking in an orbital shaker/150 rpm for 1 h, for gliceolin extraction.

Subsequently, the phytoalexins (gliceolin) were measured by supernatant reading in a spectrophotometer at 285 nm. The cotyledons were washed with distilled water, dried up and weighed on an analytical balance. The gliceolin content in the sample was obtained by the absorption value divided by the cotyledons’ mass (ABS/gmf^{-1}). As treatment control, a solution buffer Tris-HCl 0.05 M was used, and as induction treatment pattern, the *Saccharomyces cerevisiae* cell suspension was used (20 mg ml^{-1} of commercial product Fleischmann Fresh Biological Yeast) (Meinerz et al., 2008).

Bioassay for the production of phytoalexins in bean hypocotyls

Bean seeds (*Phaseolus vulgaris* L.) Cultivar IPR-Colibri were sanitized in sodium hypochlorite 1% for 5 min and washed with distilled water, and sowed in plastic trays containing autoclaved sand (three times for 1 h at 121°C and 1 atm). After 7 days, etiolated hypocotyls segment with 0.5 cm were detached from the seedlings, washed with distilled water and dried up over a sterile paper filter. Four (4) segments of hypocotyls were placed in a microcentrifuge tube and immerged in 500 µl of the purified fractions. The tubes were kept at 25°C in the dark for 48 h. The treated hypocotyls were transferred to vials containing 4 ml of ethanol 80%, kept at 4°C, for 48 h, and shaken (orbital shaker/150 rpm) for 1 h for phaseolin extraction. Next, the phytoalexins (phaseolin) were measured by supernatant reading in a spectrophotometer at 280 nm. Afterwards, the hypocotyls were washed in distilled water, dried up and weighed on an analytical balance. The content of phaseolin in the sample was obtained by the cotyledons’ mass (ABS/gmf^{-1}). As treatment control, a solution buffer Tris-HCl 0.05 M was used, and as induction treatment pattern the plant defense inductor acibenzolar-s-methyl (50 mg $i.a.L^{-1}$) (Bion ™) was used (Bailey and Burden, 1983).

Statistical analysis

The phytoalexins assays were conducted by completely randomized design, with 12 treatments (fractions) for IEC and sixty-three fractions for GFC, with four repetitions. The data were submitted to analysis of variance and Scoot-Knott test ($P \leq 0.05$) was executed. The homogeneity of the variances was determined by the Liliefors test. Data transformation was used when made necessary. The software used for statistical analysis was Genes (Cruz, 2006).

RESULTS

The majority of peaks for purified carbohydrates presented little magnitude, however, the presence of a peak of glicidic nature of higher absorbency was registered (0.132 nm), not coinciding with the protein peak, which corresponds to the material retained in the resin. As an anionic exchange, resin was used, the carbohydrates which remained attached to it presented negative liquid charge; thus they needed to be eluted in NaCl conditions. For the other protein peaks which coincided with the glicidic ones, they were considered to be glycoproteins (Figure 1). The molecule purification of female nematodes *M. javanica* by IEC that presented fractions with similar spectrophotometric aspect were collected in three fractions of glycoprotein nature (4, 5, 6) and three of glicidic nature (fractions: 1, 2, 3) to those not attached to the resin (eluted against a NaCl concentration gradient and subsequently, dialyzed with distilled water) three fractions of glycoprotein nature were registered (10, 11, 12) and three of glicidic nature (7, 8, 9,) (Figure 1, Table 1).

Bioassay of phytoalexins in bean hypocotyls

The fractions purified by IEC from females of *M. javanica* and tested in the production of phaseolin phytoalexin in bean hypocotyls are presented on Table 1. For the Scoot-Knott test, fractions 1, 6 and 5 (not attached to the resin) were grouped in the 1st batch, which presented elicitor aspect, with activating potential of phaseolin phytoalexin, of which, the total protein concentrations detected were 0.000 μl^{-1}, 0.412 µg ml^{-1}, and 0.343 µg ml^{-1}, respectively. For carbohydrate contents, 1.249, 2.529, and 2.214 µg ml^{-1} were obtained, respectively. Also, for fraction 3, which responded similarly to acibenzolar-s-methyl (ASM), the total protein and carbohydrates were 0.000 and 0.838 µg ml^{-1}, respectively. It should be pointed out that, for these fractions, as well as for the ones grouped in other batches, the carbohydrate concentrations presented themselves to be always higher when compared with the ones of total protein.

The sample quantity used to accomplish the total protein analysis was 50 µl, due to the amount available for running the tests. This amount, in turn, may exercise an influence on the reading and not represent the real quantity of protein existing in the sample. Fractions 1, 6 and 5 inducted the phaseolin synthesis in 35.17, 29.05 and 28.44%, respectively, values superior to the ASM induction standard treatment. In relation to fraction 3, it delivered the same phaseolin induction level as the standard treatment. Regarding fractions 7, 10, 4 and 2, no influence was verified on the phytoalexin induction, being their values similar to the Tris HCl 0.05 M control treatment. As to fractions 11, 8, 12 and 9, they presented suppression effect on the phaseolin phytoalexin induction,

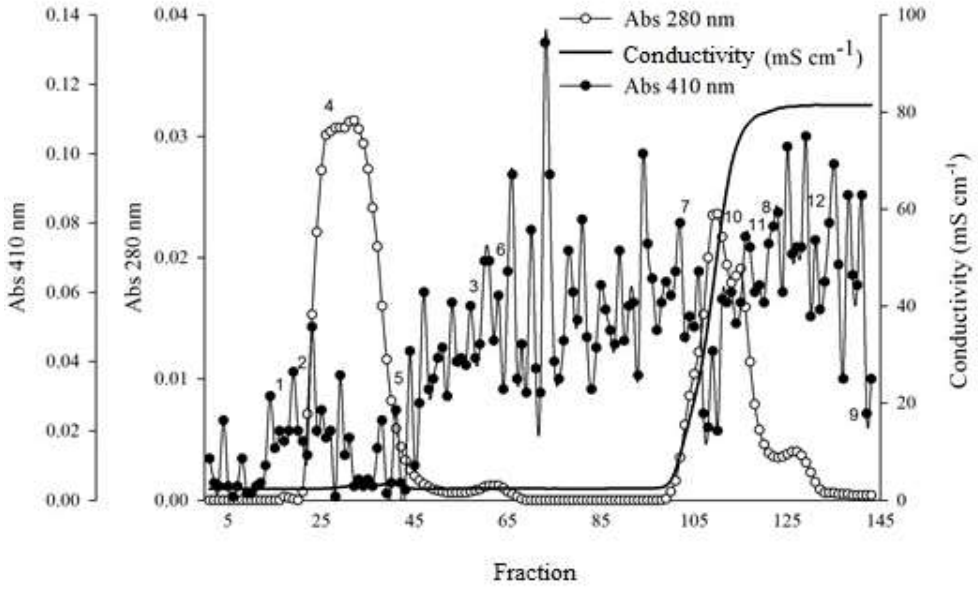


Figure 1. Ion exchange chromatography from samples of females of the nematode Meloidogyne javanica. A sample of 1.5 ml was applied over a glass column (5 cm × 20 cm) fulfilled with anion-exchange resin (UNOsphere™ Q Strong Anion Exchange) balanced and eluted with Tris-HCl 0.025 (pH 7.5) buffer, being collected fractions of 6 ml, in a 1.5 ml min^{-1} flux. The absorbed material was eluted with a NaCl linear gradient (0 to 100%) on the same buffer. Protein (○) was determined in 280 nm and carbohydrates (●) by the Lever method (1972). Concentrations of NaCl (-) were determined indirectly by conductivity.

Table 1. Fractions, nature, total protein content (µg mL^{-1}), carbohydrate content (µg mL^{-1}) and the phaseolin accumulation in bean hypocotyls (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from ion exchange chromatography (IEC), from *Meloidogyne javanica.*

Fractions of IEC	Nature	Total protein content (µg protein mL^{-1})	Carbohydrate content (µg glucose mL^{-1})	Phaseolin (280 nm $g.m.f.^{-1}$)
1	Glicidic	0.000	1.249	17.68^{A}
6	Glycoprotein	0.412	2.529	16.88^{A}
5	Glycoprotein	0.343	2.214	16.80^{A}
3	Glicidic	0.000	0.838	15.01^{B}
7	Glicidic	0.000	1.683	11.83^{C}
10	Glycoprotein	0.786	1.852	11.32^{C}
4	Glycoprotein	0.878	1.877	10.90^{C}
2	Glicidic	0.000	1.128	10.73^{C}
11	Glycoprotein	0.117	1.732	7.64^{D}
8	Glicidic	0.000	0.789	6.69^{D}
12	Glycoprotein	0.343	2.456	6.30^{D}
9	Glicidic	0.000	1.828	5.74^{D}
ASM *	--	--	--	13.08^{B}
Tris HCl **	--	--	--	10.62^{C}
CV %				15

*Acibenzolar-S-methyl (50mg i.a. L^{-1}), used as inducting standard treatment; ** Buffer solution used as mobile phase in the chromatography (control treatment); Scoot-Knott test at 0.05%.

with a reduction of 28.06, 37.00, 40.68 and 45.95%, respectively, in relation to the control treatment.

In the attempt of purifying new fractions of females of *M. javanica* with phytoalexin potential inductor, those obtained by IEC were subjected to GFC and it was possible to obtain different peaks. It is observed that the GFC patterns are represented on Table 2, whose total collected fractions, according to the elution pattern, resulting in 63 fractions. The molecule batch purified by the technic presented glycoprotein and glicidic nature with known molecular masses. The majority of purified fractions presented in their composition a higher concentration of carbohydrates than of proteins, except fractions 7 (10.873 and 3.326 µg ml^{-1}), 33 (7.102 and 3.085 µg ml^{-1}), 20 (4.848 and 3.930 µg ml^{-1}), 26 (6.833 and 3.374 µg ml^{-1}), 36 (4.614 and 3.423 µg ml^{-1}) and 5 (2.793 and 2.674 µg ml^{-1}) in which the protein concentration was superior to the carbohydrate, all of them being from glycoprotein nature.

The utilization of GFC proved to be efficient to the purification of compounds coming from IEC, with potential for activity over bean hypocotyls, although not all fractions presented the same property (Table 3). Through the Soot-Knott test, the fractions 6, 15, 17, 2 and 22 were grouped in the batch I, and indicated a greater potential of phaseolin inducting activity, characterized as being elicitors of glycoprotein natures, with average values of 124.59, 98.68, 94.06, 86.14 and 77.56, respectively, greater than the ASM standard treatment. These fractions correspond to those obtained at IEC and not attached to the resin (positive charge).

IEC fractions 1 and 5, which presented phaseolin phytoalexin inducting activity, when submitted to GFC resulted in the fractions 15, 17 and 2, which inducted defense responses in bean hypocotyls, with molecular masses around 329.31, 78.89 and 107.62 kDa, respectively. As to fraction 2 (positive liquid charge molecules) and 7 (negative liquid charge molecules) purified by IEC they did not deliver phaseolin inducting effect, however when submitted to GFC it resulted in fractions 6 and 22 of elicitor aspect, with molecular masses of 114.51 and 188.25 kDa, respectively.

Regarding fractions 7 (glycoprotein nature and molecular mass of 69.66 kDa), 56 (glicidic nature) and 3 (glycoprotein nature and molecular mass 61.52 kDa) obtained via GFC, these also presented phaseolin inducting potential, with 45.05, 44.39 and 38.94%, respectively, compared to ASM treatment. Fraction 7, which demonstrated inducting potential, corresponds to fraction 2 from IEC, which did not deliver phaseolin inducting effect, whereas, fraction 56, corresponding to fraction 9 (negative liquid charge molecules) from IEC, presented phytoalexin suppressing aspect. Yet, fraction 3 obtained at GFC, corresponding to fraction 1 (positive liquid charge molecules) from IEC, inducted phytoalexin synthesis.

Fractions 33, 20, 27, 34, 16, 26, 29, 36, 55, 1, 54, 45, 24, 42, 40, 41, 21, 38 and 25 presented the same level of phaseolin induction as ASM standard treatment, so that, for those of glycoprotein nature, molecular masses are known. The other fractions presented did not induct phytoalexins, being the values similar to Tris HCl 0.05 M standard control.

Bioassay of phytoalexin on soybean cotyledons

The fractions purified by ion exchange chromatography from females of *M. javanica* and tested in the production of gliceolin phytoalexin on soybean cotyledons are presented on Table 4. Based on the Scoot-Knott test, fraction 12 (attached to the resin), of glycoprotein nature, grouped in the 1st batch, delivered total protein and carbohydrate concentrations in 0.343 and 2.456 µg ml^{-1}, respectively. Fractions 8 and 9 (not attached to the resin), both from glicidic nature, were grouped in the 2nd batch, having as contents of total protein 0.0 and 0.0 µg ml^{-1} and as contents of carbohydrates 0.789 and 1.828 µg ml^{-1}, respectively. In addition to this, for all fractions tested at the phytoalexin assay, carbohydrate concentrations presented to be always higher in relation to the total protein values (Table 4).

The inducting standard treatment (*Saccharomyces* cerevisiae), known as phytoalexin inductor in soybean, produced a higher level of induction of gliceolin. Therefore, fractions 12, 8 and 9 also presented phytoalexin inducting effect, however with values 17.39, 56.52 and 60.87%, respectively, lower than the standard treatment. Total protein and carbohydrate contents found for fraction 8 were 0.000 and 0.789 µg ml^{-1}, respectively, and for fraction 9 were 0.000 and 1.828 µg ml^{-1}. Yet, it is verified that, for both of them, there were not registered total protein contents, but only carbohydrate content, probably for being glicidic in nature. In relation to fractions 11, 5 and 4, it was not observed gliceolin induction effect, these values being similar to the Tris HCl 0.05 M control treatment. Fractions 2, 7, 6, 10, 3, 1, suppressed the gliceolin phytoalexin induction in 39.28, 58.93, 62.50, 96.46, 96.43, 98.21%, respectively, when compared to the control treatment.

In soybean cotyledons, the GFC fractions also delivered to be efficient, as fractions 7, 17 and 26 were inserted in batch I, according the Scoot-Knott test, and presented gliceolin activity superior to the *S. cerevisiae* inducting standard treatment at 421.05, 410.53 and 289.47%, respectively (Table 5). Fractions 7 and 17 recorded molecular masses of 69.66 and 78.89 kDa and correspond to fractions 2 and 5 (not attached to the resin from IEC), which did not induct phytoalexin synthesis. Regarding fraction 26, of molecular mass of 176.91 kDa, it corresponds to fraction 9 from IEC, which was, initially, attached to IEC resin. The results indicate that for this fraction there was no elicitor compound loss during the dialysis by membrane phase, once its molecular exclusion limit is from 12 to 16 kDa, which usually may occur during the sample salt removing procedure (Table 5).

Table 2. Glycoprotein and glicidic fraction chromatograms from ion exchange chromatography (IEC) with their corresponding fractions obtained by gel filtration chromatography (GFC), from *Meloidogyne javanica*.

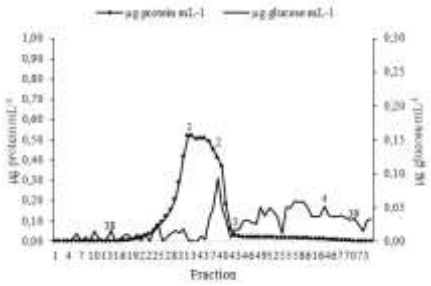
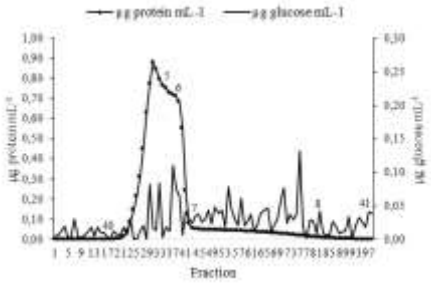
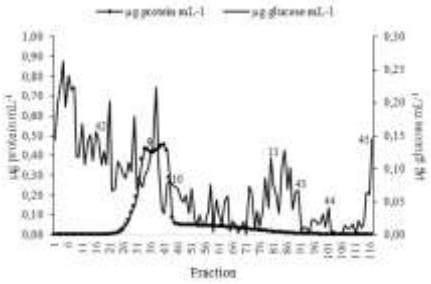
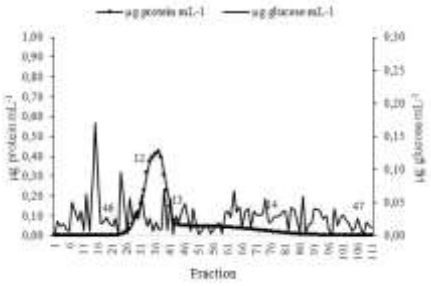
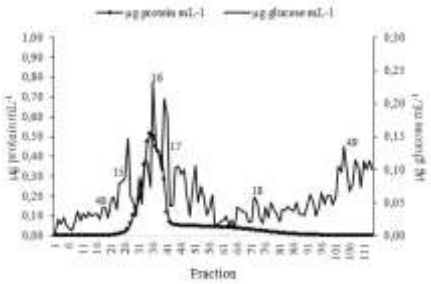
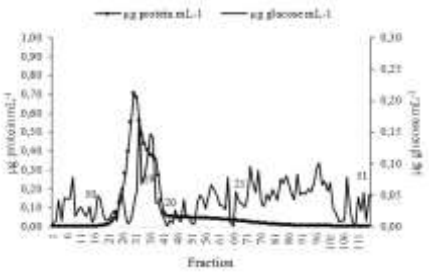
Fractions IEC	Fractions GFC	Profile
1	38 1 2 3 4 39	
2	40 5 6 7 8 41	
3	42 9 10 11 43 44 45	
4	46 12 13 14 47	
5	48 15 16 17 18 49	

Table 2. Contd.

	50 19 20 21	
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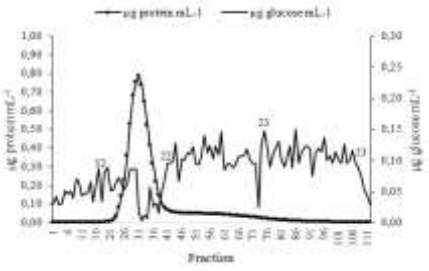
	51	
	52 22 23	
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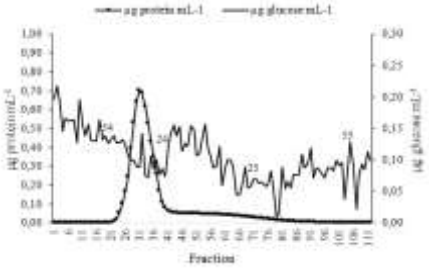
	53	
	54 24 25	
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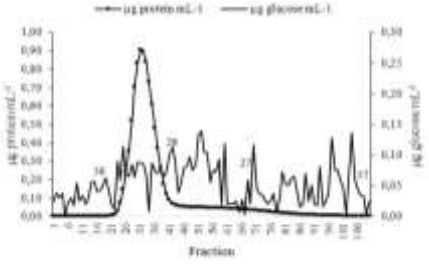
	56 26 27	
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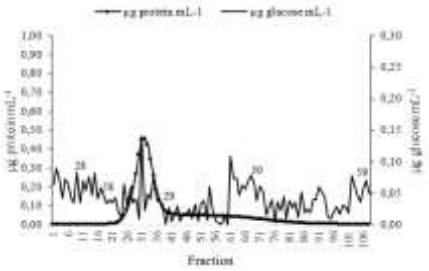
	28 58 29 30	
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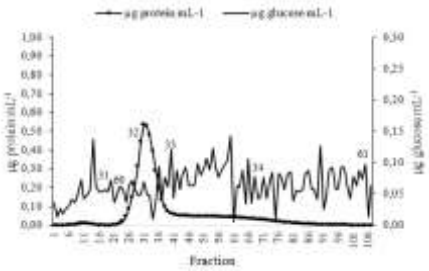
	31 60 32 33 34	
11		
	61	

Table 2. Contd.

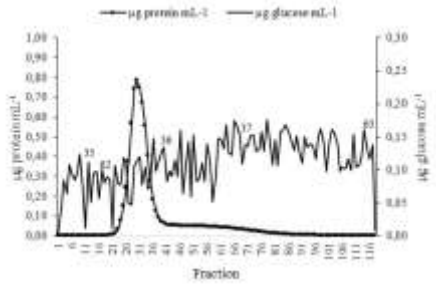
12	35	

Table 3. Fractions, nature, total protein content (µg mL^{-1}), molecular mass (kDa), carbohydrate content glucose (µg mL^{-1}), and the phaseolin accumulation in bean hypocotyls (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from gel filtration chromatography (GFC), from *Meloidogyne javanica*.

Fractions of GFC	Nature	Total protein content (µg protein ml^{-1})	Molecular mass (kDa)	Carbohydrate content (µg glucose ml^{-1})	Phaseolin (280 nm $g.m.f.^{-1}$)
6	Glycoprotein	1.142	114.51	3.229	13.61^{A}
15	Glycoprotein	0.159	329.31	3.882	12.04^{A}
17	Glycoprotein	10.873	78.89	3.326	11.76^{A}
2	Glycoprotein	0.505	107.62	2.626	11.28^{A}
22	Glycoprotein	3.869	188.25	4.220	10.76^{A}
7	Glycoprotein	1.280	69.66	3.471	8.79^{B}
56	Glicidic	0.000	--	3.471	8.75^{B}
3	Glycoprotein	0.067	61.52	2.457	8.42^{B}
33	Glycoprotein	7.102	166.25	3.085	7.44^{C}
20	Glycoprotein	4.848	101.13	3.930	6.69^{C}
10	Glycoprotein	0.820	57.81	4.002	6.44^{C}
27	Glycoprotein	0.659	37.42	4.147	6.41^{C}
34	Glycoprotein	0.159	54.33	3.519	6.21^{C}
16	Glycoprotein	1.392	114.51	3.664	6.19^{C}
26	Glycoprotein	6.833	176.91	3.374	6.13^{C}
29	Glycoprotein	0.113	176.91	3.302	5.94^{C}
36	Glycoprotein	4.614	213.16	3.423	5.93^{C}
55	Glicidic	0.000	--	2.940	5.84^{C}
1	Glycoprotein	0.243	156.24	2.698	5.69^{C}
54	Glicidic	0.000	--	3.205	5.58^{C}
45	Glicidic	0.000	--	3.278	5.55^{C}
24	Glycoprotein	1.964	176.91	3.205	5.52^{C}
42	Glicidic	0.000	--	3.060	5.43^{C}
40	Glicidic	0.000	--	2.795	5.39^{C}
41	Glicidic	0.000	--	2.867	5.30^{C}
21	Glycoprotein	0.947	47.98	2.988	5.27^{C}
38	Glicidic	0.000	--	3.109	5.26^{C}
25	Glycoprotein	0.059	47.98	3.471	5.24^{C}
46	Glicidic	0.000	--	2.795	5.21^{D}
49	Glicidic	0.000	--	2.360	5.21^{D}
39	Glicidic	0.000	--	3.205	5.09^{D}
8	Glycoprotein	0.455	42.37	4.002	5.02^{D}
37	Glycoprotein	0.059	54.33	3.616	4.99^{D}
12	Glycoprotein	0.021	107.62	3.036	4.99^{D}
47	Glicidic	0.000	--	3.616	4.99^{D}

Table 3. Contd.

4	Glycoprotein	0.044	33.04	4.002	4.95^{D}
52	Glicidic	0.000	--	2.964	4.95^{D}
31	Glycoprotein	0.417	2.123.97	3.640	4.87^{D}
61	Glicidic	0.000	--	0.790	4.85^{D}
30	Glycoprotein	0.113	54.33	3.036	4.85^{D}
35	Glycoprotein	0.466	2.989.25	4.002	4.81^{D}
13	Glycoprotein	0.021	89.31	3.616	4.75^{D}
53	Glicidic	0.000	--	2.529	4.73^{D}
59	Glicidic	0.000	--	2.481	4.70^{D}
18	Glycoprotein	0.521	42.37	3.688	4.68^{D}
50	Glicidic	0.000	--	2.577	4.65^{D}
57	Glicidic	0.000	--	3.930	4.61^{D}
44	Glicidic	0.000	--	2.981	4.59^{D}
5	Glycoprotein	2.793	188.25	2.674	4.57^{D}
32	Glycoprotein	0.348	188.25	3.254	4.55^{D}
28	Glycoprotein	0.267	2.723.24	2.988	4.49^{D}
14	Glycoprotein	0.036	42.37	3.809	4.45^{D}
63	Glicidic	0.000	--	1.200	4.42^{D}
23	Glycoprotein	0.924	42.37	3.205	4.40^{D}
51	Glicidic	0.000	--	2.601	4.31^{D}
19	Glycoprotein	0.735	200.32	3.688	4.29^{D}
60	Glicidic	0.000	--	2.360	4.22^{D}
11	Glycoprotein	0.498	29.19	2.263	4.18^{D}
62	Glicidic	0.000	--	1.635	4.17^{D}
48	Glicidic	0.000	--	2.432	4.16^{D}
58	Glicidic	0.000	--	2.312	4.07^{D}
43	Glicidic	0.000	--	3.713	4.07^{D}
9	Glycoprotein	0.659	121.86	3.785	3.96^{D}
ASM*	--	--	--	--	6.06^{C}
TrisHCl*	--	--	--	--	4.75^{D}
CV %					16

*Acibenzolar-S-methyl (50 mg i.a. L^{-1}), used as inducting standard treatment; ** Buffer solution used as mobile phase in the chromatography (control treatment); Scoot-Knott test at 0.05%.

Fraction 3, from glycoprotein nature (61.52 kDa), grouped in the 2nd batch, also presented gliceolin inducting response, with average value 284.21% higher than *S. cerevisiae* standard treatment. It corresponds to IEC fraction 1, in which any phytoalexin inducting activity was not observed, but rather suppressing (Table 4). In relation to the 3rd group, there are fractions (all of them with their corresponding molecular masses): 2 (107.62 kDa), 33 (166.25 kDa), 13 (89.31 kDa), 6 (114.51 kDa), 36 (213.16 kDa), 10 (57.81 kDa), 22 (188.25 kDa), 24 (176.91 kDa), 20 (101.13 kDa), 5 (188.25 kDa) and 29 (176.91 kDa), which delivered gliceolin inducting activity, with 231.58, 221.05, 189.47, 184.21, 136.84, 78.95, 63.16, 42.10, 26.31 and 15.79%, respectively, greater than the *S. cerevisiae* treatment. IEC fractions corresponding to these ones are presented on Table 2. It is worth mentioning that GFC fractions 36 and 37 resulted in a lesser gliceolin activity when compared to fraction 12 initially obtained by IEC, which delivered the same level of induction as the standard treatment. Thus, the defense response provided after the separation process by GFC resulted in fractions of low efficiency at gliceolin production.

Fractions 16, 19, 25, 23, 97, 14 and 30 delivered the same level of gliceolin induction as the standard treatment. In addition, all fractions of phytoalexin induction mentioned presented glycoprotein nature as a characteristic. The other fractions, grouped in the 5th batch, proved to be inefficient at induction of phytoalexin synthesis in soybean cotyledons, these values being similar to Tris-HCl 0.05 M control treatment (Table 5).

DISCUSSION

Among the methods deployed on the molecule

Table 4. Fractions, nature, total protein content (µg ml^{-1}), carbohydrate content (µg ml^{-1}) and the gliceolin accumulation in soybean cotyledons (nm $g.m.f^{-1}$) treated with the glycoprotein and glicidics fractions from ion exchange chromatography (IEC), from *Meloidogyne javanica*.

Fractions of IEC	Nature	Total protein content (µg protein ml^{-1})	Carbohydrate content (µg glucose ml^{-1})	Gliceolin (285 nm $g.m.f.^{-1}$)
12	Glycoprotein	0.343	2.456	0.19^{B}
8	Glicidic	0.000	0.789	0.10^{B}
9	Glicidic	0.000	1.828	0.09^{B}
11	Glycoprotein	0.117	1.732	0.06^{C}
5	Glycoprotein	0.343	2.214	0.06^{C}
4	Glycoprotein	0.878	1.877	0.06^{C}
2	Glicidic	0.000	1.128	0.03^{D}
7	Glicidic	0.000	1.683	0.02^{D}
6	Glycoprotein	0.412	2.529	0.02^{D}
10	Glycoprotein	0.786	1.852	0.02^{D}
3	Glicidic	0.000	0.838	0.02^{D}
1	Glicidic	0.000	1.249	0.01^{D}
Tris HCl*	--	--	--	0.56^{C}
Saccharomyces cerevisiae **	--	--	--	0.23^{A}
CV %				15

Buffer solution used as mobile phase in chromatography (control treatment); ** *Saccharomyces cerevisiae* (Fleischmann Fresh Biological Yeast) (20 mg ml^{-1}), used as induction standard treatment; Scoot-Knott Test at 0.05%.

Table 5. Fractions, nature, total protein content (µg ml^{-1}), molecular mass (kDa), carbohydrate content (µg ml^{-1}), and the gliceolin accumulation in soybean cotyledons (nm $g.m.f^{-1}$), treated with the glycoprotein and glicidics fractions from gel filtration chromatography (GFC), from *Meloidogyne javanica.*

Fractions of GFC	Nature	Total protein content (µg protein ml^{-1})	Molecular mass (kDa)	Carbohydrate content (µg glucose ml^{-1})	Gliceolin (285 nm $g.m.f.^{-1}$)
7	Glycoprotein	1.280	69.66	3.471	0.99^{A}
17	Glycoprotein	10.873	78.89	3.326	0.97^{A}
26	Glycoprotein	6.833	176.91	3.374	0.74^{A}
3	Glycoprotein	0.067	61.52	2.457	0.73^{B}
2	Glycoprotein	0.505	107.62	2.626	0.63^{C}
33	Glycoprotein	7.102	166.25	3.085	0.61^{C}
13	Glycoprotein	0.021	89.31	3.616	0.55^{C}
6	Glycoprotein	1.142	114.51	3.229	0.54^{C}
36	Glycoprotein	4.614	213.16	3.423	0.45^{C}
10	Glycoprotein	0.820	57.81	4.002	0.45^{C}
22	Glycoprotein	3.869	188.25	4.220	0.34^{C}
24	Glycoprotein	1.964	176.91	3.205	0.31^{C}
20	Glycoprotein	4.848	101.13	3.930	0.27^{C}
5	Glycoprotein	2.793	188.25	2.674	0.24^{C}
29	Glycoprotein	0.113	176.91	3.302	0.22^{C}
16	Glycoprotein	1.392	114.51	3.664	0.21^{D}
19	Glycoprotein	0.735	200.32	3.688	0.21^{D}
25	Glycoprotein	0.059	47.98	3.471	0.18^{D}
23	Glycoprotein	0.924	42.37	3.205	0.17^{D}
37	Glycoprotein	0.059	54.33	3.616	0.17^{D}
14	Glycoprotein	0.036	42.37	3.809	0.16^{D}
30	Glycoprotein	0.113	54.33	3.036	0.16^{D}
8	Glycoprotein	0.455	42.37	4.002	0.15^{E}

Table 5. Contd.

21	Glycoprotein	0.947	47.98	2.988	0.14^{E}
39	Glicidic	0.000	--	3.205	0.14^{E}
41	Glicidic	0.000	--	2.867	0.14^{E}
18	Glycoprotein	0.521	42.37	3.688	0.14^{E}
42	Glicidic	0.000	--	3.060	0.14^{E}
27	Glycoprotein	0.659	37.42	4.147	0.14^{E}
12	Glycoprotein	0.021	107.62	3.036	0.13^{E}
43	Glicidic	0.000	--	3.713	0.13^{E}
34	Glycoprotein	0.159	54.33	3.519	0.12^{E}
11	Glycoprotein	0.498	29.19	2.263	0.12^{E}
40	Glicidic	0.000	--	2.795	0.12^{E}
44	Glicidic	0.000	--	2.891	0.12^{E}
32	Glycoprotein	0.348	188.25	3.254	0.12^{E}
53	Glicidic	0.000	--	2.529	0.11^{E}
59	Glicidic	0.000	--	2.481	0.11^{E}
51	Glicidic	0.000	--	2.601	0.11^{E}
48	Glicidic	0.000	--	2.432	0.11^{E}
55	Glicidic	0.000	--	2.940	0.11^{E}
63	Glicidic	0.000	--	1.200	0.11^{E}
9	Glycoprotein	0.659	121.86	3.785	0.11^{E}
49	Glicidic	0.000	--	2.360	0.11^{E}
4	Glycoprotein	0.044	33.04	4.002	0.11^{E}
28	Glycoprotein	0.267	2.723.24	2.988	0.10^{E}
52	Glicidic	0.000	--	2.964	0.10^{E}
50	Glicidic	0.000	--	2.577	0.10^{E}
38	Glicidic	0.000	--	3.109	0.10^{E}
57	Glicidic	0.000	--	3.930	0.10^{E}
46	Glicidic	0.000	--	2.795	0.10^{E}
54	Glicidic	0.000	--	3.205	0.10^{E}
61	Glicidic	0.000	--	0.790	0.10^{E}
62	Glicidic	0.000	--	1.635	0.10^{E}
31	Glycoprotein	0.417	2.123.97	3.640	0.09^{E}
1	Glycoprotein	0.243	156.24	2.698	0.09^{E}
58	Glicidic	0.000	--	2.312	0.09^{E}
45	Glicidic	0.000	--	3.278	0.09^{E}
35	Glycoprotein	0.466	2.989.25	4.002	0.09^{E}
56	Glicidic	0.000	--	3.471	0.09^{E}
15	Glycoprotein	0.159	329.31	3.882	0.09^{E}
47	Glicidic	0.000	--	3.616	0.09^{E}
60	Glicidic	0.000	--	2.360	0.08^{E}
Tris HCl*	--	--	--	--	0.09^{E}
Saccharomyces cerevisiae **	--	--	--	--	0.19^{D}
CV %					16

Buffer solution used as mobile phase in chromatography (control treatment); ** *Saccharomyces cerevisiae* (Fleischmann Fresh Biological Yeast) (20 mg mL^{-1}), used as induction standard treatment; Scoot-Knott Test at 0.05%.

purification from microorganisms, it is important to highlight the chromatography, a technique used in the present study, which allowed the separation of molecules present in females of nematodes, molecules with relevant characteristics for the study of resistance mechanisms in soybean and bean seedlings. It is worth mentioning that the majority of the fractions collected in this study presented a glycoprotein nature, that is, presence of

proteins and carbohydrates in the fractions. This information corroborates the description by Braga (2008), in which in the majority of the purified elicitor molecules, carbohydrates are found as a compound of purified fractions, together with proteins, forming thus, glycoprotein nature elicitors.

The *M. javanica* chromatographic fractions of IEC, tested in bioassays with bean hypocotyls, delivered different responses at the induction of plant defense mechanisms, as it was observed in the presence of eliciting molecules, which resulted in the accumulation of phytoalexin. Such result is tied to the molecule joint activity from the obtained peaks. Suppressing activity fraction was also found. Therefore, the IEC fractions, when submitted to GFC, were also efficient and resulted in components with individual activity capable of inducing phytoalexin into bean seedlings. Regarding the bioassay on soybean cotyledons, from IEC fractions, there was no observed presence of eliciting molecules capable of inducing a higher level than *S. cereviviae*, a treatment used as an induction pattern. Yet, the presence of suppressing molecules for gliceolin synthesis was reported. However, when submitted to GFC, there were obtained fractions of eliciting character. Therefore, the presence of molecules of differentiated characteristics is suggested as inductors and suppressors in females of *M. javanica* for tested condition.

The results corroborate the description of Smith (1996), in which elicitors are constituted of a broad molecule chemical nature, and consequently there is not one single structural characteristic that can define its eliciting activity, similar to Dixon and Lamb (1990), there may be two or more eliciting molecules acting together and simultaneously. Therefore, the method used proved to be valid. The fact that some fractions purified in IEC did not deliver gliceolin and phaseolin inducting effect, but when subjected to GFC they did, indicates that, probably, for these molecules to be acknowledged as elicitors by the plant there is the need of individual activity by themselves. For IEC fractions, which were effective at the induction of phytoalexin and were purified by GFC they did not present the same response; it is believed that there is the need of the molecules to be acting together for such phenomenon to occur. Thus, the fractions resulting from IEC and their subsequent separations by GFC resulted in new fractions with differentiated molecule characteristics and groupings, not only for proteins but also for carbohydrates.

In a study conducted by Zanardo et al. (2009), supernatant fractions obtained from the ethanolic precipitation of the aqueous gross extract of the *Saccharimyces cerevisiae* presented resistance inducting activity in cucumber plant cotyledons. The presence of these fractions, separated by ion exchange chromatography, demonstrates the method efficiency as to the process of purification molecules with eliciting activity for studies of resistance induction. Yet, in a complementary manner, the authors verified that the fractions delivered a higher carbohydrate concentration in relation to the proteins in purified samples. These results confirm the observation in this study, in which the minority of the fractions purified by IEC and GFC presented a higher concentration of carbohydrates than of proteins. There is limitedr eports of obtaining purified fractions from females of phytopathogenic nematodes, but ion exchange chromatography and molecular exclusion techniques deserve to be highlighted for their importance and relevance regarding the research objective to purify and characterize molecules from other agents and, thus, study its resistance inducting potential in plants against diseases.

The recognition of these molecules by plants culminates in their reaction against pathogenic agents by the production of O_2 radicals, production of structural barriers and toxins that block the pathogen activity (Jones and Dangl, 2006). These defense responses may be induced when the plant recognizes molecular patterns associated to pathogens (PAMP's). PAMP's are molecules conserved by pathogens that use them to their own survival (Gheysen and Jones, 2013). According to Haegeman et al. (2012), the proteins present in nematode secretions are destined to protect the proteins from the host's defense responses. As an example, it is worth highlighting the gluatathione-S-transferase (GST) expressed in *M. incognita*s pharynx gland (Dubreuil et al., 2007). However, the authors also verified the GST acting on the detoxification of secondary metabolisms that the plant uses to prevent pathogen invasion.

In *Gobodera rostochiensis* it was identified the glutathione peroxidase, secreted and expressed in its hypoderm, however, its production in plants was also reported, which can be related to plant defense signaling pathways (Gheysen and Jones, 2013), as related in the present study, in which purified fractions posteriorly tested in assays of phaseolin and gliceolin phytoalexin induction, demonstrating phaseolin and gliceolin phytoalexin inducting potential. The hypothesis of the presence of elicitors in the female nematodes' cuticle composition and cell wall, as well as protein secretion by second-stage juvenile (J2) was based on the purified fractions capacity of inducing the gliceolin and phaseolin phytoalexin synthesis. Facing that, when performing the fraction partial purification from female nematodes of *M. javanica* via the IEC technique, followed by GFC, the action mechanism and the effect of these fractions on phytoalexin inducting activity on soybean cotyledons and bean hypocotyls were pointed out in the bioassays. Furthermore, there were elicitors, glycoproteins, on the anterior region of the nematode, capable of inducing the phytoalexin synthesis (Faria et al., 2003).

Phytoalexin synthesis play a fundamental role within the resistance induction studies, since this secondary metabolism, when synthesized by the plant, can affect the nematode functions, such as the rupture of

plasmalemma and vascular membranes, mitochondrial breathing inhibition and can also impair its own mobility (Giebel, 1973). Kaplan and Keen (1980) demonstrated that the oxygen absorption inhibition by J2 in *M. incognita* was due to gliceolin accumulation. Thus, the inducting characteristics of purified fractions may be tied to the molecules present in the nematodes, since for Ferraz and Monteiro (2011), the cuticle which involves the nematode body wall is a metabolically active structure and it is basically composed of proteins, therefore important to resistance induction studies, once they are still unknown.

Though nematodes have sub ventral esophageal glands, which produce proteins that act on the formation of feeding sites. During the eclosion period and during stage J2 these glands with highly functional cell are found, which play a fundamental role at the beginning of parasitism in the plant nematode, which in turn, also is worthy of attention as they act on the signaling paths of the plant defense mechanisms (Jones et al., 2003). The importance of knowing these proteins present in nematodes is due to the fact that during the infection phase, when penetrating the stiletto in the plant cell, the nematode injects proteins that culminate in cell physiological and morphological alterations and, as a result, originates giant cells that will produce more proteins to be used in the nematode feeding (Mitkowskli and Abawi, 2003). Therefore, the presence of these proteins, which constitute the females, and are part of their cuticle composition, as well as J2 glands, still in the egg interior, can justify the fact that some fractions selected in this study present the capacity of inducing cotyledons and bean hypocotyls to produce phytoalexins. Jones et al. (2000) and Robert et al. (2000) remark that a nematode cuticle is basically constituted by proteins, many of them important to the parasitism have already been identified and, besides that, carbohydrates and lipids were in the composition of their structures. These proteins may or may not result in the induction of the plant defense mechanisms, as to Temporal (2014) there are specific elicitors that activate signaling paths and, thus, induce the expression of specific genes. To Graham (1995), elicitors may induce precursors of plant tissue to produce phytoalexins, next to the infection area or where treatment was applied. The induction of genes associated to plant defense against pathogenic agents was reported by Manosalva et al. (2015), which aim to evaluate the plant perception and defense response as to the presence of signaling molecules, produced by plans parasite nematodes. These molecules, called ascarosides, are glycoside compounds that when undergoing hydrolysis generate sugars and, according to the authors, can be perceived by plants that is, receptors that activate defense response to several pathogens.

Still, for the present study, in the case of the fractions that have not induced phytoalexins, there are two ways in which the results can be based. The first is related to the volume of treatment used, because Hahn (1996) suggests that the low yield of purified fractions can mask the responses of defense or the activation of the routes of signalling. Although, Bonaldo et al. (2007) found that when the crude extract of eucalyptus was diluted, even so, there was induction of the phytoalexins, deoxiantocianidine in sorghum mesocotyls. Bonaldo (2005) also observed that the dilutions 10, 100, 1,000 and 10,000 times of preparation of *S. cerevisiae* induced phytoalexins in sorghum mesocotyls.

In the present study, it must be taken into consideration that during the dialysis procedure in reactions eluted in NaCl there may be compound losses by the dialysis membrane. According to Queiroz et al. (2001) the low molecular mass compounds can permeate the membrane and/or experiment dilutions. Based on this information, the possible lack of eliciting ability in purified molecule can also be highlighted. A second hypothesis would be fractions presenting phytoalexin suppressors, for in this study, beside inductors, inhibitors also were found, and that, according to Temporal (2014), the suppressing molecule grouping can result in fractions of low phytoalexin and enzyme inducting activity phytoalexin and enzyme induction. The suppression of plant defense responses may be related to the presence of proteins, called effectors, according to Kamoun (2006) and Kvitko et al. (2009) which are molecules released or associated to the organism and that can modify the physiology of another organism. These proteins can regulate the plant defense mechanisms, when inducing the plant cells to maintain or forge new feeding sites (Gheysen and Mitchum, 2011), for they interact with and affect the regulation of gene expression that culminate in alterations in the host cell functions (Gao et al., 2003).

When a purified fraction present elicitors able of inducing plant defense mechanisms and are recognized by cell receptors, many signals are forwarded, and then, converted into cell responses. At that point, activation genes induce a synthesis of many enzymes and among them there are some genes that produce phytoalexin. However, gliceolin and phaseolin synthesis was not always observed in the present study, and this result may be related to the activation of the mechanisms which were not analyzed in this study, but that do not discard the possibility of studying other host resistance activation paths.

In another study carried out by Gheysen and Mitchum (2011), the authors highlight that the nematode effecting proteins can avoid or suppress the defense responses, and that when interacting with the plant proteins they begin to control their development, such as cell growth and auxin transporting. Doyle and Lambert (2003) reported that the protein chorismate mutase, from which phytoalexins is derived, salicylic acid and auxins produced via the shikimate, was found in the *M. incognita* secretion and, as a result, there were alterations in the secondary metabolism of host cells. According to Davis et al. (2008), the majority of these proteins are produced

in the nematode esophageal glands (subventral and dorsal), and injected into the plant cytoplasm with the aid of a stiletto.

M. incognita proteins, involved in parasitism, have been purified and analyzed as to their capacity of suppressing the host defense responses and causing an infection (Bellafiore et al., 2008). The nematode effectors interact with the host proteins and manipulate the plant physiology (Quentin et al., 2013), as well as the signaling processes and hormonal and cellular balances (Wang et al., 2011), a fact which may be related to the suppressing responses found in the present study.

Proteins constitute a great percentage of mass of living organisms, and Castagnone-Sereno et al. (2011) reported that several protease genes were identified that compose the genome of gall nematodes. The authors affirm that, also, these proteins function as object of studies on phytopathogenic nematode control. This characteristic reinforces the importance of the present study and of new researches turned to the purification of eliciting molecules of plant defense inducing aspect from nematodes.

Therefore, while some effecting proteins suppress the plant defense response others can participate in the defenses signaling of host plants and/or fostering the formation of nematode feeding sites (Haegeman et al., 2012).

Conclusion

The chromatographic pattern indicated the presence of glycoprotein and glicidic elicitors capable of inducing phytoalexin synthesis. The suppression of gliceolin and phaseolin induction was verified for some of the fractions tested in the bioassays. Fractions obtained via ion exchange chromatography and posteriorly submitted to filtration chromatography influenced in the production of phytoalexins for some molecules shall act jointly, so there is eliciting effect and some others act separate, so that they may induce plant defense response.

Conflict of Interests

The authors have not declared any conflict of interests.

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