ASSOCIATION OF ISOFLAVONOIDS WITH THE INCOMPATIBLE RESPONSE OF SOYBEAN ROOTS TO *MELOIDOGYNE INCOGNITA* RACE 3

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ABSTRACT

Carpentieri-Pípolo, V., J. M. G. Mandarino, M. C. Carrão-Panizzi, and A. Souza. 2005. Association of Isoflavonoids with the Incompatible Response of Soybean Roots to *Meloidogyne incognita* Race 3. Nematropica 35:103-110.

The accumulation of isoflavonoids has been associated with a incompatible response of soybean roots to infection by the root-knot nematode. Soybean isoflavonoids have been proposed to have many effects on host-pathogen interactions. The phytoalexin accumulated in soybean tissues in response to nematode infection is glyceollin, which is derived from the isoflavonoid precursor daidzein. Accumulation of the isoflavonoids genistin and daidzin and their aglycones genistein and daidzein in soybean roots following inoculation with *Meloidogyne incognita* race 3 was determined in the whole root system by high performance liquid chromatography (HPLC). The roots were harvested from controls and nematode-inoculated seedlings 1, 3, and 10 days after inoculation. Extractions were made from roots with ethanol and the extracts were analyzed for isoflavonoids by HPLC. There was no significant difference between susceptible cultivar Pickett 71 and the resistant cultivar FT-Cometa one day after inoculation for all isoflavonoids. Daidzein and genistein were detected for all evaluated cultivars inoculated and non-inoculated. The resistant cultivar FT-Cometa showed a higher concentration of daidzein than the Pickett 71 cultivar ten days after inoculation, which ranged from 0.181/100g of root at the first day after inoculation to 1.025 mg/100g of root at tem days after inoculation.

Key words: Glycine max, Meloidogyne spp., phytoalexins, resistance.

RESUMO

Carpentieri-Pípolo, V., J. M. G. Mandarino, M. C. Carrão-Panizzi, and A. Souza. 2005. Associação entre Isoflavonóides e Resistência da Soja à *Meloidogyne incognita* Raça 3. Nematropica 35:103-110.

O acúmulo de isoflavonóides tem sido associado à resistência da soja à infecção por nematóide de galhas. A fitoalexina acumulada nos tecidos de soja em resposta ao ataque de patógenos é a gliceolina, cujo isoflavonóide precursor é a daidzeína. O acúmulo dos isoflavonóides genistina, daidzina e suas agliconas, genisteína e daidzeína nas raízes de soja, seguidas da inoculação com *Meloidogyne incognita* raça 3, foi determinada por cromatografia líquida de alto desempenho (HPLC). As raízes foram colhidas das plântulas controles e das inoculadas com o nematóide após um, três e dez dias da inoculação. Os isoflavonóides foram extraídos com etanol e submetidos à HPLC. Não houve diferença significativa entre a cultivar suscetível Pickett 71 e a resistente FT-Cometa após um dia da inoculação. Daidzeína e genisteína foram detectadas em todas as cultivares avaliadas, inoculadas ou não-inoculadas. A cultivar resistente FT-Cometa apresentou maior concentração de daidzeína que a cultivar Pickett 71 dez dias após a inoculação, a qual variou de 0,181 mg/100g de raiz no primeiro dia após a inoculação até 1,025 mg/100g de raiz após dez dias da inoculação.

Palavras-chave: Glycine max, Meloidogyne spp., fitoalexina, resistência.

INTRODUCTION

Brazil is the world's second largest soybean producer, with a yield above 41 million tons in the 2002/2003 cropping season (Problemas climáticos, 2002). The greatest difficulty to overcome in the intensive utilization of this crop is an increase in the inoculum of pathogens that cause diseases, including plant-parasitic nematodes.

Among gall-forming nematodes, *Meloidogyne incognita* and *Meloidogyne javanica* are the two most important species worldwide, corresponding to about 86% of the species found in tropical and subtropical areas (Taylor *et al.*, 1982). They have extensive distribution in Brazil and represent a serious problem for soybean production in the country. It is estimated that the damage caused by these nematodes in soybean during the 1999/2000 cropping season amounted to $52.2 million (Yorinori, 2000).

Cultivars that are resistant to gall-forming nematodes showed yield 10% to 16% higher than susceptible cultivars in the presence of the pathogen; this is the cheapest and easiest-to-adopt method that can be used by soybean producers (Arantes *et al.*, 2000; Silva, 2001).

Nematode resistance can occur either in the soil environment, in the root pre-infection, or inside the root post-infection. The most common type is post-infection resistance, manifested when some host factors undermine the establishment of the relation between host and nematode (Pipolo, 1990). Phytoalexins are among the biochemical products formed after infection; these are compounds with low molecular weight and antibiotic properties which accumulate in plant tissues as a response to infection (Paxton, 1981). They play an important part in restricting pathogen growth and in conveying resistance to the host tissue.

Isoflavonoids are flavonoids whose most common representatives in soybean are the glucosides genistin and daidzin and their aglycones, genistein and daidzein (MacLeod and Ames, 1988). The malonyl forms of these glucosides have also been reported (Kudou *et al.*, 1991). The phytoalexins that accumulate in soybean tissues correspond to glyceollins, belonging to the class of pterocarpans, whose precursor is daidzein (Ebel, 1986). Analytical studies have determined that genistein and daidzein are the most important isoflavonoids present in soybean seeds, representing 64% and 23% of all isoflavonoids, respectively (Naim *et al.*, 1974; Carrão-Panizzi and Kitamura, 1995; Carrão-Panizzi, 1996). These isoflavonoids are the most effective compounds in antixenotic and antibiotic relations (Fisher *et al.*, 1990; Graham and Graham, 1991).

Graham (1991) studied the distribution of flavonoids and isoflavonoids and their conjugates during soybean development in seedling, root, and seed tissues. The author predominantly found daidzein and its conjugates in all root sections, particularly at radicle tips, where the highest concentration of the substance was found.

Kaplan *et al.* (1980a) studied the association between glyceollin and soybean root resistance to *Meloidogyne incognita*. They reported that resistant cultivar Centennial accumulated glyceollin two to three days after inoculation with *M. incognita*, differently from the susceptible cultivar Pickett 71 which did not accumulated glyceollin. Both cultivars were susceptible to *M. javanica* and neither cultivar, with or without nematodes, accumulated a significant amount of glyceollin over the same time period. Significant glyceollin concentrations were detected in the central cylinder region, indicating that there was a hypersensitivity response from cultivar Centennial in those tissues. The glyceollin effect

was nematostatic rather than nematicidal on *M. incognita* juveniles. A significant reduction in the number of eggs per female, and in the number of females per root was observed in the resistant cultivar. A similar result was obtained by Veech (1982), in which healthy roots of soybean cultivars Pickett 71 and Centennial showed a concentration of 15 µg glyceollin/g root. After inoculation with *M. incognita*, an increase in glyceollin concentration was only detected in cultivar Centennial, reaching 40 µg/g root after three days, and above 70 µg/g root after seven days.

Kaplan *et al.* (1980a and 1980b) studied glyceollin's mechanism of action in the incompatible response of the nematode *M. incognita* to soybean roots. The authors reported that glyceollin inhibited *M. incognita* mobility, but did not inhibit *M. javanica* mobility. Glyceollin also prevented oxygen absorption by *M. incognita* in resistant plants. According to the authors, the possible role of the compound in the incompatible response of soybean roots to *M. incognita*, is a localized hypersensitive response which has been associated with inhibited nematode development.

Liu *et al.* (1992) evaluated the contents of the phytoalexins glyceollin and coumestrol in different soybean genotypes. The insect-resistant PI 227687 cultivar produced significantly more phytoalexins than susceptible cultivar Davis. The authors also observed that glyceollin was the best resistance induction indicator, and that the concentration of phytoalexins in soybean seedlings can be used to identify insect-resistant materials in cultivar development programs.

The objective of this work was to evaluate the relationship between isoflavonoid concentration and soybean resistance to *M. incognita* race 3, in order to develop a method that would allow the concentration of isoflavonoids in roots to be used as a parameter for the selection of soybean materials resistant to root-knot nematodes.

MATERIALS AND METHODS

The experiment was carried out in a greenhouse in the experimental area of the Agronomy Department of Universidade Estadual de Londrina, in Londrina, PR, Brazil, from September 1999 to March 2000. The *M. incognita* inoculum was reared on 'Rutgers' tomato. Eggs and juveniles were extracted from tomato root systems according to the blender method and by centrifugation with a sucrose and kaolin solution (Coolen and D'Herde, 1972). Seeds of soybean cultivars Pickett 71 and FT-Cometa, which are susceptible and resistant to *M. incognita*, respectively (Kaplan *et al.*, 1980a; Embrapa, 1995), were germinated in paper towel rolls in an incubator at 25°C for 72 hr. The seedlings were transplanted to 500-mL plastic pots containing a mixture of soil and sand at a 3:1 proportion, previously treated with methyl bromide. Five thousand juveniles per pot were inoculated when the first pair of unifoliolate leaves appeared. The inoculum suspension was applied with a pipette into 3-cm deep holes, near the seedling's root collar.

A completely randomized design was used, with six replicates for each treatment. The seedlings were harvested 1, 3, and 10 days after inoculation. Treatments without inoculum were applied to the cultivars as controls. The seedling roots were carefully washed and dried in a forced-air oven at 50°C for 4 hr.

Isoflavonoid extraction and quantification was performed at the Plant Breeding Laboratory of Embrapa's Centro Nacional de Pesquisa de Soja (Embrapa-Soja) using high performance liquid chromatography (HPLC). For extraction, the root system of each seedling was ground in a porcelain

mortar with a pestle and placed in a 10 mL test tube containing 4.0 mL of a solution consisting of 70% ethanol and 0.1% acetic acid for 12 hr at room temperature. A 1.5 mL aliquot of the extract was centrifuged at 15,000 rpm for 4 min. After centrifugation, 40 µl of the supernatant from the filtered extract were transferred into the automatic sampling injection vials of a model 2690 Waters liquid chromatograph equipped with a model 996 Waters photodiode array detector and a reverse-phase column (ODS-C18) with a diameter of 4.6 × 250 mm.

The quantitative analysis of isoflavonoids was performed based on their spectra and retention times, according to method described by Kudou *et al.* (1991). The genistein, daidzein, genistin, daidzin, malonyl genistin, and malonyl daidzin standards were obtained from Sigma-Aldrich Chemical Co. The column was initially balanced with a 80% aqueous solution gradient of 0.1% acetic acid and 20% acetonitrile with 0.1% acetic acid. The acetonitrile concentration was high, reaching 45% after 30 minutes (complete elution of isoflavonoids), 80% at 33 minutes (for elution of all remaining compounds) and finally 20% at 35 minutes, for analysis of the next sample. Flow was set at 1.0 mL/minute, and a photodiode array detector adjusted at a wavelength of 260 nm was used for isoflavonoid detection.

The data obtained were subjected to analysis of variance and the isoflavonoid concentration means were compared using Tukey's test at 5% probability.

RESULTS AND DISCUSSION

Although there was a difference in the concentrations of the isoflavonoid malonyl-genistin ($P < 0.05$) among the evaluated treatments, the main source of variation was represented by sampling time (ST), $P < 0.01$ for all isoflavonoids evaluated (Table 1). The treatment × sampling time interaction showed a highly significant effect ($P < 0.01$) for daidzin, malonyl-genistin, daidzein, and genistein, and a significant effect ($P < 0.05$) for genistin and malonyl-daidzin. The significant effect of the treatment × time interaction thus evidenced that the cultivars showed a differential behavior with respect to isoflavonoid concentration in different sampling times, and that there was a difference between treatments within the same sampling time.

In the presence of the nematode, cultivars FT-Cometa and Pickett 71 showed significant differences ($P < 0.01$) in all evaluation times for all isoflavonoids. There was no significant difference ($P < 0.01$) in daidzein concentration for Pickett 71 without nematodes in any of the sampling times. FT-Cometa without nematodes only showed significant differences ($P < 0.01$) for malonyl-daidzin and genistein.

The mean isoflavonoid concentrations did not show significant differences between treatments until the third day after inoculation. However, ten days after inoculation it was possible to detect significant differences ($P < 0.01$) between treatments for all isoflavonoids. This fact agrees with results by other authors (Kaplan *et al.*, 1979; Kaplan *et al.*, 1980a), who detected glyceollin accumulation two to three days after inoculation with the pathogen, and attributed this period to the time necessary to begin giant cell development and the subsequent response process by the host.

The relationship between sampling time and isoflavonoid concentration was observed in this study. The cultivar FT-Cometa, at 10 days, in the absence of nematodes showed the lowest levels of accumulation of the evaluated isoflavonoids (Figs. 1, 2, and 3). Except for malonyl-daidzin, the concentration of all isoflavonoids in cultivar FT-Cometa, at 10 days, was differ-

Table 1. Mean squares for the concentrations of daidzin, genistin, malonyl-daidzin, malonyl-genistin, daidzein, and genistein, in the roots of soybean cultivars FT-Cometa and Pickett 71, after one, three, and ten days from inoculation with *M. incognita* race 3.

		MS (mg/100g root)					
Source	DF	Daidzin	Genistin	M-Daidzin	M-Genistin	Daidzein	Genistein
Treatment (T)	3	0.19114	0.76483	0.00364	0.02305*	0.12068	0.68191
Sampling time (ST)	2	3.34203**	26.50432**	0.20060**	0.47140**	1.04266**	19.53692**
T × ST	6	0.23023**	1.60216*	0.00659*	0.02963**	0.16742**	1.04222**
FT-Cometa with	2	1.56053**	9.00303**	0.08557**	0.15329**	0.79136**	11.04202**
FT-Cometa without	2	0.01862	0.23609	0.01315**	0.01055	0.12480	2.81103**
Pickett 71 with	2	1.08680**	8.00431**	0.03549**	0.17306**	0.53088**	4.70865**
Pickett 71 without	2	1.36677**	14.06737**	0.08615**	0.22339**	0.09789	4.10189**
1 day	3	0.07646	0.55372	0.00145	0.00937	0.05196	0.05465
3 days	3	0.02712	0.20792	0.00200	0.00602	0.13951*	0.82451*
10 days	3	0.39958**	3.17000**	0.01198**	0.06682**	0.22777**	1.60645**
Residue	49	0.04737	0.37237	0.00227	0.00709	0.04688	0.25197
Mean		0.68939	2.15948	0.15271	0.33489	0.5899	1.51037
CV%		31.57	28.25	31.22	25.15	36.70	33.23

*, **Significant at 5% and 1%, respectively, by F test.

ent in treatments with and without nematodes ($P<0.05$) (Figs. 1, 2, and 3). Cultivar Pickett 71 had a different behavior, and no significant differences were observed between treatments with and without nematodes for any of the isoflavonoids. This is an indication that the inoculation of *M. incognita* in the susceptible cultivar does not produce a higher accumulation of isoflavonoids. Similar results were obtained by Kaplan *et al.* (1980a) and by Huang and Barker (1991), who observed a greater concentration of glyceollin in the roots of resistant soybean cultivars inoculated with *M. incognita* and with *Heterodera glycines*, respectively.

Graham *et al.* (1990) proposed that daidzein, genistein, daidzin, genistin, and their malonyl forms can contribute toward resistance against fungal infection in soybean seeds and seedling tissues. In this study, accumulation of daidzin, genistin, and their malonyl forms during the inoculation period did not allow the susceptible cultivar Pickett 71 to be differentiated from the resistant cultivar FT-Cometa. Morris *et al.* (1991), in their study on the identification and accumulation of isoflavonoids in soybean as a response to *Phytophthora megasperma* f. sp. *glycinea*, indicated that isoflavonoids may or may not have an antimicrobial effect, and that this effect would depend on the host × pathogen interaction.

According to several authors (Ebel, 1986; Kochs *et al.*, 1987; Zacahrius and Kalan, 1990; Abbasi and Graham, 2001), the presence of daidzein is the first step for

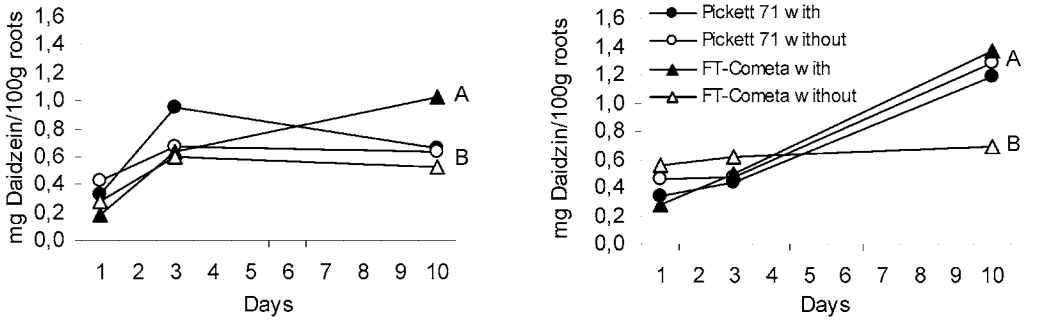

Fig. 1. Concentration means of daidzein and daidzin (mg/100 g root) in roots of cultivars FT-Cometa and Pickett 71, with and without inoculum of *M. incognita* race 3. Means followed by the same letter are not different among themselves by Tukey test at 5%.

the potential biosynthesis of glyceollin, the main phytoalexin with antimicrobial properties present in soybean. Three days after inoculation, cultivar Pickett 71 cultivar showed higher genistein concentration than cultivar FT-Cometa ($P < 0.05$) (Fig. 3). Although cultivar Pickett 71 with nematodes had showed 0.9988 mg/100 g root of daidzein, there was no significant difference of this with all others evaluated cultivars with and without nematodes which had daidzein concentration ranged from 0.6039 mg/100 g root to 0.6691 mg/100 g roots.

At 10 days after inoculation, in the presence of nematodes, the resistant cultivar FT-Cometa was different ($P < 0.05$) from the susceptible Pickett 71 with and without nematodes, with a higher concentration of daidzein, varying from 0.181 mg/100 g root at 1 day after inoculation to 1.025 mg/100 g root at 10 days after inoculation (Fig. 1), and of genistein, varying from 0.329 mg/100 g root at 1 day after inoculation to 3.33 mg/100 g root at 10 days after inoculation (Fig. 3). This is confirmed in studies by Graham *et al.* (1990), Rivera-Vargas *et al.* (1993), Graham and Graham (1999), and Abbasi and Graham (2001), where they reported that preformed daidzein and genistein conjugates are hydrolyzed in the region of infection in resistant plants, with the release of a large amount of free daidzein and genistein. The rate of release of these com-

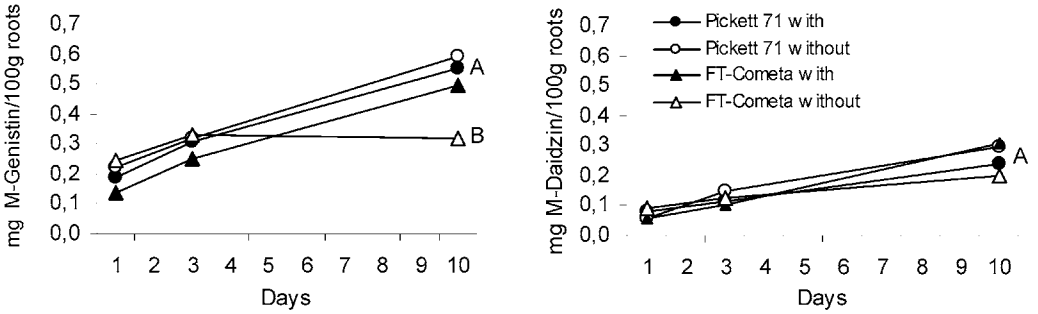

Fig. 2. Concentration means of malonyl-genistin and malonyl-daidzin (mg/100 g root) in roots of cultivars FT-Cometa and Pickett 71, with and without inoculum of *M. incognita* race 3. Means followed by the same letter are not different among themselves by Tukey test at 5%.

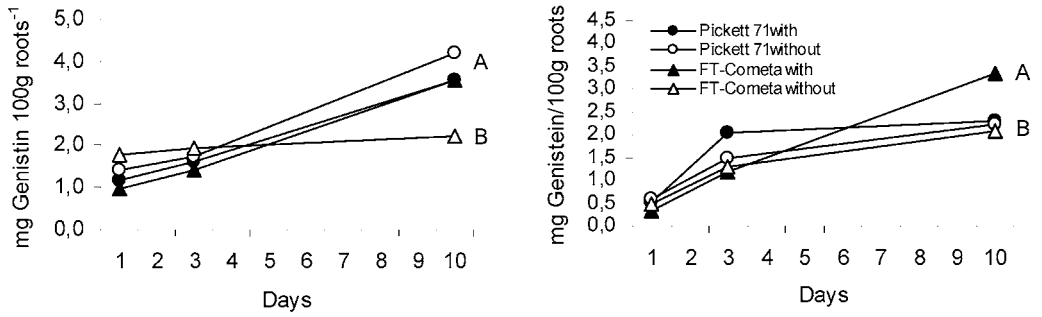

Fig. 3. Concentration means of genistin and genistein (mg/100 g root) in roots of cultivars FT-Cometa and Pickett 71, with and without inoculum of *M. incognita* race 3. Means followed by the same letter are not different among themselves by Tukey test at 5%.

pounds would be conditioned to incompatibility with the infecting pathogen and would have a positive impact on glyceollin accumulation.

According to the results obtained in this study, it was observed that it is possible to use the accumulation of isoflavonoids daidzein and genistein in soybean roots inoculated with the pathogen as an auxiliary tool in the process of selection of soybean genotypes resistant to *M. incognita* race 3.

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