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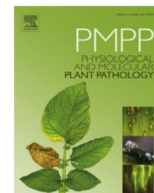

Principal Response Curves analysis of polyphenol variation in resistant and susceptible cotton after infection by a root-knot nematode (RKN)

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The root-knot nematode (RKN), *Meloidogyne incognita*, attacks cotton root system. This study aimed to compare the induction of phenolic compounds over time in two cotton genotypes, resistant (TX-25) and susceptible (FM966), by RKN. Chemical profiles of cotton roots were obtained by HPLC, NMR, and colorimetric methods at four different time intervals. The Principal Response Curves analysis, a time-dependent, multivariate method, showed consistent variability over time in the profile of phenolic compounds between treatments for both genotypes. The variables that most contributed to the divergence between damaged and undamaged cotton roots were dimethoxylated and non-methoxylated gossypols, total flavonoids, and total phenols.

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1. Introduction

Cotton (*Gossypium hirsutum* L.) is a crop of great economic importance worldwide. Since 1965 breeding programs have developed resistant varieties to the root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid and White) Chitwood. This nematode attacks young roots and causes harmful effects to the plant with significant yield losses for cotton crops [1]. Cotton resistant strains TX-25 and M-315 RNR were able to limit RKN reproduction to less than 5 eggs g^{-1} fresh roots at 120 days after inoculation with 10,000 eggs per plant, whereas susceptible cultivars FM966 and AS0190 presented over 5000 eggs g^{-1} fresh roots in the same experiment [2]. A higher number of RKN eggs imposes an enormous amount of damage to susceptible cotton seedlings, as well as increases the severity of other soilborne diseases caused by different fungi [3].

The search for chemical factors of cotton resistance to RKN, wireworm, tobacco budworm, and fungal diseases has shown the involvement of phenolic compounds in defense mechanisms. Several reports have demonstrated the induction of gossypol and related terpenoid aldehydes, hemigossypol, 6-methoxyhemigossipol, and 6-methoxygossipol in response to wireworm (*Agriotes lineatus* L.) and RKN root attacks [1,4,5], as well as fungal infections caused by *Fusarium oxysporum* f. sp. *vasinfectum* and *Rhizoctonia solani* K. [1,6,7]. Gossypol-type terpenoids were also responsible for the resistance of *G. hirsutum* to tobacco budworm (*Heliothis virescens* F.), whereas for *Gossypium arboreum* flavonoids gossypetin-8-O-glucoside and gossypetin-8-O-rhamnoside were the main source of resistance to this insect [8].

Induction responses of secondary metabolites to herbivore or pathogenic fungi attacks are usually monitored at regular time intervals. Most studies apply the standard univariate statistical analysis (ANOVA) to assess overall changes in the phenolic composition of cotton-infected tissues over time [1,3,4,6,9]. However, a multivariate statistical analysis may also be used to detect overall trends in time at chemical level, and its advantage over the univariate method is that it uses and summarizes all information simultaneously [10]. Therefore, the effects of the treatment on chemical variables are assessed together during analysis.

Following our previous study on the histological characterization of defense mechanisms in cotton genotypes TX-25 and FM966 [2], this work aimed to carry out a chemical examination of both

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strains during 35 days after RKN inoculation. Phenolic compounds, sugars, and organic acids extracted from inoculated and non-inoculated roots were analyzed by ^{1}H NMR spectroscopy, UV-liquid chromatography, and colorimetric methods to compare the level of chemical defenses in each genotype. The change in the chemical profile of damage and undamaged cotton plants over time was assessed using Principal Response Curves (PRC) analysis, a time-dependent multivariate analysis [10,11].

2. Material and methods

2.1. *Gossypium hirsutum* genotypes

FiberMax 966 (FM966), *G. hirsutum* race latifolium, commercial cultivar from Bayer Crop Sciences Fibermax Division, was used as a susceptible strain, whereas wild accession TX-25, *G. hirsutum* race punctatum, Mexico's origin (NPGS PI no. 154035), was used as a resistant strain. The accessions used in this study were obtained from Embrapa's germplasm collections in Brasília, Brazil.

2.2. Nematode inoculation

Meloidogyne incognita race 3 population was collected in Londrina (Paraná State, Brazil); species and race identification have been described by Mota et al. [2]. Prior to inoculation, the population was multiplied on tomato (*Solanum lycopersicum* cv. Kada) for 90 days under greenhouse conditions. Eggs were extracted from infected roots using 0.5% NaOCl according to the method proposed by Hussey and Barker [12]. Freshly hatched second-stage juveniles (J2) were collected using modified Baermann funnels. Egg and J2 counting was performed using a light microscope and Peter's slides.

Individual plants of each genotype were grown in plastic pots (1 L) filled with autoclaved sand under greenhouse conditions, totaling 21 plants for each genotype. Twenty days after seed emergence, three plants from each genotype were uprooted (Time zero, T0) and half of the other plants were inoculated with 5000 *M. incognita* race 3 eggs and J2 by pipetting the nematode suspension around the stem base. Plants were arranged in a randomized block design with three replications and then kept in a greenhouse at a temperature ranging between 25 and 30 °C, and were watered and fertilized according to necessity. Plants were uprooted after 8 (T1), 24 (T2), and 35 (T3) days after inoculation (DAI), then roots were rinsed under tap water, freeze-dried, cut in pieces, and weighed.

2.3. Extraction and colorimetric assays of phenolic compounds

Freeze-dried roots (0.1 g) were homogenized with 5.0 mL of ethanol 96% in a test tube and sonicated for 15 min. The extract was separated from the solid residue by centrifuging at 4000 rpm for 5 min and transferred to a 10.0 mL volumetric flask. The same procedure was repeated two more times with 3.0 mL and 2.0 mL of ethanol 96% for 15 min each. Extracts were combined in a final 10.0 mL volume and prepared in triplicate.

Total phenols (phenolic acids, flavonoids, tannins and gossypols) were quantified by the Folin-Ciocalteu method described by Escarpa and Gonzalez [13]. Total flavonoid content was determined by a modification of the Pharmacopoeia Helvetica method [14]. All samples in the two assays were analyzed in duplicates; the standard curve for total phenolics and total flavonoids was constructed with gallic acid (Merck) and rutin (Sigma-Aldrich), respectively. Results were expressed as mg gallic acid equivalent/g dry root and mg rutin equivalent/g dry root, respectively.

2.4. ^{1}H NMR analysis

Ethanolic root extract (4.0 mL) was first concentrated until dryness under reduced pressure and then dissolved with 0.58 mL of methanol-d_4 (CIL). The solution was placed in a 5 mm NMR tube, and 20 μL of gallic acid solution (1% w/v methanol-d_4) was added as internal standard for quantitative analysis. NMR spectra of ^{1}H, COSY, and HSQC were recorded on a Bruker AVANCE III 500 spectrometer, operating at 500.13 MHz for ^{1}H and at 125 MHz for ^{13}C, using TMS as chemical shift reference ($\delta = 0$). The following parameters were applied to ^{1}H NMR spectra: the spectral window was 10 ppm and data was collected into 65 k data points after 48 scans; the recycle delay was 5 s and had a flip angle of 90°, with an acquisition time of 4.06 s at a fixed temperature of 25 °C. Data were analyzed by the TopSpin 2.1 software (Bruker BioSpin Corp., MA, USA). Seven metabolites were quantified by measuring the peak area ratio of their signals in the ^{1}H NMR spectrum relative to gallic acid.

2.5. HPLC's extract profile

HPLC followed the method described by Dowd and Pelitire [15]. The chromatographic system used was a Shimadzu LC-10AVP with two LC-10ADvp solvent delivery units (Shimadzu Corp., Japan) connected to an SPD-10AVvp ultraviolet photodiode array detector. Chromatographic separations were performed using a LiChrospher 100 RP-18 (5 μm), 250 mm × 4.0 mm i.d. (Merck Millipore, Billerica, MA, USA). The isocratic mobile phase consisted of 60/40 acetonitrile/phosphate buffer (10 mM, pH 3). Analyses were conducted using a 1.0 mL min^{-1} flow rate, detector wavelength at 235 nm, and sample injection volume of 20 μL. Ethanolic extracts from cotton roots (0.5 mL) were filtered with a 0.22 μm membrane (Millipore-LCR, PTFE hydrophilic) prior to HPLC. Class-VP software, version 5.02, was used to determine the relative percentage of separated compounds.

2.6. Statistical analysis

Average multiple comparisons were performed by analysis of variance (two-way ANOVA) using SAS GLM analyses (Statistical Analysis System, version 6.12, SAS Institute Inc., Cary, NC, 1996), with cultivar control (two levels) and time (four levels) as factors, as well as cultivar treatment (two levels) and time (four levels). All data was checked for normal distribution by the Kolmogorov-Smirnov test and for homogeneity of variance using Hartley's and Cochran's tests. Whenever these tests revealed significant departures from the basic assumption, variables were rank, angular or log-transformed. In addition, whenever a difference was established in ANOVA, a *post-hoc* Tukey test was performed. Results are shown as mean values and $p < 0.05$ was regarded as significant.

To analyze differences in chemical contents between the data sets from the two non-inoculated cotton lines, as well as between each inoculated (treatment) and non-inoculated (control) accession, a multivariate PRC technique was performed using the Canoco software package [10,16].

PRC is based on the Redundancy Analysis method (RDA), the constrained form of Principal Component Analysis (PCA), and it is applied to investigate the effects of variables (chemical contents) and their changes over time. Sampling time was used as a categorical covariable and the interaction between sampling time and treatment was used as an explanatory variable. The analysis produces a diagram showing the time gradient on the x-axis and the first or second PCs of the differences in chemical structure (between treated and non-treated cotton) on the y-axis [17], which are expressed as canonical coefficients (Cdt). The line at $y = 0$

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