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Article

NMR-Based Metabolomic Screening for Metabolites Associated with Resistance to *Meloidogyne javanica* in *Annona muricata* Roots

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The resistance of *Annona muricata* roots to the nematode *Meloidogyne javanica* was investigated by nuclear magnetic resonance (NMR)-based metabolomics in combination with principal component analysis (PCA). Metabolic changes in roots exposed and not exposed to the nematode were evaluated and compared. In addition, the presence of nematicidal compounds in the root extracts was investigated through *in vitro* assay against *Meloidogyne javanica* second-stage juveniles. Plants exposed to nematodes showed significant changes in their metabolism after 24 h. Several resistance-related metabolites, including dopamine, xanthine and aromatic compounds, could be identified in the roots with the joint analysis of 1D/2D NMR and the loadings of PC3 (17.8%). *A. muricata* root chloroform extract, containing mainly acetogenins, has shown nematostatic activity against *M. javanica*, suggesting that a pre-formed defense mechanism can support the reported resistance. For the first time, metabolomic studies allowed to identify induced and pre-formed defense mechanisms and their related metabolites in *Annona muricata*.

Keywords: Annonaceae, plant resistance, nematode, ^{1}H NMR, chemometrics

Introduction

All the harvestable plants can be attacked by nematodes, whose presence in the soil may remain undetected due to their small size and the fact that visible symptoms are not always present in the infected plants. Root-knot nematodes (*Meloidogyne* spp.) are the most common ones. They are responsible for economic losses in different crops, and the use of synthetic nematicides as a mean of control has been causing serious environmental damage. Since current nematode control methods present many disadvantages, such as difficulty of implementation in some areas and high cost, there is an increasing demand for new safe methods that prevent human and environmental contamination with toxic substances.[1,2] Several nematicidal substances have been isolated from plants and can be considered possible alternatives.[1] Secondary metabolites reported as potential phytonematode controlling agents may present unique and complex chemical structures belonging to different classes of natural products, such as alkaloids, terpenes, tannins, flavonoids glycosides, among others.[3]

The Annonaceae family comprises a group of tropical and subtropical fruit plants of economic importance in Brazil and in other regions of the world. More than 2,000 species are known for their highly commercial edible fruits, folk medicinal use, industrial and reforestation potential, as well as for their exotic plants.[4] Some Annonaceae, such as *Annona coriacea*

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and *A. muricata*, are resistant to nematodes of the genus *Meloidogyne*.[5] This resistance mechanism might be very complex, include physical structures or barriers, and even produce secondary metabolites with repellent or harmful action to the nematode. This metabolite production can either be a pre-formed defense mechanism or start after signals alerting about possible nematode penetration.[6] Nematicidal evaluations of *Annona* extracts have been performed.[7] However, there are no metabolomic studies at the moment that correlate the resistance of *Annona* to nematodes with any particular metabolite. The metabolomic approach has brought a faster and more holistic view on natural products produced by a living organism under certain conditions.[8] Changes in the metabolome can be monitored using different analytical techniques affording a general view of all the metabolites involved in the understanding of the plant-nematode relationship as well as the defense mechanism used by these nematode-resistant species.

The nuclear magnetic resonance (NMR)-based metabolomic analysis of inoculated and non-inoculated plants may assist the development of new methods for controlling nematodes. NMR permits not only a simultaneous detection of metabolites from diverse classes, but also their total identification and quantification into complex matrices without previous separation.[8] In this work, ^{1}H NMR experiments combined with principal component analysis (PCA),[9] an exploratory and unsupervised chemometric classification method, were applied to evaluate the metabolic responses of *A. muricata* plants exposed to the nematode *Meloidogyne javanica*. In order to evaluate the presence of nematicidal compounds in the roots of the *A. muricata*, root extracts were submitted to *in vitro* nematicidal assay against second-stage juveniles of *M. javanica*.

Experimental

Annona muricata seedlings

Sixty seedlings were acquired from the Instituto Estadual de Florestas (Minas Gerais, Brazil) with homogeneous height and 90 days of germination. They were grown in plastic trays containing substrate and kept in a greenhouse during the entire experiment.

Nematode inoculum

Roots of tomato plants infested with *M. javanica* were carefully washed and cut into 1 cm pieces. They were then ground in a blender for 40 s in a sodium hypochlorite 0.5% (m/m) solution.[10] The eggs retained in the sieve (25 μm pore diameter) were separated and placed to hatch in a Baermann funnel. The second-stage juveniles (J2) were collected after 48 and 72 h, discarding the first 24 h collection. An aqueous suspension containing 500 J2 *per* mL was prepared using a Peters plate and an optical microscope.

Experiments with *Annona muricata* seedlings

An aqueous suspension (2.0 mL) containing 1000 J2 of *M. javanica* was added to the substrate of each *A. muricata* seedling, through four equidistant cylindrical holes. At 1, 2, 4, 20 and 30 days after the nematode inoculation, the aerial parts of the plants were cut and the root systems were carefully separated from the substrate and washed with water. Then, the roots were frozen in liquid nitrogen and lyophilized. As control, plants without inoculation were submitted to the same procedure.

Root extract preparation for metabolic profile screening

A mixture of 0.75 mL of KH_2PO_4 buffer solution in D_2O (pH 6.0), containing 0.01% (m/v) 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) as an NMR internal standard, and 0.75 mL of methanol-d_4 was added to each of the three biological replicates of the roots of *A. muricata* from the control and inoculated groups (50 mg), both collected at 1, 2, 4, 20 and 30 days of growing.[8] The samples were vortexed for 1 min, placed in an ultrasound bath for 20 min and centrifuged at 17,000 rpm. Then, 800 μL of supernatant extract were transferred to 5 mm diameter NMR tubes.

Nuclear magnetic resonance

All 1D and 2D NMR experiments were performed on a Bruker AVANCE DRX400 spectrometer at the High-Resolution Magnetic Resonance Laboratory (LAREMAR, Chemistry Department, UFMG, Belo Horizonte, Brazil).

^{1}H NMR spectra were acquired at 25 °C with a 16 ppm spectral width, 128 scans, 32,000 data points, with or without pre-saturation of the HDO signal, with 2.0 and 2.6 s relaxation delays, respectively. The raw data were processed using 0.3 Hz line broadening in the Fourier transform. Phase and baseline were corrected and finally the spectra were calibrated on the TSP-d_4 signal at 0.00 ppm. 2D *J*-resolved NMR experiment were performed with 8 scans *per* 32 increments for F1, and 16,000 data points for F2, using spectral widths of 6.6 kHz in F2 (chemical-shift axis), and 66 Hz in F1 (spin-spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a

total acquisition time of 20 min. Data sets were zero-filled to 512 points in F1 prior to magnitude mode Fourier transformation with a sine shape window functions in both dimensions. The resulting frequency domain data were tilted by 45°, and then symmetrized along the F2 dimension (F1 = 0 Hz) and referenced according to internal TSP. From the resulting 2D *J*-resolved spectra 1D-projection along the F2 dimension were extracted using the built-in positive projection routine in Topspin (Bruker Biospin Corporation). ^{1}H-^{1}H correlation spectroscopy (COSY) experiment was acquired with a relaxation delay of 1.0 s. A data matrix of 1024 × 2048 points covering 8 kHz of spectral width in both dimensions was recorded with eight scans for each increment. The data was zero-filled to 2048 × 2048 points prior to 2D Fourier transformation with Qsine shaped window function applied in both dimensions. The heteronuclear single quantum coherence (HSQC) experiment was performed with a relaxation delay of 1.5 s, 20 kHz spectral width in F1, and 8 kHz in F2. A total of 1024 increments in F1 with 16 scans of 2048 data points for each increment were recorded, and a Qsine shaped window function was applied in both dimensions for the 2D Fourier transformation.

Multivariate analysis

^{1}H NMR spectra were converted to ASCII files by the ACD NMR Processor (ACD Lab, Toronto, Canada). Spectral intensities were scaled to total intensity. The matrix was then subjected to multivariate data processing by PCA, using the Pirouette 3.11 software.[11] Data were previously mean-centered, and the regions δ 4.75-4.90 and 3.20-3.40 were excluded from the model, due to the presence of residual signals of water and methanol, respectively.

Root extracts preparation for activity evaluation against *M. javanica*

In order to evaluate the activity of the roots, new extracts were obtained from roots of *A. muricata* grown after 90 days. The extracts were obtained based on a modification of the method described by Chatterjee *et al.*[12] Powdered roots (400 mg) were macerated with 5 mL of hexane and filtered. This process was repeated three times, and the combined filtrates were concentrated to dryness in a rotary evaporator. The plant residue was further extracted three times with 5 mL of a mixture of methanol/water (9:1). This hydromethanolic fraction was concentrated in a rotary evaporator and then partitioned with $CHCl_3$ (three times). The chloroform and hydromethanolic phases were separated, and the solvents were removed under reduced pressure.

The chloroform extract was analyzed by ^{1}H NMR in $CDCl_3$ and high-resolution atmospheric pressure chemical ionization mass spectrometry (HR-APCIMS, Supplementary Information (SI) section) using a Shimadzu LCMS-IT-TOF spectrometer.

In vitro nematostatic activity of root extracts

For the study of the J2 mobile and immobile activities, the hexane, chloroform and hydromethanolic extracts were frozen and lyophilized. Then, they were dissolved in 1% (m/v) Tween 80 aqueous solution at a concentration of 2 mg mL^{-1}. In ELISA plates with 300 µL wells, 20 µL of the aqueous suspension containing 20 to 30 *M. javanica* J2 nematodes were deposited and then 100 mL of the extracts were added. Four replicates *per* sample were performed. As a control, the solvent of the samples (1% m/v Tween 80) and water were used. The plate was kept in biochemical oxygen demand (BOD) at 25.5 °C for 48 h. All J2 mobile and immobile were counted with the aid of an inverted microscope at magnification 100×. The obtained values were transformed into percentage of J2 immobile and subjected to variance analysis, using the SISVAR[13] computer program. Means were compared by the Scott-Knott test.

Results and Discussion

Metabolic profile screening

Firstly, the assignment of the hydromethanolic root extract spectra obtained with methanol-d_4 and KH_4PO_4 solution in D_2O (pH 6) was performed for the control group. Despite various overlapped signals, some metabolites were identified by comparing the spectra with the appropriate NMR data from the literature.[8,14-17] 2D NMR techniques, such as 2D *J*-resolved, COSY and HSQC, were applied in order to provide additional information for the spectral interpretations as well as to confirm signal assignments.

From the signals in the region between δ 0.80 and 4.00, it was possible to identify amino acids and organic acids, such as alanine, threonine, glycine, 4-aminobutyric acid, inositol and succinic acid, as well as betaine and choline. In the region from δ 4.0 to 5.5, which is characteristic of carbohydrate resonances, the anomeric proton signals of sucrose δ 5.39 (d, 3.9 Hz), α-glucose δ 5.17 (d, 3.8 Hz) and β-glucose δ 4.58 (d, 7.9 Hz) were also observed.

Dopamine was also identified throughout signals at δ 6.68 (H-8, dd, 8.0 and 2.0 Hz), δ 6.78 (H-4, d, 2.0 Hz) and δ 6.82 (H-7, d, 8.0 Hz). The HSQC experiment allowed the unequivocal assignment of the chemical shift values of dopamine hydrogenated carbons (Figure 1). Purinic

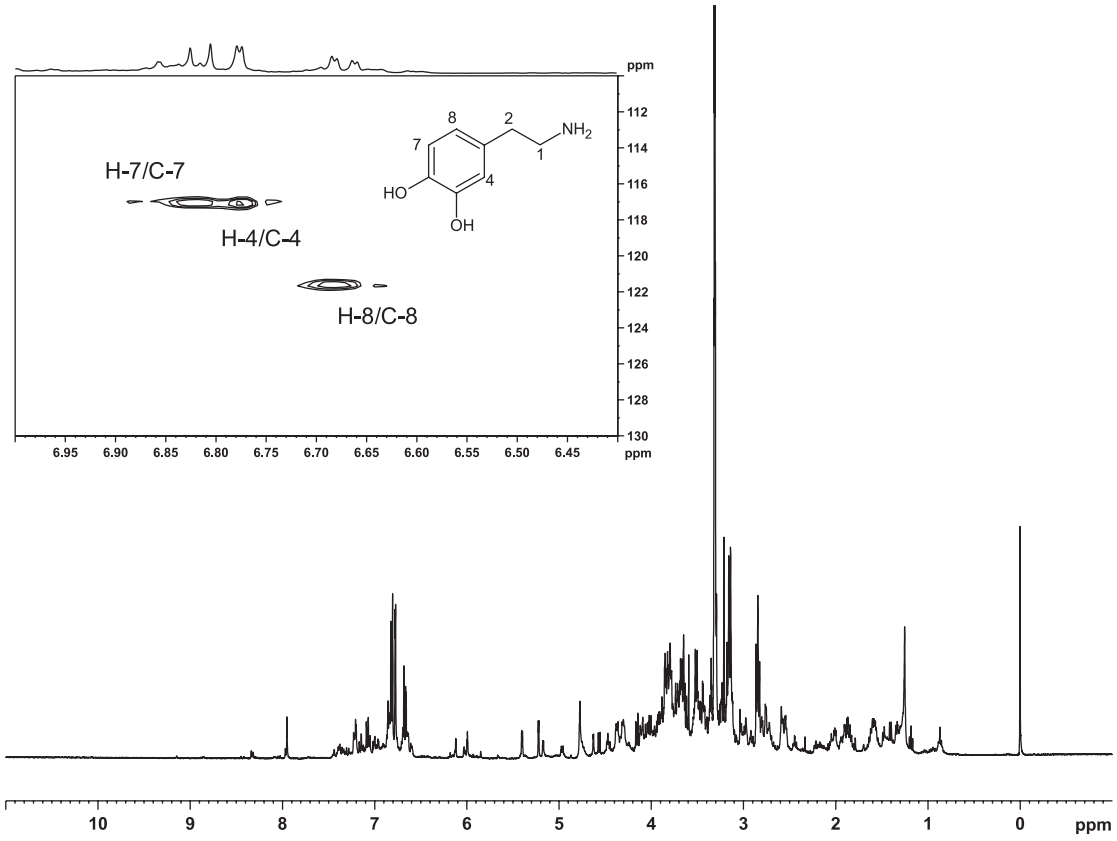

Figure 1. Typical ^{1}H NMR spectrum of the *A. muricata* root extract (400 MHz, methanol-d_4 and KH_4PO_4 solution in D_2O, pH 6). The inset shows the expansion from the ^{1}H-^{13}C HSQC in the range of δ 5.0-7.0 for ^{1}H NMR and δ 110-124 for ^{13}C NMR.

compounds and trigonelline were also identified and confirmed by 2D-analysis. Among the substances detected by ^{1}H NMR, trigonelline may induce plant resistance to parasites.[18] The detected metabolites and their chemical shifts are listed in Table 1.

^{1}H NMR spectrum of chloroform extract (Figure S1, SI section) showed signals at δ 7.18, 6.98, 5.05, 3.80, 3.4 and 1.40, which were assigned as typical of those of Annonaceous acetogenins, common metabolites in the Annonaceae family.[19,20] These compounds are derived from long chain fatty acids combined with a 2-propanol unit, apparently of polyketide origin (C35-C37), possessing a γ-lactone terminal group and probably tetrahydrofuran or tetrahydropyran rings. The liquid-liquid extraction was able to enrich the chloroform fraction in acetogenins, which were further characterized by HR-APCIMS (Figure S1, SI section).

Table 1. ^{1}H chemical shifts of metabolites identified in *Annona muricata* root extracts obtained with the mixture of methanol-d_4 and KH_2PO_4 buffer solution in D_2O (1:1, pH 6) (400 MHz)

Substance	Chemical shift (δ, in ppm) (multiplicity, scalar coupling constant (J / Hz))
Glycine	3.55 (s)
Threonine	1.32 (d, 6.6)
Choline	3.24 (s)
Sucrose	5.39 (d, 3.9), 4.17 (d, 8.5)
α-Glucose	5.17 (d, 3.8)
β-Glucose	4.58 (d, 7.8)
Betaine	3.25 (s)
Malic acid	4.31 (dd, 6.6 and 4.7), 2.74 (dd, 16.6 and 4.7), 2.68 (dd, 16.6 and 6.6)
4-Aminobutyric acid	2.30 (t, 7.2), 3.01 (t, 7.5)
Succinic acid	2.53 (s)
Trigonelline	9.14 (s), 8.87 (m)
Purinic compound (xanthine)	7.92 (s)
Dopamine	6.82 (d, 8.0), 6.78 (d, 2.0), 6.68 (dd, 8.0 and 2.0), 3.18 (m), 2.86 (m)

Metabolomic analysis of *A. muricata* roots exposed and non-exposed to *Meloidogyne javanica*

^{1}H NMR spectra of root extracts were used for monitoring metabolic changes in *Annona muricata* plants exposed and non-exposed to the nematode *Meloidogyne javanica*. For the obtained data set, a PCA model with three principal components (PCs) accounted for 71.17% of the total variance.

It is essential to analyze simultaneously the scores, which provide information about the samples, and the loadings, which provide information about the variables/spectra, for the interpretation of a PCA model. The scores of the first two PCs (Figure 2A) described the plant natural maturation, a process that is usually accompanied by significant metabolic changes. PC1 (30.08%) modeled the plant ageing from the positive to the negative score values, contrasting newer (2-4 days) and older (20-30 days) plants. PC2 (23.34%) clearly discriminated the very new plants (1 day), which were projected on its more negative scores, from the others. It is important to note that the first two PCs did not provide any discrimination between inoculated and non-inoculated plants, modeling the natural maturation as independent factors. Thus, the analysis of only the two first PCs could suggest that the nematodes had not properly penetrated in the plant in order to cause significant changes in its metabolism. For detecting the most important metabolites associated to the plant maturation, the most negative loadings of PC1, positively correlated to the negative scores (older plants), should be identified (Figure 2B). The most negative loadings are in the spectral region from δ 3.00 to 5.50, characteristic of methinic carbohydrate and anomeric hydrogen signals of α-glucose (δ 5.17) and sucrose (δ 5.39). The identification of carbohydrates in both positive and negative loadings of PC1 is coherent with the changes in the content of sugars occurring during the plant growth in both directions.[21,22]

Other important negative loadings on PC1 are in the region of δ 6.66-7.30, characteristic of phenolic compounds, which are a widespread group of defensive substances with an important role in phytoparasitic defense.[2,8] A signal at δ 7.92 was observed and could be assigned to xanthine.[23] Besides being related to the maturation process of this plant, these kinds of compounds are powerful natural allelochemicals that can repel and inhibit the phytoparasite development.[24]

The score plot of PC1 *versus* PC3 (Figure 3A) allowed to discriminate the inoculated plants on PC3 (17.75%) because of the most negative values presented by the scores corresponding to these samples, in opposition to the positive and slightly negative scores of the control plants. This trend is much more pronounced for the samples analyzed one day after the nematode inoculation (I_1 in Figure 3A), which formed a group with the most negative scores, and it became less intense in the following days with the plant maturation. Another remarkable aspect is that this discrimination is modeled by a lower amount of variance in comparison with the effect of plant maturation (53.42%, PC1 + PC2), what strengthens the need of using multivariate analysis for data interpretation.

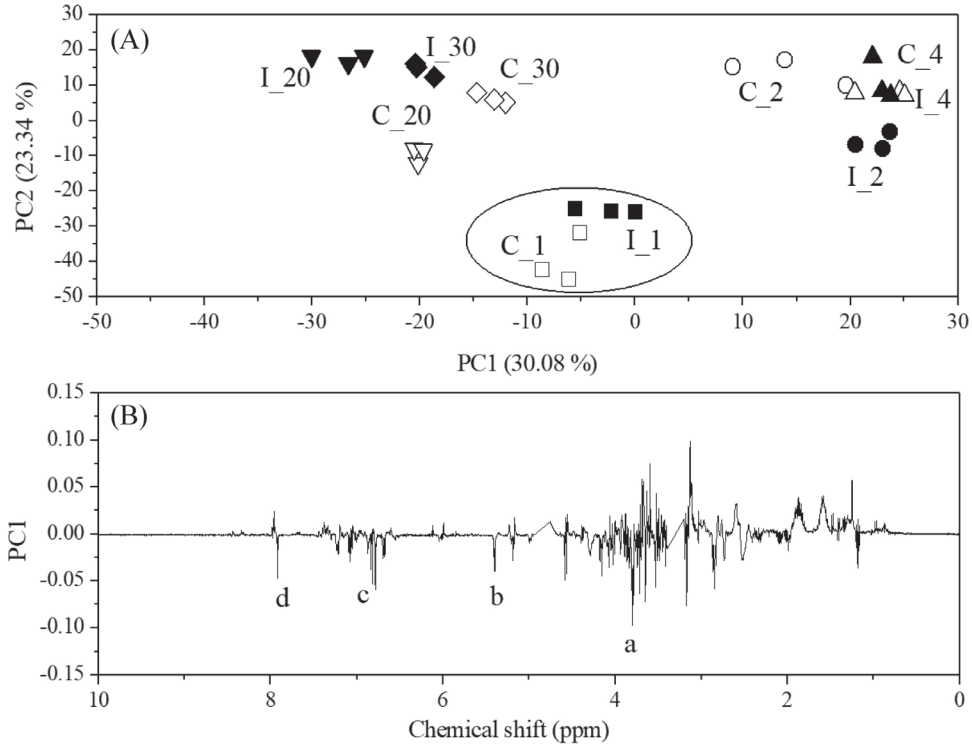

Figure 2. (A) PC1 *versus* PC2 score plot. White icons stand for the control plants and black icons stand for the inoculated plants. The shape of the icons indicates the days after inoculation: □/■ 1 day, ○/● 2 days, △/▲ 4 days, ▽/▼ 20 days and ◇/◆ 30 days; (B) loading plot of PC1 as a function of the chemical shift: (a) methinic carbohydrate signals; (b) sucrose anomeric hydrogen signal; (c) phenolic compounds signals; (d) purine compound signal.

The main chemical shifts that contribute to this discrimination can be attributed by observing the negative loadings on PC3 (Figure 3B). It must be stressed that the signals in the region of the phenolic substances and a signal at δ 7.95 could be associated with the plant response on the first day after inoculation.

In the region of the phenolic substances, dopamine signals (δ 6.68, 6.78, 6.82, 3.18 and 2.86) were identified from the most negative corresponding loadings, and high levels of this metabolite were found in the roots of plants exposed to the nematodes. Wuyts *et al*.[25] have also observed high levels of dopamine in the roots of banana cultivars (*Musa* spp.) resistant to nematode attacks, characterizing this metabolite as a potential chemical barrier. Furthermore, dopamine is a good substrate for polyphenol oxidases, which are enzymes that catalyze two different reactions: the hydroxylation of monophenols to *o*-diphenols; and their further oxidation to the corresponding *o*-quinones. These quinones are more toxic to the microorganisms than phenolic compounds.[26] Additionally, polyphenol oxidases are also stimulated by reactive oxygen species (ROS), which can lead to a systemic acquired resistance (SAR). It is also worth mentioning that dopamine is an intermediate in the biosynthetic route of isoquinoline alkaloids, which may present nematicidal activity.[27]

The signal at δ 7.95 may be associated to an aromatic or purinic compound involved in the response defense. Possibly, this compound derived from xanthine metabolism since a positive loading peak at δ 7.92, attributed to xanthine, was previously observed on PC1 related to the plant natural maturation. The comparison of loading plots showed differences in the metabolic profile (Figure 4).

These observations may suggest that the compounds possibly involved in the defense mechanism were rapidly synthesized or released as a response after one day at the nematode presence. This hypothesis may be strengthened by the analysis of the PC3 loadings (Figure 3B). It is known that many plant species are not host of nematodes, because they liberate repellent or toxic compounds into the soil, preventing nematodes from migrating in their direction and establishing parasitism.[1]

In vitro activity evaluation of root extracts against *Meloidogyne javanica*

In order to evaluate the presence of a pre-defense mechanism against nematode attacks in the roots of *A. muricata*, their extracts were submitted to *in vitro* nematicidal assay against second-stage juveniles of *M. javanica*. Although several compounds with potential nematicidal activity were identified in polar extracts of

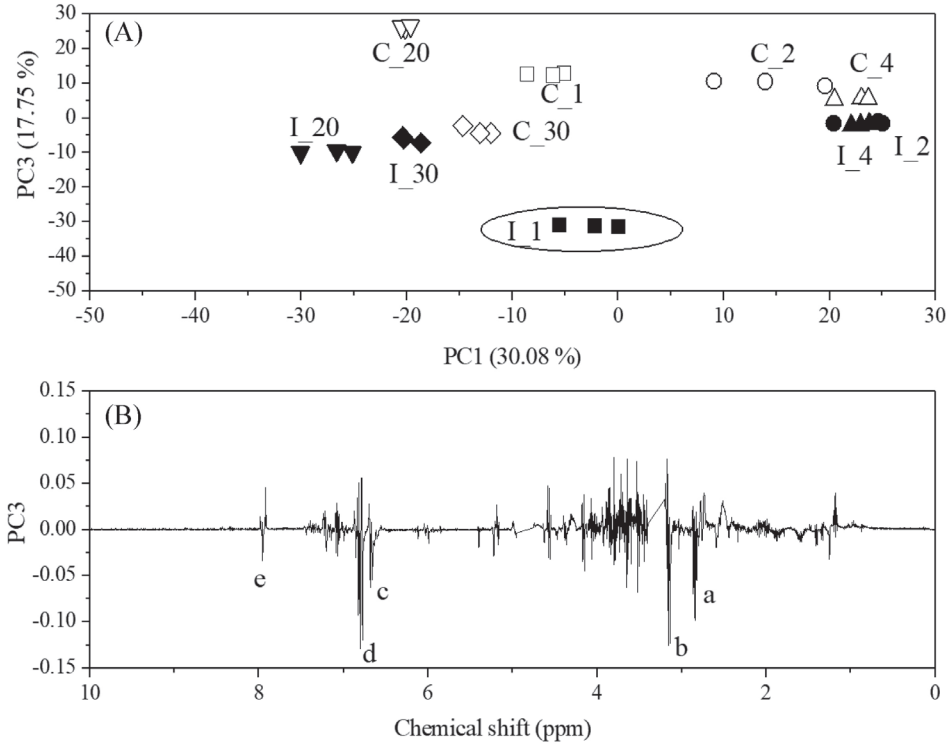

Figure 3. (A) PC1 *versus* PC3 score plot. White icons stand for the control plants and black icons stand for the inoculated plants. The shape of the icons indicates the days after inoculation: □/■ 1 day, ○/● 2 days, △/▲ 4 days, ▽/▼ 20 days and ◇/◆ 30 days; (B) loading plot of PC3 as a function of the chemical shift: (a, b) methylenic signals of dopamine; (c, d) aromatic signal of dopamine; (e) aromatic compound signal.

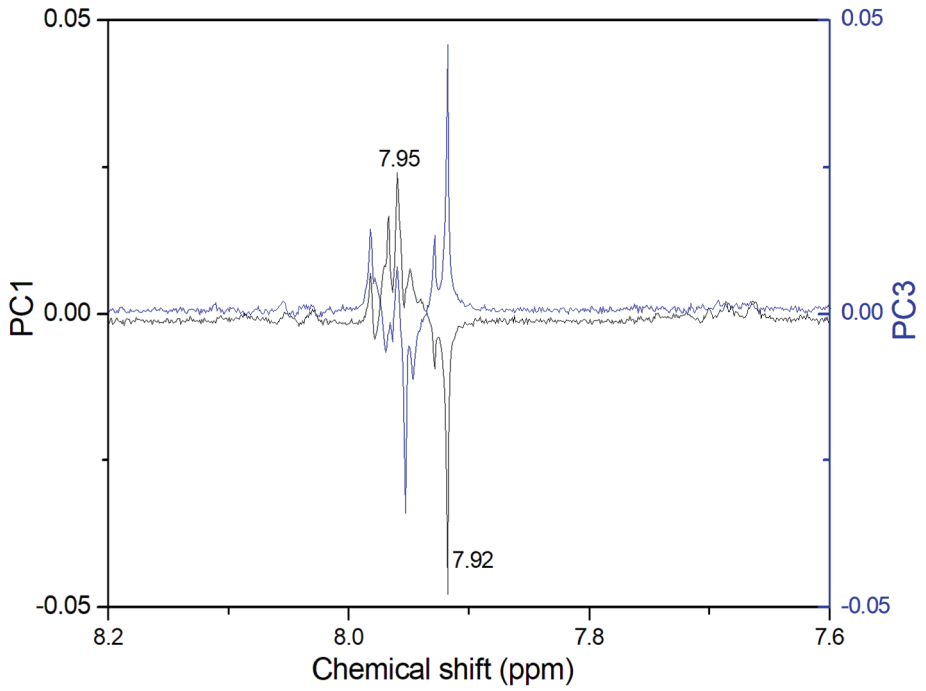

Figure 4. Expanded loading plots as a function of the chemical shift: black line of PC1 and blue line of PC3 in the range of δ 7.8-8.20.

A. muricata roots, these extracts showed no nematicidal activity. However, they presented nematostatic effect (Table 2) when subjected to the *M. javanica* mortality test.

Table 2. *In vitro* nematostatic effect of *Annona muricata* root extracts on *Meloidogyne javanica* second-stage juveniles

Extract	Immobile nematode[a] / %
Hexane	3.5[A]
Chloroform	77.1[C]
Hydromethanolic	28.0[B]
Tween 80 at 1% m/v	4.1[A]

[a]Means with the same letter do not differ significantly according to the Scott-Knott test ($p \leq 0.05$).

Among all the tested extracts, only the chloroform extract showed the highest toxicity to *M. javanica* J2, presenting the best nematostatic action. The metabolic profile of the chloroform extract revealed the presence of Annonaceous acetogenins, metabolites mainly found in Annonaceae family. These compounds exhibit remarkable antitumor and pesticidal activity. Some of them even display activity against the *Aedes aegypti* mosquito larvae, the corn borer, beetles and nematodes.[28,29] Therefore, these results suggested that, besides the production of ROS and dopamine, Annonaceous acetogenins may also be involved in defense mechanisms against the nematode *M. javanica*. Thus, it is possible that the *M. javanica* J2 have not penetrated or established a feeding site on the *A. muricata* roots, since no significant changes were observed in the plant metabolism after the second day of inoculation.

Conclusions

A metabolomic study associated with multivariate data analysis (PCA) allowed the screening for metabolic alterations in the *A. muricata* plant until after 24 h of exposure to the nematode *M. javanica*. This effect was modeled by a principal component (PC3) accounting for a lower amount of variance (17.8%) than the components (PC1 and PC2) that described the natural maturation of the plant. A rapid defense response was observed and associated with increased levels of an aromatic or purinic compound and dopamine (assigned based on PC3 loadings), present mainly in the metabolome of inoculated plants when compared to the non-inoculated ones. The presence of Annonaceous acetogenins and their toxicity to the nematodes were also detected in the root, suggesting a synergistic plant protection mechanism to nematode attacks. For the first time, a systematic metabolomic study aiming to evaluate the resistance of *A. muricata* to the nematode *M. javanica* was carried out and suggested that induced and pre-formed defense mechanisms may be involved. It also allowed the identification of some metabolites without need of an extensive chromatographic purification. Further studies are being conducted to fully elucidate the *A. muricata* resistance mechanisms to the *M. javanica* nematode.

Supplementary Information

Supplementary data (liquid chromatography-mass spectrometry and NMR spectra) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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