Heliyon 9 (2023) e17778

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Secondary metabolites related to the resistance of *Psidium* spp. against the nematode *Meloidogyne enterolobii*

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ARTICLE INFO

Keywords:
Guava tree
Meloidoginosis
Nematodes
Secondary metabolites

ABSTRACT

The guava tree (*Psidium guajava*) is a tropical species native to South America and is recognized as the 11th most economically important fruit tree in Brazil. However, the presence of the nematode *Meloidogyne enterolobii* and the fungus *Fusarium solani* in the roots of guava plants leads to the development of root galls, causing significant damage. In contrast, the species *P. guineense* and *P. cattleianum* have been identified as resistant and immune to the nematode, respectively. In this study, the researchers aimed to compare the metabolomic profiles of infected and uninfected roots of *P. guajava*, *P. cattleianum*, and *P. guineense* using mass spectrometry coupled with liquid chromatography (LC-MS). The goal was to identify secondary metabolites that could potentially be utilized as biochemical resources for nematode control. The findings of the study demonstrated that the plant metabolism of all three species undergoes alterations in response to the phytopathogen inoculation. By employing molecular networks, the researchers identified that the secondary metabolites affected by the infection, whether produced or suppressed, are primarily of a polar chemical nature. Further analysis of the database confirmed the polar nature of the regulated substances after infection, specifically hydrolysable tannins and lignans in *P. guineense* and *P. cattleianum*. Interestingly, a group of non-polar substances belonging to the terpene class was also identified in the resistant and immune species. This suggests that these terpenes may act as inhibitors of *M. enterolobii*, working as repellents or as molecules that can reduce oxidative stress during the infection process, thus enhancing the guava resistance to the nematode. Overall, this study provides valuable insights into the metabolic alterations occurring in different *Psidium* spp. in response to *M. enterolobii* infection. The identification of specific secondary metabolites, particularly terpenes, opens up new possibilities for developing effective strategies to control the nematode and enhance guava resistance.

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[1] This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ, EMBRAPA, and the Conselho Nacional de Desenvolvimento Científico (CNPq). The first author was supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) MSc research grant.

https://doi.org/10.1016/j.heliyon.2023.e17778
Received 15 March 2023; Received in revised form 15 June 2023; Accepted 16 June 2023
Available online 4 July 2023

1. Introduction

The Myrtaceae family belongs to the eudicotyledonous clade and encompasses 132 botanical genera. Within this family, the *Psidium* genus consists of 150 species. Among them, *Psidium guajava* L., commonly known as guava, holds significant importance [1,2]. Globally, the major guava producers include Pakistan, Brazil, Mexico, Italy, Greece, and other Mediterranean countries [3].

The guava tree, particularly the cultivar "Paluma," holds the position of being the 11th most economically important fruit tree in Brazil [4]. In addition to its nutritional value, the guava fruit contains high levels of sugars, retinol (vitamin A), and various B vitamins such as thiamine (vitamin B1) and niacin (vitamin B3). It is also a notable source of essential minerals including phosphorus, potassium, iron, and calcium [5].

P. guajava has been gaining significant biotechnological interest due to its reported pharmacological properties, which include antioxidant, anti-inflammatory, antibacterial, antifungal, antidiabetic, antihyperlipidemic, cardioprotective, antimutagenic, hepatoprotective, and larvicidal actions. The leaves of *P. guajava* are particularly noteworthy as they contain an essential oil that is rich in phenolic compounds from the tannin and flavonoid groups. Additionally, the leaves contain alkaloids and numerous terpenoids, including saponins. One prominent triterpenoid found in *P. guajava* is corosolic acid [6,7]. In 2020, there were many promising studies related to metabolites obtained by *P. guajava*, including the secondary metabolites flavonol morin-3-*O*-arabinoside and isoquercetin, involved in binding to three key coronavirus disease associated protein from SARS-CoV-2 [8].

However, the guava is attacked by 80 different species of pests and diseases, including the root-knot nematode, *Meloidogyne enterolobii*. This is the main agent causing plant death in cultivated areas in Brazil [9].

Nematodes play a crucial role in all trophic levels of the soil food chain, making them the most abundant animals on Earth. The Phylum Nematoda represents a total biomass of approximately 300 million tons of nematodes residing in surface soils worldwide. They exhibit the highest abundance in subarctic regions, accounting for 38% of the total biomass. Nematodes are also plentiful in temperate regions (24%) and tropical regions (21%). These statistics emphasize the widespread distribution and ecological significance of nematodes in soil ecosystems [10].

In the list of the 10 major phytonematodes, *Meloidogyne* spp., which belongs to the Chromadorea class of the Meloidogynidae family, holds a leading position [11]. This genus exhibits a global distribution, primarily in tropical climate regions, and it parasitizes nearly all higher plant species [12].

Meloidoginosis, also known as root-knot disease, in guava is a complex interaction between the nematode *Meloidogyne enterolobii* and the fungus *Fusarium solani*. When guava plants are infected by this nematode, they exhibit several symptoms. These include stunted growth, yellowing of leaves, reduced number and size of flowers and fruits, the presence of large and multiple galls on the roots, underdeveloped root systems, and in severe cases, the eventual death of the tree [13]. It is important to note that the formation of galls on the roots and leaf discoloration are primary symptoms that are further aggravated by the rot caused by the phytopathogenic fungus [14].

In Brazil, the hybrid 'BRS Guaraçá', registered by the Ministry of Agriculture, Livestock and Supply (MAPA) is used as a guava rootstock, resistant to *M. enterolobii*. It was originated from a cross between a mother plant (*P. guajava* – susceptible species) and a parent plant, an araçazeiro (*P. guineense* – resistant species). However, for guava cultivation areas that have been infected by nematodes, the crop rotation strategy is not recommended, as the perennial characteristic of the plant species limits the success of this control practice [15].

Plants activate immune responses against the attacks of these invaders [16–18]. Using signals designed to the recognition of the pathogen, plants induce a complex defense process through hypersensitive response (HR), systemic acquired resistance (SAR), the induction of pathogenesis-related proteins (PR proteins) and the production of signaling compounds, such as salicylic acid and hydrogen peroxide [18,19].

The first line of the plant defense system, also called innate immunity or basal immunity, includes physical barriers such as the cell wall, waxes and cuticles; and chemical ones, including enzymes and secondary metabolites [20]. Receptors located in plant cell membranes, called pattern recognition receptors (PRRs), usually of the kinases type (RLKs), recognize molecules derived from microorganisms, called molecular patterns associated with pathogens ("pathogen-associated molecular pattern" - PAMPs) or molecular patterns associated with injury ("damage-associated molecular patterns" - DAMPs) [18]. When nematodes invade and migrate within plant roots, cell wall fragments are generated in the form of oligogalacturonides (OGs) that can act as molecular patterns associated with damage and activate host defense responses [21].

This recognition triggers a defense response known as PTI ("PAMP-triggered immunity" (PTI). Some pathogens have the ability to suppress PTI by producing molecules called effectors. The related response due to the action of these effectors is called effector-triggered susceptibility (ETS) leading to activation of innate immunity resulting from recognition of foreign macromolecules [22–24].

However, resistant plants can recognize these molecular patterns by activating effector-triggered immunity (ETI). In general, the ETI and PTI response pathways are similar, but ETI is more rapidly activated and mediated by a single R resistance gene. Therefore, it is related to plant resistance, with an incompatible interaction. PTI, on the other hand, is mediated by several genes and responses are delayed, enabling pathogen infection, thus, with compatible interaction [23–25].

> Recently, scientists have identified several effectors secreted by root nematodes. These plant parasites produce a conserved family of pheromones that regulate the development and communication of the phytonematoid with other organisms [26], acting as a signature that elicits the plant immune responses [23].

The most abundant ascaroside secreted by different phytonematoid species, ascr #18 [27], has been discovered. Ascarosides are the

only PAMPs described so far that activate the initial pattern-triggered immunity responses and are recognized by plants as nematode-associated molecular patterns (NAMPs) [20].

Through chemosensory perception, nematodes locate their host through root exudate signals, indicating that specific metabolites act as attractants or repellants, stimulating or inhibiting egg incubation, or exhibiting nematicidal properties. Plant secondary metabolites, including fatty acid derivatives, terpenoids and phenolics (flavonoids, tannins) contribute independently or jointly to many biological processes [28–30].

In this context, this work aimed to analyze the metabolic profiles of roots of *Psidium* spp. control and test conditions obtained from mass spectrometry coupled to liquid chromatography (LC-MS) in order to identify classes of secondary metabolites differentially produced in response to inoculation by the *M. enterolobii*.

2. Materials and methods

2.1. Biological material

Seedlings of *P. guineense* (resistant to the nematode) [42], of *P. guajava* cv. Paluma (susceptible to the nematode) [44] and of *P. cattleianum* (immune to the nematode) [46] were obtained by germinating seeds in a greenhouse at Embrapa Semiarid, Petrolina, PE (Latitude: 9°09′S, Longitude 40°22′W, at an altitude of 365.5 m) with proper cultural practices.

At 30 days after planting, inoculation was performed by applying a suspension containing 10,000 eggs and second-stage juveniles in two 2 cm deep pits on the soil, at a 2 cm distance from the seedling stem. After inoculation, the pits were filled with the soil of the experimental plot, followed by light irrigation.

After 20 days of inoculation (DAI), samples of roots were collected and washed in running water to remove the substrate. The samples were lyophilized and root sample pools of the three studied species, before and after nematode infection, were prepared, totaling six samples.

2.2. Analysis of metabolomic profiles by mass spectrometry coupled to liquid chromatography (LC-MS)

2.2.1. Extraction of secondary metabolites

The extraction step was performed at the Laboratory of Natural Products and Biological Tests at the Federal University of Rio de Janeiro (LaProNEB-UFRJ).

For each test condition, 100 mg of sample powder was added by 2 mL of solvent, and vortexed for 15 s. The tube was allowed to rest, and the supernatant was poured into another previously weighed dry tube (initial weight). This procedure was performed three times for each solvent, following the order of chemical affinity: hexane, dichloromethane, ethyl acetate, and methanol. Samples were left to dry at room temperature under airflow, resolubilized in the respective extraction solvent and filtered through cotton before transferred back to the original flask. At the end of metabolite extraction, flasks with each of the four fractions, one for each solvent, were obtained for all samples. The flasks were weighed again after drying (final weight). In this methodology, the hexane solvent served the initial cleaning function for removing vegetable waxes. Finally, the extracts obtained were dichloromethane, ethyl acetate, and methanolic.

The masses of the crude extracts were calculated, and a solvent solution (1:1 methanol/water) was used to resolubilize the extracted material, in a final concentration of 1 mg/1 mL.

3. Analytical method in LC-MS

The sample extracts for the identification of secondary metabolites were analyzed using liquid chromatography equipment (Dionex Ultimate 3000) coupled to a Mass Spectrometer (Q Exactive Hybrid Quadrupole-Orbitrap), both from Thermo Fisher Scientific. The analysis was performed on an Ascentis Express C18 column (100 mm × 4.6 mm x 2.7 μm) with a guard column. The flow rate was set at 0.5 mL min^{-1}.

For injection into the LC-MS, 1 mL of the resolubilized extracts were transferred to a vial tube using a disposable PVDF membrane. The gradient elution mode used was as follows: 0–1 min - 15% mobile phase B; 1–16 min - 15–95% mobile phase B; 16–21 min - 95% mobile phase B; 21–22 min - 95-15% mobile phase B; 22–30 min - 15% mobile phase B.

The mobile phase A consisted of 0.1% ammonium formate in water (w/v), while mobile phase B consisted of acetonitrile with 0.1% formic acid (w/v). The oven temperature was maintained at 40 °C during the analysis. The spray ionization was set at 3.9 kV with a capillary temperature of 300 °C. Both positive and negative full scans were performed, and the normalized collision energy (NCE) was adjusted between 35 and 50%.

3.1. Processing and treatment of chromatographic data

After obtaining the metabolic fingerprints, the data were converted from raw format to mzML format using the Proteowizard software. The chromatograms were aligned, centered, and normalized. Noise removal, appropriate baseline setting, extraction, and export were performed using the mzMine software (version 2.53).

3.2. Identification of metabolites

The study employed a Venn Diagram to perform an initial statistical analysis of the data on the substances found in the sets of extracts using the three different solvents (dichloromethane, ethyl acetate, and methanol) at each tested condition (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Furthermore, dendrograms were generated to group the metabolites extracted from various samples of *Psidium* species using the different solvents.

Molecular networking was utilized to calculate the spectral similarity between mass spectrometry fragmentations, enabling an automated search in a library of spectra and expediting the metabolite annotation process. In this study, the online GNPS platform (https://gnps.ucsd.edu) was employed to load the data and visualize the molecular networks in Cytoscape 3.10.0 [31]. This approach facilitated the identification of groups of differentially produced metabolites based on their chemical and structural nature, as well as their potential connections within metabolic pathway synthesis and performance.

The data were processed using The Global Natural Product Social Molecular Networking (GNPS) program, and molecular networks were generated using the Cytoscape software. With the aid of the generated data, a comparison was made between the spectral data and literature data through the search for the molecular formula of the substances in SciFinder [32], which led to the identification of metabolites.

To further analyze the chromatograms, the XCMS Online program (https://xcmsonline.scripps.edu) was utilized, which provided tables for each species and test condition containing molecular mass data of the precursor ions and mass fragments. Subsequently, the obtained data were subjected to analysis in the MetaboloAnalyst 5.0 program (https://www.metaboanalyst.ca) to generate heatmaps. For metabolite identification, the chromatograms were visualized in the mMass program (http://www.mmass.org), and the files were exported and compared against the NIST libraries.

4. Results and discussion

4.1. Molecular networking analysis

The study identified a total of 10,406 substances in GUAJ_CONT, 10,231 in GUAJ_20DAI, 11,045 in GUIN_CONT, 10,237 in

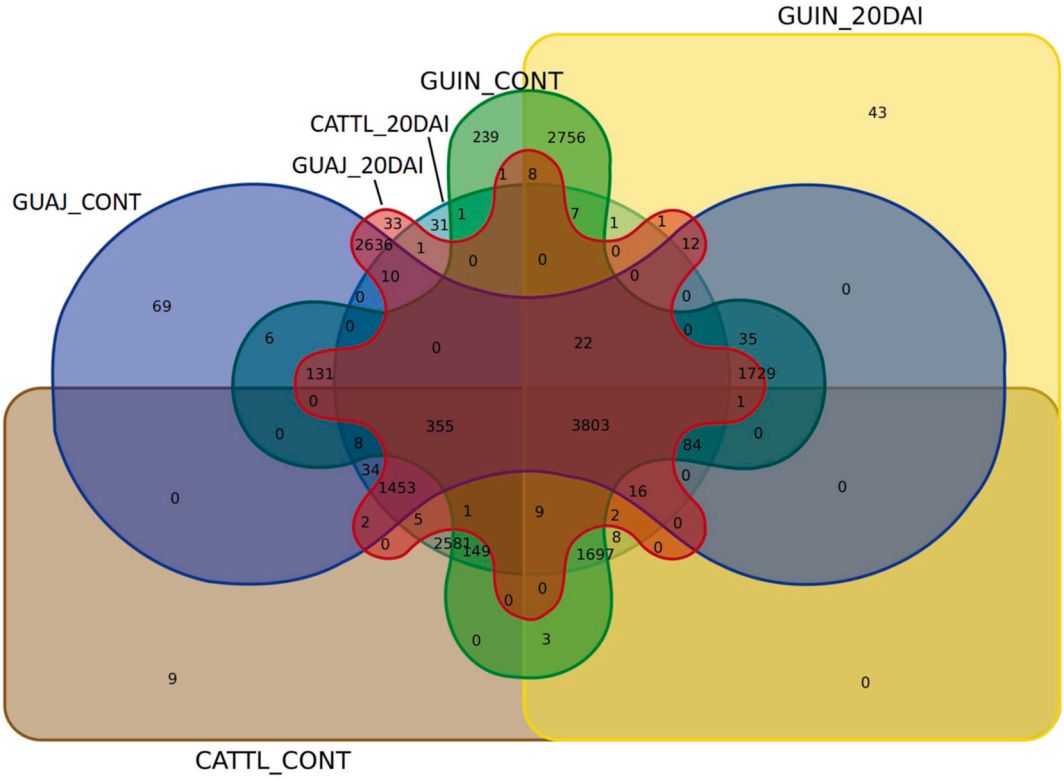

Fig. 1. Non-symmetric Venn diagram generated from the lists of metabolites extracted from the three solvent fractions (dichloromethane, ethyl acetate and methanol): GUAJ_CONT – *Psidium guajava* control; GUAJ_20DAI - *Psidium guajava* 20 days after inoculation; GUIN_CONT - *P. guineense* control; GUIN_20DAI - *Psidium guineense* 20 days after inoculation; CATTL_CONT - *P. cattleianum* control; CATTL_20DAI - *Psidium cattleianum* 20 days after inoculation.

GUIN_20DAI, 10,220 in CATTL_CONT, and 10,278 in CATTL_20DAI when considering the extracted substances in the three solvent fractions. To analyze the substances shared between the three species (*P. guajava, P. guineense*, and *P. cattleianum*), a non-symmetrical Venn Diagram (Fig. 1) was generated. The detailed information about the substances and their distribution is available in the supplementary material in Table 1S.

Interestingly, the substance M486T0 was absent only in CATTL_20DAI, while the substances M753T24, M710T24, M492T25_2, M440T25_1, M399T26_1, M431T26_2, M444T26_1, M482T26_1, and M709T24 were missing only in GUAJ_CONT. Furthermore, the substances 377T21_1, M599T26, M597T25_3, M610T25_1, M518T26_1, M595T24, M697T25, and M559T23 did not appear in GUIN_CONT and the two GUAJ conditions, suggesting their association with resistance and immunity to the phytopathogen.

The substance M356T26_1 was found exclusively in CATTL_20DAI and GUIN_20DAI, indicating its potential relationship with the resistance response of the plant individuals. Additionally, some substances were unique to specific test conditions: 69 substances were found only in GUAJ_CONT, 33 in GUAJ_20DAI, 239 in GUIN_CONT, 43 in GUIN_20DAI, 9 in CATTL_CONT, and 31 in CATTL_20DAI.

LC-MS analyses of root metabolites from all three species, under different treatments and extracted by the different solvents were compared using dendograms (Fig. 2).

Through the analysis of the branches formed by the sample groups of the species *Psidium guajava* (Fig. 2A), it is possible to observe that the secondary metabolites after infection (produced or suppressed) are mostly of polar chemical nature. The control (CONT) and twenty days after infection (20DAI) samples differed when MeOH (methanol) and AcOEt (ethyl acetate) extracts were analyzed,

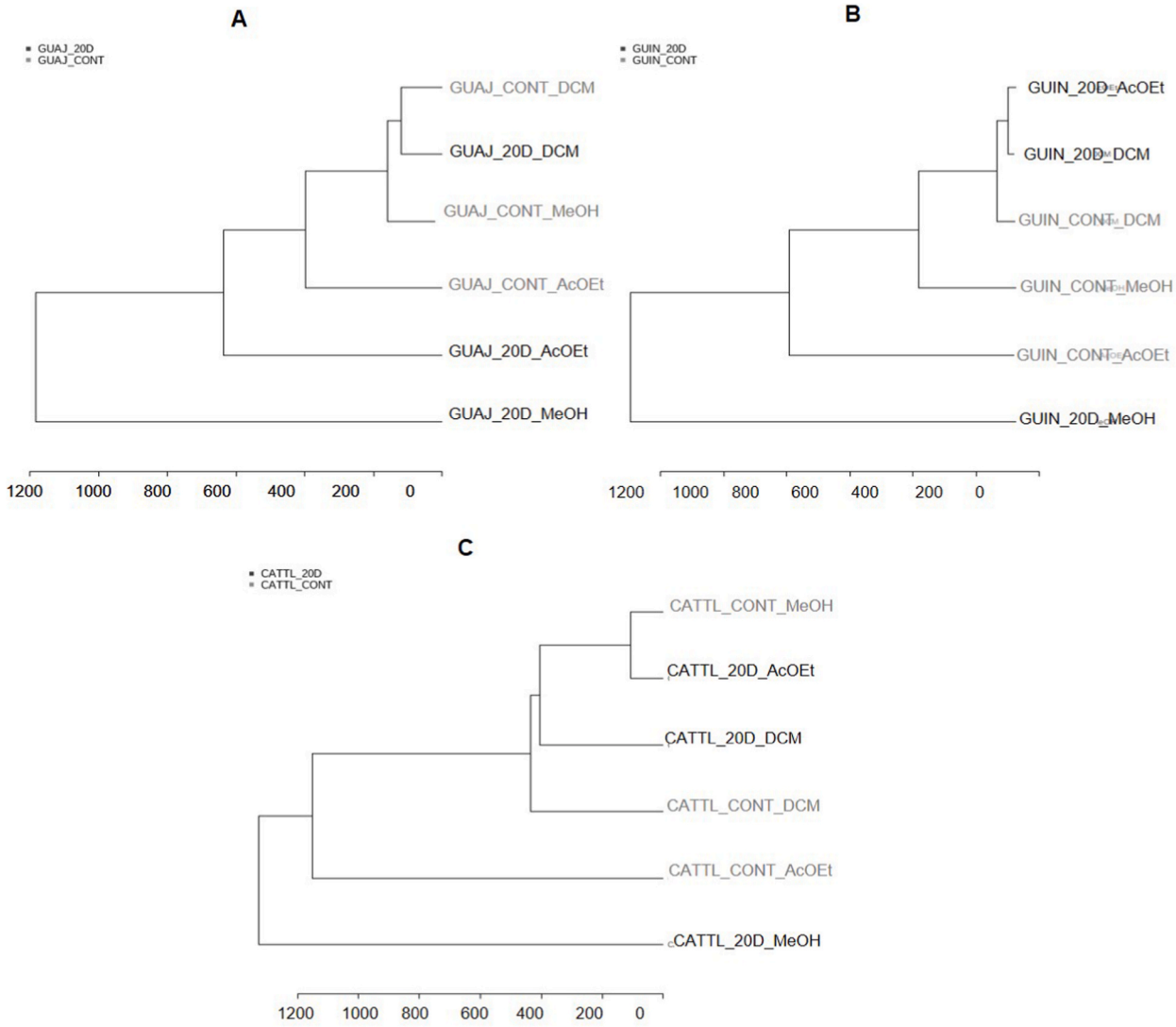

Fig. 2. Dendrograms for comparison of the analytical profiles of extractions with dichloromethane (DCM), ethyl acetate (AcOEt) and methanol (MeOH) solvents: *P. guajava* (A), *P. guineense* (B) and *P. cattleianum* (C). Graphs obtained after data treatment in XCMS Online and generated in MetaboloAnalyst Software. X-Axis – measurements of relative distance between the clades.

considering that they are not in close branches in the dendrogram. The samples in DCM (dichloromethane) are similar, showing that the metabolites produced as a result of the infection possibly do not have an affinity for this solvent. This observation also occurs for the other two species, *Psidium guineense* (Fig. 2B) and *Psidium cattleianum* (Fig. 2C), where the branches of the DCM CONT and 20DAI samples are closer in the dendrogram, and, therefore, with greater similarity. In general, it is possible to see that there is a difference in the pattern of metabolites produced by the control and infected groups, in all three species, and that this difference can be mainly noticed after extraction with methanol and ethyl acetate.

Comparing the spectral generated data with the literature, through the search for the molecular formula of the substances in SciFinder, a resource from the Chemical Abstracts Service (CAS), the metabolites were identified. The graphical representations of the networks, obtained in Cytoscape 3.10.0 (Fig. 3), show four differentially generated clusters, named A, B and C. The data of the substances and clusters generated by the Cytoscape are presented in Table 2S (Suppl.).

In cluster A, substance C20H16O12 (substance 2) was similar to ellagic acid-rhamnoside, from *Nymphaea alba* [33], phenolic compounds from *Eucalyptus* spp. and industrial cork [34], methylellagic acid-pentose, described in *Eucalyptus* globulus [35], 3-methylellagic acid-3′-*O*-β-D-xylopyranoside with antioxidant and anti-inflammatory effects obtained from *Poncianella pyramidalis* [36], eschweilenol C (an ellagic acid derivative) of *Terminalia albida* and *Eugenia moraviana* [37,38], and ellagic acid-deoxyhexoside of *P. guajava* [39], were found. Ellagic acid-rhamnoside is a positional isomer of eschweylenel C, in which there is a single change in the absolute configuration of the C1 of rhamnose. So, both are ellagic acid rhamnosides. Alpha-L-rhamnose is the most common in nature, suggesting that substance 2 is ellagic acid 3-*O*-α-rhamnopyranoside. Substance C21H18O12 (substance 4) was similar to 4-*O*-methylellagic acid 3-*O*-L-rhamnopyranoside, reported in the study of phenolics from *Canarium pimelae* leaves with antioxidant activity [40]. It has a similar structure to substance 2 with 14 more mass units, indicative of the presence of a methyl group. With the mass spectrum alone, it is not possible to assert the position of this methyl in the structure of ellagic acid. No references were found for substances originating from natural products for the molecules C20H19NO12, C21H21NO12, C22H21NO1 and C23H23NO13, named substances 1, 3, 5 and 6, respectively. Substance 1 is similar to 2, with a difference of 17 mass units. Comparing their molecular formulae, there is an indication of an amination in sugar of substance 1, as the aglycone, a non-glycidic grouping that forms part of a glycoside, being the same as substance 2. Similar correlation can be done between substances 3 and 4. Substance C23H20O13 (substance 7) showed

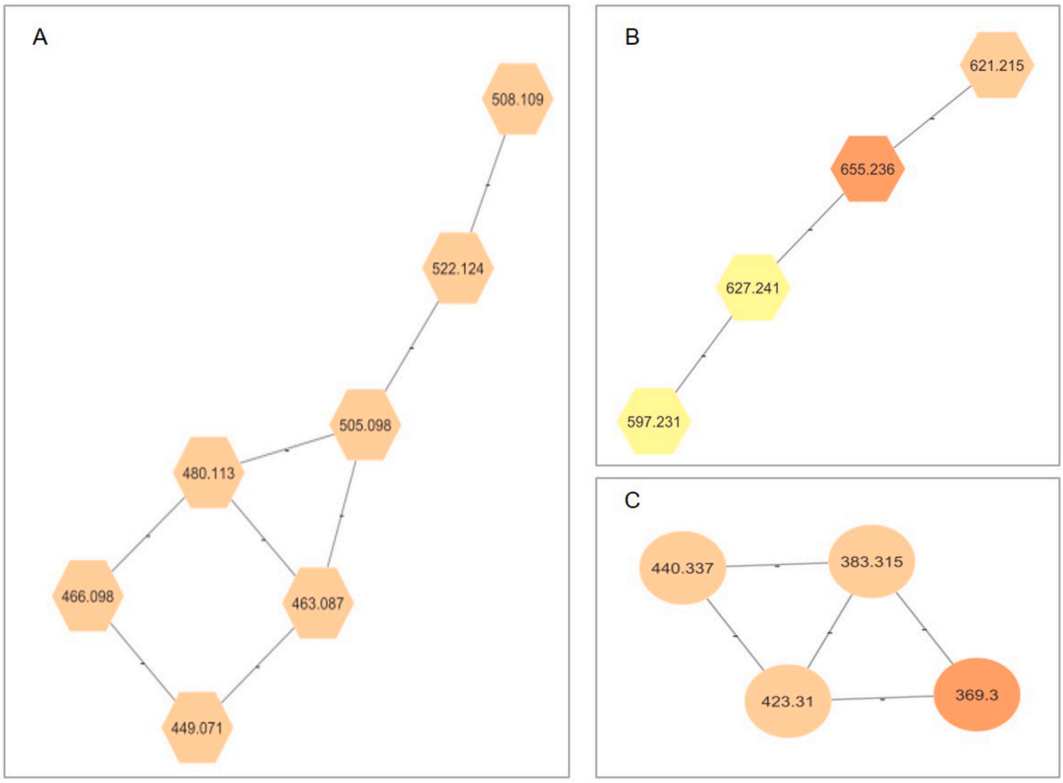

Fig. 3. Molecular networks of the extracted metabolites divided in groups: A – substances 1 to 7, B – substances 8 to 11, C – substances 12 to 15. Symbols and colours: diamond – presence in control only; ellipse – presence only after 20 days of infection; hexagon – presence in control samples and after 20 days of infection; yellow – presence in *P. cattleianum*; rose –in *P. guajava*; light orange – in *P. guineense*; green in both *P. cattleianum* and *P. guajava*; red – in both *P. guineense* and *P. cattleianum*; dark orange – in both *P. guineense* and *P. guajava*; and gray – in the three *Psidium* species. The numbers shown on the nodes refer to the molecular mass of the respective chemical. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

similarity with O-methylellagic acid correlated with acetyl-rhamnose, derived from ellagic acid from fruits of *Eucalyptus globulus* Labill [41] with a structure similar to substance 4, but with acetylated rhamnose.

Thus, it is suggested that the cluster A is formed by substances from the class of hydrolysable tannins, with ellagic acid as its representative. These molecules were differentially produced in the resistant species, *P. guineense*, before and after infection by *M. enterolobii* and extracted by methanol. Tannins are polyphenolic compounds present in various plant tissues, playing protective functions against herbivory and regulating plant growth. The biosynthetic pathways of hydrolyzable tannins (THs) include the biosynthesis of one of the intermediates, the ellagitannins (or ellagic acid) [42]. These molecules synthesized in the shikimate pathway are strong inhibitors of digestive proteases [43]. Some studies have shown that tannins reduce the hatch rate, inactivate *Meloidogyne* spp. and consequently reduce the number of galls [44–47].

In cluster B, the substance C28H38O14 (substance 8) showed similarity to the substances lignan, described in a study of Chinese herbs profiles [48], dendromoniliside E, from *Ilex pubescens* extract and in the metabolomic study of *Dendrobium officinale* [49,50], picraquasioside C, of the botanical genus *Plantago* [51], bioactive lignan ligraminol B, from rhizomes of *Acorus gramineus* [52], iso-eleutoside C, a polyketide from *Eleutherine bulbous* [53] and glucopyranosides [54,55]. Substance C30H38O11 (substance 9) showed similarity to the substance leptolepisol B, among the seven new neuroprotective sesquineolignans isolated from the seeds of *Crataegus pinnatifida* [56], to new lignans from the roots of *Datura metel* [57], and to lignans isolated from the roots of *Solanum melongena* [58]. No references were found for substances originating from natural products for the molecules C28H44O13 and C30H34NO11, named substances 10 and 11, respectively. In this cluster, substance 8 is present in *P. guineense*, while substance 9, in *P. cattleianum*. Substance 11, not identified, belongs to the species *P. guajava* and *P. guineense*, representing a connection node between substances 8 and 9. It is noteworthy that all substances in this cluster B are present in the methanol extract at the control times and after 20 days of nematode infection, as well as in the cluster A.

From the data found in the literature, it is suggested that the molecules in cluster B belong to the class of lignans. Lignans form the building blocks of plant cell walls. Among these, a subgroup of non-flavonoid polyphenols that have insecticidal activity and emerged during the co-evolution of plants and insects, providing plant protection against diseases, have been described [59]. Lignin synthesis increases plant defense to root-knot nematode infections and decreases nematode population densities [60]. A recent study has shown that, in tomatoes, responses to oligogalacturonides (OGs) trigger increased systemic resistance to pathogens. With this, there is the regulation of signaling pathways by jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET), and the activation of the main metabolic pathways for the biosynthesis of antimicrobial metabolites, such as alkaloids, flavonoids and lignans [61]. In a study in which pepper, garlic, maize and sesame seeds were added to the planting soil of tomato plants, grown under the stress of *M. incognita* infection with ground sesame seeds, rich in lignans, the authors observed the values for maximum plant length, fresh mass and total shoot dry mass, and highest percentage reduction in the number of nematode egg masses and galls [62].

In cluster C, the substance C30H46O (substance 12) presented similarity to the substances calotroprocerol A, a new ursano-type triterpene from the root bark of *Calotropis procera* [63], to cornusalterin D, a tirucallane triterpenoid from *Cornus walteri* [64], to cucurbite-1,5,22,24-tetraen-3-ol, an antioxidant triterpenoid from *Momordica charantia* [65], to taraxast-1,20(30)-dien-3-one, a triterpene from *Sida acuta* [66], to 3,4-secocycloarthane, an exudated triterpene from *Gardenia urvillei* [67], to olean-9(11), 12-dien-3-one, from Winteraceae species, with an inhibitory effect on *Trypanosoma cruzi* [68]. Substance C22H40O4 (substance 15) showed similarity to the substances: 3-methyl-3-hydroxy-2-hexadec-7-enyl-4-oxopentanoate, cytotoxic sesquiterpenoid from *Zygogynum* spp. (Winteraceae), from the tropical rainforest of *Caledonia* [42]. No references were found for substances originating from natural products for the molecule C25H45NO5 (substance 13).

All substances in cluster C are present in *P. guineense*, only in samples obtained after 20 days of infection. It is important to highlight that the substances in this cluster were extracted by dichloromethane, suggesting their nonpolar chemical nature. The cluster C is formed, from substances 12 to 15, by a group of terpenes, having the oleans as representatives. Terpenes play an important role in plant defense against biotic and abiotic stresses. The main identified plant volatiles after herbivory include volatile terpenoids, volatile phenylpropanoid (indole), fatty acid derivatives and nitrogen compounds. Elevated levels of terpenes in plants, responsible for parasitoid attraction after an attack by herbivores, are documented in many plant species. One of the examples of indirect plant defense is the release of a mixture of volatiles, which attract predator carnivores, after plant injury caused by herbivores [69,70]. *M. javanica* direct contact with these terpenes caused the death of all J2 juveniles and inhibited egg hatching [71].

As for cluster D (available in Table 2S; suppl.), there were no references for substances originating from natural products for molecules (substances 16 to 40). Among these, only substances 33, 34 and 39 were present in the susceptible species, *P. guajava*. The others are present in resistant and immune species, *P. guineense* and *P. cattleianum*, respectively. Most of the substances were produced in *P. guineense* after infection with the nematode and extracted in dichloromethane fraction, demonstrating the nonpolar chemical affinity of these molecules. The substances 22, 31, 35 and 40 are present only in the methanolic fraction of *P. cattleianum*; substances 33 and 39 are present in the ethyl-acetate extraction of *P. guajava*; substance 32 is in dichloromethane and ethyl acetate fractions, in *P. guineense* and *P. cattleianum* species, and substance 34 is present in methanol and ethyl acetate fractions of both *P. guajava* and *P. cattleianum* species.

4.2. Heatmap analysis with identification by NIST libraries

From the chromatograms data (Figs. 1S–3S Suppl.), the fifty substances of higher concentrations, extracted by DCM, AcOEt and MeOH solvents. represented by the higher peaks, were analyzed through heatmap representations (Figs. 4–6). For all *Psidium* species, it

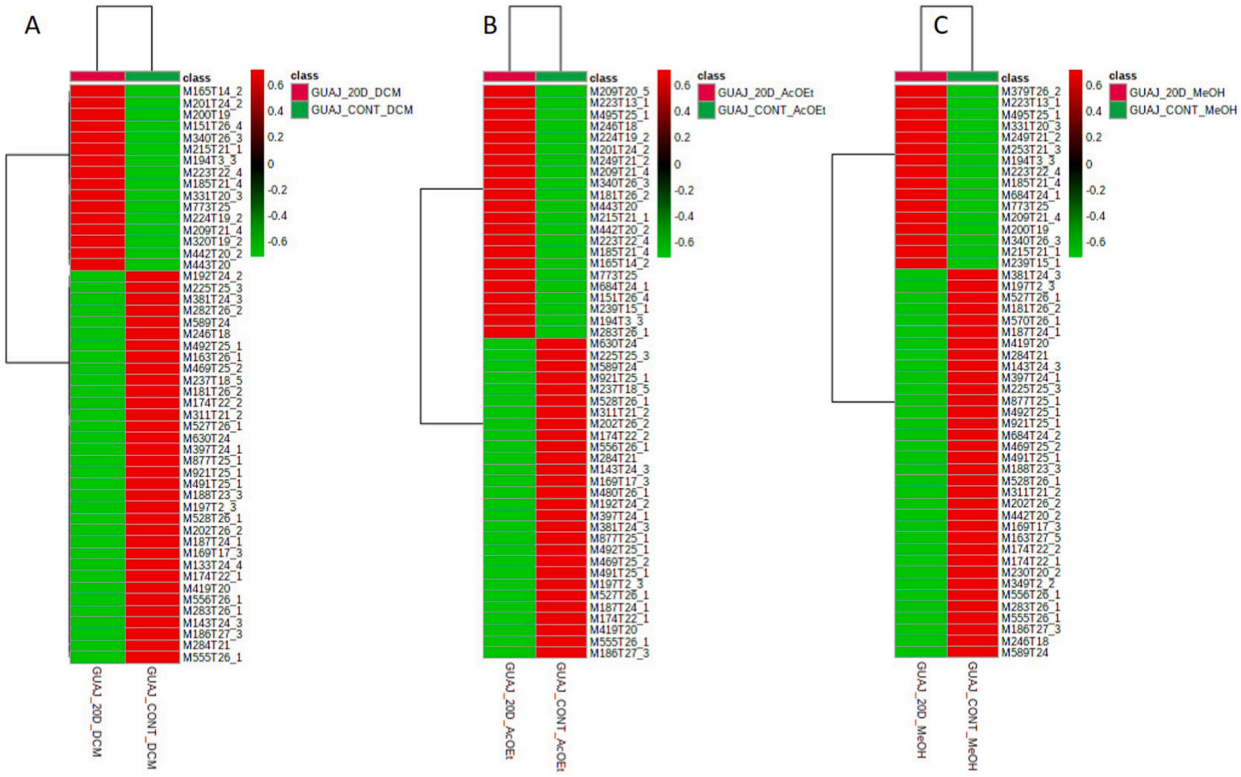

Fig. 4. Heatmaps obtained by the MetaboloAnalyst 5.0 software of the fifty substances with the highest intensity peaks from samples extracted by dichloromethane (DCM) (A), ethyl acetate (AcOEt) (B) and methanol (MeOH) (C), obtained by analysis of *P. guajava* under control conditions (GUAJ_CONT, in gray) and after 20 days of infection (GUAJ_20DAI, in black).

is observed that there was a change in the metabolome, where some substances concentrations increased after 20 days of infection, while others reduced.

In *P. guajava* (Fig. 4), substances whose concentrations increased after nematode infection (black squares left column) were: in DCM (Fig. 4A), of 32%; in AcOEt (Figs. 4B), 44%; and in MeOH (Figs. 4C), 32%. In the three extractions (DCM, AcOEt and MeOH), the substances M174T22_1 and M174T22_2, present only in *P. guajava* (susceptible species), showed a decrease after the period of infection with the nematode *M. enterolobii*. In this same species, M194T3_3 showed an increase in the same test condition.

In *P. guineense* (Fig. 5), substances whose concentrations increased after nematode infection (black squares left column) were: in DCM (Fig. 5A), of 52%; in AcOEt (Figs. 5B), 42%; and in MeOH (Figs. 5C), 68%. In this species, M303T22_4 and M303T24_3, relative to the AcOEt fraction, showed an increase after the infection period. In the three fractions of this species, the substances M128T21 and M128T25_2 presented reductions in the same post-infection test condition.

In *P. cattleianum* (Fig. 6), increased substances after the period of nematode infection (black squares left column) were: in DCM (Figs. 6A), 52%; in AcOEt (Figs. 6B), 44%; and in MeOH (Figs. 6C), 48%. In the three extractions, M195T21_2 showed an increase in *P. cattleianum* and M195T23_1 an increase in *P. guineense*. *P. guajava*, M223T22_4 showed an increase in production after infection. In the three extraction fractions, M223T11_1 showed a reduction in *P. guineense* after the infection period. Stress processes and the plant defense responses stimulate metabolic changes that can result in the biosynthesis of bioactive compounds. The accumulation of a secondary metabolite in a plant at higher levels may indicate the expression of specific genes and the increase in metabolic pathways for their biosynthesis in cells [72]. Studies performed with different plant metabolites, including alkaloids, flavonoids and phenolic compounds; report the plant potential to fight pathogens [73].

Thus, in this work it is possible to suggest that secondary metabolites are involved in the plant defense mechanisms of *Psidium* species against the nematode *M. enterolobii* mainly by regulating the shikimate pathways with hydrolysable tannins and lignans, mevalonate and methylerythritol phosphate with terpenes.

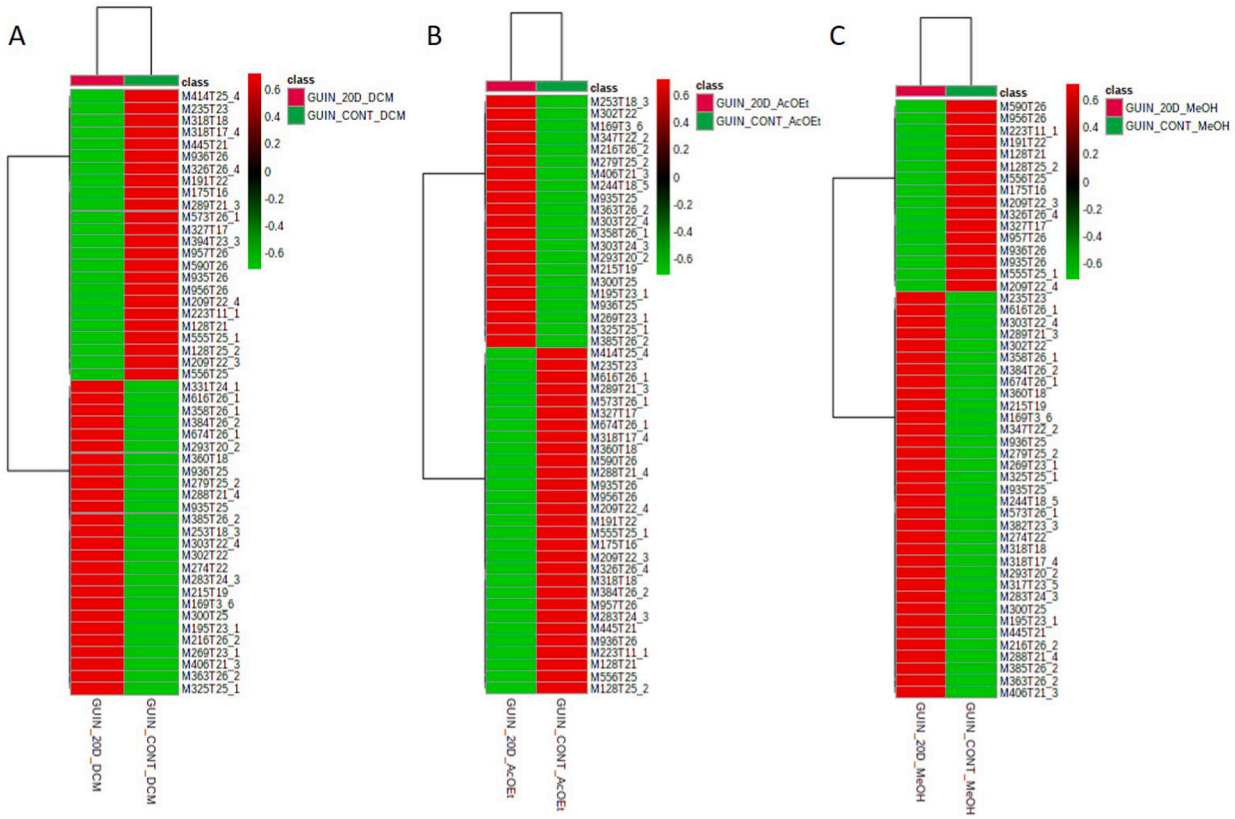

Fig. 5. Heatmaps obtained by the MetaboloAnalyst 5.0 software of the fifty substances with the highest intensity peaks from samples extracted by dichloromethane (DCM) (A), ethyl acetate (AcOEt) (B) and methanol (MeOH) (C), obtained by analysis of *P. guineense* under control conditions (GUIN_CONT, in gray) and after 20 days of infection (GUIN_20DAI, in black).

5. Conclusions

In this study, the metabolic responses of three different *Psidium* species, namely the susceptible species (*P. guajava*), the resistant species (*P. guineense*), and the immune species (*P. cattleianum*), to the nematode *M. enterolobii* were compared. This strategy aimed at the understanding of how the plant metabolism is altered upon inoculation with the phytopathogen and how these alterations relate to the susceptibility or resistance of these species to the nematode.

The findings revealed that the metabolism of all three species was indeed altered upon inoculation with the nematode. Both production and suppression of certain substances were observed, suggesting that susceptibility to the nematode may be associated with either the absence of resistance molecules or the presence of other molecules that attract the nematode.

Through the use of molecular networks, secondary metabolites altered by the infection were predominantly of a polar chemical nature. This observation indicated their involvement in the shikimate pathway, which is responsible for the regulation of phenolic compounds known to be involved in oxidative stress responses, particularly in the context of biotic factors.

Further analysis of the database revealed that the substances regulated upon infection in *P. guineense* and *P. cattleianum*, the resistant and immune species, were primarily hydrolysable tannins, lignans and also some terpenes, which may also act as inhibitors of *M. enterolobii* inoculation.

The identification of these terpenes in the resistant and immune species opens up new possibilities for developing biotechnological products that utilize these volatile substances as repellents against the nematode. Additionally, these terpenes may reduce oxidative stress during the infection process, thereby enhancing the resistance of guava to the nematode.

Overall, this study provides valuable insights into the metabolic alterations occurring in *Psidium* spp. upon infection with *M. enterolobii.* It highlights the importance of understanding the complex interactions between secondary plant metabolism and nematode infestation. The findings offer new directions for future research aimed at developing effective strategies for nematode control in guava crops, whether through repellent biotechnological products or the manipulation of oxidative stress pathways.

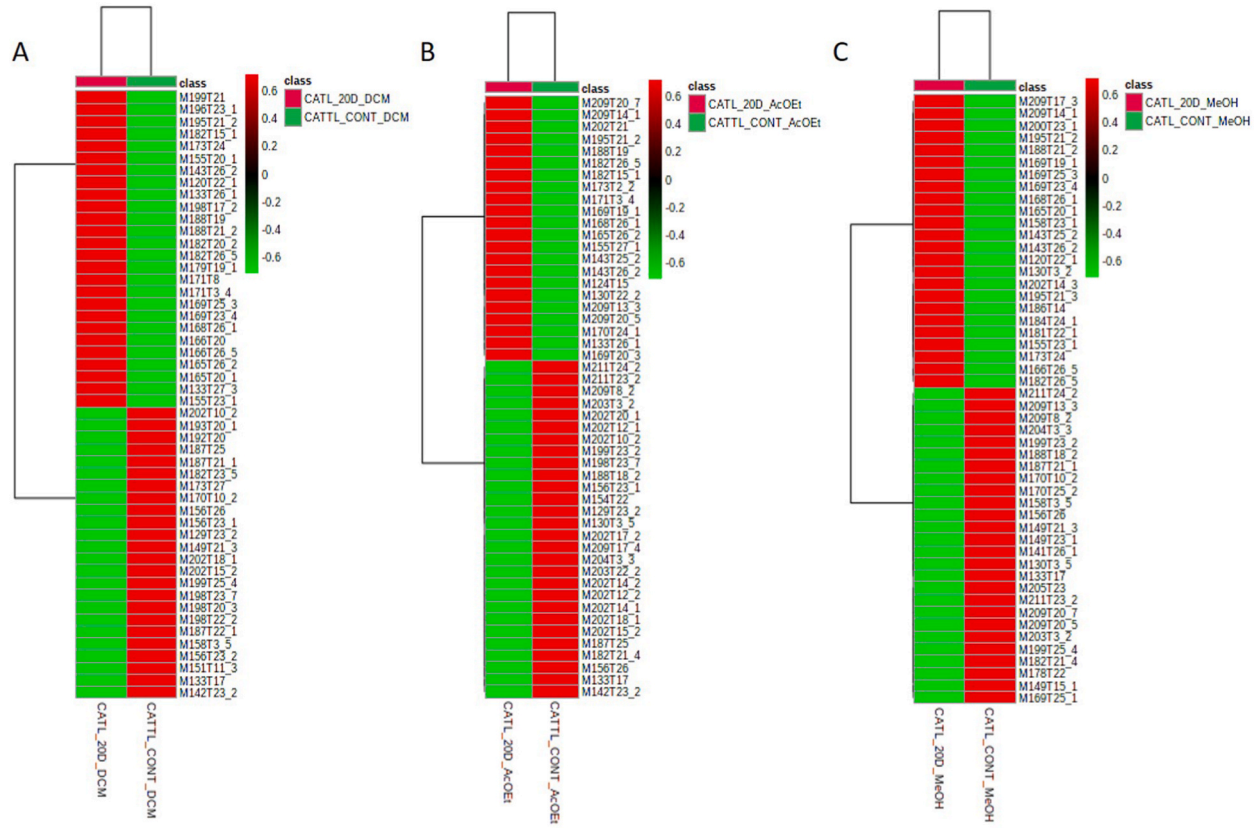

Fig. 6. Heatmaps obtained by the MetaboloAnalyst software of the fifty substances with the highest intensity peaks from samples extracted by dichloromethane (DCM) (A), ethyl acetate (AcOEt) (B) and methanol (MeOH) (C), obtained by analysis of *P. cattleianum* under control conditions (CATTL_CONT, in gray) and after 20 days of infection (CATTL_20DAI, in black).

Author contribution statement

Sara Nállia de Oliveira Costa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Marcos Vinicius Toledo e Silva: Performed the experiments; Analyzed and interpreted the data.

Juliana Martins Ribeiro: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

José Mauro da Cunha Castro: Conceived and designed the experiments; Performed the experiments.

Michelle Frazão Muzitano; Rafael Garret Costa; Antônia Elenir Amâncio Oliveira: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kátia Valevski Sales Fernandes: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

Supplementary content related to this article has been published online at [URL].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17778.

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