https://dx.doi.org/10.21577/0103-5053.20200173

J. Braz. Chem. Soc., Vol. 32, No. 2, 237-248, 2021
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Article

Mapping Biochemical Pathways in *Maytenus ilicifolia* (Celastraceae) through Integrated Proteomics and Histochemistry

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Maytenus ilicifolia (Celastraceae) is a medicinal plant that is native to southern Brazil and is popularly known as "espinheira-santa". From a biosynthesis perspective, this species accumulates quinonemethide triterpenes and sesquiterpene pyridine alkaloids as major secondary metabolites that exhibit interesting biological properties, with antitumoral and antiprotozoal activities, respectively, being the most frequently reported. Additionally, the restricted accumulation of such compounds in the roots raises questions about the expression of proteins involved in such compartmentalization and their possible biological and/or ecological role in *M. ilicifolia*. Thus, this article describes the use of shotgun proteomics and histochemical studies for the characterization of the main biosynthetic pathways involved in the regulation of the metabolism in *M. ilicifolia* roots. This combined approaches also resulted in the identification of a series of proteins involved in the quinonemethide triterpenes and sesquiterpene pyridine alkaloids, providing evidences of their differential compartmentalization.

Keywords: shotgun proteomic approach, histochemical analysis, quinonemethide triterpenes, sesquiterpene pyridine alkaloids, *Maytenus ilicifolia*, Celastraceae

Introduction

Plant growth and development are regulated by phytohormones and mediate responses to biotic and abiotic stresses.[1] In response to stress, plants synthesize and accumulate a wide diversity of secondary metabolites, which are involved in biological and ecological functions.[2,3]

Many of these compounds have shown biological activity and are considered to be prototypes for the synthesis of a large number of drugs,[4] accounting for more than 50% of all drugs employed in modern therapies.[5]

The biosynthesis of these metabolites is performed by specific pathways and involves complex interactions between proteins/enzymes, transport mechanisms, compartmentalization, and integration with primary metabolism at the molecular and cellular levels.[3,6]

Maytenus ilicifolia Mart ex Reissek is a medicinal plant native to southern Brazil that is popularly known as "espinheira-santa". Since 1988, this plant has been included as herbal medicine in the phytotherapy program, coordinated by the Brazilian Ministry of Health's Medicines Center (CEME), and was included in the National List of Medicinal Plants of Interest for the Brazilian Unified Health System (SUS).[7]

The structural richness of *Maytenus ilicifolia* roots is exemplified by quinonemethide triterpenes and sesquiterpene pyridine alkaloids (Figure 1), which are considered chemotaxonomic markers of the Celastraceae species.[8] Several biological activities have been described for these two classes of metabolites, including antioxidant, antifungal, antiproliferative, antiprotozoal, antitumoral, antimicrobial, and inhibitor of hepatitis C virus, antiprotozoal, relating to quinonemethide triterpenes and sesquiterpene pyridine alkaloids, respectively.[9-19]

Thus, this study aimed to (*i*) localize the metabolites through histochemical tests, (*ii*) characterize the main proteins involved in primary and secondary metabolism

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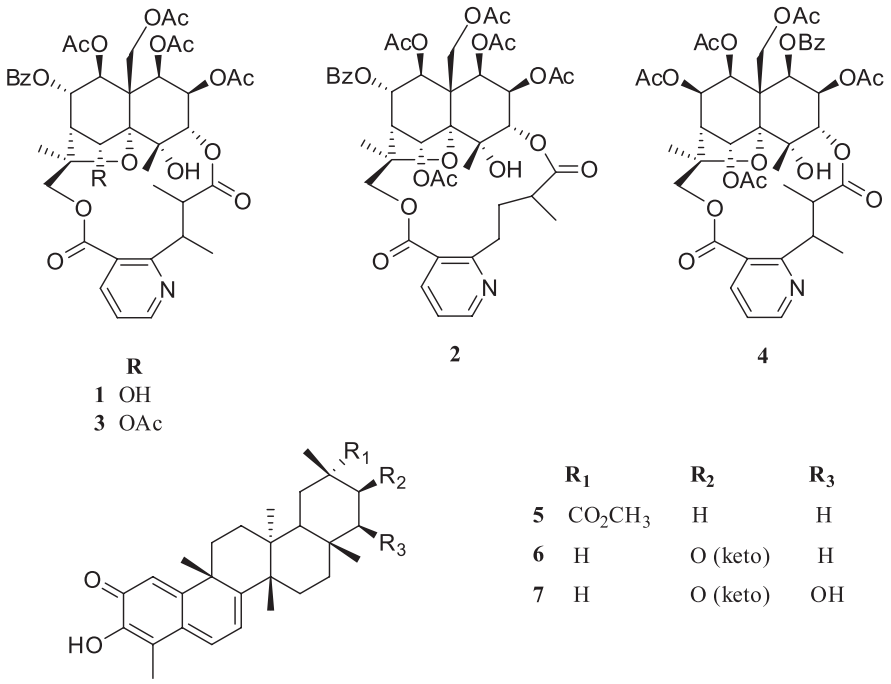

Figure 1. Chemical structures of sesquiterpene pyridine alkaloids: ilicifoliunine A (**1**), ilicifoliunine B (**2**), aquifoliunine E-I (**3**) mayteine (**4**) and quinonemethide triterpenes: pristimerin (**5**), maytenin (**6**), 22β-hydroxy-maytenin (**7**), isolated from *Maytenus ilicifolia*.

by means of proteomic studies, and (*iii*) combine the histochemical and proteomic data. These approaches aimed to identify the main biosynthetic pathways active in *M. ilicifolia* roots, determine and provide evidence of a differential distribution of the metabolites and to address the possible biological and ecological functions that these compounds serve in the roots of *M. ilicifolia*.

Experimental

Plant material

Young plants (five years old) of *Maytenus ilicifolia* ware collected in the district of Juruce, municipality of Jardinópolis, state of São Paulo (21°04'18.8''S; 47°44'08.8''W) in March 2013. After collection, the plants were transported and maintained in a greenhouse at the Instituto de Química de Araraquara, state of São Paulo (21°48'26.62''S; 48°11'33.5''W).

The botanical identification was provided by Prof Rita Maria de Carvalho-Okano. A voucher specimen (00755) was deposited in the Herbarium of the Department of Medicinal Plants, University of Ribeirão Preto, São Paulo, Brazil (HPM-UNAERP).

Histological analysis

For the histochemical tests, lateral roots were selected. Cross-sections were made manually and transferred to a watch glass containing deionized water. Later, these cross-sections were selected using a magnifying glass to obtain the thinnest cuts and cuts with correct orientation. After the selection of the cuts, some of them were kept unstained to verify the natural appearance of the cellular content, and the other part was submitted to different reagents, aiming to achieve *in situ* detection of different classes of metabolites. The following reagents were used for the histochemical tests: Sudan III (Sigma-Aldrich, St. Louis, USA) for lipophilic compounds (lipids, terpenoids, suberin and cutin), ferric chloride (Fisher Scientific, Waltham, USA) for phenolic compounds, and Dragendorff reagents for alkaloids.[20,21] The cuts were then mounted between a slide of glass and coverslip and analyzed under a microscope. The observations and photomicrographs were obtained from a Leica DFC 320 digital camera coupled to a Leica (Wetzlar, Germany) DM 5000 microscope. Autofluorescence was monitored by an epifluorescence microscope equipped with a mercury lamp and green light (excitation, 488 nm; emission longpass filter, 520 nm).

Proteomic analysis

Protein extraction

The total proteins were extracted and precipitated according to the methodology developed by Hurkman and Tanaka[22] with some modifications. Approximately 7 g of the roots were ground to form a thin paste. Subsequently, the material was transferred to centrifuge tubes containing 60 mL of

extraction buffer containing this buffer: 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in isopropanol, 50 mM ethylenediaminetetraacetic acid (EDTA) and 1% (m/v) polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, USA). The mixture was homogenized at 4 °C for 30 min on an orbital shaker.

Then, the same volume of phenol equilibrated with a solution of 10 mM Tris-HCl (Sigma-Aldrich, Saint Louis, USA) pH 7.9 ± 0.2 and 1 mM EDTA (Sigma-Aldrich, St. Louis, USA) was added. The mixture was further stirred for 30 min and finally centrifuged at 4 °C and 10000× g for 30 min. Then, the supernatant (upper phase or phenolic) was transferred to a new tube, and 1 volume of extraction buffer with 0.15 g of PVPP was added. The samples were homogenized in the same manner with the aid of an orbital shaker for 30 min at 4 °C. Subsequently, the samples were subjected to centrifugation at 4 °C and 10000× g for 30 min. The upper phase was again collected and added (1 volume) extraction buffer but without the addition of PVPP. Again, the upper phase was homogenized and centrifuged. The supernatant was recovered, and 5-6 volumes (25-30 mL) of precipitation buffer (100% methanol + 0.1 M ammonium acetate) was added, and samples were kept frozen at –20 °C overnight. After this time, the samples were centrifuged at 7000× g for 40 min at 4 °C, and the supernatant was discarded. Then, proteins were washed with the addition of 5-6 volumes of ice-cold methanol and then washed with cold acetone (–20 °C). The process of washing and centrifugation of the proteins was repeated two times. In each step, proteins were washed and left for 1 h at –20 °C and centrifuged at 7000× g for 40 min at 4 °C. The protein pellets were dried at 4 °C in a desiccator connected to a vacuum pump. The protein concentration was determined as described by Bradford using a protein assay kit Bio-Rad (Hercules, CA, USA) and bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, USA) as a standard. Exudate protein samples were stored at –80 °C.[23]

Tryptic in-solution digestion

Proteins were solubilized in 50 µL ammonium bicarbonate (NH_4HCO_3, Sigma-Aldrich, St. Louis, USA) 50 mM, pH 7.9, containing 7.5 M urea (Sigma-Aldrich, St. Louis, USA) for 1 h at 37 °C and then reduced with 10 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, USA) at 37 °C for 1 h. After this treatment, the proteins were alkylated with 40 mM iodoacetamide (IAA) at 25 °C for 1 h in the dark. Subsequently, the samples were diluted five times with ammonium bicarbonate 100 mM, pH 7.8, and calcium chloride at a concentration of 1 mM. Trypsin (Promega, Madison, WI, USA) was added to the denatured protein solution (1:50 m/m trypsin:protein) for 18 h at 37 °C. The reaction was terminated with the addition of 5 µL formic acid.

The digested samples were desalted using a Sep-Pak tC18 1 cm^3 Vac Cartridge (Waters, Milford, MA, USA) column. The tryptic peptides were solubilized in 50% acetonitrile (ACN) and subjected to analysis in a liquid chromatography coupled with time-of-flight and ion trap mass spectrometry (LCMS-IT-TOF) and MS^n system.

LCMS-IT-TOF

The samples were analyzed using the LCMS-IT-TOF system. The UFLC (ultra fast liquid chromatograph) system (Shimadzu, Kyoto, Japan) coupled directly (online) to the mass spectrometer, with two LC-20AD pumps, an SPD-M20A diode array detector, a SIL-20AHT autosampler and a CTO-20A oven column was used. The analysis was carried out under a gradient of acetonitrile (ACN) from 5 to 90% (v/v) containing 0.05% trifluoroacetic acid (TFA) (v/v) over 90 min using a C18 Shim-pack XR-ODS column (3 × 100 mm, 120 A, 2.2 µm) (Shimadzu). The elution of components was monitored by ultraviolet absorbance at 214 nm through a UFLC system with a flow rate of 0.2 mL min^{-1} for 90 min. The eluents were analyzed in continuous positive mode (ESI^+) throughout the experiment.

The mass spectra were obtained on a mass spectrometer with an ESI source, the type "ion trap-time of flight" (IT-TOF Shimadzu). LCMS solution (Shimadzu) was used to control the acquisition and data analysis. During all experiments, the temperature of the curved desolvation line (CDL) and the interface was maintained at 200 °C, the voltage on the needle was maintained at 4.5 kV, and the cone voltage was maintained at 3.5 V. The flow of drying gas (nitrogen) was 100 L h^{-1}, and the nebulizer gas flow (nitrogen) was 1.5 L h^{-1}. The detection in the mass spectrometer was made to scan in the range *m/z* 200-4000 with a resolution of approximately 15000, and the data acquisition system was continuously operated in positive mode.

The sequential mass spectrometry or fragmented peptide (MS^2) experiments were carried out using the same parameters as the MS experiments. Argon was used as the collision gas at a pressure of 100 kPa. The ions produced by the MS^2 (and MS^3) experiments were imprisoned and retained for 50 ms in ion trap using a collision energy of 50% and frequency of 30 kHz.

The mass spectra obtained were first analyzed with the tools of LCMS Solution (Shimadzu) software to control data acquisition and analysis.

Protein identification

The MS and MS^n data were combined to search with a taxonomic restriction of Viridiplantae (Green Plants)

against the National Center for Biotechnology Information nonredundant protein database (NCBInr)[24] and Swiss-Prot[25] protein database using the Mascot algorithm 2.2.06 (Matrix Science, London, UK).[26]

The parameters used to search the databases were a mass tolerance of 0.5 Da and a peptide mass tolerance of 0.8 Da peptide fragments, and the number of missing cleavages was set to 1. Modifications of the peptides were considered: carbamidomethyl (C) and oxidation of methionine and tryptophan. The values of "ion score" and "protein score" were considered significant values preset by the bioinformatic tool used. The FDR (false discovery rate) was calculated using the original decoy FDR approach from Mascot, provided by Matrix Science.[26] The FDR thresholds for protein and peptides were selected between 0.1 and 0.01 and ion score values were set to > 19. FDR was calculated based on the Mascot Score.[26] Proteins were accepted if they were confidently identified at ≥ 99.0% probability as assigned by the Protein Prophet algorithm incorporated in the software.

Functional and gene ontology (GO) analysis

The proteins identified by Mascot[26] were further annotated with Gene Ontology (GO) terms according to UniProt Knowledgebase (UniProtKB)[27] and mapped against the GO database by using the Blast2GO software.[28,29] Additionally, the proteins with Enzyme Commission (EC) numbers obtained from the Blast2GO software were mapped onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database.[30] A complete list of all identified proteins is provided in the Supplementary Information (SI) section.

Results and Discussion

Histochemical analysis

The analysis of the anatomical sections showed roots in secondary structure, with secondary xylem, secondary phloem, remains of a cortex and periderm (phellem, phellogen and phelloderm), in which a large number of cells containing starch grains was observed (Figures 2a-2b).

The quinonemethide triterpenes were predominantly evidenced on phellem cells when cross-sections of the roots were subjected to reagent Sudan III (Figure 2c). The quinonemethide triterpenes also showed compartmentalization on cortex cells and secondary xylem cells (Figure S1, SI section). In addition, the suberin shows orange coloration when submitted to treatment with Sudan III (Figure 2c).

Additionally, the histochemical analysis enabled us to locate the quinonemethide triterpenes in the outermost layers that make up the periderm (phellem, phellogen and phelloderm), more precisely the phellem, which is characterized by having its cell walls suberized (Figure 2c). These results are consistent with a recent report,[31] which showed that celastrol and demethylzeylasteral, two quinonemethide-type triterpenes, accumulate in suberized cell walls.

The presence of alkaloids was confirmed by treatment of the sections with Dragendorff reagent. These alkaloids were observed in the phellem cells and in the cortex cells and fibers of secondary xylem stored in the cytoplasm, showing a granular appearance (Figures 2e-2f), corroborating a previous work.[31]

Phenolic compounds were observed in the cortical cells after staining with ferric chloride (Figure 2d). The detection of autofluorescence was monitored by epifluorescence microscopy, corroborating the accumulation of terpenes in the phellem cells and the phenolic compounds in the walls of some cells of the cortex and secondary xylem (Figures 2g-2h).

The presence of terpenes, alkaloids and phenolic compounds in the roots of *M. ilicifolia* is not associated with specialized structures in the secretion (processes of production and release) of secondary metabolites, such as secretory structures, but rather with cells nonspecialized in secretion.

Histochemical tests indicated that the root cells contain a diversity of metabolites, including terpenes, alkaloids, flavonoids and starch. This analysis obtained novel results for *M. ilicifolia* and strongly corroborated the chemical,[9] biological,[11-13] biosynthetic[32] and proteomic studies.

Proteomic analysis

The shotgun proteomic analysis of the soluble fractions of roots from *M. ilicifolia* led to the identification of 436 proteins using the Mascot algorithm against the NCBInr and Swiss-Prot databases, of which 83 proteins were common to both databases. After the identification of 364 proteins, from the total of 436 proteins (UniProt database), they were submitted to the Blast2GO bioinformatics tool, of which 351 proteins were mapped and annotated (Table S1, SI section).

All of the identified proteins were categorized according to biological process, cellular component and molecular function at the third level (Figure 3).

According to the biological process categorization (Table S2, SI section), the groups with the highest number of proteins identified are related to organic metabolism

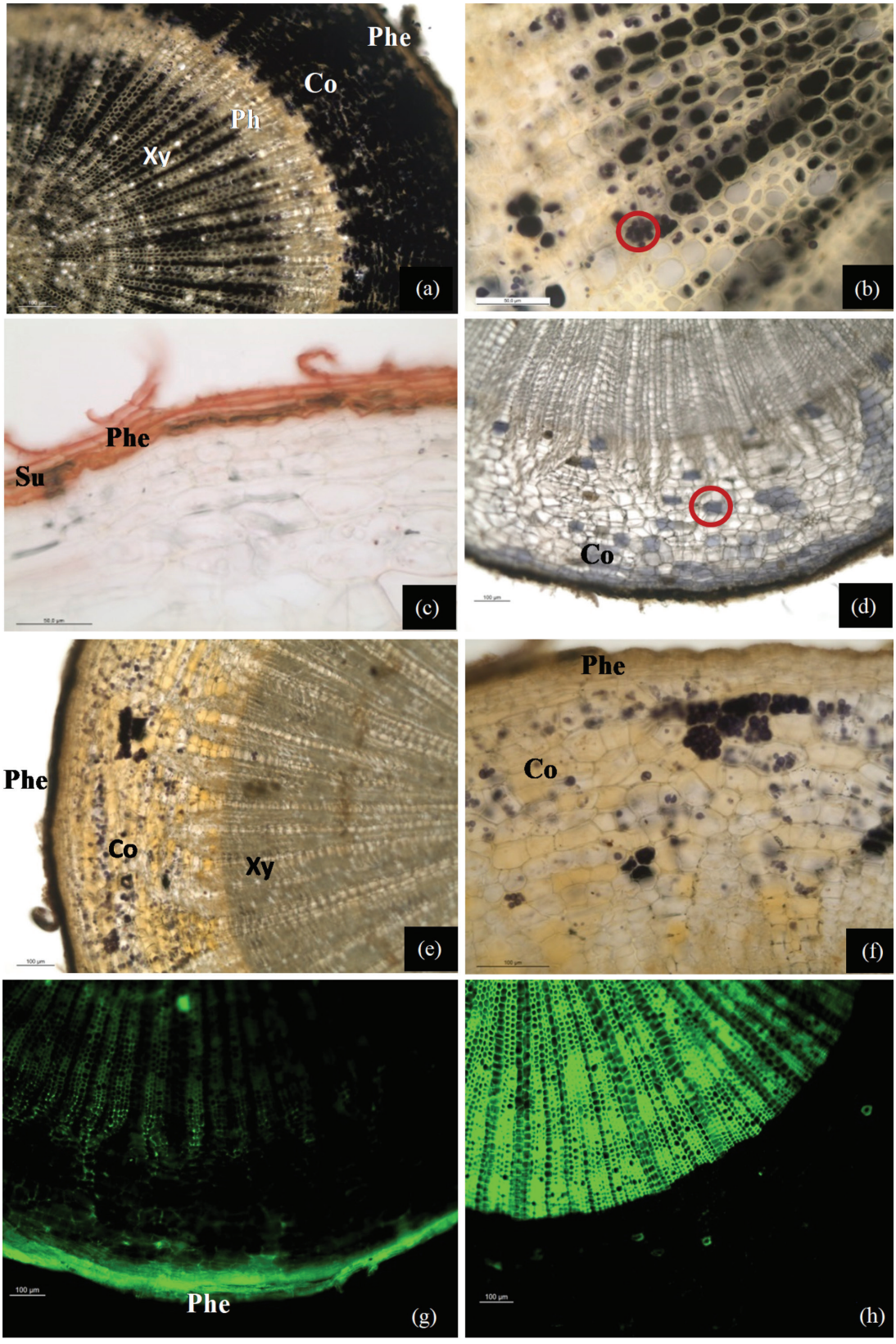

Figure 2. Photomicrographs of cross-sections of the root in secondary structure of *M. ilicifolia*. (a) Presence of starch grains (in black) in the secondary xylem (Xy), secondary phloem (Ph), cortex (Co), and phellem (Phe). (b) Featured (in black) starch grains in the fibers of secondary xylem. (c) Presence of terpenes (in orange-red) and suberin (in orange) in the phellem cells (Phe). (d) Presence of phenolic compounds (bluish black) in the cortical parenchymatic cells (Co). (e, f) Presence of alkaloids (dark brown) in phellem cells (Phe), cortical parenchymatic cells (Co), and secondary xylematic cells (Xy). Epifluorescence: (g) terpenes located in the phellem cells and (h) lignin in the cell walls of cortex and secondary xylem. Bars = 100 µm (a, d, e, f and g) and 50 µm (b and c).

process (299), primary metabolic process (286), cellular metabolic process (259) and single-organism metabolic process (228) followed by biosynthetic process (173), single-organism cellular process (123), nitrogen compound metabolic process (114), response to stress (99) and catabolic process (65) (Figure 3a).

Regarding the categorization of cellular components, intracellular (286), intracellular part (280), and intracellular organelles (222) were found followed by membrane-bound organelles (196), cell peripheries (68), plasma membranes (57), nonmembrane-bound organelles (40) and protein complexes (39) (Figure 3b). The main bonds and activities observed to molecular functions are related to ion binding (269), transferase activity (144), oxidoreductase activity (127), organic cyclic compound binding (126), heterocyclic compound binding (126), hydrolase activity (96), protein binding (73) and small molecule binding (66) (Figure 3c).

As this work aims to integrate histochemical and proteomic data to characterize the compartmentalization

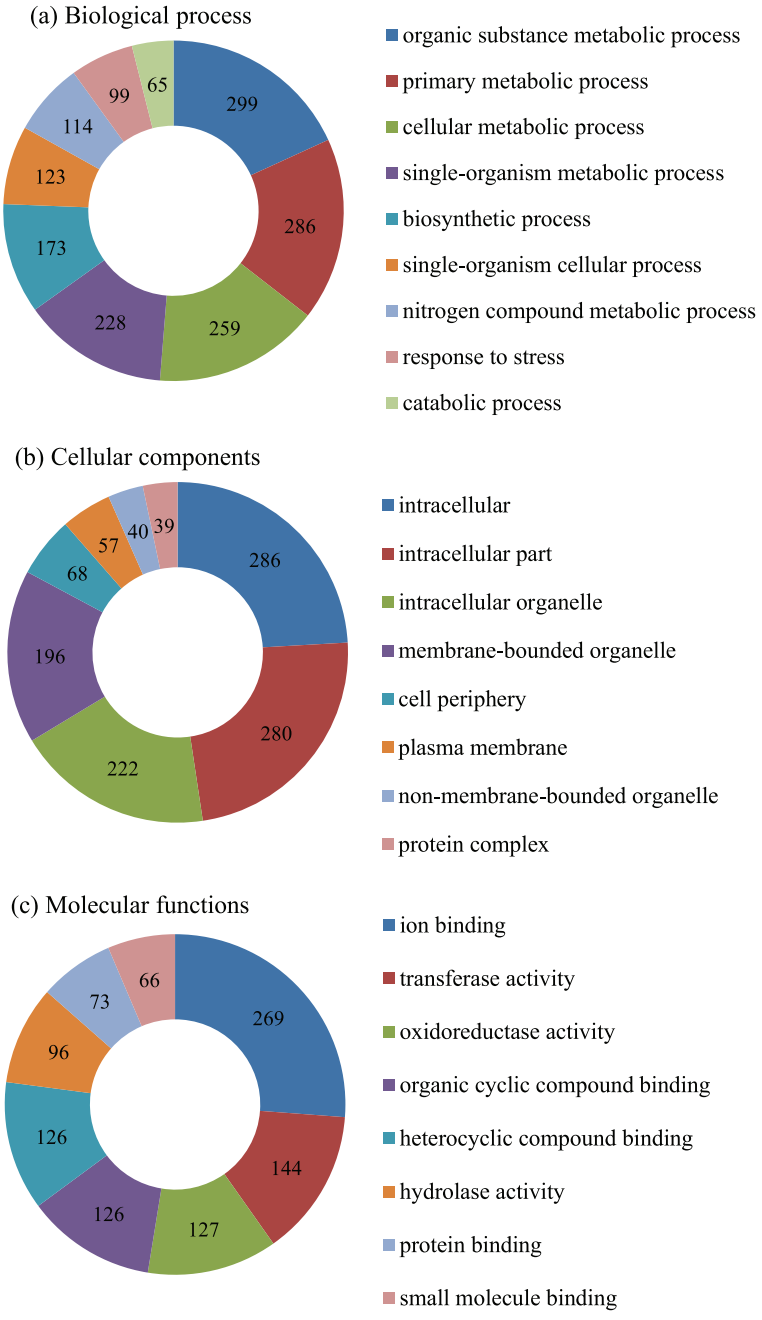

Figure 3. Functional category distribution of the identified proteins from *M. ilicifolia*. The functional annotation of the proteins was based on gene ontology (GO). Proteins were annotated according to biological processes (a), cellular components (b) and molecular functions (c) at the third level, using the Blast2GO tool. The distribution of the proteins in each category is indicated in the sectors of the circle.

and expression of proteins related to primary and secondary metabolism, the category "biological process" and the subcategories biosynthetic process/response to stress were analyzed (Table S2, SI section). The proteins identified in the subcategory biosynthetic process consist of proteins related to the biosynthesis of phytohormones and secondary metabolites.

Proteins involved in the biosynthetic process and response to stress

The shotgun proteomic analyses led to the identification of important proteins involved in the biosynthetic pathway of phytohormone auxin, including anthranilate synthase and indole-3-pyruvate monooxygenase (Table 1). This phytohormone is involved in many aspects of plant growth and development, including controlling root development.[33]

Important proteins involved in the biosynthesis of jasmonic acid, such as lipoxygenase and allene oxide synthase, were also identified (Table 1). Oxylipins originate from α-linolenic acid (EC number and KEGG map, Table S3 and Figure S2, SI section), many of which act as an important class of signaling molecules in defense responses to pathogens, insect herbivory and abiotic stresses.[34] In our study, we identified many proteins related to pathogenesis, such as nucleotide binding leucine-rich repeat (NB-LRR), pathogenesis related (PR) (osmotin, defensin, peroxidase, chitinase), thaumatin-like, and callose synthase.

It was also possible to show the occurrence of enzymes involved in the biosynthesis of phytohormones, cytokinin, abscisic acid, gibberellins and strigolactone (Table 1). These proteins interact either synergistically or antagonistically with auxin to trigger cascades of events leading to root morphogenesis and development.[35]

A large number of receptor kinases play an important role in a variety of biological processes, including growth, development, involvement in the phytohormone signaling pathway, and plant-microbe interactions.[36]

This analysis allowed us to identify many antioxidant enzymes that protect the cells and subcellular compartments of the plant from reactive oxygen species (ROS) effects, such as peroxidases and superoxide dismutase. ROS interact with phytohormones and are involved in growth and development processes and plant defense responses.[37]

The proteomic analysis culminated in the identification of several proteins that are located at a point of branching between the primary and secondary metabolisms, as well as in processes/mechanisms of defense of the plant against biotic and abiotic stresses. A number of enzymes related to the biosynthesis of phenylpropanoids were found (EC number and KEGG map, Table S3 and Figure S3, SI section), including phenylalanine ammonia-lyase (PAL), flavone 3'-*O*-methyltransferase and cinnamyl alcohol dehydrogenase (Table 1). Phenylpropanoids, such as flavonoids, anthocyanins, phytoalexins, plant hormones and lignins, play important roles in plant growth, development and pathology.[38]

Also, this analysis was enabled to identify enzymes involved in the mevalonate pathway, including isopentenyl pyrophosphate (IPP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA). Important enzymes, such as *R*-linalool synthase and (+)-alpha-pinene synthase (Table 1), were also described. The systemic emission of these volatiles in the root could play a key role in the attraction of nematodes in the rhizosphere under attack by herbivores.[39]

The enzymes 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) and farnesyl diphosphate synthase (FPP) can be involved in the formation of the sesquiterpene unit of the dihydro-β-agarofuran of the sesquiterpene pyridine alkaloids (**1**-**4**, Figure 1) via the mevalonate pathway (MVA). These enzymes are also essential in the biosynthesis of triterpenoids and must be directly involved in the biosynthetic steps of 2,3-oxidoesqualene, a precursor of the quinonemethide triterpenes accumulated in *M. ilicifolia.*[40-42]

In addition, important enzymes are involved in the cyclization of 2,3-oxidosqualene (oxidosqualene cyclases (OSCs)), including lupeol and cycloartenol synthases, leading to the formation of lupeol and cycloartenol. Cycloartenol is a fundamental precursor in the biosynthesis of the phytohormone brassinosteroid, which plays diverse roles in plant growth and development.[43] Triterpenes and steroids are oxidized by one or more cytochrome P450-type oxidoreductase enzymes. In our study, we identified cytochrome P450 85A, which is involved in the C6-oxidation step in brassinosteroid biosynthesis pathways (Table 1).

Additionally, proteomic analyses led to the identification of several enzymes involved in carbohydrate metabolic pathways. Interestingly, the alkaline/neutral invertase CINV2, which can cleave sucrose into glucose and fructose, has been suggested to be a target for signaling pathways that coordinate carbohydrate availability in growth and development in nonphotosynthetic organs.[44]

It was also possible to identify the occurrence of proteins that promote relaxation and cell wall extension in plants, such as expansins. These proteins play important roles in almost every phase of plant growth and confer tolerance in response to biotic and abiotic stresses.[45]

In addition, important proteins that make up the plant cytoskeleton, including actin and tubulin, were also identified. Cytoskeletal proteins are involved in many

Table 1. Some proteins involved in biosynthetic processes identified in the root of *M. ilicifolia* using shotgun proteomic analysis

Accession number	Protein	Biological process
P29976	phospho-2-dehydro-3-deoxyheptonate aldolase 1	chorismate biosynthetic process, aromatic amino acid family biosynthetic process
P42738	chorismate mutase 1, chloroplastic	chorismate metabolic process, aromatic amino acid family biosynthetic process
I3ZNU8	anthranilate synthase alpha-subunit 1	tryptophan biosynthetic process
G7J5E0	anthranilate phosphoribosyltransferase	tryptophan biosynthetic process
Q8VZ59	indole-3-pyruvate monooxygenase YUCCA6	auxin biosynthetic process
Q7XH05	probable aldehyde oxidase 1	auxin biosynthetic process, abscisic acid biosynthetic process
Q852M1	probable aldehyde oxidase 2	auxin biosynthetic process, abscisic acid biosynthetic process
Q7G9P4	abscisic-aldehyde oxidase	auxin biosynthetic process, abscisic acid biosynthetic process
Q9C5X8	molybdenum cofactor sulfurase	abscisic acid biosynthetic process
P27481	linoleate 9*S*-lipoxygenase	oxylipin biosynthetic process
P93184	lipoxygenase 2.1, chloroplastic	oxylipin biosynthetic process
Q9LNR3	lipoxygenase 3, chloroplastic	oxylipin biosynthetic process
Q9FNX8	lipoxygenase 4, chloroplastic	oxylipin biosynthetic process
Q8H016	probable lipoxygenase 6	oxylipin biosynthetic process
Q69TI2	putative 12-oxophytodienoate reductase 6	oxylipin biosynthesis process
P09918	seed linoleate 9*S*-lipoxygenase-3	oxylipin biosynthesis process
Q7Y0C8	allene oxide synthase 1, chloroplastic	jasmonic acid biosynthetic process, oxylipin biosynthetic process
Q93ZC5	allene oxide cyclase 4, chloroplastic	jasmonic acid biosynthetic process
Q9SB60	adenylate isopentenyltransferase 4	cytokinin biosynthetic process
Q5BPS0	cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG2	cytokinin biosynthetic process
Q43578	lycopene beta cyclase, chloroplastic	carotenoid biosynthetic process
Q40424	lycopene beta cyclase, chloroplastic/chromoplastic	carotenoid biosynthetic process
Q8VY26	carotenoid cleavage dioxygenase 8, chloroplastic	strigolactone biosynthetic process
M1PK29	3-hydroxy-3-methylglutaryl-coenzyme A reductase 3	isoprenoid biosynthetic process, coenzyme A metabolic process
Q7XYS9	farnesyl diphosphate synthase 1	farnesyl diphosphate biosynthetic process, geranyl diphosphate biosynthetic process
Q84KL6	(–)-alpha-pinene synthase, chloroplastic	alpha-pinene biosynthetic process, geranyl diphosphate metabolic process
A7IZZ2	(+)-alpha-pinene synthase	alpha-pinene biosynthetic process
Q9SPN0	*R*-linalool synthase QH1, chloroplastic	terpenoid biosynthetic process, geranyl diphosphate metabolic process
G5CV46	viridiflorene synthase	terpenoid biosynthetic process
F1CKJ1	(+)-sabinene synthase, chloroplastic	metabolic process, terpene synthase activity
Q8SA63	beta-caryophyllene synthase	terpene synthase activity
O82139	cycloartenol synthase	pentacyclic triterpenoid biosynthetic process
O82330	cytosolic sulfotransferase 10	brassinosteroid metabolic process, response to cytokinin
Q69F95	cytochrome P450 85A	oxidoreductase, catalyzes the C6-oxidation step in brassinosteroids biosynthesis
A8CDT3	lupeol synthase	triterpenoid biosynthetic process
Q9FZI2	lupeol synthase 5	multifunctional enzyme that converts oxidosqualene to tirucalla-7,21-diene-3beta-ol and two other triterpene monoalcohols
Q9LS68	putative pentacyclic triterpene synthase 7	triterpene synthase
Q42858	phenylalanine ammonia-lyase	cinnamic acid biosynthetic process, phenylpropanoid metabolism
P37114	*trans*-cinnamate 4-monooxygenase	*trans*-4-coumarate biosynthesis, phenylpropanoid metabolism
P37115	*trans*-cinnamate 4-monooxygenase	*trans*-4-coumarate biosynthesis, phenylpropanoid metabolism
Q6ETN3	probable 4-coumarate-CoA ligase 3	phenylpropanoid metabolic process
B8RCD3	*trans*-anol *O*-methyltransferase 1	phenylpropanoid biosynthetic process
Q9CAI3	probable cinnamyl alcohol dehydrogenase 1	lignin biosynthetic process
Q05964	naringenin,2-oxoglutarate 3-dioxygenase	flavonoid biosynthetic process
Q84V83	leucoanthocyanidin reductase	proanthocyanidin biosynthetic process
Q9LRC8	baicalin-beta-*D*-glucuronidase	flavonoids
Q9FK25	flavone 3'-*O*-methyltransferase 1	flavonol biosynthetic, lignin biosynthetic process

aspects of plant cell growth and development, including fundamental processes, such as cell division, cell expansion, intracellular organization, and motility, as well as regulators and targets of biotic interactions.[46]

The proteomic study allowed the identification of several proteins involved in important metabolic pathways (Figure 4) and defense responses to biotic and abiotic stresses, which are vital to the growth and development of the plant.

Combined use of histochemical and proteomic studies

Given the biological importance of quinonemethide triterpenes and sesquiterpene pyridine alkaloids, as well as the restricted accumulation in the roots of *M. ilicifolia*, the combined use of histochemical and proteomic approaches can notably characterize differential metabolite and protein distribution, especially to address possible biological and ecological functions.

In this study, histochemical analysis contributed to the localization of flavonoids in the cortex (Figure 2d). In addition, shotgun proteomic analysis led to the identification of proteins involved in the biosynthesis of flavonoids (EC number and KEGG map, Table S3 and Figure S4, SI section) and strigolactones (Table 1).

Flavonoids are synthesized in the cytosol, accumulate in vacuoles and can be transported for exudation into the rhizosphere. In the rhizosphere, flavonoids play important roles in biological communications with rhizobia, arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria, pathogens, nematodes, and allelopathic interactions between plants.[47]

In addition, strigolactones are also present in exudates and are proposed as essential signal molecules in the establishment of arbuscular mycorrhiza symbiosis.[48] These symbiotic associations are of considerable ecological importance once they regulate nutrient and carbon cycles and influence soil structure and fertility, as well as stress resistance and tolerance.[49]

According to our results, it is suggested that the flavonoids located in cortical cells act as signaling molecules to establish interactions between the microbial communities of the rhizosphere, microorganisms that inhabit the soil, and other plants.

In addition, histochemical analysis showed a large number of cortical parenchyma cells and secondary xylem cells containing many starch grains (Figures 2a-2b). Our proteomic data revealed enzymes involved in the biosynthesis of starch (EC number and KEGG map, Table S3 and Figure S5, SI section), phytohormone auxin, and flavonoids.

Starch is the major energy storage polysaccharide and carbon source in plants. Starch has emerged as a key molecule in mediating responses to abiotic stresses.[50] In nonphotosynthetic cells, starch is synthesized in amyloplasts of root-cap columella cells and is important for the perception of gravity.[51] The stimulus response to gravity is mediated by the asymmetric distribution of the phytohormone auxin. There is evidence that flavonoids modulate auxin transport and tropic responses.[52]

The insertion of our data points to the important biological function of starch as a reserve of carbon and energy, in the perception of gravity and responses to abiotic stresses.

Additionally, the histochemical analysis contributed to the localization of the quinonemethide triterpenes in the cells of the phellem, which is characterized by having its cell walls suberized (Figure 2c). The suberized cell walls are primarily composed of suberin associated with waxes, cutin and lignin, forming a protective barrier that plays important biological roles in protecting in response to biotic and abiotic stresses.[53,54]

In this study, proteomic analyses contributed to the identification of enzymes that are involved in the lignin biosynthetic process (Table 1) and a cytochrome P450-dependent fatty acid oxidase, CYP86A2, involved in the synthesis of cutin. These enzymes should be directly involved in the biosynthetic processes of the monomers that constitute the suberin aromatic and aliphatic domains.[55]

Additionally, enzymes that are essential in the biosynthesis of triterpenes and that are directly involved in the biosynthesis and cyclization of 2,3-oxidosqualeno, a precursor of the quinonemethide triterpenes, were also identified (Table 1).

Some quinonemethide triterpenes are known for their cytotoxicity and antimicrobial activity, and the presence of these compounds has been reported in several *Maytenus* species (Celastraceae).[16,17,56] Some works[3,6] have shown that to avoid the autotoxicity of some lipophilic compounds, plants usually sequester and store these compounds in the cuticle in ducts or dead resin cells, which are lined with an impermeable solid barrier.

Thus, we speculate that the function of suberin may be the storage of the quinonemethide triterpenes. The location of the quinonemethide triterpenes in the outer layers of the periderm (phellem) associated with their antimicrobial activity[16,17] makes it possible to speculate that the quinonemethide triterpenes play an important role in the first line of defense against pathogenic microorganisms that inhabit the soil.

The histochemical analysis also contributed to the localization of the sesquiterpene pyridine alkaloids in

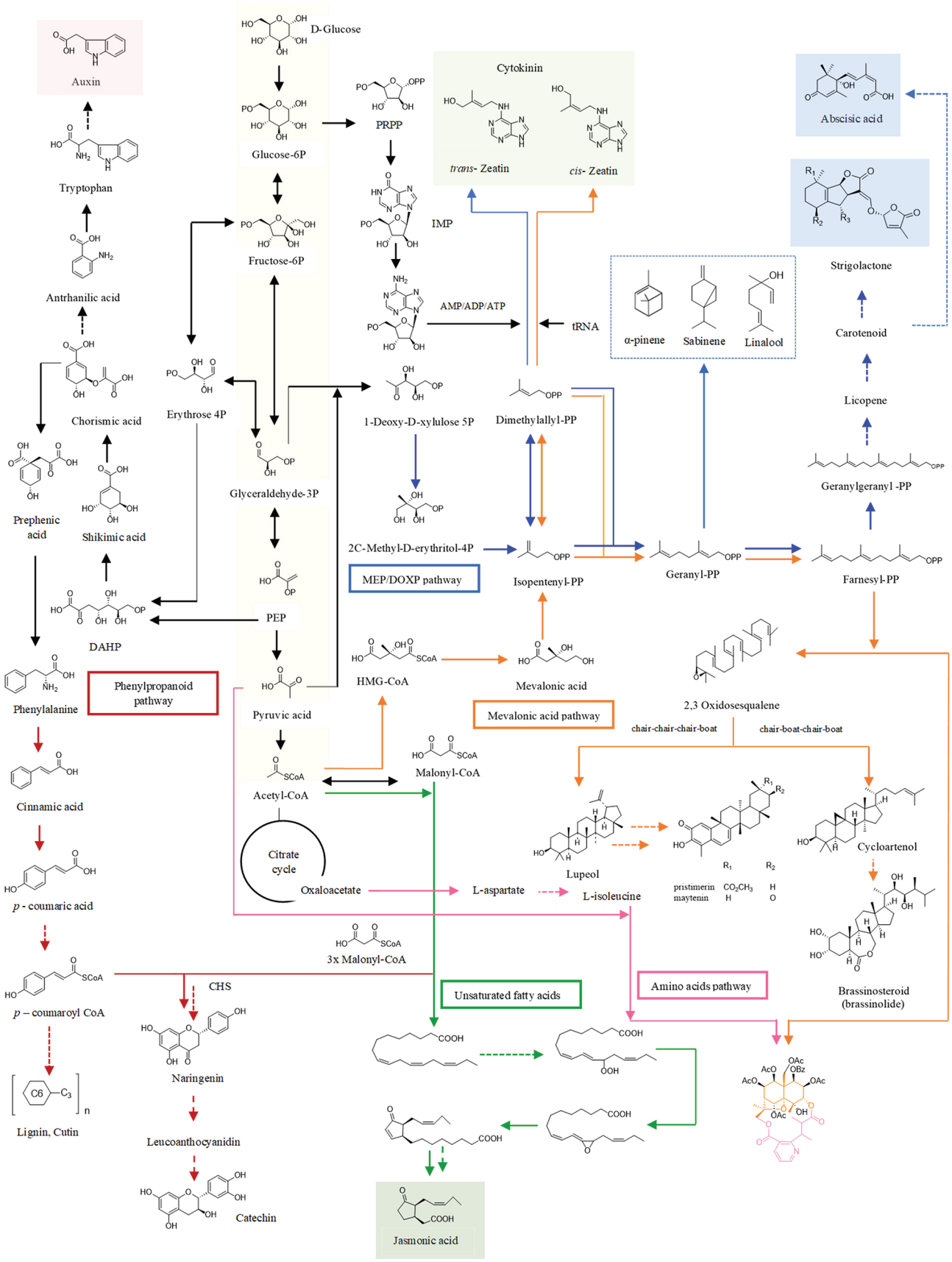

Figure 4. Summary of metabolic pathways predicted from proteomic analysis in roots of *M. ilicifolia*. Blue line: methylerythritol phosphate (MEP), orange line: mevalonate (MVA), rose line: aminoacids, red line: phenylpropanoid, green line: unsaturated fatty acids.

the cells of the phellem, the cortex, and the secondary xylem (fibers) (Figures 2e-2f). Alkaloid biosynthesis and accumulation are associated with a diversity of cell types; for example, putrescine *N*-methyltransferase (PMT) is the first enzyme committed for the biosynthesis of nicotine and tropane alkaloids. However, the expression of PMT transcripts is confined to the roots of tobacco and localized in young roots, specifically in cortical cells, including the endodermis, and in xylem cells.[57]

Many of the sesquiterpene pyridine alkaloids isolated from the roots of Celastraceae species showed cytotoxic activity.[58] Generally, alkaloids accumulate and are stored in specific cell types because of their cytotoxicity, and their location is crucial for their effectiveness in plant defense responses.[59,60] These results are in keeping with the data obtained by a previous report,[61,62] which showed that alkaloids accumulate with a variety of cell types, suggesting their involvement in plant interaction/or defense with/against microorganisms that inhabit the soil.

In addition, the shotgun proteomic analysis led to the identification of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) and (2*E*,6*E*)-farnesyl diphosphate synthase, the main precursors of the dihydro-β-agarofuran type sesquiterpene (Table 1). The sesquiterpene pyridine alkaloids are characterized from a biosynthetic point of view by a mixed route, with the sesquiterpene unit coming from the mevalonate (MVA) pathway and the alkaloid moiety from glyceraldehyde 3-phosphate and amino acid precursors.[63]

Conclusions

This study showed the combined use of histochemical and proteomic tools in the roots of *M. ilicifolia in natura*, allowed a better understanding of the location of the major metabolites and enabled the large-scale acquisition of information on the repertoire of proteins involved in primary and secondary metabolism. Furthermore, our study is the first report of a repertoire of proteins involved in the regulation of secondary metabolism in *M. ilicifolia* roots.

Finally, these results regarding primary and secondary metabolites analyses may provide a reference for further research investigating the biological and ecological functions of these compounds.

Supplementary Information

Supplementary information (protein identification data: accession number, peptide sequence, ion score, protein score, biological process, functional category distribution based on biological process, KEGG pathway, EC number, KEGG maps), is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This work was supported by the São Paulo Research Foundation (FAPESP), grant numbers: CIBFar-2013/07600-3, INCT-FAPESP-2014/50926-0. This study was also financed by INCT-CNPq-2014/465637-0. V. A. F. F. M. S. thanks FAPESP and CNPq for the provision of fellowships (2011/0154-9 and 150201/2015-0, respectively). M. F., S. P. T., and M. S. P. thank CNPq for their fellowship. V. A. F. F. M. S. is also grateful to the Pro-Rectory for research of UNESP (PROPe) for financial support.

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Submitted: May 8, 2020
Published online: August 25, 2020