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Article

Agrobacterium rhizogenes-Mediated Transformation of *Crotalaria ochroleuca*: Production of Flavonoids from Hairy Roots

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Crotalaria ochroleuca (Fabaceae) is rich in bioactive compounds used for nematode control. Transformed root culture allows the production of a large amount of hairy root biomass, conditioned by auxin biosynthesis-related genes integrated via the process of *Agrobacterium rhizogenes* infection, which is known to cause a hairy root phenotype. We transformed *A. rhizogenes* to increase the biomass of hairy roots and optimized the process of production of bioactive compounds. The transformed nature of hairy roots was confirmed by polymerase chain reaction, which confers kanamycin resistance. The transformed roots were cultured in full-strength in liquid media to realize the growth of hairy roots and the production of bioactive compounds. The transformed roots were grown in a culture medium supplemented with elicitors to produce total phenols, and flavonoids. Chromatographic analysis of transformed roots revealed the presence of flavonoids apigenin-6,8-*C*-diglucoside and luteolin-6-*C*-glucoside. The results were obtained by conducting physiological and biochemical studies with the flavonoids and studying the pathways that led to the production of large amounts of bioactive compounds from the hairy roots of *C. ochroleuca*. It was observed that the extraction of the compounds significantly affected nematodes and insect larvae, resulting in significantly high levels of economic damage to crops.

Keywords: transformed root culture, Ri plasmid, tissue culture, UPLC-QTOF-MS^E, flavonoids, *Crotalaria*

Introduction

The genus *Crotalaria* (Fabaceae), with comprises approximately 600 species, is found in Africa, India, Mexico, and Brazil.[1] Phenolic compounds and alkaloids are the primary bioactive compounds found in varying proportions in the seeds, leaves, and flowers of *Crotalaria*. The amounts of the bioactive compounds in the different parts of the plants are influenced by the season, harvest time, place of collection, and plant development stage.[1-6]

Crotalaria is cultivated in Brazil and is used as a rotation crop to improve soil conditions, reduce the extent of erosion, control nematodes, and inhibit the growth of insects and larvae.[3,7-9] The growing demand for *Crotalaria* has resulted in the exploitation and sometimes extinction of certain crops.[10] Therefore, it is important to develop biotechnological processes for establishing *in vitro* cultivation methods that can be used to realize the continuous production of high levels of secondary metabolites of *Crotalaria* under monitored conditions. Tissue culture is a reliable option for minimizing the exploitation of plants with high added value.[11]

In vitro culture methods can effectively reduce the uncontrolled exploitation of natural resources to produce natural compounds with therapeutic potential efficiently.[11] Currently, several studies are being conducted following the process of tissue culture using explants, such as meristems,

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leaves, roots, and stem segments, to produce biomass and extract secondary metabolites of interest that can be used in the biomedical, agronomic, pharmaceutical, and food industries.[12,13] The method has been widely explored to scale up the bioreactor-based production of compounds to improve the production of secondary metabolites significantly.[11,14,15] The production of bioactive compounds accumulated in roots has been realized, and biosynthesis pathways have been explored by studying root cultures. Biotechnological approaches involve the use of elicitors to stimulate the production of secondary metabolites. These methods also involve the addition of biosynthetic precursors and genetic manipulation to activate genes that regulate biosynthesis pathways.[8,16-22]

The process of introducing *Agrobacterium* into target species is one of the most frequent approaches used to explore plant-microorganism interactions. The injection of *Agrobacterium* promotes the induction of enzyme responses or the reduction in the expression levels of key enzymes that regulate metabolic pathways associated with the production of macromolecules with newly identified biological properties.[23] Advancement in the field of biotechnological production of high-value-added secondary metabolites through tissue culture has highlighted the fact that these processes are attractive alternatives to the currently used methods to address the problems associated with the overuse of wild plants as a source of bioactive compounds.[18,24-26]

Agrobacterium-rhizogenes is a soil bacterium that can transfer a portion of its deoxyribonucleic acid (DNA) to the cells of the host plant species, resulting in the genetic transformation of the plants following the process of horizontal gene transfer.[15,27-30] The transformation results in phenotypic variations in plants, and hairy roots are developed at the site of infection. The biomass and secondary metabolite content increases under these conditions, and the results indicate that secondary hairy roots can be used for the controlled production of bioactive compounds.[27,29,31]

The composition of the secondary metabolites in plants can be tuned by adding elicitor compounds. Elicitors are substances that can alter the production of specific secondary metabolites by inducing enzymatic pathways.[32-35] Elicitation can be induced by molecules of biological origins, such as carbohydrates (e.g., chitosan) and phytohormones.[36] Phytohormones (such as salicylate and abscisic acid) induce the generation of a significantly high level of response in the field of plant immunity. However, the regulation pattern may vary among different species and plant organs.[37] The addition of chitosan in the culture medium stimulates the production of secondary metabolites, the production of large amounts of phenolic compounds and flavonoids, and the induction of antioxidant activity.[38,39]

The objective of this study was to generate transformed roots of *Crotalaria ochroleuca*, study the influence of culture medium and elicitors on the production process, study the effects of the culture medium and elicitors on the content of phenols and flavonoids, and identify the extracts using ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS^E) technique. There are no reports on hairy root cultures associated with *Crotalaria* spp., indicating the need to develop biotechnological processes that can be used to establish *in vitro* technologies for the continuous production of high contents of secondary metabolites of interest from the species and the production of target compounds under monitored conditions.

Experimental

Plant material

Crotalaria ochroleuca seeds (BRseeds, São Paulo, Brazil) were sterilized under aseptic conditions by immersing them in 70% (v/v) ethanol for 1 min. Following this, the samples were treated with sodium hypochlorite (commercial bleach; 2.5% active sodium hypochlorite content; SuperGlobo®, Rio de Janeiro, Brazil), containing 0.1% (v/v) Tween™ 20 for 20 min. Subsequently, the samples were immersed in commercial sodium hypochlorite solution with 2.0% active chlorine (v/v) (Super Globo®, Rio de Janeiro, Brazil) for 5 min. Finally, the treated seeds were rinsed four times in sterile distilled water. Three seeds were inoculated *per* test tube (150 × 25 mm) containing 10 mL of Murashige and the Skoog[40] medium supplemented with sucrose (30 g L^{-1}), myo-inositol (0.1 g L^{-1}), and agar (5.5 g L^{-1}) (PhytoTechnology Laboratories, USA). The pH of the media was adjusted to 5.8 ± 0.1 with an aqueous solution of NaOH (0.1 mol L^{-1}) or an aqueous solution of HCl (0.1 mol L^{-1}), and the media were autoclaved at 120 °C and 108 kPa for 20 min.

The seeds were germinated in a culture room at 25 ± 2 °C in the dark over 15 days and subjected to irradiation conditions (16-h photoperiod). The samples were irradiated (50 μmol m^{-2} s^{-1}) using two 40W/750 white fluorescent bulbs (T10 plus, TL, Philips, São Paulo, Brazil) over 7 days. Subsequently, the seedlings were used as explants.

Root segments (average length: 1 cm) were inoculated into Erlenmeyer flasks containing 100 mL aliquots of either liquid medium to evaluate the effects of basal media formulations. The media contained half-or full strength

of the salt concentration and were supplemented with sucrose (30 g L^{-1}), vitamins, and myo-inositol (0.1 g L^{-1}). The pH of the media was maintained at 5.7.[41] The cultures were subjected to conditions of orbital agitation (80 rpm). The light and growing conditions were the same as those described above. Four treatment methods were established in a completely randomized manner. Initially, non-transformed roots were grown in two different media. Five replicates of each treatment batch, containing an average of five root segments *per* flask, were used for analysis.

Agrobacterium rhizogenes strain

The strains of *A. rhizogenes* R1601 were streaked from a previously prepared glycerol stock maintained at –80 °C. The samples were streaked on solidified Luria-Bertani (LB) medium (5 g L^{-1} of yeast extract, 10 g L^{-1} of tryptone, and 10 g L^{-1} sodium chloride) and cultured at 28 °C for 48 h.[42] The isolated colonies were selected and mixed with 50 mL of the liquid LB selective medium. The samples were inserted in a shaking incubator operated at 28 °C until the optical density $(OD)_{600\ nm}$ value was in the range of 0.5-0.6. The *Agrobacterium* suspension was centrifuged at 3500 rpm for 10 min at 4 °C. The pellet was resuspended in 50 mL of the MS medium supplemented with sucrose (30 g L^{-1}) and myo-inositol (100 mg L^{-1}). The pH was adjusted to 5.8 ± 0.1, and the medium was autoclaved at 120 °C and 108 kPa for 20 min. The $OD_{600\ nm}$ value was recorded to be 0.5.

Plant transformation

A kill curve for kanamycin was plotted to determine the appropriate concentration for selecting the transformed roots. Untransformed root segments (average length: 1 cm) were inoculated in the MS liquid medium, following the process described above, and supplemented with kanamycin (0, 25, 50, or 100 mg L^{-1}).

Seedlings grown *in vitro* were used as the source of hypocotyl segments. Segmented hypocotyls (average length: 15 mm) were punctured 5 times in the distal region using a hypodermic syringe needle containing *Agrobacterium* suspension (1 mL; $OD_{600nm} = 0.5$). Wounded stem segments were cultured upright in the physiological polarity orientation in 250 mL glass bottles containing 45 mL of the MS medium. The samples were cultured over 20 days under the conditions previously described.[41] After 20 days, the explants with adventitious roots were transferred to the semisolid MS-based selective medium supplemented with cefotaxime (250 mg L^{-1}) and kanamycin (50 mg L^{-1}). These explants were grown for 60 additional days and re-cultured in a fresh selective medium every 15 days. After 60 days, hairy roots were used as the source of explants to determine the influence of elicitors on the process of transformed root culture.

The plant material obtained post root transformation was macerated in liquid nitrogen, and genomic DNA was extracted following the Doyle and Doyle[43] methodology. The process involved the addition of 2% polyvinylpyrrolidone (PVP) to the cetyltrimethylammonium bromide (CTAB) buffer. DNA integrity and quality were analyzed using a denatured 1.5% (m/v) agarose gel system, stained with gel red (Biotium, St. Louis, Missouri, USA). The samples were quantified using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Ribonucleic acid (RNA) was digested in DNA samples using RNA A (Sigma-Aldrich®, St. Louis, Missouri, USA) following the instructions outlined by the manufacturers. For the polymerase chain reaction (PCR)-based studies, 2.0 µL of DNA (25 ng μL^{-1}), 2.5 µL of buffer, 0.75 µL of Mg, 0.5 µL deoxynucleotide triphosphates (dNTPs), 0.25 µL of each primer (forward and reverse), and 0.2 µL of the Platinum II Taq Hot-Start DNA Polymerase (Invitrogen, Waltham, Massachusetts, USA) were used. The reaction was carried out in a C1000 Touch Thermal Cycler thermocycler (Bio-Rad; conditions: denaturation at 94 ºC; 35 cycles conducted at 94 ºC (15 s), 55 ºC (15 s), 68 ºC (15 s); final extension at 68 ºC (5 min)). The primers used for neomycin phosphotransferase (*npt*II) gene amplification have been presented: forward (5'-TCAGCGCAGGGGCGCCCGGTT-3' and reverse 5'-GCGGTCAGCCCATTCGCC-3').

Influence of elicitors on hairy root culture

Segments of transformed roots (length: 1 cm) were cultivated in Erlenmeyer flasks containing 100 mL of the liquid MS supplemented with methyl salicylate (methyl-SA), chitosan, and abscisic acid (ABA). These cultures were maintained in a growth room under previously described light and temperature conditions,[36] and the samples were agitated at 80 rpm. A randomized design was followed for the experiments, and the experiments were conducted with five replicates *per* treatment. Each experimental unit was composed of five hairy roots *per* Erlenmeyer flask. The cultures were analyzed after 30 days to study growth patterns and determine antioxidant activity. The samples were analyzed using the chromatography (UPLC-QTOF-MS^E) technique. The data were submitted to analysis of variance (ANOVA), and the means were compared by conducting the Tukey's test ($p \geq 0.05$).

Quantification of oxidative stress enzymes

The hairy root samples were macerated and extracted using an extraction solution (1 mL; 0.1 M potassium phosphate buffer, pH 6.8; 0.1 mM ethylenediaminetetraacetic acid; 1 mM phenylmethylsulfonyl fluoride; and 1% m/v polyvinylpolypyrrolidone) to evaluate enzymatic activity. The extract was then centrifuged at 12,000 × g for 15 min at 4 °C, and the obtained supernatant was used for protein quantification. It was used as a crude enzymatic extract to perform the enzyme assays. The entire procedure was conducted on ice.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by analyzing the crude enzymatic extract and reaction medium (100 mM phosphate buffer; pH 7.8; 13 mM methionine; 75 µM *p*-nitro tetrazolium blue (NBT); 0.1 mM EDTA (ehylenediamine tetraacetic acid); 2 µM riboflavin; distilled water). The reaction was conducted at 25 ºC in a reaction chamber under the illumination of a 15 W fluorescent lamp. The absorbance at 560 nm was subtracted from that of the illuminated sample. The absorbance of the reaction medium at 560 nm was the same as that reported previously. The samples and the controls were kept in the dark for the same time period. The SOD unit (U) is defined as the amount of enzyme needed to inhibit the photoreduction of NBT by 50% and is expressed in the units of U min^{-1} mg $prot^{-1}$.[44]

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by conducting an assay using the plant extract and reaction medium (50 mM phosphate buffer; pH 6.0; 1 mM ascorbic acid; 20 mM H_2O_2). The decrease in absorbance at 290 nm (temperature: 25 °C) was measured for 5 min at an interval of 30 s, and the APX activity was determined based on the slope of the line. The enzymatic activity was determined based on the molar extinction coefficient of the system (2.8 mM cm^1). APX activity was expressed in the units of µmol^{-1} min^{-1} g^{-1} protein.[45]

The activity of peroxidase oxidoreductase (POD), (EC1.11.1.7) was determined by analyzing the crude enzymatic extract and reaction medium (50 mM phosphate buffer; pH 7.0; 20 mM H_2O_2; 20 mM pyrogallol; distilled water). Following the completion of the reaction, 10 readings were taken over 5 min at an interval of 30 s (wavelength: 420 nm). POD activity was expressed in the units of µmol^{-1} min^{-1} g^{-1} protein.[46]

Quantification of total phenols and flavonoids compounds

Hairy root samples were macerated, freeze-dried, and subjected to conditions of methanolic extraction. Phenolic compounds and total flavonoids were quantified by conducting colorimetric reactions. The extract was added to a reaction medium (Folin-Ciocalteu reagent 2% (v/v), Na_2CO_3 10% (v/v), and distilled water) to determine the total phenol content. The absorbance was read at 760 nm following a 2 h reaction in the dark, and gallic acid (GAE) was used as the standard.[47]

The methanolic extract was added to the reaction medium ($NaNO_2$ 5% (v/v); $AlCl_3$ 2.5% (v/v); 1 M NaOH; and distilled water) to quantify total flavonoids. After the reaction in the dark, the absorbance was read at 500 nm, and catechin (CE) was used as the standard.[48]

Extraction methodology for UPLC analysis

Methanol extracts from untransformed and transformed roots were sprayed in liquid nitrogen and placed in a vial containing 30 mL of methanol to quantify oxidative stress enzymes. The hairy roots were crushed, and the samples were filtered. Following this, 30 mL of methanol was added to the samples. This procedure was performed 3 times to extract the bioactive compounds efficiently. The extract was then concentrated using a rotary evaporator. The solid part (hairy roots) was separated and transferred to an Erlenmeyer flask (VidroLabor, Brazil) containing methanol (100 mL), and the samples were packed and refrigerated for 7 days. The samples were then filtered, and the extract was concentrated in a rotary evaporator.

Analysis by UPLC-QTOF-MS^E

The analysis was performed using an Acquity UPLC (Waters, Milford, Massachusetts, USA) chromatographic system coupled to a quadrupole/time of flight (QTOF, Waters) system. Chromatographic runs were performed on a Waters ACQUITY UPLC BEH (150 mm × 2.1 mm, 1.7 µm) system at a fixed temperature of 40 °C. The binary gradient elution system consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The UPLC elution conditions were optimized as follows: linear gradient from 2 to 95% B (0-15 min), 100% B (15-17 min), 2% B (17.01 min), and 2% (17.02-19.01 min). The flow rate was maintained at 0.4 mL min^{-1}, and the sample injection volume was 5 µL.

The chemical profiles of the samples were determined by coupling a Waters ACQUITY UPLC system with a QTOF mass spectrometer (Waters, Milford, MA, USA). The electrospray ionization interface (ESI) in the positive ionization mode was used for sample analysis. The ESI^+ mode was used to acquire data in the range of 110-1180 Da in MS and the range of 50-1180 Da in MS^2. The source

temperature was maintained at 120 °C, and the desolvation temperature was 350 °C. The rate of desolvation gas flow was 350 L h^{-1}, and the experiments were conducted under the ESI^+ mode. The capillary voltage was set at 3 kV. Leucine-enkephalin was used as the lock mass (MS mode: Xevo G2-XS QTof). The spectrometer was operated under conditions of MS^E centroid programming (tension ramp: 20-40 V). The data were analyzed using MassLynx 4.1 (Waters Corporation, USA).

Compound identification

The dataset was imported to the Mass Spectrometry-Data Independent Analysis software (MS-DIAL 4.60) to implement functions required for untargeted metabolomics. This software was used to analyze deconvoluted spectra and for peak alignment and filtering. Thus, MS-DIAL is a prerequisite for compound identification.[49-51] The unknown metabolites can be identified based on their elemental formulae and by analyzing the *in silico* mass spectral fragments with MS-FINDER 3.50.[49,50] Structural elucidation and metabolite identification processes were based on the molecular formulae of the samples. MS/MS fragmentation was achieved with activated heuristic rules.[52,53] The putative identification of the compounds was performed after obtaining the MS/MS spectral profiles. The data were compared with the data in the databases such as the KNApSAcK Core System database, Human Metabolite Database (HMDB), the Kyoto encyclopedia of genes and genome database (KEGG), SciFinder, PubChem, and ChemSpider. Putative identification was achieved based on the parameters associated with the metabolic standards initiative (MSI) level 2.1. The parameters included the data on the molecular formula and MSE fragments. In addition, it is important to mention that chemical identification was based on chemotaxonomy (family, genus, and species).[53]

Results and Discussion

A survival curve for non-transgenic roots was initially generated in the MS liquid culture medium containing different concentrations of kanamycin to transform the roots. Mortality was induced in all the roots when the kanamycin concentration was 100 mg L^{-1}. This concentration was used for the selection of transgenic roots (Figure 1a). Roots emerged from the hypocotyl explants 30 days after transformation (Figure 1b). The formation of

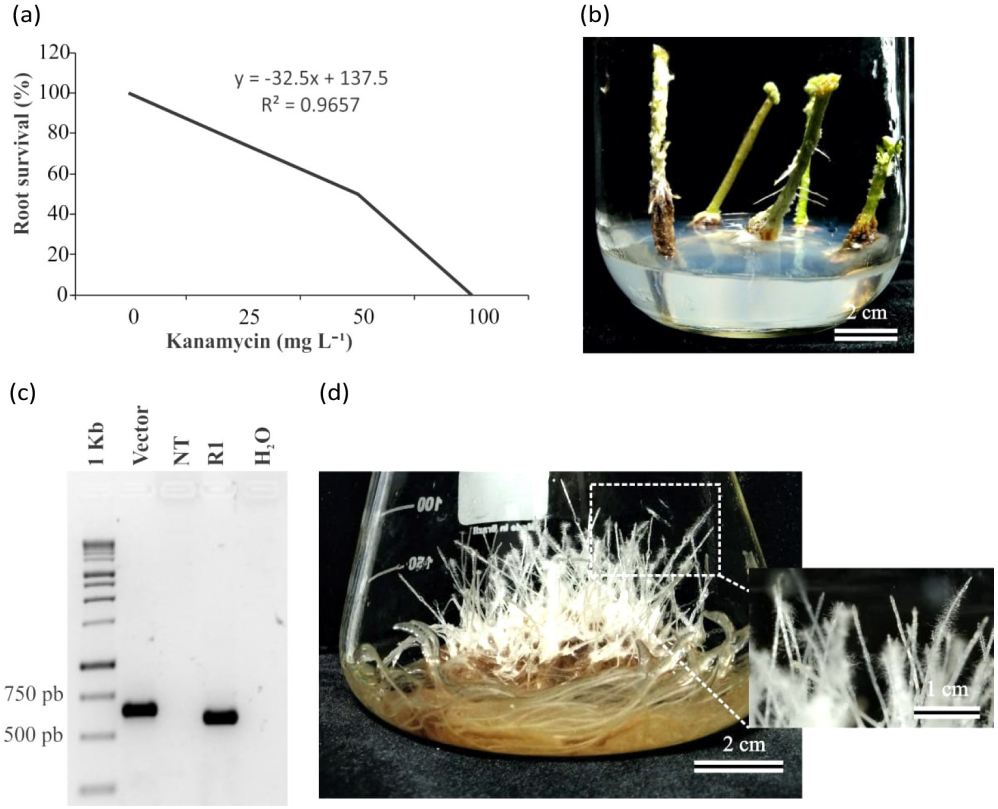

Figure 1. Production of hairy roots of *Crotalaria ochroleuca*. (a) Kanamycin kill curve for non-transformed roots, indicating the concentration used for the selection of transgenic roots. (b) Hypocotyl explants after 30 days of transformation. (c) PCR confirmation of transgenic roots showing amplification of the *npt*II gene in the vector and transgenic root (R1). (d) Characteristics of transformed root cultures.

transgenic roots was confirmed by PCR (Figure 1c), and the roots were cultivated in a semisolid selective medium over 30 days. The roots were then cultivated in a liquid medium for another 30 days. Root cultures exhibited negative geotropic growth, which is a characteristic of transformed root cultures (Figure 1d).

The development of transgenic and non-transgenic roots in JADS and MS media was evaluated to establish the best conditions for the culture medium. The degree of increase in the biomass of the transgenic roots grown in the MS-based medium (after 30 days of cultivation) was higher than the degree of increase in the biomass of the samples recorded in other media. The MS medium contained a higher concentration of macronutrients and micronutrients than the JADS medium, which mainly contained calcium and magnesium ions.[54] However, the JADS medium had twice the concentration of P and Fe compared to the MS medium. The concentration of Zn and Mn in the medium was low.[41] Therefore, the differences in the composition of the culture media significantly affected the growth responses of the non-transgenic and transgenic roots. Initially, the experiment was tested with MS basal medium. However, the roots were very thin and the growth seemed poorly sustained and stopped growing. That is why, after comparing MS and JADS medium, the latter enabled a good root growth dynamic with nice biomass in the flasks.

The novelty of the study lies in the fact that it standardizes the production process of secondary metabolites from *C. ochroleuca*. To the best of our knowledge, this is the first attempt to realize the root transformation of *C. ochroleuca* to obtain plant biomass and natural secondary metabolites. These results provide an optimized methodology that can be used to scale up the production of the compounds in bioreactors without the loss of the biosynthetic potential of cultures.[55] Hairy roots grow fast without the need for hormone supplements. Hairy root cultures are desirable owing to their high growth rates and ease of cultivation. They may also recapitulate the biosynthetic capacity of whole plants and are amenable to gene overexpression and suppression technologies.[56] Additionally, hairy roots can be used for the sustainable production of high-value metabolites.[57]

A. rhizogenes-based genetic transformation promotes the induction or reduction of key enzymes that regulate the biosynthetic pathways of specific metabolites.[21,22] Genetic manipulation results reveal genetic and biochemical stability, and the results indicate the development of a novel method for the biosynthesis of secondary metabolites identified in plant extracts. Various biotechnological processes have been developed to explore the biochemical and molecular aspects of interactions in plant-microorganism. These processes also help produce high-value-added secondary metabolites and enzymes with applications in academia and industry.[58-61]

The chemical identification of the transformed roots has opened new perspectives for identifying the stages that determine the speed and regulatory mechanisms associated with the synthesis and accumulation of secondary metabolites. The versatility of hairy roots can be exploited to promote changes in cellular metabolism and study the influx of compounds of interest into biosynthetic pathways.

Transgenic and non-transgenic roots cultivated in a medium containing elicitor compounds showed marked differences in bioactive compounds (Figure 2). The maximum content of phenolic and flavonoid compounds was recorded in transgenic roots, except for ABA, which showed high phenolic content in the non-transgenic roots. ABA induced a significant reduction in the content of phenolic compounds in transgenic roots, and the phenolic content in these roots was lower than the content in non-transgenic roots. In addition, the elicitors induced a significant reduction in flavonoid content.

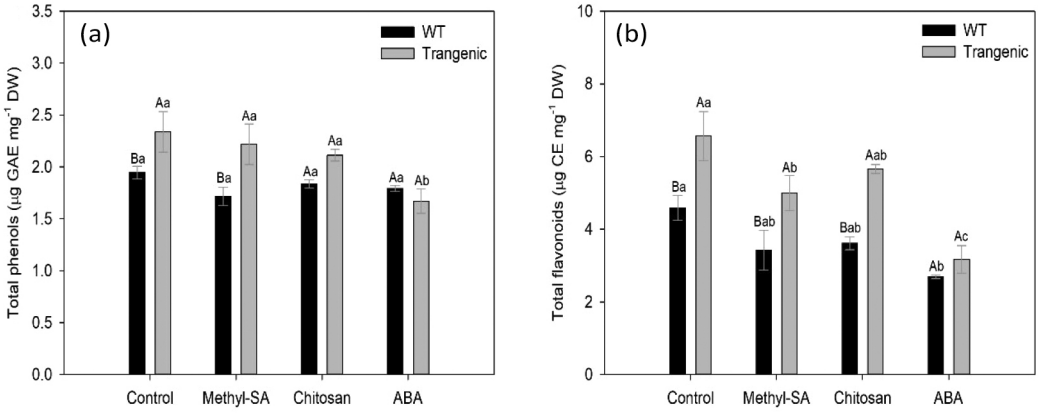

Figure 2. The effect of methyl-SA, chitosan, and ABA on total phenolic (a) and total flavonoid (b) contents in *Crotalaria ochroleuca* hairy roots. Aa, Ba, Ab, Aab, Bab, Ac corresponding means followed by the same letter do not differ in the least significant difference Tukey's test ($p < 0.05$).

Elicitation is a technique in which elicitors alter the content of the bioactive secondary metabolites in plants by inducing enzymatic pathways.[32] The results reported herein indicate that methyl-SA, chitosan, and ABA influence the total phenolic and total flavonoid contents. Some researchers have reported the biosynthesis pathways associated with the production of phenolic compounds. These pathways were associated with the increase in flavonoid contents under the influence of transformed hairy roots.[62,63] The accumulation of *in vitro* cultivated plant flavonoids can be induced under conditions of exogenous supplementation of precursors.[64-70]

The culture medium can be manipulated by adding suitable growth regulators, nutrients, and elicitors to realize maximum production efficiency during *in vitro* cultivation.[70] Chitosan induced an increase in the concentrations of phenolic compounds. Researchers conducting *in vitro* studies have reported the addition of chitosan to stimulate the production of secondary metabolites. These processes result in the production of high levels of phenolic compounds and flavonoids and induce excellent antioxidant activity.[38] Chitosan has been used as it is cost-effective and exhibits low toxicity to plants. Moreover, it generates a good response during the production of secondary metabolites.[38]

The secondary metabolites in the methanolic extracts of transformed and untransformed *C. ochroleuca* were identified using UPLC-QTOF-MS^E. The results were arrived by analyzing the spectral profiles and main peaks in the chromatograms recorded for the samples. The correlation of data published in the literature for the genus *Crotalaria* was also analyzed to arrive at the results. Chemical identification of extracts from the roots of *C. ochroleuca* and genetically transformed roots revealed that chemical compounds that were identified under the positive ionization mode were present (Table S1, Supplementary Information (SI) section).

UPLC-QTOF-MS^E analysis was performed to annotate and compare the major chemical components present in the plant extracts. Our results annotated four phytochemical compounds, mainly flavonoids and other unidentified compounds. Base peak chromatography was conducted under the positive ionization mode (Figures 3 and 4 and S1-S3, SI section). The structure of each compound was proposed based on the detected *m/z*, error (ppm), calculated molecular formula, and MS fragment data. The compounds were annotated and characterized by comparing the MS and MS spectra. The fragmentation mechanism and reference data obtained from Poaceae, Nitrariaceae, Bromeliaceae, Araceae, Cucurbitaceae, in SciFinder, ScienceDirect, ChemSpider, PubChem, and Human Metabolome databases were analyzed to arrive at the results.

Researchers have used UPLC-QTOF-MS^E to identify metabolites in plant extracts.[71] In this study, four simple

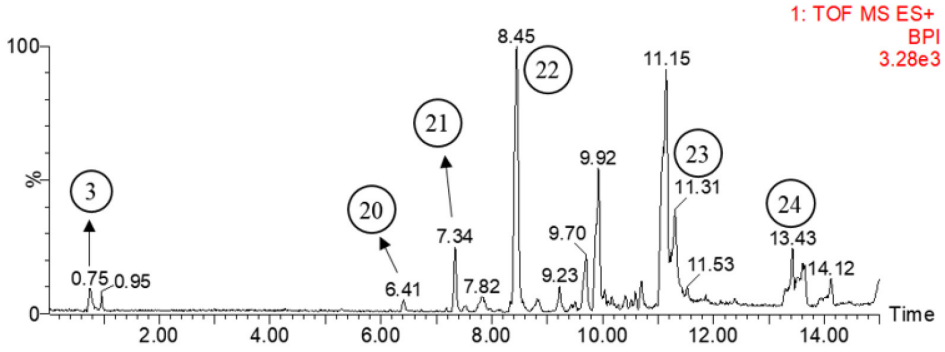

Figure 3. Chromatogram generated for the untransformed root sample of *C. ochroleuca*.

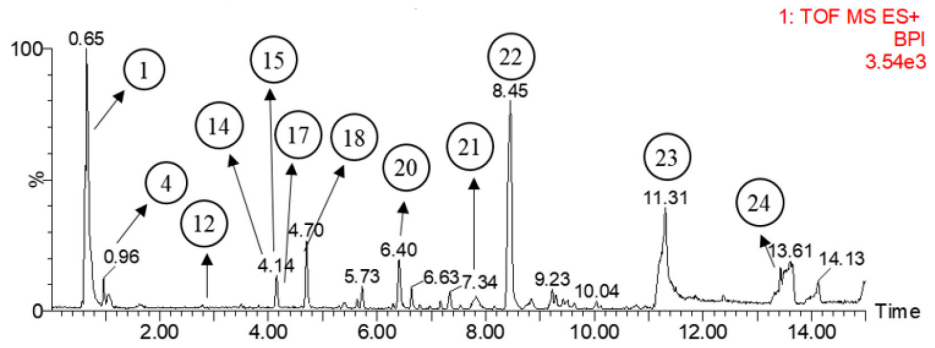

Figure 4. Chromatogram corresponding to the root transformed sample of *C. ochroleuca.*

flavonoids have been annotated. The hairy root transgenic extracts contained apigenin-6,8-*C*-diglucoside, luteolin-6-*C*-glucoside (or luteolin-8-*C*-glucoside), and apigenin.[72] Apigenin was also found in the non-transgenic root extracts. The peak at retention time (t_R), t_R = 7.34 min was attributed to the presence of apigenin. The presence of this peak can also be observed in Figure 4, which shows the chromatogram recorded for the hairy root transgenic sample. The peak located at t_R = 4.29 min can be attributed to the presence of apigenin-6,8-*C*-diglucoside. The peak at 4.61 min was attributed to luteolin-6-*C*-glucoside (or luteolin-8-*C*-glucoside). The flavonoid content in the extracts of the hairy roots of transgenic *Crotalaria ochroleuca* was higher than the contents in the extracts obtained from other parts, demonstrating that genetic transformation can be effectively used to increase the production of bioactive compounds in *C. ochroleuca* roots.

The results obtained from chromatogram analysis revealed that apigenin, a flavone, was present in both the methanolic extracts of the transformed roots and the methanolic extracts of the untransformed roots of *Crotalaria ochroleuca*. This bioactive compound was isolated from the *Crotalaria pallida* extracts.[72] Flavones have already been identified in *Crotalaria lachnophora*,[73] *Crotalaria micans*,[74] *Crotalaria pallida*, *Crotalaria retusa*,[74] and *Crotalaria sessililflora*.[75] Meanwhile, apigenin-4',7-diglycoside, has been isolated from the seed extracts of *Crotalaria juncea*.[75,76] Several flavonoids were identified and isolated from the extracts of *C. sessiliflora* L.: 2',4',5,7-tetrahydroxyisoflavone, 2',4',7-trihydroxyisoflavone, 4',7-dihydroxyflavone and isovitexin..[77] Taxifine, naringenin, quercetin-7-*O*-β-D-glycopyranoside, and naringenin-7-*O*-β-D-glycopyranoside were isolated from the seed extracts of *C. assamica*.[78] Flavonoids are responsible for protecting plants from ultraviolet radiation, insects, and diseases caused by microorganisms.[79]

In addition to flavonoids, several secondary metabolites, including alkaloids, such as quinolizidines and pyrrolizidines, as well as amines, polysaccharides, chalcones, and tannins, have been isolated, identified, and characterized. The biological activities of these compounds were also analyzed.[2]

3α-Hydroxy-arbor-12-ene-28-carboxylic acid and 2β,3β,21-trihydroxy-arbor-12-ene-28-carboxylic acid, both triterpenes were isolated from the extracts of leaves of *C. emarginella*. The samples were characterized.[80] Euctomic acid, hydroxyeuchomic acid, hydroquinone, vitexin, orientin, isoorientin, (2*R*)-eriodictiol-7-*O*-β-D-glycopyranoside, and epigallocatechin gallate were isolated from the ethanolic extracts of *C. sessiliflora* leaves.[75]

The presence of flavonoids in *Crotalaria* extracts has been attributed to several biological activities of these plants. It has been reported that these compounds impart anti-inflammatory and antioxidant properties to plants.[75,77,79]

The activities of the SOD antioxidative enzymes increased post chitosan treatments (Figure 5), and the activities recorded under these treatment conditions were higher than the activities recorded for the control group. The values were higher than 60 g^{-1} mg^{-1} of protein for transformed and untransformed roots. The values recorded following the treatment of the samples with methyl-SA were lower than those recorded for the control group. This value was comparable to that of the control group for transgenic roots. Similar results were obtained under conditions of ABA treatment.

The mean POD enzyme activity for both cultivars recorded under conditions of chitosan treatment (Figure 5) was slightly lower than the activity recorded for the control for both non-transgenic and transgenic roots. The values recorded for the transgenic roots under ABA treatment conditions were lower than those recorded for the non-transgenic roots. The maximum value was recorded when the transgenic roots were subjected to conditions of methyl-SA treatment.

The APX enzyme activity for both cultivars recorded under conditions of chitosan treatment (Figure 5) was slightly lower than that recorded for the control for the non-transgenic and transgenic roots. The activity recorded under conditions of ABA treatment for the transgenic roots was lower than the activity recorded under the same conditions for the non-transgenic roots. The maximum value was recorded when methyl-SA was used to treat transgenic roots.

The pharmacological properties are influenced by the contents of isolated compounds, such as apigenin, which is the major compound in the bark extracts of *C. pallida* and *C. assamica.*[73,79] This compound exhibits anti-inflammatory activity.[73,79] Extracts of *C. pallida* present antifungal and antibacterial activities against the filamentous fungus *Fusarium oxysporum*, and *Proteus* sp., a Gram-negative bacterium.[81]

Species of the genus *Crotalaria* are widely used in agriculture as forage plants, for green fertilization, and in crop consortia, as they help address erosion problems and improve soil fertility. The aerial parts of these plants improve soil fertility and participate in nitrogen fixation in the soil.[82] The nematicide activity of *Crotalaria* has been attributed to the presence of the monocrotaline compound.[2]

Bioactive compounds isolated from *Crotalaria* should be studied, and the biological activities associated with these

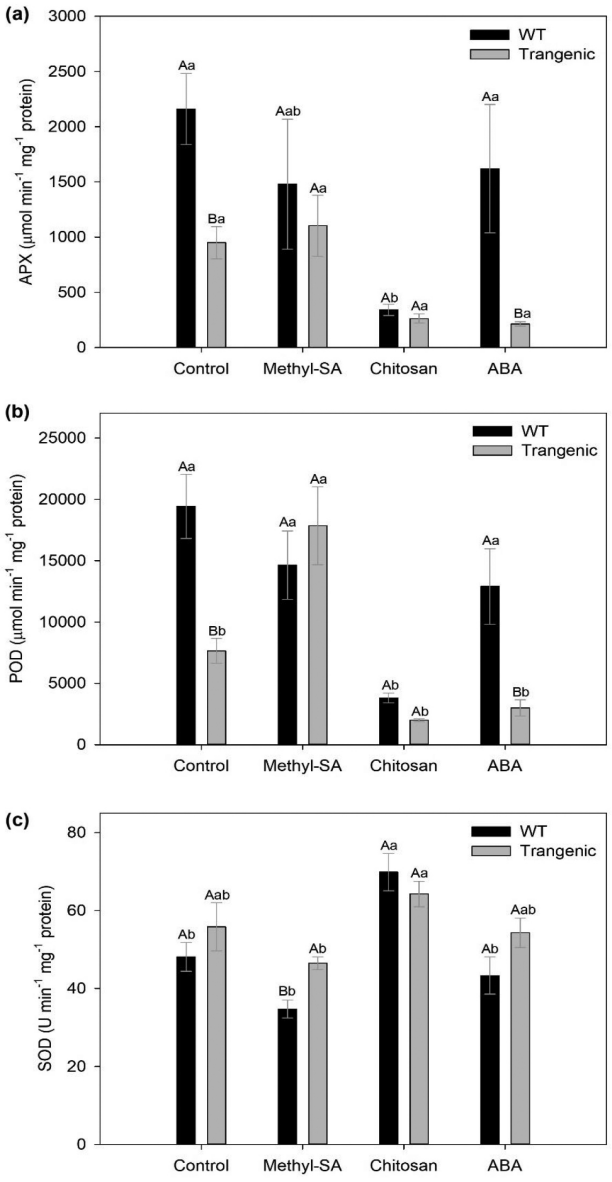

Figure 5. Effect of methyl-SA, chitosan, and ABA on the antioxidant enzymes activities for *Crotalaria ochroleuca* hairy roots; ascorbate peroxidase (APX) (a), peroxidase (POD), (b) and superoxide dismutase (SOD) (c). Aa, Ab, Aab, Bb corresponding means followed by the same letter do not differ in the least significant difference Tukey's test ($p < 0.05$).

substances should be analyzed as these compounds may prove beneficial for the treatment of various diseases. It can be inferred that *in vitro* cultivation, chemical identification of *C. ochroleuca* extracts, and isolation of bioactive compounds can contribute to the development of biotechnological, pharmacological, and bioinsecticide products.

Conclusions

The interactions between plant microorganisms, especially the co-cultivation of *C. ochroleuca* with *A. rhizogenes*, can be exploited as excellent tools for inserting genes of interest into plants to produce secondary metabolites of interest. Transformed hairy root extracts contained apigenin-6,8-*C*-diglucoside, luteolin-6-*C*-glucoside (or luteolin-8-*C*-glucoside), and apigenin. Apigenin was also found to be present in untransformed root extracts. Based on these results, innovative strategies to integrate metabolomic data can be developed to elucidate the biosynthetic pathways of secondary metabolites in *Crotalaria* spp. Bioprocesses that involve the use of precursors can help expand our knowledge of the regulation of the biosynthetic pathways for promising compounds in *C. ochroleuca* extracts.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Antonio J. Demuner was associated with obtaining resources, supervision, writing, revision, and project administration; Daiane E. Blank, Jilma L. B. Carvalho, Maria J. M. Firmino, Tainá S. Figueiredo and Gustavo S. F. Souza were associated with investigation and designing methodology; Daniele Vidal, Lorena Vieira, Jessica R. Soares, Evandro A. Fortini and Marcia A. C. Santos performed *in vitro* cultivation and visualization; Wagner Otoni was associated with obtained resources, supervision, and visualization of *in vitro* cultivation results; Guilherme J. Zocolo and Jhonyson A. C. Guedes chemically analyzed samples using UPLC-QTOF-MSE.

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