0971-4693/94 Euro 10.00
Allelopathy Journal 35 (2): 237-248 (2015) *International Allelopathy Foundation* 2015
Tables: 2, Figs : 6

Effects of leaf extracts of *Myrcia guianensis* (Aubl.) DC.: on growth and gene expression during root development of *Sorghum bicolor* (L.) Moench

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(Received in revised form: February 25, 2015)

ABSTRACT

The allelopathic potential of leaf extracts from the medicinal plant *Myrcia guianensis* (Aubl.) DC. was studied in Petri dish bioassays on sorghum and determined the seed germination, germination rate index (GRI), root growth, secondary root number, the genes involved in root development (*SHR, PHB, PHV* and *REV*) and microRNA 166 that regulates these genes. The hydroalcoholic extract was more inhibitory than methanol extract (moderate inhibition) and aqueous extract at 25 and 100% concentration were least inhibitory. Application of higher dose of hydroalcoholic *M. guianenesis* leaf extracts on sorghum seeds, inhibited the root development and changed the expression of *SHR* and *PHB* genes and microRNA 166. This suggested that the expression of these genes could be indicator of allelopathic potential for inhibition of root development in sorghum.

Key words: Allelopathic potential, gel permeation chromatography, germination, growth, inhibition, leaf extracts, microRNA 166, Molecular biology, *Myrcia guianensis*, Myrtaceae, *Phabulosa* gene, *Phavoluta* gene, *Revoluta* gene, root development, *Short-root* gene, *SHR, HD-ZIP III*, sorghum.

INTRODUCTION

Allelochemicals accumulate in all plant organs and the highest concentrations are in leaves (25,27). After release, allelochemicals can cause different effects on organisms and the environment (9,17). The direct action occurs through interference in plant growth and development, changes occur at cellular level and in membrane function, phytohormone balancing, photosynthesis and nutrient absorption (18,22,28). For example, Golisz *et al*. (10) used DNA microarray analysis to show that 110 and 69 genes were up- and downregulated, respectively, in *Arabidopsis* after a 6-hour exposure to L-DOPA. The expression pattern of various genes that are correlated with the adjustment of indeterminate root growth has been identified in *Arabidopsis*. The SHORT-ROOT (SHR) protein, an important component of the developmental pathway, regulates the specification of meristematic cells in roots (2,20).

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The expression of HD-ZIP III transcription factors specifies the differentiation of xylem into protoxylem or metaxylem (4,24). Specifically, an increase in *PHB* expression results in primary morphological changes in the pericycle that occur after the beginning of cell division until the emergence of secondary roots (11). Transcripts of *HD-ZIP III* genes are subject to post-transcriptional regulation via microRNAs and function as targets of microRNAs 165 and 166 (13). The miRNAs are a group of small non-coding RNAs (21-22 nt) that act in post-transcriptional gene regulation in animals and plants (14,15,26).

The Myrtaceae family [*Eucalyptus*, *Eugenia*, *Campomanesia* and *Myrcia* genera], comprises of several species with allelopathic activity (12). The genus *Myrcia* contains more than 300 species that are distributed across Brazil (16), many of them have medicinal properties (23). Aromatic and medicinal plants contain biologically active substances with structures that resemble allelochemical substances and therefore, have allelopathic potential (19). *M. guianensis* is most abundant Cerrado species and shows allelopathic activities of essential oils and purified fractions containing gallic and protocatechuic acids (8,31).

Classical studies have resulted in considerable progress in allelopathy, but advances in molecular biology have necessitated new investigations for a better understanding of mechanism of action of allelochemicals, mainly on root development. The integrity of root tissue depends on the combined action of different genes. The inhibition of root development due to allelopathic activity is initially caused by alterations in gene expression. Thus, this work aims to evaluate the phytochemical profile of *Myrcia guianensis*. Specifically, biological assays containing extracts and biologically active fractions of *M. guianensis* were used to determine the allelopathic effects on sorghum germination and initial seedling growth. The development parameters analysed were: germination rate index, root growth and secondary root number. In addition, the expression of genes related to the signalling of initial root development was studied and determined the possibility of these genes as molecular markers to identify the allelopathic potential.

MATERIALS AND METHODS

I. Plant material and extract preparation

Mature leaves of *M. guianensis* were collected from adult trees in vegetative stage in January 2010 from the *"Cerrado sensu stricto"* physiognomy, Forest Institute, Assis Municipality, São Paulo, Southeastern Brazil (22º35'65.32"S 50º22'97.55"W).

Aqueous extracts: Leaves were dried in oven at 40°C and for 48 h. Then, powdered leaves were extracted using water at 25°C and 100°C in ratio of 1:10 (W/V) for 2 h. After the material was filtered and the procedure was repeated twice, obtaining aqueous leaf extracts in water at 25°C (AMLE25) and aqueous leaf extracts in water at 100°C (AMLE100).

Methanol and hydroalcoholic extracts: Leaves were dried in oven at 40°C for 48 h. The separately powdered leaves (2-kg) were extracted by percolation for 5-days using 2-organic solvents: 5 L methanol (100%) and 5 L ethanol: water (7:3 v/v) at room

temperature. The solvent was evaporated under reduced pressure in a rotary evaporator and subsequently lyophilised to produce methanol leaf extract (MMLE) and hydroalcoholic leaf extract (HMLE).

II. HPLC-PAD Analysis

The hydroalcoholic leaf extract (HMLE) was injected into an HPLC-PAD-ESI/MS. Analyses were performed using an Accela High Speed LC (Thermo Scientific®, San Jose, CA, USA) with a Phenomenex® Luna C18 column model (250 x 4.6 mm, 5 µm, 100 Å) and a Phenomenex® guard column (4 x 3 mm). A gradient of eluents A (MeOH + 0.1% Formic acid) and B (H_2O + 0.1% Formic acid) consisted of 80% A and 20% B (time = 0 min) and 20% A and 80% B (time = 40 min) at a flow of 1 ml/min. The mass spectra were obtained on an LCQ Fleet mass spectrometer (Thermo Scientific®) coupled to an Accela LCQ Fleet equipped with an electrospray ionisation (ESI) source and a 3D ion trap (IT) analyser (Thermo Scientific®). Generation and analysis of first-order mass spectra in negative mode were performed under following conditions: capillary voltage: -4 V; voltage: 5 kV; spray temperature: 280°C; capillary gas drag (N_2) flow: 60 (arbitrary units). The range of acquisition was 50-1000 *m/z*; two or more scanning events were performed simultaneously using the mass spectrometer. The constituents present in the HMLE extract were identified by comparing their MS/MS^n data with data from the literature (29).

III. Fractionation by GPC

HMLE (7.0 g) was solubilised in MeOH 100% (10 ml) in ultrasonic bath for 10 min and then solution was centrifuged for 15 min to separate the precipitate from the supernatant. The supernatant was filtered and subjected to gel permeation chromatography (GPC) using a Sephadex LH-20 column (1 m x 0.06 m; SR 25/100 Column, GE Healthcare). The fractions (7 ml) were collected using an automatic fraction collector (Frac-920, GE Healthcare) at a flow rate of 400 drops per minute. The fractions were analysed using thin layer chromatography (TLC) on silica gel with liquid phase 85:15 ($CHCl_3$:MeOH), 5:6:1:4 ($CHCl_3$: MeOH: n-propanol: water), revealed using H_2SO_4/*p*-anisaldehyde and heated to 105°C until maximum visualisation of spots. Then, the fractions were grouped based on the Rf (ratio to front) and staining of their spots.

IV. Bioassay

Sorghum seeds were placed in 2% sodium hypochlorite solution for 2-min and then washed using distilled water. Stock solutions (1 g dry weight/10 ml water) of AMLE25 and AMLE100 were prepared and diluted to concentrations of 5, 10, 20, 40, 80 and 100%; MMLE and HMLE were diluted to concentrations of 100, 200, 400, 800, 1600 and 3200 mg L^{-1} and the fractions obtained by GPC were diluted to 1 g L^{-1} concentration. The sorghum seeds were germinated in Petri dishes (9 cm Ø) containing 10 ml of solution as per treatments and the control contained deionised water. The seeds were placed in growth chamber [12:12-h light/dark photoperiod at 25°C] for 5 days. The parameters determined were: germination rate index (GRI), root growth, number of secondary roots and relative quantification of gene expression. The GRI was calculated using the following equation:

$$\text{GRI} = \left(\frac{\text{G1}}{\text{N1}}\right) + \left(\frac{\text{G2}}{\text{N2}}\right) + \left(\frac{\text{G3}}{\text{N3}}\right) + \cdots + \left(\frac{\text{Gn}}{\text{Nn}}\right)$$

Where, G1, G2, G3, ..., Gn = Number of seedlings in first, second, third and last count. N1, N2, N3, ..., Nn = Number of days from seeding to first, second, third and last count.

V. Gene expression analysis using qRT-PCR

Sorghum roots exposed to HMLE at 400 mg L^{-1} concentration were collected for gene expression analysis. Total RNA isolation was done using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, United States of America). Three treated and control replicates, each containing 20 roots seedlings, were ground using liquid nitrogen. Genomic DNA contamination was eliminated using an on-column DNase I Digestion Set kit (Sigma-Aldrich). First-strand cDNA was synthesised using the stem-loop pulsed RT-PCR technique (34) and 2 µg of total RNA purified using the Improm-II Reverse Transcriptase Kit (PROMEGA) following standard recommendations. The RT-PCR was performed on a 7300 Real-Time PCR System (APPLIED BIOSYSTEMS) using a GoTaq qPCR Master Mix kit (PROMEGA). Gene expression was calculated relative to the reference gene *rRNA18S* and the control that contained plants treated with deionised water.

The primers used for qRT-PCR were designed using sorghum genes obtained from the *Sorghum bicolor* Genome Data Base (http://www.plantgdb.org/SbGDB/) (Table 1). The *Sorghum bicolor* (L.) Moench Genome Initiative locus identifier numbers for the genes investigated in this study were: *SHR* (Sb02g037890), *PHB* (Sb08g021350), *PHV* (Sb01g013710), *REV* (Sb01g013710), *rRNA18S* (Sb02g027580) and *miR166* (MIMAT0001394).

Statistical analysis: The results were presented as the means ± standard deviations (SD) expressed as percentage of control. Statistical comparisons were done using a one-way analysis of variance followed by Tukey's test or the Kruskal-Wallis test. Statistical significance was set at $P < 0.05$. Sigma Plot (version 12.0) was used for graphic design and statistics. We did principal components analysis (PCA) on the bioassay results of purified fractions using XLstat version 2014.1.10.

RESULTS AND DISCUSSION

Chemical characterisation

The HMLE analysed using HPLC-PDA-ESI/MS, was characterised by 5-main Peaks (Figure 1 chromatogram). Peak **1** (R_t = 2.10, λ_{max} = 272 nm) with *m/z* 169 $[M - H]^-$ was related to gallic acid, Peak **2** and **3** (R_t = 3.93, λ_{max} = 272 nm) with *m/z* 179 $[M - H]^-$ and 191 $[M - H]^-$ were related to derivatives of caffeic and quinic acids, respectively and Peak **4** (R_t = 15.17, λ_{max} = 260, 350 nm) with *m/z* 463 $[M - H]^-$ and Peak **5** (R_t = 17.72, λ_{max} = 258, 356 nm) were related to glycosylated flavonoid derivatives (1).

The HMLE subjected to gel permeation chromatography (GPC) using Sephadex resulted in 14-final fractions that were used in bioassays. TLC plates sprayed with anisaldehyde/H_2SO_4 revealed the purplish and yellow/orange bands, which suggested the

Table 1. Gene's access and primers used to qRT-PCR analysis

Gene Name	Identification Number	Forward Primer	Reverse Primer
SHR (Short-root)	Sb02G037890	TGCCCCAACAACTTTAGTCC	CACAATCGAACCTCCACCTT
PHB (*Phabulosa*)	Sb08G021350	TTCACTCCACTCCCCACTC	ACCGCCTAGTAGTGCAGCAG
PHV (Phavoluta)	Sb01G013710	CACTCGGACTCGATCATGTG	TCAAGGGAGATGTCCTGGAG
REV (*Revoluta*)	Sb01G013710	AGGCATTATTTGTGCGAAGG	GAAGCCAAATACGCATCCAT
rRNA 18S (*18S*)	Sb02G027580	GGCTCGAAGACGATCAGATACC	TCGGCATCGTTTATGGTT
Sbi-mirR166 (miRNA166)	MIMAT0001394	CCTGAGTCGGACCAGGCTTCA	-
Sbi-mirR166 (miRNA166) for reverse transcriptase	MIMAT0001394	-	GTCGTATCCAGTGCAGGGTC-CGAGGTATTCGCACTGGATA-CGACGGGGAA

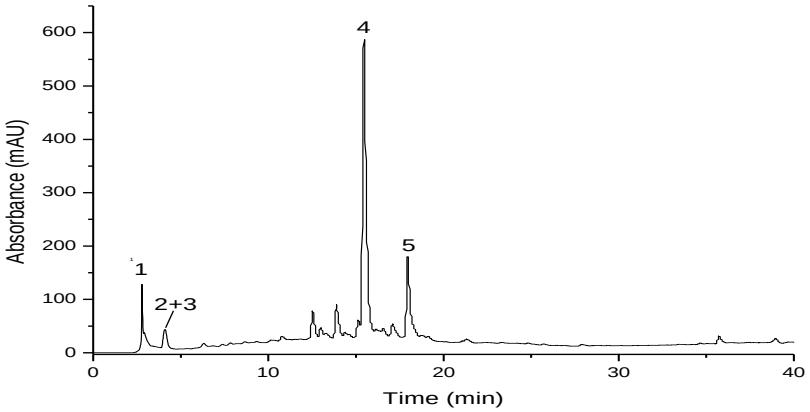

Figure 1. HPLC-PAD analytical chromatogram of HMLE leaves extract of *M. guianensis* with identified peaks. Experimental conditions: Eluents **A** (MeOH + 0.1% Form. ac.) and **B** (H_2O + 0.1% Form. ac.). Gradient system: 20–80% of **A** in **B** in 40 min. Column: Phenomenex® Luna C_{18} (250 × 4.6 mm i.d., 5 μm). Injection volume: 20.0 μL; λ = 254 nm; Column temperature: 25 °C; Flow ratio: 1.0 mL·min^{-1}.

presence of phenolic acid and flavonoid derivatives. Fractions 3,4,5,6 and 7 contained mostly phenolic acids [gallic acid and derivatives of caffeic and quinic acids] that were associated with Peak 1,2 and 3, respectively, observed in the HPLC-PDA analysis. Fractions 8 to 14 contained glycosylated flavonoids that were observed as Peak 4 and 5 in the HPLC-PDA analysis. Souza Filho *et al.* (31) identified two phenolic acids, i.e., gallic acid and protocatechuic acid, from ethyl acetate leaf extracts of *M. guianensis*. In addition, Saldanha *et al.* (29) and Vareda *et al.* (33) characterised 70% EtOH extracts of *Myrcia bella* and showed the presence of phenolic acids and glycosylated flavonoid derivatives from myricetin and quercetin.

Germination and root development

The seeds germination (%) was not affected by HMLE, MMLE, AMLE25 or AMLE100. The Germination Rate Index (GRI) was affected by HMLE, slightly by MMLE and only at the highest dose of AMLE100 (Figure 2).

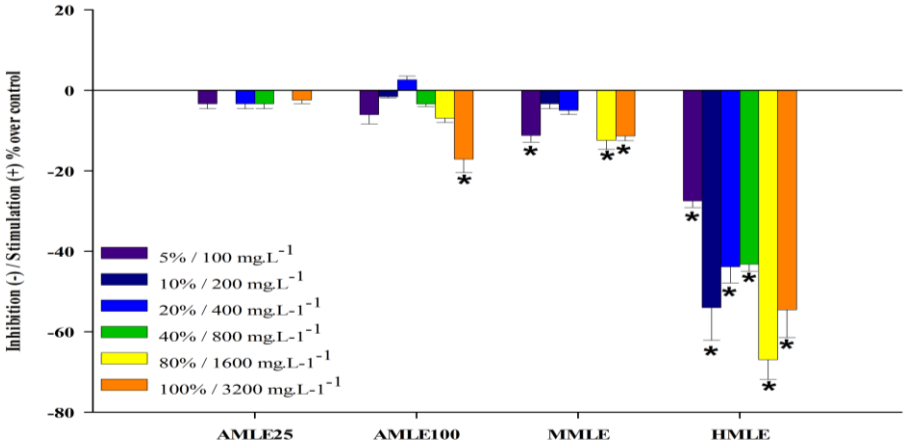

Figure 2. Effects of various extracts and concentration on the Germination rate index (GRI) of sorghum seeds. Results are shown relative to control. *Level of significance p <0.05 by ANOVA followed by Tukey test. AMLE25: Aqueous *Myrcia* leaves extract with water at 25°C; AMLE100: Aqueous *Myrcia* leaves extract with water at 100°C; MMLE: Methanol *Myrcia* leaves extract; HMLE: Hydroalcoholic *Myrcia* leaves extract.

HMLE was more inhibitory than other extracts. The germination (%) was not sufficient for the analysis of seedling development, which demonstrated the importance of abortion assessment to check the performance of germination stage. Among all leaf extracts, the highest concentration (100% for aqueous extracts and 3200 $mg.L^{-1}$ for methanol and hydroalcoholic extracts) exhibited maximum inhibitory effects on sorghum. Sorghum seeds were more susceptible to HMLE action. Souza Filho *et al.* (31) demonstrated that germination of *Mimosa pudica* and *Senna obtusifolia* seeds exposed to *M. guianensis* essential oil were inhibited and stimulated, respectively.

Seed germination was not significantly affected by any fraction (Table 1). This is a common finding in studies conducted on other species (7,36). The GRI values indicate that fraction 6 (phenolic acids) showed high inhibitory activity, especially of crude extracts. Allelopathic studies on physiological effects have shown that in addition to germinated seeds (%), other factors must be used to interpret these effects viz., GRI and root growth (7,21,35). Moreover, the inhibition of root system development of neighbouring species leads to reduction in the competitive pressure of a plant, which favours its development.

By contrast, root length and the number of secondary roots were drastically affected by extracts at different doses and in some cases there was total inhibition of roots, which was significantly correlated with the extract doses (Figure 3 and Figure 4). In

addition, the extracts changed the number of secondary roots produced by seedlings (Figure 4). The development of secondary roots was very poor and under the effect of HMLE, the number of secondary roots was nil. Fraction 14 showed greater inhibitory activity on the root length compared with other fractions. When subjected to fractions 3, 4 and 6, seeds showed a similar response; however, fractions 10 and 11 induced root growth. Moreover, when subjected to fractions 2, 4, 6, 7, 13 and 14, the secondary root growth was completely inhibited (Table 2).

Table 2. Allelopathic activity of fractions from HMLE obtained in GPC. *: Significance value obtained by Kruskal-Wallis test. ns : Non-significant. HMLE : Hydroalcoholic *Myrcia* leaves extract

Fractions	Germination	GRI	Root growth	Secondary roots
Fr 1	0.0 ns	1.7 ns	-43.6 ns	44.4 ns
Fr 2	-6.7 ns	-18.6*	-45.2 ns	-100*
Fr 3	-10.0 ns	-22.0*	-64.8*	-88.9*
Fr 4	0.0 ns	-6.8 ns	-60.4*	-100*
Fr 5	-3.3 ns	-5.1 ns	-42.4 ns	-77.8*
Fr 6	-6.7 ns	-27.1*	-54.3*	-100*
Fr 7	-3.3 ns	-10.2 ns	-40.3 ns	-100*
Fr 8	-6.7 ns	-6.8 ns	28.4 ns	0.0 ns
Fr 9	-3.3 ns	-3.4 ns	28.0 ns	-100*
Fr 10	0.0 ns	1.7 ns	93.0*	11.1 ns
Fr 11	-3.3 ns	-3.4 ns	82.8*	11.1 ns
Fr 12	0.0 ns	1.7 ns	-15.4 ns	-55.6 ns
Fr 13	0.0 ns	1.7 ns	19.6 ns	-100*
Fr 14	0.0 ns	-11.9	-85.8*	-100*

Borella and Pastorini (3) reported that tomato seeds and *Bidens pilosa* L. root length were affected by aqueous extracts of *Phytolacca dioecious* L. leaves and the reduction in root length was related to the extract concentration. The roots were withered, defective or even absent. Imatomi *et al.* (12) showed that Myrtaceae family species, [including species of the *Myrcia* genus (i.e., *Myrcia bella*, *M. lingua*, *M. multiflora*, *M. splendens*, *M. tomentosa*)], showed the allelopathic inhibitory effects on root and shoot growth.

A PCA was performed to group the variables germination, GRI, root growth and number of secondary roots (Figure 5). The fractions with greater activity were grouped together (Fr 2, 3, 4, 5, 6 and 7). Thus, we can infer that extract HMLE had a phenolic acid fraction that is responsible for the grouping of active fractions (Fr 1 to Fr 7).

HMLE effects on gene expression

The hydroalcoholic extraction (HMLE) was more inhibitory to root growth compared with other extraction methods. The intermediate dose of 400 mg L^{-1} of this extract did not reduce seed germination but decreased the germination rate (GRI) and root growth. This dose was selected for gene expression analysis in sorghum roots. The analyses indicated differential expression of these genes than control group (Figure 6).

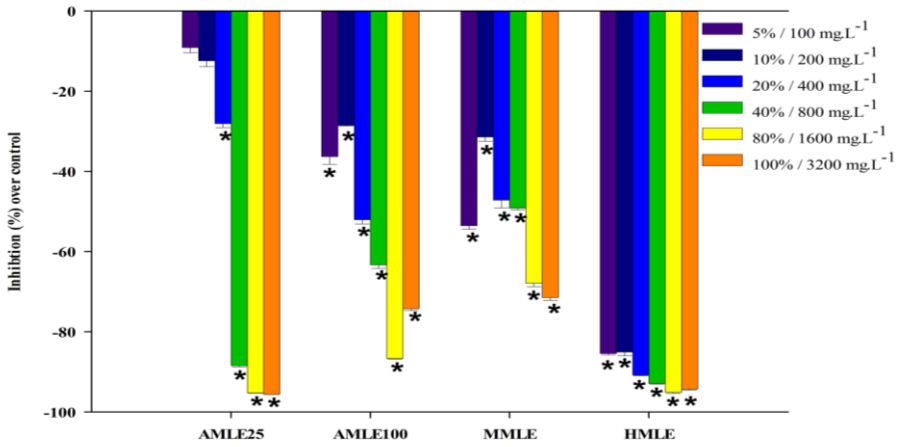

Figure 3. Effects of various extracts and concentration on the root growth of sorghum seeds. Results are shown relative to control. *Level of significance p <0.05 by ANOVA followed by Tukey test. AMLE25: Aqueous *Myrcia* leaves extract with water at 25°C; AMLE100: Aqueous *Myrcia* leaves extract with water at 100°C; MMLE: Methanol *Myrcia* leaves extract; HMLE: Hydroalcoholic *Myrcia* leaves extract.

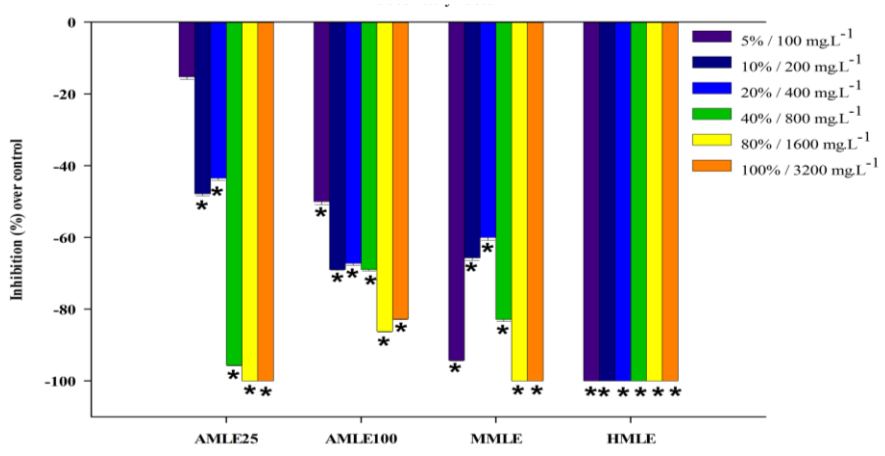

Figure 4. Effects of various extracts and concentration on the secondary root number of sorghum seeds. Results are shown relative to control. *Level of significance p <0.05 by ANOVA followed by Tukey test. AMLE25: Aqueous *Myrcia* leaves extract with water at 25°C; AMLE100: Aqueous *Myrcia* leaves extract with water at 100°C; MMLE: Methanol *Myrcia* leaves extract; HMLE: Hydroalcoholic *Myrcia* leaves extract

The miRNA166, *SHR* and *PHB* genes showed 2.9, 3.2 and 2.2-folds increases in their expressions relative to the control, respectively. The expression of *PHV* and *REV* genes did not differ significantly from control. These genes, are expressed in the root, are

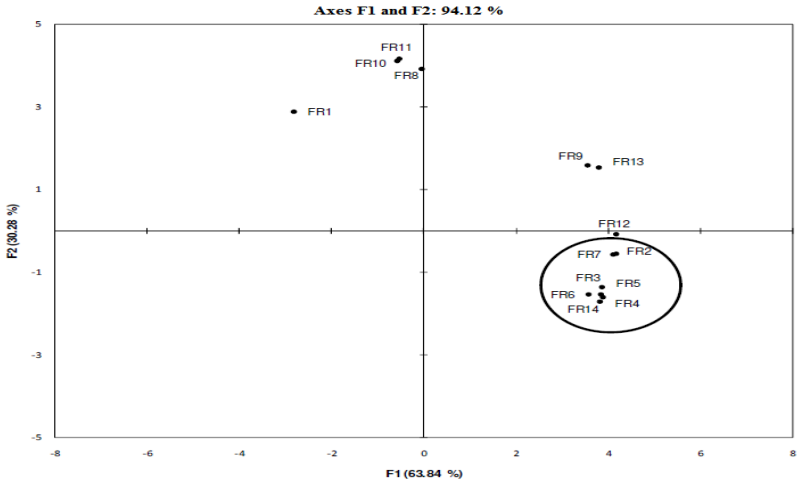

Figure 5. Pattern of biological activity of GPC fractions (FR) obtained by HMLE. Circled fractions showed significant inhibition pattern. Fractions 2 to 7 contains mostly phenolic acids compounds and fraction 14 contains predominant flavonoids compounds. HMLE: Hydroalcoholic *Myrcia* leaves extract.

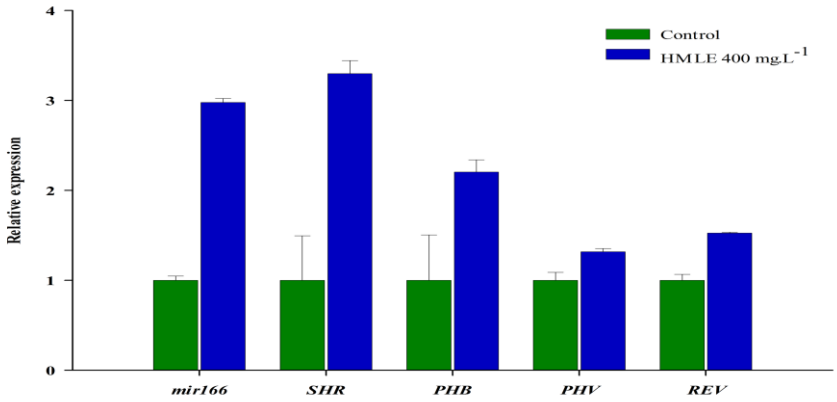

Figure 6. Effects of HMLE 400 mg.L^{-1} expression relative to control of miR166, *SHR*, *PHB*, *PHV* and *REV* genes in sorghum root. Gene expression is represented on the Y-axis with ratio (relative expression) of absolute value of expression value of each gene-by-gene expression normalizer *18S* ribosomal RNA. X-axis are represented each group evaluated and vertical bars represent the standard deviation of three biological samples. HMLE : Hydroalcoholic *Myrcia* leaves extract.

related to the initial development of root tissue and have stimulatory and regulatory effects on cellular differentiation. The protein encoded by *SHR* gene is related to the differentiation of meristematic cells that form the cortical tissue in the root; *shr* mutants of

Arabidopsis have very small primary roots (2,20). However, the balance between SHR and SCR (Scarecrow) results in the specific characteristics of cortical cells (i.e., the cortex and endodermis) and *shr* mutants of *Arabidopsis* possesses a single layer of cortical cells and endodermis was absent (30). Thus an increase in *SHR* expression may lead to modification of endodermis, which may hinder the entry of allelochemicals.

The transcription factors of HD-ZIP III family members (the *PHB*, *PHV* and *REV* genes) are correlated with the formation of vascular tissues (metaxylem and protoxylem arches) in the root (4). The expression of *PHB* gene is related to the formation of secondary roots, even when expressed in tandem with the genes of the KANADI family (11). The development of secondary roots is a morphological mechanism to tolerate various types of biotic or abiotic stresses and an increase in PHB expression indicates a molecular mechanism that stimulates the development of secondary roots.

However, the family members of HD-ZIP III transcription factors described above are subjected to post-transcriptional regulation via regulatory RNAs. Transcripts of HD-ZIP III genes are targets of microRNAs 165 and 166 (13). The increased expression of miR166 regulates the transcripts of the HD-ZIP III family, including *PHB*, which is correlated with the poor development of secondary roots and even the differential expression of miR166 - an indicator of stress in roots. Sunkar *et al.* (32) reported a reduction in the expression level of miR166 in *Hordeum vulgare* under drought stress compared with an increase in the expression level in *Arabidopsis thaliana* root under temperature and UV-B stress conditions. However the phenolic acids from rice had inhibitory allelopathic activity but increased miRNA expression in *Echinochloa crus-galli* (6). The allelochemical L-Dopa can alter the expression of genes related to metabolism, cellular division and protein function (10). Plant extracts with allelopathic potential reduced the root growth and can alter the root development related to *SHR* and *HD-ZIP III* transcription factors, which influence the regulatory network of root tissues.

CONCLUSIONS

Leaf extracts of *M. guianenesis* drastically inhibited the sorghum root development and changed the expression of *SHR* and *PHB* genes and microRNA 166, which can be used as indicators of allelopathic potential on root development. The extraction method interfered with the inhibitory potential of *M. guianenesis*. The different types of extractants exhibited variability in potential activity and HMLE was most inhibitory. These result indicated that an ethanol:water (70:30) mixture can extract compounds with high inhibitory potential from the *M. guianensis* leaves.

ACKNOWLEDGEMENTS

Author are thankful to *Fundação de Amparo à Pesquisa do Estado de São Paulo* – FAPESP/BIOTA (Process No. 2009/52237-9) for financial support.

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