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Phytotoxic effects of aqueous leaf extracts of four Myrtaceae species on three weeds

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ABSTRACT. Research on allelopathic interactions can be useful in the search for phytotoxins produced by plants that may be employed as natural herbicides. The aim of this study was to assess the phytotoxic action of aqueous leaf extract of *Blepharocalyx salicifolius*, *Myrcia multiflora*, *Myrcia splendens* and *Myrcia tomentosa* on the germination and development of three weeds. The working hypothesis was that leaf extracts of Myrtaceae may negatively influence the development of weed species. Aqueous leaf extracts at 5 and 10% (g mL^{-1}) were tested on the germination and growth of *Euphorbia heterophylla*, *Echinochloa crus-galli* and *Ipomoea grandifolia* and compared with the herbicide oxyfluorfen and distilled water (control). The most extracts caused pronounced delays in seed germination and inhibited the growth of seedlings of *E. heterophylla*; *I. grandifolia* and *E. crus-galli*, with the last target species had no growth shoot inhibited by the extracts. In this study, the potential and efficiency of the tested aqueous leaf extracts were evident because they were more phytotoxic to the weeds than the herbicide. Thus, the aqueous extracts of leaves from Myrtaceae species show potential for the isolation of active compounds that can be used for the production of natural herbicides in the future.

Keywords: allelopathy, germination, growth, herbicide.

Efeito fitotóxico de extrato foliar aquoso de quatro espécies de Myrtaceae sobre três espécies infestantes de cultura

RESUMO. Estudos relacionados à interações alelopáticas podem ser úteis na busca por fitotoxinas produzidas por plantas que podem ser empregadas como herbicidas naturais. O objetivo deste trabalho foi avaliar a atividade fitotóxica de extratos aquosos de folhas de *Blepharocalyx salicifolius*, *Myrcia multiflora*, *Myrcia splendens* e *Myrcia tomentosa* sobre a germinação e o desenvolvimento de três plantas infestantes de culturas agrícolas. A hipótese testada neste trabalho foi que extratos foliares de Myrtaceae podem influenciar negativamente sobre o desenvolvimento de espécies infestantes de cultura. Foram testados os extratos foliares aquosos a 5 e 10% (g mL^{-1}) sobre a germinação e crescimento de três espécies infestantes de cultura e comparados com o herbicida oxyfluorfen e água destilada (controle). A maioria dos extratos promoveram atrasos acentuados na germinação de sementes e inibiram o crescimento de plântulas de *E. heterophylla*; *I. grandifolia* e *E. crus-galli*, apenas esta última espécie alvo não apresentou inibição no crescimento de sua parte aérea pelos extratos. O presente estudo comprovou o potencial e a eficiência dos extratos, uma vez que foram mais fitotóxicos às espécies infestantes que o herbicida. Assim, extratos foliares aquosos de espécies de Myrtaceae apresentam potencial para isolamento de compostos que poderão ser usados para produção de herbicidas naturais no futuro.

Palavras-chave: alelopatia, germinação, crescimento, herbicida.

Introduction

According to the National Health Surveillance Agency (ANVISA), Brazil has been the greatest consumer of pesticides in the world since 2008, with herbicides accounting for 50% of all the pesticides used. Indeed, pesticide consumption in Brazil has grown by 190% the last ten years. Because the large-scale use of pesticides has led to environmental problems and risks to human health alternative control methods are necessary (CARNEIRO et al., 2012).

Allelopathy has became an important tool to identify plants with bioactive compounds (OLIVEROS-BASTIDAS, 2008) for use in the development of natural herbicides that are more specific and cause less environmental damage (MACÍAS et al., 1998). The concept of allelopathy describes the influence of one plant on others in an ecosystem mediated by biomolecules (allelochemicals) produced by the plant, which may damage or promote the growth of the target plant (RIZVI et al., 1999). Research on the allelopathic process has mainly focused on its use in agriculture to indicate species with phytotoxic activity.

The excessive use of herbicides might cause soil and water pollution and damage to human health. The herbicide Goal BR (240 g L^{-1} of oxyfluorfen) is widely used for weed control in crops and is classified as selective and non-systemic in action, belonging to the chemical group diphenyl ethers. Oxyfluorfen is indicated for the control of weeds (monocots and eudicots) and can be used pre- and post-emergence. However, the herbicide is considered harmful to the environment, highly persistent and toxic to aquatic organisms (D'AMATO et al., 2002). Natural compounds have advantages over synthetic compounds due to the absence of halogenated molecules, a lower half-life (DUKE et al., 2000) and (in most cases) solubility in water, providing inhibitory activity at lower concentrations (OLIVEROS-BASTIDAS, 2008).

Myrtaceae is one of the most abundant and diversified plant families in Brazilian ecosystems (MORI et al., 1983). The Brazilian flora, published by Brazilian Institute of Geography and Statistics (IBGE), indicates that this is one of the most representative families of the Cerrado (Brazilian savannah). Although such genera as *Eucalyptus*, the most studied in this family, have been widely reported to show allelopathic effects (FANG et al., 2009), there have been few studies concerning the phytotoxic potential of Myrtaceae from the Cerrado. Thus, the objective of this study was to determine whether aqueous leaf extracts of four species of Myrtaceae from the Cerrado inhibit the growth of weeds and may be used to replace commercial herbicides. Aqueous leaf extracts of Myrtaceae species have phytotoxic activity on bioindicator species (IMATOMI et al., 2013a and b). Therefore, it has been hypothesised that leaf extracts of Myrtaceae may influence the development of weed species. The main objective of this study was to assess the phytotoxic activity of aqueous leaf extracts of *Blepharocalyx salicifolius*, *Myrcia multiflora*, *Myrcia splendens* and *Myrcia tomentosa* and their effects on the germination and development of three weed species: *Echinochloa crus-galli*, *Euphorbia heterophylla* and *Ipomoea grandifolia*.

Material and methods

Collection area

Leaves were randomly gathered from plants in the Cerrado area of Universidade Federal de São Carlos (UFSCar) in São Carlos, São Paulo State, Brazil (21º 58' to 22º 00' S and 47º 51' to 47º 52' W). The climate is Cwa-type by Koeppen's classification (upland tropical). The vegetation is characterised by a woody layer formed by small trees and bushes that protrude above a well-defined dense herbaceous layer (RIBEIRO; WALTER, 1998).

Biological material

Extracts were selected from the following species: *Blepharocalyx salicifolius* Kuth O. Berg., *Myrcia multiflora* DC., *Myrcia splendens* DC. and *Myrcia tomentosa* DC. Exsiccates of each specimen were deposited in the Herbarium of the Universidade Federal de São Carlos (HUFSCar) under accession numbers 8308 (*B. salicifolius*), 8316 (*M. multiflora*), 8317 (*M. splendens*) and 8318 (*M. tomentosa*).

The Myrtaceae specimens used were marked and observed *in situ* until the period of flowering and fruiting, enabling the species to be identified. The leaves of each species were randomly collected from at least five plants in the vegetative stage during the dry season (July to October 2008). The collected leaves were dried in an oven (for 48h, at 40°C), powdered using a Willey mill (Mesh 14) and stored in plastic bags at room temperature (± 25°C).

The phytotoxic activity of aqueous leaf extracts of the donor species was tested on diaspores of three weeds: barnyard grass (*Echinochloa crus-galli* (L.) Beauv., Poaceae), wild poinsettia (*Euphorbia heterophylla* L., Euphorbiaceae) and morning glory (*Ipomoea grandifolia* L., Convolvulaceae). The morning glory diaspores were scarified with concentrated sulphuric acid (98%) for five minutes to break the mechanical dormancy and then washed in distilled water, dried on filter paper and immediately used in bioassays (VOLL et al., 2010).

Preparation of plant extracts

The aqueous leaf extract were prepared by mixing leaf powder with distilled water at 10% (g mL^{-1}) and leaving the mixture in the fridge (4°C) for 12h. After this period, the extract was filtered by vacuum through qualitative filter paper using a Büchner funnel. A 5% extract was produced by diluting the 10% extract with distilled water (GATTI et al., 2004).

The effects of the aqueous leaf extracts at 10 and 5% were compared with two control groups: distilled water (negative control) and commercial herbicide (positive control), Goal BR (240 g L^{-1} of oxyfluorfen) at 10 and 5% of the manufacturer's recommended dose (720 g.i.a.ha^{-1}).

Germination bioassay

The diaspores of target species were sown in 9 cm Petri dishes, with two filter papers moistened with 5 mL aqueous leaf extract, oxyfluorfen herbicide (positive control) or distilled water (negative control). The experiments were performed in four replicates of 20 diaspores per Petri dish. The dishes were maintained in a germination chamber (B.O.D) under a 12:12-hour photoperiod at 27ºC; the conditions were confirmed by a pretest.

The germinated diaspores were counted at 12-hour intervals until 15 days after sowing. Germination was considered as the protrusion of one part of the embryo from the seed coat (BORGHETTI; FERREIRA, 2004).

Seedling growth bioassay

The diaspores used in this bioassay were pre-germinated in distilled water (2 to 4 mm of radicle) and then distributed in plastic boxes (14 x 10 cm) lined with two filter papers moistened with 13 mL aqueous leaf extract, herbicide at 5 and 10% (positive control) or distilled water (negative control).

The experiments were performed in four replicates of 10 diaspores per plastic box. The boxes were maintained in a germination chamber (B.O.D.) under a 12:12-hour photoperiod at 27ºC. The length of the shoot and primary root were measured using a digital calliper, and abnormal seedlings were evaluated after five days. Four types of anomalies caused by extracts were identified as damaging or weakening seedling development: 1) root atrophy; 2) early secondary root development; 3) gravitropic plant inversion and 4) root necrosis, according to Regras para Análise de Sementes (BRASIL, 2009).

Physicochemical characteristics of the extracts

The osmotic potential (OP, mOsm kg^{-1}) was measured using an automatic osmometer (μOsmotte 5004), and the values of OP were converted to MPa (LARCHER, 2004). Subsequently, the germination and growth bioassays were performed using a previously described method with polyethylene glycol 6000 (PEG-6000), in accordance with the specifications given by Sun (2002), to simulate the osmotic potential of the aqueous leaf extracts.

The pH of the aqueous leaf extracts was measured using a pH meter. The pH of all the extracts remained between 5.8 and 7.1, within the tolerance limits for germination and development of the target species (LARCHER, 2004); thus, pH-related bioassays were not performed.

Mathematical and statistical data analyses

For the germination bioassay, the germination rate (*G*, in percentage), average germination time (*AT*, in hours), informational entropy (*H*, in bits) (RANAL; SANTANA, 2006) and index of allelopathic effects (*RI*) (ZHANG et al., 2010) were calculated. *RI* is a qualitative index, with negative values indicating inhibitory activity, and was calculated as follows: $RI = (T.\ C^{-1}) - 1$, where T and C are the speed of germination (seeds germinated per day) of seeds subjected to the leaf extract and control, respectively. The shoot and root lengths of the seedlings were converted to percent deviation from control. Thus, zero indicated the same length as the control, positive values indicated stimulation, and negative values inhibition (MACÍAS et al., 2006). Seedling anomalies were presented as a cumulative percentage.

The laboratory experimental design was completely randomised, with four replicates per treatment. Data normality was analysed by the Lilliefors test (Kolmogorov-Smirnoff). The statistical significance of the differences between the treatments (including positive control) and negative control was tested by the Student *t*-test for normal data or by the Mann-Whitney test for non-normal data, both at the 5% level. All analyses were performed in Bioestat 5.0.

Results and discussion

The osmotic potential of the aqueous leaf extracts and the herbicide ranged from -0.10 (*Myrcia splendens*) to -0.19 MPa (*M. multiflora*). The equivalent PEG-6000 solutions did not have significant effects on the germination rate, average germination time or initial growth of the weeds (Figures 1 and 2), corroborating other studies showing that only extreme osmotic potentials affect the germination and growth of plants (GRISI et al., 2011; ZHANG et al., 2010).

Neither the aqueous leaf extract of any donor species nor the herbicide had a significant effect on the germination rates of the target species (Figure 1). With regard to the average germination time (*AT*), the wild poinsettia diaspores showed a significant increase when treated with the aqueous leaf extracts of all the donor species at both concentrations tested, thus delaying the germination process (Figure 1). All the aqueous leaf extracts and the herbicide, at both concentrations, significantly increased the barnyard grass *AT* (Figure 1). The *M. splendens* and *M. tomentosa* extracts at both concentrations and the *M. multiflora* extract at 10% significantly increased the morning glory *AT* (Figure

1). Studying the same weed species, Matsumoto et al. (2010) found that the ethyl acetate fraction of *Annona glabra* (Annonaceae), as obtained by liquid-liquid partition with hexane and ethyl acetate, was unable to reduce the germination rate of *Echinochloa crus-galli*, *Euphorbia heterophylla* or *Ipomoea grandifolia*; although the fraction retarded the germination of *E. crus-galli*, it did not affect the average time of *E. heterophylla* and *I. grandifolia*. Seyyednejad et al. (2010) evaluated the allelopathic effect of aquatic hull extract of 13 rice cultivars (*Oryza sativa*) on *E. crus-galli*, observed that none of the extracts significantly reduced the seed germination rate, corroborating the results of the present work.

Although the final germination rate was not affected by the aqueous leaf extracts, the extracts did inhibit the average germination time more than the herbicide. Sometimes the allelopathic effect is not evident in the final germination rate but in the average time or other parameters of the germination process (FERREIRA, 2004). According to Fenner (2000), the average time is a crucial factor for seedling survival, influencing their growth and performance in subsequent stages of development. Plants that germinate slowly could be reduced in height (JEFFERSON; PENNACCHIO, 2003) and, consequently, may be more susceptible to stress and predation and have lower success in competition for resources.

Deviations from standard germination parameters may result from physiological processes in the seed that are affected by phytotoxins, the most reported being the suppression of enzyme activities and / or phytohormones related to the hydrolysis of the reserve materials of the embryo at the beginning of development (SINGH et al., 2009). In addition, other metabolic processes are affected, including respiration, photosynthesis, xylem element flux, membrane permeability, cell division and development and protein synthesis (HAIG, 2008).

Increases in informational entropy indicate changes in the synchrony of metabolic reactions that occur during the germination process (RANAL et al., 2009). According to the results obtained in the present study, the values of informational entropy of the wild poinsettia and barnyard grass diaspores treated with the extracts did not differ from the control, indicating synchrony in the germination process (Figure 1). The morning glory diaspores showed an increase in entropy when subjected to the aqueous extracts of *B. salicifolius*, *M. splendens* and *M. tomentosa*, at both concentrations, and *M. multiflora* at 10% (Figure 1).

The assessment of phytotoxic effects on diaspore germination should not be based only on the final number of diaspores germinated. In addition to this, the average time, homogeneity and synchrony of germination are variables that express the rate and degree of organisation or disorder in the chemical reactions that occur in seeds during germination and, thus, should be analysed together with the germinability (SANTANA et al., 2006). The index of allelopathic effects (RI), an important indicator for allelopathic effects, was calculated from the germination speed of the control and treatment groups (GAO et al., 2009). All the diaspores treated with the extracts showed negative RIs, indicating the presence of phytotoxic activity in the extracts. Both herbicide concentrations inhibited the diaspores of the target species, and the RI value was small for the wild poinsettia diaspores at the 10% concentration (Figure 1).

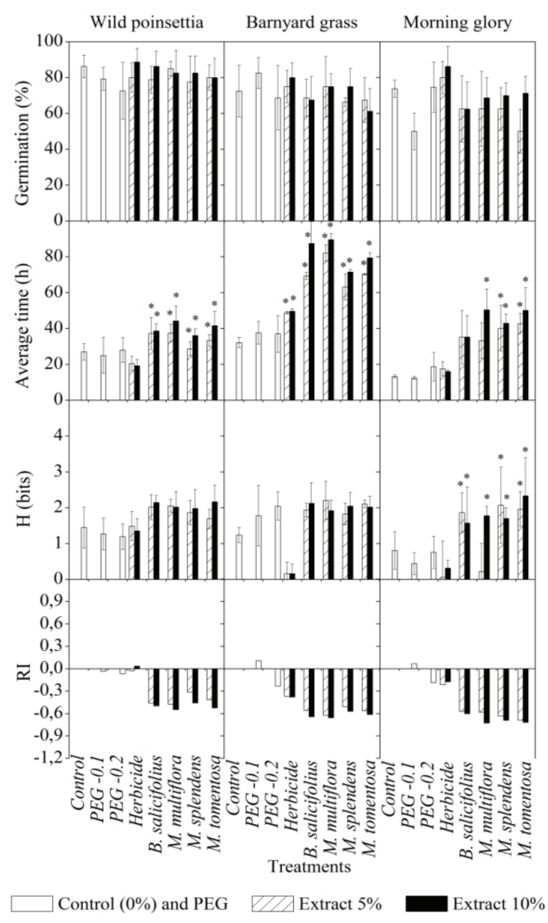

Figure 1. Average values (columns) and standard deviation (bars) of germination rate, average time, informational entropy (H) and index of allelopathic effects (*RI*) of wild poinsettia (*Euphorbia heterophylla*), barnyard grass (*Echinochloa crus-galli*) and morning glory (*Ipomoea grandifolia*) subjected to aqueous leaf extracts at 5 and 10%, herbicide (positive control) and distilled water (negative control) (*t*-test and Mann-Whitney). *indicates a significant difference from the control ($p < 0.05$).

Allelochemicals also interfere directly with the physiological and biochemical reactions involved in the growth and development of plant organs (WEIR et al., 2004). All the 10% aqueous Myrtaceae species leaf extracts reduced the shoot and root lengths of the wild poinsettia seedlings, and the 5% *B. salicifolius* and *M. tomentosa* extracts inhibited the shoot length, as did the herbicide at both concentrations tested. The root lengths of the barnyard grass seedlings were inhibited by the aqueous leaf extracts at both concentrations, yet the *M. tomentosa* extract at 5% increased the shoot and root length. The herbicide was the only treatment that inhibited the shoot and root lengths of the barnyard grass seedlings at both concentrations, and the shoot length showed a hormetic response to the extracts from all the donor species tested. Only the *M. tomentosa* extract significantly stimulated the shoot length. The morning glory seedling shoot length was inhibited by the 10% *B. salicifolius*, *M. splendens* and *M. tomentosa* extracts, and the root length was inhibited by both concentrations of all the leaf extracts tested and the herbicide (Figure 2).

Root length was more affected by the extracts than the shoot length (Figure 2). Phytotoxins from plant extracts can be more associated with root growth processes, such as cellular division, hormone production, membrane permeability, mineral absorption, enzymatic activity and water relations (GNIAZDOWSKA; BOGATEK, 2005). Furthermore, the roots of the seedlings remained in direct contact with the extracts, which may well damage this organ more rapidly than other organs. Other authors have reported similar results with other species, for example, Al-Sherif et al. (2013) studying water, ethanol and chloroform extracts of *Brassica nigra* on two crops, *Trifolium alexandrinum* and *Triticum aestivum*, and two weeds, *Phalaris paradoxa* and *Sisymbrium irio*, showing that root growth was more affected than shoot growth.

The results of the present study also showed that the seedlings of wild poinsettia and morning glory were more affected than those of barnyard grass (Figure 2). Such species-dependent responses to allelochemicals can influence the plant species composition of natural ecosystems and can be used to design selective herbicides in agroecosystems (IMATOMI et al., 2013b).

Allelochemicals can induce the appearance of abnormal seedlings, and the root is the organ that responds most dramatically (GRISI et al., 2012). Thus, necrosis is the most evident symptom, inhibiting the development of the target plant (FERREIRA; ÁQUILA, 2000). In the present study, necrosis was observed in all the seedlings treated with all the extracts tested, whereas the herbicide caused necrosis only in the wild poinsettia seedlings (Figure 3). With injury to the root cap, the levels of cytokinin, which controls the root geotropic curvature, will be reduced and will induce gravitropic inversion (ALONI et al., 2006). This reduction in cytokinin combined with other hormones may cause the appearance of early secondary roots (FUKAKI; TASAKA, 2009). The early appearance of secondary roots, which are shorter and thicker, compromises the absorption of nutrients by the plant and normal development (SCHMIDT et al., 2010).

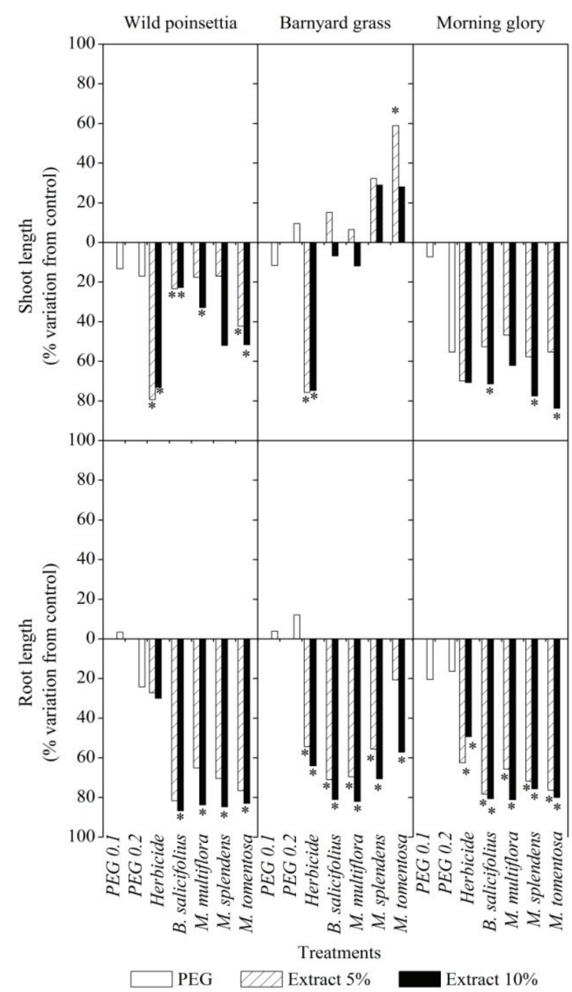

Figure 2. Average values of inhibition / stimulation of shoot and root length of wild poinsettia (*Euphorbia heterophylla*), barnyard grass (*Echinochloa crus-galli*) and morning glory (*Ipomoea grandifolia*) seedlings treated with aqueous leaf extracts of donor species and herbicide at 5 and 10% compared to the control (*t*-test and Mann-Whitney). *indicates a significant difference from the control ($p < 0.05$).

The wild poinsettia seedlings exhibited root necrosis, gravitropic inversion, early secondary root and atrophy, with the exception of those treated with the *B. salicifolius* extract and herbicide at 5 and

10% (Figure 3). When treated with the herbicide, these seedlings showed root necrosis and early secondary root development and also showed atrophy when treated with the *B. salicifolius* extract. The barnyard grass seedlings displayed root necrosis and early secondary root development when subjected to the extracts; the morning glory seedlings treated with the extracts showed all these anomalies (Figure 3).

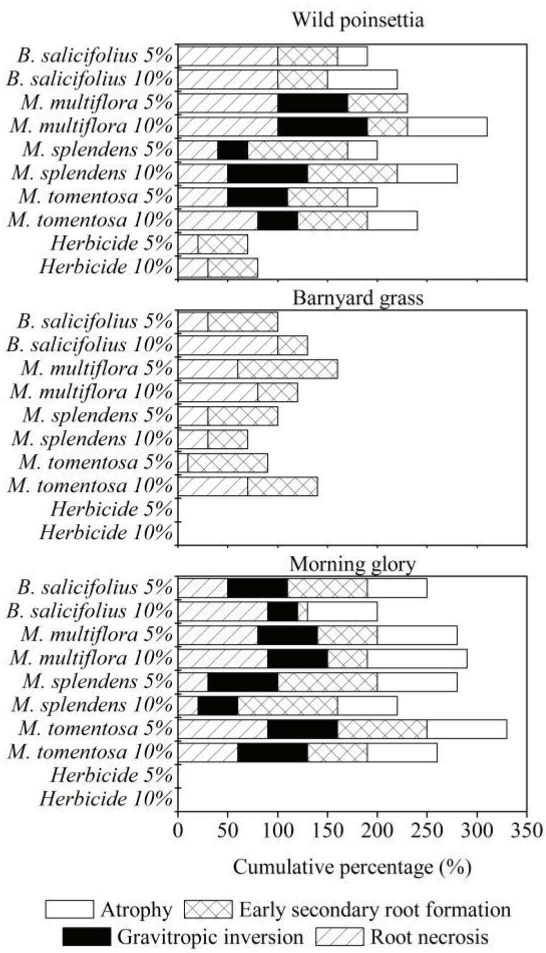

Figure 3. Cumulative percentage of anomalies induced in wild poinsettia, barnyard grass and morning glory seedlings by Myrtaceae aqueous leaf extracts and the herbicide oxyfluorfen, both at 5 and 10%.

The development and survival of plants are based on the ability to perceive and respond to environmental changes, and these responses are often modulated by hormones. Cytokinin and auxin are key hormones that regulate root development, vascular tissue differentiation and root gravitropism; together with ethylene, these hormones also regulate secondary root initiation (ALONI et al., 2006; FUKAKI; TASAKA, 2009). The cytokinins produced in the root cap are considered the primary signals for the gravitropic response, and the absence of this phytoregulator alters the normal curvature of the root. Thus, changes in the phytohormone concentrations may be responsible for the abnormal gravitropic responses reported in this study (Figure 3). In addition, this gravitropic inversion might be useful to the seedlings by delaying the contact of the secondary roots with the extract. Secondary root formation is regulated by a complex process and involves the interaction of auxin with cytokinin and ethylene. At the secondary root initiation stage, the pericycle cells are stimulated by a high auxin concentration and inhibited by elevated cytokinin and ethylene concentrations. Accordingly, the root cap necrosis in this study may have limited cytokinin production and, thus, stimulated early secondary root formation.

The inhibition of weed growth by various plant extracts is well documented (DUKE et al., 1998; OLOFSDOTTER et al., 2002; EL-ROKIEK et al., 2006, 2009, 2010), but there are no reports regarding the potential use of Myrtaceae species from the Brazilian savannah. The considerable potential and efficiency of these extracts was revealed in the present study because the extracts were as phytotoxic to the weeds as the herbicide.

Conclusion

All the aqueous leaf extracts studied were more phytotoxic to weeds that the herbicide. Thus, the aqueous extracts of Myrtaceae species leaves show potential for the isolation of active compounds that can be used for the production of natural herbicides in the future.

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