Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v35i2.16166

Phytotoxicity of leaf aqueous extract of *Rapanea umbellata* (Mart.) Mez (Primulaceae) on weeds

Paula Novaes[1*], Maristela Imatomi[2], Maria Augusta Ferraz Machado Miranda[2] and Sonia Cristina Juliano Gualtieri[2]

*[1]Grupo de Alelopatia, Departamento de Química Orgánica, Universidad de Cádiz, Av. Republica Saharauí, s/n, 11510, Puerto Real, Cádiz, España. [2]Departamento de Botânica, Universidade Federal de São Carlos, São Carlos, São Paulo, Brazil. *Author for Correspondence. E-mail: paulanovaes.botanica@gmail.com*

ABSTRACT. Allelopathic substances can be used to develop weed control alternatives based on natural products. The objective of this study was to compare the phytotoxic activity of aqueous leaf extracts of *Rapanea umbellata* with the toxicity of a synthetic herbicide on the germination and growth of weed species. The weeds species barnyard grass (*Echinochloa crus-galli*), wild poinsettia (*Euphorbia heterophylla*) and morning glory (*Ipomoea grandifolia)* were used. The effects of the aqueous leaf extract of *R. umbellata* at concentrations of 10% and 5% (g mL^{-1}) were compared to the control (distilled water) and to the synthetic herbicide oxyfluorfen. The average weed germination time was significantly lower ($p < 0.05$) in control than in extract and herbicide treatments. The herbicide had more significant effects than the extract on the initial growth of the aerial part. However, the initial growth of the root part was significantly more affected by the leaf extract than by the herbicide. The extract also caused many disorders in weed root anatomy. Therefore, the leaf aqueous extract of *R. umbellata* showed important results that indicate that it should be bioprospected and that its allelochemicals should be purified for the discovery of natural-origin herbicides.

Keywords: allelopathy, germination, growth, initial length, oxyfluorfen.

Fitotoxidade do extrato aquosos foliar de *Rapanea umbellata* (Mart.) Mez (Primulaceae) sobre plantas infestantes de culturas agrícolas

RESUMO. Substâncias alelopáticas podem ser utilizadas para o desenvolvimento de produtos alternativos baseados em compostos naturais para o controle de espécies infestantes. O objetivo deste trabalho foi comparar o efeito do extrato aquoso foliar de *Rapanea umbellata* com um herbicida sintético sobre a germinação e o crescimento inicial de espécies infestantes das culturas agrícolas. As espécies infestantes estudadas foram: capim-arroz (*Echinochloa crus-galli*), amendoim-bravo (*Euphorbia heterophylla*) e corda-de-viola (*Ipomoea grandifolia*). Os efeitos do extrato foliar aquoso de *R. umbellata* nas concentrações 10 e 5% (g mL^{-1}) foram comparados com controle (água destilada) e o herbicida sintético oxifluorfem. O tempo médio de germinação das espécies infestantes foi significativamente menor ($p < 0,05$) no controle do que em extrato foliar e o herbicida. O comprimento da parte aérea foi significativamente mais afetado pelo herbicida do que pelo extrato. Porém, o comprimento da parte radicular foi mais afetado pelo extrato do que pelo herbicida. O extrato também causou vários tipos de danos na anatomia das raízes das infestantes. O extrato foliar de *R. umbellata* apresentou resultados promissores que indicam que deve ser bioprospectado e seus aleloquímicos purificados para a descoberta de novos herbicidas de origem natural.

Palavras-chave: alelopatia, germinação, crescimento, comprimento inicial, oxifluorfem.

Introduction

Weed management has been a problem since the beginning of agriculture. In general, weeds show higher specific leaf area and are more competitive than other plant species because they are better colonizers, reproduce themselves faster and survive in more adverse situations (COLAUTTI et al., 2006; HAMILTON et al., 2005). Furthermore, they are frequently annual or biannual, smaller and more toxic to herbivorous species (SUTHERLAND, 2004). Considerable increases in weed distribution and abundance can occur because of the low regulation by natural predators, pathogens and other competing species (CALLAWAY et al., 1999) associated with monocultures such as soybean, sugar cane and rice plantations.

Conventionally, different types of synthetic herbicides are used to control weeds. Synthetic herbicides are highly effective in low concentrations, show good selective action and are relatively cheap to produce (DAYAN et al., 2009). In 2008, Brazil has become the higher consumer of pesticides in the world, and it is responsible for 84% of Latin America's utilization (IBAMA, 2010). The indiscriminate and largely inadequate use of these compounds has been intoxicating human population and contaminating biological communities (PERES; MOREIRA, 2007).

In addition, weeds have developed resistance to conventional synthetic herbicides (DUKE et al., 2000). Some of these resistances originate from their improper use (PRESTON, 2005). Therefore, the search for natural herbicides that are biodegradable and do not produce contamination as synthetic herbicides is very important (SOUZA FILHO et al., 2006). Furthermore, there is an increase in the worldwide consumption of products from 'organic agriculture', which does not allow synthetic herbicide use (DAYAN et al., 2009).

Allelopathy is considered as a phenomenon that occurs between donor and target organisms by which plants, algae, bacteria and fungi can release chemical substances (allelochemicals) into the environment, influencing the growth and development of biological systems (MALLIK, 2008; RICE, 1984). The negative influence of the allelopathy of some plants on others has been described mainly in crops (MACÍAS et al., 2001; MALLIK, 2008). The allelopathic effects of many species have been studied to control weeds (VYVYAN, 2002), and allelochemicals can be used as a resource for natural herbicide development (BAGCHI et al., 1997; MACÍAS et al., 2001, 2008). For example, acetic acid, eugenol, oleic acid, pelargonic acid (COPPING; DUKE, 2007), triketone, cinmethylin, bialaphos and glufosinate (DUKE et al., 2000), sorgoleone (EINHELLIG; SOUZA, 1992), arteether (BAGCHI et al., 1997) and heliannuols are all allelochemicals that have already been identified as potential herbicides.

The use of herbicides from allelochemicals has several advantages. They are water soluble and free from halogenated molecules, and they have alternative paths of action, specific interactions with target plants, activity at lower concentration and less damaging environmental effects compared to conventional synthetic herbicides (DUKE et al., 2000; MACÍAS et al., 2008; OLIVEROS-BASTIDAS, 2008). Allelochemicals can affect target species through their interference in respiration, photosynthesis, enzymatic activity, water relations, stomatal aperture, phytohormone levels, and mineral availability in addition to cellular elongation and division, membrane structure and the permeability of the plasmalemma and cell wall (CRUZ-ORTEGA et al., 1998; FERREIRA; AQUILA, 2000; GNIAZDOWSKA; BOGATEK, 2005).

Allelopathic research must be grounded in the evidence of allelopathy in field, the isolation and characterization of allelochemicals and their mechanisms in target organisms (INDERJIT; WESTON, 2000). Phytotoxicity bioassays can be carried out in laboratory to simulate some of the factors that could occur in the environment. The most commonly used biological assays are seed germination and seedling growth in Petri dishes lined with moist filter paper containing extracts of the plant under study at different concentrations (VYVYAN, 2002). These assays are economical and quick (DUKE et al., 2000). In this way, phytotoxicity is an important component of allelopathy studies.

Rapanea umbellata (Mart.) Mez (Primulaceae) is a species that occurs in the Cerrado (Brazilian savanna) with high abundance and distribution in São Paulo State, Brazil (WANDERLEY et al., 2005). It is an arboreal evergreen species (PINHEIRO; CARMO, 1993). Evergreen plants can present higher phenol quantities because such compounds remain in leaves for the longest possible period of time due to their elevated nutritional cost (COLEY et al., 1985). Furthermore, *R. umbellata* shows canals and small punctuations sparsely distributed in the abaxial leaf face, which could accumulate terpenes or tannins (MAUSETH, 1988).

Therefore, this work was based on the hypothesis that *R. umbellata* leaf aqueous extract should show phytotoxic activity on weeds. The aim of this study was to compare the effects of *R. umbellata* leaf aqueous extract with the effects of a synthetic herbicide on the germination and growth of weeds: wild poinsettia, barnyard grass, and morning glory.

Material and methods

Donor species, geographic and meteorological characterization of collection area

Leaves were randomly gathered from plants in the area of Cerrado (a Brazilian neotropical savanna) of Universidade Federal de São Carlos (UFSCar) São Carlos *campus*, São Carlos, São Paulo State, Brazil (21° 58' to 22° 00' S and 47° 51' to 47° 52' W). Healthy, fully expanded leaves of *Rapanea umbellata* (Mart.) Mez (Primulaceae Batsch ex Borkh.) were collected from ten mature individuals during the dry season (July 2008). The individuals of *R. umbellata* had a diameter at ground level raging from 3.0 to 15.5 cm and presented reproductive structures in the stems. The *R. umbellata* exsiccates were deposited in the Herbarium of the Department of Botany of UFSCar – Brazil (vouchers 7276).

The aforementioned Cerrado area has Red-Yellow Alic Latosol with slightly undulating topography at 845 m asl (LORANDI et al., 1999). The climate is CWA according to Köppen's classification, tropical with a dry winter (June to September) and wet summer (December to March) (TOLENTINO, 1967). The historical annual average values of rainfall, relative humidity, temperature and vapor pressure deficit are 1506 mm, 74, 21.0 and 0.72 kPa, respectively (DAMASCOS et al., 2005).

Weed species

Wild poinsettia (*Euphorbia heterophylla* L., Euphorbiaceae) and barnyard grass (*Echinochloa crus-galli* (L.) P. Beauv., Poaceae) were used in this study because they are weeds that have showed resistance to conventional herbicides (JULIANO et al., 2010; VIDAL et al., 2007). Morning glory (*Ipomoea grandifolia* (Drammer) O'Donnell, Convolvulaceae) was also used because it shows widespread infestation in Brazilian crop fields (KISSMANN; GROTH, 1992). Wild poinsettia and morning glory are eudicots native to the American continent, and barnyard grass is a monocot native from Europe and Asia that mainly affects rice plantations (KISSMANN; GROTH, 1992, 1999). Wild poinsettia diaspores were scarified in pure sulfuric acid for five seconds and then washed in running water to standardize the seed integuments (AZANIA et al., 2003).

Extract preparation

The aqueous leaf extract was prepared as described by SOUZA et al. (2010) and used in weed germination and growth bioassays. The *R. umbellata* leaves were dried in a stove (for 72h, at 40°C) and powdered in a Willey mill (Mesh 14). Aqueous leaf extract were prepared by mixing leaf powder with distilled water, at the concentrations of 10 or 5% of weight volume^{-1} (g mL^{-1}). This mixture remained for 12h inside a refrigerator at approximately 4°C. After this period, the extract was filtered through a Buckner funnel. This funnel was over a kitasato connected to a vacuum bomber.

The effects of leaf aqueous extract at 10% and 5% were compared to two control groups: one negative, containing distilled water, and one positive, containing the synthetic herbicide oxyfluorfen (240 g L^{-1}) at 10% and 5% (1.5 10^{-5} and 0.75 10^{-5} g mL^{-1}, respectively) of the manufacturer's recommendation (5 L ha^{-1}).

Weed germination bioassays

Germination bioassays were performed as described by Gatti et al. (2004), using 5 mL of leaf extract at 10 and 5%, oxyfluorfen herbicide solution at 10 and 5% of the manufacturer's recommendation and distilled water (negative control) to humidify two filter papers lining sterilized Petri dishes. This bioassay was conducted using four replicas of 20 diaspores of weeds per dish. These replicas were maintained in a germination chamber (B.O.D) with a photoperiod of 12 hours at the optimum temperature of 28ºC, confirmed by a pre-test. The count of the germinated diaspores was carried out at 24-hour intervals (FERREIRA; AQUILA, 2000). The ones that presented 2 mm of root protrusion were considered as germinated (RANAL; SANTANA, 2006). It was possible to calculate the germination percentage, average time and informational entropy based on these counts (RANAL; SANTANA, 2006).

Weed growth bioassays

The bioassays of the initial growth of aerial (AP) and root (RP) weed parts were conducted using four replicas of ten seeds or diaspores pre-germinated in distilled water (2-4 mm of radicle) per plastic box. These samples were maintained in a germination chamber (B.O.D.) with a photoperiod of 12 hours at the optimum temperature of 28ºC, confirmed by a pre-test. The boxes were lined with two filter papers humidified with 20 ml of leaf extract and oxyfluorfen herbicide at 10 and 5% and with distilled water (negative control). The initial growth measures were carried out after five days of incubation. The aerial part (AP) of the seedlings (mm) was considered as the distance from the lap up to the steam meristem apex, and the root part (RP) (mm) was considered as the distance from the lap up to the main root meristem apex (BRASIL, 2009).

Physical-chemical extract characteristics

The pH of the leaf extract at 10 and 5% and of oxyfluorfen herbicide solution at 10 and 5% of the manufacturer's recommendation were measured using a pH meter. The osmotic potential (OP, mOsm Kg^{-1}) was measured by an automatic osmometer (μOsmotte, modelo 5004), using 50 μL of each concentration of the extract and the herbicide, at 25°C. The values of the OP were converted to osmotic pressure by the conversion of mOsm Kg^{-1} to MPa (LARCHER, 2000). The effects of the OP on the weed germination percentage, average time, informational entropy and growth were tested using polietilenoglicol 6000 (PEG-6000) at a concentration of -0.1, -0.2 and -0.3 MPa, and using distilled water as a control.

Statistical analyses

The experimental design in the laboratory was completely randomized. In addition to the negative control group, two handlings (leaf aqueous extract and oxyfluorfen herbicide use) were divided into two treatment levels (two concentrations each, 10 and 5%). Four replicas were used for the control group and treatments.

The statistical tests were performed using the free software Bioestat 5.0. The data normality was analyzed by the Lilliefors test. Significant differences among the average values for the negative control and the same concentrations of the leaf aqueous extract and oxyfluorfen herbicide were pair evaluated by the Mann-Whitney test (when the average values were not normally distributed) and by the T test (when the average values were normally distributed), with the decision level at $p < 0.05$ (ZAR, 2010).

Results and discussion

Extract and herbicide physical-chemical characteristics

The pH values of *R. umbellata* leaf extract at 10 and 5% were 5.41 and 5.6, respectively. The pH of the oxyfluorfen herbicide at both concentrations was 5.4. All pH values were inside the limits well tolerated by vascular plants (LARCHER, 2000) and, therefore, bioassays of the pH influence of the extract and the herbicide on the weeds were dismissed. The OP values of the leaf extract at 10 and 5% were -0.21 and -0.10 MPa, respectively. The oxyfluorfen herbicide at both concentrations showed very low OP values and they could not be measured. There was no effect of PEG at -0.1 and -0.2 on the germination percentage and average time, and weeds initial growth. These results agree with Grisi et al. (2011), who also tested the effect of these values of PEG on barnyard grass morning-glory, *Lactuca sativa* (lettuce) and *Allium cepa* (onion).

Germination

There was no significant difference ($p > 0.05$) between the control and the leaf aqueous extract and oxyfluorfen herbicide on the germination percentage of the weeds (Figure 1A, C and E). There was also no influence of the extract or herbicide on wild poinsettia germination average time (Figure 1B).

The barnyard grass and morning glory germination average time were significantly lower ($p < 0.05$) in the control group than in the extract and herbicide at both concentrations (Figure 1D and F). The extract and the herbicide significantly increased the barnyard grass germination average time (Figure 1D). The morning glory average time was significantly higher with the use of the extract than with the herbicide, at both studied concentrations (Figure 1F).

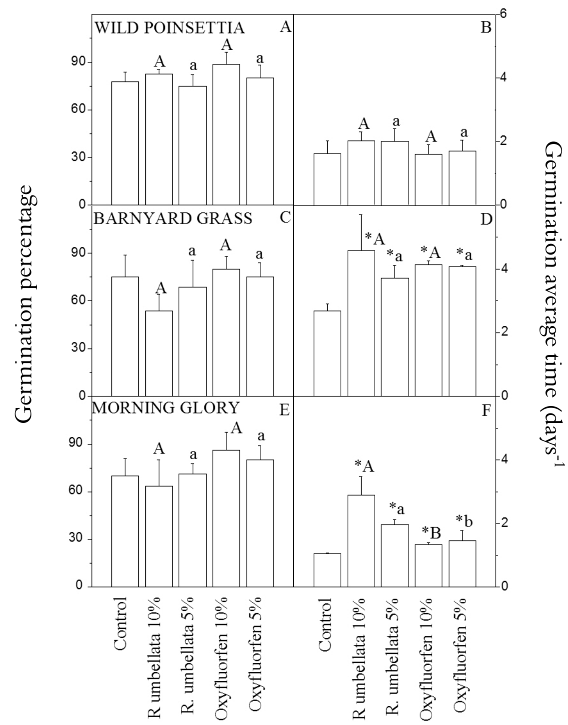

Figure 1. Average values (columns) and standard deviation (bars) of germination percentage and germination average time of wild poinsettia (*Euphorbia heterophylla*), barnyard grass (*Echinochloa crus-galli*), and morning glory (*Ipomoea grandifolia*) in distilled water (control), *Rapanea umbellata* leaf aqueous extract and oxyfluorfen synthetic herbicide at concentrations of 10% and 5%. Asterisks over columns indicate a significant difference from the control ($p < 0.05$); lower case indicates a significant difference between the extract and the herbicide at 10%; and capital letters indicate a significant difference between the extract and the herbicide at 5%.

The wild poinsettia germination informational entropy did not show a significant difference between values coming from the control and from the extract or herbicide concentration values (Figure 2A). Only the concentrations of the herbicide significantly reduced barnyard grass's germination informational entropy. The barnyard grass diaspores germinated synchronously (approximately 4 days), and therefore, they showed reduced germination informational entropy despite the increase in germination average time.

The morning glory germination informational entropy was significantly higher when seeds were submitted to the extract and herbicide than to the control (Figure 2C), but the extract at 10% yielded values significantly higher than the herbicide.

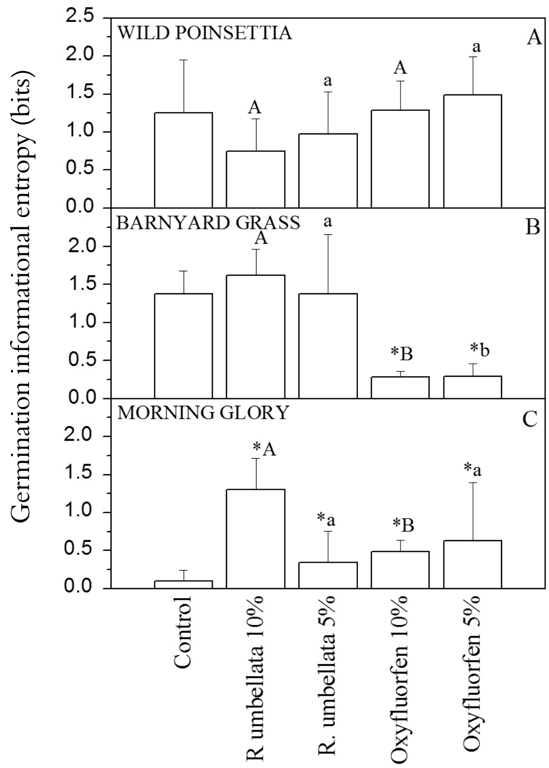

Figure 2. Average values (columns) and standard deviation (bars) of the germination informational entropy of wild poinsettia (*Euphorbia heterophylla*), barnyard grass (*Echinochloa crus-galli*), and morning glory (*Ipomoea grandifolia* Drammer) in distilled water (control), Rapanea umbellata aqueous leaf extract and oxyfluorfen synthetic herbicide at concentrations of 10% and 5%. Asterisks over columns indicate a significant difference from the control ($p < 0.05$); lower case letters indicate a significant difference between the extract and the herbicide at 10%; and capital letters indicate a significant difference between the extract and the herbicide at 5%.

The germination process of wild poinsettia was the least affected by the extract and herbicide among the weeds, as its values of germination percentage, average time and informational entropy were not altered by these solutions. Wild poinsettia is a weed that produces seeds in large quantities, with elevated viability (KISSMANN; GROTH, 1992). It shows great capacity for multiplication, grows rapidly and strongly competes for soil nutrients. For these reasons, it spreads across the subtropical areas of different continents (KISSMANN; GROTH, 1992).

Kern et al. (2009) affirmed that wild poinsettia seeds show great resistance to known allelochemicals such as rutin, quercetin, aconitic acid, ferulic acid, coumaric acid, vanillic acid and eucaliptol. Wild poinsettia was the less affected weed by coumarin among other species such as morning glory, *Bidens pilosa* L. and *Senna obtusifolia* L., even at elevated concentrations (PERGO et al., 2008). This result shows that wild poinsettia is an aggressive weed, making its control difficult even in the presence of solutions of known toxicity.

The germination average time and, consequently, the informational entropy are important factors in weed survival, as plants that germinate slowly could show reduced height, lower competition capacity and a lower chance of establishment in the environment (CALLAWAY; WALKER, 1997). The extract inhibited morning glory germination processes, the germination average time and informational entropy, more strongly than the herbicide. These results reveal the high concentration or toxicity of the allelochemicals present in *R. umbellata* leaves. The allelochemicals certainly show modes of action that are new to morning glory seeds (MACÍAS et al., 2008), giving more effective results than the herbicide. The allelochemicals could have affected the morning glory germination process. For example, there could be an increase in amylase activity and a greater release of reserves that should be provided to the embryo, extension of oxidative stress and seed dormancy through the increase of ABA production, and inhibition of water absorption via alterations in the membrane permeability (SINGH et al., 2009).

Weed growth

The wild poinsettia showed significantly lower initial growth of both AP and RP in extract or herbicide than in the control (Figure 3A). The 10 and 5% concentrations of the herbicide were significantly more active on the AP than those of the extract. On the other hand, these concentrations of extract were significantly more active on the RP than the herbicide.

Only the herbicide had a significant effect on wild poinsettia AP growth (Figure 3B). The values of the RP were significantly different from the control at both concentrations of the extract and herbicide, but the effect of the extract at 10% was significantly higher than that of the herbicide.

The leaf extract and the herbicide yielded morning glory AP and RP growth significantly lower than for the control group (Figure 3C). However, the herbicide concentration of 5% was significantly more active on the AP than the extract. The extract at 10% was significantly stronger in RP than the herbicide.

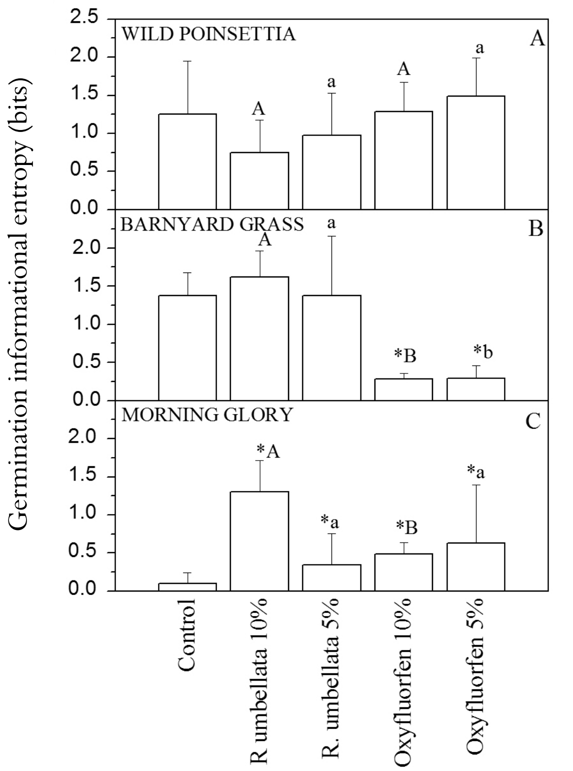

Figure 3. Average values (columns) and standard deviation (bars) of initial growth (mm) of the aerial (AP) and root parts (RP) of wild poinsettia (*Euphorbia heterophyll*), barnyard grass (*Echinochloa crus-galli*), a lnd morning glory (*Ipomoea grandifolia*) in distilled water (control), *Rapanea umbellata* aqueous leaf extract and oxyfluorfen synthetic herbicide at concentrations of 10 and 5%. Asterisks over columns indicate a significant difference from the control ($p < 0.05$); lower case letters indicate a significant difference between the extract and the herbicide at 10%; and capital letters indicate a significant difference between the extract and the herbicide at 5%.

In general, the weed AP growth was more affected by the herbicide than by the extract. Oxyfluorfen is a synthetic herbicide indicated for weed control, mono and eudicotyledonous, during pre-emergence and initial post-emergence. Chemically, oxyfluorfen is an herbicide of selective and non-systemic action that belongs to the diphenyl esthers (DPEs) group. This group needs light to be active, which suggests that its phytoxicity is related to the photosynthesis process (HESS, 2000). There are evidences that show that the DPEs are connected to the photoporphyrinogen oxidase enzyme during the chlorophyll biosynthesis, and they may destroy plant cell membranes as a result of the peroxidation of polyunsaturated fatty acid (HESS, 2000). These factors may explain the higher activity of the herbicide on the initial growth of AP in weeds.

The initial growth of the weed RP was more affected by the extract than by the herbicide. This result suggests that the substances present in the extract do not have the same mode of action as the herbicide or its chemical group. The allelochemicals in the extract may be more associated with the processes of root growth, such as cellular division, hormone production, membrane permeability, mineral absorption, enzymatic activity or water relations (FERREIRA; AQUILA, 2000; GNIAZDOWSKA; BOGATEK, 2005).

Cruz-Ortega et al. (1998), for example, observed that the aqueous extracts of *Sicyos deppei* yield tissue disorganization and a lack of cellular differentiation in bean root, evidenced by ultra-structure alterations in the plasmatic membrane, cell wall, mitochondria, endoplasmic reticulum and cell division. Singh et al. (2009) observed a reduction in protein content and in the activity of reductase nitrate in corn root tissues exposed to *Nicotiana plumbaginifolia* leachates. Modifications in mitochondrial respiration that provoke decreases in ATP liberation to cells and ion uptake by roots are other possible explanations for the reduction of growth by allelopathic extracts (GNIAZDOWSKA; BOGATEK, 2005). These activities may explain the interference of *R. umbellata* extract on initial weed RP growth.

Aside from the slower RP growth, the concentrations of the extract caused significantly higher necrosis of the main root tip and early secondary root formation than the control in all studied weeds (Figure 4). Gravitropic plant inversion occurred significantly more often than for the control in wild poinsettia exposed to the extract at 10% and in morning glory exposed to both concentrations. There was a significant occurrence of root atrophy in morning glory when the extract was used at 10%.

The auxin transport from weed AP to RP might remain high, as there was no damage in the AP when the plants were exposed to the extracts. When auxins are present in the root, they act hand in hand with columel statoliths (which are sensitive to gravity), leading to positive geotropic growth (ALONI et al., 2006; MORITA, 2010). However, disordered root growth and negative gravitropism should occur with the necrosis of the root cap and, consequently, in statoliths (BURGOS et al., 2004; TANIMOTO et al., 2008).

Cytokinins are produced in the cap of the main root and, like the auxins, they positively control the root geotropic curvature (MIYAWAKI et al., 2004). With root cap necrosis, the levels of cytokinin will be reduced, and there will be no induction of negative root gravitropism (ALONI et al., 2006). Gravitropic inversion is useful to seedlings in this situation because it could delay the contact of newly forming secondary roots with the extract.

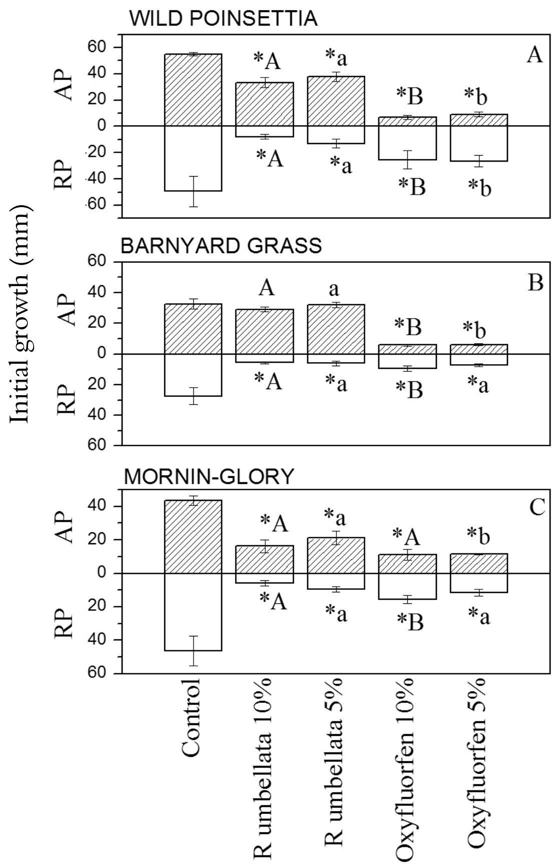

Figure 4. Root anomalies observed in seedlings of wild poinsettia (*Euphorbia heterophylla*), barnyard grass (*Echinochloa crus-galli*), and morning glory (*Ipomoea grandifolia*) in distilled water (control) and in *Rapanea umbellata* aqueous leaf extract. Asterisks over columns indicate a significant difference from the control ($p < 0.05$).

Secondary root formation is regulated by different hormones, such as auxin, ethylene, and cytokinin. Elevated levels of auxins should increase the ethylene production in weeds, as they increase the ACC synthetase activity (KONDO et al., 2009; STEPANOVA et al., 2007). In addition, the stress induced by the extract in the weed RP could have prompted an increase in ethylene and a reduction in cytokinins (ZHANG et al., 2003). Reduced levels of cytokinins and elevated levels of ethylene and auxin could have stimulated the early secondary root formation (ALONI et al., 2006; DAYAN et al., 2000).

Modes of actions different from conventional herbicides are desirable characteristics for a plant extract to be used in further studies of natural herbicides. The aqueous leaf extract of *R. umbellata* showed a different mode of action from the oxyfluorfen herbicide on morning glory germination and on the initial RP growth of all studied weeds. Therefore, it has the potential to be used in biodirected fractionation studies so that the allelochemicals present in *R. umbellata* leaves could be synthesized as natural-origin, biodegradable herbicides.

Conclusion

R. umbellata leaf aqueous extract was more active than the herbicide oxyfluorfen on germination average time and informational entropy of wild poinsettia and barnyard grass, but the effect is most clearly evident in morning glory. The growth of aerial weed part was more affected by the herbicide than by the extract. However, the growth of root part was more inhibited by the extract, showing root cap necrosis, early secondary root formation, gravitropic inversion and atrophy in all weeds. Therefore, the results indicate that the extract of *R. umbellata* should be bioprospected and its allelochemicals be purified to discover natural-origin herbicides.

Acknowledgements

The authors are thankful to Dr. Luis Carlos Bernacci, a researcher of IAC (Instituto Agronômico de Campinas, Brazil), for the identification of plant material. This work was supported by the Brazilian agencies CNPq (Conselho Nacional de Pesquisa, Brazil) for the doctorate and research productivity scholarships awarded to Paula Novaes and Sonia Cristina Juliano Gualtieri, respectively, and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível superior, Brazil) for the financial support.

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Received on February 27, 2012.
Accepted on May 13, 2012.