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Allelopathic potential of bark and leaves of *Esenbeckia leiocarpa* Engl. (Rutaceae)

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ABSTRACT – (Allelopathic potential of bark and leaves of *Esenbeckia leiocarpa* Engl. (Rutaceae)). We investigated the inhibitory potential of aqueous extracts of bark and leaves of *Esenbeckia leiocarpa* Engl. on lettuce germination and early seedling growth. We compared the effects of four concentrations (100, 75, 50 and 25%) of each extract to water and polyethylene glycol (PEG 6000) solution controls for four replicates of 50 seeds for germination and four replicates of ten seedlings for seedling growth. The inhibitory effects of *E. leiocarpa* extracts on the percentage of germination and on the germination speed seemed to be more than simply an osmotic effect, except for the percentage of seeds germinated in bark extracts. When compared to water control, both bark and leaf extracts delayed germination, and leaf extracts also affected the percentage of germinated seeds. Leaf extracts of all concentrations strongly inhibited the development of seedlings and caused them some degree of abnormality; bark extracts also caused abnormalities and reduced seedling growth. Root development was more sensitive to the extracts than hypocotyl growth. The negative effects of leaf extracts on germination and seedling growth were more pronounced than those of bark extracts, and the overall effects of both extracts were positively correlated with extract concentrations.
Key words: allelopathy, germination, growth, inhibition, plant-plant interaction

RESUMO – (Potencial alelopático de folhas e cascas de *Esenbeckia leiocarpa* Engl. (Rutaceae)). Neste trabalho, nós investigamos o potencial inibitório de extratos aquosos de folhas e cascas de *Esenbeckia leiocarpa* Engl. na germinação e no crescimento inicial de plântulas de alface. Nós comparamos os efeitos de quarto concentrações (100, 75, 50 e 25%) de cada extrato a um controle em água e outro em uma solução de polietilenoglicol (PEG 6000), com quatro repetições de 50 sementes para o experimento de germinação e quatro repetições de 10 plântulas para o experimento de crescimento. Os efeitos inibitórios dos extratos de *E. leiocarpa* na porcentagem e na velocidade de germinação foram mais do que um efeito do potencial osmótico das soluções, exceto para a porcentagem de sementes germinadas nos extratos de casca. Ambos os extratos causaram atrasos na germinação, sendo que os extratos de folha afetaram também a porcentagem de sementes germinadas. Os extratos de folha, em todas as concentrações, inibiram fortemente o desenvolvimento das plântulas e causaram a todas elas algum grau de anormalidade; os extratos de casca também causaram anormalidades e reduziram o crescimento das plântulas. O desenvolvimento da radícula foi mais sensível à ação dos extratos do que o crescimento do hipocótilo. Os efeitos negativos dos extratos de folhas foram mais pronunciados do que os causados pelos extratos de casca e os efeitos de ambos os extratos foram sempre positivamente correlacionados com sua concentração.
Palavras-chave: alelopatia, crescimento, germinação, inibição, interação planta-panta

Introduction

Tropical forests are known for their high diversity, and the countless interactions of all organisms with biotic and abiotic factors in these forests still make the understanding of the processes responsible for community organization a complex and intriguing subject.

Among the factors that determine community structure and dynamics are the positive and negative plant-plant interactions (Callaway & Walker 1997; Lortie *et al.* 2004; Michalet *et al.* 2006). One of the several ways by which plants can interact is through the release of secondary metabolites in the environment, which may cause direct or indirect interference of one plant on another, by a process called allelopathy (Rice 1984). Although most commonly reported as having a negative effect, the allelopathic process can also result in benefits to the organism that receives the allelochemical (e.g., Parvez *et al.* 2004; Orr *et al.* 2005; Dorning & Cipollini 2006).

In plants, allelopathy is often related to harmful effects on germination and seedling growth (Kato-Noguchi 2003; Oliveira *et al.* 2004; Herranz *et al.* 2006; Cavieres *et al.* 2007; Zhang *et al.* 2007). Some authors suggest that allelopathic effects can also contribute to promote shifts in density, dominance and spatial patterns of plant populations (Rice 1984; Wardle *et al.* 1998; Chou 1999; Ridenour & Callaway 2001). Thus, allelopathic plants may have a differential role in species coexistence (Inderjit & Callaway 2003) and in forest succession (Peng *et al.* 2004). Despite its importance, allelopathy is a poorly studied mechanism among tropical tree species, and the relevance of this process has probably been underestimated in the theories about the driving forces of ecological processes in tropical forests.

Evidence of allelopathy can be obtained by observing spatial patterns in the field (Inderjit & Callaway 2003). In some cases, there are zones of growth inhibition beneath or around the canopy of an allelopathic plant, where individuals or species are suppressed (see examples in Rice 1984, Wardle *et al.* 1998; Inderjit & Callaway 2003). Moreover, the changes in the environment and in the neighbouring vegetation caused by an allelopathic plant can favour the establishment of conspecifics (Wardle *et al.* 1998).

Esenbeckia leiocarpa Engl. is a tropical tree species that occurs in Seasonal Semideciduous Forests. It is a

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deciduous and autochorous tree (Morellato 1991) and reaches up to 18 m in height (Pirani & Skorupa 2002). In addition to the fact that this species is frequently found in clusters (Seoane *et al.* 2000), adult individuals (diameter at breast height - DBH ≥ 4.8 cm) of *E. leiocarpa* were found to be more abundant under the canopies of individuals of *E. leiocarpa* than under eight other canopy species (F. M. Souza *et al.*, unpublished data). These data suggest that *E. leiocarpa* may inhibit other plant species or facilitate the establishment of their own individuals. The presence of compounds with allelopathic potential in roots and leaves of this species (Delle Monache *et al.* 1989; Delle Monache *et al.* 1990; Nakatsu *et al.* 1990; Michael 1993), as well as the existence of phytotoxic substances in another species of the genus *Esenbeckia* (Mata *et al.* 1998) strengthen the idea that *E. leiocarpa* may be allelopathic.

In this study, we carried out laboratory experiments to investigate the effects of aqueous extracts of bark and leaves of *E. leiocarpa* on seed germination and early seedling growth of the test-plant *Lactuca sativa* L. as evidence of allelopathy. Despite the known limitations of laboratory experiments to show the occurrence of allelopathy under natural conditions (Stowe 1979; Inderjit & Callaway 2003), they are very useful as an initial investigation of allelopathy, in addition to having the advantages of low cost, rapid execution and easy replication (Leather & Einhellig 1986; Weidenhamer *et al.* 1989; Inderjit & Weston 2000). We also discuss the ecological implications of the results for the vegetation patterning of Tropical Seasonal Semideciduous Forests.

Material and methods

Aqueous extracts - At the beginning of the rainy season (November 2004), we collected mature healthy leaves and bark of ten individuals of Esenbeckia leiocarpa at Caetetus Ecological Station, a Seasonal Semideciduous Forest Reserve located in Central Western São Paulo state, Brazil (22°41'S; 49°10'W). The collected material was frozen in a domestic refrigerator (-10 °C) until use.

The collected material was dried in an oven at 80°C until reaching a constant weight and then ground in a blender in a proportion of 7.1% (w/v) for each material (50 g of dried leaves in 700 mL of distilled water and 15 g of dried bark in 210 mL of distilled water). After that, the extracts were centrifuged for three minutes, collected by decantation and then vacuum-filtered through one layer of filter paper to obtain the most concentrated extract (100%) of each material. We diluted these solutions in distilled water to achieve 75 (~5.3% w/v), 50 (~3.5% w/v) and 25% (~1.8% w/v) concentrations and stored the extracts at -10°C until use.

Germination – We evenly placed 50 seeds of lettuce (Lactuca sativa L. var. Grand Rapids) in 9 cm-diameter Petri dishes lined with two layers of previously autoclaved filter paper and moistened with 5 mL of each extract (n = four replicates). We then placed the Petri-dishes in a chamber with an exhaust fan for five hours and remoistened them with 5 mL of distilled water.

As controls, we used distilled water and a Polyethylene glycol 6000 (PEG 6000) solution. This procedure was carried out to assess the influence of the osmotic potential of the extracts on seed germination. The osmotic potential of the most concentrated (100%) extract of each material was first measured using a FISKE OS Osmometer, with samples of 250 µL of each extract previously defrosted at room temperature (bark extract = - 0.06 MPa; leaf extract = - 0.31 MPa). The recommended amount of PEG (Villela *et al.* 1991) was then diluted in distilled water and shaken for 24 hours to obtain PEG solutions with the same osmotic potential of each 100% extract. The Petri dishes were sealed with parafilm and placed randomly in a growth chamber (FANEM 347) in the dark at 26-28°C. The germinated seeds (2 mm of radicle protrusion) were counted and removed every 12 hours up to the seventh day and the percentage of germination and germination speed were calculated according to the formulae presented by Labouriau (1983).

Growth – Lettuce seeds of 4-6 mm radicle length that had been previously germinated for 27 hours in distilled water (germination procedures were the same as those described in the previous item, except for the 12-hour photoperiod) were transferred to plastic boxes lined with two layers of filter paper. Ten germinated seeds were evenly distributed in each box (n = four replicates), and the filter paper was moistened with 12 mL of each extract or distilled water as a control. The plastic boxes were sealed with transparent thick plastic bags with sparse holes and placed randomly in a growth chamber (FANEM 347) at 26-28°C under a 12-hour photoperiod. After seven days, the seedlings were classified as normal or abnormal, according to Brasil (1992), and the hypocotyls and the roots of the normal seedlings were measured with a digital caliper.

Data analysis - The percentage of germination and the germination speed of bark and leaf 100% extracts were compared to their equivalent PEG solutions with a t test, assuming unequal variances. In cases of significant results, an analysis of variance (ANOVA) was used followed by a Tukey test to look for differences among all treatments (the four concentrations of bark and leaf extracts and water control). This analysis was also performed to compare the proportion of abnormal seedlings among treatments. Differences in the root and hypocotyl length of normal seedlings were analysed by a Kruskal-Wallis test, followed by the Dunn test for multiple comparisons, since the variances of those variables were not homogeneous (Zar 1999). When the variables presented only one observation, the single value was compared to the 95% confidence interval for the mean of the control. Linear regression was used to evaluate the dependence of each variable (percentage of germination, germination speed, hypocotyl and root length) on the concentration of the extracts. Statistical tests were performed with 5% of significance.

All the proportions were transformed using a slight modification of the Freeman and Tukey transformation, since it provides better results for small and large proportions than the most commonly used formulae (Zar 1999).

Results

Germination –The lettuce seeds germinated slower in the 100% bark extract than in the equivalent PEG solution (t = - 5.02; d.f. = 3.58; p = 0.01; Tab. 1). Since the percentage of germinated seeds did not differ between this extract and the PEG (t = - 1.33; d.f. = 4.98; p = 0.24; Tab. 1), this variable was not compared among the four concentrations and the water control. The germination of the seeds in the 100% leaf extract was close to zero, with only two seeds germinated in one of the replicates, differing from the equivalent PEG solution (t = - 11.71; d.f. = 3.80; p < 0.001; Tab. 1). The germination speed in this single replicate (0.18 days^{-1}) was outside the confidence limit of the PEG solution (lower limit = 0.63 and upper limit = 0.92 days^{-1}), indicating that the germination speed in the bark extract was lower than in the PEG solution, allowing the continuation of the subsequent analysis.

The number of germinated seeds in the leaf extracts was lower than those in the water control (F = 153.58; d.f. = 4; p < 0.001; Fig. 1) and inversely proportional to the extract concentrations (R^2 = 0.98; d.f. = 18; p < 0.001; Fig. 1). The germination speeds of the seeds in the 75, 50 and 25% leaf extracts and in the 100 and 75% bark extracts were lower than those observed in the water control (F = 87.50; d.f. = 7; p < 0.001; Fig. 2); the value recorded in the 100% leaf extract (0.18 days^{-1}) was lower than the lower limits of the confidence

Table 1. Percentage and germination speed of lettuce (*Lactuca sativa* L.) seeds in the most concentrated (100%) aqueous extracts of *Esenbeckia leiocarpa* Engl. barks and leaves and in the PEG (polyethylene glycol 6000) solutions with osmotic potential equivalent to the extracts (mean ± SE).

Treatment	Germination (%)	Germination speed ($days^{-1}$)
Leaf 100%	0.5 ± 0.5 [a]	0.18 [a]
PEG (- 0.28 MPa)	65.0 ± 6.6 [b]	0.78 ± 0.05 [b]
Bark 100%	75.5 ± 4.6 [a]	0.65 ± 0.01 [a]
PEG (- 0.12 MPa)	85.0 ± 6.1 [a]	0.86 ± 0.04 [b]

Different superscript letters within each column indicate that the means were significantly different ($p < 0.05$) between the extract and its corresponding PEG solution.

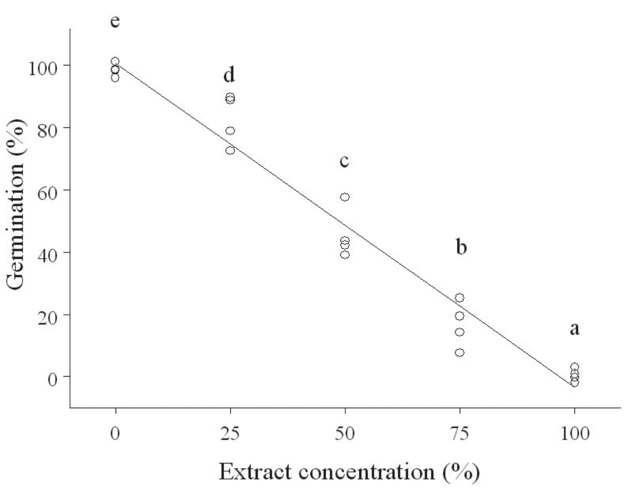

Figure 1. Percentage of germination of lettuce (*Lactuca sativa* L.) seeds in different concentrations of leaf aqueous extracts of *Esenbeckia leiocarpa* Engl. (0 = water control; $R^2 = 0.98$; $p < 0.001$). Different letters indicate significant differences among concentrations by Tukey test ($p < 0.05$).

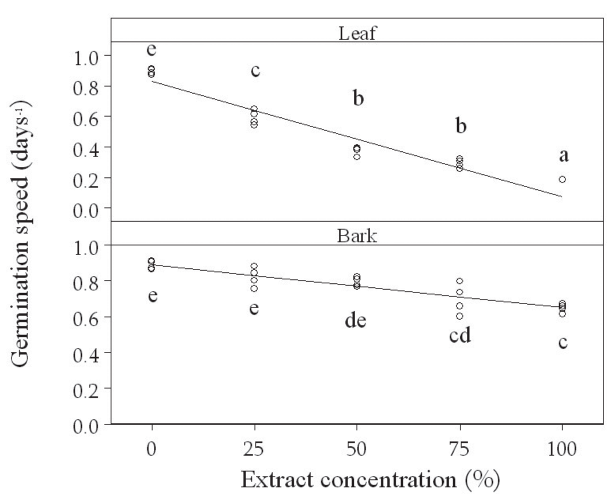

Figure 2. Germination speed of *Lactuca sativa* L. seeds in different concentrations of aqueous extracts of bark and leaves of *Esenbeckia leiocarpa* Engl. (0 = water control; bark: $R^2 = 0.78$; $p < 0.001$; leaf: $R^2 = 0.93$; $p < 0.001$). Different letters indicate significant differences among treatments ($p < 0.05$).

intervals of all other treatments (leaf 75% = 0.25; leaf 50% = 0.34; leaf 25% = 0.51; bark 100% = 0.61; bark 75% = 0.57; bark 50% = 0.75; bark 25% = 0.73; water control = 0.87). The germination speed was also inversely proportional to both leaf ($R^2 = 0.93$; d.f. = 15; $p < 0.001$; Fig. 2) and bark ($R^2 = 0.78$; d.f. = 18; $p < 0.001$; Fig. 2) extract concentrations.

The reduction in the percentage of germination and in the germination speed ranged from approximately 15% (25% leaf extract) to almost 100% (100% leaf extract), and from approximately 20% (75% bark extract) to 80% (100% leaf extract), respectively. The leaf extracts affected the germination speed more than the bark extracts in all concentrations (Fig. 2). The differences between the materials were more pronounced between the 100% extracts and diminished as the concentration decreased (Fig. 2).

Growth – Both leaf and bark extracts induced the occurrence of abnormalities in the seedlings. The most frequent problems were the weak development and the disproportion between the root and the hypocotyl. In most cases, roots were more damaged than the aerial part of the seedlings. In the former, the most common abnormalities were the oxidation of the cap, longitudinal cracks and negative geotropism (Fig. 3); in the latter, the most common problems were twists and small cotyledonary necrosis (Fig. 3).

All of the seedlings grown in the leaf extracts showed some degree of abnormality. The proportion of abnormal seedlings grown in the leaf extracts was significantly higher than in the control for all concentrations, whereas only the proportion of abnormal seedlings grown in the 100 and 75% bark extracts were higher than in the control ($F = 15.38$; d.f. = 8; $p < 0.001$; Tab. 2). The proportion of abnormal seedlings grown in the bark extracts was positively related to the concentration ($R^2 = 0.77$; d.f. = 17; $p < 0.001$; Tab. 2).

Since all of the seedlings grown in the leaf extracts were abnormal, we analysed the root and hypocotyl length of the seedlings grown only in bark extracts. In the 100% extract, only one out of the 40 seedlings was normal. The root length of this single seedling (10.9 mm) was comprised of the confidence limits of the 75% bark extract (lower limit = 9.3; upper limit = 16.6), but was lower than the lower limit of the confidence interval of all the other treatments (50% bark = 16.2; 25% bark = 27.3; water = 40.9). The hypocotyl length of this seedling (3.6 mm) was lower than the lower limit of the confidence interval of all the other concentrations and the water control (75% bark = 4.1; 50% bark = 7.4; 25% bark = 7.1; water = 8.2).

The root length of the normal seedlings grown in other concentrations also differed from the control (Kruskal-Wallis $H = 85.48$; d.f. = 3; $p < 0.001$; Tab. 2) and was inversely proportional to the extract concentration ($R^2 = 0.69$; d.f. = 111; $p < 0.001$; Tab. 2; Fig. 4). Mean root length was reduced by at least 34% (in the slightest concentrated extract) and by up to 75% (in the 100% extract). Hypocotyls of seedlings grown in 75% extracts differed from the control (Kruskal-Wallis $H = 13.87$; d.f. = 3; $p = 0.003$; Tab. 2; Fig. 4); the reduction in mean hypocotyl length ranged from 40% (in the 75%) to 59% (in the 100% extract).

Table 2. Percentage of abnormal seedlings, root length and hypocotyl length of normal seedlings of lettuce (*Lactuca sativa* L.) grown in aqueous extracts of barks of *Esenbeckia leiocarpa* Engl. (mean ± SE; concentration "0" = water control).

Extract concentration (%)	Abnormal seedlings (%)	Root length (mm)	Hypocotyl length (mm)
100	96.7 ± 3.3 [b]	10.9 [a]	3.6 [a]
75	82.5 ± 8.5 [b]	12.9 ± 1.5 [ab]	5.4 ± 0.5 [b]
50	32.5 ± 22.9 [a]	17.0 ± 0.4 [b]	8.2 ± 0.4 [c]
25	5.0 ± 2.9 [a]	29.3 ± 1.0 [c]	8.1 ± 0.5 [c]
0	0 [a]	44.3 ± 1.7 [d]	8.8 ± 0.3 [c]

Different superscript letters within each column indicate that the means were significantly different ($p < 0.05$) among extract concentrations.

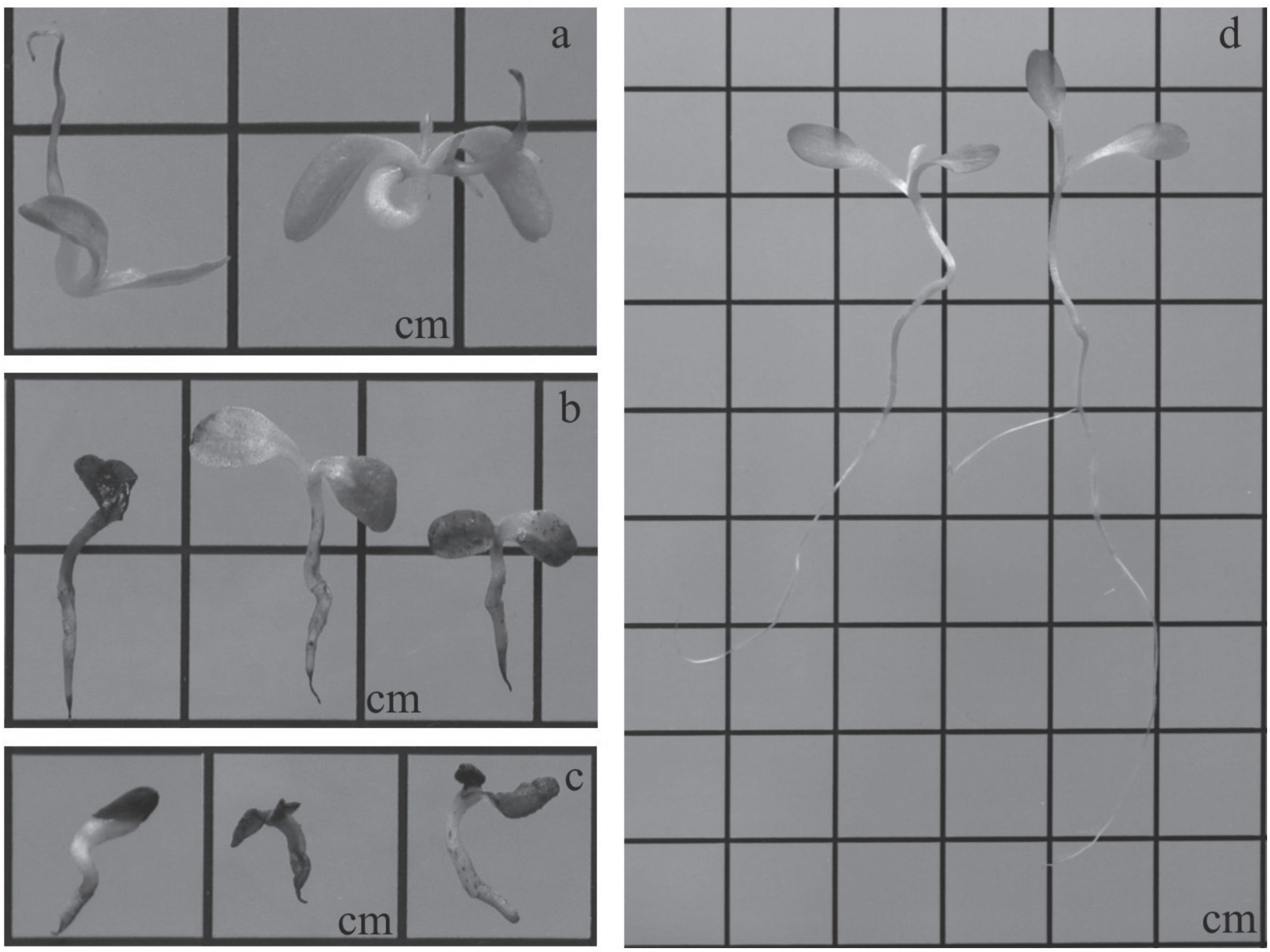

Figure 3. Abnormalities of *Lactuca sativa* L. seedlings grown in aqueous extracts of bark and leaves of *Esenbeckia leiocarpa* Engl. – (a) negative geotropism and twisted hypocotyl (75% bark extract); (b) cotyledonary necrosis, root longitudinal cracks and oxidation of the root cap (75% leaf extract); (c) rottenness and weak development (100% leaf extract); (d) normal seedlings (water control).

Discussion

The differences in the percentages of germination and germination speed between the 100% leaf extract and PEG 6000 suggest that the inhibition of germination was not only an effect of the osmotic potential. The same can be said for the germination speed in the bark extract, but not for the percentage of germination, in which the reduction seems to be exclusively an osmotic effect.

Both leaf and bark extracts were phytotoxic and potentially inhibitors of seed germination, they also induced abnormalities in seedling structures and reduced the growth of normal seedlings. The germination was delayed rather than reduced; seedling development was more sensitive to the extracts than germination, and root elongation was even more affected than that of the hypocotyl. In general, the negative effects increased with the concentration of the extracts. This pattern of

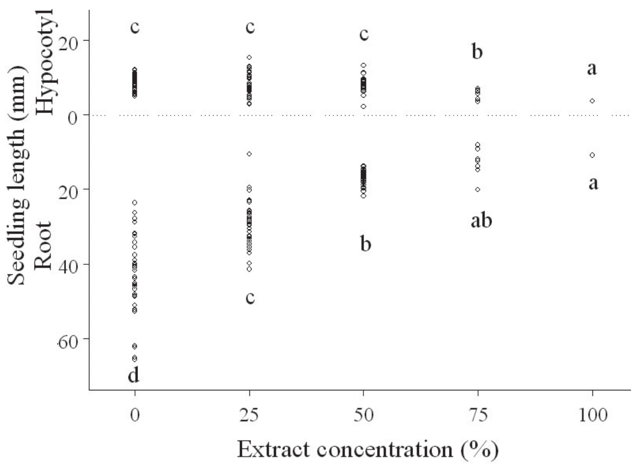

Figure 4. Root and hypocotyl length of normal seedlings of *Lactuca sativa* L. grown in aqueous extracts of bark of *Esenbeckia leiocarpa* Engl. (0 = water control; $R^2 = 0.69$; $p < 0.001$). Different letters within each seedling structure indicate that the means differed significantly among concentrations.

negative effects (effects on speed > percentage; growth > germination; radicle > hypocotyl) is the same as those reported by several studies concerning other plant species around the world (*e.g.*, Kato-Nogushi 2003, Dorning & Cipollini 2006; Zhang *et al.* 2007).

Leaf extracts affected both germination and seedling development more strongly than bark extracts and caused damage that seemed severe enough to prevent seedling establishment, even at the lowest concentrations. Injuries resulting from bark extracts were less intense and occurred mostly at the highest concentrations. However, the hypothesis that the reduction of root growth caused by the bark extracts may also be sufficient to hinder the future seedling development should not be dismissed.

Studies regarding the consequences of alterations in germination process on the performance of individuals in natural forests are still lacking, which limits our discussion about the ecological implications of our findings. We hypothesize that the slow seed germination and the low germinability could affect the uptake of vital resources, such as light, water and nutrients. Similarly, root and shoot damages could probably delay or even prevent seedling development, which in turn would increase seedling vulnerability and competitive ability, decreasing the chances of a seedling to survive, grow and reach maturity.

However, to confirm the occurrence of the allelopathic activity, it is necessary to verify whether, in natural conditions, the compounds are released and accumulated in the environment at levels which could actually affect the individuals of the community (Putnam & Tang 1986; Inderjit & Callaway 2003). This stresses the importance of field experiments for the understanding of how, and to what extent, an allelopathic species may affect the community (Harborne 1997; Inderjit & Weston 2000). On the other hand, in field conditions, it is almost impossible to isolate the allelopathic interference from the myriad of factors that are part of forest dynamics, such as competition or the activity of herbivores and pathogens (Putnam & Tang 1986; Inderjit & Del Moral 1997; Wardle *et al.* 1998).

Many factors can influence the allelopathic activity from the donor perspective, as well as influence the response of the receptor organism. The concentration of the compounds, for instance, may vary along the day and season, or may be influenced by environmental conditions (light, water, temperature and nutrients), genetic factors or even by the age of the plant or the organ (Rice 1984; Larcher 1995). Herbivores, pathogens and microorganisms can also increase or reduce the concentration of the allelochemicals (Rice 1984). In addition, the magnitude of the allelopathic effect may be species-specific and may vary according to the density of individuals receiving the compounds (Weidenhamer *et al.* 1989; Orr *et al.* 2005).

For *E. leiocarpa*, leaf age and season are factors particularly relevant for consideration in future studies, since this is a deciduous species. Taking this into account, it is necessary to ascertain whether the results observed for adult leaves are the same for recently fallen and for senescent leaves, and whether they vary with the season, since the leaves fall most abundantly during the drought period (Morellato 1991). Experiments to investigate the effects of *E. leiocarpa* on seeds of native species, as well as the production of stimulatory effects on its own seeds are essential to provide stronger evidence of an allelopathic potential and to better understand the role of this species in structuring plant communities.

Substances broadly recognized for their allelopathic potential, such as alkaloids and coumarins (Rice 1984; Michael 1993; Larcher 1995) were already reported as components of *E. leiocarpa* leaves (Delle Monache *et al.*1989; Delle Monache *et al.*1990; Nakatsu *et al.* 1990; Michael 1993). Although the constitution of the bark of *E. leiocarpa* is not known, substances of the same group were found in bark of another two *Esenbeckia* species (Oliveira *et al.* 1996), which suggests the presence of these compounds in the *E. leiocarpa* bark.

Although our data do not provide conclusive information about the allelopathic effects of *E. leiocarpa* bark and leaves, they showed the potential of this species to delay seed germination and strongly inhibit the seedling growth of lettuce seedlings. Whether or not this event occurs under natural conditions still remains to be investigated, but if the effects of *E. leiocarpa* on germination and growth of native species are similar to those reported here, this species may have an important role in the community organization and diversity of Tropical Seasonal Semideciduous Forests.

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