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Phytotoxic effects of *Morus nigra* aqueous extract on germination and seedling growth of *Lactuca sativa*

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Abstract

Some exotic species threat the integrity of natural environments due to their invasive potential. They can affect other species by releasing secondary compounds in the soil. *Morus nigra* (Moraceae) is an invasive species of riparian forests in southern Brazil. The objective of this study was to verify if the aqueous extracts of fruit, fresh and dry leaves of *M. nigra* show phytotoxic effects on germination, seedling growth and membrane integrity of seedlings of *Lactuca sativa*. Extract concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5 and 10% were tested. Germination rate and speed of germination were determined. Effects on initial growth were evaluated by measuring seedling root and shoot length, and membrane integrity was assessed by conductivity tests. Results showed the phytotoxicity of *M. nigra* aqueous extracts, causing significant inhibition on germination and seedling growth. The fruit extract was generally less phytotoxic than extracts from fresh and dry leaves. Moreover, the extracts induced changes in membrane integrity and caused morphological deformities in seedlings, such as necrotic roots and chlorotic plants. The high phytotoxicity of fruit, dry and fresh leaf extracts of *M. nigra* was evidenced, indicating the allelopathic potential of the species.

Key words: allelopathy, invasive species, membrane integrity, phenolics.

Resumo

Algumas espécies exóticas vêm ameaçando a integridade dos ecossistemas naturais pelo seu potencial invasor. Elas podem ser favorecidas pelo lançamento de compostos secundários no solo. *Morus nigra* (Moraceae) é uma espécie invasora de florestas ribeirinhas no sul do Brasil. O objetivo do estudo foi verificar se o extrato aquoso dos frutos e folhas frescas e secas apresentam efeitos fitotóxicos sobre a germinação, o crescimento e a integridade de membranas de plântulas de *Lactuca sativa*. Foram testadas as concentrações 0,1; 0,25; 0,5; 0,75; 1; 2,5; 5; 7,5 e 10%. A taxa e o índice de velocidade de germinação foram definidos. O crescimento inicial foi obtido com as medidas do comprimento de raiz e parte aérea. A integridade das membranas foi estimada através de medidas de condutividade. Os resultados evidenciaram os efeitos fitotóxicos dos extratos aquosos, causando a inibição significativa da germinação e no crescimento inicial das plântulas. Os efeitos fitotóxicos dos extratos das folhas frescas e secas foram superiores ao do fruto. Além disso, os extratos causaram alterações na estrutura das membranas e deformações morfológicas nas plântulas, como radículas necrosadas e plantas acloríticas. A alta fitotoxidez dos extratos *M. nigra* foi evidenciada, indicando o seu potencial alelopático.

Palavras-chave: alelopatia, espécies invasoras, integridade de membrana, fenólicos.

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Introduction

Allelopathy consists in the interaction, either beneficial or harmful, among plants through the production of chemical compounds released in the environment (Rice 1984). Considering this plant interaction, a donor plant is the one that produces and releases allelochemicals, and a target or recipient organism is the one that suffers allelochemicals' effects (Rivzi *et al.* 1999). Allelochemicals can be released by tissue leaching, volatilization, root exudation and by decomposition of plant structures (Einhellig 1986; Rice 1984; Weir *et al.* 2004). These allelochemicals can be responsible for several interactions among organisms (Gotllieb 1982; Weidenhamer 1996).

Phytotoxins released in soil by exotic species can inhibit native species germination and development, contributing to invasion success of exotic ones. This can occur due to the lack of adaptation of native species to allelochemicals from exotic species (Bais *et al.* 2003). Consequently, when exotic species succeed to establish in different biogeographic regions, they can threat natural ecosystem integrity by changing plant communities' structure (Kennedy *et al.* 2002).

Morus nigra L. (Moraceae) is an invasive exotic species in southern Brazil. It is a tree species 7–10 meters high, with simple leaves, long and slender petioles and which produces a great quantity of aggregate fruit (Chan *et al.* 2016; Ercisli & Orhan 2007) of the drupe type (Judd *et al.* 2009), constituting an infructescence. The fruit presents reddish color in the beginning of maturation and black when mature. Leaves and fruit accumulate in large quantities under the plant, as *M. nigra* is a deciduous species. The soil, in general, is bare due to the presence of only few individuals of other plant species (personal observation). The presence of few individuals under the species may indicate that leaf and mature fruit accumulated on soil could release secondary metabolites that cause allelopathic effects on other plants.

Therefore, it is relevant to comprehend whether allelopathy can be a mechanism associated to *M. nigra* invasion. The objective of the present study was to evaluate the phytotoxic effect of aqueous extracts of leaves and fruit of *M. nigra* on germination and seedling growth, using *Lactuca sativa* L. as the recipient species.

Material and Methods

Plant collection and extract preparation

Leaves and fruit of *M. nigra* were collected from individuals situated at the margins of two rivers (29°30'4"–29°29'55"S and 51°58'9"–51°57'32"W) in southern Brazil, which vegetation type is inserted in the Atlantic Forest biome. Fertile material from a specimen from each of the collection sites was inserted in the HVAT Herbarium of the Universidade do Vale do Taquari - Univates under registers 5,129 and 5,130. In order to obtain samples from the whole branch, leaves were collected from the base to the tip of branches. After collection, part of leaves were immediately manually macerated (fresh leaves), while the remaining leaves were kept in a drying oven at 40 °C for 48 hours, and then also macerated (dry leaves). Mature fruit (with dark color) was collected.

Leaf extracts (fresh and dry) were obtained by the infusion of macerated leaves in deionized water (1:10 w:v), heated at 90 °C. The fruit extract was obtained by trituration followed by filtration and storage in ultra-low-temperature freezers (-80 °C) for 12 hours. Lyophilization of all extracts was carried out at -50 °C with 760 mmHg of pressure. For germination and growth bioassays, concentrations of the three extracts were obtained by diluting the crude extract in distilled and autoclaved water, resulting in the following treatments: 0% (control); 0.1%; 0.25%; 0.5%; 0.75%; 1%; 2.5%; 5%; 7.5% and 10%.

Germination assays

Germination assays were conducted with *L. sativa* (*var*. Grand Rapids) as the recipient species. Each repetition comprised 25 *L. sativa* cypselae placed on filter paper in a Petri dish (9.0 mm), with 8 ml of each treatment. For each concentration of extract, four repetitions were established. Plates were kept in a growth room with 16 hours/light photoperiod and temperature of 25 °C (± 2 °C). These conditions were maintained by white fluorescent lamps (40 W), with average irradiance of 91 $\mu mol.m^2.s^{-1}$. The experimental design was completely randomized.

In total, six days were needed to finish the bioassay, with daily counting at each 12 hours. Germinated cypselae were considered those with root protrusion of 2 mm. Germination rate (GR) and speed of germination index (SGI) were

determined for each group. Germination rate was calculated as the percentage of germinated seeds in the last counting. GSI value was calculated by the formula GSI = (G1 / N1) + (G2 / N2) +...+ (Gn / Nn), where: G1 = number of germinated seeds in the first counting; N1 = number of hours elapsed until the first counting; G2 = number of germinated seeds in the second counting; N2 = number of hours elapsed until the second counting; and n corresponds to the last counting (Maguire 1962).

Initial growth assays

Effects of the aqueous extracts on root and shoot growth were evaluated with *L. sativa* seedlings. For the pre-treatment period, the cypselae were kept in Petri dishes with filter paper and distilled water. After primary root growth and the emergence of cotyledonary leaves (36 h), seedlings were transferred to plates containing the treatments, in the same conditions of the germination assays. For each extract and its respective concentrations, four repetitions of 25 seedlings were set. After five days in a growth room, seedlings were photographed and the values of root and shoot length were obtained with the software Image J.

Establishment of pH controls

Changes in extract pH can affect germination and seedling growth, and thus, effects of extracts can be erroneously attributed to allelochemicals (Einhellig 1986). Effects of pH of *M. nigra* aqueous extracts were assessed on germination and growth of *L. sativa*. Water pH and pH of the highest concentration of each extract (10%) were measured with a pHmeter. Aqueous extracts showed pH values of 5.97 for fruit, 7.04 for fresh leaves, 6.89 for dry leaves, and water pH was 7.02. Controls were established in solutions of distilled water and HCl 1M to adjust pH to the same value of each extract. Effects of these pH controls on germination and seedling growth were tested and compared to control (water), as described above.

Membrane integrity evaluation

Effects of *M. nigra* aqueous extracts on membrane integrity were evaluated by measuring the electrolyte leakage of seedling membranes. At the end of the early growth experiments, seedlings (0.2 g) from each plate were placed in tubes with distilled water (10 mL). After 24 h, the initial electrical conductivity of the medium (EC1) was measured using a digital conductivity meter (METRHOM 856). Samples in the tubes were frozen for total releasing of cell electrolytes, unfrozen and, after 24 hours, the final electrical conductivity (EC2) was measured. In order to calculate the relative electrolyte leakage (REL), the formula $REL(\%) = E_1/(E_1+E_2)*100$ was used.

Total phenolic content

Total phenolic content in the aqueous extracts was assessed by Folin-Ciocalteau colorimetric method (Singleton *et al.* 1999). An aliquot of 100 µL from the extract sample was mixed with 7.0 mL of distilled water, followed by addition of 500 µL of Folin-Ciocalteau reagent. After five minutes, 1.5 mL of a solution of sodium carbonate and water (2:10) was added to the sample. The absorbance was measured after 120 minutes at 760 nm with a spectrophotometer (UV-VIS). For each extract concentration, samples were made in triplicate. Tannic acid was used as the reference phenolic, in nine concentrations ranging from 0.1 to 10 mg/mL ($R^2 = 0.98$). Results were expressed in mg tannic acid equivalent/mL of extract.

Statistical analysis

For all the experiments, the measured parameters for each recipient species (germination rate, speed of germination, shoot length, root length and electrolyte leakage) were compared between groups by univariate analysis of variance with randomization (PERMANOVA). When analysis of variance indicated significant differences between groups, contrast analyses were used for pairwise comparisons (Pillar & Orlóci 1996). Randomization tests are not based on assumptions of normality and homogeneity of variances (Anderson 2001). Tests were conducted with 10,000 bootstrap iterations, using Euclidean distance as the dissimilarity measure, and considering a significance level of $p \leq 0.05$. Analyses were performed with Multiv software (Pillar 2009).

Results

Extracts of fruit, fresh and dry leaves of *M. nigra* caused inhibitory effects on *L. sativa* germination. All the three extracts significantly reduced the germination rate of the recipient species from 0.25% (Fig. 1a). Regarding the speed of germination, the extracts of fresh and dry leaves were inhibitory from 0.25%, while the fruit extract only affected this parameter from 2.5% (Fig. 1b).

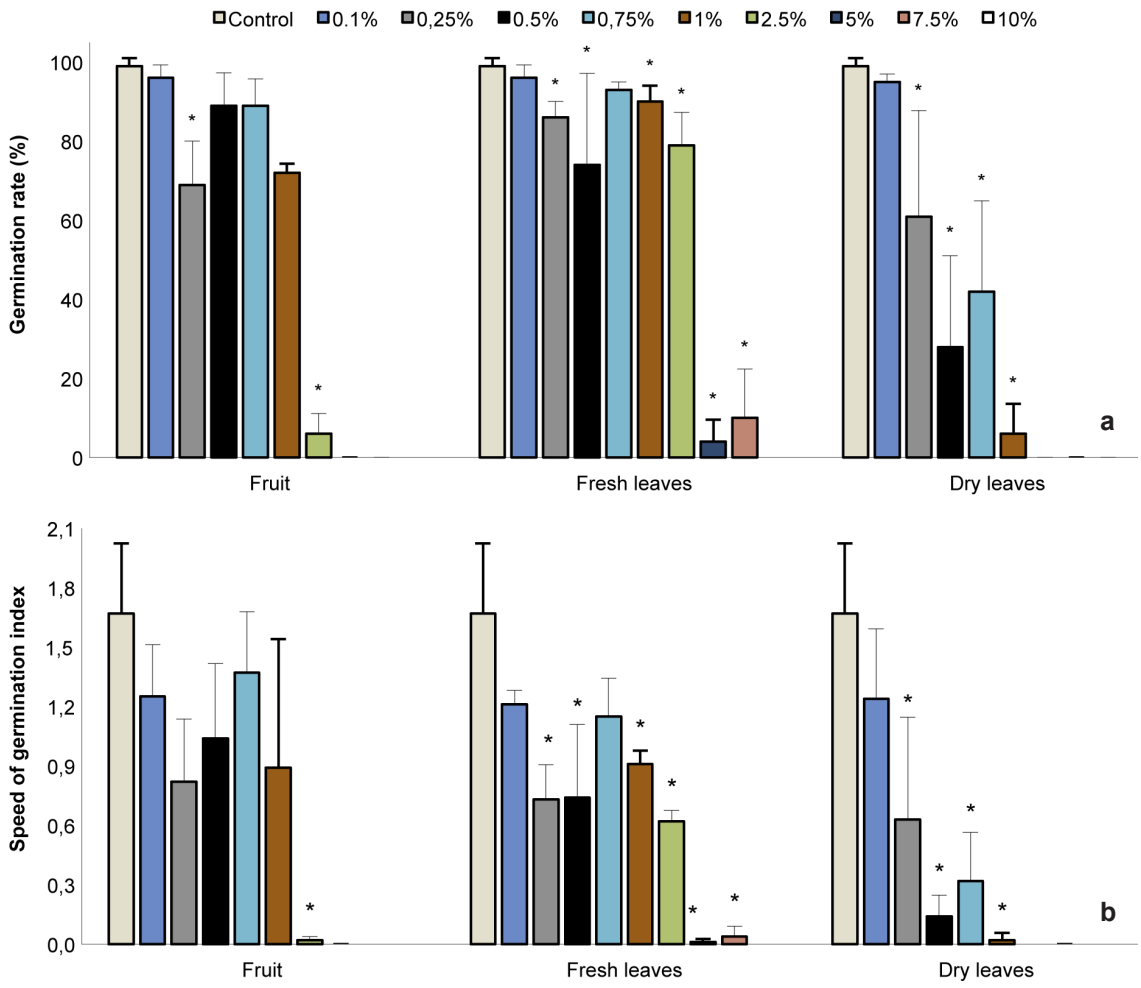

Figure 1 – Effects of aqueous extracts of fruit, fresh and dry leaves of *Morus nigra* on (a.) germination rate and (b.) speed of germination of *Lactuca sativa*. Data are presented as mean + standard deviation, with n = 4. (*) Significantly different from control, according to PERMANOVA, at $p \leq 0.05$.

Total inhibition of germination rate and speed of germination of *L. sativa* was observed from 2.5%, 5% and 10% for the extracts of dry leaves, fruit and fresh leaves, respectively.

The aqueous extracts of *M. nigra* negatively affected seedling growth of *L. sativa*, with a stronger effect on root length. Extracts of dry and fresh leaves of *M. nigra* inhibited root growth of the recipient species from 0.1%, and the fruit caused inhibition from 0.5% (Fig. 2a). Shoot growth was affected by extracts of fresh leaves, dry leaves and fruit from 0.25%, 0.5% and 7.5%, respectively (Fig. 2b). Some seedlings were so damaged that they were decomposing, breaking at the slightest handling and thus could not be measured, which occurred for extracts of dry (10%) and fresh leaves (from 5 to 10%). Fruit extract of *M. nigra* at 0.75% and 1% promoted *L. sativa* shoot growth. In addition, necrotic roots were observed for seedlings exposed to the extracts of fresh and dry leaves from 0.75% and to the fruit extract from 2.5%. Seedlings were chlorotic (whitish) from concentrations of 5%, 7.5% and 10% for extracts of fresh leaves, dry leaves and fruit, respectively.

The pH control of the extracts did not cause significant inhibition in germination rate and speed of germination, when compared to the control treatment (Tab. 1). The same result was observed in relation to root and shoot length, as the treatments

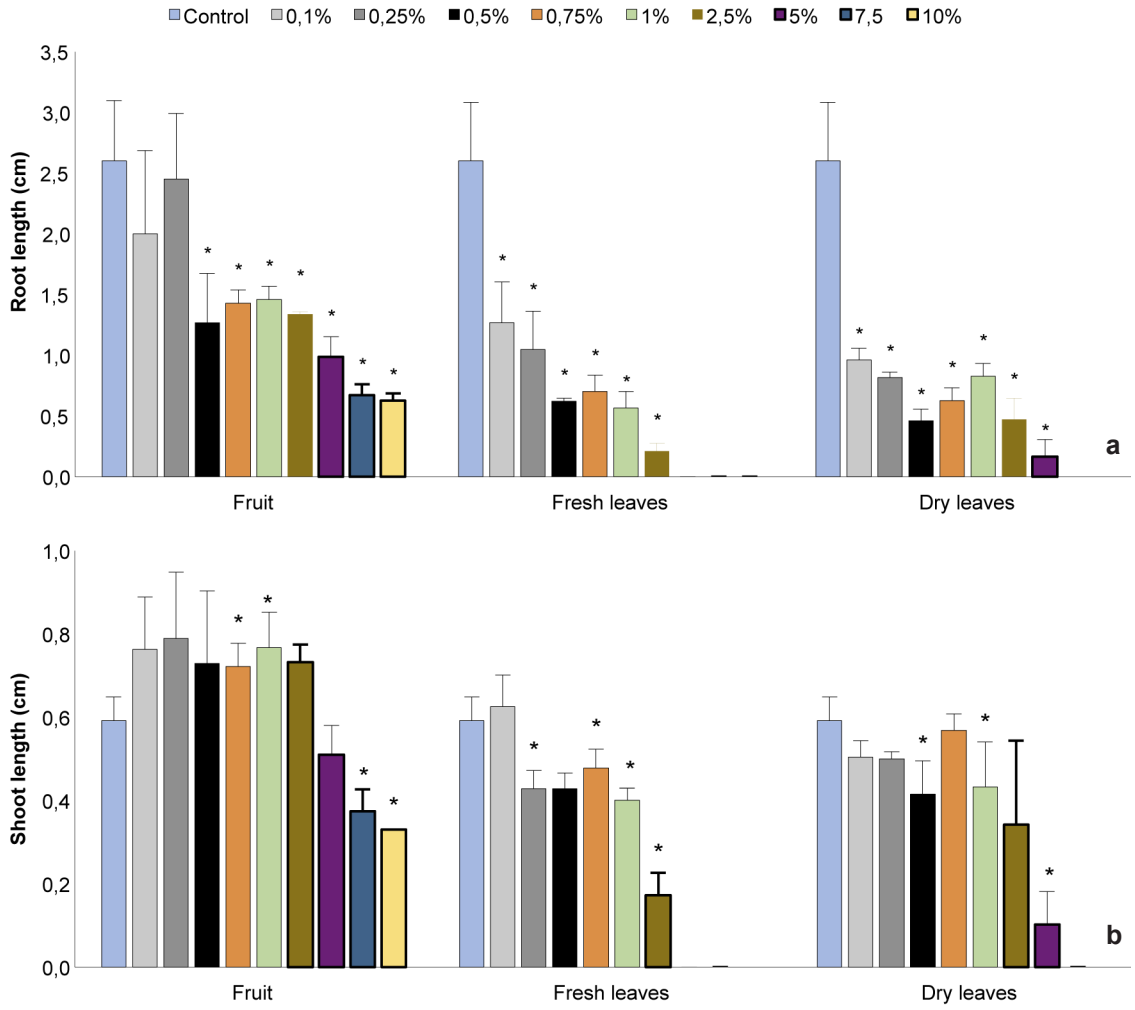

Figure 2 – Effects of aqueous extracts of fruit, fresh and dry leaves of *Morus nigra* on (a.) root length and (b.) shoot length of *Lactuca sativa*. Data are presented as mean + standard deviation, with n = 4. (*) Significantly different from control, according to PERMANOVA, at $p \leq 0.05$.

with the same pH of the extracts did not differ in relation to control.

Integrity of seedling membranes was affected by the three extracts, and the highest electrolyte leakage was observed in seedlings submitted to the fruit aqueous extract (Fig. 3). Electrolyte leakage of seedlings exposed to the fruit extract was significantly higher than the control from 0.1%. Dry and fresh leaf extracts affected electrolyte leakage from 0.5% and 2.5%, respectively. However, evaluation of membrane integrity was not possible for concentrations from 5 to 10% of fresh and dry leaf extracts for the same reason of growth measurements (seedling decomposition).

The aqueous extracts showed high phenolic content (Fig. 4), especially the fruit extract, which had the highest value at 10% (1.37 mg tannic acid equivalent/mL of extract). The aqueous extracts of dry and fresh leaves at 10% reached, respectively, 1.01 and 0.50 mg tannic acid equivalent/mL of extract.

Discussion

The aqueous extracts of *M. nigra* inhibited germination and growth of *L. sativa* seedlings even in low concentrations, which evidences the high phytotoxicity of the extracts. Furthermore, the extracts caused morphological changes in seedlings

Table 1 – Effects of pH controls for aqueous extracts of fruit, fresh and dry leaves of *Morus nigra* on germination and seedling growth of *Lactuca sativa*. Controls were established in solutions of distilled water and HCl 1M in the same pH of the highest concentration of each extract (10%). Data are presented as mean + standard deviation, with n = 4. No treatment differed from the control (water), according to PERMANOVA, at $p \leq 0.05$.

	Control	Fruit extract pH	Fresh leaf extract pH	Dry leaf extract pH
Germination rate (%)	99 ± 2	99 ± 2	100	99 ± 2
Speed of germination	1.4 ± 0.09	1.18 ± 0.13	1.22 ± 0.12	1.19 ± 0.11
Root length (cm)	2.90 ± 0.81	2.65 ± 0.32	3.10 ± 0.46	2.85 ± 0.45
Shoot length (cm)	0.64 ± 0.05	0.63 ± 0.06	0.66 ± 0.03	0.60 ± 0.01

(chlorotic seedlings and necrotic roots). All the extracts showed similar phytotoxicity regarding germination rate (inhibitory effects starting from 0.25%). The fruit extract was less phytotoxic on speed of germination, root and shoot growth and morphological alterations than the leaf extracts, but it was more phytotoxic on integrity of seedling membranes. Thus, although the fruit extract was in general less phytotoxic, this is not a rule for all parameters. This shows the importance of evaluating several parameters in phytotoxicity studies, which allows a better comprehension of variability in effects. Additionally, the fruit extract in low concentrations caused stimulatory effects on shoot growth of the recipient species. This may have occurred as low concentrations of allelochemicals can stimulate effects that high concentrations inhibit (Rice 1984).

Extracts of fruit, dry and fresh leaves of *M. nigra* affected integrity of seedling membranes, which may have been related to the inhibition in seedling growth. Effects on membranes were so pronounced that at concentrations above 2.5% of fresh and dry leaf extracts, seedlings decomposed. Changes in integrity, fluidity and permeability of membranes can be initially caused by production and accumulation of reactive oxygen species (ROS), which can affect the membranes and result in DNA and protein damages, leading to cell death (Testa & Caldwell 1995; Qian *et al.* 2009).

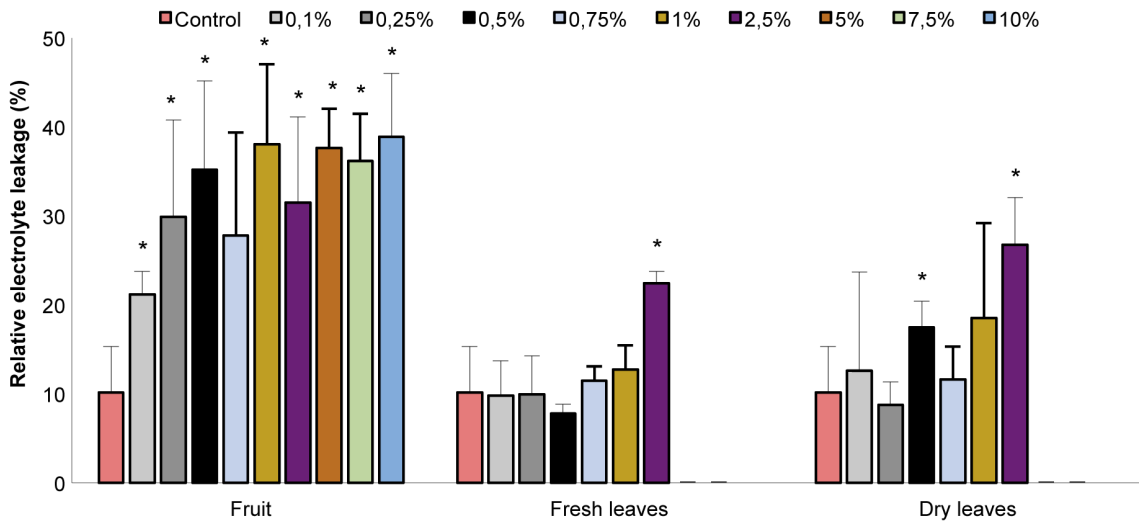

Figure 3 – Effects of aqueous extracts of fruit, fresh and dry leaves of *Morus nigra* L. on electrolyte leakage of membranes of *Lactuca sativa* L. seedlings. Data are presented as mean + standard deviation, with n = 4. (*) Significantly different from control, according to PERMANOVA, at $p \leq 0.05$.

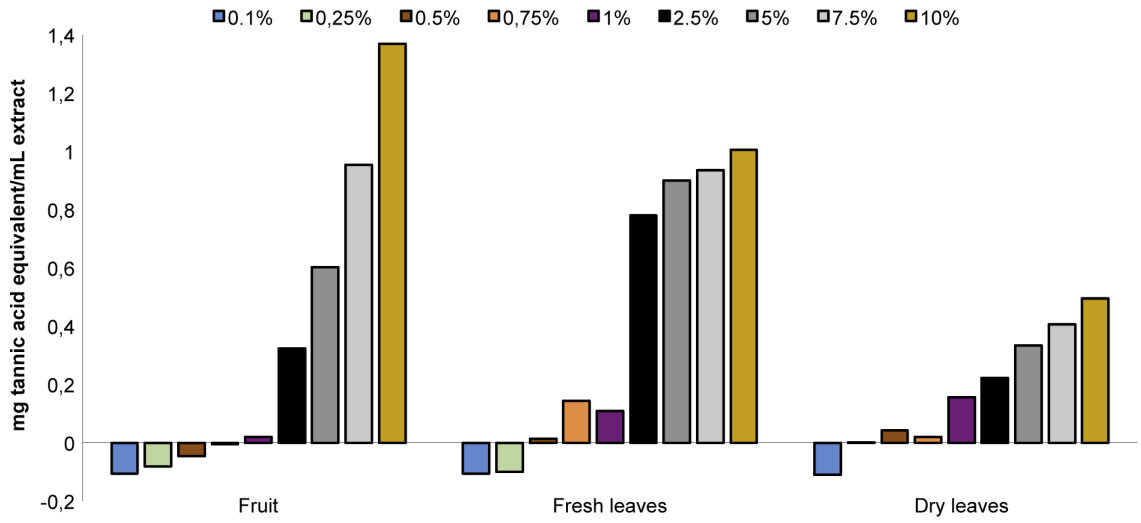

Figure 4 – Total phenolic content of aqueous extracts of fruit, fresh and dry leaves of *Morus nigra*, based in Folin-Ciocalteau method and using tannic acid as the standard phenolic compound. Data are presented as mean + standard deviation, with n = 3.

The overproduction of ROS has been considered one of the initial mechanisms of allelochemical effects on germination and seedling growth (Yu *et al.* 2003). Moreover, the chlorotic appearance of *L. sativa* seedlings was observed when they were submitted to high concentrations of *M. nigra* extracts, indicating degradation of chlorophyll molecules or inhibition of their synthesis (Rice 1984). Changes on chlorophyll content due to exposure to allelochemicals can interfere in photosynthesis, affecting plant development (Rice 1984; Inderjit & Dakshini 1996; Chou 1999). Thus, the effects of *M. nigra* extracts on germination and seedling growth may have been caused by ROS accumulation that caused membrane damages, and by chlorophyll reduction, consequently decreasing photosynthesis.

Changes in extract pH did not inhibit germination and seedling growth, indicating that the observed effects were due to allelochemicals. Total phenolic content was higher in the fruit extract than in the extracts of fresh and dry leaves. However, the fruit extract was not more phytotoxic than the leaf extracts, suggesting the existence of another class of compounds with phytotoxic action. Further studies are necessary in order to elucidate the chemical aspects of *M. nigra* phytotoxicity. Phytochemical studies of species from *Morus* genus indicate that their fruit show phenolic compounds with a wide biochemical activity (Pérez-Gregório *et al.* 2011; Toshio *et al.* 2005; Wang *et al.* 2011), which are found in greater quantities in *M. nigra* than in *M. alba* and *M. rubra* (Memon *et al.* 2010; Ercisli & Orhan 2007; Nakamura *et al.* 2003). Sývacý & Sökmen (2004) and Dugo *et al.* (2001) recorded high content of total phenolics in phytochemical studies with *M. alba* and *M. nigra* branches in a certain seasonal period for both species. Moreover, some classes of phenolic compounds which are often found in decomposing plant material and widely distributed inside the plant (Kujala *et al.* 2000) are known by their potential to inhibit seed germination (Rice 1984). Thus, even if the phenolic compounds are probably associated to the phytotoxic effects of *M. nigra*, other classes of chemical substances are also possibly related to its phytotoxicity.

In spite of being an initial study, the high phytotoxicity of *M. nigra* extracts was observed herein. This suggests the possibility of associating allelopathy with the invasive potential of the species. Nevertheless, by the extraction process used for the aqueous extracts, the maceration may release compounds that would not be active in natural processes, such as leaf fall on soil and microorganism degradation (Inderjit & Dakshini 1996). Thereby, further studies must consider using plant tissues from intact leaves and fruit of *M. nigra* in order to better comprehend the potentiality of this species to release allelochemicals.

Even though the high phytotoxicity of *M. nigra* extracts has been observed, it is important to highlight that this study only shows that the species causes phytotoxic effects in laboratory conditions. The evidence of these effects on germination and growth of species that are sensitive to allelochemicals needs to be tested in natural conditions, and using soil. Moreover, it is relevant to comprehend the mechanisms of allelopathic action of the species in seasonal periods. The species can release its compounds through tissue lixiviation, with the leaf attached to the branch or during decomposition in the soil. Therefore, it is possible that *M. nigra* affects other species, mainly the native ones, increasing its propagation.

In conclusion, *M. nigra* fruit and leaf extracts are phytotoxic, affecting germination and initial growth of *L. sativa*, and also the integrity of seedling membranes. In general, the fruit extract was less phytotoxic than the fresh and dry leaf extracts. This indicates the allelopathic potential of the species, possibly through decomposition and lixiviation by rain of allelochemicals from leaf and fruit. However, field studies are still necessary to comprehend whether allelopathy is indeed involved in the invasiveness of *M. nigra.*

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