Allelopathic Effects of Aqueous and Ethanolic Leaves Extracts of *Schinus molle* L. under Different Kinds of Pruning

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Abstract: Secondary metabolites produced by plants can be used in popular medicine, as well they can interact with plants and other organisms, in which case they are called allelochemicals and influence the neighboring ecosystem. This work aimed to evaluate the allelopathic effects of *Schinus molle* L. species widely used in reforestation and urban afforestation. Therefore, leaves of *S. Molle* were collected from different populations located at Alfenas and Nepomuceno in Minas Gerais, Brazil. Cypsela of lettuce (*Lactuca sativa* L.) were germinated in biochemical oxygen demand (BOD) chamber at 25 °C with a photoperiod of 12 h under different extracts concentrations. The experiment was performed in factorial design (2 × 4) with two kinds of extracts (aqueous and ethanol) and four concentrations (2.5, 5, 10 and 20 mg/mL) in randomized blocks. The variables analyzed were germination (%), germination speed index (GSI), % of normal seedlings, root length, fresh biomass and dry biomass. Changes in the cell cycle in meristematic cells of the used model were also evaluated. Concentration-dependent effect on all parameters was observed, with the exception of dry biomass exposed to ethanol extract. The root elongation parameter was different between the extracts even in lower concentrations, thus indicating that this is the most sensitive parameter of this species. Toxic effect from *S. molle* extracts was observed in all parameters regardless the kind of pruning management or method of extraction (water or ethanol).

Key words: Secondary metabolites, allelopathic effect, *Schinus molle* L., phytotoxicity, cytogenetics.

1. Introduction

Many plants are used by people indiscriminately for medicinal, including *Schinus molle* L., popularly known in Brazil as Aroeira-Salsa. *S. molle* L. produces leaves and fruits of spicy essential oils, which are widely used for containing metabolites with antimicrobial, antifungal, anti-inflammatory, antispasmodic, antipyretic and cicatrizing properties [1]. Besides great importance for these purposes, these metabolites have physiological function in the metabolism of plants and can interact with other plants, which impacts the surrounding environment. In this ecological context, these chemicals are called

allelochemicals and have great importance in the adaptation of species and organization of plant communities [2]. These allelochemicals are responsible for an ecological phenomenon called allelopathy, which can refer to any direct or indirect effect, harmful or beneficial among plants and other organisms [3].

The production of allelochemicals may vary in response to various factors and environmental conditions [4], such as climatic factors, solar intensity, temperature and rainfall [5]. The low nutrient concentration in soil can also induce the production of certain substances, or enlarge the production of others compost which are normally produced in low amounts [6]. Physiological factors, such as photosynthetic rate,

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stomatal behavior, mobilization reserves, leaf expansion, reproduction and growth, can be altered by some kinds of stress and consequently lead to changes in the secondary metabolism [7]. Collection season is also a factor that interferes with the secondary metabolism, since the amount and sometimes even the nature of allelochemicals are not constant throughout the year and may have seasonal variations, which should be considered in studies of the effect of these substances [8]. Also, mechanical damages, such as pruning and herbivorous, lead to changes in the composition of secondary metabolites [9, 10]. In this sense, this study aimed to evaluate the allelopathic effect of different extracts of populations of *S. molle* under different pruning regimes and environmental conditions on *Lactuca sativa*.

2. Materials and Methods

2.1 Plant Materials and Extracts

The used plant materials were completely expanded leaves of *S. molle* collected in October 2010, January, April and July 2011 (in order to eliminate possible existing seasonal effects in the production of allelochemicals) of the two distinct populations with different pruning schemes located in the cities of Alfenas (21°26' S and 45°56' W) and Nepomuceno (21°15' S and 45°15' W), Minas Gerais, Brazil. The population of Alfenas, Minas Gerais suffers the effect of constant pruning by the city government in order to not disturb the electricity network in the region, while the population in Nepomuceno does not suffer any kind pruning during the year. Dried material was deposited in the herbarium of the Federal University of Alfenas (UALF) under registration No. 2530 and 2439, respectively.

The leaves were oven dried at 40 °C until stabilization of the masses, crushed, sieved in 20 mesh and followed by the ethanol and water extracts preparation. The ethanolic extract was prepared by maceration process, adding absolute ethanol in the plant material until the complete exhaustion of the

mash. After this process, the extract was concentrated in rotary evaporator. The aqueous extract was prepared by the infusion method at a concentration at 5% according to Ref. [11] and subsequently lyophilized.

2.2 Phytotoxic Tests

Phytotoxicity tests were made according to Refs. [12, 13] with some adaptation, using three replicates of 30 cypselsae of lettuce (*L. sativa* cv. Grand Rapids) in Petri dishes containing 3 mL of different concentrations (0, 2.5, 5, 10 and 20 mg/mL) of ethanol and aqueous extracts of *S. molle*.

The bioassay was carried out in biochemical oxygen demand (BOD) growth chamber at 25 °C with a 12 h photoperiod. Germination rate was evaluated every 12 h for a period of 7 d in order to get the germination speed index (GSI), as Eq. (1):

$$GSI = (G_1/N_1) + (G_2/N_2) + \dots + (G_n/N_n) \quad (1)$$

where, G_1 = number of germinated seeds in the first count; N_1 = the first count; G_2 = number of germinated seeds in the second count; N_2 = the second count and n = the last count.

On the 7th day, germination rate, normal seedlings (NS%), root elongation (RE) and fresh biomass (FB) were evaluated. Essential organs in perfect stage of development were considered as normal seedlings. For RE, roots of 10 normal seedlings were chosen and measured randomly in each Petri dish. After weighing FB, the seedlings were placed in an oven with air circulation at 45 °C until mass stabilization in order to evaluate the total dry biomass (DB). For evaluations of FB and DB, 10 seedlings were weighed in each Petri. Although on some concentration, not all seed were germinated; in these cases, all normal seedlings were weighed and the values divided by the number of available seedlings.

2.3 Cytogenetics Tests

The study of effect of extracts of *S. molle* on the cell cycle and in the chromosome complement of the test plant was adapted from Refs. [12, 14]. Thirty

lettuce cypselaes were incubated in Petri dishes containing 3 mL of ethanol and aqueous extracts of *S. molle*, respectively, under the same experimental conditions described above.

Three thousands cells/treatment were evaluated in order to evaluate the mitotic index (MI) and chromosomal abnormalities (CA), according to Ref. [15]. The CA quantified was: chromosomal bridges, micronuclei, lost chromosomes, C-metaphase and sticky chromosomes.

2.4 Statistics Analysis

The experimental design was a completely randomized blocks in a factorial scheme (2×4), with aqueous and ethanol extraction in four concentrations (2.5, 5, 10 and 20 mg/mL) and three replicates. The data were analyzed in relation to the results obtained in the control.

The results, except for germination, were subjected to analysis of variance (ANOVA), with all variables attended the normality assumptions and/or homogeneity. To compare the averages of the different extracts, Scott-Knott test was used, and to evaluate the effect of concentrations, regression

models were adjusted. All analyzes were performed using the statistical software R version 3.0.2 [16], and adopted for all tests at the significance level of 5%. The results about germination rate did not attend the normality and/or homogeneity, and were submitted to Kruskal-Wallis test. The averages for this parameter were evaluated by Student-Newman-Keuls (SNK) test at 5% significance level.

3. Results

3.1 Phytotoxic Results

3.1.1 Toxicity from Nepomuceno Population Extracts (No Pruning Effects)

When lettuce seeds were exposed to leaf extracts of *S. molle* from Nepomuceno population, the interaction effect between type of extract and concentration on GSI ($P < 0.0001$), NS ($P < 0.0001$), RE ($P = 0.0073$) and DB ($P = 0.0332$) can be observed (Table 1). For FB, MI and CA, there was no interaction between type of extract and concentration ($P = 0.59$; $P = 0.102$; $P = 0.262$, respectively), but were significant in both FW and MI for the variable concentrations ($P < 0.0001$; $P = 0.00002$, respectively) and type of extract ($P = 0.039$;

Table 1 GSI (%), NS (%), RE (%) and DB (%) of *L. sativa* seeds exposed to different concentrations of aqueous and ethanolic extracts of two *S. molle* populations.

Extract	Concentrations (mg/mL)							
	2.5		5		10		20	
	P ₁	P ₂	P ₁	P ₂	P ₁	P ₂	P ₁	P ₂
GSI								
Aqueous	98.85 ^a	97.80 ^a	93.99 ^a	94.39 ^a	82.36 ^a	76.27 ^a	37.45 ^b	29.80 ^b
Ethanolic	94.23 ^a	92.28 ^a	89.44 ^b	87.21 ^a	83.63 ^a	78.76 ^a	69.40 ^a	59.20 ^a
NS								
Aqueous	100.17 ^a	99.60 ^a	96.51 ^a	100.46 ^a	95.55 ^a	88.26 ^a	14.21 ^b	20.90 ^b
Ethanolic	100.76 ^a	100.21 ^a	98.76 ^a	100.18 ^a	98.80 ^a	96.79 ^a	89.34 ^a	73.50 ^a
RE								
Aqueous	42.40 ^b	41.65 ^b	31.46 ^b	25.51 ^b	19.97 ^b	18.88 ^b	5.20 ^b	7.33 ^a
Ethanolic	75.54 ^a	67.13 ^a	73.11 ^a	71.12 ^a	66.77 ^a	46.84 ^a	24.91 ^a	17.47 ^a
DB								
Aqueous	10.91 ^a	10.91 ^a	9.83 ^a	11.13 ^a	10.56 ^a	10.48 ^a	4.69 ^b	7.87 ^b
Ethanolic	10.01 ^a	10.23 ^a	10.14 ^a	10.12 ^a	9.18 ^a	10.64 ^a	8.53 ^a	9.80 ^a

P₁ = Nepomuceno; P₂ = Alfenas; GSI: germination speed index; NS: normal seedlings; RE: root elongation; DB: dry biomass. Means followed by the same letter in the columns do not differ by the Scott-Knott test at 5% significance level.

Table 2 Average of FB (%), MI (%) and CA (%) of *L. sativa* seeds exposed to aqueous and ethanolic extracts from two populations.

Extract	FB		MI		CA	
	P ₁	P ₂	P ₁	P ₂	P ₁	P ₂
Aqueous	8.59 ^a	8.03 ^a	91.06 ^a	85.64 ^a	58.44 ^a	65.04 ^a
Ethanolic	7.63 ^b	7.59 ^a	56.64 ^b	65.15 ^b	68.75 ^a	70.60 ^a

P₁ = Nepomuceno; P₂ = Alfenas; FB: fresh biomass; MI: mitotic index; CA: chromosomal abnormalities.

Means followed by the same letter in the columns do not differ by the Scott-Knott test at 5% significance level.

$P < 0.0001$, respectively). Difference between the tested concentrations was observed in the parameter CA ($P = 0.0153$) (Table 2, Fig. 1).

The aqueous extract showed greater reduction in GSI, NS and DB, regardless of the population studied compared with the ethanolic extract at the concentration of 20 mg/mL. However, at the concentration of 5 mg/mL from Nepomuceno population, the lower GSI was observed in the ethanol extract (Table 1).

3.1.2 Toxicity from Alfenas Population Extracts (Pruning Effects)

Similar pattern observed in extracts from Nepomuceno population was observed for extracts from Alfenas population, with interaction on GSI ($P = 0.000034$), NS ($P < 0.0001$), RE ($P = 0.0236$) and DB ($P < 0.0001$) (Table 1), and no interaction was detected in the measured parameters FB ($P = 0.3935$), MI ($P = 0.5313$) and CA ($P = 0.208$). In the parameter MI, differences were observed between concentrations ($P = 0.0147$) and types of extracts ($P = 0.00238$) independently. For FB and CA, only difference between the concentrations was observed ($P < 0.0001$; $P = 0.043$, respectively) (Table 2).

According to the results, RE parameter was the most sensitive to the present compounds in the aqueous extract, as it showed greater reductions than in the ethanol extract even in the lowest concentration, regardless of the population extract source. However, when extracts obtained from Alfenas population at a concentration of 20 mg/mL was evaluated, no difference between the extraction methods was observed (Table 1).

For FB, there were no differences observed in the

two populations tested, since in Alfenas population no difference was observed between the two extraction methods tested and in Nepomuceno population aqueous extract showed less interference than ethanol extract (Table 2). Similar behavior was observed for DB. When lettuce cypselsae were exposed to Alfenas population extracts (population who suffered constant pruning effect), no difference was observed among the ethanolic extract concentrations in relation to DB (Fig. 2).

3.2 Germination Rate

The germination from Nepomuceno and Alfenas population did not present normal distribution (both $P < 0.0001$) or homogeneity ($P < 0.0001$, $P = 0.0232$), so it was necessary to use Kruskal-Wallis test (non-parametric test) and Student-Newman-Keuls test (average analysis) at 5% significance level (Table 3).

Although germination parameter is often used to study allelopathy, in this present work, little variation was observed between the tested concentrations. Even at the highest concentration, the germination rate was very close to the rate in the control treatment. The lowest percentage of germination was observed in cypselsae exposed to aqueous extract of Alfenas population at concentration of 20 mg/mL, which showed 87% germination compared to control treatment (Table 3).

3.3 Cytogenetic Evaluation

In relation to MI, the behavior of the two tested populations was similar, with the largest reductions observed in the ethanolic extract compared to aqueous extract (Table 2). When cypselsae of lettuce were exposed

Fig. 1 Relationship among different extracts of *S. molle* collected in a population located in Nepomuceno (no pruning effect).

Y_1 : equation of aqueous extracts; Y_2 : equation of ethanol extracts.

Fig. 1a: $Y_1 = 100.738 - 0.57554x - 0.129315x^2$, $R^2 = 0.99$; $Y_2 = 97.19518 - 1.38833x$, $R^2 = 0.99$.

Fig. 1b: $Y_1 = 88.4735 + 4.6395x - 0.4177x^2$, $R^2 = 0.99$; $Y_2 = 102.86 - 0.6337x$, $R^2 = 0.91$.

Fig. 1c: $Y_2 = 12.99 - 0.776x + 0.0181x^2$, $R^2 = 0.99$.

Fig. 1d: $Y_1 = 12.2143 - 0.3427x$, $R^2 = 0.83$; $Y_2 = 9.469$.

Fig. 1e: $Y_1 = 43.62029 - 2.01127x$, $R^2 = 0.95$; $Y_2 = 73.601 + 0.96x - 0.1695x^2$, $R^2 = 0.99$.

Fig. 1f: $Y_2 = 98.179 - 4.1698x$, $R^2 = 0.99$.

Fig. 1g: $Y_2 = 81.51 - 3.07x$, $R^2 = 0.99$.

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Fig. 2 Relationship among concentrations of different extracts from *S. molle* collected in Alfenas population.

Y_1 : equation of aqueous extracts; Y_2 : equation of ethanol extracts.

Fig. 1a: $Y_1 = 112.185 - 4.012x$, $R^2 = 0.98$; $Y_2 = 96.985 - 1.87x$, $R^2 = 0.99$.

Fig. 1b: $Y_1 = 96.222 + 2.2356x - 0.3x^2$, $R^2 = 0.99$; $Y_2 = 107.58 - 1.59x$, $R^2 = 0.91$.

Fig. 1c: $Y_2 = 11.4242 - 0.385x$, $R^2 = 0.97$.

Fig. 1d: $Y_1 = 11.854 - 0.1866x$, $R^2 = 0.91$; $Y_2 = 10.2029$.

Fig. 1e: $Y_1 = 39.428 - 1.7155x$, $R^2 = 0.85$; $Y_2 = 79.74 - 3.104x$, $R^2 = 0.97$.

Fig. 1f: $Y_2 = 92.96 - 3.012x$, $R^2 = 0.98$.

Fig. 1g: $Y_2 = 90.27 - 3.84x$, $R^2 = 0.98$.

Table 3 Germination rate (%) of *L. sativa* exposed to different concentrations of aqueous and ethanol extracts of two populations.

Extract	Concentration	Germination rate (%)	
		P ₁	P ₂
Aqueous	2.5	99.90 ^a	100.18 ^a
	5	100.18 ^a	100.18 ^a
	10	99.63 ^a	99.63 ^a
	20	90.16 ^b	87.37 ^b
Ethanolic	2.5	100.18 ^a	99.90 ^a
	5	99.90 ^a	99.63 ^{ab}
	10	99.62 ^{ab}	98.79 ^{ab}
	20	97.95 ^{ab}	90.45 ^b

P₁ = Nepomuceno; P₂ = Alfenas.

Means followed by the same letter in the columns do not differ by Student-Newman-Keuls test at 5% significance level.

to aqueous and ethanolic extracts of *S. molle* from Nepomuceno population, a concentration-dependent effect can be observed, i.e., increasing concentrations is related to an increasing in the toxicity. This behavior was different only on the data obtained from DB of lettuce exposed to ethanolic extract, which showed no difference between the tested concentrations, and the average of DB was 9.46% in relation that observed in the control (Fig. 1).

4. Discussion

The chemical compounds produced by plants can present high, medium or low polarity. Compounds with high polarity in general tend to be more active in the plants, although this effect can be more or less accentuated in relation to the studied parameter, like germination, size and even genetic parameters [17]. In this aspect, different type of plant extracts can have a different activity in the plants, affecting sometimes more on physiological parameters and in other occasions more on cytological parameters. In this sense, the present study evaluated the effect of two extracts with high polarity (aqueous and ethanolic), which proved the allelopathic effect of *S. molle*, with the stronger effect of ethanolic extract in MI and FB, while the aqueous extract showed more evident effect on germination rate index, NS, RE and DB. The effect of these parameters was possible to be adjusted the linear regression model, showing

concentration-dependent effect, i.e., when the concentration increased, there was a decrease of the analyzed response. The same pattern was observed by Borella et al. [18], who observed a proportional reduction in the first count and GSI. It was observed a smaller interference in germination rate compared with the others tested parameters. Compared to the control, most of the treatments presented around 100% of germination rate. This fact was not observed in any of the other parameters. This observation comes from the fact that germination is a discrete phenomenon and the other parameters evaluate a more complex process [2].

Mechanical damage, such as pruning (suffered by Alfenas population), can lead to a similar response to which occurs by herbivores in plants as an increase in the production to secondary metabolites in order to combat this attack [9, 10]. However, as in the present study, there was no greater allelopathic activity in the population with pruning management. This pattern can be a consequence of the fact that plants with different management of pruning are in similar soil and weather conditions, rainy season and radiation temperature, and these conditions can interfere in the production of secondary metabolites [5]. The obtained results suggested that these conditions influence more strongly in the production of these compounds than the stress caused by mechanical pruning.

RE is a result of a combination of different factors

among division and cell elongation [19]. Plants, which undergo a stress imposed by the different chemical compounds present in the extracts, can respond to them in several different forms affecting the elongation and division, thus leading to changes in cell differentiation [20]. Therefore, RE results are closely linked to cell division results [14, 15]. However, the data presented in this work showed that the ethanolic extract lead a greater reduction in cell division, which was not observed for RE parameter, wherein the aqueous extract showed a greater reduction than ethanolic extract. This fact can be explained by that despite the compounds present in the ethanolic extract reduce germination, they do not reduce the cell elongation as the compounds in aqueous extract. The aqueous extract, although showing a less effective reduction in cell division, showed a more pronounced reduction in RE, possibly a result of increased interference in cell elongation and not in division.

5. Conclusions

From the results of this study, it can be concluded that the different managements of pruning had no influence on the toxicity of *S. molle* extracts. In general, the kind of vegetal substances extraction (water or ethanol) has also no difference in this toxicity. In both methods for both populations, there was a concentration-dependent effect observed in *L. sativa* seeds.

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