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Seed size correlation with phytotoxic effects of *Baccharis psiadioides* essential oil during seeds germination

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ABSTRACT

We studied the sensitivity of 21-accessions, from 6-botanical families (Asteraceae, Brassicaceae, Fabaceae, Lamiaceae, Pedaliaceae, Poaceae) of different seed mass, to essential oil of *Baccharis psiadioides* (Less.) Joch. Mull during its germination. The essential oil was obtained from fresh leaves by distillation and was used as allelochemical. Seeds were submitted to a dose-response experiments to determine the sensitivity of each accession. Germination inhibition was calculated with the effective dose (ED_{50}). We found a positive correlation between the seed size and ED_{50}. Smaller seeds required less quantity of essential oil to inhibit their germination than larger seeds. Effects reported herein may be a pattern for action of other essential oils, however, others studies may be conducted relating to other seed attributes as seed reserve or embryo size.

Key-words: *Baccharis psiadioides*, crop seeds, dose-response analysis, essential oils, germination inhibition, seed size, terpenes.

INTRODUCTION

Seed size, an important functional trait in plant ecology has been much investigated (13,27,31,34,37,52,60). As lineages evolve and speciate, angiosperms moved out of tropics and shifted from being predominantly small-seeded, to much wider range of seed sizes (from the dust seeds of orchids up to the 20 kg seeds of double coconut) (14,19,37,38). Seed size is related with the amount of nutrients reserve provided in the embryo by the mother plant. The small-seeded species generally have less reserves to support the growing embryo and seedling respiration during the periods. Furthermore, small seeds have greater root length per unit of root mass, providing more absorptive surface area, through which phytotoxins might enter and cause subsequent damages.

Small-seeded species produce more seeds for a given quantity of maternal reproductive effort than the large-seeded species (20,27,37). The smaller seeds are incorporated into the soil seed bank and dispersed more easily, increasing their persistence (17,27,36). However, larger seeds are better able to tolerate stresses (Competition, shading, mineral nutrients shortage, defoliation and buried deeper in soil or litter) during the seedling establishment. The larger seed have better seed quality and more vigorous seedlings than smaller seed (11,22,35).

Most biotic and abiotic factors (temperature, light, water and salinity) can influence

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the seeds germination (2,5,7,15,64). The biotic factors have been investigated especially by allelopathy bias (33,48,49,55), wherein effects of chemical compounds released by organisms into the environment are studied. The effects of volatile essential oils from plants have been most studied on seeds germination (3,30,41,42,54,59,63), due to its relevance in plant ecology and potential implications for weed management in agroecosystems (12). Some studies have shown that small seeds are more sensitive to phytotoxins than large seeds during the germination (29,44,68,69). However, these studies have investigated few compounds, especially aqueous extracts with isoflavonoids, phenolic acid derivatives, isothiocyanates and coumarins, even without osmotic and pH control. In addition, these studies did not report the effects of volatiles terpenes, these have distinct way of action than aqueous extracts, owing to their partial solubility in water and have specific mechanism of penetration (3,16,65).

Baccharis psiadioides (Less.) Joch. Mull. (Asteraceae) is shrub growing in the grasslands and shrublands in Uruguay and South Brazil, often forming dense and dominant populations (57). This pattern of establishment indicates that the species release phytotoxic compounds into the environment inhibiting the sympatric species through allelopathy (58). The species produces large quantity of volatile compounds, easily noticeable in field due to their strong smell. Essential oil of *B. psiadioides* have already been characterized (23,62) and some of these compounds are very phytotoxic to cell division (54), seed germination and early growth (23,24,59).

This study aimed to determine the sensitivity of 21 seeds accession from 6-families (Asteraceae, Brassicacae, Fabacae, Limiacae, Pedaliacae, Poacae) to a phytotoxic essential oil duing germination.

MATERIALS AND METHODS

The test seeds were obtained from commercial seed dealers, other research groups and our own collections. We choose crop species without dormancy and with uniform germination to minimize the effects of other factors unrelated to seed size. In some species more than one cultivar were obtained. Thus, we refer to each species or cultivar as an accession. Asteraceae and Poaceae accessions refer to their fruits (cypsela and caryopsis, respectively) these are treated as seeds here.

To determine the mean seed mass of each accession, three samples of 100 seeds were dried at 105 Cº for 24 h and then weighed (100-seed mass - HSM).

Moisture (%) was calculated as: {[(Fresh weight - Dry weight)/Fresh weight] x 100}.

Plant material and essential oil

Leaves of *B. psiadioides* were collected between September and October 2015 from the natural populations at Morro Santana (30°03'S, 51°07'W), in city of Porto Alegre (Rio Grande do Sul State, Brazil). A voucher specimen was deposited in the Herbarium ICN of Federal University of Rio Grande do Sul (ICN 175007). The collected material was dried at room temperature for 2-weeks. Then, essential oil was obtained from the leaves by steam distillation from 6 kg dry leaves using an inox extractor with a steam flow rate of 3 L/h for 1 h, yielding 0.46% (w/v). Anhydrous sodium sulfate was used to remove residual water from essential oil samples. Essential oil was stored in glass vials (5 mL), wrapped in aluminum foil and stored in ultra freezer (-80 °C) prior to use.

Bioassays

To determine the sensitivity level of each accession to phytotoxic essential oil, we submitted each accession to dose-response experiments. The dose-response experiments consisted of 8-10 treatments and four replicates of each treatment per accession, in completely random design. The essential oil doses in treatments varied from zero (control) to 150 µL (Table 1) of pure essential oil, being the range of doses studied for each accession in pilot tests.

Thirty seeds were used in each replicate. Bioassays of Accessions with HSM < 2,000 mg (small seeds) were conducted in glass Petri dishes (10 cm x 1.5 cm); and accessions with HSM > 2,000 mg (large seeds) were conducted in germination boxes (gerbox) with glass cover (11 cm x 11 cm x 3.5 cm). Seeds were placed on one layer of quantitative filter paper with 7 mL of distilled water into Petri dishes and 10 mL into germination boxes. Because the larger seeds did not germinate in the Petri dishes.

Essential oil was added onto a cotton ball attached with double-sided tape to the inner face of the container lid (gerbox or Petri dishes) (Fig. 1) without direct contact between seeds and water, but allowing the compounds to volatilize into the airspace within the box. Control treatments did not receive oil. The gerboxes and petri dishes were sealed with plastic film to minimize loss of the volatiles and incubated in germination room at 22 ºC (+/- 2ºC), with long-day photoperiod (16 h light). The illumination was provided by white fluorescent lamps (20W), with an irradiance of 48 µmol m^{-2} sec^{-1}.

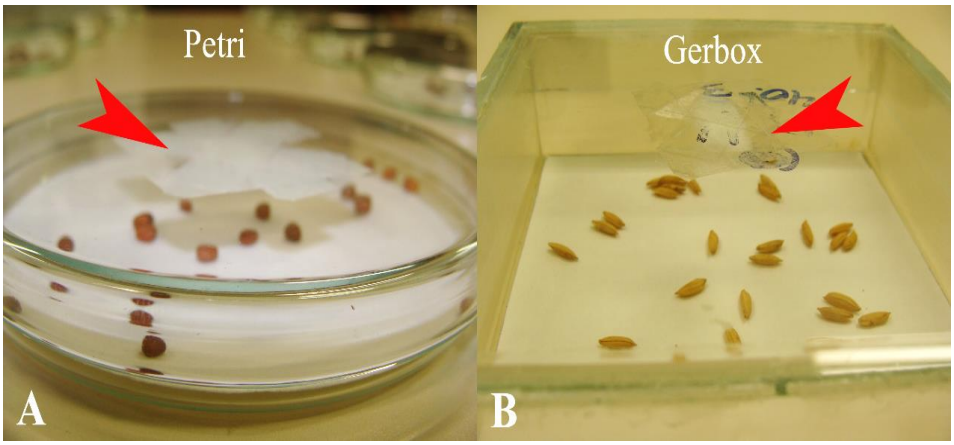

Figure 1. Petri and Gerbox dishes used for the bioassays. Essential oil was added onto a cotton ball attached with double-sided tape to the inner face of the container lid (see the red arrows).

The seeds were kept in the germination chamber for three weeks. The number of germinated, seeds in each replication was counted on the first, third and last day of the experiment. Seeds were considered germinated when they had a radicle longer than 2 mm. Control of each accession usually reached the highest germination rate in the first 5-7 days, but all treatments were maintained until end of experiment to determine the inhibition of germination. Experiments were conducted between January and December 2016.

Each accession germination inhibition by the essential oil was calculated with the mean effective dose (ED_{50}), which corresponds to the dosage of essential oil capable of inhibiting the germination of 50% population than control of each accession in the same conditions. The ED_{50} was calculated by a nonlinear regression using open-source statistical environment R (47), with the add-on package *drc and* three-parametric log-logistic model as previously proposed (50,51).

Statistical analysis

The ED_{50} were subjected to analysis of covariance using the log_e-transformed mean 100-seed mass (HSM - in milligrams) of each accession as a quantitative explanatory factor. Log_e-transformation was necessary to meet normality requirements for generalized linear model. We also compared the ED_{50} average of smaller and larger accession between groups by univariate analysis of variance with randomization (PERMANOVA) with 10,000 bootstrap iterations, using Euclidean distance as dissimilarity measure. Correlations and regressions between ED_{50} and HSM were performed using the software InfoStat (21) and PERMANOVA was performed using the software MULTIV (45).

RESULTS AND DISCUSSION

Accessions from six angiosperm botanical families: Asteraceae, Brassicaceae, Fabaceae, Lamiaceae, Pedaliaceae and Poaceae varied in their seeds sizes (mass) from 8.4 mg to 32,781.5 mg. Hence, these accessions also differed in their germination susceptibilities to phytotoxic essential oil, with ED_{50} varying from 15.86 µL to 114.9 µL (Table 1). This difference was corroborated by significant difference between mean ED_{50} from smaller and larger seeds groups (Fig. 2).

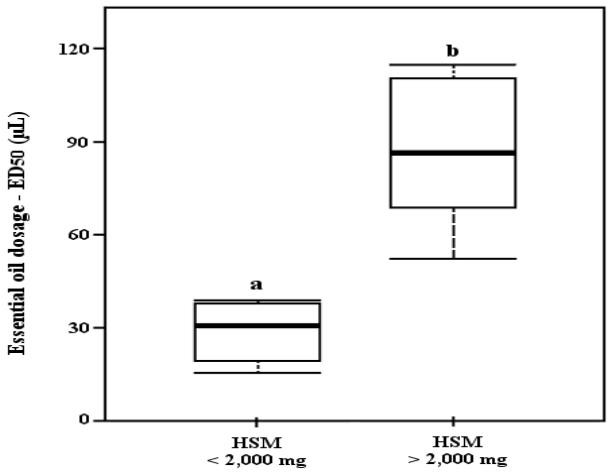

Figure 2. Mean ED_{50} from smaller and larger seeds groups in box plot with upper and lower limits from each group. Groups differed significantly according to PERMANOVA with randomization at $p < 0.001$ level.

Correlation between the ED_{50} and HSM (r = 0.85, P < 0.001) (Fig. 3), indicated that during the germination smaller seeds were more sensitive to phytotoxic effects of essential oil seeds than larger seeds. Larger seeds required higher dose of essential oil to inhibit their germination. For example, the germination of *Triticum* and *Zea* accessions was inhibited at 60 µL and 112 µL, respectively, whereas, 19 µL inhibited the germination of *Origanum* and *Matricaria* compared with control. No correlation was observed between the seed moisture and phytotoxicity susceptibility.

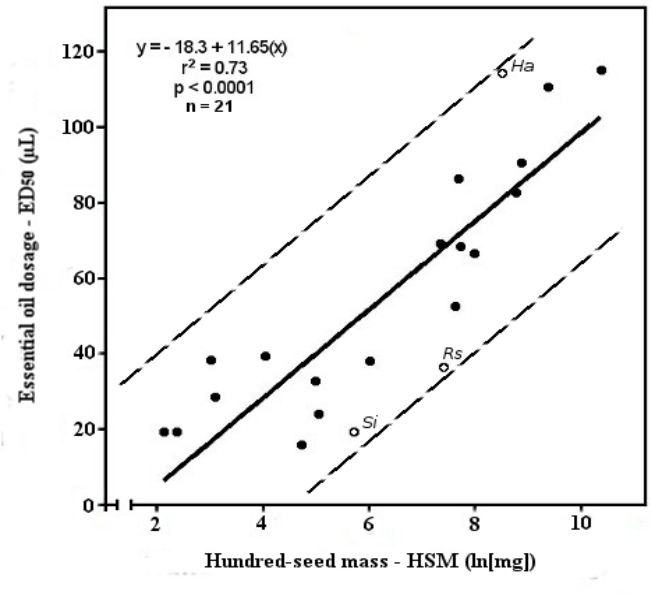

Figure 3. Effective essential oil dosage (ED50) of each accession as a function of seed mass. Twenty one accessions differing in their seed size were exposed to a dose-response experiment to determine essential oil dose capable inhibit germination of 50 % of population compare to each control treatment. The dashed lines represent an approximate 95% confidence interval of the regression as a function of the proposed model. See text for methods of bioassays, calculating ED_{50} and data analysis.

Three accessions caught attention to its limit border using the adjusted regression model: in the lower limits, *Sesamun indicum* L. (red) and *Raphanus sativus* L. (blue) had an ED_{50} under the expected as function to their seed mass; alternatively, *Helianthus annuus* L. (green) had an ED_{50} almost above the limits margin of the predictive equation (Fig. 2). These cases may be related to other seed attributes: differences in seed reserves might contribute to variation among species in their ability to mobilize reserves, respond to temperature and also detoxify the phytotoxins (6,18). The three accessions are widely recognized as oleaginous seeds (9,10,43) and lipid content of seeds may have a specific lipophilic interaction with essential oil, although this hypothesis still has to be investigated with an appropriate experimental design.

Table *1*. Seed species used in bioassays, 100-seeds mass (mg), moisture content, range of essential oil doses used in bioassays (excluding control - 0 µL) and ED_{50}.

Species	100-seed mass 'HSM' (mg)	Moisture control (%)	Range of essential oil doses used (µL)	ED_{50} (µL)
Family : Asteraceae				
Helianthus annuus L. 'Multissol'	5043.5	5.93	0, 40, 50, 60, 70, 80, 90, 100, 110, 120	114.08
Matricaria recutita L.	10.7	20.74	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	19.16
Family : Brassicaceae				
Brassica oleracea L.'Bortytis'	418.3	6.94	0, 15, 25, 30, 35, 40, 45, 50, 55, 60, 70	38.49
Eruca sativa Mill.	147.9	6.21	0, 10, 25, 30, 35, 40, 45, 50, 55, 60	32.78
Nasturtium officinale R.Br.	20.4	9.13	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	38.21
Raphanus sativus L. 'Acanthiformis'	1637.9	4.40	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	36.46
Family : Fabaceae				
Cajanus cajans (L.) Millsp) 'IAPAR 43'	6540.1	10.53	0, 25, 35, 45, 55, 60, 70, 80, 90	82.45
Crotalaria spectabilis Roth	1556.7	9.30	0, 15, 25, 35, 40, 45, 50, 60, 65, 70	69.20
Lens culinaris Medik.	7167.6	16.72	0, 40, 50, 60, 70, 80, 90, 100	90.44
Lotus corniculatus L.	114.6	11.14	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	15.86
Trifolium repens L.	57.25	7.47	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	39.25
Family : Lamiaceae				
Origanum vulgare L.	8.4	7.18	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	19.18
Family : Pedaliaceae				
Sesamun indicum L. 'IAC Ouro'	301.4	4.48	0, 5, 10, 15, 20, 25, 30, 35, 40, 45	19.26
Family : Poaceae				
Eragrostis plana Nees	22.05	8.79	0, 5, 10, 15, 20, 30, 35, 40, 45, 50	28.55
Oryza sativa L. 'IAC 500'	2205.4	10.38	0, 40, 50, 60, 70, 80, 90, 100	86.19
Oryza sativa L. 'BRS Nipon'	2299.5	9.86	0, 40, 50, 60, 70, 80, 90, 100	68.32
Paspalum notatum Flüggé	156.2	10.94	0, 10, 15, 20, 25, 30, 35, 40, 45, 50	24.02
Triticum aestivum L. 'TBIO Parrudo'	2975.8	10.91	0, 40, 50, 60, 65, 70, 80, 90	66.58
Triticum aestivum L. 'TBIO Sossego'	2075.4	10.80	0, 30, 35, 40, 50, 60, 70, 80	52.39
Zea mays L. 'IAC 125 Pipoca'	11830.5	9.89	0, 60, 90, 95, 100, 105, 110, 120,	110.50
Zea mays L. 'IAC 8390'	32781.5	10.17	0, 60, 90, 100, 105, 110, 120, 130	114.90

Between the accessions from the same species that varied in cultivars, slightly difference in ED_{50} were found: larger cultivar *Zea mays* 'IAC 8390' needed up to 114.9 µL, whereas, smaller cultivar 'IAC 125 Pipoca' needed 110.5 µL. In *Triticum aestivum*,

the seeds of heaviest cultivar 'TBIO Parrudo' had 66.5 µL, whereas, lighter 'TBIO Sossego' had 52.3 µL. This pattern of seed size variation within same species has been explored by Williams and Bartholomew (68,69). These authors used a mixed seed size population to separate a large population of seeds from two single species (*Vicia villosa* Roth. and *Raphanus sativus* L.) into three size classes (large, medium and small). This allowed the analysis of the effects of a given phytotoxin (coumarin) directly as a function of seed size and confirmed statistically, that smaller seeds are more sensitive to phytotoxic aqueous extract of coumarin in germination response to coumarin at 10^{-3} and 10^{-5}.

Our results are consistent with other researchers working with non-essential oil phytotoxins and target species representing a range of seed masses. Firstly, Putnam and DeFrank (46) reported that in field experiments, the cover crop residues of barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor* L.) suppressed the germination and growth of smaller-seeded vegetables (e.g., radish, (*R. sativus*), lettuce (*Lactuca sativa* L.) and weeds (e.g., smooth crabgrass (*Digitaria ischaemum* Schreb ex Muhl.), pigweed (*Portulaca oleraceae* L.), but did no effect the growth of larger-seeded vegetables (bean, *Phaseolus vulgaris* L., pea, *Pisum sativum* L.). Burgos and Talbert (8) found that inhibition of root and shoot growth was more in small-seeded species than in larger-seeded species, when aqueous extracts of rye were applied to seeds of six crop and nine weed species in Petri dish bioassay. Likewise, Petersen (44) used five weed species and wheat seeds placed on filter paper in Petri dishes, exposed to methanol-water solutions containing isothiocyanates released by turnip-rape mulch (*Brassica napus* L.) and observed a negative correlation between the seed mass and germination percentage, with smaller seeds being more inhibited than larger seeds. Finally, Liebman and Sundberg (29) studied many groups of weed and crop species (62 in total) spanning three orders of magnitude in mass. They quantified relationships between the seed size and susceptibility to phytotoxins derived from aqueous extracts of red clover shoots (*Trifolium pratense* L.) in laboratory bioassays using filter paper and Petri dishes. Greater germination and radicle inhibition occurred in lighter seeds and least in heavier seeds. Additionally, drastic radicle inhibition was observed in monocotyledonous than in dicotyledonous species, when seed size was similar.

Small-seeded species were more susceptible to phytotoxins than large-seeded species for reasons that have been discussed within seed ecology. The seed size is related to the amount of nutrients reserve provided for embryo by mother plant. Thus, small-seeded species generally have less reserves to support the growing embryo and seedling respiration, during periods of stress-induced by carbon deficit, suffering reductions in early embryo growth (28,29,67). Furthermore, small-seeded species have longer roots per unit of root mass (56), providing higher absorptive surface area through which phytotoxins might enter and cause subsequent damages. Moles and Westoby (36) explored relationships between the seed mass, tolerance to various stresses (nutrient deficits, drought, burial, shading, defoliation and resource competition) and fecundity. They concluded that the less stress-tolerance of smaller-seeded species is offset by their ability to produce more seeds. Therefore, small-seeded species, which were highly susceptible to phytotoxins, in the present study, might persist abundantly in an ecological system with released phytotoxins, unless their survival and fecundity were greatly diminished.

Essential oil of *B. psiadioides* has been characterized (23,53,62) and the differences in the oil composition are known. The main constituents have been identified as monoterpenes hydrocarbons, followed by sesquiterpenes hydrocarbons, oxygenated monoterpenes and oxygenated sesquiterpenes, with monoterpene β-pinene being the major compound in the oil (23,53); whereas Suyenaga *et al*. (62) characterized the oil specially by sesquiterpenes hydrocarbons, the major compound being the germacrene D and oxygenated monoterpenes were not identified. Although no attempt was made in the present study to isolate or to characterize the chemical agents present in *B. psiadioides* essential oil, previous investigations lead us to believe that observed effects might be caused by synergistic activity exerted by the compounds present in the essential oil and not just by its major compounds (54).

Mode of action of phytotoxins derived from terpenoid pathway is not fully understood, due to numerous constituents, essential oils do not have specific cellular targets (4). Mono and sesquiterpenes reduces the mitotic activity of root cells - apparently by impeding the cell nuclear and organellar DNA synthesis, to inhibit the mitochondrial respiration of isolated organelles, to increase reactive oxygen species production and to disrupt the cell membranes (4,32,40,61). The *B. psiadioides* essential oil decreased the mitotic activity and chromosomal abnormalities in root meristematic cells in onion and lettuce seedlings (54). Furthermore, oxidative damage with H_2O_2 accumulation occurred (24) and was related with depigmentation of cotyledons in *Arabidopsis thaliana* (L.) Heynh seedlings. In fact, oxidative molecules can degrade the chlorophyll or inhibit its synthesis and consequently affects the photosynthesis (1,66). In the present study, cotyledons of seedlings accessions that germinated became whitish after exposure to the oil and at the end of bioassays, germinated and non-germinated seeds showed the disintegrated structure. Therefore, morphological effects observed can be assigned to the potential of oil to cause damages in mitosis, to affect the photosynthesis mediated by oxidative damage and also to disrupt the cell membranes.

Effects reported herein may be a pattern for action of other essential oils. Preliminary tests with essential oil from other species have shown the same results: smaller seeds were more sensitive to phytotoxic effects (data not shown). However, others studies may be conducted including other seeds - spanning different orders of magnitude or different botanical families - or relating other attributes as seed reserve or embryo size.

CONCLUSIONS

Seed size is important trait in plant ecology, with real implications in allelopathy. Small-seeded species tends were more sensitive to an allelochemical during its germination than larger-seeded. The sensitivity of the seeds to an allelochemical may also be related to others seeds attributes as seed reserve.

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