Brazilian Journal of Biology
ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

https://doi.org/10.1590/1519-6984.178222
Original Article

Germination constraints of dicarpic cypselae of *Bidens pilosa* L.

P. R. M. Souza-Filho[a]* and *M. Takaki*[b]

[a]Centro Multidisciplinar da Barra, Universidade Federal do Oeste da Bahia – UFOB, Avenida 23 de Agosto, s/n, CEP 47100-000, Barra, BA, Brasil

[b]Laboratório de Fotomorfogênese de Plantas, Departamento de Botânica, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Avenida 24-A, 1515, CEP 13506-900, Rio Claro, SP, Brasil

*e-mail: paulorob2000@gmail.com

Received: April 10, 2017 – Accepted: January 12, 2018 – Distributed: August 31, 2019
(With 6 figures)

Abstract

Bidens pilosa L. is a heterocarpic weed species with two cypselae types that present morpho-physiological differences, being the peripheral type smaller and slower to germinate than the central one. We aimed to verify how the germination mechanism varied between types. We focused on two mechanisms: (1) pericarp constraints (physical and chemical) and (2) hormonal stimulation (Abcisic acid [ABA] and Gibberellin [GA]). Both cypselae types are physically constrained by the pericarp, for when it is excised both seed types increase their germination, but behavioral differences still remain. The pericarp of the peripheral type also has chemical inhibitors that effectively inhibited germination of the intact central cypsela. To test the hormonal effects, we focused on the ABA:GA control. Both cypselae responded to an exogenous ABA concentration gradient, however there is no variation between types on the sensitivity to it. Also, both cypselae types were indifferent to Fluridone (ABA inhibitor), which indicates that the dormancy is not maintained by *de novo* ABA synthesis. Cypselae types had different sensitivity to an exogenous GA_3 gradient, the central type being more sensitive to the treatment than the peripheral one. But when the endogenous GA synthesis was blocked by Paclobutrazol, both types responded equally to same GA_3 concentrations. This indicates that endogenous GA synthesis may be related to differences observed on germination of cypselae types. To conclude, seed types differ on their growth potential to overcome the pericarp resistance: while the inhibitor in the peripheral pericarp reduces growth potential, GA increases it.

Keywords: hormonal controlling, pericarp inhibition, heteromophic diaspores.

Restrições a germinação de cipselas dicárpicas de *Bidens pilosa* L.

Resumo

Bidens pilosa L. é uma espécie de planta daninha heterocarpica com dois tipos de cipselas que possuem diferenças morfofisiológicas, sendo o tipo periférico de menor tamanho e com germinação lenta se comparado com o central. Nosso objetivo foi verificar como o mecanismo de germinação varia entre os tipos. Focamos em dois mecanismos: (1) restrição causada pelo pericarpo (física e química) e (2) estímulo hormonal (Ácido abscísico [ABA] e Giberelina [GA]). Os tipos de cipselas são fisicamente limitados pelo pericarpo, pois quando ambos os tipos de sementes são excisados há um aumento na germinação, contudo as diferenças no processo se mantém. O pericarpo do tipo periférico ainda possui inibidores que efetivamente retardam a germinação das cipselas centrais intactas. Para testar os efeitos hormonais, nós focamos no controle pelo ABA:GA. Ambas cipselas responderam ao gradiente de concentração de ABA exógeno, contudo não houve variação na sensibilidade entre os tipos. Ainda, ambos tipos de cipselas foram indiferentes à Fluoridona (inibidor de ABA), que indica que a dormência não é mantida pela nova síntese de ABA. Tipos de cipselas apresentam diferentes sensibilidades ao gradiente exógeno de GA_3, com o tipo central sendo mais sensível ao tratamento que o periférico. Mas quando a síntese endógena de GA foi bloqueada pelo Paclobutrazol, ambos os tipos responderam de forma similar às concentrações de GA_3. Isso indica que a síntese de GA endógena pode estar relacionada com a diferença observada na germinação dos dois tipos de cipselas. Para concluir, os tipos de sementes diferem no potencial para superar a resistência do pericarpo, sendo o inibidor no pericarpo da cipsela periférica o redutor do potencial de crescimento, enquanto a GA aumenta esse potencial.

Palavras-chave: controle hormonal, inibição do pericarpo, diásporos heteromórficos.

1. Introduction

Many higher plants produce diaspores with two or more morpho-physiological traits to favor seed establishment and plant dispersion, a strategy known as seed heteromorphism. This reproductive strategy is found in many plant families and it is particularly common in Asteraceae (Imbert, 2002; Baskin et al., 2014). In *Bidens*, the accepted dicarpy model is that the central cypsela is bigger and germinates faster than the peripheral ones (Forsyth and Brown, 1982; Rocha, 1996; Amaral and Takaki, 1998; Brändel, 2004). They follow the H/L-L/H strategy according to Baskin and collaborators (2014). Thus, the central type has more chances to widen its spatial dispersal area. On the other hand, the germination delay allows the peripheral one to increase its temporal spread.

To avoid early establishment and environmental threats, fresh seeds germinate after some requirements are found, which vary among species populations. This barrier is alleviated by environmental signals that trigger inner embryo mechanisms provoking root protrusion, which is germination *stricto sensu* (Finch-Savage and Leubner-Metzger, 2006; Martins et al., 2015). In many cypselae (or achenes) species, the pericarp restricts embryo growth by two main ways: mechanical restriction and/or chemical inhibition. The pericarp may limit water and air exchanges, causing a delay in seed germination (Venable and Levin, 1985; Aguado et al., 2011). It also impedes embryo growth mechanically through the presence of fiber layers or hard tissues (McEvoy, 1984). Also, the pericarp may contain chemical inhibitors that block the progress of embryo development (Matilla et al., 2005); those soluble leachates retard cypsela germination (Forsyth and Brown, 1982; Beneke et al., 1993).

Bidens pilosa L. is an Asteraceae species that has crop weed importance and it is worldwide distributed. Its germination was investigated previously, which indicated its dicarpic behavior (Forsyth and Brown, 1982; Rocha, 1996; Amaral-Baroli and Takaki, 2001). However, the mechanism which induces different physiological behaviors in each cypsela type is under discussion. Rocha (1996) suggests the peripheral cypsela pericarps are thicker than the central ones. Forsyth and Brown (1982) showed an inhibitor effect from the peripheral type cypsela. Amaral-Baroli and Takaki (2001) proposed that both types have different environmental sensitivity.

The main control mechanism of germination is the hormonal balance between Abscisic acid (ABA) and Gibberellins (GA). The first one is related to dormancy maintenance, the second triggers the embryo growth. The relation between the concentration (synthesis or degradation) and the sensing of each hormone will trigger the germination process (Finch-Savage and Leubner-Metzger, 2006; Barua et al., 2012). The hormonal balance can be influenced by secondary compounds which may maintain the dormancy (Matilla et al., 2005).

The aim of this work was to study the germination behavior of dicarpic *B. pilosa* via hormonal stimuli and pericarp removal manipulations. Initially we focused on verifying the behavioral variation: (1) germination of four populations and (2) their ABA sensibility, via exogenous ABA and Fluridone gradients. We verified the pericarp removal manipulations for: (1) isolated seed performance; (2) effect of pericarp leachate on isolated seed or intact cypsela germination; (3) exogenous GA sensitivity of intact and pericarp removed seeds. Our hypothesis is that the pericarp inhibitor on the peripheral cypsela will induce ABA synthesis on the peripheral cypselae, which moves the hormonal balance to a dormant state. On the other hand, the absence of that inhibitor makes the central type germination faster than peripheral ones.

2. Material and Methods

2.1. Cypselae collection

Cypselae of *Bidens pilosa* L. were collected from populations growing in rural or urban sites at Rio Claro, SP, Brazil. The distances between populations varies from 5.21 to 13.5 km (Table 1). The ripened capitulum was collected from, at least, twenty five plants. The cypselae of each plant, or small group (when they were tangled), were stored in paper bags under room conditions (approx. 25 ± 2 °C) for less than one month. When needed for a new experiment, fresh cypselae were collected at the same site to avoid after-ripening effects on stored seeds.

2.2. Germination variation among populations

To analyze physiological variations among populations, we carried out germination assays with freshly collected cypselae (one day storage). Prior to the assay, cypselae were selected by each type, and then twenty-five of them were put inside 90 mm Petri dishes lined with two layers of filter paper (Prolab, Brazil). Then the dishes were moistened with 5 mL deionized water and kept under constant fluorescent white light stimulus (approx. 30 μmol. $m^2.s^{-1}$) or in the dark (inside black polystyrene boxes) at 25 ± 1 °C in a germination room. Five replicates were made for each treatment. Germinated seeds were scored every day for light treatment and weekly for the dark treatment, the latter occurring in a dark room under dim green safe

Table 1. Location and features of population sites.

Population	Coordinates	Features
Cana	22°26'7.42"S; 47°37'54.05"W	Roadside near a sugar cane crop.
Horto	22°25'28.99"S; 47°30'5.92"W	Site surrounded by sugar cane crop.
Ajapi	22°20'49.36"S; 47°32'42.87"W	Site surrounded by sugar cane crop.
11-B	22°24'20.57"S; 47°32'52.31"W	Urban wasteland site.

light. At the end of the assay (after 3 weeks), the viability of non-germinated cypselae was tested with tetrazolium (0.5% in phosphate buffer kept at 41 ± 1 °C for 24h).

2.3. Variation of sensitivity to abscisic acid

To evaluate the effect of ABA on the germination of cypsela types, we applied exogenous ABA or Fluoridone (FLU). Twelve cypselae of each type were placed inside 50 mm Petri dishes lined with two layers of filter paper (Prolab, Brazil) moistened with 3 mL of different solutions. Four replicates were made for each treatment. Assays were performed for the four populations. Solutions were: 0.05, 0.5, 5 µM ABA (Sigma-Aldrich Co., US); 0.1, 1, 10 µM FLU (Fluka; Sigma-Aldrich Co., US), and both stock solutions were diluted in ethanol (max. 0.1%); concentrations were chosen based on previous assays and literature (Barua et al., 2012). Petri dishes were maintained under the same conditions (light and darkness) described above. Every week, cypselae were placed into dishes with a newly prepared solution to ensure constant hormonal concentration. Censuses and dish exchange were performed daily at room temperature for light treatments, and in the darkroom for dark treatments. The assays lasted three weeks and the viability of non-germinated cypselae was tested, as mentioned before.

2.4. Pericarp influence on seed germination

To analyze the role of pericarp in seed germination, we used the "Horto" population due to high cypsela availability and their physiological responses. Freshly collected cypselae were kept for 4 to 12h in darkness in a humid chamber (no direct contact with liquid water) prior to pericarp removal. Then, in the dark room, under dim green safe light, the pericarps of 12 cypselae per Petri dish were carefully separated from the seeds (testa, endosperm and embryo) using forceps and a stereoscope. Treatments were: (1) intact cypselae; (2) intact cypselae with crushed pericarp of another type (crossed fruits influence); (3) excised seeds; and excised seeds of both types with crushed central (4) and peripheral (5) pericarps. Pericarps were crushed with a mortar, and silica sand was added to help with the crushing. The cypselae or seeds were put inside 50 mm Petri dishes, to optimize the leachate effect, lined with one layer of filter paper (Prolab, Brazil), under which one layer of silica sand (treatments 1 and 2) and powdered pericarps (treatments 3 and 4) were added. All dishes were moistened with 3 mL deionized water and maintained under the same conditions (light and darkness) such as detailed above. Germination time was recorded for further analysis twice a day for the light treatments, to verify for subtle differences, and once a week for the dark treatment.

2.5. Gibberellic acid effects on germination

For the first assays, we used the "Horto" population. Four replicates of 30 cypselae of each type were put inside 90 mm Petri dishes lined with two layers of filter paper (Unifil, Germany) moistened with 7 mL of GA_3 solution. The GA_3 (VETEC, Brazil) work solutions were diluted in ethanol and deionized water was added to reach final concentrations of 50, 100, 500, and 1000µM, determined via previous tests.

The assays were maintained under the same conditions mentioned above (light and darkness); dishes were renewed twice a week, to ensure constant hormonal concentration, and scored daily for both treatments. Seeds in darkness treatment were counted daily to account for its germination behavior. At the end of the assay (after 30 days), the remaining non-germinated cypselae were tested with tetrazolium. The dead seeds were excluded from the statistical analysis.

2.6. Sensitivity to exogenous gibberellic acid

To assess the sensitivity for exogenous GA, "Horto" and "Ajapi" populations were used in this second assay. Four replicates with 12 cypselae or excised seeds of each type were placed in 50 mm Petri dishes lined with one layer of filter paper to favor the pericarp leachate diffusion. The manipulation treatments were: (1) intact cypsela, (2) isolated seeds, and (3) seeds with crushed pericarp. Fruit removal followed the protocol described above. All dishes were then moistened with 3 mL of solution or deionized water for the control. Solutions were Paclobutrazol (Pestanal®; Sigma-Aldrich Co., US) 200 µM diluted in acetone (PAC), and 10, 100 µM of GA_3 diluted in ethanol. The final concentrations of the work solution were 0.03% acetone and 0.1% ethanol (to 100µM GA_3), and it may have had toxic effects for some seeds. We used an endogenous GA inhibitor (PAC) to avoid the bias caused by GA synthesis variation among embryo types (detailed below). The dishes were renewed weekly to ensure constant solution concentrations due to GA stability.

2.7. Statistical analysis

We tested germination behaviors such as (1) final germination proportion and (2) germination rate, by two modeling approaches: (1) Generalized Linear Mixed Models (GLMM) and (2) Time-to-event Analysis, respectively, both focusing on cypselae or seeds as sampling units. These modeling methods were preferred to deal with individual seed behavior rather than with the population inside dishes (Sileshi, 2012). All analyses were performed with R (R Development Core Team, 2013).

The final germination proportion of the treatments was analyzed by multiple regression generated by GLMM or GLM (General Linear Models), the latter when the random effect was not significant. To make the models we used Binomial distribution of the errors with log link ('lme4' package (Bates et al., 2014)). Model building followed Zuur et al. (2009) recommendations.

When chosen, via model comparison excluding the non-significant variables, the final model coefficients (β) were analyzed by multiple pairwise Z-test comparison via least-square means ('multcomp' package (Hothorn et al., 2008) and 'lsmeans' package (Lenth, 2016)).

For germination behavior responses, we used Time-to-Event analysis, also known as survival analysis

('survival' package (Therneau and Grambsch, 2000)), which allows analysis of events such as germination (that occurs over time), and quantification of effects of contributing factors (Onofri et al., 2010). The germination times of the individual seeds were used to calculate the probability that one seed may germinate after a specific time t once the assay began. The time the seeds take to germinate is preferred to final germination proportions to detect variations in seed behaviors. The censuses were considered to yield "exact data" due to daily verification. Non-parametric Kaplan-Meyer estimator was made before modeling to estimate germination probability. Parametric Accelerated Failure Time (AFT) modeling was then run to estimate the best distribution (Weibull, log logistic, logarithmic or exponential). Based on Akaike´s Information Criterion we chose the model that best fitted the non-linear regression model of cumulative germination data. The AFT is known as fully parametric because the survival functions follow the parameters of the chosen distributions. It also assumes there is a linear relationship between the logarithm of time to germinate and the analyzed factors. The latter accelerates or decelerates germination curves according to a coefficient (b). For the models, we used fixed explanatory variables: cypsela types, light treatments, the assay treatment (depending on the assay), and all interactions. Replicates and/or populations were tested as clustering random effects and included in the model using the frailty function of the survival package with Gamma distribution, to check if it had significance within the model; if not, then it was removed from the model (Onofri et al., 2010; Délye et al., 2013).

The F-statistics were used to verify the significance of the variable inputted in the model. When ready, the final model was Z-tested for factor effects on the germination curve coefficients via multiple comparison, with Bonferroni p-value adjustment.

For both model-building procedures, the non-viable cypselae, which were tested with tetrazolium at the end of each assay, were excluded from the analysis. We assumed that their embryos were either undeveloped or damaged, but it wasn't possible to detect which because of the pericarp structure.

3. Results

3.1. Germination variation among populations

Germination proportion varied for *B. pilosa* cypsela types, with the peripheral cypselae presenting lower germination proportion when compared to the central ones for the whole model (Figure 1A, $\beta = -2.27 \pm 0.71$, $z = -321$, $p = 0.001$). Also, both cypselae types showed a significant requirement for white light to trigger its germination ($\beta = 6.80 \pm 0.73$, $z = 9.27$, $p<0.001$), even though there is some germination for two populations. "Cana" and "Horto" populations presented no germination for both types under the darkness treatment. The final germination proportion model included cypselae types, light treatment, population, cypselae types x population and light treatment x population interactions as fixed effects. The model distribution was binomial linked by log logistic function.

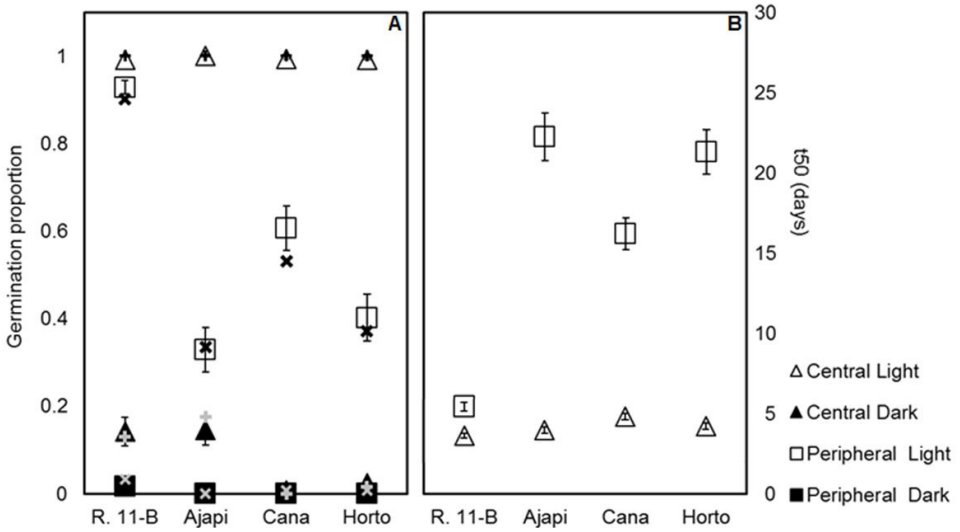

Figure 1. Estimated values of final germination proportion (A) and Time to 50% germination (t50) of *B. pilosa* cypsela (B). The symbols represent estimated values for cypselae types (*types*) and the light treatment (*light*) among the collected populations (*populations*). The binomial model accounted for *types*, *light*, *populations*, *types* x *light* and *types* x *populations* as fixed effects. For graphical purpose, the random effect was not considered. Crosses represent the real data results. The time-to-event analysis model (AFT) of the germination time generated accounts for *types*, *light*, *populations* and all the interactions. For graphical purposes, random effects were not considered. Error bars = standard error. The "+" and "x" represent real data results.

Under white light, cypselae types differed for all populations, represented for its t_{50} (i.e. time to 50% germination). Central cypsela type germinated faster than peripheral ones (b = 0.42 ± 0.17, z = 2.39, p = 0.002). The model estimated around 4 days to accomplish 50% germination of central cypselae, while for the peripheral ones the model estimated between 5 to 22 days. The germination behavior among populations varied for peripheral cypsela, with "11-B" having a significant faster germination than other populations (p<0.001), while the central ones did not differ. The AFT model for the germination rate was built considering populations, cypselae types and their interaction as fixed variables, and the dishes were considered as random effects, and the model was log logistic distributed.

3.2. Variation of sensitivity to abscisic acid (ABA)

The increased ABA concentration reduced germination of the cypselae (Figure 2A, β = -2.68 ± 0.74, z = -3.59, p<0.001, 0 vs. 5 µM of ABA). Cypselae types (β = -1.09 ± 0.32, z = -3.36, p<0.001) and light treatment (β = 4.09 ± 0.39, z = 10.54, p<0.001) were also significantly different. However, the triple interaction was not significant for the model, thus it is not possible to test for the pairwise comparison. The ABA sensitivity assay model was made with three main effects, i.e. cypsela type, light and hormone treatments, and the interaction of the cypsela type:light treatment and hormone:light treatment. Also, populations were added to the model as random effect. The model distribution was binomial linked by log function.

Both main effects, cypsela type (Figure 2C, b = 0.79 ± 0.05, z = 15.68, p<0.001) and hormonal concentration (Figure 2C, b = 0.12 ± 0.01, z = 9.77, p<0.001), were significantly different. In addition, the interaction showed variation between cypselae types within the ABA gradient (b = 0.09 ± 0.02, z = 4.49, p<0.001). It is probably related to intrinsic cypsela germination delay. Thus, ABA reduces the germination timing at a similar proportion for both types. For the AFT germination rate model all treatments and their interactions were significant: cypsela type, hormone and interaction, by log logistic distribution. The population was added as random effect.

To verify variation among populations, we made a new GLMM model with just the 5µM ABA concentration. There is a difference on the population responsiveness, while we did not detect variation on cypsela type germination for the whole model (Figure 2B, β = -0.76 ± 0.50, z = -1.52, p = 0.13). However, through pairwise comparison, peripheral types of all populations presented slower germination than central ones, except for the 11-B population (b = 0.75 ± 0.42, z = 1.77, p = 0.08). For that population model, cypselae types and their interactions were the fixed effects variables and the dishes were considered random effects, and it was log logistic distributed.

Both cypselae types were insensitive to FLU, indicating that *de novo* ABA synthesis neither influenced final germination proportion (Figure A1A of Annex A, β = 0.25 ± 0.21, z = 1.22, p = 0.22) nor rate (Figure A1B of Annex A, β = 0.00 ± 0.005, z = 0.02, p = 0.99) for cypselae of *B. pilosa*. For the germination proportion model (Binomial distributed), the main effect of FLU concentration increase was not significant, i.e. there is no seed behavior variation. Also, the AFT model showed no response to FLU. The yellowish seedling color observed may indicate that there is no ABA *de novo* synthesis that inhibits embryo germination for both types.

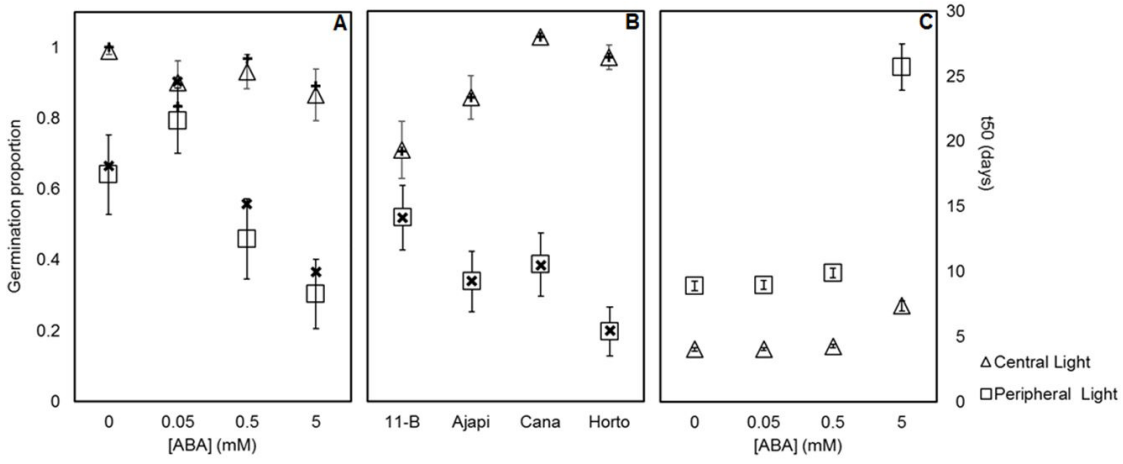

Figure 2. Estimated values of final germination proportion on exogenous Abscisic acid gradient (A), final germination proportion of the populations submitted to 5 µM of ABA (B) and time to 50% germination (t50) of the ABA gradient of *B. pilosa* cypsela (C). The symbols represent estimated values for cypselae types (*types*) among different ABA concentrations (*hormone*) and collected populations (*populations*). The binomial model for the ABA concentrations accounted for *types*, *hormone*, *types* x *hormone* as fixed effects, and the *populations* as random effect. The binomial model for the populations on 5 µM of ABA accounted for *types*, *populations*, *types* x *populations* as fixed effects, and the replicates as random effect. The time-to-event analysis model (AFT) of the germination time generated accounts for types, hormone *and* types *x* hormone. For graphical purposes both random effects were not considered. Error bars = Standard error. The "+" and "x" represent real data results.

3.3. Pericarp influence on seed germination

According to the model, the mechanical removal of the pericarp increased the germination proportion (Figure 3A, β = 5.42 ± 1.35, z = 4.02, p<0.001, Intact vs. No fruit), which was evident for the treatment under darkness. Chemical restriction was not observed for the intact cypsela (β = -15.57 ± 1009.57, z = -0.02, p = 0.99, Intact vs. Crossed), neither for the isolated seed in central (β = -1.37 ± 0.91, z = -1.50, p = 0.13, Intact vs. Central) nor peripheral (β = -1.37 ± 0.91, z = -1.50, p = 0.13, Intact vs. Peripheral) leachate treatments. The influence of the pericarp in the final model accounted for the cypsela type, light treatment, pericarp restriction treatments, and cypsela type:light and cypsela type:pericarp restriction treatment interactions. The model distribution was binomial linked by logarithmic function.

The pericarp removal treatment affected germination timing just for the peripheral type (Figure 3B, b = 0.64 ± 0.12, t = 5.57, p<0.001, Intact vs. No fruit for peripheral). The crossed chemical treatment on the intact cypsela was effective to slow down just the central cypsela germination rate (b = -0.29 ± 0.10, t = 2.92, p = 0.03, Intact vs. Crossed for the central). On the other hand, isolated seeds were neither affected by the central (b = 0.05 ± 0.10, z = 0.49, p = 0.62, No fruit vs. Central Fruit) nor peripheral (b = -0.12 ± 0.09, z = -1.24, p = 0.21, No fruit vs. Peripheral Fruit) fruit extracts. AFT model for the pericarp influence accounted for the two main variables: treatment and cypsela type, and their interaction.

3.4. Response of germination to hormonal promoter

The rise in GA_3 concentration increased the germination proportion of both cypsela (β = 1.59 ± 0.15, z = 10.59, p<0.001) (Figure 4A). The global model did not present significant difference for cypsela type (β = -0.39 ± 0.26, z = -1.47, p = 0.14) or light treatments (β = 23.98 ± 41.96, z = 0.006, p = 0.99). However, under darkness, hormone addition overcame the light requirement for germination for both cypselae types and their germination varied along the gradient (β = 2.03 ± 0.26, z = 7.69, p<0.001). The model for the GA_3 sensitivity assay considered three main variables: cypsela type, light and hormonal treatments, and their interactions, except for the triple interaction. The random effect of the replicates was also considered. The model distribution was binomial linked by log link.

The germination probability differed between type (b = 0.38 ± 0.08, z = 4.73, p<0.001), light (b = -2.41 ± 0.06, z = -42.88, p<0.001) and GA_3 concentration treatments (b = -0.001 ± 0.00, z = -18.82, p<0.001) (Figure 4B). Under white light, germination rate differed for both types (b = -0.36 ± 0.04, t = -10.30, p<0.001), as well as under darkness (b = -0.47 ± 0.05, t = -8.79, p<0.001). The AFT model was made with the same variables as in the previous model, i.e. cypsela type, light and hormonal treatments, and all their interaction. This model was log logistic distributed.

3.5. Sensitivity to exogenous gibberellic acid

This assay was carried out to verify GA influence on germination progress of intact cypselae and excised seeds. It differs from the previous GA_3 sensitivity assay because

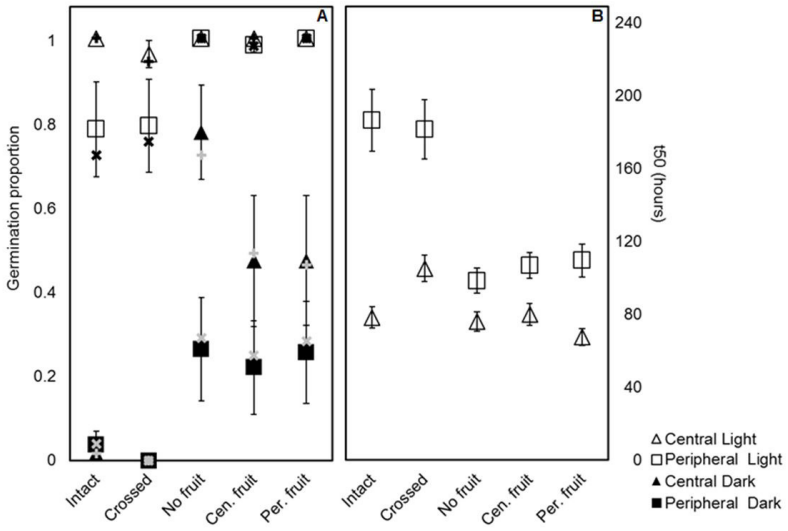

Figure 3. Estimated values of final germination proportion (A) and time to 50% germination (t50) of the *B. pilosa* cypsela pericarp manipulations (B). The symbols represent the estimated values for cypselae types (*types*) among different cypsela manipulations (*treatments*): Intact - control, Crossed - cypsela with the powdered pericarp from another cypsela, No fruit - pericarp removed, Cen. fruit and Per. Fruit - the isolated seed with central and peripheral powdered pericarp, respectively. The binomial model accounted for *types*, *treatments*, *types* x *treatments* as fixed effects, and the replicates as random effect. The time-to-event analysis model (AFT) of the germination time generated accounts for *types*, *treatments* and *types* x *treatments*. For graphical purposes, random effects were not considered. Error bars = Standard error. The "+" and "x" represent real data results.

we excluded internal GA production, thus our focus was the difference of the amount of GA required to actually induce germination. Cypselae types slightly differed on their responses in the model (Figure 5, $\beta = -0.91 \pm 0.23$, $z = -4.01$, $p<0.001$). For all manipulations, higher GA_3 levels increased germination proportion ($\beta = 2.47 \pm 0.57$, $z = 4.35$, $p<0.001$, PAC vs. PAC+100 µM GA_3). Removal of the pericarps increased seed germination (Figure 5B, $\beta = 2.97 \pm 0.77$, $z = 3.85$, $p<0.001$). On the other hand, the addition of the powdered fruit did not affect GA_3 sensitivity ($\beta = 0.63 \pm 0.53$, $z = 1.18$, $p = 236$), thus we considered that the fruit leachate may not be related to GA sensing pathway. At the end of the assay, the remaining ungerminated seeds were 78.74% viable for solution treated cypselae against 89.43% for the control, thus we considered the treatment of low toxic effect. The model GLMM accounted for seed types, pericarp manipulations and hormonal treatments (PAC; PAC+10µM GA_3; PAC+100µM GA_3 and the control) as categorical variables, with the interaction being homone:manipulation. Both dishes and populations were added as random effects. This model distribution was binomial linked by log function.

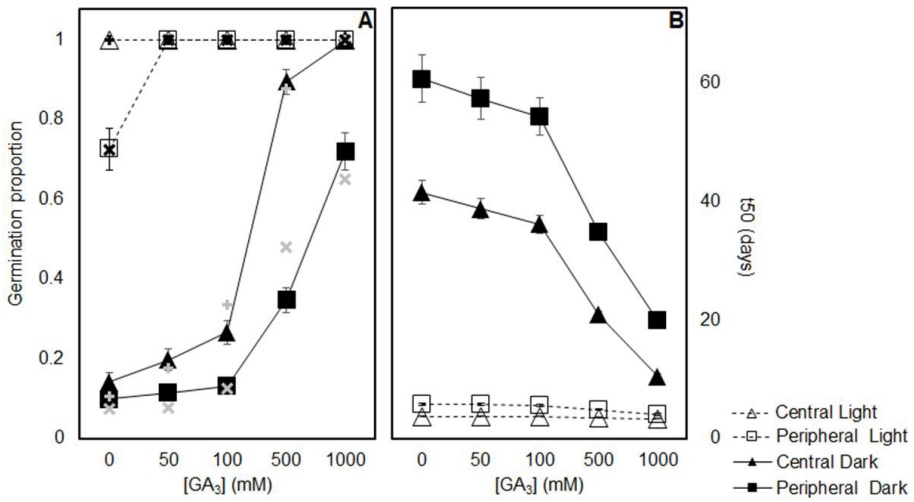

Figure 4. Estimated values of final germination proportion on exogenous Gibberellic acid (GA_3) gradient (A) and time to 50% germination (t50) of the GA_3 gradient (B) of *B. pilosa* cypsela. The symbols represent the estimated values for cypselae types (*types*) among the different GA_3 concentrations (*hormone*). The binomial model for the GA_3 concentrations accounted for *types*, *hormone*, *types* x *hormone* as fixed effects, and the dishes as random effect. The time-to-event analysis model (AFT) of the germination time accounts for *types*, *hormone* and *types* x *hormone*. Error bars = Standard error. The "+" and "x" represent real data results.

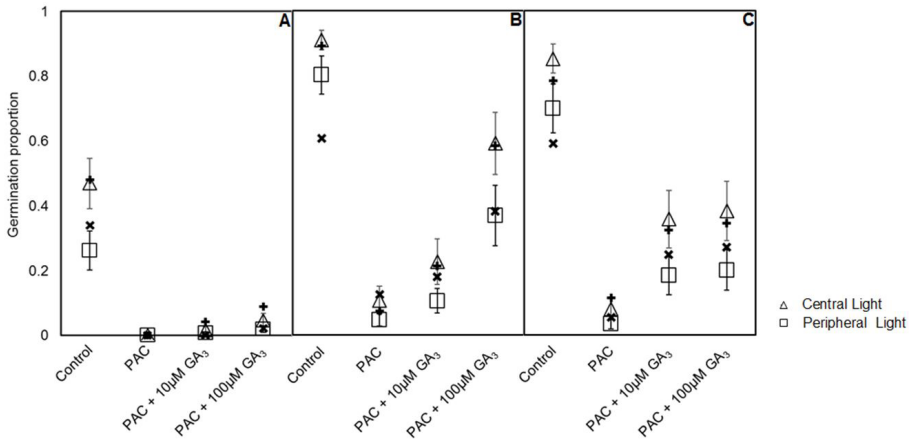

Figure 5. Estimated values of final germination proportion of the intact cypselae (A), excised seeds alone (B) and seeds plus powdered pericarps (C) of *B. pilosa* submitted to hormonal treatments. The symbols represent estimated values for manipulated cypselae or seeds (*manipulation*) from different types (*types*) among 200 µM of PAC and different GA_3 concentrations (*treatments*). The binomial model for essay accounted for *types*, *treatments*, *manipulation, and treatment* x *manipulation* as fixed effects, and the collected populations and dishes as random effects. Error bars = Standard error. The "+" and "x" represent real data results.

4. Discussion

The dimorphic behavior of *Bidens pilosa* has been previously reported in other works (Forsyth and Brown, 1982; Rocha, 1996; Amaral and Takaki, 1998); however, our effort was the first to investigate the nature of the germination constraints on cypselae types. Despite the fact that the pericarp may not influence the dicarpic seed germination (Souza Filho and Takaki, 2011), there are species in which different mechanical constrains are imposed by the pericarp (Imbert, 2002). Still, few reports presented chemical constraints on the germination of heterocarpic species. Our experiments using germination active compounds and pericarp manipulation points that both mechanical and chemical constraints are influences for the dicarpy of *B. pilosa*.

The dicarpic behavior of *B. pilosa* cypsela might vary among different populations. No previous work reported that difference on the dicarpic behavior for *B. pilosa*. Under laboratory conditions, the central cypsela germinated 2 to 4 times faster than the peripheral ones. The germination timing variability is attributed to the peripheral cypselae rather than the central ones, which for the 11-B population was faster than for the populations from the other sites. In *Crepis sancta* populations, the germination rate of both cypsela types varied (Dubois and Cheptou, 2012). Variation in peripheral cypsela delay can be related to a strategy to maintain the populations (Rocha, 1996). Germination delay is a response to environmental effects on the mother plant. This plasticity optimizes sibling establishment and guarantees the maternal population maintenance (Donohue, 2005; Wang et al., 2012).

The physiological mechanisms for seed germination can be detected via hormonal sensitivity (Barua et al., 2012), so we tried to detect variations among cypselae types or populations. The inhibitory effects of ABA on seed germination delay radicle expansion and weaken the endosperm, in addition to enhancing the expression of transcription factors (Miransari and Smith, 2014). This signaling can be affected in other ways that can increase or release seed dormancy deepness. Some *Arabidopsis* ecotypes presented different ABA sensitivity, via DOG1 (Delay of Germination) expression, with seed dormancy increasing with higher expression. On the other hand, there is little variation in exogenous ABA response among *Arabidopsis* genetic backgrounds, and their responsiveness seems to be related to gibberellins pathway (Barua et al., 2012). The exogenous ABA decreased seed germination in *B. pilosa*. Also, responsiveness varied among populations. Variation of the ABA response related to the peripheral ones may be related to its maintenance strategy, where this type should "fit better" to the surrounding environment. Endogenous ABA content, maintained by its anabolism, during seed imbibition, is responsible for dormancy maintenance in several species (Finch-Savage and Leubner-Metzger, 2006). However, it was not the case with mature fresh seeds of *B. pilosa,* where ABA anabolism is not related to the maintenance of germination delay, evidenced by inefficiency of Fluridone. However, we do not discard that a secondary dormancy or dormancy cycling is present, caused by unsuitable environmental conditions (Footitt et al., 2013).

Root protrusion occurs when the increase in embryo growth potential overcomes pericarp blockage (Sun et al., 2009). Mechanical constraints were observed just for the peripheral cypsela due to the increase in germination after the pericarp removal. In *Hemizonia increscens*, the pericarp of peripheral cypsela physically impedes embryo growth and, when excised or nicked, germination is quicker, whereas removing the pericarp of central cypselae has no effect (Tanowitz et al., 1987). Pericarp structure cause a barrier effect for *Senecio jacobaea* (McEvoy, 1984), *Heterotheca latifolia* (Venable and Levin, 1985) and *Anthemis chrysantha* (Aguado et al., 2011). Thus, the pericarp of the dormant cypsela type acts as a barrier for embryo growth via development of sclerenchymatous cell layers. In *Senecio jacobaea* the dormant marginal cypselae presented lower seed:cypselae ratio than central ones (McEvoy, 1984). Thus we consider that the peripheral cypselae demand a higher environmental stimuli than central ones, via GA signaling, to gain growth potential to overcome mechanical constraints and actually germinate (Amaral-Baroli and Takaki, 2001).

Some chemical inhibitors can be present in the external diaspore layers that can reduce the growth potential of the embryos (Beneke et al., 1993; Jia et al., 2012). Forsyth and Brown (1982) observed that ethanolic extracts of peripheral cypselae contained unknown compounds (or groups) that reduce the germination of the central type. We also obtained similar results in our manipulations. The intact central cypselae suffered a slight reduction in the germination timing when exposed to the peripheral pericarps. However, the excised seeds were less responsive to that chemical effect. The main compounds pointed out were some flavonoids and coumarins (data not shown). Some secondary compounds influence seed dynamics, e.g. proanthocyanidins, which are present in the testa of *Arabidopsis thaliana*. They induce ABA anabolism in the embryo, thus maintaining dormancy (Jia et al., 2012). Nevertheless, this pathway was not observed in the germination mechanism of *B. pilosa* seeds, because FLU was not effective to alleviate dormancy. Whitaker et al. (2010) showed that the germination of peripheral cypselae of *B. pilosa* is stimulated by some reactive oxygen species donors, which alleviate their dormancy. Superoxide (methyl viologen dichloride hydrate [MV]) affects both after-ripening dormancy and light requirement processes. On the contrary, hydroxyl radical generators, i.e. hydrogen peroxide and Fenton reagent, induce little germination. Thus, the antioxidant action of the flavonoids of the pericarp can scavenge the endogenous superoxide from the embryo and inhibit its germination. The addition of peripheral pericarp leachates on intact central cypselae may also affect germination via superoxide scavenging (Rice-Evans et al., 1997; El-Maarouf-Bouteau et al., 2015). In *Dimorphotheca sinuate* (monocarpic), *D. polyptera*,

(dicarpic) and *Artotis fastuosa* (dicarpic), the addition of pericarp leachate inhibited their growth germination, described as condensed tanniferous substances (proanthocyanidins). In addition, the partial removal of the pericarp released cypselae from dormancy. For those species, dormancy maintenance is attributed to both mechanical and chemical germination resistance (Beneke et al., 1993).

GA triggers various mechanisms that allow germination *sensu stricto*, or root protrusion: both embryo growth and endosperm weakening overcome the mechanical resistance of the envelope and need GA anabolism (Yamaguchi and Kamiya, 2000). GA also down-regulates ABA-up-regulated genes: (1) GA reduces ABA levels by affecting ABA biosynthesis; (2) GA negatively regulates the ABA response pathway; and (3) GA and ABA signals are targeted independently of the distinct cis-regulatory sequences of a single gene (Ogawa et al., 2003). In ungerminated seeds, GA concentrations are maintained by endogenous homeostasis, which limits them. In *A. thaliana*, these mechanisms are controlled by GA_{20}-oxidases and GA_3-oxidases that are negatively regulated by GA activity through feedback inhibition, whereas GA up-regulates the genes encoding GA-deactivating GA_2-oxidases through a positive feed forward loop (Olszewski et al., 2002). The addition of exogenous GA_3 to the medium increases GA concentration and forces GA-mediated genes to express and trigger root protrusion. In cypselae of *B. pilosa*, exogenous GA increased the peripheral type germination rate under light, and the germination proportion and rate in darkness. However, we observed that the central type is more responsive to GA_3 than the peripheral one.

To check for the difference on GA sensitivity among the types we manipulated the growth potential of the embryos via excluding the endogenous GA production using paclobutrazol. The PAC treatment inhibits the mono-oxygenases involved in the oxidation of ent-kaurene into ent-kaurenoic acid - a GA pathway precursor (Jacobsen and Olszewski, 1993). The 200 µM of PAC solution was effective to inhibit germination and maintained seed viability of intact cypsela; however, the removal of the pericarp facilitates the embryo growth, showing slight germination. Comparing the types, we verified little difference in germination, so we consider that the growth potential imposed by the variation on GA production is the main reason for different germination behavior. The pericarp acted as a blockage for both cypsela types on the GA sensitivity assay corroborating the hypothesis that endogenous GA plays a role causing different germination behavior.

For both *B. pilosa* cypselae, pericarps act mechanically to inhibit seed germination, however the peripheral type is relatively more impeded. Felippe (1978) also showed that alleviating the pericarp mechanical constraints through apex removal of *B. pilosa* cypselae overcame photodormancy. The difference in cypsela germination is not directly related to pericarp mechanical constraints alone.

Our efforts revealed the nature of *B. pilosa* to be dicarpic, with the behavior of cypselae types depending on two pericarp components: (1) mechanical and (2) chemical. Both negatively interacted with the embryo growth potential to block the germination *sensu stricto* and, depending on the intensity, generated a deviation between cypselae types, and that can vary among populations. The mechanical constraints seem to affect similarly both cypselae types, however the embryo growth of the peripheral ones are depreciated by the inhibitor leachate from the pericarp. Also, the chemical does not affect the GA-ABA regulation directly, because there was no detection of *de novo* ABA synthesis and no difference on GA sensitivity.

Acknowledgements

The authors thank "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (GD 147810/2010-8), by ways of a grant to P.R.M.S.F.

References

AGUADO, M., MARTÍNEZ-SÁNCHEZ, J.J., REIG-ARMIÑANA, J., GARCÍA-BREIJO, F.J., FRANCO, J.A. and VICENTE, M.J., 2011. Morphology, anatomy and germination response of heteromorphic achenes of *Anthemis chrysantha* J. Gay (Asteraceae), a critically endangered species. *Seed Science Research*, vol. 21, no. 4, pp. 283-294. http://dx.doi.org/10.1017/S0960258511000183.

AMARAL, A. and TAKAKI, M., 1998. Achene dimorphism in *Bidens pilosa* L. (Asteraceae) as determined by germination test. *Brazilian Archives of Biology and Technology*, vol. 41, no. 1, pp. 10-15. http://dx.doi.org/10.1590/S1516-89131998000100002.

AMARAL-BAROLI, A. and TAKAKI, M., 2001. Phytochrome controls seed germination in *Bidens pilosa* L. (Asteraceae) by very low fluence response. *Brazilian Archives of Biology and Technology*, vol. 44, no. 2, pp. 121-124. http://dx.doi.org/10.1590/S1516-89132001000200002.

BARUA, D., BUTLER, C., TISDALE, T.E. and DONOHUE, K., 2012. Natural variation in germination responses of Arabidopsis to seasonal cues and their associated physiological mechanisms. *Annals of Botany*, vol. 109, no. 1, pp. 209-226. http://dx.doi.org/10.1093/aob/mcr264. PMid:22012958.

BASKIN, J.M., LU, J.J., BASKIN, C.C., TAN, D.Y. and WANG, L., 2014. Diaspore dispersal ability and degree of dormancy in heteromorphic species of cold deserts of northwest China: a review. *Perspectives in Plant Ecology, Evolution and Systematics*, vol. 16, no. 2, pp. 93-99. http://dx.doi.org/10.1016/j.ppees.2014.02.004.

BATES, D., MAECHLER, M., BOLKER, B. and WALKER, S., 2014 [viewed 10 April 2017]. *lme4: linear mixed-effects models using Eigen and S4. R package version 1.0-6* [software]. Vienna: R Foundation for Statistical Computing. Available from: http://CRAN.R-project.org/package=lme4

BENEKE, K., VAN ROOYEN, M.W., THERON, G.K. and VAN DE VENTER, H.A., 1993. Fruit polymorphism in ephemeral species of Namaqualand: III. Germination differences between the polymorphic diaspores. *Journal of Arid Environments*, vol. 24, no. 4, pp. 333-344. http://dx.doi.org/10.1006/jare.1993.1029.

BRÄNDEL, M., 2004. Dormancy and germination of heteromorphic achenes of *Bidens frondosa*. *Flora*, vol. 199, no. 3, pp. 228-233. http://dx.doi.org/10.1078/0367-2530-00150.

DÉLYE, C., MENCHARI, Y., MICHEL, S., CADET, E. and LE CORRE, V., 2013. A new insight into arable weed adaptive evolution: mutations endowing herbicide resistance also affect germination dynamics and seedling emergence. *Annals of Botany*, vol. 111, no. 4, pp. 681-691. http://dx.doi.org/10.1093/aob/mct018. PMid:23393095.

DONOHUE, K., 2005. Seeds and seasons: interpreting germination timing in the field. *Seed Science Research*, vol. 15, no. 3, pp. 175-187. http://dx.doi.org/10.1079/SSR2005208.

DUBOIS, J. and CHEPTOU, P.O., 2012. Competition/colonization syndrome mediated by early germination in non-dispersing achenes in the heteromorphic species *Crepis sancta. Annals of Botany*, vol. 110, no. 6, pp. 1245-1251. http://dx.doi.org/10.1093/aob/mcs203. PMid:23022677.

EL-MAAROUF-BOUTEAU, H.A.Y.A.T., SAJJAD, Y., BAZIN, J., LANGLADE, N., CRISTESCU, S.M., BALZERGUE, S., BAUDOUIN, E. and BAILLY, C., 2015. Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. *Plant, Cell & Environment*, vol. 38, no. 2, pp. 364-374. http://dx.doi.org/10.1111/pce.12371. PMid:24811898.

FELIPPE, G.M., 1978. Estudos de germinação, crescimento e floração de *Bidens pilosa* L. *Revista do Museu Paulista*, vol. 25, pp. 183-217.

FINCH-SAVAGE, W.E. and LEUBNER-METZGER, G., 2006. Seed dormancy and the control of germination. *The New Phytologist*, vol. 171, no. 3, pp. 501-523. http://dx.doi.org/10.1111/j.1469-8137.2006.01787.x. PMid:16866955.

FOOTITT, S., HUANG, Z., CLAY, H.A., MEAD, A. and FINCH-SAVAGE, W.E., 2013. Temperature, light and nitrate sensing coordinate Arabidopsis seed dormancy cycling, resulting in winter and summer annual phenotypes. *The Plant Journal*, vol. 74, no. 6, pp. 1003-1015. http://dx.doi.org/10.1111/tpj.12186. PMid:23590427.

FORSYTH, C. and BROWN, N.A.C., 1982. Germination of the dimorphic fruits of *Bidens pilosa* L. *The New Phytologist*, vol. 90, no. 1, pp. 151-164. http://dx.doi.org/10.1111/j.1469-8137.1982.tb03248.x.

HOTHORN, T., BRETZ, F. and WESTFALL, P., 2008. Simultaneous inference in general parametric models. *Biometrical Journal. Biometrische Zeitschrift*, vol. 50, no. 3, pp. 346-363. http://dx.doi.org/10.1002/bimj.200810425. PMid:18481363.

IMBERT, E., 2002. Ecological consequences and ontogeny of seed heteromorphism. *Perspectives in Plant Ecology, Evolution and Systematics*, vol. 5, no. 1, pp. 13-36. http://dx.doi.org/10.1078/1433-8319-00021.

JACOBSEN, S.E. and OLSZEWSKI, N.E., 1993. Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. *The Plant Cell*, vol. 5, no. 8, pp. 887-896. http://dx.doi.org/10.1105/tpc.5.8.887. PMid:8400871.

JIA, L., WU, Q., YE, N., LIU, R., SHI, L., XU, W., ZHI, H., RAHMAN, A.N.M., XIA, Y. and ZHANG, J., 2012. Proanthocyanidins Inhibit seed germination by maintaining a high level of abscisic acid in *Arabidopsis thaliana. Journal of Integrative Plant Biology*, vol. 54, no. 9, pp. 663-673. http://dx.doi.org/10.1111/j.1744-7909.2012.01142.x. PMid:22765383.

LENTH, R.V., 2016. Least-squares means: the R package lsmeans. *Journal of Statistical Software*, vol. 69, no. 1, pp. 1-33. http://dx.doi.org/10.18637/jss.v069.i01.

MARTINS, J.R., EDVALDO, A.A.S., ALVARENGA, A.A., RODRIGUES, A.C., RIBEIRO, D.E. and TOOROP, P.E., 2015. Seedling survival of *Handroanthus impetiginosus* (Mart ex DC) Mattos in a semi-arid environment through modified germination speed and post-germination desiccation tolerance. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 75, no. 4, pp. 812-820. http://dx.doi.org/10.1590/1519-6984.23413. PMid:26675900.

MATILLA, A., MATILLA, A., GALLARDO, M. and PUGA-HERMIDA, M.I., 2005. Structural, physiological and molecular aspects of heterogeneity in seeds: a review. *Seed Science Research*, vol. 15, no. 1, pp. 63-76. http://dx.doi.org/10.1079/SSR2005203.

MCEVOY, P.B., 1984. Dormancy and dispersal in dimorphic achenes of tansy ragwort, *Senecio jacobaea* L. (Compositae). *Oecologia*, vol. 61, no. 2, pp. 160-168. http://dx.doi.org/10.1007/BF00396754. PMid:28309405.

MIRANSARI, M. and SMITH, D.L., 2014. Plant hormones and seed germination. *Environmental and Experimental Botany*, vol. 99, pp. 110-121. http://dx.doi.org/10.1016/j.envexpbot.2013.11.005.

OGAWA, M., HANADA, A., YAMAUCHI, Y., KUWAHARA, A., KAMIYA, Y. and YAMAGUCHI, S., 2003. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell*, vol. 15, no. 7, pp. 1591-1604. http://dx.doi.org/10.1105/tpc.011650. PMid:12837949.

OLSZEWSKI, N., SUN, T.P. and GUBLER, F., 2002. Gibberellin signaling biosynthesis, catabolism, and response pathways. *The Plant Cell Online*, vol. 14, suppl. 1, pp. S61-S80. http://dx.doi.org/10.1105/tpc.010476. PMid:12045270.

ONOFRI, A., GRESTA, F. and TEI, F., 2010. A new method for the analysis of germination and emergence data of weed species. *Weed Research*, vol. 50, no. 3, pp. 187-198. http://dx.doi.org/10.1111/j.1365-3180.2010.00776.x.

R DEVELOPMENT CORE TEAM, 2013 [viewed 10 April 2017]. *R: a language and environment for statistical computing* [software]. Vienna: R Foundation for Statistical Computing. Available from: http://www.R-project.org/

RICE-EVANS, C., MILLER, N. and PAGANGA, G., 1997. Antioxidant properties of phenolic compounds. *Trends in Plant Science*, vol. 2, no. 4, pp. 152-159. http://dx.doi.org/10.1016/S1360-1385(97)01018-2.

ROCHA, O.J., 1996. The effects of achene heteromorphism on the dispersal capacity of *Bidens pilosa* L. *International Journal of Plant Sciences*, vol. 157, no. 3, pp. 316-322. http://dx.doi.org/10.1086/297351.

SILESHI, G.W., 2012. A critique of current trends in the statistical analysis of seed germination and viability data. *Seed Science Research*, vol. 22, no. 3, pp. 145-159. http://dx.doi.org/10.1017/S0960258512000025.

SOUZA FILHO, P.R.M. and TAKAKI, M., 2011. Dimorphic cypsela germination and plant growth in s*ynedrella nodiflora* (L.) Gaertn. (Asteraceae). *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 71, no. 2, pp. 541-548. http://dx.doi.org/10.1590/S1519-69842011000300027. PMid:21755175.

SUN, H.Z., LU, J.J., TAN, D.Y., BASKIN, J.M. and BASKIN, C.C., 2009. Dormancy and germination characteristics of the trimorphic achenes of *Garhadiolus papposus* (Asteraceae), an annual ephemeral from the Junggar Desert, China. *South African Journal of Botany*, vol. 75, no. 3, pp. 537-545. http://dx.doi.org/10.1016/j.sajb.2009.05.001.

TANOWITZ, B.D., SALOPEK, P.F. and MAHALL, B.E., 1987. Differential germination of ray and disc achenes in *Hemizonia increscens* (Asteraceae). *American Journal of Botany*, vol. 14, no. 3, pp. 303-312. http://dx.doi.org/10.1002/j.1537-2197.1987.tb08612.x.

THERNEAU, T.M. and GRAMBSCH, P.M., 2000. *Modeling survival data: extending the cox model*. 1st ed. New York: Springer, 350 p. http://dx.doi.org/10.1007/978-1-4757-3294-8.

VENABLE, D.L. and LEVIN, D.A., 1985. Ecology of achene dimorphism in *Heterotheca latifolia*: I. achene structure, germination and dispersal. *Journal of Ecology*, vol. 73, no. 1, pp. 133-145. http://dx.doi.org/10.2307/2259774.

WANG, L., BASKIN, J.M., BASKIN, C.C., CORNELISSEN, J.H.C., DONG, M. and HUANG, Z., 2012. Seed dimorphism, nutrients and salinity differentially affect seed traits of the desert halophyte *Suaeda aralocaspica* via multiple maternal effects. *BMC Plant Biology*, vol. 12, no. 1, pp. 170. http://dx.doi.org/10.1186/1471-2229-12-170. PMid:23006315.

WHITAKER, C., BECKETT, R.P., MINIBAYEVA, F.V. and KRANNER, I., 2010. Alleviation of dormancy by reactive oxygen species in *Bidens pilosa* L. seeds. *South African Journal of Botany*, vol. 76, no. 3, pp. 601-605. http://dx.doi.org/10.1016/j.sajb.2010.04.014.

YAMAGUCHI, S. and KAMIYA, Y., 2000. Gibberellin biosynthesis: its regulation by endogenous and environmental signals. *Plant & Cell Physiology*, vol. 41, no. 3, pp. 251-257. http://dx.doi.org/10.1093/pcp/41.3.251. PMid:10805587.

ZUUR, A., IENO, E.N., WALKER, N., SAVELIEV, A.A. and SMITH, G.M., 2009. *Mixed effects models and extensions in ecology with R*. 1st ed. New York: Springer, 574 p. http://dx.doi.org/10.1007/978-0-387-87458-6.

Annex A. Exogenous fluridone gradient essay on *B. pilosa cypsela.*

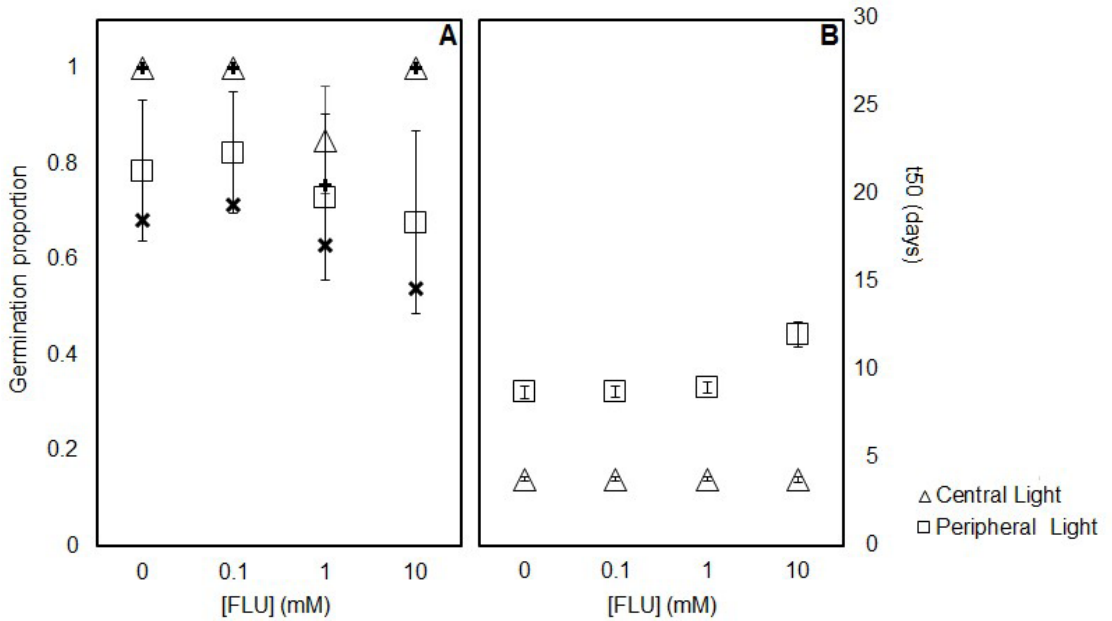

Figure A1. Estimated values of final germination proportion on exogenous Fluridone gradient (A) and time to 50% germination (B) of the FLU gradient (t50) of *B. pilosa* cypsela. The symbols represent estimated values for cypselae types (*types*) among the different FLU concentrations (*hormone*) and collected populations (*populations*). The binomial model for the FLU concentrations accounted for *types*, *hormone*, *types* x *hormone* as fixed effects, and the *populations* as random effect. The time-to-event analysis model (AFT) of the germination time generated account for *types*, *hormone* and *types* x *hormone*. For graphical purposes random effects were not considered. Error bars = Standard error. The "+" and "x" represent real data results.