



## To resist or to germinate? The effect of fire on legume seeds in Brazilian subtropical grasslands

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### ABSTRACT

Fire plays an important role in several grassland ecosystems in the world. Fire can trigger germination in several species, by breaking the physical dormancy of their seeds. Thus, we hypothesized that exposure to high temperatures during fire would break seed dormancy and enhance germination. We tested the effect of high temperatures on the germination of six species of legumes from Brazilian subtropical grasslands. We used heat shock experiments with the following treatments: 60, 90, 120 and 150°C for one minute. Seeds were then placed to germinate for 60 days in 12/12 hours light/dark and 20/30°C. Germination was generally low for all study species. Most species was not affected by heat shock treatments. However, *Stylosanthes montevidensis* was the only species that had its physical dormancy broken when exposed to 120°C. The seeds of all the other species were neither stimulated nor killed by high temperatures. Although the exposure to high temperatures did not affect the germination of the study species (except for one), it also did not kill seeds, thereby showing that seeds are resistant to fire. Therefore, the rapid passage of fire in these grasslands is not sufficient to break the dormancy of most of the studied species of legumes.

**Keywords:** Brazilian Campos grasslands, Fabaceae, fire-resistance, germination percentage, heat shock experiments

Germination triggered by fire cues is an important trait in fire-prone environments, enabling plant population to regenerate after disturbance (Keeley & Fotheringham 2000; Keeley *et al.* 2011). Hard-coated seeds, for an example, remain dormant until suitable environmental conditions take place (Baskin 2003; Willis *et al.* 2014). The exposure to high temperatures (e.g. Moreira & Pausas 2012), as well as daily fluctuating temperatures (see Santana *et al.* 2013), are major factors related to break of physical dormancy in fire-prone ecosystems. Increasing temperature promotes the breaking of dormancy through cracking the seed coat, allowing imbibition to occur, and promoting germination (Keeley 1991; Morrison *et al.* 1998; Baskin & Baskin 2014).

In fire-prone ecosystems, studies focusing on the effects of heat shock on dormancy break and germination are of great importance. Fire-related germination is found in different types of vegetation, but most studies describe this relationship in Mediterranean ecosystems (see Reyes & Trabaud 2009; Moreira *et al.* 2010) and in Australia (see Auld & O'Connell 1991; Ooi *et al.* 2014). Keeley *et al.* (2011) concluded thus, that breaking the dormancy or stimulating the germination by fire cues should be considered as evidence for selective pressures in plant evolution. In a plant functional approach, researchers have classified physically dormant seeds as having pyrogenic obligate or facultative dormancy-breaking mechanisms (Ooi *et al.* 2014). On the

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other hand, fire may be irrelevant for some species, so they survive high temperatures but do not show a specific dormancy-breaking bounded by higher temperatures (see Hanley & Lamont 2000; Fichino *et al.* 2016).

Since heat shock may play a major role in breaking physical dormancy in fire-prone ecosystems, it is critical to determine its role as a fire-related cue in hard-coated seeds from Brazilian subtropical grasslands. Fires in these grasslands are very fast, occurring with low temperatures and intensity (Fidelis *et al.* 2010a). Moreover, vegetation dynamics are affected by fire, maintaining open physiognomies and promoting plant diversity (Overbeck *et al.* 2007; Fidelis *et al.* 2012). Earlier studies found little evidence that germination could be enhanced by seed exposure to high temperatures (Overbeck *et al.* 2006; Fidelis *et al.* 2010b). However, recent studies (Silveira & Overbeck 2013) have demonstrated that heat exposure at 80°C led to increased germination by a grassland legume, *Desmodium barbatum*.

Our study addresses the role of fire temperature (dry heat shocks) to influence seed germination across six Fabaceae species from Brazilian subtropical grasslands, in order to test if high temperatures would break seed. We hypothesize that heat shock would be sufficient to break the dormancy of seeds, or at least that they would survive to the exposure to higher temperatures. The species used in our study belonged to three subfamilies in the Fabaceae family: *Chamaecrista repens* (Vogel) H.S.Irwin & Barneby (forb, Caesalpinioideae); *Desmanthus tathuyensis* Hoehne (forb, Mimosoideae); *Collaea stenophylla* (Hook. & Arn.) Benth (shrub), *Crotalaria tweediana* Benth. (forb), *Stylosanthes montevidensis* Vog (forb), and *Zornia reticulata* Sm. (forb, all Papilionoideae). Seeds from Fabaceae are known to have impermeable seed coats (Baskin & Baskin 2000; Jayasuriya *et al.* 2013) and therefore, we chose species from this family to test our hypothesis.

Seeds of each the six legume species were collected on different individuals and populations found in natural grasslands, located on Morro Santana, a granitic hill located in Porto Alegre (Southern Brazil, 30°03' S, 51°07' W; 311 m a.s.l.). The climate of this region is subtropical humid (Köppen classification Cfa), with annual precipitation of 1350 mm and mean temperatures of 22°C (Livi 1999). Seeds were collected during the dispersion period (from October to February) and kept at room temperature in paper bags. We conducted heat shock experiments two months after seed collection.

Subtropical grasslands are very rich in plant species (450-500 species; see Aguiar 1986) and they are composed of a mixture of C<sub>3</sub> and C<sub>4</sub> grasses, which are characteristic in Southern Brazil (Fidelis 2010). Fire has been present in the study area since 1200 BP (Behling *et al.* 2007) with fire frequency estimated between 2-5 years. Further, fires are mostly set by local residents to clean fields and to stimulate resprouting of medicinal plants. Fire is fast and temperatures at soil surface range from 48 to 537°C and

temperatures >150°C never lasted more than 60s (Fidelis *et al.* 2010a). Although temperatures below soil surface (where seeds from the seed bank are) have not been measured, we believe that they are much lower than at soil surface, as observed by Miranda *et al.* (1993) for Cerrado areas. In fact, Bradstock & Auld (1995) registered temperatures from 0-3cm soil depth ranging from 60°C to 120°C. Moreover, we used temperatures similar to the ones used by Overbeck *et al.* (2006) and Silveira & Overbeck (2013) in studies on seed germination of Brazilian subtropical grasslands. Therefore, we chose the range from 60°C (which is usually measured in the upper layers of soils during fire) to 150°C (extreme temperatures) to test our hypothesis.

Seeds were exposed to different heat shock temperatures lasting one minute: 60°, 90°, 120°, 150°C and control (no heat shock treatment). Each species and treatment had five replicates (except for *Chamaecrista repens* and *Collaea stenophylla*, with three replicates each). We used 20 seeds/replicate, except for *Chamaecrista repens* (15 seeds/replicate). Such differences in seed numbers and replicates across species were related to constraints on seed availability.

We applied heat shock treatments using a preheated oven, with insertion and removal of replicates in aluminium dishes. Each replicate was heated separately to avoid pseudoreplication (Morrison & Morris 2000). Both treated and non-treated seeds were placed in Petri dishes, on three layers of sterilized filter papers. All dishes were moistened with distilled water during 60 days in germination chamber. Since this study aimed to reproduce real conditions from the field, 20°/30°C temperature, 12/12 hours dark/light conditions were chosen. Seeds were kept moistened while we tracked germination rates weekly. When radicle and/or cotyledons could be observed, they were counted and removed from Petri dishes.

By the end of the germination tests, non-germinated seeds (except for *Desmanthus tathuyensis* and *Collaea stenophylla*) were submitted to a tetrazolium viability test, in order to test if seeds were still viable after treatments. Some species showed several seeds damaged by fungi, and thus, we also counted the damaged seeds and used only the non-damaged seeds for the tetrazolium test. Since the seeds did not show any sign of imbibition, we punctured them and left to imbibe before they were cut and soaked in a 2,3,5-triphenyl-tetrazolium chloride solution (0.075%; Brasil 2009), at 30°C for four hours. When embryos stained red, seeds were considered to be viable, and viable seeds (%) were considered the germinated + stained seeds at the end of the experiment.

We used a one-way analysis of variance (ANOVA) to test whether there were statistical differences of germination percentages across the different temperatures (main fixed factor) for each study species. We used randomization tests (Euclidean distances between replicates, 10000 iterations), since there is no restriction about normal distribution of



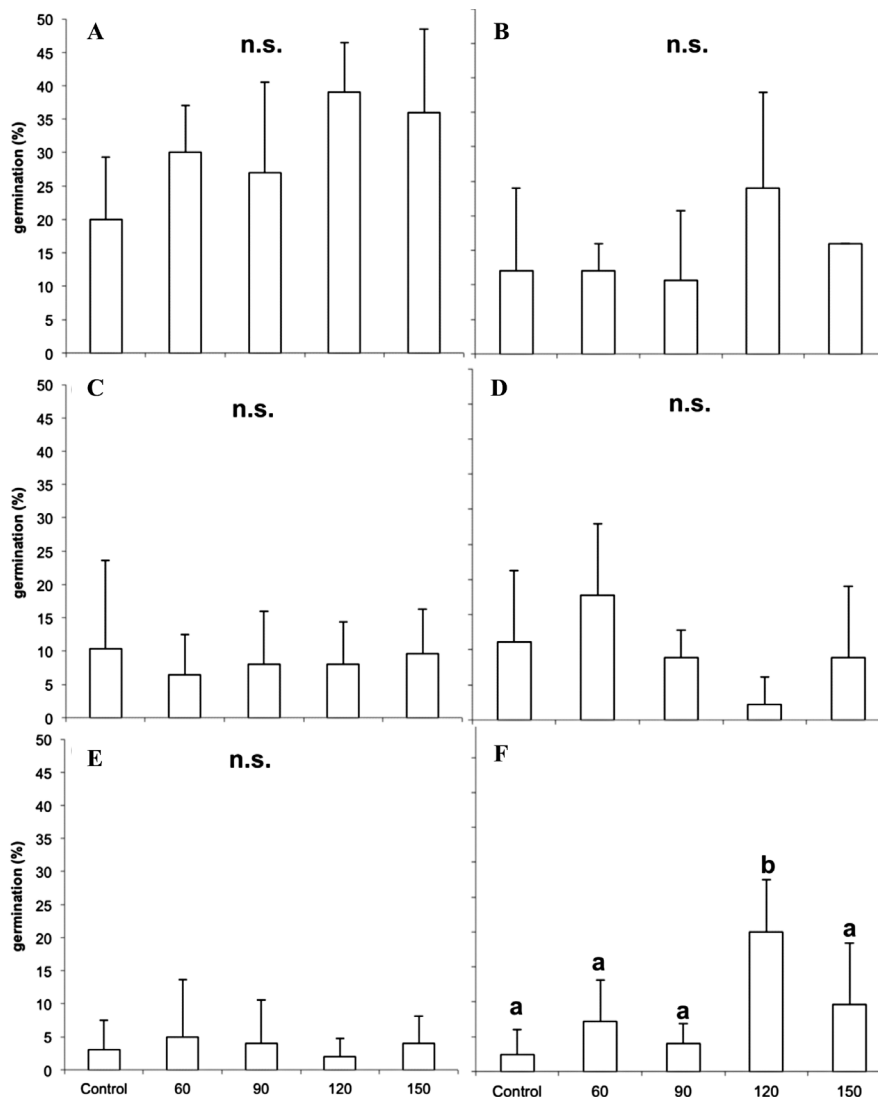
data (for more details, see Manly 2007). All statistical analyses were performed using MULTIV (Pillar 2008).

Most of the species germinated less than 20% across all treatments, remaining visibly impermeable by the end of the tests (e.g., no changes in seed size). In *Crotalaria tweediana*, we observed ca. of 20% of germinable seeds in the control treatment; and although the species almost doubled germination with the heat shocks, differences between treatments were not found (Fig. 1A,  $P > 0.05$ ). Also an increase in germination was observed with the high temperatures in *Collaea stenophylla* (Fig. 1B), but differences were not statistically significant ( $P > 0.05$ ). Seeds of *Desmanthus tathuyensis* showed low germination percentages in all treatments (<10%) and the exposure to high temperatures did not affect germination ( $P > 0.05$ , Fig. 1C) *Chamaecrista repens* seeds tended to germinate more after being exposed to 60° (Fig. 1D,  $P > 0.05$ ). Seeds

of *Zornia reticulata* (Fig. 1E) also did not show any response to the heat shocks, with germination percentages <10% in all treatments ( $P > 0.05$ ). The only species affected by the heat shock treatments was *Stylosanthes montevidensis*: a higher germination percentage was observed after seeds were exposed to 120°C (20%) in relation to the control (2.4%,  $P = 0.01$ , Fig. 1F).

Seed total viability (germinated + stained seeds) was higher in *Zornia reticulata* and *Crotalaria tweediana* (Tab. 1), presenting a low percentage of damaged seeds. On the other hand, 88% and 86.6% of non-germinated seeds of *S. montevidensis* and *C. repens* were damaged by fungi and thus, they also had low viability (Tab. 1).

The increase in germination after fire, both due to heat shock or smoke, is considered an adaptation to fire by some authors (e.g. Keeley *et al.* 2011). Several species from Mediterranean ecosystems show fire-related cues to germi-



**Figure 1.** Germination percentages (mean±1SE) of legumes submitted to heat shock treatments (60°C, 90°C, 120°, and 150°) and control (no exposure to high temperatures) for 1 minute. A) *Crotalaria tweediana*, B) *Collaea stenophylla*, C) *Desmanthus tathuyensis*, D) *Chamaecrista repens*, E) *Zornia reticulata* and F) *Stylosanthes montevidensis*. Different letters mean significant differences between temperature treatments ( $P \leq 0.05$ ).



**Table 1.** Percentage of viable (germinated + stained seeds) and damaged by fungi seeds of legume species from Brazilian subtropical grasslands after the experiment for all treatments: Control, 60°C, 90°C, 120°C and 150°C.

Species	Control		60°C		90°C		120°C		150°C	
	viable	damaged	viable	damaged	viable	damaged	viable	damaged	viable	damaged
<i>Crotalaria tweediana</i>	55.0±13.7	22.0±5.7	60.0±13.2	10.0±6.1	53.0±10.9	14.0±11.9	64.0±13.8	14.0±5.5	68.0±11.5	13.0±9.7
<i>Chamaecrista repens</i>	11.1±10.2	88.9±10.2	22.2±7.7	75.6±10.2	15.6±7.7	84.4±7.7	6.6±7.7	93.3±6.7	8.9±10.18	91.1±10.18
<i>Zornia reticulata</i>	50.0±7.9	8.0±1	48.0±7.6	5.2±3.1	23.0±14.4	9.2±4.8	31.0±13.8	7.8±3.1	30.0±7.9	11.0±2.5
<i>Stylosanthes montevidensis</i>	2.4±3.5	96.8±4.4	11.2±5.9	88.8±5.9	8.8±5.2	91.2±5.2	20.8±8.2	79.2±8.2	11.2±7.7	88.8±7.7

nation (see Moreira *et al.* 2010), being fire-dependent, while other species can germinate after temperature fluctuations (gap-dependent, Ooi *et al.* 2014). Our study showed that only seeds of *Stylosanthes montevidensis* had a fire-related increase in germination after the exposure to 120°C, showing breakage of physical dormancy of at least 20% of the seeds. For most of the species, there was no significant effect in germination after the exposure to high temperatures.

In general, all species showed very low germination percentages and high percentage of hard seeds after experiments, confirming their impermeable coats. Although Moreira & Pausas (2012) showed that dormancy break is fire-related in physically dormant seeds in the Mediterranean Basin, in the present study, the exposure to high temperatures was not sufficient to break the dormancy for most of the studied legume species. However, all of them showed to be resistant to fire, showing no significant decrease in germination when exposed to 120°C and 150°C. Since seeds can survive fire, other mechanisms should be involved in the dormancy-breaking process. Jaganathan (2015) argued that, since seeds have a specific mechanism of losing physical dormancy related to 'water gaps', that open as a response to an environmental sign of fluctuating temperatures. Seed responses to fire might be actually exaptations, instead of adaptations *sensu stricto*. In other words, heat-induced traits did not properly evolve related to fire as a selective pressure, being result of a 'shift' in its function through the evolutionary process in seasonal ecosystems (Bradshaw *et al.* 2011). Santana *et al.* (2013) showed that seeds of *Ulex parviflorus* had their dormancy broken when exposed to daily fluctuating temperatures, arguing that fire was not the main factor affecting plant germination.

Auld & O'Connell (1991) analysed 35 species of Fabaceae in Australia, and they concluded that the time of exposure was less important than the temperature to germination of these species. Although *Stylosanthes montevidensis* had the highest germination percentages after the exposure to 120°C, showing that this temperature broke the dormancy of part of the seeds (20%), that means that 80% fraction of the population remained dormant or did not germinate for another reason. In the case of our study, 78% of the remaining seeds were damaged by fungi and thus, it is not possible to affirm if they would have ger-

minated in the absence of the fungi. Silveira & Overbeck (2013) showed that a grassland species, *Desmodium barbatum*, increased the germination when exposed to 80°C, but it is not clear if the remaining seeds were dormant or dead, since the authors did not mention a viability test by the end of experiments. Most studies addressing the effects of high temperatures on the germination of Fabaceae species found peak germination between 70 – 100°C (Martin *et al.* 1975; Auld & O'Connell 1991; Williams *et al.* 2003). Luna *et al.* (2007) reported that maximum germination for Mediterranean plants was after the exposure to 80°C, and that these temperatures can be reached by the soil during summer, being these species classified as having facultative pyrogenic dormancy, or as gap dependent (Ooi *et al.* 2014).

Some authors found that long exposure to high temperatures decrease germination or even kill seeds (e.g. Keeley 1991; Tarrega *et al.* 1992; Herranz *et al.* 1998). However, our study showed that after the exposure to high temperatures, seeds still germinated. Those results are very consistently with what has been reported to some grass species from the Brazilian subtropical grasslands (Overbeck *et al.* 2006) and herbaceous species in the Cerrado ecosystem (Fichino *et al.* 2012; 2016; Le Stradic *et al.* 2015). When species from Cerrado were compared to forest species, the exposure to high temperatures did not affect negatively the Cerrado species, while forest species showed lower germination percentages (Ribeiro *et al.* 2013; Ribeiro & Borghetti 2014). Therefore, even with no evidence of fire-related cues to germination, seeds can resist the passage of fire and this trait (fire resistance) should also be considered as an important trait in flammable ecosystems (Fichino *et al.* 2016).

We showed that the exposure to high temperatures did not break dormancy of legume species in Brazilian subtropical grasslands, except for one species: *Stylosanthes montevidensis* (20% of seeds). Therefore, other factors related to breaking of dormancy (e.g. temperature fluctuations) might play a more important role than the direct effect of heat shocks. Additionally, we highlight the importance of resistance of seed propagules as a plant functional trait in fire-prone ecosystems.



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## References

- Aguiar L. 1986. Estudo preliminar da flora e vegetação de morros graníticos da Região da Grande Porto Alegre, Rio Grande do Sul, Brasil. *Iheringia série Botânica* 34: 3-38.
- Auld TD, O'Connell MA. 1991. Predicting patterns of post-fire germination in 35 eastern Australian Fabaceae. *Australian Journal of Ecology* 16: 53-70.
- Baskin C. 2003. Breaking physical dormancy in seeds—focusing on the lens. *New Phytologist* 158: 229-231.
- Baskin CC, Baskin JM. 2000. Ecology and evolution of specialized seed dispersal, dormancy and germination strategies. *Plant Species Biology* 15: 95-96.
- Behling H, Pillar VD, Müller S, Overbeck GE. 2007. Late-Holocene fire history in a forest-grassland mosaic in Southern Brasil: implications for conservation. *Applied Vegetation Science* 10: 81-90.
- Bradshaw SD, Dixon KW, Hopper SD, Lambers H, Turner SR. 2011. Little evidence for fire-adapted plant traits in Mediterranean climate regions. *Trends in Plant Science* 16: 69-76.
- Bradstock RA, Auld TD. 1995. Soil temperatures during experimental bushfires in relation to fire intensity: consequences for legume germination and fire management in south-eastern Australia. *Journal of Applied Ecology* 32: 76-84.
- Brasil. Ministério da Agricultura, Pecuária e Abastecimento. 2009. Regras para análise de sementes. Brasília, MAPA/ACS.
- Fichino B, Fidelis A, Schmidt I, Pivello VR. 2012. Efeitos de altas temperaturas na germinação de sementes de capim-dourado (*Syngonanthus nitens* (Bong.) Ruhland, Eriocaulaceae): implicações para o manejo. *Acta Botanica Brasílica* 26: 508-511.
- Fichino B, Pivello VR, Fidelis A. 2016. Does fire trigger seed germination in the Neotropical Savannas? Experimental tests with six Cerrado species. *Biotropica* (in press).
- Fidelis A. 2010. South Brazilian Campos grasslands: biodiversity, conservation and the role of disturbance. In: Runas J, Dahlgren T. (eds.) *Grassland biodiversity: habitat types, ecological processes and environmental impacts*. New York, Nova Science Publisher. p. 223-239.
- Fidelis A, Blanco CC, Müller SC, Pillar VD, Pfoadenhauer J. 2012. Short-term changes caused by fire and mowing in Brazilian Campos grasslands with different long-term fire histories. *Journal of Vegetation Science* 23: 552-562.
- Fidelis A, Delgado-Cartay MD, Blanco CC, Müller SC, Pillar VD, Pfoadenhauer J. 2010a. Fire intensity and severity in Brazilian campos grasslands. *Interciencia* 35: 739-745.
- Fidelis A, Müller SC, Pillar VD, Pfoadenhauer J. 2010b. Population biology and regeneration of forbs and shrubs after fire in Brazilian Campos grasslands. *Plant Ecology* 211: 107-117.
- Hanley M, Lamont BB. 2000. Heat pre-treatment and the germination of soil- and canopy-stored seeds of south-western Australian species. *Acta Oecologica* 21: 315-321.
- Herranz JM, Ferrandis P, Martínez-Sánchez JJ. 1998. Influence of heat on seed germination of seven Mediterranean Leguminosae species. *Plant Ecology* 136: 95-103.
- Janagathan GK. 2015. Are wildfires an adapted ecological cue breaking physical dormancy in the Mediterranean basin? *Seed Science Research* 25: 120-126.
- Jayasuriya KMG, Wijetunga ASTB, Baskin JM, Baskin CC. 2013. Seed dormancy and storage behavior in tropical Fabaceae: a study of 100 species from Sri Lanka. *Seed Science Research* 23: 257-269.
- Keeley JE. 1991. Seed germination and life history syndromes in the California Chaparral. *The Botanical Review* 57: 81-116.
- Keeley JE, Fortheringham CJ. 2000. Role of fire in regeneration from seed. In: Fenner M. (ed.) *Seeds: the ecology of regeneration in plant communities*. Wallingford, CABI. p 311-330.
- Keeley JE, Pausas JG, Rundel PW, Bond WJ, Bradstock RA. 2011. Fire as an evolutionary pressure shaping plant traits. *Trends in Plant Science* 16: 406-411.
- Le Stradic S, Silveira FAO, Buisson E, Cazelles K, Carvalho V, Fernandes GW. 2015. Diversity of germination strategies and seed dormancy in herbaceous species of *campo rupestre* grasslands. *Austral Ecology* 40: 537-546.
- Livi FP. 1999. Elementos do clima: o contraste de tempos frios e quentes. In: Menegat R, Porto ML, Carraro CC, Fernandes LAD. (eds.) *Atlas ambiental de Porto Alegre*. Porto Alegre, Editora da Universidade Federal do Rio Grande do Sul. p. 73-78.
- Luna B, Moreno JMM, Cruz A, Fernández-González F, Fern F. 2007. Heat-shock and seed germination of a group of Mediterranean plant species growing in a burned area: An approach based on plant functional types. *Environmental and Experimental Botany* 60: 324-333.
- Manly BFJ. 2007. Randomization, bootstrap, and Monte Carlo methods in biology. Boca Raton, Chapman & Hall/CRC.
- Martin RE, Miller RL, Cushwa CT. 1975. Germination response of legume seeds subjected to moist and dry heat. *Ecology* 56: 1441-1445.
- Moreira B, Pausas JG. 2012. Tanned or Burned: The Role of Fire in Shaping Physical Seed Dormancy. *PLoS ONE* 7: e51523. 10.1111/j.1600-0706.2011.20258.x.
- Moreira B, Tormo J, Estrelles E, Pausas JG. 2010. Disentangling the role of heat and smoke as germination cues in Mediterranean Basin flora. *Annals of Botany* 105: 627-35.
- Morrison DA, McClay K, Porter C, Rish S. 1998. The role of the lens in controlling heat-induced breakdown of testa-imposed dormancy in native Australian legumes. *Annals of Botany* 82: 35-40.
- Morrison DA, Morris EC. 2000. Pseudoreplication in experimental designs for the manipulation of seed germination treatments. *Austral Ecology* 25: 292-296.
- Ooi MKJ, Denham AJ, Santana VM, Auld TD. 2014. Temperature thresholds of physically dormant seeds and plant functional response to fire: variation among species and relative impact of climate change. *Ecology and Evolution* 4: 656-671.
- Overbeck GE, Müller SC, Fidelis A, et al. 2007. Brazil's neglected biome: The South Brazilian Campos. *Perspectives in Plant Ecology, Evolution and Systematics* 9: 101-116.
- Overbeck GE, Müller SC, Pillar VD, Pfoadenhauer J. 2006. No heat-stimulated germination found in herbaceous species from burned subtropical grassland. *Plant Ecology* 184: 237-243.
- Pillar VD. 2008. Multiv: Multivariate exploratory analysis, randomization testing and bootstrap resampling. Porto Alegre, Universidade Federal do Rio Grande do Sul.
- Reyes O, Trabaud L. 2009. Germination behaviour of 14 Mediterranean species in relation to fire factors: smoke and heat. *Plant Ecology* 202: 113-121.
- Ribeiro LC, Borghetti F. 2014. Comparative effects of desiccation, heat shock and high temperatures on seed germination of savanna and forest tree species. *Austral Ecology* 39: 267-278.
- Ribeiro LC, Pedrosa M, Borghetti F. 2013. Heat shock effects on seed germination of five Brazilian savanna species. *Plant Biology* 15: 152-7.
- Santana VM, Baeza MJ, Blanes MC. 2013. Clarifying the role of fire heat and daily temperature fluctuations as germination cues for Mediterranean Basin obligate seeders. *Annals of Botany* 111: 127-134.
- Silveira FS, Overbeck GE. 2013. Effect of high temperature on germination of four legumes from a forest-grassland mosaic in Southern Brazil. *Biota Neotropica* 13: 331-335.
- Tarrega R, Calvo L, Trabaud L. 1992. Effect of high temperatures on seed germination of two woody Leguminosae. *Vegetatio* 102: 139-147.
- Williams PR, Congdon RA, Grice AC, Clarke, PJ. 2003. Fire-related cues break seed dormancy of six legumes of tropical eucalypt savannas in north-eastern Australia. *Austral Ecology* 28: 507-514.
- Willis CG, Baskin CC, Baskin JM, et al. 2014. The evolution of seed dormancy: environmental cues, evolutionary hubs, and diversification of the seed plants. *New Phytologist* 203: 300-309.

