

Clinal variation in seed dormancy, germination and regeneration of *Boronia*



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Summary

*Physiologically dormant species dominate most of the world's ecosystems, and those from fire-prone vegetation in Australia have been described as 'difficult to germinate' and remain poorly understood. Funding from the Australian Flora Foundation contributed to the establishment of several experiments designed to understand the ecology of *Boronia*, a physiologically dormant genus, and how germination response varies at the inter- and intra-specific level. Key findings were that fire season and fire intensity affected both the magnitude and timing of seedling emergence in wild populations, with important implications for current fire management practices and for population persistence under climate change. Additionally, response to smoke, which acts as a germination cue in physiologically dormant species, varied between individuals, highlighting the potential for germination in response to variability in the fire regime. Finally, comparison of populations from high and low elevations found variation between populations, but less clear effects of local adaptation of germination temperature requirements.*

Introduction

Seed banks are a key life-history stage of plants, yet data are often limited or missing from predictive models developed to determine population persistence (Menges 2000; Walck *et al.* 2011; Ooi 2012). Plasticity and heritability of dormancy traits, seedling survival and recruitment have direct consequences on population persistence, and are all critical factors in determining seed bank dynamics. Understanding the mechanisms of key traits such as these, that drive plant population dynamics, can contribute to strategies addressing threats to biodiversity, such as from climate change.

The Rutaceae are a significant component of understorey species in Australian sclerophyll vegetation. A total of 78 native taxa occur in the Sydney region, in heaths and woodlands on nutrient-poor soils (Auld 2001). Fifteen species are rare or threatened at the NSW or national level (Benson and McDougall 2001). Despite this, the ecology of the Rutaceae remains poorly understood and their seeds are considered difficult to germinate (Dixon *et al.* 1995; Martyn *et al.* 2009). This limits effective management of species within the family, and the ability to utilise *ex situ* seed collections for conservation and horticultural purposes (Auld 2001).

The aim of this project was to understand how key traits, including seed dormancy, seed bank longevity and seedling growth and survival, vary for species from the poorly understood genus *Boronia* (Rutaceae). To achieve the objectives of this Australian Flora Foundation funded study, we proposed several aims (some modified over the course of the project), including to:

- (1) Assess how seed dormancy and germination are controlled and can vary,

- (2) Measure seed bank longevity under current and future climatic conditions, and
- (3) Assess how seed dormancy and seedling survival varies between populations along an altitudinal gradient from Sydney to the upper Blue Mountains.

This final experiment will allow assessment of whether there is any local adaptation in these key life history traits or evidence of phenotypic plasticity in response to parental conditions, utilising a climatic gradient. So far, we have addressed aim (1) by an extensive study of the seasonal temperature and fire-related requirements for germination (Mackenzie *et al.*, 2016; Ma *et al.*, in prep.). We have also achieved aim (2) by utilising climatic differences between common gardens in coastal and western Sydney. Experiments related to Aim (3) are ongoing, and have been modified due to a failed seed cohort in the first year of study.

Following is a report of the findings from our work for the completed experiments.

Methods

Dormancy and germination of Boronia – Intra-specific variation

The role of seasonal temperatures as germination cues in fire-prone habitats is often overlooked due to a focus on direct fire cues such as heat shock and smoke (Ooi *et al.* 2006; Mackenzie *et al.*, 2016). Additionally, little is known about the combined effects of multiple fire-related cues and environmental cues as these are seldom assessed in combination. We aimed to improve understanding of the germination requirements of species with physiological dormancy in fire-prone floras, and determine the level of variation at the intra-specific level, by investigating germination responses across members of the Rutaceae from south eastern Australia. We used a fully factorial experimental design to quantify the individual and combined effects of heat shock, smoke and seasonal ambient temperatures on germination of freshly dispersed seeds of seven species of *Boronia* (Table 1). The individual and combined effects of heat shock, smoke and seasonal ambient temperatures on the germination of fresh seed were tested in a fully orthogonal laboratory experiment with 12 possible factorial combinations of heat shock (2 levels: heated, unheated), smoke (2 levels: smoked, unsmoked), and seasonal temperature (3 levels: representing summer, autumn/spring, and winter). Heat shock treatments were applied by placing seeds in aluminium foil cups and exposing them to 90°C in a pre-heated oven for 10 minutes. This temperature and duration of heating falls within the range of conditions experienced by seeds in the upper layers of the soil during the passage of fire, and has been reported to promote germination of a wide range of local species, including several Rutaceae. Smoke treatments were applied by placing seeds in aluminium

Table 1. Attributes of the study species and their study sites

Species	Habitat ¹	Fire Response	Regional Significance ¹	Study Site	Elevation (m)
<i>Boronia anemonifolia</i> subsp. <i>anemonifolia</i>	Among rocks in open forest and heath	killed by fire ² (may resprout ²)	widespread on coast and ranges	Evans Lookout, Blue Mountains NP	890
<i>Boronia floribunda</i>	Ridgetops and rock outcrops in open forest and heath	resprouts ^{1,2,3}	local endemic	Scouters Mountain, Heathcote NP	60-90
<i>Boronia fraseri</i>	Gullies in moist eucalypt open forest	killed by fire ²	local endemic	Campfire Creek, Blue Mountains NP	140-180
<i>Boronia ledifolia</i>	Ridges and rocky outcrops in woodland	killed by fire ^{1,2,3}	widespread on coast & ranges	Warumbul Road, Royal NP	150
<i>Boronia pinnata</i>	Ridges and plateaus in eucalypt forest and heath	killed by fire ^{2,3} (may resprout ^{1,2,3})	chiefly coastal	Narrowneck Peninsula, Blue Mountains NP	950
<i>Boronia serrulata</i>	Rock outcrops and platforms in moist heath and woodland	killed by fire ^{1,2,3}	local endemic	Scouters Mountain, Heathcote NP	180
<i>Boronia thujona</i>	Gullies, creeks, clifflines in moist eucalypt open forest	killed by fire ^{1,2}	northern limit of distribution	Flat Rock Creek Royal NP	80

¹ Benson and McDougall (2001); ² NSW Fire Response Register; ³ BDE Mackenzie (pers. obs.)

Table 2. Meteorological data derived from Australian Bureau of Meteorology (BOM) weather stations closest to the seed collection sites.

Parameter	Lucas Heights	Springwood	Mount Boyce
Mean summer max/min temp. (°C)	26/17 ^a	27/16 ^a	23/13 ^b
Mean autumn/spring max/min temp. (°C)	22/12 ^b	22/12 ^b	17/8 ^c
Mean winter max/min temp. (°C)	16/7 ^c	16/7 ^c	10/3 ^d
Mean annual rainfall (mm)	1015	980*	960
Elevation (m)	140	320	1080
Relevant study species/collection sites	<i>B. floribunda</i> <i>B. ledifolia</i> <i>B. serrulata</i> <i>B. thujona</i>	<i>B. fraseri</i>	<i>B. anemonifolia</i> <i>B. pinnata</i>

^{a,b,c,d} denote four ambient seasonal maxima/minima distinguishable between the study sites.

* rainfall data derived from the Glenbrook weather station as it is closest to the *B. fraseri* site but does not provide temperature data

foil cups inside a 60 L plastic chamber and piping in aerosol smoke for 10 minutes. Smoke was generated using a bee smoker to combust fresh leaves, dry litter and fine fuels collected from sclerophyll woodland. This method and duration of smoke exposure is reported to enhance germination of a number of species in the region. Replicate heat shock and smoke treatments, respectively, were applied independently to avoid pseudoreplication, and heat shock treatments were applied first in the case of combined heat shock and smoke treatments (from Mackenzie *et al.*, 2016).

Replicates of seeds were placed on moistened filter paper in 9 cm Petri dishes. Dishes were sealed with plastic wrap to reduce desiccation and then placed inside temperature- and light-controlled incubators set to one of three seasonal temperature regimes, depending on location (Table 2) on a 12h/12h light/dark and maximum/minimum temperature cycle. The position of dishes within each incubator was rotated weekly to minimise the chance of any position effect. Filter paper was kept moist by watering with distilled water as required. Germination was scored weekly on emergence of the radicle for 14 weeks with the exception of weeks 8, 11 and 12 in which trays went uncensused. Ungerminated seeds were assessed for fill and viability post-trial (see Mackenzie *et al.*, 2016).

Inter-specific variation in response to smoke

Based on findings from the above, we focused on a single species, *Boronia floribunda*, to assess how much variation occurs between individuals. It is currently unknown whether within-population variation in germination response to smoke, at the individual level, occurs for species in fire-prone systems. Using seeds from six individual plants, we established an experiment by applying smoke treatments. Six replicates of 20 seeds per individual were placed in 9-cm Petri dishes. Half the replicates were then assigned to a smoke treatment, and the others left as Controls, which amounted to 36 replicates (6 individuals \times 2 treatments (control and smoke) \times 3 replicates). Experiments were carried out in a temperature controlled incubator at 17/6°C 12/12 h dark/light diurnal cycle for 56 days. Germination was checked every two days.

To assess the response of individuals to different smoke concentrations, smoke water was prepared by burning green and dried leaf litter in the bee smoker, and pumping the smoke through 1L of distilled water for 30 minutes. This solution was then divided into three flasks, with one lot kept at 100% and the other two diluted to 50% and 10%. Distilled water was used for the control. For this experiment, sufficient numbers of seeds were only available for four individuals. For each individual, 20 seeds in each of three replicate Petri dishes were used for each smoke water concentration per individual. Approximately 2 mL of smoke solution was added to Petri dishes for each corresponding treatment, and seeds were incubated as described above. Germination was checked every two days.

Clinal variation in dormancy and seedling performance

Again using *B. floribunda* as our study species, we collected seeds from two sites at each of two elevations (high > 950m and coastal < 100m). Four replicates of 20 seeds were placed on moistened filter paper in 9 cm Petri dishes per treatment combination (4 seasonal temperatures x 2 germination cues (smoke vs control) x 4 sites = 32 combinations). Dishes were sealed with plastic wrap to reduce desiccation and then placed inside temperature- and light-controlled incubators set to one of four seasonal temperature regimes (25/18°C, 22/12°C, 17/6°C, 11/3°C) on a 12h/12h light/dark and maximum/minimum temperature cycle, representing a range from summer temperatures at lower elevations down to winter temperatures in the upper mountains. The position of dishes within each incubator was rotated weekly to minimise the chance of any position effect. Filter paper was kept moist by watering with distilled water as required. Germination was scored weekly for 14 weeks.

Once the radicle of germinated seeds had reached 1 cm, seedlings were placed in tubestock pots in a 5:1 sand:vermiculite mixture, and fertiliser added. When seedlings had reached approximately 1 – 2cm in height, they were placed in a growth chamber for 6 weeks, set at either mean warm diurnal temperatures (25/15°C) or more stressful conditions (25/15°C plus two 1 week periods of 35/25°C representing heat waves in Weeks 2 and 5). Height and number of leaves at the beginning and end of the experiment were recorded, and Relative Growth Rates (RGR) calculated for each of these factors.

Analyses

Germination was recorded on emergence of the radicle, and calculated as a proportion of viable seeds. Germination was analysed using generalised linear models (GLMs) with logit link functions. For seedling data, RGR was calculated for height and number of leaves using the equation:

$$\ln(a_2) - \ln(a_1) / t_2 - t_1$$

where a_2 = final value and a_1 = initial value of each factor divided by time (t) of the duration of the experiment. Using RGR allowed for examination of relative increases of these traits, and was analysed using a GLM with Gaussian distribution. Analyses were conducted using R 3.2.2.

Results and Discussion

Dormancy and germination of Boronia – (i) Intra-specific variation

This experiment identified that variation exists at the intra-specific level, both in the seasonal temperature requirements for germination and the response to fire cues. This work has been published (Mackenzie et al., 2016) below:

RESEARCH ARTICLE

The Effect of Seasonal Ambient Temperatures on Fire-Stimulated Germination of Species with Physiological Dormancy: A Case Study Using *Boronia* (Rutaceae)

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Abstract

Dormancy and germination requirements determine the timing and magnitude of seedling emergence, with important consequences for seedling survival and growth. Physiological dormancy is the most widespread form of dormancy in flowering plants, yet the seed ecology of species with this dormancy type is poorly understood in fire-prone vegetation. The role of seasonal temperatures as germination cues in these habitats is often overlooked due to a focus on direct fire cues such as heat shock and smoke, and little is known about the combined effects of multiple fire-related cues and environmental cues as these are seldom assessed in combination. We aimed to improve understanding of the germination requirements of species with physiological dormancy in fire-prone floras by investigating germination responses across members of the Rutaceae from south eastern Australia. We used a fully factorial experimental design to quantify the individual and combined effects of heat shock, smoke and seasonal ambient temperatures on germination of freshly dispersed seeds of seven species of *Boronia*, a large and difficult-to-germinate genus. Germination syndromes were highly variable but correlated with broad patterns in seed morphology and phylogenetic relationships between species. Seasonal temperatures influenced the rate and/or magnitude of germination responses in six species, and interacted with fire cues in complex ways. The combined effects of heat shock and smoke ranged from neutral to additive, synergistic, unitive or negative and varied with species, seasonal temperatures and duration of incubation. These responses could not be reliably predicted from the effect of the application of single cues. Based on these findings, fire season and fire intensity are predicted to affect both the magnitude and timing of seedling emergence in wild populations of species with physiological dormancy, with important implications for current fire management practices and for population persistence under climate change.

Inter-specific variation in response to smoke

After six weeks, seed germination differed significantly among the six individuals treated with the same smoke treatment ($df=5, \chi^2=40.621, p<0.001$). For Individuals 1 and 6, germination was lower than 10% and significantly less than the other four individuals, which showed germination of 30% or more (Fig. 1). Individuals 1, and 6 germinated less than 10% while the remaining individuals reached approximately 40%.

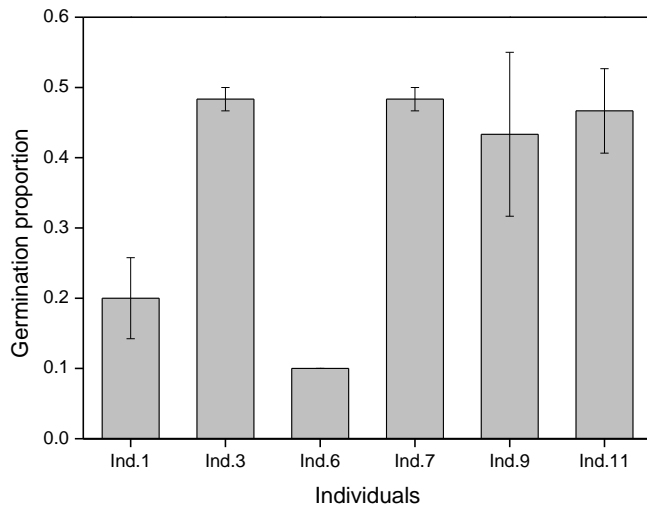


Figure 1
Seed germination proportions of different individuals in response to the same smoke cue

Seed germination of *B. floribunda* was significantly affected by the concentration of smoke water with a significant interaction between smoke concentrations and individual ($df=6, \chi^2=28.323, p<0.001$) highlighting that the pattern of response differed within the population. Two of the four Individuals (9 and 12) produced their highest germination at 100% smoke water concentrations, however there were no significant differences between the 10% and 100% concentrations for Individuals 3 and 11 (Fig. 2)

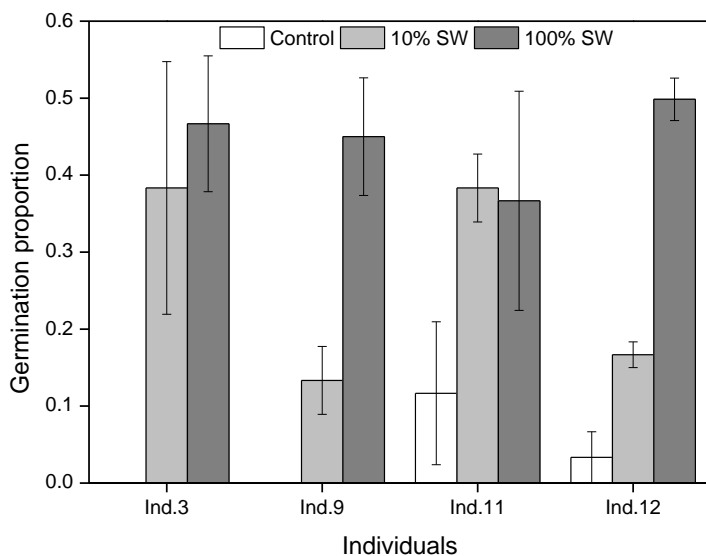


Figure 2
Seed germination of different individuals in response to different smoke concentrations

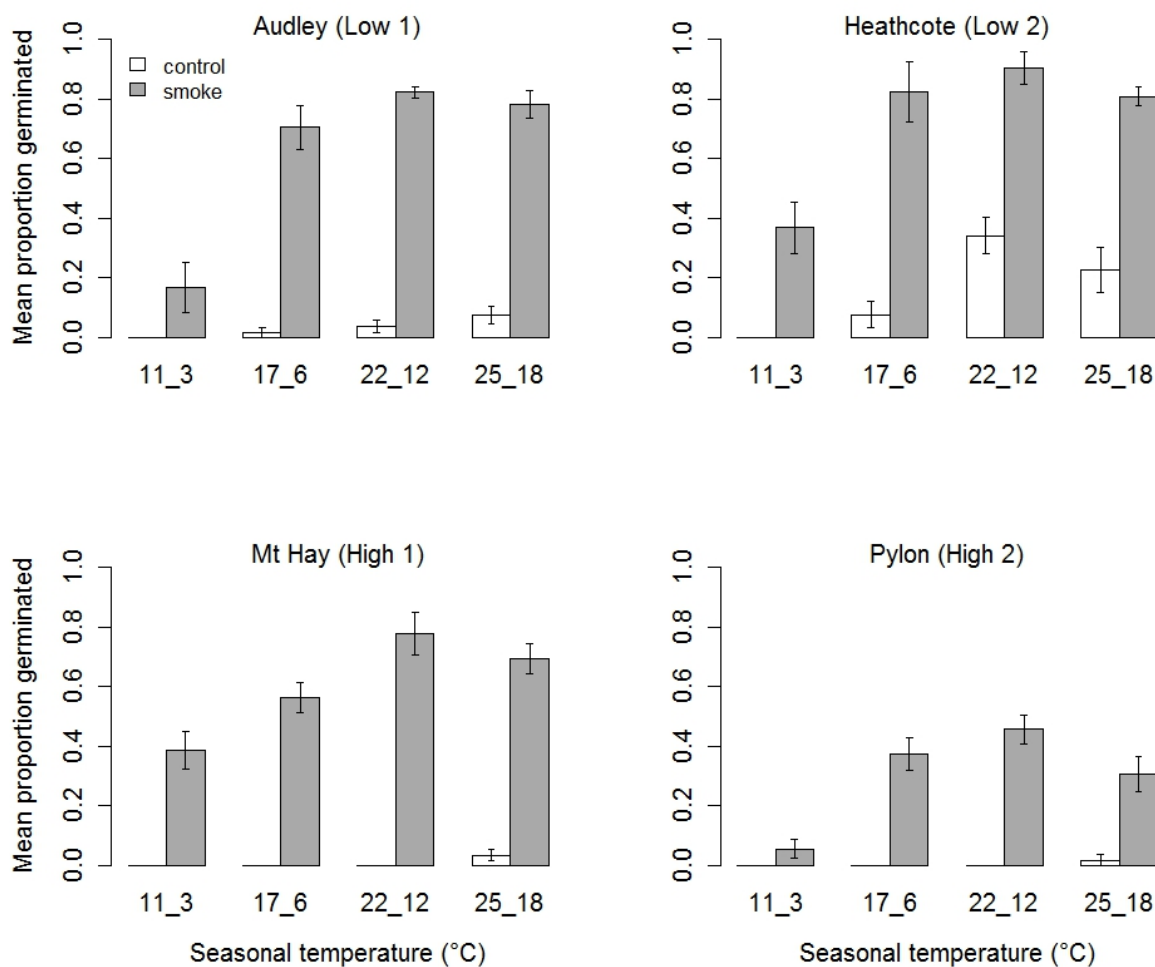
Variation in germination response to fire cues is hypothesised to contribute towards species coexistence and has been identified for physically dormant species, where heat is the main cue breaking dormancy. This ensures that some germination can occur in response to the variation of heat produced by fire in the soil (Auld, 1986; Penman and Towerton, 2008), with a proportion of seeds able to germinate under either high or low soil temperatures (Trabaud and Oustric, 1989; Ooi *et al.*, 2014). The results from our study (currently being developed for a publication in 2017) show that this variation can also occur in smoke responsive species, and highlights a similar mechanism.

Clinal variation in dormancy and seedling performance

For most locations, there was little germination without smoke (but see Heathcote site Fig. 3).

Analysis focused on the smoke-treated seeds only, and seed germination differed significantly across temperatures ($df=3, \chi^2=160.78, p<0.0001$) and among sites ($df=3, \chi^2=124.31, p<0.0001$) (Fig. 3).

Figure 3. *Boronia floribunda* germination for seeds from four sites (2 x low elevation and 2 x high elevation) incubated at four seasonal temperature regimes.



The seedling performance experiment suffered from low survival. The original design intended to incorporate 40 seedlings per site, however this was reduced at some sites to 10. As a result, there was considerable variation around the means (Fig. 4).

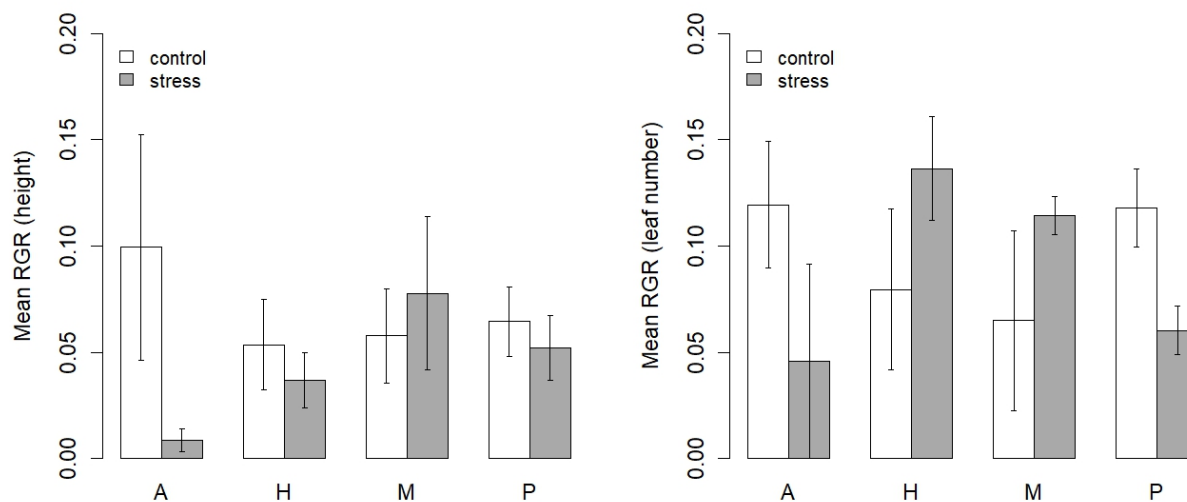


Figure 4. Relative Growth Rates (RGR) for maximum height and number of leaves produced throughout the experiment, under consistent warm season conditions (control) and heat wave conditions (stress).

Conclusions

Species with physiological seed dormancy are dominant throughout the world, however an understanding of their ecology in Australian fire-prone ecosystems is still surprisingly limited. While perhaps not unexpected, this study has clearly shown that variation in dormancy-breaking requirements and/or in response to germination cues occurs at both the inter- and intra-specific level for taxa from the genus *Boronia* in the Rutaceae family. This is similar to that reported for numerous hard-seeded species which display variation in response to heat shock for overcoming dormancy. Although these dormancy types have vastly different mechanisms, and therefore evolved independently, we consider that the similarity in their response to fire cues represents convergent evolution (Keeley & Bond, 1997). Less clear from our results is any evidence for strong local adaptation of dormancy cues for populations from different climatic maternal environments. While germination varied, considerably more work is required to identify potential maternal effects.

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