Within population variation in germination response to smoke cues: convergent recruitment strategies and different dormancy types

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Abstract

Aims Maintaining variation in germination response provides a selective advantage, by spreading risk during recruitment. In fire-prone regions, physically dormant (PY) species vary their response to dormancy-breaking fire-related heat cues at the intra-population level. However little is known about physiologically dormant (PD) species, which respond to smoke cues. These contrasting dormancy types reflect different evolutionary developmental pathways and we considered whether intra-population variation in germination of *Boronia floribunda* (PD) occurs in response to smoke.

Methods Seeds were collected from individual plants. We assessed germination magnitude and rate of seeds from each individual in response to a single aerosol smoke treatment, and three concentrations of smoke water, using replicate seed lots in temperature-controlled incubators.

Results The magnitude and onset of germination differed significantly among individuals in response to the same smoke treatment. Seeds from different individuals varied in their sensitivity to smoke water concentration, with some responding to very low doses, and others obligated to high doses.

Conclusions Variation in germination response to smoke highlights a mechanism by which PD species spread risk, by allowing some seeds to emerge quickly, while others remain dormant in the soil seed bank. The similarity to heat-cued variation displayed by PY species suggests that this could represent a convergent functional response.

Keywords: convergent evolution; fire; physiological dormancy; Rutaceae; smoke; soil seed bank

Introduction

Fire drives plant population dynamics and community diversity in a range of ecosystems around the world (Bond and Keeley 2005; Pausas and Keeley 2009; Wilgen et al. 2010). Recruitment from seeds is an important life-history stage for many species from such systems, and seedlings take advantage of post-fire conditions which are conducive to establishment and growth, ensuring that those individuals killed by the passage of fire are replaced and populations can persist. Dormancy and germination of soil-stored seeds are therefore linked to fire cues, including heat and smoke, so that seedling emergence coincides with fire (Auld and O'Connell 1991; Dixon et al. 1995; Ooi et al. 2014). There is a selective advantage to maintaining high levels of variability in germination timing, to spread risk during recruitment (Donohue et al. 2005), however there are few studies identifying the mechanisms by which this is achieved in fire-prone species, and functional similarities of species with different seed dormancy mechanisms have not been compared.

A key function of seeds of species from fire-prone ecosystems is to maintain variation in response to fire cues, to enable risk-spreading. Fire-related germination cues are inherently variable, and maintenance of within-population variation in germination response has previously been identified as an important characteristic contributing to population persistence in fire-prone regions (Bradstock et al. 1992; Liyanage and Ooi 2015). Furthermore, studies investigating both heat- and smoke-responsive species have found that variability is higher within compared to between regions (e.g. Moreira et al. 2012). A mechanism for maintaining variation in response to fire cues has been identified for species with physical seed dormancy (Ooi et al. 2015). Seeds can vary in the heat shock temperature thresholds they require (i.e. heat shock that can range from 40°C to > 80°C) to break the impermeable seed coat (Auld and O'Connell 1991; Liyanage and Ooi 2015). This is well studied, and hypothesised to contribute towards species coexistence (inter-specific variation), ensuring that some germination can occur in response to the variation of heat in the soil produced by fire (Auld 1986; Bradstock et al. 1992; Penman and Towerton 2008). This is enabled by a proportion of seeds committing to germinate under either high or low soil temperatures (Trabaud and Oustric 1989; Moreno and Oechel 1991; Ooi et al. 2014). However, the majority of plant species have physiological seed dormancy (Ooi 2007; Baskin and Baskin 2014), and smoke is an important germination cue once this type of dormancy is overcome by seasonal cues (Ooi et al. 2006; Merritt et al. 2007; Ooi 2010; Thompson and Ooi 2010, 2013). Smoke responsive species are found within key physiologically dormant family groups, including the Asteraceae, Bruniaceae, Ericaceae, Restionaceae and Rutaceae (Dixon et al. 1995; Brown and Botha 2004; Ooi et al. 2006; Mackenzie et al. 2016; Moreira and Pausas 2016).

Physiologically dormant seeds have been shown to vary in the period of after-ripening or stratification required to overcome dormancy at the intra-specific level (e.g. Hume 1994; Cruz et al. 2003; Baskin and Baskin 2014; Lu et al. 2017). However, in many physiologically dormant species from fire-prone habitats, seeds still require smoke to cue germination. In some cases, species are obligated to such cues which include chemicals such as the butenolide KAR_1 (karrikinolide) (Flematti et al. 2004; Chiwocha et al. 2009), cyanohydrins (glyceronitrile) (Flematti et al. 2011), and nitrates (Keeley and Fotheringham 1997) present in smoke, after sufficient after-ripening or stratification (Mackenzie et al. 2016; Collette and Ooi 2017). Unlike variation in soil heating during fire, relatively few studies have identified how smoke cues may vary in the field. The germination-promoting compounds in smoke, including KAR_1 and glyceronitrile (Flematti et al. 2004, 2011; Downes et al. 2013), are water soluble (Keeley and Pizzorno 1986; van Staden et al. 2000; Stevens et al. 2007). This means that the timing and magnitude of rainfall, as well as soil depth, will have a strong effect on the concentration of smoke cues that can reach seeds post-fire, in addition to the variation in the amount of smoke produced during fire (Stevens et al. 2007; Ghebrehiwot et al. 2011, 2013). Thus, like physically dormant species and heat shock response, it would be advantageous for physiologically dormant species to maintain variability in their response to smoke, with this range of sensitivities contributing to population persistence by allowing some germination even in the presence of low smoke concentrations. There is also an identified pattern of increasing germination response with increasing smoke cue concentration, with inhibitory effects possible at high concentrations (Soós et al. 2012; Downes et al. 2013). Maintaining some seeds which can respond to higher concentrations, and not be inhibited, would therefore similarly contribute to recruitment under this variable germination cue. This would contribute to a populations' ability to spread germination or bet-hedge against the failure of a seedling cohort.

Variation in germination response to smoke has been identified at the inter-population level for several species including the agricultural weed *Brassica tournefortii* (Stevens et al. 2007) and the Western Australian native endemic, *Anigozanthos manglesii* (Tieu et al. 2001). However intra-population variation could occur between individuals and, along with intra-individual variation, contribute to persistence of plant populations in fire-prone systems and promote species coexistence, as has been described for physically dormant species (Moreira et al. 2012; Tavşanoğlu and Çatav 2012; Liyanage and Ooi 2015). The vastly different mechanisms of heat-responsive physically dormant and smoke-responsive physiologically dormant species reflect the different evolutionary pathways followed during the development of these dormancy types (Vandelook et al. 2012; Baskin and Baskin 2014). We hypothesise that similar functional responses to fire cues by physiologically dormant species would represent convergent evolution, but it is currently unknown whether within-population variation in germination response to smoke, at the individual level, occurs for species in fire-prone systems.

Recent work by Mackenzie et al. (2016) identified a native Australian species, *Boronia floribunda*, with a clear smoke response. This species is suitable for use as a case study to investigate variation in germination response to smoke, both between individuals and under different smoke concentrations. Previously, model species used for bioassay assessment, such as 'Grand Rapids' lettuce (*Lactuca sativa*), have been used to ascertain the effects of smoke. The use of a native species from a fire-prone plant community provides a more direct link to understanding the potential variation in response and its ecological consequences. *Boronia* sits within the cosmopolitan Rutaceae and represents the largest genus within this family in Australia. The Rutaceae has representatives in fire-prone systems around the world (Roche et al. 1997; Auld 2001; Brown and Botha 2004). *Boronia* seeds are physiologically dormant (PD) and have persistent soil seed banks (Auld 2001; Mackenzie et al. 2016). Fire-related cues are required for germination for several species, with smoke being the dominant cue (Dixon et al. 1995; Roche et al. 1997; Mackenzie et al. 2016). The shrub *Boronia floribunda* occurs in heath and dry sclerophyll forest on sandstone, chiefly in the Sydney region of south eastern Australia (Auld 2001), and responds to smoke primarily when incubated at winter seasonal temperatures (Mackenzie et al. 2016). Using *B. floribunda* as our model species, we set out to address several key questions regarding variation of the smoke-cue response. These were: 1) Do individuals from the same population differ in their sensitivity to smoke as a germination cue? 2) Does germination increase with greater concentrations of smoke? 3) How can variation in smoke response at the intra-population level contribute to persistence? 4) Do similarities in functional responses by different dormancy types represent convergent evolution?

Methods

Seed collection

Mature seeds of *Boronia floribunda* were collected from 12 individual plants in February 2014, within a single population occurring on well-drained sandstone-derived soils in Heathcote National Park (34° 07′S, 150° 58′E). All individuals were located on the same gentle south-facing slope covering an area 60 m by 50 m, with each individual approximately 10 m from any other study plant, to minimise microclimatic differences. *Boronia floribunda* is a shrub of 0.5–1.0 m high and seeds are ballistically dispersed. Mesh bags were therefore placed on multiple branches of each individual plant after flowers had been pollinated via natural mechanisms. After maturation, bags were collected and seed lots from each of the 12 individuals kept separately. Seeds were stored in paper bags in laboratory conditions (~22°C) until experiments commenced in July 2014. Seed mass was weighed with a digital balance using approximately 30 replicate seeds (each weighed separately) per individual.

Comparison of germination response to aerosol smoke between individuals

Six individuals produced sufficient seeds to conduct intra-population germination experiments.

Six replicates of 20 seeds per seed lot were each placed in 9-cm Petri dishes. Half the replicates were then assigned to a smoke treatment, which was applied by putting opened dishes in a 100cm × 50cm × 40cm polyethylene box. Smoke was generated by burning green and dry native vegetation in a bee smoker, and was pumped into the box for 10 min, as this period of time is reported to enhance germination for species from the region (Ooi et al. 2006). During this time, the box was filled with thick smoke. This process was repeated three times, so that each replicate of each individual received a separate smoke treatment. After 10 min, seeds were removed and each replicate placed in new Petri dishes containing one layer of filter paper (Double Ring® qualitative 101 paper). Distilled water was added to all of the 36 replicates [6 individuals ×2 treatments (control and smoke) ×3 replicates] to moisten the filter paper, and water was added when necessary throughout the experiment. Petri dishes were kept in a temperature controlled incubator at 17/6°C with a 12/12 h dark/light diurnal cycle for 56 days. Germination was checked every two days and scored on emergence of the radicle. Viability of seeds was assessed using a cut test, was uniformly high, and did not differ significantly among the six individuals (df = 5, $\chi^2 = 9.333$, p = 0.097) (see Supplementary Figure).

Germination response to different smoke water concentrations

Smoke-water was prepared by burning green and dried leaf litter in the bee smoker, and pumping the smoke through 1 L of distilled water for 30 min (Jäger et al. 1996). This solution was then used at full-strength (100%), or in diluted form (10% v/v). Distilled water was used for the control. For this experiment, sufficient numbers of seeds were only available for four individuals and the mixed seed lot. For each individual, 20 seeds were placed in each of three replicate Petri dishes for each treatment. The treatments comprised of two smoke water concentrations (10% and 100%) and a water control. Approximately 2 mL of smoke solution was added to each Petri dish for the corresponding treatments, and seeds were incubated as described above. Germination was checked every two days for eight weeks and scored on emergence of the radicle.

Statistical analyses

Mean germination time (MGT) was calculated for each *B. floribunda* individual using the formula:

$$\mathrm{MGT}=\frac{\sum nT}{\sum n}$$

(Ellis and Roberts 1980), where *n* is the number of seeds newly germinated at time *T*, and *T* is the days from the beginning of the germination test. Onset of germination was recorded as the number of days to the first germinant in each replicate.

Seed mass among different individuals were compared by one-way analysis of variance (ANOVA). Final germination was calculated based on the number of viable seeds. Generalized linear models (GLMs) with a binomial error structure and logit link function were used to compare the proportional data for the final germination of individuals for both smoke experiments. The MGT and onset of germination were analysed using GLMs with a Poisson error structure and log link function. Tukey's test was used for multiple comparisons when the final germination among individuals was significant. We used linear regression to examine the relationship between seed mass and germination parameters.

Results

Seed mass of different individuals

Seed mass of *B. floribunda* showed significant differences among individuals (F = 11.537, df = 11, $p < 0.001$). The highest median mass was found for Individuals 1, 4 and 6 (>1.3 mg) and the lowest (< 1 mg) for Individuals 2, 7 and 11. Within each individual, seed mass also showed considerable variation (Fig. 1).

Comparison of germination response to aerosol smoke between individuals

After six weeks, seeds that were not treated with smoke (control seeds) had not germinated, except for negligible germination for seeds from Individual 9 (~2%) and were excluded from further analysis. Germination of smoked seeds differed significantly among the six individuals treated with the same smoke treatment (df = 5, $\chi^2 = 37.723$, $p < 0.001$). 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. 2).

For seeds treated with aerosol smoke, the MGT did not differ significantly among individuals (df = 5, $\chi^2 = 6.823$, $p = 0.234$), although the mean of Individual 11 (34.5±1.2 days) was approximately 13 days shorter than that of Individual 1 (Fig. 3a). The onset of germination did differ significantly among individuals (df = 5, $\chi^2 = 8.819$, $p = 0.117$), with Individuals 1 and 6 significantly slower than all other individuals (Fig. 3b).

There was a strong negative correlation between seed mass and the proportion of seeds germinating (n = 6, p = 0.018, $r^2 = 0.736$), with the two individuals producing the heaviest seeds germinating to the lowest proportions. There was no relationship between seed mass and MGT (p = 0.507), but a strong positive relationship with the number of days to the onset of germination (n = 6, p = 0.003, $r^2 = 0.882$), where the individuals with the heaviest seeds were slowest to germinate (Fig. 4).

Germination response to different smoke water concentrations

Seed germination of *B. floribunda* was positively affected by the application of smoke-water, with all individuals producing significantly greater response compared to the control seeds (Fig. 5). A significant interaction between smoke-water concentration and individual (df = 6, $\chi^2 = 28.323$, $p < 0.001$) indicated 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. 5).

The speed of germination, as demonstrated by MGT and onset of germination, also differed significantly among smoke-water concentrations (Table 2), with higher concentrations leading to much faster rates of germination. The mean germination time was significantly affected by smoke-water concentrations (df = 1, $\chi^2 = 5.746$, p = 0.017), but not by individuals (df = 4, $\chi^2 = 3.090$, p = 0.543). The onset of germination day was significantly different among individuals (df = 4, $\chi^2 = 10.199$, p = 0.037) and the smoke-water concentration (df = 1, $\chi^2 = 12.885$, $p < 0.001$).

Discussion

Variability in germination stimulation by both heat and smoke has previously been identified to be higher within, than between regions (Moreira et al. 2012). In our study, we found clear differences among individuals, providing evidence that this variability can be driven, at least in part, by varying response to smoke at the intra-population level. Variation occurred in the magnitude and timing of onset of germination, despite seeds being treated with the same smoke cues. Similarly, seeds from different individuals displayed varying sensitivity to smoke, with some obligated to germinate only in response to higher smoke doses. The patterns observed highlight a mechanism by which populations of smoke-responsive species may function during the recruitment phase, with different individuals producing seeds that would allow for variation in the magnitude of seed bank response. The depth from which seeds could potentially emerge would also vary between individuals, as those with more smoke-sensitive seeds could respond to the lower smoke concentrations that reach greater depths. The intra-population variation of germination response to smoke is functionally similar to that displayed by physically dormant species, which respond to heat cues (Liyanage and Ooi 2015), suggesting that this is an example of convergent evolution in response to the selective pressure of fire.

Variation in germination response to smoke provides species with some capacity to regenerate under the variable cues resulting from fire. This theory has been developed primarily from observations of variation in temperature thresholds of heat responsive physically dormant species, at the inter-specific (Trabaud and Oustric 1989; Auld and O'Connell 1991; Moreno and Oechel 1991), inter-population (Moreira and Pausas 2012; Ooi et al. 2012), and intra-population (Moreira et al. 2012; Tavşanoğlu and Çatav 2012; Liyanage and Ooi 2015) levels, and has been proposed to contribute to species co-existence and community assembly (Jiménez-Alfaro et al. 2016). Variation in the magnitude of germination in response to smoke within a population could be a form of bet-hedging, with some seeds able to take advantage of the one fire event, while other seeds are maintained in the seed bank. Spreading the onset of emergence (i.e. temporal variation) is another key risk-spreading strategy, where some seeds are able to emerge later on in case of failure of the initial post-fire cohort.

Smoke-responsive species are primarily physiologically dormant (Merritt et al. 2007; Ooi 2007) and several studies have identified within- and between-plant differences for this dormancy type (e.g. Cruz et al. 2003; Lu et al. 2017). However, while sensitivity to smoke cues is functionally analogous to dormancy-breaking temperature threshold variation in species from fire-prone regions, there have been surprisingly few studies investigating this variation in species from fire-prone systems. One of the few studies is from Western Australia, where Tieu et al. (2001) found inter-population differences in smoke response of *Anigozanthos manglesii* seeds, but no difference at the intra-population level. The authors noted that further testing was required, and it is possible that any effects could have been masked by the bulking of seeds from multiple individuals within stands. It is highly likely that variation between individuals in their response to smoke is common to many physiologically dormant species, but further work is required to confirm this.

The differing sensitivity of seeds to smoke is an important finding for understanding how populations respond to the variable concentrations of smoke produced during fire (Stevens et al. 2007; Ghebrehiwot et al. 2011). Maintaining individuals that produce highly sensitive seeds would allow some germination post-fire, even if smoke concentrations produced during the fire were low. The water soluble nature of many of the active smoke compounds (Keeley and Pizzorno 1986; Stevens et al. 2007) also means that being able to respond to low concentrations would vary the depth at which seeds in the seed bank could respond, with more sensitive seeds cued to germinate from greater depths. This again may represent a form of risk-spreading, with seedlings from deeper within the soil profile less susceptible to desiccation.

Seed size is often considered to be an important general determinant of colonisation and recruitment (Eriksson 1999). The extent to which the patterns observed in our study is driven by seed mass was highlighted by strong correlations with germination onset and magnitude (Fig. 4). While some care in interpretation has to be taken because of the small sample size, it was the two individuals with the heaviest mean seed mass that produced the lowest amounts and slowest germination. These results are similar to those found for the physically dormant *Cistus salviifolius* (Cistaceae) in the Mediterranean Basin (Tavşanoğlu and Çatav 2012), and for a mixed group of species in southeastern Australia (Moles and Westoby 2004). Small seeds are likely to benefit from faster germination and quick establishment because they have less resources available within their seed reserves. However, opposing hypotheses have been developed based on the idea that larger seeds are more at risk from predation and need to germinate quickly in order to reduce this threat (Norden et al. 2009). Results from studies investigating seed size and germination speed are inconsistent (Moles and Westoby 2004), and it is clear that further work is required to understand the evolutionary basis of this relationship.

There was little difference in maternal environmental conditions during seed development between the individuals used here, and studies of physiologically dormant species from other regions have found similar results (Donohue et al. 2005; Bischoff and Müller-Schärer 2010; Lu et al. 2017). While maternal environment effects have been shown to clearly influence seed dormancy (Fenner 1991; Gutterman 2000; Galloway 2001; Bischoff and Müller-Schärer 2010), our results and other studies suggest that genetic differentiation can play a part in determining both the depth of physiological dormancy and the individual's sensitivity to smoke.

Similarities in germination behaviour across different fire-prone vegetation communities have been described as convergent evolution (Keeley and Bond 1997). The variation in germination response found in our study highlight a mechanism by which physiologically dormant species spread risk, contributing to maintenance of populations by allowing some seeds to take advantage of a fire event more quickly, or of the one fire event, while other seeds are maintained in the seed bank. The similarity that these smoke-responsive species display in recruitment patterns to their physically dormant heat-responsive counterparts suggests that there has been convergent evolution in this functional strategy, by species with disparate dormancy mechanisms.

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Figure captions

Figure 1. Seed mass (mg) for each of the 12 individuals originally collected. Boxplots show median mass.

Figure 2. Germination proportions of seeds from six different *Boronia floribunda* individuals in response to aerosol smoke treatment, after incubation at 17/6 °C for 56 days.

Figure 3. (a) Mean germination time (MGT) and (b) time to onset of germination of six different *Boronia floribunda* individuals treated with aerosol smoke, after incubation at 17/6 °C for 56 days.

Figure 4. The linear relationship between the mean seed mass (mg) of the six different *Boronia floribunda* individuals treated with aerosol smoke and (a) Proportion of seeds germinated after incubation at 17/6 °C for 56 days, (b) mean germination time (MGT), and (c) time to onset of germination.

Figure 5. Germination proportions of seeds from four different *Boronia floribunda* individuals in response to smoke-water treatments (100%, or 10% dilution), after incubation at 17/6 °C. NB SW=smoke water