Variation in seed dormancy among populations of the fire-ephemeral flannel flower, *Actinotus helianthi*

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Dormancy is a necessary mechanism to prevent seeds from germinating during unfavourable external environmental conditions. For the Sydney flannel flower (*Actinotus helianthi* Labill.) it is not clear how the environment influences the erratic variation documented in published germination trials. This study examined differences in dormancy and viability among several wild populations of *Actinotus helianthi*. Mature seeds were collected from four populations across the Greater Sydney region. Germination of seeds was assessed at 15^{o}C and seeds were pre-treated with deionised water or 1% smoke-water (1% has been previously demonstrated to improve germination in *Actinotus leucocephalus*). Poor viability ranging from 40% to 58% was identified across all populations, producing low numbers of germinated seeds. Significant variation in germination percentage among populations was exhibited in seeds treated with smoke-water. This variability in germination recorded among populations is most likely an adaptive response to the fire history of the area, giving varying levels of smoke sensitivity. Therefore, to help reduce the amount of germination variation recorded in this study and previous studies, seed from flannel flower populations should be collected, stored and germinated separately. This might also be necessary for other Australian species.

Keywords: *Actinotus helianthi*, germination, seed dormancy, smoke-water

Introduction

Storage of seed for horticultural, restoration or conservation purposes assumes that seeds can be germinated whenever needed and that all seeds have an equal potential to germinate and establish as seedlings. This assumption requires rigorous testing as seed dormancy, and the corresponding cues to stimulate germination, are dependent on the local environmental conditions (Gutterman, 1992, Tieu et al., 2001), genetic factors (Roach and Wulff, 1987) and the interactions between these two sources of variation. Plant populations might experience different local environments because of climatic differences over wide geographic distributions (Tieu et al., 2001), soil and other habitat differences. Disturbances such as fire (Baker et al., 2005d) or clearing may change the local microclimate over time so that some years are more suitable for germination and seedling establishment. The link between local environments and germination implies that variation among populations is an important consideration for *ex situ* seedbanks.

Seed banking is the most cost-efficient and surest method of conserving flora *ex situ* (Cochrane and Monks, 2006, Offord and Meagher, 2009). Seedbanks do not simply conserve the biological diversity of a species or ecosystem, but have the potential to provide a source of genetically diverse, reproductive material. Genetic diversity provides a resource that enables a species to adapt to a changing environment, and is usually ascertained by sampling from multiple populations. Seedbanks must understand the different environmental cues required for germination or significant proportions of *ex situ* seed collections may be wasted.

Dormancy can be defined as a viable seed that does not have the ability to germinate during a specific time-frame under environmental conditions that are favourable for germination. Dormancy is of critical importance to the success of conservation and horticultural practices and is regulated by the embryo and/or the outer seed coat (testa/pericarp). There are two documented stages of dormancy: primary and secondary. Primary dormancy occurs during seed maturation on the parent plant. Secondary dormancy occurs when seeds are released from primary dormancy and unfavourable environmental conditions trigger the seeds to re-enter a dormant state (Merritt and Rokich, 2006, Turner and Merritt, 2009). The induction and release of secondary dormancy may then occur for several seasons until environmental conditions are sufficiently favourable for the germination of a particular seed – a process known as dormancy cycling.

In its simplest form, the level of dormancy for any batch of seeds can be identified by: (total-germinated seeds) / total seeds tested = proportion of dormant seeds (equation [1]). Equation [1], however, fails to take into account the proportion of viable or living seeds. Viability is known to vary among species and, like dormancy, the number of viable seeds may vary between years. Therefore, we can modify equation [1] to include seed viability: (total-germinated seeds) / (total seeds tested – number of non-viable seeds [determined by a chemical staining test]) = proportion of dormant seeds (equation [2]). This gives a more accurate measure of the level of dormancy as well as viability of a seed batch.

In addition to equation [2], the level of dormancy may be influenced by pre-treating seeds prior to sowing. One commonly used pre-treatment is smoke that has been bubbled through distilled water (or smoke-water), and it is well documented that the inclusion of smoke increases the germination of many Australian species (Dixon et al., 1995, Roche et al., 1997, Read and Bellairs, 1999b), including the West Australian *Actinotus leucocephalus* (Baker et al., 2005a, Baker et al., 2005c). However, not all seeds will germinate with the presence of smoke-water. These seeds often have a stronger level of dormancy and require other external pre-treatments or cues (possibly in conjunction with smoke water), such as heat shock to induce germination (Baker et al., 2005d, Clarke and French, 2005, Read et al., 2000, Thomas et al., 2003, Thomas et al., 2007). Furthermore, it possible that different species respond in a dose-dependent fashion and usually only one concentration was tested in screening studies. So it is not surprising that there are several cases where smoke exposure has inhibited germination (Dixon et al., 1995). For example, smoke partially inhibited the germination of the native grass *Stipa scabra* subsp. s*cabra* by around 10% (Read and Bellairs, 1999a). The native Cumberland Woodland species *Aristida vegans* was also reported to have around 6% less germination when treated with smoke derived from local vegetation (Clarke and French, 2005).

The effectiveness of smoke-water as a germination stimulant, particularly in Australian and South African plants, is a consequence of an evolutionary adaptation to fire. When a fire occurs, the smoke permeates through the seed, stimulating germination-inducing mechanisms inside the seed. Specific chemicals that are found in smoke have been now demonstrated to be the catalyst in breaking dormancy and allowing the seed to germinate (Flematti et al., 2004, Chiwocha et al., 2009). However, the biological reasons why these chemicals should stimulate germination are not yet known. In a recent study, seeds of *Tersonia cyathiflora* were observed to be responsive to smoke, but not the smoke-stimulant butenolide (Downes et al., 2010). Therefore, it is likely that there are multiple germination-inducing chemicals within smoke and some could be species specific.

Variation among populations could significantly influence the outcomes of germination studies, particularly those using single populations, by preventing accurate interpretation and modeling of dormancy trends (Andersson and Milberg, 1998). Selection acts on existing variation and if the match of the phenotype to the environment leads to a higher fitness through increased survival or reproduction then there will be a change in the population composition to increase the frequency of individuals that exhibit the more fit phenotype. If this variation has an underlying genetic basis (heritability) then the population will evolve a new distribution of that trait. Therefore, studies must examine and define the inherent variation in germination of seeds by sampling numerous populations of a single species. This is particularly important for species which show temporal and/or geographical variation as it would improve seed collection strategies and the protocols for *ex situ* seed storage. These improvements will in turn maximize germination rates as well as optimize the efficient use of seed collections for rehabilitation, restoration or horticultural practices.

Actinotus helianthi is a relatively short-lived perennial species in the Apiaceae. It is a fire ephemeral (germinate in abundance after fire), found in fire-prone sclerophyll vegetation with a geographic distribution along the eastern coast of Australia between central Queensland (QLD) and Jervis Bay on the New South Wales (NSW) south coast. Flowers are produced from late October to late February and mature seeds are formed by early November and are still present by March. Seeds are produced in compound umbels surrounded by several cream-coloured bracts on branched inflorescences that are produced in sequences of maturity referred to as umbel order. *A. helianthi* is an excellent model species to examine variability in dormancy among populations. Seeds lacking an endosperm and embryo can account for a large proportion of seeds in many Apiaceae. Thomas (1996) found 25% of parsley seeds from primary umbels had shriveled or lacked an embryo, while Lee (1995) found 0-16% of *A. helianthi* seeds lack an embryo. This could be due to poor pollination or a consequence of mass seed production (i.e. the inability of the parent plant to allocate resources evenly) and complicates the interpretation of the variability in germination. Therefore, this study aims to examine whether variation in germination percentages among populations of *A. helianthi* is associated with dormancy or with viability.

A. helianthi has potential in the horticultural industry as a cut-flower crop and potted plant, however erratic germination results has prevented full scale practices from being undertaken. Because *A. helianthi* populations are located in fire-prone habitats, it is expected that dormancy might be partly broken by the addition of a smoke-water pre-treatment for conservation and horticultural practices. A greater understanding of the variability in dormancy of *A. helianthi* and the underlying mechanisms will provide a model for other species that are difficult to germinate.

Methods

Fresh, mature seeds of *A. helianthi* were harvested towards the end of the flowering season between 25 February 2009 and 28 March 2009 in the Greater Sydney Region from populations at Kenthurst (33^{o}38’06”S 150^{o}57’12”E), Kentlyn (34^{o}02’54”S 150^{o}53’35”E), Royal National Park (RNP; 34^{o}03’44”S 151^{o}03’43”E) and Manly Dam Reserve (M. Dam; 33^{o}46’09”S 151^{o}14’02”E). Post-harvest seed samples were cleaned manually, counted and subsequently stored in paper bags and kept dry during the period between collection and the initiation of laboratory experiments on 29 April 2009.

Climate data (mean annual temperature and precipitation) for each population was obtained from the Bureau of Meteorology's website (Table 1).

Variation in dormancy and viability among populations was examined using 160 seeds from each population, giving 640 seeds in total. For each population, half of the seeds were soaked in deionised water and the second half in 1% aqueous smoke-water, made from a smoke concentrate (Seed Starter, Kings Park and Botanic Garden, Perth). A 1% (1:100) smoke-water dilution was used as it has been effective in promoting germination in *Actinotus leucocephalus* (Baker et al., 2005a). Seeds were then drained and sown on water agar (7 g L^{-1}) in petri dishes. Each dish contained 20 seeds with a sample size of n = 4 for each population and pre-treatment combination. Petri dishes were sealed with a plastic wrap to prevent moisture loss and placed in a germination incubator at 15°C (the optimum temperature for *A. helianthi*; Lee, 1995) with a 12 h light/12 h dark light regime. Seeds were first checked for germination after 14 days and subsequently checked every 2-3 days for 60 days. Seeds with a radicle protrusion of ≥2 mm were scored as germinated, counted and then removed from the dish. After the completion of each experiment, non-germinated seeds were examined for a viable embryo using a biochemical tetrazolium (TZ) test (Miller, 2005). Germination percentages were adjusted for each replicate based on the numbers of viable seeds.

Prior to analyses, the germination and viability proportion data were arcsine square-root transformed (Sokal and Rohlf, 1995). A two-factor analysis of variance (ANOVA), using Minitab 15.1.1 (Minitab Inc., Pennsylvania, USA), was performed with germination modeled as a response to two factors, smoke-water and populations as the fixed factor. A one-factor ANOVA was used to determine whether the proportion of viable seeds among populations was consistent. Fisher's LSD tests were undertaken as post-hoc analyses to identify which populations (if any) differed significantly. Results were deemed significantly different when $P < 0.05$. Annual temperature and rainfall were obtained from the Bureau of Meteorology to briefly examine environmental variation.

Results

There was no significant interaction between populations and pre-treatment ($F_{3,24} = 2.16$, $P = 0.12$). Overall, there was some difference in final germination among populations, however this was not significant ($F_{3,24} = 2.$ $P = 0.052$). A highly significant difference was recorded between the two pre-treatments ($F_{1,24} = 137.66$, $P < 0.001$) with final germination being higher in each population for seeds treated with smoke-water (Figure 1).

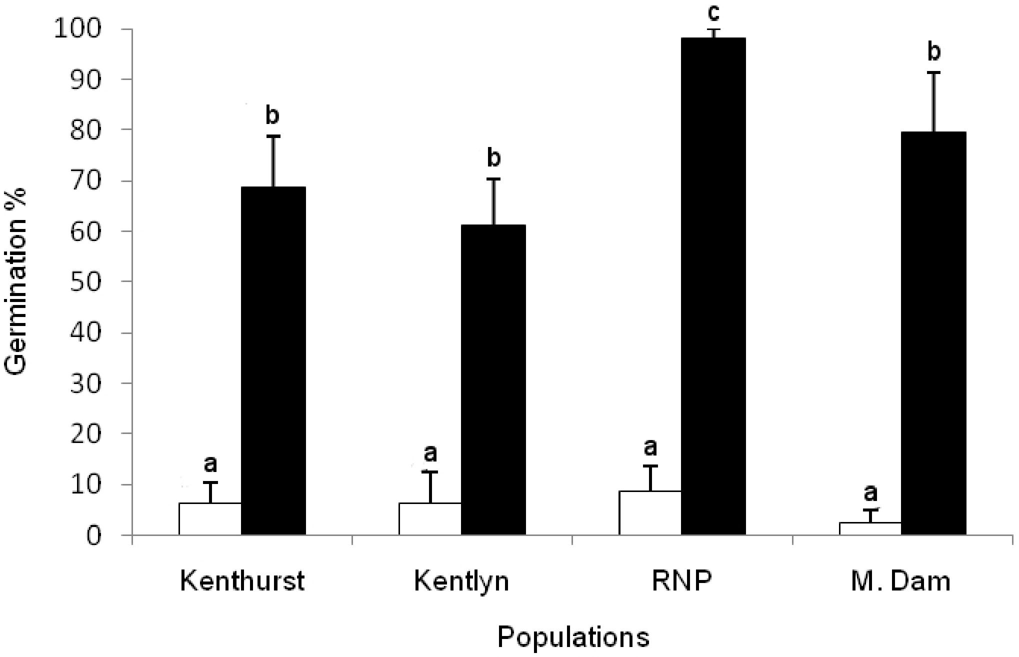

Figure 1. Percentage germination (mean + S.E.) of *Actinotus helianthi* tertiary seeds across four populations when adjusted for viability. Seeds were germinated at 15°C and pre-treated with a 24 h soak in deionised water (open bars) or 1% smoke-water (filled

bars). Different lower-case letters indicate significant differences ($P < 0.05$) in germination among populations indicated by Fishers LSD test.

Table 1. Mean annual temperature and rainfall data of the four study sites. Climate data from the Bureau of Meteorology (2009).

Site	Closest weather station	Mean annual minimum temperature (°C)	Mean annual maximum temperature (°C)	Mean annual rainfall (mm)
Kenthurst	Richmond (RAAF)	11.0	23.7	809.3
Kentlyn	Campbelltown	10.9	23.3	829.1
Royal National Park	Lucas Heights	12.3	21.4	1014.7
Manly Dam Reserve	Manly Town Hall	13.3	21.4	1218.9

From the post-hoc analyses, the four populations varied significantly when pre-treated with smoke-water ($F_{3, 15} = 4.31$, $P = 0.028$), as RNP seeds germinated to a significantly higher percentage than the other three populations tested (Figure 1). Seeds which were not treated with smoke-water produced consistently low germination percentages ranging from 2% (M. Dam) to 9% (RNP) and did not differ significantly among populations ($F_{3, 15} = 0.27$, $P = 0.845$; Figure 1). Viability of seeds ranged from 40% (Kentlyn), 46% (Kenthurst and M. Dam) and 58% (RNP) and did not differ significantly among populations ($F_{3, 31} = 2.40$, $P = 0.089$).

The four populations of *A. helianthi* sampled in this study experience similar mean annual temperatures (Bureau of Meteorology, 2009; Table 1). Rainfall, however, does vary among the four populations, with Manly Dam and Royal National Park receiving over 1000 mm annually (Bureau of Meteorology, 2009; Table 1). On the other hand, the populations at Kenthurst and Kentlyn receive around 800 mm annually (Bureau of Meteorology, 2009, Table 1).

Discussion

Actinotus spp. are classified as fire ephemerals, preferentially germinating from the soil seedbank after fire (Baker et al., 2005b, Keighery, 1982, Baker et al., 2005a). In this study, seed germination percentages were improved overall with the inclusion of 1% smoke-water, however the final germination percentage was high (>90%) only for seeds from RNP (Figure 1). These results indicate that smoke-water improves germination of *Actinotus helianthi* and closely reflects the highly variable response of seeds to smoke previously reported by Offord and Tyler (1993) (68% to 96%). This and previous germination trials were undertaken in an attempt to establish germination assays to produce maximum return from collected seed for use in horticultural practices. No consistent germination assays have been established yet and the optimal smoke-water concentration had not been identified. A 1% smoke-water concentration was shown to be optimum for *Actinotus leucocephalus* and was adopted for this study. Since significant variation was observed, it is difficult to determine if this concentration is optimal without a dose-response analysis. Such an experiment would also determine whether other populations of *A. helianthi* exhibited maximal germination at different concentrations.

The differences in germination observed among populations might be related to the site's fire history, as frequent exposure to fire *in situ* may induce a greater release from dormancy as an adaptive response to smoke. Seed from Kenthurst for example, was harvested from private property, which has been protected from fires for nearly a decade (J. Wait, pers. comm. 2009). This could contribute to the lower response of seeds to smoke-water from this population (68%) compared to Royal National Park (98%), which has experienced several fires over the last two decades. Furthermore, because there are a small proportion of seeds that germinate without smoke (Figure 1), smoke responsiveness might diminish with time while the ability to germinate without smoke is favoured if plants are not exposed to fire. In contrast, the lack of variation documented in seeds pre-treated with water in this study might be partially explained by late season collection and low viability.

It might be possible to select for seed with a higher germination potential in response to smoke, but not water alone, if differences among populations are related to their fire history. It is probable however, that the evolutionary response to fire in many Australian species has evolved over a period of several thousand years since indigenous Australians used firestick farming. However, short-term fire history cannot be ignored, particularly if increased fire frequency influences a population's health and ability to reproduce (Roy and Sonie, 1992). Identifying the correlation between these factors in longitudinal studies of *A. helianthi* would help to test this hypothesis.

The effect of smoke-water in promoting germination of *A. helianthi* could also be associated with the removal of groundcover after a fire, resulting in less competition for resources (i.e. light and soil nutrients) with other plant species. The fact that *A. helianthi* populations used in this study were located along edges of clearings (fire trails) gives strength to this argument. Populations of pre-reproductive individuals in the Blue Mountains around Blackheath were found in north-facing, exposed locations which had been burnt in the past 3-5 years. If light and inter-specific competition are important factors influencing the establishment of plants, then this would explain the trend observed in population localities where groundcover is substantially reduced by fire or clearing. There was no evidence of recent fire from the four populations collected. Populations, therefore, might have become established after clearing, causing responses in germination similar to a fire disturbance, as distinct from the Blue Mountains populations. This has also previously been reported by Lee (1995).

The four populations of *A. helianthi* sampled in this study experience similar mean annual temperatures, therefore the variation recorded cannot be related to this environmental characteristic (Bureau of Meteorology, 2009). Rainfall, however, does vary among the four populations, with Manly Dam and Royal National Park receiving around 200 mm more rain annually than Kenthurst and Kentlyn (Bureau of Meteorology, 2009; Table 1). Thus, the differences in annual rainfall could explain the germination differences seen in this study (Figure 1), as *A. helianthi* prefers habitats with >900 mm annual rainfall (Benson and McDougall, 1993). Dodd and Bell (1993) reported that many sub-shrubs like *A. helianthi* have shallow root systems. Limited water or micro-organism availability during seed maturation may decrease the proportion of viable and/or dormant seed (Bell et al., 1993). While not examined in this study, the correlation between germination and annual rainfall is an important environmental parameter that warrants further research.

The release of dormancy is also partly associated with seed maturity, particularly in species that have compound inflorescences (Gray and Thomas, 1982, Gutterman, 1992, Roach and Wulff, 1987). Whether seed maturity contributes to variation in germination for *A. helianthi* was not directly addressed in this study. In carrot seeds, embryo length was found to increase and germination improved when time of harvesting was delayed (Gray and Steckel, 1983). This might also occur in *A. helianthi* seeds, as Lee (1995) reported inconclusive results and stated that seed maturity was not always an important germination requirement. Seeds collected from Kenthurst were stored at room temperature for almost two months before the commencement of experiments. Variation as a consequence of seed maturity, therefore, could have been altered during this storage period as seeds would have begun to after-ripen. It is arguable, however, that germination tests using fresh seeds provide little insight when comparing results with seeds *in situ*. Once shed from the maternal plant, most seeds will remain in the soil seedbank for at least a month before germination commences. Therefore, differences in dormancy may be alleviated as the seeds experience some degree of after-ripening during this period. Given this, the results here may portray more accurate representations of germination *in situ*.

The seed is considered the reproductive unit of the plant only when it contains an embryo, otherwise it is referred to as a capsule. In this study the high proportion of capsules to seeds can be accounted for by three hypotheses. Firstly, resource depletion towards the end of the flowering season may cause the maternal plant to produce fewer viable seeds. A lack of available resources might be an important factor if nutrient and/or pollen availability is also low. This then leads into the second hypothesis that seed viability could be related to the age structure of individuals that comprise a population. When nutrient availability is restricted, older plants may not have photosynthetic reserves to complete reproduction during the flowering season (Bell et al., 1993). It was noted that mature plants at Kentlyn had the lowest recorded seed viability of 40%, and personal observations in late September 2009 have found the majority of the population has since died. Thirdly, the production of capsules might be an evolutionary strategy to lower predation risk by ants and other insects. In *Juniperus osteosperma*, for example, a correlation was identified between the level of predation an individual experiences and the proportion of parthenocarpic or otherwise empty seeds (Fuentes and Schupp, 1998). A similar correlation might also exist in *A. helianthi*. It is important to collect from populations known to produce high proportions of viable seeds to maximise germination success and future research into the factors that influence viability are desired.

The results from this study indicate that populations differ in their response to smoke-water and, therefore, their level of morphophysiological dormancy. This suggests that conclusions about the germination biology of *A. helianthi* can only be made if tests include seed from multiple populations. Given the variation identified in natural levels of dormancy among populations of *A. helianthi*, it may be possible to select for lower dormancy by collecting from populations known to have higher germination percentages. Germination success will also differ between years if the level of dormancy is related to environmental adaptation and/or the age structure of a population. Seed should be collected, stored and germinated separately by population in order to determine which seed-lots have the highest germination potential. Ongoing research is examining the seasonal and annual variation in dormancy as well as the environmental and/or genetic factors that could be correlated with the release of dormancy.

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