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The influence of ethanol as a solvent on the gibberellic acid-induced germination of *Brachyscome* and *Allittia* (Asteraceae) seeds

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Abstract. Gibberellic acid (GA_3) is routinely used as a germination stimulant for seeds. However, the methods used to dissolve GA_3 powder – particularly if using organic solvents – have the potential to affect germination outcomes. In this study we examined the influence of the solvent ethanol, used to dissolve GA_3, on the seed germination of 14 species of *Brachyscome* and two species of *Allittia*. These species are important Australian native composites with potential for use in habitat restoration. Seeds of 11 of these species were found to be particularly responsive to GA_3. However, the use of a low concentration of ethanol (0.5%) to dissolve GA_3 affected subsequent germination outcomes, with four species responding positively and eight negatively to this solvent. The pure effect of GA_3 was therefore masked by the presence of small concentrations of ethanol, whose effects varied between the species examined. Because of these potentially confounding effects of ethanol, we recommend the use of pure water for dissolution of GA_3 when testing seed germination responses in these genera.

Additional keywords: *Allittia*, *Brachyscome*, *Brachycome*, GA_3, gibberellin, seed testing methods.

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Introduction

Gibberellic acid (GA_3) is an endogenous plant hormone that plays an important role in the control of seed germination, and is routinely applied to stimulate seed germination or to alleviate seed dormancy of many plant species (Karssen *et al*. 1989). Reagent grade gibberellic acid is formulated as a powder and needs to be dissolved before application to seeds at desired concentrations in aqueous media. Although GA_3 is difficult to dissolve in water (solubility 5g L^{-1} at 20°C), it is freely soluble in ethanol. One quick and effective method for dissolution is to dissolve the required amount of GA_3 in a small quantity of ethanol and then make up to volume with water. However, the potential effects of this small concentration of ethanol in the treatment solution on seed germination often fail to be considered. Ethanol itself has been shown to relieve dormancy in the seeds of various species of *Cucumis* (Sreenivasulu and Amritphale 2000), *Helianthus* (Nasreen *et al*. 2015), *Lycopersicon* (Afzal *et al*. 2013), *Oryza* (Miyoshi and Sato 1997), *Lactuca*, *Hordeum*, *Avena* (Black *et al*. 2006) and several other Poaceae (Taylorson and Hendricks 1979), and to inhibit germination in *Euphorbia heterophylla* (Kern *et al*. 2009). Most seed studies involving GA_3 focus either on alleviation of seed dormancy or stimulation of seed germination, so our primary focus here was to determine the effects of ethanol when used as a solvent in either type of study.

The effects of ethanol on seed germination are dependent on many variables including temperature, ethanol concentration, length of exposure, and stage of seed development at which ethanol is applied and removed. Much of the literature on this topic has examined relatively high concentrations of ethanol, ranging between 1 and 75% (v/v). For example, Taylorson and Hendricks (1979) found that 3% ethanol over 1–3days overcame dormancy in several weedy grass species. Miyoshi and Sato (1997) showed that concentrations of 1–5% ethanol over 10 days produced maximum germination of *Oryza sativa* L. *Lycopersicon esculentum* L. primed for 24 h with 2–4% ethanol improved germination (Afzal *et al*. 2013). Higher concentrations of 25% ethanol for 15 min, or 25–75% ethanol for 10–120 min were used to overcome dormancy of *Helianthus* (Nasreen *et al*. 2015) and *Corylopsis* (Kim *et al*. 2016) species respectively.

Negative effects of ethanol have been found to result from high concentrations or long exposures. For example, injury is more likely to occur if seeds remain exposed to ethanol after radicle elongation has begun (Taylorson and Hendricks 1980). Concentrations of ethanol above 200 mM (~1.2%) were toxic for *Avena sativa* L. (Corbineau *et al*. 1991) and *Avena fatua* L. (Adkins *et al*. 1984*a*) seeds. In rice (*Oryza*) germination was inhibited with ethanol concentrations greater than 5% (Miyoshi and Sato 1997), and in tomato (*Lycopersicon*) ethanol concentrations above 6% proved toxic to germination (Afzal *et al*. 2013). Many seeds undergo a period of anoxia during imbibition, leading to the production of endogenous ethanol from the process of fermentation, and if unregulated can result in soaking injury or death (Morohashi and Shimokoriyama 1972). However, seeds that are tolerant of anoxia are able to limit ethanol production through regulation of glycolysis (Crawford 1977; Small *et al*. 1989).

When ethanol is used to dissolve gibberellin, its final concentration in solution is generally far lower than those discussed in the previous two paragraphs (Marth *et al*. 1956). However, in much of the literature in this field, the method of gibberellin dissolution is not reported, or if an alcohol is used as a solvent, the concentration is not specified, an appropriate alcohol control is not included, and potential influences of the alcohol on germination are ignored. We decided to examine the influence of the solvent ethanol at an appropriate concentration to dissolve GA_3, on germination of 14 species of *Brachyscome* and two species of *Allittia*. The genus *Brachyscome* is endemic to Australasia, with many species rare or endangered, and some proving very difficult to germinate (Aleman *et al*. 2011). However, many South Australian *Brachyscome* and other closely related composite species have a particularly strong germination response to GA_3 (Bunker 1994; Jusaitis *et al*. 2004; Aleman *et al*. 2011). This study aimed to examine the effects of a low concentration (0.5%) of ethanol when used as a solvent to dissolve GA_3, on seed germination in *Brachyscome* and *Allittia*.

Materials and methods

Seed collection

Seeds of fourteen *Brachyscome* and two *Allittia* (formerly *Brachyscome*) species native to South Australia were tested in these experiments: *B. campylocarpa* J.M.Black, *B. cuneifolia* Tate, *B. dentata* Gaudich., *B. diversifolia* (Graham ex Hook.) Fisch. & C.A.Mey. var. *diversifolia*, *B. exilis* Sond., *B. goniocarpa* Sond. & F.Muell., *B. graminea* (Labill.) F. Muell., *B. iberidifolia* Benth., *B. muelleri* Sond., *B. parvula* Hook.f. var. *parvula*, *B. perpusilla* (Steetz) J.M.Black, *B. rara* G.L.R.Davis, *B. tatei* J.M.Black, *B. xanthocarpa* D.A.Cooke, *A. cardiocarpa* (F.Muell. ex Benth.) P.S.Short and *A. uliginosa* (G.L.R.Davis) P.S.Short. These species cover a broad spectrum of Australian ecosystems, ranging from arid to wetland habitats. Seeds were collected from within the native range of each species between 2004 and 2011 and were stored at 15°C and 15% RH until used in experiments in 2011. Cut tests and tetrazolium assays performed following harvest showed that each collection contained a high proportion of full and viable seeds.

Agar germination media

Preliminary experiments with several *Brachyscome* species indicated that 250 mg L^{-1} GA_3 was the optimal concentration to stimulate germination, and ongoing exposure to GA_3 through agar was more effective than pre-soaking seeds in a GA_3 solution for 24 h. Thus, germination was tested on agar plates prepared with the appropriate concentration of GA_3 and/or ethanol. Micropropagation grade agar powder

(PhytoTechnology, St Lenexa, KS, USA, gel strength 1100 g cm^{-2}) was added to sterile reverse osmosis (RO) water to make a 1% (w/v) solution, heated in a microwave oven until boiling, then allowed to cool to 50°C in a water bath. Absolute ethanol (undenatured, analytical reagent grade) or pre-dissolved gibberellic acid (analytical reagent grade, Merck, Hohenbrunn, Germany) treatments were added to the agar solution while at this temperature, and then thoroughly mixed. The molten agar solution was then dispensed aseptically into 55 mm sterile disposable Petri dishes (10 mL per dish) and allowed to solidify.

To examine the effects of 250 mg L^{-1} GA_3 and 5 mL L^{-1} (0.5%) ethanol on germination, four germination media were prepared as follows: (1) GA_3 (250 mg) dissolved in ethanol (5 mL) before adding to agar solution to make 1 L final volume; (2) GA_3 (250 mg) dissolved in sterile RO water (50 mL) at room temperature, requiring at least 40 min stirring with a magnetic stirrer, before adding to agar solution to make 1 L final volume; (3) water agar containing 5 mL L^{-1} ethanol; and (4) water agar.

In order to duplicate the protocols of most seed germination trials, the natural pH of these treatments was not adjusted, resulting in a pH of 2.9 for the GA_3 treatments and 6.0 for the latter two water (± ethanol) treatments.

Seed germination

Seeds of each species of *Brachyscome* or *Allittia* were dispersed evenly onto the agar surface in each Petri dish (25 seeds per dish) and the dishes were sealed with plastic film. The experiment was replicated four times, with each dish being equivalent to a single experimental unit. Dishes were incubated under spring/autumn conditions (12 h day^{-1} photoperiod, 22°C/10°C (±1°C) day/night temperature), representing South Australian climatic conditions under which seeds were likely to germinate (Aleman *et al*. 2015). Lighting was provided by GE Polylux XL F36W/840 fluorescent tubes delivering a photosynthetic photon flux density of 50 μmol m^{-2} s^{-1}. Germination (radicle emergence to at least half the length of the seed) was recorded at weekly intervals over 8 weeks.

Statistical analysis

The experiment was constructed as a completely randomised design with two levels of GA_3 (0 and 250 mg L^{-1}) and two levels of ethanol (0 and 0.5%), tested over 16 species. Germination counts were converted to percentages and transformed using an arcsine square-root transformation to normalise variation. Standard error of the means was calculated for final germination data. The final germination data for each species were then subjected to two-way ANOVA to separate the effects of GA_3 and ethanol on germination. Where there was no significant interaction effect, the mean of each factor over both levels of the other was determined. If the interaction effect was significant, Tukey's *post-hoc* test was used to compare differences between means. Data are presented as untransformed means. All statistical tests were performed using Stata 12 software (StataCorp 2011).

Results

The individual germination responses (over time) of all species tested are shown in Fig. 1. Germination percentage varied significantly between species, with some (*B. perpusilla* and *B. xanthocarpa*), showing very low germination percentages. The rate of germination also varied markedly between species; *Brachyscome dentata*, *B. exilis*, *B. iberidifolia*, *B. muelleri*, *B. rara* and *B. tatei* all germinated rapidly, particularly in treatments without ethanol. When comparing the effects of individual treatments, ethanol had little effect on, or stimulated germination of five species (*B. campylocarpa*, *B. goniocarpa*, *B. iberidifolia*, *B. perpusilla* and *A. uliginosa*), whereas inhibiting germination of the remainder (Fig. 1). Highest final germination percentages were observed with *B. diversifolia* (80%), *B. rara* (87%) and *B. tatei* (91%) seeds incubated with GA_3 dissolved with water and *B. rara* (89%) seeds on pure water agar. *Allittia uliginosa* only germinated if gibberellin was present in the treatment medium.

Analysis of variance of final germination figures (week 8) showed significant effects of ethanol and gibberellin for the majority of species, but *Brachyscome iberidifolia* was the only species to show a significant interaction effect between these two factors. Tukey's *post-hoc* test revealed that all treatments (ethanol, GA_3 and GA_3 + ethanol) significantly ($P<0.001$) increased germination of *B. iberidifolia* to about the same level above water agar controls (Fig. 2).

For the remaining species there was no significant interaction between ethanol and GA_3, so the mean germination for each factor was calculated separately over both levels of the other. The pooled germination data for each of these species in response to ethanol or gibberellin treatment is shown in Figs 3 and 4 respectively. Agar treatments containing ethanol significantly reduced germination of *B. dentata*, *B. diversifolia*, *B. graminea*, *B. muelleri*, *B. parvula*, *B. rara*, *B. tatei* and *A. cardiocarpa* (Fig. 3). The largest reductions occurred with *B. muelleri* (75%), *B. tatei* (87%) and *A. cardiocarpa* (77%) seed. Germinants of *B. rara* in the ethanol-dissolved GA_3 treatment were particularly inhibited, showing cotyledonary expansion but no root development, resulting in eventual seedling mortality. Ethanol significantly increased germination of *B. campylocarpa*, *B. goniocarpa* and *B. perpusilla*, but showed no significant effect on seeds of *B. cuneifolia*, *B. exilis*, *B. xanthocarpa* and *A. uliginosa* (Fig. 3).

Gibberellic acid had an overall positive effect on germination in the majority of species examined. Comparing the effects of GA_3 on those species with no interaction effect, GA_3 significantly enhanced germination of *B. campylocarpa*, *B. cuneifolia*, *B. dentata*, *B. diversifolia*, *B. exilis*, *B. goniocarpa*, *B. graminea*, *B. muelleri*, *B. parvula* and *A. uliginosa*, whereas having no significant effect on *B. perpusilla*, *B. rara*, *B. tatei*, *B. xanthocarpa* and *A. cardiocarpa* (Fig. 4). Species with the largest increases in germination with GA_3 were *B. diversifolia* (61%), *B. goniocarpa* (80%) and *B. muelleri* (61%).

Discussion

The genus *Brachyscome* includes many annual and perennial herbaceous plants with potential for commercialisation (Bunker 1995) or use in habitat restoration (Jusaitis *et al*. 2004; Aleman *et al*. 2013), and also includes many species of conservation significance (Jusaitis *et al*. 2004; Aleman *et al*. 2011). However, little is known about the specific dormancy-breaking and germination requirements of many *Brachyscome* species, so it is an appropriate genus with which to examine the role of gibberellin in germination and to evaluate potential effects of small concentrations of ethanol when used as a solvent to dissolve gibberellin.

Gibberellic acid proved to be a highly effective germination stimulant for the majority of *Brachyscome* and *Allittia* species examined here. Of the 16 species tested, 11 showed significantly enhanced germination with gibberellin, whereas only five species showed no significant effect. Dissolving GA_3 in water rather than in ethanol yielded significant improvements in germination of six species above all other treatments. Gibberellin did not inhibit germination in any of the species tested. Earlier studies have confirmed the activity of gibberellin in stimulating germination in species of *Brachyscome* (Bunker 1994; Jusaitis *et al*. 2004; Aleman *et al*. 2011), and the hormone has also been shown to stimulate germination in other genera within the Asteraceae (Plummer and Bell 1995; Afolayan *et al*. 1997; Merritt *et al*. 2006).

Ethanol produced negative effects on germination in eight of the 16 species tested. Even though it may be time consuming to completely dissolve GA_3 in pure water, the addition of ethanol as a solvent to speed dissolution added significant variability to the germination results, and in at least one species tested, produced damaging morphological effects. *Brachyscome rara* showed abnormal development of germinants, even though the ethanol concentration used here was relatively low (0.5%) compared with that used in other studies. The continuous exposure to ethanol may have contributed to this outcome, as toxic effects of ethanol on germination increased with increased length of exposure, particularly once radicle elongation had begun (Taylorson and Hendricks 1980). Ethanol was also found to inhibit phytochrome-enhanced germination of *Rumex crispus* seeds (Taylorson 1984), and to interact with phytochrome in the promotion of seed germination by light in other species, suggesting that it may act on phytochrome through perturbation of cellular membranes (Taylorson and Hendricks 1980). Kern *et al*. (2009) suggested that ethanol-induced inhibition of *Euphorbia heterophylla* germination resulted from cellular damage caused by the conversion of ethanol to acetaldehyde.

Interestingly, four species in the present study significantly increased their germination in response to ethanol (*B. campylocarpa*, *B. goniocarpa*, *B. iberidifolia* and *B. perpusilla*). These species produced their highest germination in the presence of both GA_3 and ethanol, suggesting a possible synergistic effect of these two chemicals. Adkins *et al*. (1984*a*) suggested that the promotive effect of ethanol on

germination may in fact be gibberellin-dependent, since the effect was blocked in *Avena fatua* by an inhibitor of gibberellin biosynthesis. Whatever its mode of action may be, these examples again show the potential for small quantities of ethanol to mask the true effect of the hormone and present yet another reason to refrain from using ethanol as a solvent for GA_3 in seed testing.

Most documented effects of exogenously applied ethanol on seed germination have been in relation to its effects on breaking seed dormancy. Early studies on the use of ethanol for removing dormancy in seeds found that many substances that have anaesthetic properties in animals can also overcome seed dormancy through an effect on cell membranes (Taylorson and Hendricks 1979, 1980; Taylorson 1982, 1988). The effect is unlikely to be on the membrane lipid composition (Jackson and St John 1984), but it has been suggested that amphipathic molecules such as ethanol break dormancy by partitioning into cell membranes to affect phospholipid head group spacing and thereby facilitate membrane protein-regulated pathways essential for germination (Hallett and Bewley 2002). Other evidence has suggested that ethanol may break dormancy by acting as a substrate for respiration thereby stimulating glycolysis (Adkins *et al*. 1984*b*; Corbineau *et al*. 1991). One recent study postulated that soaking seeds in an ethanol solution may alter the morphology of the seed coat, thus causing inhibitory chemicals such as abscisic acid to be leached from seeds, resulting in increased germination (Kim *et al*. 2016).

We did not determine the mechanism or modes of ethanol action for the species that responded to the alcohol in this study. Alcohol is readily metabolised in plant cells (Taylorson 1984), being rapidly converted in *O. sativa* embryos to a carboxylic acid, and it is thought that the weak acid, rather than the alcohol, produces the germination response (Cohn 2002). Thus, the treatment that is applied to the seed may not necessarily be the chemical that is breaking dormancy or inhibiting germination (Cohn 2002). Quite possibly, ethanol may act in distinct ways for different species. We suggest further research to gain an understanding of the effects of ethanol on the *Brachyscome* and *Allittia* genera, including whether factors such as ethanol concentration (Afzal *et al*. 2013), the duration of exposure to ethanol (Taylorson and Hendricks 1979) and the level of available oxygen (Miyoshi and Sato 1997) interact with ethanol to influence germination response. Also, it is unclear whether ethanol is breaking dormancy or simply stimulating germination in the species examined here. As two of the highly dormant species in our study (*B. goniocarpa* and *B. perpusilla*) responded to ethanol, it seems that at least for these species, ethanol may be acting to alleviate dormancy.

Extensive studies on a range of seeds have shown that ethanol has dormancy breaking activity (Footitt and Cohn 2001) or other effects on seed germination (Adkins *et al*. 1984*a*; Corbineau *et al*. 1991; Miyoshi and Sato 1997). We have shown that even at low concentrations it can interfere with seed germination, either positively or negatively depending on the species. Our research demonstrates some effects that commonly used solvents such as ethanol can have on germination, and highlights the importance of precise reporting of detailed methods used in testing seed germination chemicals. To avoid confounding results, we recommend that for germination trials involving gibberellin, the hormone is dissolved in pure water rather than in ethanol. Ethanol is also routinely used at high concentrations as a pre-treatment to sterilise seeds against fungal contamination (Oyebanji *et al*. 2009; Nasr *et al*. 2013), and it is quite possible that such treatments could also influence germination outcomes.

Conflicts of interest

The authors declare no conflicts of interest.

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Figures

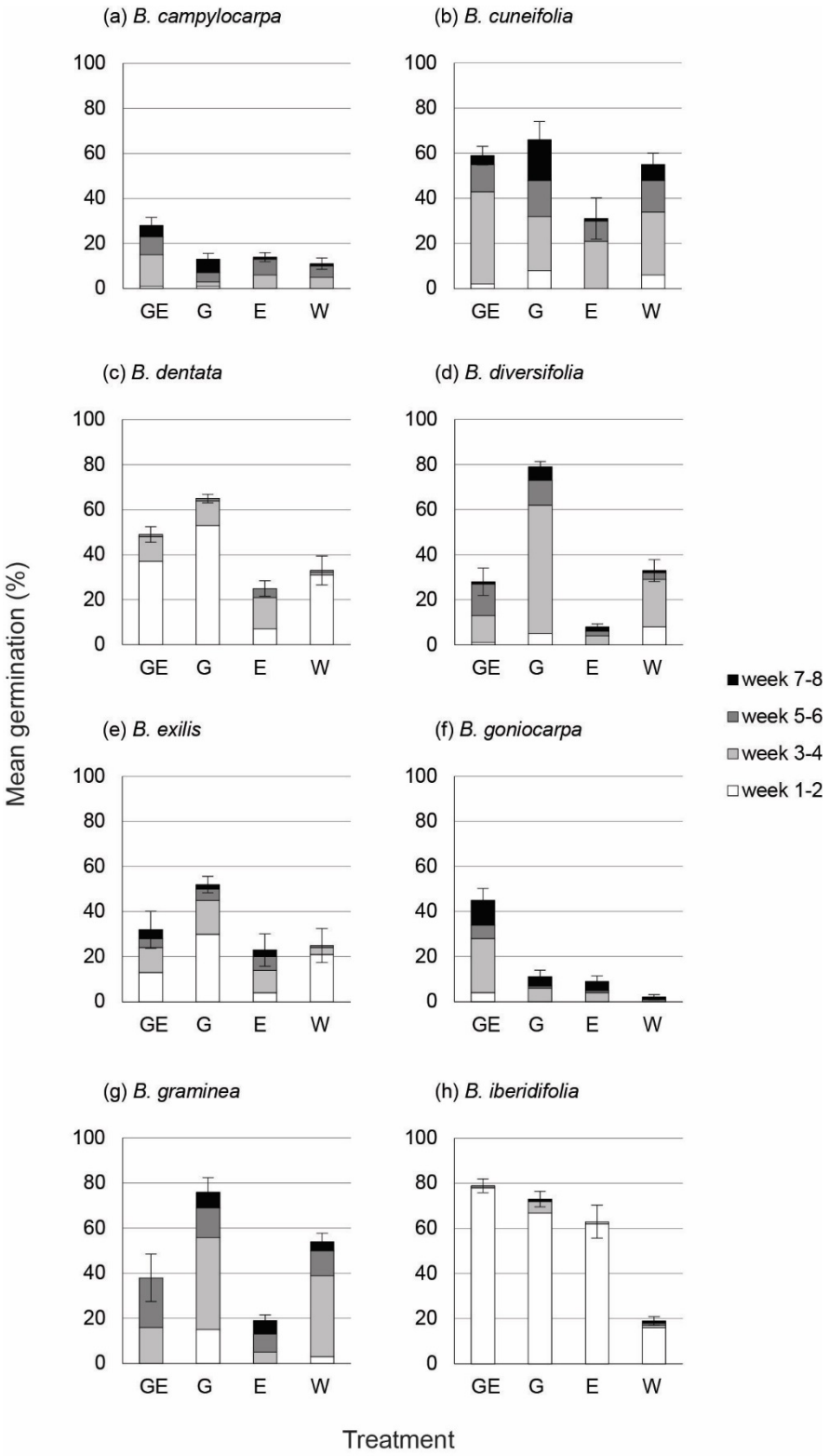

Fig. 1. Mean germination of *Brachyscome* and *Allittia* seeds in response to four seed treatments: GE, GA_3 + ethanol; G, GA_3; E, ethanol; W, water. Error bars indicate ±s.e. for final germination (n = 4).

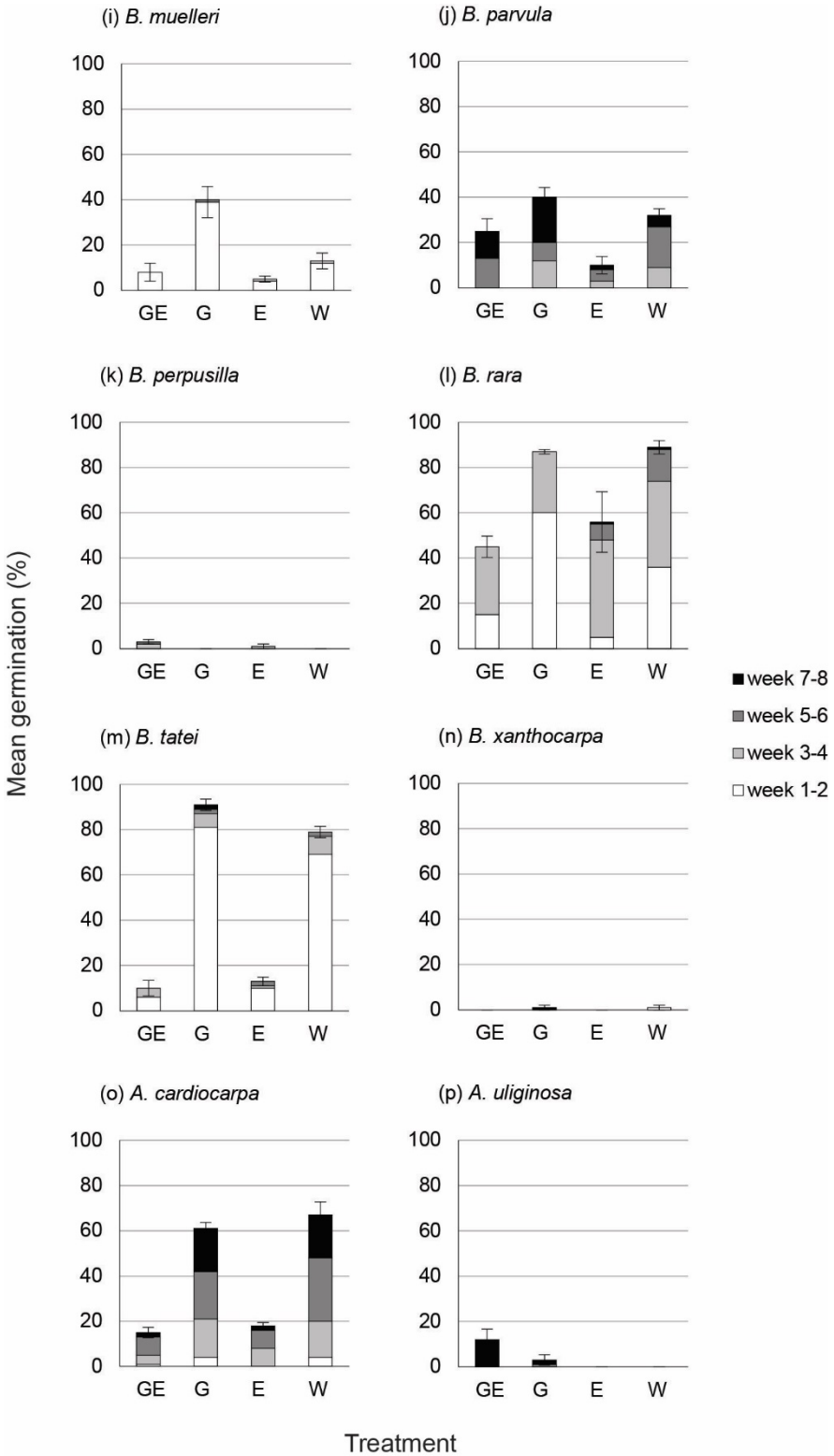

Fig. 1. (cont'd) Mean germination of *Brachyscome* and *Allittia* seeds in response to four seed treatments: GE, GA_3 + ethanol; G, GA_3; E, ethanol; W, water. Error bars indicate ±s.e. for final germination (n = 4).

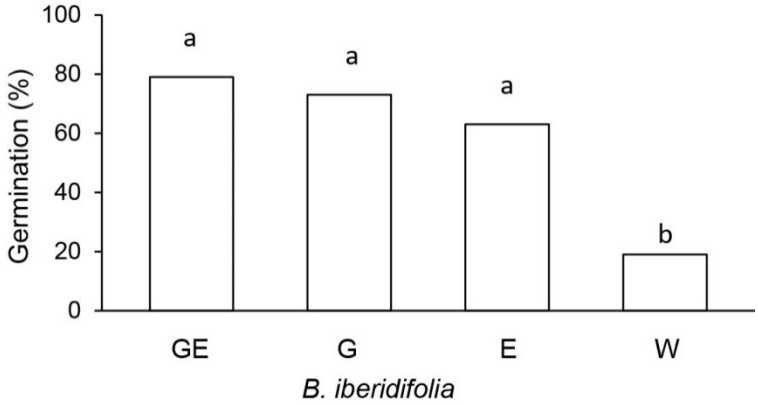

Fig. 2. The effects of individual treatments on germination of *Brachyscome iberidifolia*, the only species to show a significant interaction between ethanol and gibberellin. Bars sharing the same letter are not significantly different at the 5% level. Abbreviations (treatments): GE, GA_3 + ethanol; G, GA_3; E, ethanol; W, water.

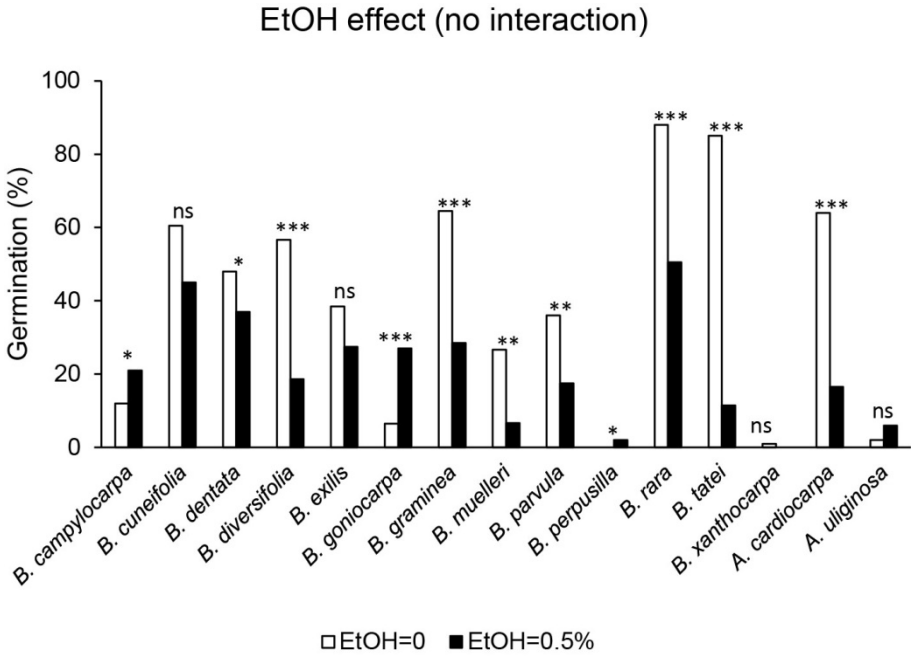

Fig. 3. The individual effects of ethanol on germination of *Brachyscome* and *Allittia* species where the ethanol × gibberellin interaction was non-significant. White bars represent the mean germination of treatments with no ethanol, and dark bars represent the mean germination of treatments with 0.5% ethanol, each calculated respectively over both levels of gibberellin. Significant difference due to ethanol are indicated above each species: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

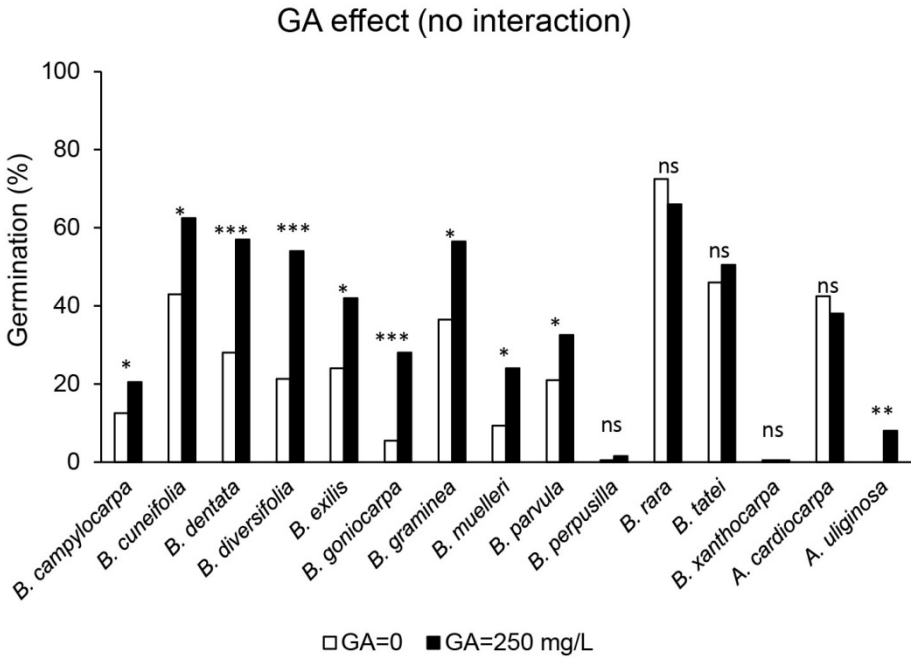

Fig. 4. The individual effects of gibberellin on germination of *Brachyscome* and *Allittia* species where the ethanol × gibberellin interaction was non-significant. White bars represent the mean germination of treatments with no gibberellin, and dark bars represent the mean germination of treatments with 250 mg L^{-1} gibberellin, each calculated respectively over both levels of ethanol. Significance difference due to gibberellin are indicated above each species: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.