SHORT COMMUNICATION

The fire ephemeral *Tersonia cyathiflora* (Gyrostemonaceae) germinates in response to smoke but not the butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one

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• *Background and Aims Tersonia cyathiflora* (Gyrostemonaceae) is a fire ephemeral with an obligate requirement for smoke to germinate. Whether it is stimulated to germinate by 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (karrikinolide, KAR_1), the butenolide isolated from smoke that stimulates the germination of many other smoke-responsive species, is tested.

• *Methods* Seeds of *T. cyathiflora* were buried in autumn following collection and were exhumed 1 year later, as this alleviates dormancy and enables seeds to germinate in response to smoke-water. Exhumed seeds were tested with smoke-water and KAR_1. Fresh preparations of these solutions were again tested on seeds exhumed 2 months later under a broader range of conditions. They were also tested on *Grevillea eriostachya* (Proteaceae) and *Stylidium affine* (Stylidiaceae) to confirm the activity of KAR_1.

• *Key Results T. cyathiflora* seeds germinated in response to smoke-water but not to KAR_1. In contrast, *G. eriostachya* and *S. affine* germinated in response to both smoke-water and KAR_1.

• *Conclusions* Although many smoke-responsive seeds germinate in the presence of KAR_1, this does not apply universally. This suggests that other chemical(s) in smoke-water may play an important role in stimulating the germination of certain species.

Key words: Butenolide, germination, karrikinolide, smoke, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, *Grevillea*, *Stylidium*, *Tersonia cyathiflora*, Gyrostemonaceae.

INTRODUCTION

Since de Lange and Boucher (1990) reported on the smoke-stimulated germination of *Audouinia capitata* (Bruniaceae), considerable research has been undertaken to determine which other species respond similarly, and to identify the bioactive chemical(s) in smoke. Species in numerous families, and from many parts of the world, have been found to germinate in response to smoke (Baldwin *et al.*, 1994; Roche *et al.*, 1997; Keeley and Fotheringham, 1998; Morris, 2000; Pérez-Fernández and Rodríguez-Echeverría, 2003; Brown and Botha, 2004; Baker *et al.*, 2005*a*; Kulkarni *et al.*, 2006).

The butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (karrikinolide, KAR_1) was identified as a highly active germination promoter in smoke (Flematti *et al.*, 2004; van Staden *et al.*, 2004). To date, almost all papers on KAR_1 have reported positive germination responses in species that respond to smoke (Flematti *et al.*, 2004, 2007; Kulkarni *et al.*, 2006; Merritt *et al.*, 2006; Stevens *et al.*, 2007; Mulaudzi *et al.*, 2009). However, KAR_1 has so far only been tested on approx. 5 % of known smoke-responsive species (Chiwocha *et al.*, 2009).

Tersonia cyathiflora (Gyrostemonaceae) is a fire ephemeral that is restricted to the sandplains north of Perth, Western Australia. This species germinates after fire, lives for a few years and then persists in the seedbank until a subsequent fire (Pate *et al.*, 1985). Previously, *T. cyathiflora* seeds have germinated in response to smoke-water, but only after a period of soil burial, with optimum germination occurring following autumn exhumation. This species appears to have an obligate requirement for smoke in order to germinate, as other treatments such as heat pulses, nitrate and light have not promoted germination (Baker *et al.*, 2005*a*, *b*). The response of *T. cyathiflora* seeds to KAR_1 has not yet been examined. The present study tests whether seeds of the smoke-responsive *T. cyathiflora* also respond to KAR_1.

MATERIAL AND METHODS

Tersonia cyathiflora fruits were collected from typical habitat, *Banksia* woodland situated on sand over limestone, in Lesueur National Park (30°3′52″S 115°5′14″E) on 13 January, 2008. The site had been burnt by wildfire 2 years prior to collection (27 January, 2006).

Germination of freshly collected seeds

Seeds were extracted from the indehiscent fruits using a nutcracker, and were surface sterilized in a solution of 2 % sodium hypochlorite with a drop of Tween 80 (polyoxyethylene sorbitan mono-oleate). The solution containing the seeds was placed under vacuum for 5 min, allowed to return to normal air pressure for 5 min and again placed under vacuum for 5 min. This was carried out in order to improve contact of seeds with the sterilizing agent by removing small air bubbles trapped on the surface of seeds. Seeds were rinsed twice with sterile deionized water and placed on two sheets of Whatman No. 1 filter paper over three 4-cm^2 pieces of 'Vileda' sponge in 9-cm Petri dishes.

For each treatment, three replicates of 50 *T. cyathiflora* seeds were used, and the filter paper was moistened with 10 mL of test solution. The test solutions were sterile deionized water (control), 10 mM potassium nitrate (KNO_3) and a 1^:^10 (v/v) dilution of smoke-water (pH = 4.05, Seed Starter, Kings Park and Botanic Garden, Perth, Western Australia). Potassium nitrate was used as it is a stimulant recommended for the germination of many species (International Seed Testing Association, 2007). The concentration applied is optimal for the germination of certain post-fire Californian species and is similar to soil nitrate levels after fire (Thanos and Rundel, 1995). Prior to dilution, the smoke-water was filtered (0.2 μm) because many insoluble compounds in smoke are deleterious to germination (Baldwin *et al.*, 1994), and the main germination-stimulating chemical in smoke is water-soluble (de Lange and Boucher, 1990; Flematti *et al.*, 2004). A 1^:^10 (v/v) dilution of the smoke-water was employed because it is optimal for the germination of other species, and has previously been shown to stimulate the germination of *T. cyathiflora* (Baker *et al.* 2005*a*, *b*, *c*).

Petri dishes were sealed with Parafilm and incubated at 15 °C, which is the optimal temperature for the germination of seeds of many species in south-western Australia (Bellairs and Bell, 1990). Seeds were exposed to two light regimes, continuous light and continuous darkness (wrapped in aluminium foil) and scored for germination after 4 weeks. Dark treatments were only scored at the completion of the trial to prevent exposure of the seeds to light. Seeds were considered to have germinated once the radicle exceeded 1 mm.

Seeds that did not germinate were dissected to determine whether they were filled and healthy, and hence potentially viable. Germination is presented as a percentage of filled, healthy seeds.

Burial trial

Seeds were buried at the site of collection in late autumn (May, 2008) in nylon mesh bags to expose them to the natural moisture and temperature regimes for 10 months. This has previously alleviated dormancy of *T. cyathiflora* seeds and allowed seeds to germinate when treated with smoke-water (Baker *et al.*, 2005*a*, *b*). Seeds were buried 2 cm beneath the soil surface, because many south-western Australia seeds naturally occur at this depth in the soil (Tacey and Glossop, 1980; Lamont *et al.*, 1993). Three burial sites were established within the population to serve as germination replicates. Seeds were exhumed in March, 2009 and the experiments undertaken on the freshly collected seeds were repeated. An additional test solution of 0.1 μM KAR_1 was included. KAR_1 was isolated and purified (99 %) from smoke-water, generated from burning *Themeda triandra* and *Passerina vulgaris* plants, as outlined in van Staden *et al.* (2004).

Additional seeds were exhumed after a further 2 months in May, 2009. Control, smoke-water and KAR_1 treatments were repeated in the light and dark to verify the results of the March, 2009 tests, and additional treatments were also conducted. These included using a higher concentration of KAR_1 (1 μM) in the light and dark at 15 °C, various treatments (control, smoke-water and KAR_1) at 25 °C in the dark, and treating seeds for 1 min in concentrated sulfuric acid (H_2SO_4) prior to treatment with water (control), smoke-water and KAR_1, in the light and in the dark at 15 °C. For the H_2SO_4 treatment, 50 seeds were placed in each of nine tea strainers and separately dipped in concentrated H_2SO_4 for 1 min (in a fumehood). Thereafter, seeds were rinsed twice in sterile deionized water before transfer to Petri dishes. Germination was scored after 3 weeks because the etiolated *T. cyathiflora* germinants from seeds exhumed in March were difficult to separate when counted after 4 weeks. Seeds were cut-tested except for those in Petri dishes in which no germination was recorded. These remained at the specified temperature and were checked again for germination after 4 months.

Verification of KAR_1 activity

Grevillea eriostachya (Proteaceae) and *Stylidium affine* (Stylidiaceae) seeds were tested to authenticate the activity of KAR_1. *S. affine* has previously shown a germination response to KAR_1 (Flematti *et al.*, 2004). *G. eriostachya*, however, has not previously been

tested in response to smoke or KAR_1, although many other *Grevillea* species are known to be smoke responsive (Roche *et al.*, 1997; Morris, 2000).

G. eriostachya seeds were collected on 12 January, 2008, between Regens Ford and Cataby, Western Australia (30°55.189′S, 115°39.204′E) and stored at ambient room conditions until germination trials commenced. *S. affine* seeds, collected near Boddington in December, 2007, were purchased from Nindethana Seed Service, Albany, Western Australia. Germination trials on the partially after-ripened seeds of both species were prepared in July, 2009 according to the germination protocol outlined above. Three treatments were tested under continuous light at 15 °C, as *Grevillea* seeds have previously been shown to germinate in the light (Morris, 2000). The treatments were control (sterile deionized water), 1^:^10 (v/v) dilution of smoke-water and 0.1 μM KAR_1. For the *S. affine* seeds, these treatments were also undertaken in the dark. For each species there were three replicates per treatment, with 25 *G. eriostachya* seeds and 50 *S. affine* seeds per replicate. The *G. eriostachya* and *S. affine* trials ran for 8 and 10 weeks, respectively.

Statistical analysis

Prior to analysis, all percentage data were converted to a proportion between 0 and 1, then arcsine square-root transformed. Comparisons of the effect on germination of light versus dark and of smoke versus KAR_1 were made using *t*-tests. Treatments were regarded as significantly different at $P < 0.05$. Statistical analyses were performed in GenStat, 12th edition (VSN International, Oxford, UK).

RESULTS

No freshly collected *Tersonia cyathiflora* seeds germinated in the control, KNO_3 or smoke-water treatments in either the light or the dark. Following burial for 10 and 12 months, *T. cyathiflora* seeds only germinated when smoke-water was applied (Table 1). Germination was negligible (≤1 %) in all other treatments undertaken following incubation for 3 or 4 weeks, including those treated with KAR_1 (Table 1). After 4 months of incubation, there was still negligible (≤1 %) germination in the non-smoke treatments (data not shown).

Grevillea eriostachya and *Stylidium affine* seeds both required either smoke-water or KAR_1 to germinate (Table 2). Final germination levels promoted by smoke-water and KAR_1 were not significantly different in *G. eriostachya* ($t = 1.10$, d.f. = 4, $P = 0.334$) or *S. affine* in the light ($t = –2.34$, d.f. = 4, $P = 0.072$) or the dark ($t = –2.23$, d.f. = 4, $P = 0.090$).

Tersonia cyathiflora seeds germinated to higher levels in the dark than the light, in the presence of smoke-water, after seeds were exhumed in March, 2009 ($t = –2.91$, d.f. = 4, $P = 0.044$) and May, 2009 ($t = 3.47$, d.f. = 4, $P = 0.026$). Similarly, *S. affine* seeds germinated to higher levels in the dark than the light in both smoke-water ($t = –7.39$, d.f. = 4, $P = 0.002$) and KAR_1 ($t = –4.89$, d.f. = 4, $P = 0.008$).

DISCUSSION

To date, almost all papers on KAR_1 have reported positive germination responses in species that respond to smoke (Flematti *et al.* 2004, 2007; Kulkarni *et al.*, 2006; Merritt *et al.*, 2006; Stevens *et al.*, 2007; Mulaudzi *et al.*, 2009). Chiwocha *et al.* (2009) report that they have identified over 60 species in 26 families that germinate in response to both smoke and KAR_1. However, this number is dwarfed by the 1200 species in 80 families for which smoke is reported to have promoted germination (Chiwocha *et al.*, 2009). The present study highlights that not all species responsive to smoke-water are necessarily responsive to KAR_1.

The results here support the suggestion that there are other chemical(s) in smoke that may stimulate seed germination. Indeed, other analogues of KAR_1 have been reported to occur in smoke-water and to stimulate germination (Flematti *et al.*, 2009; Nelson *et al.*, 2009). For example, 3,5-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one (KAR_3) is present in smoke and may contribute to the overall germination effect of smoke-water. Other analogues of KAR_1, however, are only bioactive at concentrations higher than that naturally found in smoke-water (Flematti *et al.*, 2009). These chemicals may still have a role in germination promotion, such as through synergistic interactions, but this has not yet been examined. Nevertheless, only species that respond to KAR_1, the analogue most abundant in smoke-water and the original bioactive chemical identified (Flematti *et al.*, 2004; van Staden *et al.*, 2004), have so far also been reported to respond to other karrikin analogues (Flematti *et al.*, 2007; Nelson *et al.*, 2009). The existence of bioactive smoke chemical(s) other than KAR_1 is also supported by the claim of Nelson *et al.* (2009) that a Perth collection of the holoparasite *Orobanche minor* (Orobanchaceae) was not stimulated to germinate by KAR_1, but germinated in response to smoke.

Tersonia cyathiflora is not receptive to KAR_1, and it is possible that other smoke-responsive species might also be unresponsive to KAR_1. This has important implications for possible use of KAR_1 as a field management chemical, as applications of KAR_1 might not stimulate germination of the full range of plant taxa that would be stimulated by applying smoke-water alone. However, if different smoke-responsive species have certain

requirements for germination, it would enable more specific targeting of species germination. One application could be weed management, where KAR_1 could be used to stimulate germination of the seedbank of weeds such as *Brassica tournefortii* (Stevens *et al*., 2007), which could be removed, prior to application of smoke to stimulate *T. cyathiflora* germination.

Other Gyrostemonaceae species such as *Codonocarpus cotinifolius*, *C. pyramidalis*, *Gyrostemon racemiger* and *G. ramulosus* also respond to smoke treatment (Baker *et al.*, 2005*a*; Ainsley and Thorpe, 2007), but their response to KAR_1 has not yet been tested. Studies are presently being undertaken on Gyrostemonaceae species, other than *T. cyathiflora*, to determine whether a response to smoke-water, but not KAR_1, extends beyond this monotypic genus to other genera within this Australian endemic family. This would indicate whether the response to KAR_1 is taxonomically related.

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TABLE 1. Germination of *Tersonia cyathiflora* seeds, following 10 months (exhumed March, early autumn) and 12 months (exhumed May, late autumn) of burial, when incubated for 4 and 3 weeks, respectively

Pre-treatment	Treatment	Incubation temperature (°C)	Germination (%, mean ± s.e.)	
			Light	Dark
Seeds exhumed after 10 months (germination after 4 weeks)				
None	Control (H_2O)	15	0.0 ± 0.0	0.0 ± 0.0
None	KNO_3 (10 mM)	15	0.0 ± 0.0	0.0 ± 0.0
None	Smoke-water (1^:^10, v/v)	15	22.3 ± 4.0	42.8 ± 5.8
None	KAR_1 (0.1 μM)	15	0.0 ± 0.0	0.0 ± 0.0
Seeds exhumed after 12 months (germination after 3 weeks)				
None	Control (H_2O)	15	0.7 ± 0.7	0.0 ± 0.0
None	Smoke-water (1^:^10, v/v)	15	34.9 ± 4.5	53.7 ± 2.8
None	KAR_1 (0.1 μM)	15	0.0 ± 0.0	0.0 ± 0.0
None	KAR_1 (1 μM)	15	0.0 ± 0.0	0.0 ± 0.0
None	Control (H_2O)	25	–	0.0 ± 0.0
None	Smoke-water (1^:^10, v/v)	25	–	54.0 ± 4.1
None	KAR_1 (0.1 μM)	25	–	0.0 ± 0.0
H_2SO_4	Control (H_2O)	15	–	0.7 ± 0.7

H_2SO_4	Smoke-water (1^:^10, v/v)	15	–	66.6 ± 12.4
H_2SO_4	KAR_1 (0.1 μM)	15	–	0.0 ± 0.0

TABLE 2. Germination of *Grevillea eriostachya* and *Stylidium affine* seeds incubated at 15 °C for 8 and 10 weeks, respectively

Species		Germination (%, mean ± s.e.)		
	Light/Dark	Control (H_2O)	Smoke-water (1^:^10, v/v)	KAR_1 (0.1 μM)
Grevillea eriostachya	Light	0.0 ± 0.0	52.4 ± 13.8	69.5 ± 8.0
Stylidium affine	Light	0.0 ± 0.0	12.9 ± 2.0	6.1 ± 1.8
Stylidium affine	Dark	0.0 ± 0.0	32.2 ± 1.3	24.3 ± 3.2

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