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# Evidence of different compounds in smoke derived from legumes and grasses acting on seed germination and seedling emergence

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## Abstract

Our previous study showed that smoke derived from alfalfa (*Medicago sativa*) caused different germination responses compared with that from prairie hay (*Festuca hallii*) and wheat straw (*Triticum aestivum*), but the mechanism remained unclear. In this study, we used Salad Bowl lettuce (*Lactuca sativa*) as a quick bioassay to trace the active compounds in each of these three smoke solutions. Column chromatography and high performance liquid chromatography (HPLC) were used to separate and identify active fractions. Seeds of four species from Fescue Prairie were primed for 24 h at room temperature in darkness using serial dilutions of separated active fractions, as well as karrikinolide ($KAR_1$). After priming, seeds were dried at room temperature in darkness for 7 days and subsequently incubated at 10/0°C or 25/15°C in 12 h light–12 h dark or 24 h darkness for 49 days. $KAR_1$ was in the smoke made from prairie hay, and wheat straw, but was absent in alfalfa smoke. Priming in $KAR_1$ solutions increased germination of three native species. Priming in highly concentrated $KAR_1$ reduced radicle length of *Cirsium arvense*, the only non-native species. Even though $KAR_1$ has the potential to enhance regeneration of native species in the Fescue Prairie, $KAR_1$ is not universally present in smoke derived from different plant materials. Unknown compound(s) in smoke derived from legumes remain to be identified.

**Keywords: *Festuca hallii*, germination, karrikinolide, *Medicago sativa*, seedling growth, *Triticum aestivum***

## Introduction

Historically, fire regulates plant communities in Fescue Prairie (Bailey and Anderson, 1978; Anderson and Bailey, 1980). Seedling recruitment of many species in Fescue Prairie can be promoted by fire (Romo and Gross, 2011). In fire-prone habitats, many propagules, seeds in particular, have evolved strategies to benefit from various factors associated with fire (Van Staden *et al.*, 2000). Heat, the most important physical fire cue, can fracture the hard seed coat (Brits *et al.*, 1993) or stimulate the embryo (Van de Venter and Esterhuizen, 1988; Musil and de Witt, 1991). Seed germination may also be affected by different chemical fire cues, including ethylene and ammonia (Van de Venter and Esterhuizen, 1988), nitrogen oxides (Keeley and Fotheringham, 1997), ash (Henig-Sever *et al.*, 1996), and smoke (De Lange and Boucher, 1990; Brown, 1993). Among these chemical fire cues, smoke is the most striking one, stimulating seeds with varying size, shape, and life form (Dixon *et al.*, 1995; Van Staden *et al.*, 2000).

The major active compound in plant-derived smoke is 3-methyl-2*H*-furo [2,3-c]-pyran-2-one (Flematti *et al.*, 2004; Van Staden *et al.*, 2004), known as karrikinolide ($KAR_1$) (Commander *et al.*, 2009). The promoting effects of $KAR_1$ on seed germination have been reported for various species (Merritt *et al.*, 2006; Daws *et al.*, 2007; Kulkarni *et al.*, 2007; Stevens *et al.*, 2007). $KAR_1$ can be active in stimulating seed germination at very low concentrations, it can widen the environmental conditions under which seeds can germinate (Jain *et al.*, 2006) and plays a positive role in enhancing seedling growth of weeds (Daws *et al.*, 2007) and medical plants (Kulkarni *et al.*, 2007). Fescue Prairie is well known for its adaptation to burning, indicating that seeds of species in it may be adapted to the smoke cue.

Five $KAR_1$ analogues ($KAR_2$ to $KAR_6$), also known as karrikins, were discovered and confirmed by chemical synthesis from smoke solutions (Flematti *et al.*, 2009). Although $KAR_1$ is the most important stimulant for most species, germination can be greatly affected by other analogues. For example, $KAR_2$ is the most active stimulant for germination of Arabidopsis (*Arabidopsis thaliana*) (Nelson *et al.*, 2009). Cyanohydrin glyceronitrile is another important active compound in plant-derived smoke (Flematti *et al.*, 2011). Germination of

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*Tersonia cyathiflora* responded positively to cyanohydrin glyceronitrile but not to karrikins (Downes *et al.*, 2010).

Several studies have shown that smoke produced from different plant materials has similar effects on germination response (Baxter *et al.*, 1995; Jager *et al.*, 1996; Catav *et al.*, 2012). However, over 5000 compounds may exist in smoke (Smith *et al.*, 2003) and the stimulating effects of smoke on seed germination vary among species. So it is quite possible that some active compounds that can stimulate seed germination in certain species are unidentified. Our previous study has shown that smoke derived from alfalfa (*Medicago sativa*, Fabaceae) affected germination differently compared with that produced from prairie hay (*Festuca hallii*, Poaceae) and wheat straw (*Triticum aestivum*, Poaceae), indicating that different smokes may contain different active compounds (Ren and Bai, 2016c).

The objectives of this study were to: (1) determine whether different active compounds exist in smoke originating from alfalfa, prairie hay and wheat straw; and (2) determine how active compounds in smoke derived from different plant materials and $KAR_1$ interact with temperature and light to affect seed germination and seedling growth of species in Fescue Prairie.

## Materials and methods

### *Smoke and plant materials*

Smoke solutions were produced by burning alfalfa, prairie hay and wheat straw according to Ren and Bai (2016c). Smoke made from herbaceous Fabaceae has never been tested before and alfalfa is the most popular legume forage species; testing smoke made from Prairie hay may highlight the importance of fire for the germination of species *in situ*; smoke made from wheat straw has been studied before (Abu *et al.*, 2016) and was used to compare the other two smoke types in our study. Seeds from eighteen different cultivars of lettuce were purchased from Early's Home and Garden Center in Saskatoon, SK. Cleaned seeds were kept in sealed plastic bags and stored at –20°C prior to germination experiments. A completely randomized design with five replicates was used for germination tests. Thirty seeds of each cultivar of lettuce were placed in 10-cm diameter Petri dishes lined with two layers of Whatman number 1 filter paper and moistened with 5 ml of distilled water under safe green light, and subsequently incubated at 25°C under 12 h light–12 h dark, or 24 h darkness. Petri dishes were placed in transparent zip-lock bags for those incubated in 12 h light–12 h dark. Zip-lock bags wrapped with two layers of aluminum foil were used to accommodate Petri dishes for seeds incubated in 24 h dark. Germination was recorded after 1 day of incubation (Jager *et al.*, 1996). The cultivar (*L. sativa* L. cv. Salad Bowl), which showed the greatest difference between light and dark germination, was selected as the test cultivar to compare among smoke types.

### *Salad Bowl lettuce bioassay*

For each bioassay test, a completely randomized design with five replicates was used. Thirty seeds of Salad Bowl lettuce were placed in 10-cm Petri dishes lined with two layers of Whatman number 1 filter paper and moistened with 5 ml of test solutions under a green safe light in darkness. Distilled water was used as the control for each experiment, and various dilutions of each separated fraction were used to ensure the optimum concentration range for activity. Petri dishes were sealed in plastic bags wrapped with two layers of aluminum foil and incubated in darkness at 25°C. Germination was recorded after 1 day of incubation.

### *Fractionation of smoke solutions*

The procedures for separating the active compounds involved in smoke solutions produced from alfalfa, prairie hay and wheat straw were based on modifications by Flematti *et al.* (2008). The total of 2 litres of each stock smoke solution produced from alfalfa, prairie hay and wheat straw were filtered (32 cm, Whatman number 1 filter papers) and separated. Each litre of smoke solution was exhaustively extracted with ethyl acetate (3 × 200 ml). Aqueous NaOH (1% w/v) (5 × 100 ml) was then used to fractionate the combined organic extract solution into acid (NaOH-soluble) and neutral fractions. The resulting neutral fraction solution was dried with $Na_2SO_4$, filtered, and evaporated in vacuum to remove water and to give the neutral fractions (452 mg for alfalfa, 422 mg for prairie hay, and 428 mg for wheat).

Concentrated neutral fractions were subjected to column chromatography using a 2.5 × 30 cm column packed with 50 g silica gel 60 (Merck, 0.040–0.063 mm) and eluted with a hexane: ethyl acetate gradient [hexane proportion: 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 0% (v/v); 150 ml aliquots of each mixture]. The active fraction was evaporated in vacuum and then subjected to reverse-phase (RP) C18 column (Waters Sep-Pak 12 cc Vac Cartridge), eluted with a water: methanol gradient [methanol proportion: 0, 10, 15, 20, 25 and 100% (v/v); 50 ml aliquots of each mixture]. The active fraction was evaporated to 1.5 ml in vacuum.

Part of the active fraction (20 µl) from alfalfa, prairie hay or wheat straw smoke solution was analysed with a C18-RP HPLC column (Chromolith Performance

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RP-18e 100-4.6), eluted with an acetonitrile:water gradient specified as 7%–14%–95%–7%–7% acetonitrile/water over 0–14–15–16–20 min for further fractionation. The elution rate was 2 ml/min. UV absorbance was measured at 330 nm. Fractions were collected between 3 and 12.5 min (3–5.7, 5.7–6.6, 6.6–9, 9–10, 10–11 and 11–12.5 min) based on the elution pattern. A sample of 20 µl of pure $KAR_1$ (0.1 mg/ ml) (Toronto Research Chemicals Inc.) was eluted with the same acetonitrile based method as standard. More active fractions were obtained by applying 80 µl of the fraction obtained after (RP) C18 column chromatography to the C18-RP HPLC each time, using the same methodology described above for fractionation of 10 times. In total, 18 ml (2 ml/min, 0.9 min for each run, and 10 runs) of active fractions from the smoke solutions produced from alfalfa, prairie hay and wheat straw were obtained.

### Selection of plant species from Fescue Prairie

Four Asteraceae species from Kernen Prairie in which total seedling densities were significantly increased after burning (Ren and Bai, 2016a) were chosen for this study, including fringed sage (*Artemisia frigida*), white sagebrush (*Artemisia ludoviciana*), Canada thistle (*Cirsium arvense*), and Canadian horseweed (*Conyza canadensis*). After one month after-ripening at room temperature and ambient relative humidity, cleaned seeds were kept in sealed plastic bags and stored at –20°C until they were used for germination and seedling growth experiments.

### Priming effects of active fractions on germination and seedling growth

Each active fraction (18 ml) obtained from smoke solutions produced from alfalfa, prairie hay or wheat straw was diluted in 1.07 litres of distilled water, to be equivalent to the concentration of the active compounds in each 2 litres of 1/1 v/v stock smoke solution (2 litres times 0.8 divided by 1.5, because for each type of smoke solution, in total 1.5 ml of fraction was obtained after reverse phase chromatography and 0.8 ml of this was subjected to the HPLC for further separation). In addition, to determine the effects of $KAR_1$ on seed germination, a concentration of $10^{-6}$ M of $KAR_1$ was dissolved in distilled water to be used as the 1/1 v/v $KAR_1$ solution. Each of the four different solutions (1/1 v/v), including active fractions obtained from alfalfa, prairie hay, wheat straw and $KAR_1$ solution was made into three serial dilutions, including 1/1000 v/v, 1/100 v/v and 1/10 v/v. Seeds primed in distilled water were used as the control. Each fraction (18 ml) contained 10% acetonitrile. To determine and eliminate the effects of acetonitrile on seed germination, 1.8 ml acetonitrile was dissolved in 1.07 litres distilled water and was regarded as 1/1 v/v acetonitrile solution. Various concentrated acetonitrile solutions (1/1000 v/v, 1/100 v/v, 1/10 v/v and 1/1 v/v) had no effect on germination or seedling growth of any tested species (data not shown).

Fifty seeds of each species were counted and placed in a 50 ml centrifuge tube stored vertically in perforated paper boxes. Seeds were submerged after adding 10 ml of distilled water, 1/1000 v/v, 1/100 v/v, 1/10 v/v or 1/1 v/v of each aqueous fractions, or $KAR_1$ solutions. Each centrifuge tube was sealed with a cap and kept in darkness for 24 h at 20°C. Seeds were then transferred to 10-cm Petri dishes lined with two layers of Whatman number 1 filter paper and dried for 1 week at 20°C in darkness. Seeds and filter paper in each Petri dish were then moistened with 5 ml of distilled water and incubated at 10/0°C or 25/15°C regimes under 12 h light–12 h dark or 24 h darkness. These two temperature conditions mimic average spring (April) and summer (July) daily temperatures in Keren Prairie (Environment Canada, 2012). Petri dishes were placed in transparent zip-lock bags for those incubated in 12 h light–12 h dark. Zip-lock bags wrapped with two layers of aluminum foil were used to accommodate studied Petri dishes for seeds incubated in 24 h darkness.

Germination of *A. frigida, A. ludoviciana, C. arvense* and *C. canadensis* were counted weekly for 7 weeks. Seeds with a radicle ≥1 mm were considered germinated. Distilled water was added to keep the filter paper moist. Seed germination in the 24 h darkness treatment was checked under a green safe light (Drewes *et al.*, 1995). Germinated seeds were counted weekly, and were transferred to a new Petri dish and seedlings were allowed to grow for 7 days under 12 h light–12 h dark at the same temperature for seed germination. Lengths of radicle and hypocotyl were measured after 7 days. Hence the seedling could be between 7 and 13 days old when measured.

A randomized complete block design was used for each species with five priming treatments (1/1000 v/v, 1/100 v/v, 1/10 v/v, 1/1 v/v and distilled water) for each of the four different types (alfalfa, prairie hay, wheat straw and $KAR_1$), and four replicates within each of the four germination conditions, which included 12 h light–12 h dark at 10/0°C, 24 h darkness at 10/0°C, 12 h light–12 h dark at 25/15°C and 24 h darkness at 25/15°C. The experiment was repeated once.

### Data analysis

A *t*-test was used to compare germination differences among lettuce cultivars under light and dark conditions

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at 25°C. One-way analysis of variance (ANOVA) was used to determine the effects of various fractions or smoke types on germination of Salad Bowl lettuce seeds after each separation. Treatment means were separated using Tukey's test at $P \leq 0.05$.

For the effects of separated fractions and $KAR_1$ on seed germination and seedling growth, data of total germination percentage, length of radicle, hypocotyl and total root were analysed with a randomized complete block design with two runs (four replicates for each run) using the mixed model procedure in SAS version 9.3 software (SAS Institute Inc., USA). For each species in each treatment, priming effects for each of the studied smoke types were used as independent variables. Replicates, blocks and runs were treated as random effects.

All germination data were arcsine square root transformed before analysis using *t*-test or ANOVA. Seedling length data that did not meet normality assumptions were log-transformed before analysing. The Shapiro–Wilk test was used to test data normality before and after transformation. All the data were normal distributed after transformation. Treatment means were separated using Tukey's test at $P \leq 0.05$.

## Results

### Screening L. sativa cultivars based on response to light

Seed germination of 18 different cultivars of *L. sativa* was tested in 12 h light–12 h dark and 24 h darkness at constant 25°C (Fig. 1). Germination of Salad Bowl lettuce showed the greatest difference in germination between light and dark ($P < 0.01$).

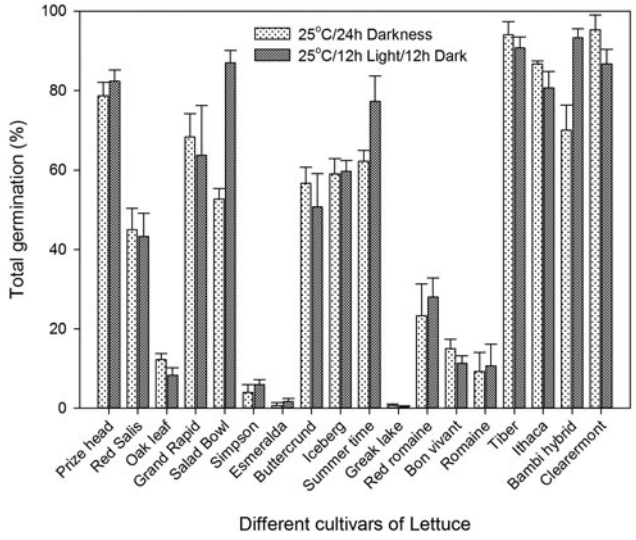


**Figure 1.** Total germination of various cultivars of *Lactuca sativa* seeds incubating at 25°C in 24 h darkness or in 12 h light–12 h dark for 1 day. Bars represent means ± SE of five replicates with 30 seeds each.

### Effects of different smoke solutions on seed germination of Salad Bowl lettuce

The stimulant effects of smoke solutions on germination of Salad Bowl lettuce varied with different smoke types and different dilutions (Fig. 2). Germination of Salad Bowl lettuce after treating with 1/5000 v/v smoke dilutions produced from prairie hay and wheat straw increased from 55 to 80% ($P < 0.01$) and 78% ($P = 0.01$), respectively. Different dilutions of smoke made from alfalfa had no effect on germination of Salad Bowl lettuce compared with the control ($P = 0.44$).

### Tracing the active compounds involved in plant-derived smoke using Salad Bowl bioassay

Ethyl acetate separated a stock smoke solution made from alfalfa, prairie hay or wheat straw into water (inorganic) and ethyl acetate (organic) fractions (Fig. 3A). The two fractions were both tested with the Salad Bowl lettuce bioassay at a series of dilutions to ensure the right concentration range for activity (data not shown). Priming with ethyl acetate fractions from prairie hay and wheat straw significantly increased seed germination of Salad Bowl lettuce from 53 to 77% and 69%, respectively ($P < 0.01$). Ethyl acetate fraction from the alfalfa solution had no effects on seed

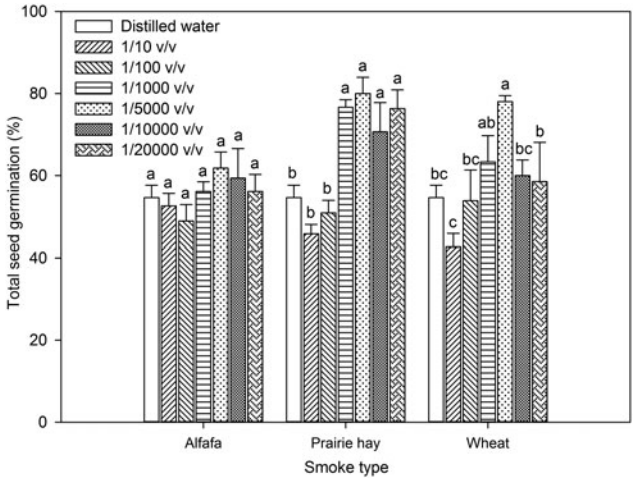


**Figure 2.** Total germination of Salad Bowl lettuce (*Lactuca sativa*) seeds after treating with serial dilutions of smoke solutions made from alfalfa, prairie hay or wheat straw, and incubated at 25°C in 24 h darkness for 1 day. Means with different letters indicate total germination of treated seeds were significantly different ($P \leq 0.05$) within smoke types. Bars represent means ± SE of five replicates with 30 seeds each.

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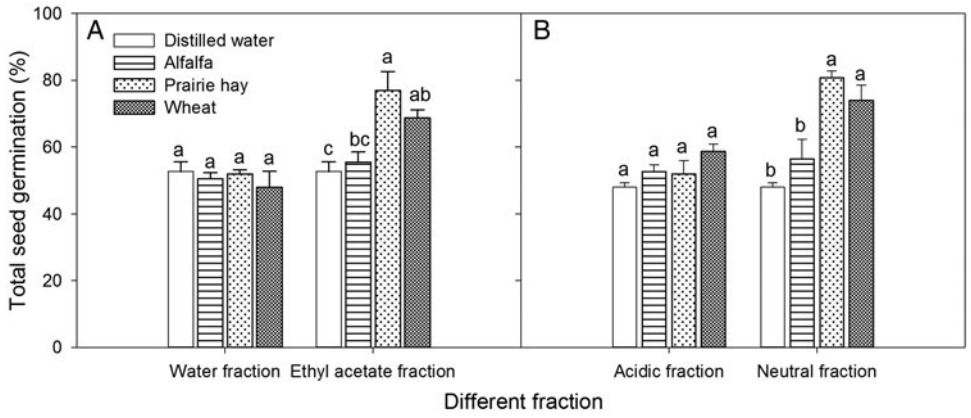


**Figure 3.** Total germination of Salad Bowl lettuce (*Lactuca sativa*) seeds after treatment with (A) water or ethyl acetate fraction and (B) neutral or acidic fraction derived from smoke solutions produced from alfalfa, prairie hay or wheat straw, and incubated at 25°C in 24 h darkness for 1 day. Means with different letters indicate total germination of treated seeds were significantly different ($P \leq 0.05$) within fractions. Bars represent means ± SE of five replicates with 30 seeds each.

germination of Salad Bowl lettuce. None of the three water fractions from different smoke types had effects on seed germination of Salad Bowl lettuce compared with the control ($P = 0.70$).

NaOH was used to fractionate organic extract into acid and neutral fractions (Fig. 3B). The two fractions were both tested with the Salad Bowl lettuce bioassay at a series of dilutions to ensure the right concentration range for activity (data not shown). NaOH fractions from prairie hay and wheat straw solutions significantly increased seed germination compared with the control ($P < 0.01$). However, NaOH fraction from alfalfa had no effects on seed germination relative to the control. None of the three acid fractions from different smoke types had effects on seed germination of Salad Bowl lettuce compared with the control ($P = 0.10$).

The concentrated neutral fraction was separated by chromatography, eluted with ethyl acetate: hexane gradient (ethyl acetate proportion: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%). Different fractions were tested with the Salad Bowl lettuce bioassay at a series of dilutions to ensure the right concentration range for activity (data not shown). The fraction with germination stimulating activity eluted in 70:30 ethyl acetate:hexane fraction occurred in all three smoke types (Fig. 4).

The 70:30 ethyl acetate:hexane fraction was separated by reverse-phase chromatography, eluted with methanol:water gradient (methanol proportion: 0, 10, 15, 20, 25 and 100%). Different fractions were then tested with the Salad Bowl lettuce bioassay at a series of dilutions to ensure the right concentration range for activity. Fractions with germination-stimulating activity were eluted in 10:90 methanol:water and obtained from prairie hay and wheat straw (Fig. 5). None of the fractions from alfalfa had effects on seed germination of Salad Bowl lettuce compared with the control ($P = 0.31$).

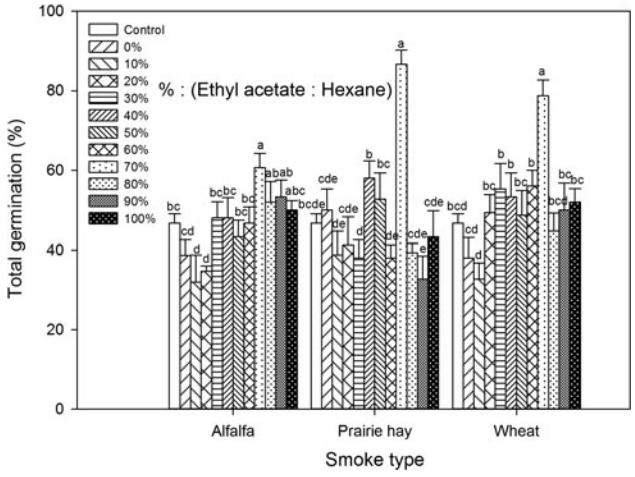


**Figure 4.** Total germination of Salad Bowl lettuce (*Lactuca sativa*) seeds after treatment with fractions derived from normal phase chromatography of the neutral fraction from alfalfa, prairie hay, wheat straw or distilled water and incubated at 25°C in 24 h darkness for 1 day. Means with different letters indicate total germination of treated seeds were significantly different ($P \leq 0.05$) within smoke types. Bars represent means ± SE of five replicates with 30 seeds each.

The 10:90 methanol:water fraction was separated using HPLC. Six parts were collected between 3 and 12.5 min (3–5.7, 5.7–6.6, 6.6–9, 9–10, 10–11 and 11–12.5 min) based on the elution pattern and were regarded as parts 1 to 6, respectively. Different parts were tested with the Salad Bowl lettuce bioassay at a series of dilutions to ensure the right concentration range for activity (data not shown). Treating seeds with the second part from prairie hay and wheat straw significantly increased seed germination from 36 to 71% and 65%, respectively ($P < 0.01$) (Fig. 6).

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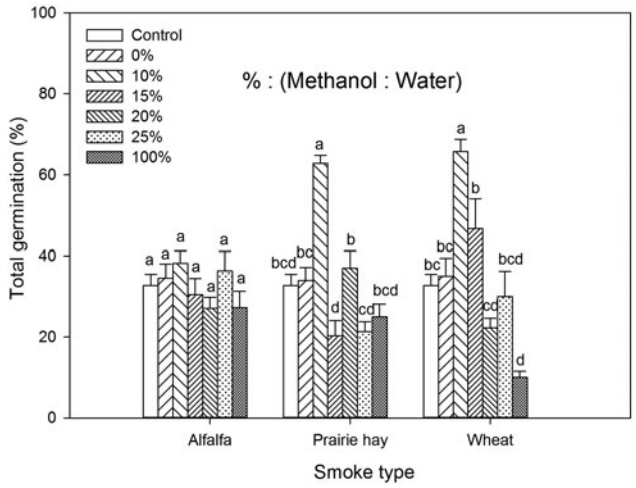


**Figure 5.** Total germination of Salad Bowl lettuce (*Lactuca sativa*) seeds after treatment with fractions derived from the reverse phase chromatography of the 70:30 ethyl acetate: hexane fraction from alfalfa, prairie hay or wheat straw or distilled water and incubated at 25°C in 24 h darkness for 1 day. Means with different letters indicate total germination of treated seeds were significantly different ($P \leq 0.05$) within smoke types. Bars represent means ± SE of five replicates with 30 seeds each.

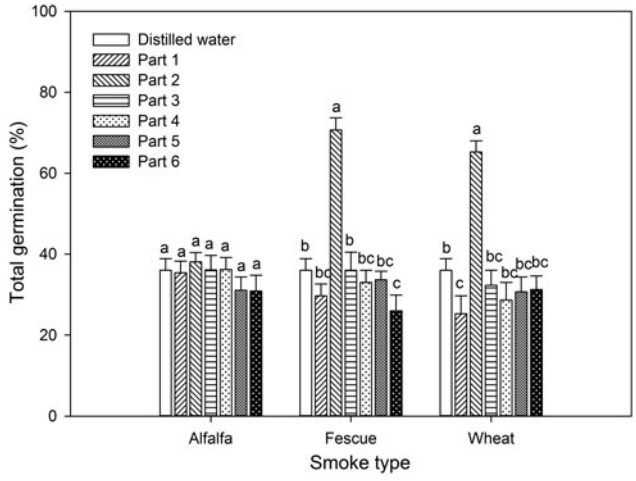


**Figure 6.** Total germination of Salad Bowl lettuce (*Lactuca sativa*) seeds after treatment with fractions derived from the HPLC separation of the 10:90 methanol:water fraction from alfalfa, prairie hay, wheat straw or distilled water and incubated at 25°C in 24 h darkness for 1 day. Means with different letters indicate total germination of treated seeds were significantly different ($P \leq 0.05$) within smoke types. Bars represent means ± SE of five replicates with 30 seeds each.

None of the six parts from alfalfa had effects on seed germination of Salad Bowl lettuce compared with the control ($P = 0.61$).

$KAR_1$ co-eluted with the active compound in the fractions obtained from smoke produced from prairie hay and wheat straw, but not in that made from alfalfa (Fig. 7).

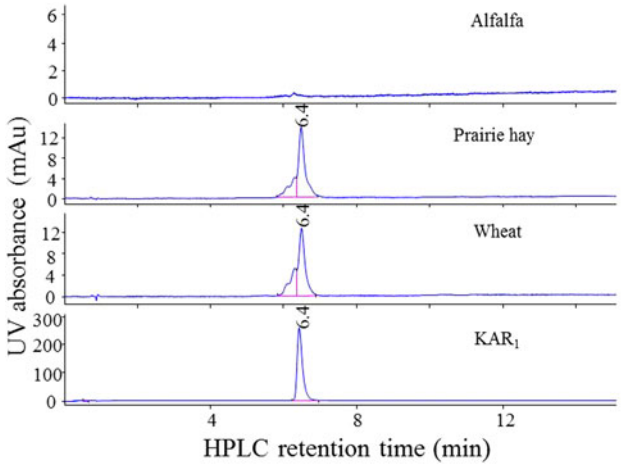


**Figure 7.** Chromatograms of the active fraction of smoke produced from alfalfa, prairie hay, and wheat, together with $KAR_1$ standard.

### *Effects of active fractions and $KAR_1$ on seed germination and seedling growth of selected species*

Total seed germination of *A. frigida* was affected by the priming effect of the active fraction from prairie hay solution ($P < 0.01$) and $KAR_1$ ($P < 0.01$) in 12 h light–12 h dark at 10/0°C (Table 1). Priming with 1/1 v/v active fraction from prairie hay significantly increased seed germination from 28 to 49%. Priming seeds with 1/10 v/v and 1/1 v/v $KAR_1$ solutions increased seed germination from 28 to 45% and 46%, respectively. Priming in various dilutions of fractions from wheat straw ($P = 0.09$) and alfalfa ($P = 0.33$) had no effect on germination of *A. frigida* compared with the control. Seed radicle, hypocotyl and total seedling lengths of *A. frigida* were not affected by priming effects of any studied solutions in any studied germination conditions (data not shown).

Total seed germination of *A. ludoviciana* was affected by priming effects of active fractions from prairie hay ($P = 0.04$) and wheat straw ($P = 0.04$) and $KAR_1$ solutions ($P = 0.02$) in 24 h darkness at 25/15°C (Table 1). Priming with 1/1 v/v fractions from prairie hay and wheat straw increased seed germination from 47 to 64% and 59%, respectively. Seed germination increased from 47 to 64% after priming with 1/1 v/v $KAR_1$ solution. Priming in various dilutions of fractions from alfalfa ($P = 0.25$) had no effect on germination of *A. ludoviciana* compared with the control. Seed radicle, hypocotyl and total seedling lengths for *A. ludoviciana* were not affected by any priming effect of any studied solutions in any studied germination conditions (data not shown).

Total seed germination of *C. canadensis* was affected by priming effects of active fractions from prairie hay ($P = 0.02$), wheat straw ($P = 0.03$) and $KAR_1$ solution

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($P = 0.02$) in 12 h light–12 h dark at 25/15°C (Table 1). Priming with the 1/10 v/v fraction from prairie hay increased seed germination from 38 to 74%. Priming with 1/1000 v/v fraction of wheat increased seed germination from 38 to 80%. Seed germination increased from 38 to 65% and 63% after priming with 1/1000 v/v and 1/100 v/v of $KAR_1$ solution, respectively. Priming in various dilutions of fractions from alfalfa ($P = 0.43$) had no effect on germination of *C. canadensis* compared with the control. Seed radicle, hypocotyl and total seedling lengths for *C. canadensis* were not affected by any priming effect of any studied solutions in any studied germination conditions (data not shown).

Total seed germination, hypocotyl and total seedling lengths of *C. arvense* were not affected by any priming effect of any studied solutions in any studied germination conditions (data not shown). Radicle length for *C. arvense* was affected by priming effect of $KAR_1$ solution in 12 h light–12 h dark at 25/15°C (Table 2). Priming with 1/1 v/v $KAR_1$ solution ($P < 0.01$) significantly reduced radicle length from 12.0 to 7.4 mm. Priming in various dilutions of fractions from alfalfa ($P = 0.28$), prairie hay ($P = 0.06$) and wheat straw ($P = 0.46$) had no effect on radicle length of *C. arvense* compared with the control.

## Discussion

### *$KAR_1$ is not universally present in smoke solutions*

A previous study has shown that active compounds in smoke solutions can substitute for light effects and stimulate germination of lettuce seeds in darkness (Drewes *et al.*, 1995). In our study, Salad Bowl lettuce was used as a rapid bioassay for the detection of germination-promoting compounds in smoke solutions because it germinated consistently better in light compared with darkness. Previous studies have used Grand Rapid lettuce for the bioassay (Flematti *et al.*, 2004; Van Staden *et al.*, 2004). However, in our study, germination of Grand Rapid lettuce seeds did not differ between light and darkness. This may be due to the fact that seeds from different sources may have different germination characteristics. Drewes *et al.* (1995) reported germination of Grand Rapid lettuce seeds from Stokes Seeds Inc. was much higher in 24 h darkness at a constant 25°C relative to that of the seeds collected from five other sources.

In our study, priming in low-concentration smoke solutions made from prairie hay and wheat straw increased germination of Salad Bowl lettuce seeds in the darkness (Fig. 2), agreeing with previous studies that germination of light-sensitive lettuce seeds (*L. sativa*) can be increased by smoke solutions (Drewes *et al.*, 1995; Jager *et al.*, 1996; Gardner *et al.*, 2001; Van Staden *et al.*, 2004). However, priming in various dilutions of smoke solution made from alfalfa did not affect seed germination of Salad Bowl lettuce. Lack of $KAR_1$ explained the neutral responses of germination of Salad Bowl lettuce seeds to the smoke made from alfalfa. $KAR_1$ was found in the smoke solutions made from prairie hay and wheat straw, but not in that made from alfalfa (Fig. 7). This is the first report that $KAR_1$ is absent in smoke solutions.

**Table 1.** Total germination (%) of *Artemisia frigida, A. ludoviciana* and *Conyza canadensis* seeds after priming in serial dilutions of separated fractions and incubating at 10/0°C or 25/15°C in 24 h darkness or in 12 h light–12 h dark

| | | | Types | | | |
|---|---|---|---|---|---|---|
| Species | Condition | Dilution | Alfalfa | Prairie hay | Wheat | $KAR_1$ |
| *Artemisia frigida* | 10/0°C Light/dark | DW | $28.0 \pm 4.5^{a}$ | $28.0 \pm 4.5^{b}$ | $28.0 \pm 4.5^{a}$ | $28.0 \pm 4.5^{b}$ |
| | | 1/1000 | $33.4 \pm 3.7^{a}$ | $32.9 \pm 5.3^{ab}$ | $27.5 \pm 4.3^{a}$ | $33.5 \pm 4.1^{ab}$ |
| | | 1/100 | $24.7 \pm 4.6^{a}$ | $27.0 \pm 4.6^{b}$ | $28.2 \pm 2.2^{a}$ | $37.7 \pm 5.4^{ab}$ |
| | | 1/10 | $28.7 \pm 3.8^{a}$ | $40.2 \pm 3.0^{ab}$ | $36.6 \pm 5.1^{a}$ | $44.6 \pm 4.1^{a}$ |
| | | 1/1 | $31.8 \pm 3.9^{a}$ | $48.8 \pm 6.0^{a}$ | $39.4 \pm 6.0^{a}$ | $46.1 \pm 3.0^{a}$ |
| *Artemisia ludoviciana* | 25/15°C Darkness | DW | $46.6 \pm 4.2^{a}$ | $46.6 \pm 4.2^{b}$ | $46.6 \pm 4.2^{b}$ | $46.6 \pm 4.2^{b}$ |
| | | 1/1000 | $54.8 \pm 4.6^{a}$ | $55.2 \pm 5.1^{ab}$ | $58.2 \pm 5.6^{ab}$ | $52.2 \pm 6.0^{ab}$ |
| | | 1/100 | $55.8 \pm 4.2^{a}$ | $51.3 \pm 3.1^{ab}$ | $56.7 \pm 4.4^{ab}$ | $51.8 \pm 6.1^{ab}$ |
| | | 1/10 | $49.6 \pm 3.8^{a}$ | $53.2 \pm 4.2^{ab}$ | $52.4 \pm 6.5^{ab}$ | $54.8 \pm 4.4^{ab}$ |
| | | 1/1 | $56.0 \pm 4.5^{a}$ | $64.0 \pm 5.2^{a}$ | $59.2 \pm 3.9^{a}$ | $64.1 \pm 4.6^{a}$ |
| *Conyza canadensis* | 25/15°C Light/dark | DW | $37.8 \pm 8.2^{a}$ | $37.8 \pm 8.2^{b}$ | $37.8 \pm 8.2^{b}$ | $37.8 \pm 8.2^{bc}$ |
| | | 1/1000 | $56.5 \pm 7.7^{a}$ | $60.5 \pm 10.3^{ab}$ | $79.7 \pm 7.6^{a}$ | $65.2 \pm 3.4^{a}$ |
| | | 1/100 | $49.2 \pm 8.2^{a}$ | $38.0 \pm 9.9^{b}$ | $75.8 \pm 8.9^{a}$ | $63.4 \pm 2.3^{a}$ |
| | | 1/10 | $56.8 \pm 9.9^{a}$ | $74.0 \pm 6.9^{a}$ | $57.9 \pm 17.7^{ab}$ | $60.0 \pm 7.6^{ab}$ |
| | | 1/1 | $43.9 \pm 9.7^{a}$ | $59.9 \pm 10.1^{ab}$ | $61.8 \pm 9.9^{ab}$ | $33.2 \pm 8.8^{c}$ |

Only the conditions in which germination was significantly affected are presented. Means with different superscript letters indicates that total germination of primed seeds were significantly different ($P \leq 0.05$) among dilutions within fractions. DW, distilled water. Values represent means ± SE.

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**Table 2.** Radicle length (mm) measured after 7–13 days of germination for *Cirsium arvense* seeds after priming in serial dilutions of separated fractions and incubating at 25/15°C in 12 h light–12 h dark

| | | Types | | | |
|---|---|---|---|---|---|
| Condition | Dilution | Alfalfa | Prairie hay | Wheat | $KAR_1$ |
| 25/15°C Light/dark | DW | $12.0 \pm 0.8^{a}$ | $12.0 \pm 0.8^{a}$ | $12.0 \pm 0.8^{a}$ | $12.0 \pm 0.8^{a}$ |
| | 1/1000 | $12.9 \pm 1.0^{a}$ | $8.9 \pm 0.9^{a}$ | $11.8 \pm 1.0^{a}$ | $11.3 \pm 1.1^{ab}$ |
| | 1/100 | $11.3 \pm 1.0^{a}$ | $9.5 \pm 1.1^{a}$ | $9.6 \pm 1.2^{a}$ | $10.5 \pm 1.0^{ab}$ |
| | 1/10 | $10.0 \pm 1.4^{a}$ | $12.0 \pm 1.1^{a}$ | $10.8 \pm 1.4^{a}$ | $8.7 \pm 1.2^{ab}$ |
| | 1/1 | $10.3 \pm 1.1^{a}$ | $12.0 \pm 0.9^{a}$ | $10.3 \pm 0.9^{a}$ | $7.4 \pm 0.7^{b}$ |

Means with different superscript letters indicate that radicle lengths of primed seeds were significantly different ($P \leq 0.05$) among dilutions within fractions. DW, distilled water. Values represent means ± SE.

Our results directly show that smoke originating from different plant materials contains different compounds. Many previous studies reported similar germination responses for smoke produced from different plant materials (Dixon *et al.*, 1995; Perez-Fernandez and Rodriguez-Echevarria, 2003; Thomas *et al.*, 2010). However, the number of plant species tested so far is limited. To our knowledge, smoke solutions made from herbaceous Fabaceae have never been tested. Species in the family of Fabaceae have unique metabolic approaches in fixing $N_2$, which may create contrasting chemical compositions compared with species from other families. However, Jager *et al.* (1996) reported that smoke produced from leaves of *Acacia mearnsii*, a leguminous tree native to Australia, had similar germination responses compared to smoke made from four other species, including *Themeda triandra*. $KAR_1$ was also found in the smoke produced from *T. triandra* (Flematti *et al.*, 2004; Van Staden *et al.*, 2004). Further studies are needed to test whether the absence of $KAR_1$ in the plant-derived smoke is a taxonomic trait.

Interestingly, priming in 70:30 ethyl acetate:hexane fraction from alfalfa after normal phase chromatography increased germination of Salad Bowl lettuce compared with the control (Fig. 5). Lack of the stimulating effects of different fractions on the germination of Salad Bowl lettuce ahead of normal phase chromatography separation may be due to existing inhibitors. Lack of response to different fractions on Salad Bowl lettuce germination after normal phase chromatography separation indicated possible synergistic effects of more than one compound that were later separated into different fractions, accounting for increased germination.

### $KAR_1$ favours seed germination of native species from Fescue Prairie

Seedling emergence of *A. frigida*, *A. ludoviciana*, *C. arvense* and *C. canadensis* in the field was positively affected by burning (Ren and Bai, 2016a). In this study, $KAR_1$ increased seed germination of *A. frigida*, *A. ludoviciana* and *C. canadensis*, highlighting the importance of smoke in affecting seed regeneration of these three species. Priming in high-concentration $KAR_1$ solutions and active fractions from prairie hay increased germination of *A. frigida* compared with distilled water at 10/0°C in 12 h light–12 h dark. Germination of *A. frigida* occurred in a wide temperature range, with 20/10°C as the optimal temperature (Wilson, 1982). Increased germination of *A. frigida* at a suboptimal temperature observed in this study was consistent with Jain *et al.* (2006), who reported that $KAR_1$ can improve seed germination of *Solanum esculentum* when using suboptimal temperatures. Our results indicated that $KAR_1$, the active compound in prairie hay, could play a critical role in enhancing the establishment of *A. frigida* after early spring burning when ambient temperatures are still low.

Priming in concentrated $KAR_1$ solutions and active fractions made from prairie hay and wheat straw increased germination of *A. ludoviciana* compared with distilled water at 25/15°C in 24 h darkness. This finding agrees with a previous study which found that $KAR_1$ stimulated seed germination of light-sensitive seeds, including *Angianthus tomentosus, Gnephosis tenuissima, Myriocephalus guerinae, Podolepis canescens* and *Rhodanthe citrina* in suboptimal light or darkness (Merritt *et al.*, 2006). $KAR_1$ may substitute for light effects and affect the conversion of $P_r$ to $P_{fr}$ in the phytochrome response (Drewes *et al.*, 1995). In addition, $KAR_1$ can alter the metabolism and perception of gibberellins (GAs) and abscicic acid (ABA) in seeds (Nelson *et al.*, 2009). Exposure to smoke solutions increased endogenous GA levels and decreased endogenous ABA levels for two positively photoblastic species, *Lactuca sativa* and *Nicotiana attenuate* (Gardner *et al.*, 2001; Schwachtje and Baldwin 2004). Increased germination of *A. ludoviciana* in darkness by $KAR_1$ partly explained the increased coverage of this species after burning in Fescue Prairie and tallgrass prairie (Bailey and Anderson, 1978; Anderson and Bailey, 1980; Collins, 1987).

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Although the active fractions produced from prairie hay and wheat straw both had $KAR_1$, priming in the concentrated active fraction from prairie hay but not wheat straw increased germination of *A. frigida* compared with distilled water at 10/0°C in 12 h light–12 h dark. Not only qualitative but also quantitative variation exists in smoke solutions produced by different material, which, in turn, may affect seed germination differently. Priming in low, but not highly concentrated active fractions made from prairie hay and wheat straw and $KAR_1$ solutions stimulated seed germination of *Conyza canadensis* in 12 h light–12 h dark at 25/15°C. Different seeds have different sensitivity to $KAR_1$ solutions. For example, germination of *L. sativa* and *Stylidium affine* can be increased after treating with $KAR_1$ solutions concentrated at $10^{-9}$ M and $10^{-7}$ M, respectively (Flematti *et al.*, 2004).

Although burning increased seedling emergence of *C. arverse* (Ren and Bai, 2016a), smoke (Ren and Bai, 2016c) and $KAR_1$ had no positive effect on seed germination of this species. In addition, ash did not increase seedling density of this species emerging from soil seed bank (Ren and Bai, 2016b), indicating that heat might be the crucial factor promoting seed germination of species. Priming in 1/1 v/v $KAR_1$ reduced radicle length of *C. arvense* compared with distilled water in 12 h light–12 h dark at 25/15°C. Although most previous studies have shown a positive or neutral effect of $KAR_1$ on the seedling growth of different species (Daws *et al.*, 2007; Kulkarni *et al.*, 2007; Stevens *et al.*, 2007), radicle length of *Eragrostis curvula* was reduced by $KAR_1$ at constant 30 and 35°C conditions, but not at constant 15, 20 and 25°C conditions (Ghebrehiwot *et al.*, 2009), indicating that effects of $KAR_1$ on seedling length are temperature and species dependent.

It is not surprising that separated fractions from alfalfa had no effect on seed germination and seedling growth of all tested species, since this fraction did not contain any detectable compound (Fig. 7). However, smoke produced from alfalfa increased germination of *C. canadensis* significantly more than that made from prairie hay and wheat straw (Ren and Bai, 2016c), indicating that unique active compound(s) may be involved in it, which deserves further study.

$KAR_1$ increased seed germination of all three native species and decreased seedling growth of the only non-native species under the germination conditions we applied. Previous studies have shown that fire could be used to restore native species and control non-native species in various grasslands (DiTomaso *et al.*, 1999; Prober *et al.*, 2005; MacDonald *et al.*, 2007). Burning increased species density and richness of native seedlings emerging from the soil seed bank in Fescue Prairie (Aran *et al.*, 2013; Ren and Bai, 2016b). Results from our study indicate that $KAR_1$ could be the key effect of burning in restoring native species in Fescue Prairie. They also show that the potential of $KAR_1$ in regulating seed regeneration in Fescue Prairie is time sensitive, because the influence on seed germination and seedling growth varied across temperature and light conditions, which varies seasonally.

It should be noted that although $KAR_1$ seemed to be the dominant active compound in the separated fractions from prairie hay and wheat straw in our study, other unidentified compounds were still involved in germination. Our methods were targeted to gross identification of the main smoke fractions. To more thoroughly understand the effects of smoke on Fescue Prairie seed regeneration, the germination responses of different species to smoke solutions and other founded active components including other karrikins (Flematti *et al.*, 2009) and glyceronitrile (Flematti *et al.*, 2011) should be tested.

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## Conflicts of interest

None.

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