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Germination Response of Dormant Tanglehead (*Heteropogon contortus*) Seeds to Smoke-infused Water and the Smoke-associated Stimulatory Compounds, Karrikinolide and Cyanide

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***Abstract*. Tanglehead (*Heteropogon contortus*) is a native Hawaiian grass that has been used in restoration and has potential for expanded re-vegetation use. Although interest and demand for tanglehead re-vegetation has increased, the supply of tanglehead seeds has remained limited as a result of a lack of seed production protocols addressing seed dormancy. Smoke water from burning vegetation may provide an economical and practical seed treatment because aerosol smoke has been reported to stimulate tanglehead seed germination. Dose rate and side-by-side comparison studies were conducted to evaluate the germination stimulation efficacy of food-grade liquid smoke, xylose smoke-infused water, tanglehead smoke-infused water, karrikinolide (KAR_1), and cyanide (i.e., mandelonitrile and potassium cyanide). Optimum smoke water dilutions were 1% v/v for food-grade liquid smoke and undiluted for xylose smoke-infused water and tanglehead smoke-infused water. KAR_1 was not stimulatory at concentrations between 0.0067 and 66.7 μM. Potassium cyanide stimulated tanglehead seed germination at concentrations between 50 to 500 μM. Germination was promoted to even greater levels with the cyanohydrin, mandelonitrile, indicating a role for benzaldehyde (a byproduct of mandelonitrile decomposition) in stimulating tanglehead seed germination. Benzaldehyde was confirmed to be stimulatory at concentrations between 50 to 100 μM. The presence of cyanide at stimulatory levels was confirmed in tanglehead smoke-infused water (i.e., ≈100 μM), but not in food-grade liquid smoke or xylose smoke-infused water. Germination with non-cyanide-containing smoke waters indicates the presence of other compounds in smoke that can stimulate tanglehead germination. In the side-by-side comparison study, food-grade liquid smoke (1% v/v) and undiluted tanglehead smoke-infused water provided consistent germination stimulation comparable to 500 μM potassium cyanide. Undiluted xylose smoke-infused water did not provide significant germination stimulation in the comparison studies. This may be the result of differences in seed batch sensitivity to the germination stimulant, seed storage duration as well as subtle differences in the preparation of xylose smoke-infused water.**

Tanglehead (*Heteropogon contortus*, Poaceae) or Pili grass (in Hawaiian) is a perennial C4 bunchgrass found on all main islands of the Hawaiian Archipelago and throughout the tropics (Wagner et al., 1999). In Hawaii, it is an important re-vegetation and restoration species as a result of its cultural and ecological significance (Daehler and Goergen, 2005) and ability to grow in low-resource conditions (i.e., low rainfall and low fertility soils) (USDA-NRCS, 2007). Over the past decade, tanglehead has been extensively used in restoration and re-vegetation of the uninhabited island of Kahoolawe (USDA-NRCS, 2007) as well as some offshore islands around Oahu (Smith, 2006). In recent years it has been incorporated into water efficient landscapes (Aldridge et al., 2009; Board of Water Supply, 2004; USDA-NRCS, 2007), streambank stabilization (Crago and Puttock, 2008), native species roadside re-vegetation (DeFrank and Lukas, 2012), and buffer strip plantings (A. Fares, personal communication).

A major hindrance to the immediate and large-scale use of tanglehead is its seed dormancy. Freshly harvested seeds exhibit poor to no germination. To remove dormancy and improve seed germination, an after-ripening period (i.e., dry storage) of 6 to 12 months is required (Baldos et al., 2014; Daehler and Goergen, 2005; Pater, 1993; Tothill, 1977; USDA-NRCS, 2007). Alternatively, dormancy of tanglehead can be relieved by soaking the seeds in 0.5% (C. Daehler, unpublished data) to 1% gibberellic acid (Tothill, 1977). This practice, however, can be costly and results in elongated seedlings (O.C. Baldos, personal observation).

The effects of smoke and aqueous smoke extracts on stimulating seed germination are well known. It has been shown to promote germination in both fire and non-fire-adapted species (Flematti et al., 2013) including a number of crops (Chumpookam et al., 2012; Drewes et al., 1995; Sparg et al., 2006; Taylor and van Staden, 1998; Thomas and van Staden, 1995). It is estimated that ≈1200 species from 80 genera are smoke-responsive under field conditions (Chiwocha et al., 2009; Dixon et al., 2009; Flematti et al., 2011a, 2013).

Recently, two a.i. in smoke responsible for improving seed germination have been isolated and characterized. The butenolide compound, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one or KAR_1, was first isolated in 2004. KAR_1 was discovered and described in Australia by Flematti et al. (2004) and was later corroborated in South Africa by van Staden et al. (2004). KAR_1 is a highly active, heat-stable, and long-lasting compound (van Staden et al., 2004) capable of stimulating germination in lettuce (*Lactuca sativa*) and in a large number of smoke-responsive species (Flematti et al., 2011a; Nelson et al., 2012). KAR_1 is a very potent germination stimulant. Species sensitive to KAR_1 can be stimulated to germinate at very low concentrations (1 ppb; 1 $\mu g \cdot L^{-1}$; 10^{-9} M) (Flematti et al., 2004, 2013). KAR_1 can be isolated from smoke-infused water prepared from the combustion of plant material, cellulose, or simple carbohydrates (Flematti et al., 2011b). A number of synthetic analogs have also been prepared (Flematti et al., 2007; Goddard-Borger et al., 2007; Scaffidi et al., 2011; Sun et al., 2008) with some of these detected in smoke (Chiwocha et al., 2009). Based on combustion experiments with pure xylose, glucose, or cellulose, it was proposed that KAR_1 is derived from a pyranose sugar (Flematti et al., 2011b; Nelson et al., 2012).

Glyceronitrile is another compound recently isolated from plant-derived smoke. It was first isolated and characterized in 2011 from smoke-infused water prepared from the combustion of oaten (*Avena sativa*) hay as well as from fresh and dried bushland shoot materials (Flematti et al., 2011a). Research that led to the isolation and characterization of glyceronitrile was spurred by the inactivity of KAR_1 on seeds of *Anigozanthos manglesii*, a smoke-responsive species (Flematti et al., 2011a). Glyceronitrile itself does not cause the observed germination stimulation in *A. manglesii* seeds. Flematti et al. (2011a) found that, in the presence of water, glyceronitrile slowly hydrolyzes to release cyanide, which in turn stimulates seed germination. This observation was later confirmed by germination assays using a number of cyanohydrins (i.e., mandelonitrile, acetone cyanohydrin, glycolonitrile, and 2,3,4-trihydroxybutyronitrile). Cyanide-stimulated germination is not a new observation (Flematti et al., 2013). It has been reported in a wide variety of plant species, including grasses (Cohn and Hughes, 1986; Flematti et al., 2013; Roberts, 1973; Siegień and Bogatek, 2006; Taylorson and Hendricks, 1973). The novelty of discovering the presence of cyanohydrins in smoke is that it establishes cyanide as an important germination stimulant in post-fire environments (Flematti et al., 2013).

Smoke-infused water and food-grade liquid smoke can offer an economical and practical seed treatment alternative for enhancing tanglehead seed germination. Because tanglehead is a fire-adapted species (Goergen and Daehler, 2001), it is assumed that smoke applications may improve the germination of dormant seeds. Campbell et al. (1996) confirmed this hypothesis through germination studies of seeds treated with cool aerosol smoke from combusted tanglehead. Assays indicate that smoked seed exhibited more than twice the germination of untreated seed. Although Campbell et al. (1996) confirmed the smoke responsiveness of tanglehead seeds, follow-up studies have yet to be conducted to further explore other smoke sources and assay the recently identified germination stimulants found in smoke (i.e., KAR_1 and cyanide). By examining the response of tanglehead seeds to these smoke and smoke-derived compounds, one may be able to elucidate the mechanisms that control dormancy and provide a basis for applied uses on direct-seeded plantings. The objectives of this study were to: 1) identify the optimum concentrations of smoke-infused water derived from burned xylose, burned tanglehead (foliar and stem tissue), and food-grade liquid smoke; 2) determine the stimulatory capability and identify the optimum concentrations of KAR_1 and cyanide; 3) compare the germination stimulation capability of the smoke sources against KAR_1 and cyanide; and 4) estimate the amount of cyanide in each smoke source using the Cyantesmo paper (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Figure 1 provides a flowchart of the experiments conducted for this study.

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Materials and Methods

Seed source. Seeds used for the assays were of a Kahoolawe Island source-identified germplasm (Accession # 9079683, HA-5748). These were obtained from irrigated field plantings at the U.S. Department of Agriculture–Natural Resources Conservation Service (USDA-NRCS) Plant Materials Center on the island of Molokai. Seed harvests were conducted in Mar. 2011 and July 2012 using a self-propelled combine harvester (Massey Ferguson MF-17/19; Kincaid Equipment Manufacturing, Haven, KS). After harvesting, the seeds were immediately transported to the University of Hawaii at Manoa campus on Oahu, where they were air-dried for 8 d to 10.3% moisture (dry weight basis). After drying, the seeds were passed through an airblast seed cleaner (Almaco, Nevada, IA) to remove chaff, awns, and empty seeds. The seeds (563,061 seeds/kg) were placed in plastic zipper bags (Hefty Slider Storage Bags; Reynolds Consumer Products Inc., Richmond, VA) and stored at 5 °C on 29 Mar. 2011 (Mar. 2011 seed batch) and 31 Aug. 2012 (July 2012 seed batch) until used. Seed viability was tested using the tetrazolium method and ranged between 83% and 89%. Table 1 lists the harvest dates and storage durations (at 5 °C) of the seeds used for each experiment. Mar. 2011 harvested seeds were used in all dose rate studies except the high-rate potassium cyanide and potassium chloride studies. Comparison studies between smoke-infused water formulations and potassium cyanide used July 2012 seeds to reduce the possible confounding effects of storage duration on dormancy status.

Smoke-infused water formulations. Dose–response studies of food-grade liquid smoke (Colgin Liquid Smoke Natural Mesquite; The Colgin Companies, Dallas, TX), xylose smoke-infused water, and tanglehead smoke-infused water were conducted between Sept. and Dec. 2012. Independently prepared solutions for each smoke water formulation were used for the first and second runs of the experiments. For food-grade liquid smoke, two bottles from different lots (i.e., two different expiration dates) were purchased. Xylose smoke-infused water and tanglehead smoke-infused water were prepared twice using the procedures of Flematti et al. (2011b). Briefly, 2.4 g of D-xylose (Sigma Aldrich, St. Louis, MO) or 2.4 g of air-dried tanglehead (i.e., leaves, stems, and seed heads cut into 1-cm pieces) was combusted in a preheated three-necked 250-mL round bottom flask. The smoke produced from combustion was bubbled through 100 mL of distilled water at 30 mL·min^{-1}. After 10 min of heating, the material was carbonized and did not produce additional smoke. Condensates that accumulated inside the connector tubes and joints attaching the erlenmeyer flask and the round-bottomed flask were collected by rinsing with the smoke-infused water and filtered through a layer of Whatman #3 filter paper. Four dilution treatments (undiluted, 1/10, 1/100, and 1/1000 v/v) and a distilled water control solution were prepared for each smoke water formulation on the day the germination assays were conducted. Excess solutions were stored in sealed, clear glass bottles at 5 °C.

KAR_1. The KAR_1 dose–response study was conducted in May (first experimental run) and Sept. 2012 (second experimental run) using independently prepared solutions. KAR_1 was kindly provided by Dr. Gavin Flematti, The University of Western Australia. A dilution series consisting of 0.0067, 0.067, 0.67, 6.67, and 66.7 μM KAR_1 was tested, including a distilled water control (Flematti et al., 2004). The KAR_1 dilutions were prepared from a 66.7 μM (10 ppm) stock solution. To make the stock solution, 1 mg KAR_1 was dissolved with 100 mL distilled water. The water was heated to 40 °C to facilitate the dissolution of KAR_1 crystals.

Cyanohydrins. Dose rate studies with mandelonitrile and potassium cyanide were conducted between Oct. 2012 and Feb. 2013. All cyanohydrin studies used independently prepared solutions for the first and second runs of the experiment. For mandelonitrile, a dilution series consisting of 1, 5, 10, 20, and 50 μM was tested alongside a distilled water control (Flematti et al., 2011a). The dilution treatments were prepared from a 100 μM (13.3 ppm) stock solution. To make the stock solution, 2.66 mg (2.38 μL) of technical-grade mandelonitrile (Sigma Aldrich) was dissolved with distilled water to a volume of 200 mL.

Because the decomposition of mandelonitrile produces both free cyanide and benzaldehyde, a germination assay was also conducted for benzaldehyde. A dilution series consisting of 1, 5, 10, 20, 50, and 100 μM was tested alongside a distilled water control. The dilution treatments were prepared from a 100 μM stock solution (10.61 ppm). To make this stock solution, 2.04 μL of technical-grade benzaldehyde (Sigma Aldrich) was dissolved with distilled water to a volume of 200 mL.

Dose rate studies with potassium cyanide were conducted at low (less than 50 μM) and high (50 to 500 μM) rates. For the low rate studies, a dilution series consisting of 1, 5, 10, 20, and 50 μM was tested alongside a distilled water control. The dilution treatments were prepared from a 100 μM (6.51 ppm) stock solution. To make the stock solution, 1.30 mg of technical-grade potassium cyanide (Sigma Aldrich) was dissolved with distilled water to a volume of 200 mL.

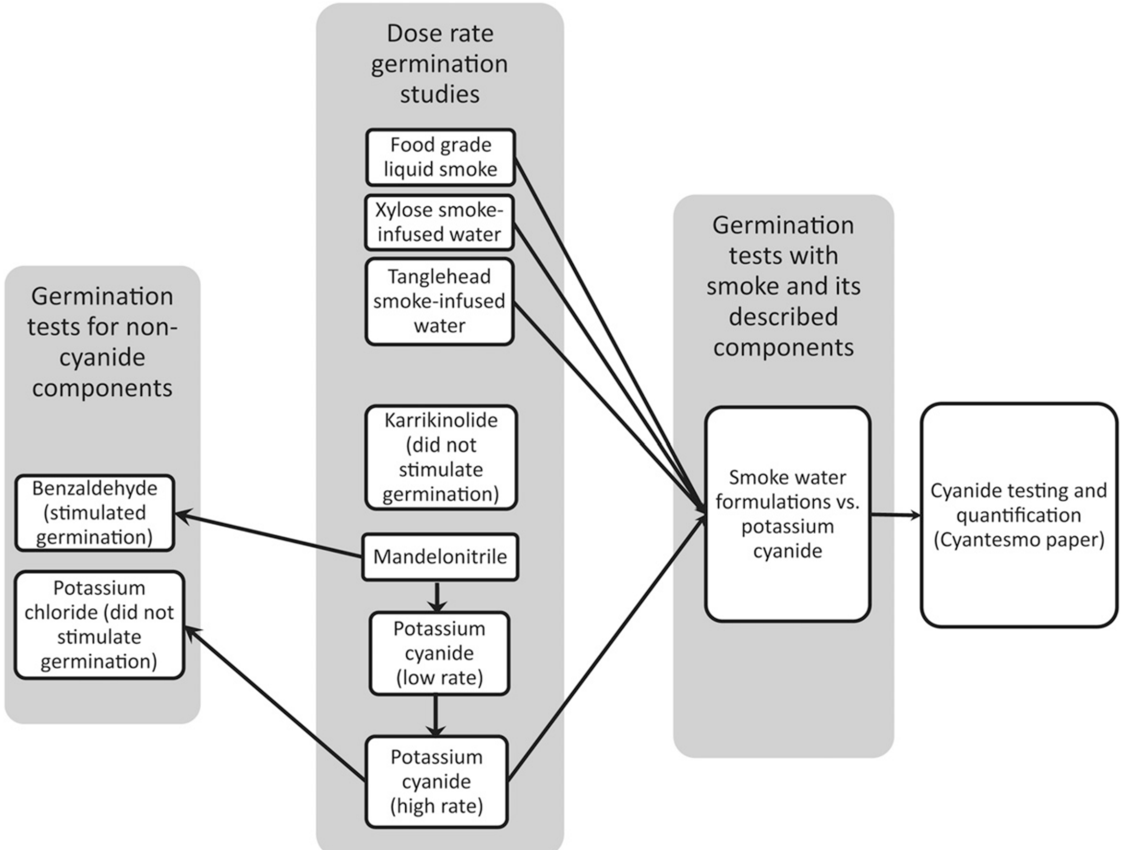

Fig. 1. Flowchart of experiments conducted to evaluate the stimulatory capability of smoke-infused waters (food-grade liquid smoke, xylose smoke-infused water, and tanglehead smoke-infused water), karrikinolide, and cyanide (mandelonitrile and potassium cyanide) on dormant tanglehead (*Heteropogon contortus*) seeds. Arrows show the follow-up studies conducted after results of the previous experiment were obtained.

Table 1. Harvest date and storage duration (at 5 °C) of tanglehead (*Heteropogon contortus*) seeds used for the dose rate and comparison experiments of smoke water formulations, karrikinolide and cyanide.

Expt.	Seed harvest	Days in storage at 5 °C: First run	Second run
Dose rate studies with smoke water formulations			
Food-grade liquid smoke[z]	Mar. 2011	524	530
Xylose smoke-infused water[z]	Mar. 2011	524	530
Tanglehead smoke-infused water	Mar. 2011	608	609
KAR_1 dose rate study			
Karrikinolide	Mar. 2011	387	533
Cyanohydrin dose rate studies			
Mandelonitrile	Mar. 2011	553	555
Potassium cyanide (low rate)	Mar. 2011	574	575
Potassium cyanide (high rate)	July 2012	137	138
Benzaldehyde	Mar. 2011	588	590
Potassium chloride	July 2012	153	154
Smoke-infused water and cyanide comparison study			
Smoke water formulations vs. potassium cyanide[z]	July 2012	174	174

[z]Experiments exhibited a significant interaction with experimental runs.

For the high rate studies, a dilution series consisting of 50, 100, 250, and 500 μM potassium cyanide was tested alongside a distilled water control. This range represents amounts typically reported in germination studies (50 μM) and the maximum concentration of cyanide that can be potentially released from smoke-infused water (300 μM glyceronitrile; Flematti et al., 2011a). The dilution treatments were made from a 500 μM (32.55 ppm) stock solution. To make the stock solution, 6.51 mg of technical-grade potassium cyanide was dissolved with distilled water to a volume of 200 mL.

Follow-up studies with potassium chloride were also conducted to determine whether the potassium ion had an effect on tanglehead seed germination. Potassium chloride was tested at 50, 100, 250, 500, and 1000 μM alongside a distilled water treatment and 500 μM potassium cyanide.

Smoke-infused water and cyanide comparison study. Optimum concentrations obtained from the dose rate studies were used to compare germination stimulation efficacy of smoke-infused water (i.e., food-grade liquid smoke, xylose smoke-infused water, and tanglehead smoke-infused water), potassium cyanide, and distilled water (control). Smoke-infused water and potassium cyanide solutions were prepared 3 d before (stored at 5 °C) the germination test according to the methods previously described. Independently prepared solutions were used for the first and second runs of the experiment.

Germination assay. Germination tests were used to evaluate all dose rate and comparison studies described previously. Treatments were replicated four times and conducted on 100 mm × 15-mm petri dishes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) lined with a layer of filter paper (Whatman #3; Whatman International, Piscataway, NJ). The filter papers were pre-moistened with ≈3 mL of treatment solution before sowing the seeds. Fifty seeds from a specified harvest batch (Table 1) were sown on each filter paper-lined petri dish. After sowing, the petri dishes were sealed along the sides with parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent drying. These dishes were then incubated at 30 °C (Tothill, 1977) and 12 h of light daily supplied by a 60-W incandescent plant light bulb (Philips Agro-Lite A/9; Philips, Andover, MA). The petri dishes were observed daily and re-moistened with treatment solution as needed. Total percent germination in each petri dish was recorded after 20 d of incubation. Seeds were counted as germinated when at least 1 mm of the radicle or shoot had emerged.

Cyanide quantification of smoke-infused water formulations using the Cyantesmo paper. Following the manufacturer's instructions, the presence of cyanide in the food-grade liquid smoke, xylose smoke-infused water, and tanglehead smoke-infused water was determined using the Cyantesmo qualitative test paper (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The presence of cyanide-generating compounds is detected when the color of the test strip in the vapor phase of the solution turns from light yellow to varying shades of blue. According to the manufacturer's instructions, the minimum sensitivity limit of the test paper is 7.4 μM (0.2 ppm) HCN after 15 min of reaction. Smoke solutions that tested positive for cyanide had its cyanide content estimated by comparing the intensity of the blue shading of the test paper in the smoke solutions with those produced by known potassium cyanide dilutions (0, 50, 100, 250, and 500 μM).

Experimental setup and statistical analysis. All germination studies were set up as a split plot with four replicates. The main effect plots were the experimental runs and the subplot effect was treatment concentration/stimulant type. Total percent germination after 20 d, except for the higher rate potassium cyanide data sets, was transformed to conform the data to the analysis of variance (ANOVA) assumptions (i.e., homogeneity of variance and normality). All germination data recorded in the dose–response studies of the three smoke-infused waters, KAR_1, mandelonitrile, and benzaldehyde, and in the comparison studies with potassium cyanide were arcsine square root-transformed. Germination data recorded in the lower rate potassium cyanide and potassium chloride dose rate studies were square root-transformed. ANOVA was conducted using Statistix 9 (Analytical Software, Tallahassee, FL). Tukey's range test was used to separate the means in all experiments except for the benzaldehyde and potassium chloride studies. Mean separation for these used the Dunnett's test because the objective for these two studies was to compare the germination of seeds in the distilled water treatment with those incubated in compounds with or without cyanide. Dunnett's test is designed specifically for these types of planned comparisons (Sileshi, 2012).

Results

Dose rate studies with smoke water formulations. ANOVA results indicated a significant interaction between food-grade liquid smoke treatments and experimental run ($P <$ 0.01). Germination in both experimental runs of the food-grade liquid smoke treatments consistently increased when concentrations were raised from 1/1000 to 1/100 (Fig. 2). At 1/10 dilution, the germination response between experimental runs was significantly different. Increasing the concentration to undiluted inhibited germination in both experimental runs. Based on these results, consistent maximum germination was determined at the 1/100 dilution of food-grade liquid smoke.

Results of the ANOVA for the xylose smoke-infused water dose rate study indicated a significant interaction between experimental run and xylose smoke-infused water treatments (P = 0.0469). Among the rates tested, only the undiluted treatment exhibited consistent germination, which is significantly higher than the distilled water treatment (Fig. 3). The undiluted treatments also exhibited the highest percent germination values.

ANOVA did not indicate a significant interaction between tanglehead smoke-infused water treatments and experimental runs, which allowed means to be pooled. Increasing concentrations of tanglehead smoke-infused water increased germination of dormant seeds (Fig. 4). The undiluted solution exhibited the highest percent germination among all treatment dilutions.

KAR_1 dose rate studies. Results of the ANOVA indicated no effects of the KAR_1 treatments (P = 0.2839). Tanglehead seeds in the KAR_1 treatments exhibited little to no germination (less than 3%, data not presented for the two runs of the experiment) indicating that tanglehead is not responsive to KAR_1 at the concentrations tested.

Cyanohydrin dose rate studies. ANOVA results for the cyanohydrins (i.e., mandelonitrile and the low and high rates of potassium cyanide) and their non-cyanide components (i.e., benzaldehyde and potassium chloride) did not indicate a significant interaction between experimental runs. This allowed pooling of the experimental runs in each dose rate study. Mandelonitrile significantly stimulated germination of Mar. 2011 tanglehead seeds at concentrations between 5 to 50 μM (Fig. 5). The highest percent germination was observed in 50 μM

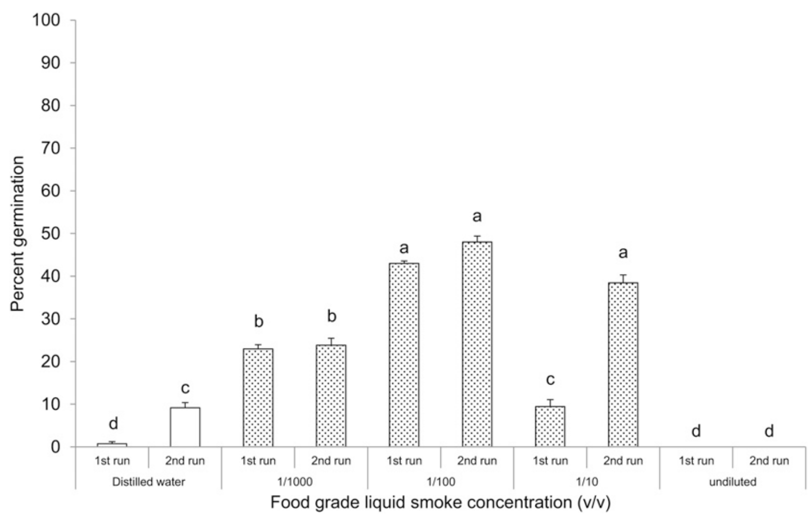

Fig. 2. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 524 and 530 d) to dilutions of food-grade liquid smoke. Germination percentages and SES presented are arcsine square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 4.

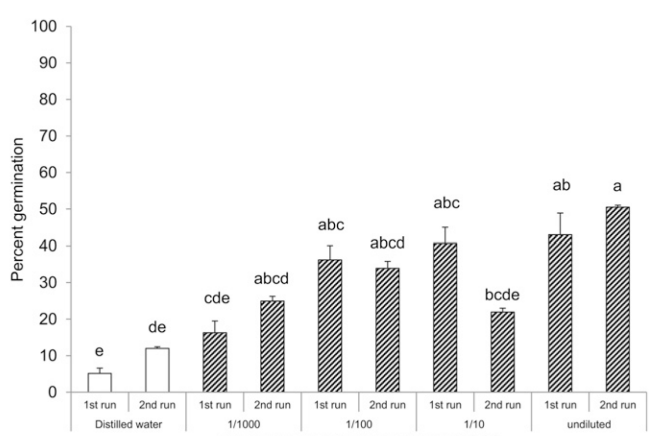

Fig. 3. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 524 and 530 d) to dilutions of xylose smoke-infused water. Germination percentages and SES presented are arcsine square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 4.

mandelonitrile. In the potassium cyanide studies, significant germination stimulation was recorded at 5 and 50 μM in the low rate study using Mar. 2011 seeds (Fig. 6) and at 100 and 500 μM in the high rate study using July 2012 seeds (Fig. 7). Benzaldehyde concentrations at 50 to 100 μM significantly stimulated germination of Mar. 2011 tanglehead seeds when compared with distilled water (Fig. 8). Interestingly, when percent germination values of Mar. 2011 seeds were observed at 50 μM potassium cyanide (14%; Fig. 6) and 50 μM benzaldehyde (28%; Fig. 8), an additive effect was observed, which corresponded to the percent germination recorded in 50 μM mandelonitrile (43%; Fig. 8). The benzaldehyde and low rate potassium cyanide studies confirmed that the mandelonitrile-stimulated germination was caused by both the products of its decomposition (i.e., cyanide and benzaldehyde).

In the potassium chloride dose rate study, ANOVA did not indicate a significant interaction between treatments and experimental runs allowing treatment means to be pooled over experimental runs. Concentrations of potassium chloride ranging from 50 to 1000 μM did not significantly improve percent germination above the distilled water treatment (Fig. 9), indicating that the potassium ion was not responsible for the observed potassium cyanide germination stimulation.

Smoke-infused water and cyanide comparison study. ANOVA indicated a significant interaction between experimental run and germination stimulant treatments ($P = 0.0116$). Undiluted tanglehead smoke-infused water, 500 μM potassium cyanide, and 1% (v/v) food-grade liquid smoke significantly increased germination of tanglehead seeds (Fig. 10). Mean comparisons between the percent germination responses of these stimulants indicated similar levels of efficacy. Incubation in xylose smoke-infused water did not increase percent germination of tanglehead seeds (Fig. 10).

Cyanide quantification of smoke-infused water formulations using the Cyantesmo paper. Colorimetric quantification of the smoke-infused water samples using the Cyantesmo qualitative test paper indicated the presence of cyanide in tanglehead smoke-infused water but not in food-grade liquid smoke or xylose smoke-infused water. The intensity of blue shading in tanglehead smoke-infused water corresponded to an aqueous solution of 100 μM of cyanide (data not presented).

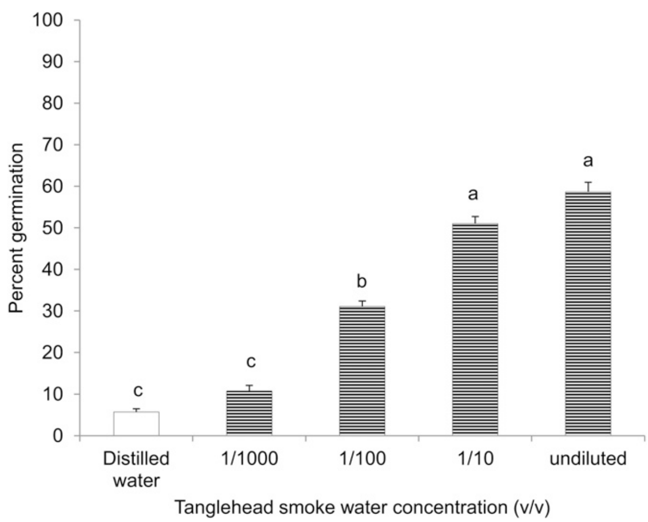

Fig. 4. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 608 and 609 d) to dilutions of tanglehead smoke-infused water. Germination percentages and SES combined across experimental runs are arcsine square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 8.

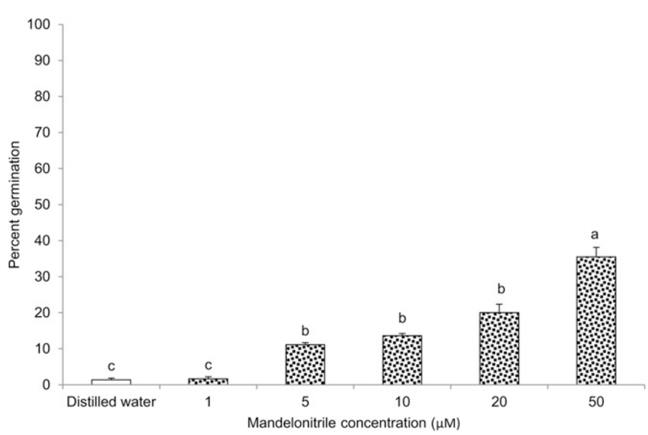

Fig. 5. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 553 and 555 d) to dilutions of mandelonitrile (less than 50 μM). Germination percentages and SES combined across experimental runs are arcsine square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 8.

Discussion

Dose–response experiments indicated that the optimum dilutions of food-grade liquid smoke, xylose smoke-infused water, and tanglehead smoke-infused water were at 1/100 v/v, undiluted and undiluted, respectively. Differences in the optimum dilutions and the observed significant experimental run-by-dilution-treatment interactions [i.e., 1/10 dilution in both food-grade liquid smoke (Fig. 2) and xylose smoke-infused water (Fig. 3)] were attributed to differences in materials combusted (Jäger et al., 1996), combustion temperature (Brown and van Staden, 1997; Jäger et al., 1996), and the balance of the germination stimulants and inhibitors produced during combustion (Light et al., 2010). In Jäger et al. (1996), smoke-infused water dilutions optimized for germination depended on the starting material and combustion temperatures. Smoke solutions derived from the combustion of different leaves (i.e., *Acacia mearnsii*, *Eucalyptus grandis*, *Hypoxis colchifolia*, and *Pinus patula*) and tissue paper exhibited maximized germination stimulation of Grand Rapids lettuce (*Lactuca sativa* 'Grand Rapids') between 1:100 or 1:10 dilution. Smoke-infused water from the combustion of *E. grandis* or tissue paper (5 g, dry weight) provided maximum germination at 1:100 dilution. For smoke-infused water derived from *A. mearnsii*, *H. colchifolia*, and *P. patula* leaves (5 g, dry weight), maximum germination was recorded at 1:10 dilution. Dilutions exceeding the optimum inhibited germination. Besides starting material, Jäger et al. (1996) also reported that increasing combustion temperature from 140 to 200 °C increased the stimulatory effects of smoke-infused water. Smoke-infused water derived from dried *Themeda triandra* leaves (5 g, dry weight) burned at 200 °C resulted in the highest germination (≈60%) of Grand Rapids lettuce (Jäger et al., 1996). Combustion temperatures exceeding 200 °C reduced the stimulatory effect of smoke-infused water. The balance between germination stimulants and inhibitors produced during combustion is another important factor that can explain the significant experimental run-by-treatment interaction in the food-grade liquid smoke dose rate study and in studies involving xylose smoke-infused water. Because smoke is a complex mixture of compounds,

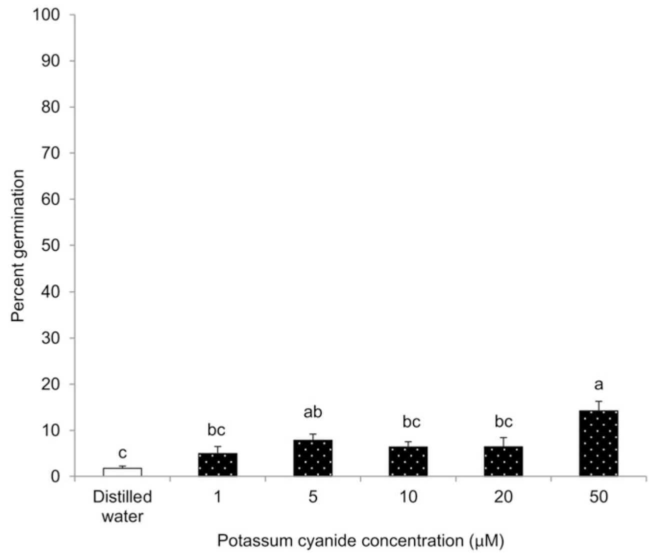

Fig. 6. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 574 and 575 d) to dilutions of potassium cyanide (less than 50 µM). Germination percentages and SEs combined across experimental runs are square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 8.

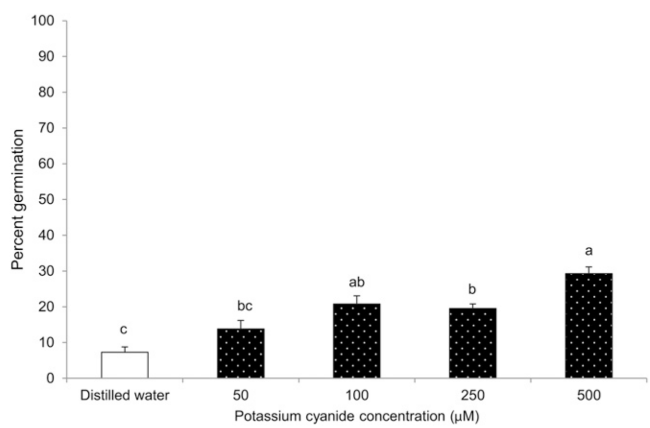

Fig. 7. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested July 2012, stored at 5 °C for 137 and 138 d) to higher concentrations of potassium cyanide (greater than 50 µM). Germination percentages and SEs presented are combined across experimental runs. Means followed by the same letters are not significantly different as determined by Tukey's range test at $P < 0.05$, n = 8.

no two batches have the same concentration or balance of compounds (Daws et al., 2007). Bottle-to-bottle variability of liquid smoke flavoring can be attributed to differences in shelf storage conditions as well as the factors described (Doherty and Cohn, 2000). Evidence of inhibitors produced in xylose smoke-infused water was observed in bioassays conducted by Flematti et al. (2011b). In this study, lettuce seed germination was inhibited in crude samples (1/10 and 1/100 dilutions) but not in solid phase extracted samples. Bioactivity-guided fractionation studies by Light et al. (2010) also showed that certain smoke water fractions (derived from combustion of *Passerina vulgaris* and *Themeda triandra*) were inhibitory to lettuce seeds. This led to the isolation of the racemic 3,4,5-trimethlyfuran-2(5*H*)-one (2,3,4-trimethylbut-2-enolide), which was inhibitory to lettuce at concentrations between 10 and 100 µM (Light et al., 2010) and *Arabidopsis thaliana* at 10 µM (Nelson et al., 2011).

Results of the KAR_1 dose rate study places tanglehead on the list of species reported to be unresponsive to this compound but responsive to smoke-infused water. To date, there are only a handful of species that possess this trait (Downes et al., 2010, 2013, 2014; Flematti et al., 2011a; Long et al., 2011) in contrast with more than 60 species that respond to both KAR_1 and smoke (Chiwocha et al., 2009). In grasses, KAR_1 stimulation was observed in seven of the 19 grass species tested for KAR_1 and smoke water or aerosol smoke (Long et al., 2011). Approximately six species were found to be stimulated by both KAR_1 and smoke solutions, whereas five species were unresponsive to KAR_1 but responsive to smoke solutions (Long et al., 2011). The KAR_1 response in grasses was observed to be dependent on a number of factors including germination temperature, concentration, and after-ripening/dormancy status. In the current study, the germination assays tested a range of concentrations typically observed as stimulatory in most species (0.0067 to 66.7 µM). However, our results did not indicate significant germination stimulation with KAR_1.

The cyanide concentrations evaluated in the current study covered the range naturally found in smoke-infused water and stimulatory to a number of species. Flematti et al. (2011a) estimated that undiluted smoke-infused water contains ≈190 to 300 µM cyanide (from glyceronitrile). Concentrations of cyanide (from glyceronitrile) that were observed as stimulatory to test plants (i.e., *A. manglesii*, *A. flavidus*, and *Rhodocoma arida*) were between 5 and 500 µM (Downes et al., 2010; Flematti et al., 2011a).

The cyanide-stimulated germination (100 to 500 µM) observed in this study was consistent with observations in other grass species, which exhibit physiological dormancy. Cyanide-stimulated germination has been observed in *Avena fatua* (Simpson, 1990), *Panicum virgatum* (200 µM; Sarath et al., 2006), *Aristida contorta* (100 to 1000 µM; Mott, 1974), and *Oryza sativa* (1000 µM; Cohn et al., 1989; Cohn and Hughes, 1986). The mode of action for cyanide-stimulated germination is believed to involve reactive oxygen species (ROS) (Iglesias-Fernandez et al., 2011; Nelson et al., 2012; Oracz et al., 2007, 2009; Siegień and Bogatek, 2006) and ethylene (Gniazdowska et al., 2010; Nelson et al., 2012; Oracz et al., 2008). Studies on dormant sunflower (*Helianthus annuus*) embryonic axes demonstrate that cyanide treatment generates ROS such as hydrogen peroxide and superoxide anions (Flematti et al., 2013; Oracz et al., 2009). Increased ROS levels inhibit the ROS scavenging enzymes, catalase and superoxide dismutase, and activates NADPH oxidase, an ROS-generating enzyme that has been implicated to play a role in seed germination in rice and warm-season grasses (Oracz et al., 2009). The increase in ROS after cyanide treatment or after-ripening can trigger carbonylation of proteins that are specifically associated with seed germination (Iglesias-Fernandez et al., 2011; Oracz et al., 2007, 2009). Besides triggering ROS production, cyanide can also activate the expression of *ERF1*, an ethylene biosynthesis transcription factor that plays a role in the cyanide signaling pathway (Flematti et al., 2013; Oracz et al., 2008).

Although the dose rate studies confirmed that cyanide can provide significant germination stimulation of tanglehead seeds, the results in the smoke-infused water and cyanide comparison studies (Fig. 10) suggested that other compounds in smoke may have worked in combination with cyanide to promote germination. Interestingly, the benzaldehyde germination study (a follow-up study after germination with mandelonitrile) confirmed benzaldehyde as stimulatory to tanglehead at concentrations between 50 and

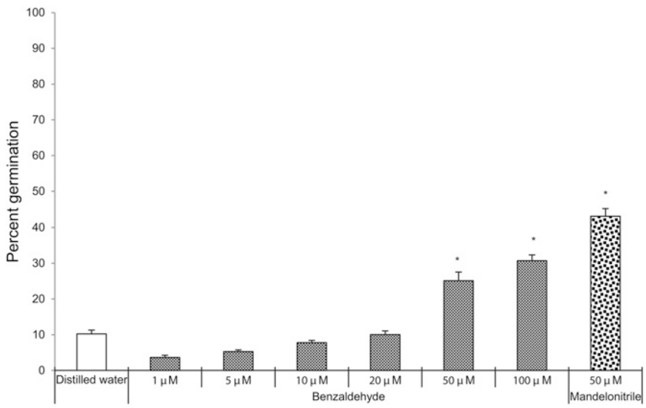

Fig. 8. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested Mar. 2011, stored at 5 °C for 588 and 590 d) incubated in dilutions of benzaldehyde and mandelonitrile. Germination percentages and SES combined across experimental runs are arcsine square root back-transformed values. Means with asterisks are significantly different from the control (i.e., distilled water) as determined by Dunnett's test at $P < 0.05$, n = 8.

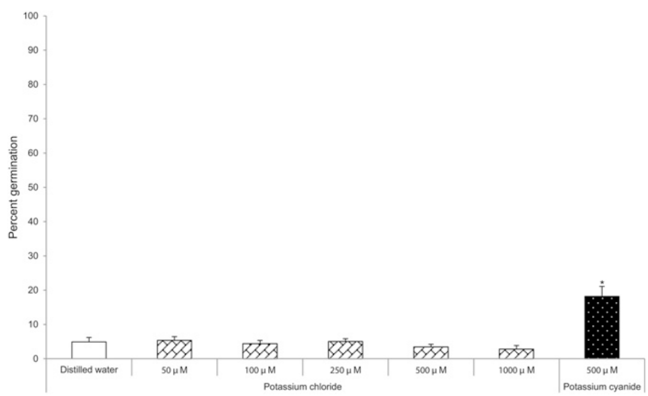

Fig. 9. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested July 2012, stored at 5 °C for 153 and 154 d) to dilutions of potassium chloride and potassium cyanide. Germination percentages and SES combined across experimental runs are square root back-transformed values. Means with an asterisk are not significantly different from the control (i.e., distilled water) as determined by Dunnett's test at $P < 0.05$, n = 8.

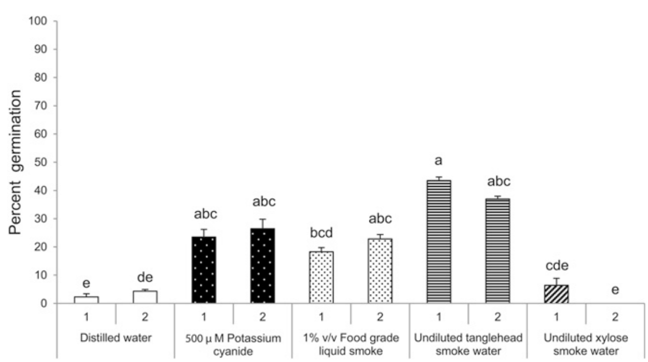

Fig. 10. Percent germination response of tanglehead (*Heteropogon contortus*) seeds (harvested July 2012, stored at 5 °C for 174 d) incubated in potassium cyanide, food-grade liquid smoke, tanglehead smoke-infused water, and xylose smoke-infused water. Germination percentages and SES combined across experimental runs are arcsine square root back-transformed values. Means followed by the same letters are not significantly different as determined by Tukey's range test $P < 0.05$, n = 4.

100 μM. Benzaldehyde has been observed to improve germination of select weed and crop species (French et al., 1986; French and Leather, 1979; Kokalis-Burelle et al., 2002) and it has been detected in food-grade liquid smoke (Hruza et al., 1974) and in smoke from combustion of a number of plant products (Edye and Richards, 1991; Guillén and Manzanos, 1999; Hedberg et al., 2002; Kataoka et al., 1997; Kleindienst et al., 1986). Although benzaldehyde has been detected in smoke, information on its quantity in these smoke sources is meager, making it difficult to establish whether the amounts present is optimal for germination stimulation to occur. Further research on quantifying the amount of benzaldehyde in smoke-infused water is therefore recommended.

In contrast to tanglehead smoke-infused water and food-grade liquid smoke, xylose smoke-infused water was not consistent in its stimulatory capability. Although the xylose smoke-infused water dose rate studies (using seeds harvested in Mar. 2011) exhibited significant germination stimulation with increased concentrations, the xylose smoke-infused water assay that used July 2012 harvested seeds did not exhibit a significant germination response. This inconsistency in germination response to xylose smoke-infused water may be the result of differences in the quality of xylose smoke-infused water solutions prepared or resulting from dormancy status of seeds imposed by factors associated with storage duration after harvest.

Variations in the quality of smoke-infused water samples produced may be caused by subtle differences in preparation, which may have affected the production of the germination stimulant. As mentioned earlier, combustion temperature can greatly influence the final products formed (Jäger et al., 1996). Because the preparation of xylose smoke-infused water used an open flame to heat the combustion flasks, precise control of the combustion temperature was not achieved. Differences in combustion temperatures may have increased or decreased the amount of germination stimulants or inhibitors in the smoke-infused water samples.

In addition to the subtle differences in the preparation of xylose smoke-infused water solutions, the observed variation in germination can also be attributed to differences in the seed dormancy status between batches. In the dose rate study where the Mar. 2011 seeds were used, significant germination stimulation in the undiluted xylose smoke-infused water was recorded (Fig. 3). In contrast, no significant germination was observed with undiluted xylose smoke-infused water where July 2012 seeds were used [i.e., the smoke and cyanide comparison studies (Fig. 10)]. Duration of storage at 5 °C with Mar. 2011 seeds was longer than the July 2012 harvested seeds (Table 1). Although storage at low temperature (10 °C) can maintain dormancy of tanglehead seeds for up to 1 year (Baldos et al., 2014), the longer storage period (i.e., greater than 1 year) for the Mar. 2011 seed batch can account for reduced dormancy status compared with the July 2012 seed batch. Differences in dormancy status of the two seed batches can be observed in the percent germination of distilled water treatments in Figures 3 and 10. These observations indicate that seeds with a reduced level of dormancy may be less sensitive to germination stimulants present in xylose smoke-infused water.

The effects of dormancy status on the responsiveness of seeds to smoke-derived germination stimulants (i.e., smoke and KAR_1) have been reported in a number of species. According to Nelson et al. (2012), the efficacy of smoke or KAR_1 depends on the dormancy state, which is in turn affected by storage conditions. For example, storage under laboratory conditions or burial in soil increased smoke responsiveness of *A. manglesii*, *Actinotus leucocephalus*, *Stylidium affine*, *Stylidium crossocephalum*, and *Tersonia cyathiflora* seeds (Baker et al., 2005; Nelson et al., 2012; Tieu et al., 2001). In seeds of *Eragrostis curvula*, KAR_1 responsiveness increased when seeds were dark-stratified at 20/10 °C (Long et al., 2011). Inconsistencies in germination response between seeds collected at different growing seasons were also observed in *Brassica tournefortii* (Stevens et al., 2007). In that study, seeds collected from the same localities for 2 consecutive years (i.e., Perth metropolitan region and Shark Bay in 2005 and 2006) exhibited differences in response to KAR_1. Less than 30% germination was recorded in the 2005-collected seeds treated with KAR_1. In contrast, seeds harvested in 2006 exhibited complete germination (i.e., 100%) with KAR_1 application.

In summary, the study indicated that food-grade liquid smoke, smoke-infused water from the combustion of tanglehead and xylose as well as cyanide increased germination of dormant tanglehead seeds. KAR_1 was not effective in stimulating germination of dormant tanglehead seeds. Increased germination with non-cyanide-containing smoke-infused water indicates that benzaldehyde and other unidentified compounds are capable of stimulating tanglehead seed germination. These and other factors such as source material, seed batch (i.e., sensitivity to the germination stimulant), and storage durations highlight the complexity of the mechanisms behind smoke-stimulated germination in dormant tanglehead seeds. From a management standpoint, the study showed that smoke-infused water can be a quick and practical alternative seed treatment compared with a 12-month dry after-ripening treatment (Baldos et al., 2014). Commercially available food-grade liquid smoke (at 1% v/v) and tanglehead smoke-infused water (undiluted) are equally effective and can be used to presoak tanglehead seeds immediately before large-scale seeding.

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