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Is 'NO' news good news? Nitrogen oxides are not components of smoke that elicits germination in two smoke-stimulated species, *Nicotiana attenuata* and *Emmenanthe penduliflora*

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Abstract

Both the California chaparral species, *Emmenanthe penduliflora* Benth. (*Hydrophyllaceae*), and a tobacco native to the Great Basin Desert of south-western Utah, *Nicotiana attenuata* Torr. ex Wats. (*Solanaceae*), germinate in response to component(s) of wood smoke. Nitrogen oxides (NO and NO_2), in amounts produced by a fire, have been proposed to be germination signals for *E. penduliflora*. We examined the germination response of dormant seeds of *E. penduliflora* and *N. attenuata* to aqueous solutions of smoke adjusted to different pHs, and two NO donors [sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP)]. The smoke solutions, at pH 4 or 5, induced the maximum germination response. Aqueous solutions of SNP and SNAP, releasing NOx as high as 42 μM, had no effect on germination. Additionally, NO_2^- could not be detected in aqueous smoke extracts derived from combusted cellulose or wood. Therefore, unidentified cellulose combustion factors, rather than NO_x, are likely to be the ecologically relevant germination signals.

Keywords: *Emmenanthe penduliflora*, *Nicotiana attenuata*, nitrate, nitrite, nitrogen oxides, smoke-induced germination

Introduction

Exposure to smoke elicits germination in species associated with rapid colonization of fire-produced habitats of the South African fynbos and savannah (van de Venter and Esterhuizen, 1988; Brown, 1993), western Australian heath (Dixon *et al.*, 1995), California chaparral (Keeley *et al.*, 1985; Keeley and Pizzorno, 1986), and the Great Basin Desert (Baldwin and Morse, 1994; Baldwin *et al.*, 1994; Preston and Baldwin, 1999), as well as many agricultural weeds (Adkins and Peters, 2001). Progress in the identification of the component(s) of smoke responsible for signalling germination in these species has been slow. Baldwin *et al.* (1994) tested 233 compounds, individually and in combination, that were identified in smoke fractions, structurally similar to those reported in smoke or reported to induce germination in other species, and none reproduced the activity of wood smoke on dormant *Nicotiana attenuata* Torr. ex Wats. (*Solanaceae*) germination. Keeley and Fotheringham (1997) demonstrated that exposure to NO_x (specifically, NO and NO_2) gases, which are reported to occur in fires, stimulated germination in dormant seeds of the smoke-responsive California chaparral species, *Emmenanthe penduliflora* Benth. (*Hydrophyllaceae*). However, the response was pH dependent, suggesting that exogenous NO_x may act either by directly oxidizing the seed coat or by oxidizing cellulose or other components in the seed coat, producing a product that subsequently elicits germination (Keeley and Fotheringham, 1998). Moreover, the increases in soil NO_x levels resulting from a fire are short lived and can not account for germination after the first growing season after a fire, but bacterial nitrification and denitrification may generate NO in the soil for more extended periods (reviewed in Stöhr and Ullrich, 2002). Nitrogen oxides within the soil may act as environmental sensors for several species of seeds, indicating proximity of competitors, nitrogen status of the potential growth environment, etc. (Giba *et al*., 2003).

In contrast, the germination signal for dormant seeds of *N. attenuata* is produced after the combustion of pure cellulose and, therefore, likely contains only C, H and O (Baldwin *et al.*, 1994; I.T. Baldwin, unpublished data). The signal is water soluble and

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long lasting. Extracts of soils collected from areas burned 7 years previously still significantly induce germination of dormant seeds (Preston and Baldwin, 1999), which is approximately 3 years longer than soil ammonium pools remain elevated after fires in these habitats (Lynds and Baldwin, 1998), and hence, biological nitrification of ammonia is not likely to produce substantial quantities of NO in these habitats. These two fire-chasing species may utilize two different mechanisms in responding to smoke signals, or NO_x may induce germination by a separate mechanism not related to smoke.

With striking similarity to its involvement in several physiological and pathological effects in animals, such as inflammation and programmed cell death, NO appears to play an important role in growth, development and pathogen defence (Durner *et al.*, 1998, 1999). For example, NO can act with reactive oxygen intermediates (ROI) in the hypersensitive response (HR) of plants to pathogen attack (Delledonne *et al.*, 1998). NO also stimulates germination of species not associated with fires, e.g. lettuce seeds (*Lactuca sativa* L. cv. Grand Rapids), under conditions that normally require light (Beligni and Lamattina, 2000; Gardner *et al.*, 2001). Recently, Light and van Staden (2003) were unable to initiate germination in the same cultivar of lettuce seeds using the NO-releasing compound SNP, but the seeds did germinate in response to smoke. Additionally, since nitrite (NO_2^-) and nitrate (NO_3^-), products of NO decomposition in water, are recognized germination signals in many other species (Hendricks and Taylorson, 1974; Pons, 1989), NO may signal germination in seeds with a variety of dormancy mechanisms. However, no detectable amount of NO_3^- was found in a commercial liquid smoke product used to stimulate germination in red rice (*Oryza sativa*) (Doherty and Cohn, 2000), suggesting that smoke-induced germination does not require NO_x. An additional test of the ecological relevance of NO as a germination signal would be to expose dormant seeds to an active release of NO into an aqueous environment, rather than to expose dry seeds to NO gas (Keeley and Fotheringham, 1998).

Here, we examine the role of NO in triggering germination in dormant seeds of two fire-chasing species, *N. attenuata* and *E. penduliflora*. To simulate natural conditions, seeds were exposed to NO in aqueous solutions using two NO donors [sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP)], and the germination responses of NO_x-exposed seeds were compared with those of seeds exposed to aqueous smoke solutions derived from the combustion of hardwood and pure cellulose. Because NO is rapidly oxidized to NO_2^- in aqueous solutions, we determined NO_2^- spectrophotometrically to estimate the NO dynamics of all treatments.

Materials and methods

Germination bioassay

Germination was characterized as previously described (Baldwin and Morse, 1994; Baldwin *et al.*, 1994; Preston and Baldwin, 1999). Briefly, three replicate bioassays, each with 20 *N. attenuata* seeds or 20 *E. penduliflora* seeds, were used for each treatment. The *N. attenuata* seeds originated from a collection near Flagstaff, Arizona, in 1996 and were selfed in the glasshouse for three generations, while the *E. penduliflora* seeds were from a field collection provided by Keeley and Fotheringham in 1998. The seeds of both species were stored at room temperature and ambient relative humidity. Because *N. attenuata* genotypes vary in their dormancy, with non-dormant genotypes germinating in response to water and dormant genotypes responding to combinations of smoke and nitrate (Baldwin *et al.*, 1994; Krock *et al.*, 2002), we chose a dormant genotype that responded only to smoke (KR4). To determine the maximum and minimum germination percentages of the dormant *N. attenuata* and *E. penduliflora* seeds, the experiment included three replicate smoke-controls, in which seeds were exposed only to a 1:300 dilution of the liquid smoke (derived from the combustion of hickory wood) condiment (House of Herbs, Passaic, New Jersey, USA) or smoke derived from pure cellulose (see below), and three replicate water-controls, in which seeds were treated only with distilled water. In addition, three replicate 100 µM NO_2^- and three replicate 100 µM NO_3^- treatments were used for each species. In water, NO_2^- and NO_3^- are normal oxidation products of NO (Cotton and Wilkinson, 1962). After a 3–4 h exposure to the treatment solutions, seeds were transferred with the treatment solution to soufflé cups [Solo 1 oz (29.6 ml), P100, Urbana, Illinois, USA] containing approximately 3 g of sterile sand (Merck, Darmstadt, Germany) and subsequently saturated with distilled water to ensure adequate moisture. The cups were sealed with transparent lids (Solo PL1 lids, Urbana, Illinois, USA). The *N. attenuata* seeds were placed in a growth chamber (Snijders Scientific, Tilburg, Holland; model S 1084) with a photoperiod of 16 h light:8 h dark (16L:8D) and 32°C day, 27°C night temperature cycle. The *E. penduliflora* seeds were placed in a growth room with a 16L:8D photoperiod and 27°C day, 23°C night temperature cycle. Seeds were examined after 72 h and were monitored daily for 7 d for radicle emergence. To determine seed viability, 250 µl of 1 mM gibberellic acid (GA_3; Duchefa, Haarlem, The Netherlands) was applied to each bioassay container on day 8. Seeds were re-examined on day 10 for radicle emergence in response to the GA_3 application. Previous trials with *N. attenuata*

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seeds determined that all viable seeds germinate in response to this GA_3 treatment (T. Krügel and C.A. Preston, unpublished data). *N. attenuata* seeds used in the experiment further investigating germination responses to longer SNP, nitrite and nitrate exposures were treated similarly, but response to the initial treatment was evaluated over 10 d. GA_3 was then applied on day 10 and seeds were re-examined for radicle emergence on day 13.

Preparation of smoke treatments

We pyrolysed pure α-cellulose (Sigma, Steinheim, Germany) and bubbled the resulting smoke through water to determine if NO or NO_2^- was produced during combustion and, therefore, could be responsible for the smoke-induced seed germination of *N. attenuata* and *E. penduliflora*. The aqueous smoke extract was prepared by pyrolysing 150 g of α-cellulose in a stainless-steel funnel supported by a perforated stainless-steel plate. The funnel was connected to an atomizer submerged in 1.5 litres of distilled water in a 2-litre Erlenmeyer flask, and smoke was drawn through the water by a vacuum pump. A magnetic stirring bar dispersed the smoke into the water. To remove particulate material, the smoke extract was filtered through Whatman No. 1 filter paper (Whatman Intl, Maidstone, England) before use.

Preparation of chemical treatments

All treatments were made fresh on the day of the experiment, with the exception of the sodium nitrite ($NaNO_2$; Merck, Darmstadt, Germany) and sodium nitrate ($NaNO_3$; Merck, Darmstadt, Germany), which were made 1 week previous for preliminary experiments; measures of their NO_2 and NO_3 concentrations found them to be stable. All solutions were prepared using double-distilled water. Preliminary experiments demonstrated that the release of NO from the sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP), and its subsequent conversion to NO_2^-, occurs quickly after solution preparation (see Fig. 2 for kinetics). Therefore, SNP and SNAP solutions were prepared immediately before application to the seeds. SNP and SNAP were obtained from ICN Biomedicals, Inc. (Aurora, Ohio, USA).

Spectrophotometric determination of NO_2^-

NO_2^- concentrations were determined with modifications of the Jaworski (1971) method. An external standard calibration curve was determined by plotting absorption versus known concentrations of NO_2^-. First, 300 µl of 1% sulfanilamide in 3 M HCl and 300 µl of 0.02% *N*-1-naphthylethylenediamine were added to 400 µl of each test solution. These mixtures were allowed to react for 20 min, after which, 4 ml of double-distilled water were added and the optical density at 540 nm was measured with a Perkin Elmer Lambda 16 UV/VIS Spectrometer (Bodenseewerk Perkin-Elmer GmbH, Überlingen, BRD).

pH effects on seed germination

To disentangle the effects of acidity and smoke 'cues' on germination, we compared germination of seeds exposed to either liquid smoke or cellulose smoke at their initial acidities and after adjusting to pH 7. The NO_2-induced germination in *E. penduliflora* is pH dependent (Keeley and Fotheringham, 1998). In a preliminary trial, we measured the pH of all the treatments. Liquid smoke at a 1:300 dilution and cellulose smoke at 1:100 dilution (amount known to stimulate maximum germination in *N. attenuata*; Baldwin *et al*., 1994; Baldwin and Morse, 1994; Preston and Baldwin, 1999; R. Becker, unpublished data) had pH values of 4 and 5, respectively. The pH was adjusted to 7 in both samples by the addition of sodium hydroxide. The remaining treatments had initial pH values near 7. All pH adjustments were made just before treatments were applied to the seeds.

Statistical analysis

The percentage of all seeds germinating in each bioassay container at each observation was arcsine-transformed for normality. Unpaired *t*-tests were used to analyse pH effects on seed germination. Paired *t*-tests were used to analyse germination responses within each treatment to the application of GA_3, comparing data from day 7 and day 10. Repeated-measure ANOVAs on the transformed percentages were used to analyse main effects. There was insufficient variation in the *E. penduliflora* germination values for a repeated-measures ANOVA over the entire 7 d. Therefore, only data from the counts on days 3, 4 and 5 were used in the repeated-measures ANOVA. Fisher's protected least significant difference (PLSD) *post-hoc* tests were performed on data from the final day of counts before GA_3 treatment, and the germination values at 2 or 3 d following GA_3 treatment, to compare individual treatments. Analyses were performed with the STATVIEW 5.0 statistical package (Abacus Concepts, Berkeley, California, USA).

Results

pH effect on smoke-induced germination

The germination responses in both *E. penduliflora* and *N. attenuata* were significantly greater in all the smoke

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treatments as compared to their respective water controls (Fig. 1; one-way ANOVA: $F_{4,10}$ = 23.0, 52.7, respectively; $P < 0.0001$). *Post-hoc* tests revealed that significantly more *E. penduliflora* and *N. attenuata* seeds germinated in the liquid smoke at pH 4 compared to the pH 7 solution ($P = 0.010$ and 0.016, respectively). There was no significant difference in the germination response of *E. penduliflora* or *N. attenuata* seeds when exposed to either pH 5 or pH 7 cellulose smoke treatments ($P = 0.095$, 0.311, respectively), although the trends of the data suggest that pH values less than 5 might enhance germination. We conclude that while pH has some effect on germination, germination is also due to cue(s) found in the smoke solutions, which act independently of the acidic solution. To avoid any variations in germination due to variations in pH, we used smoke solutions that were adjusted to pH 7 for the remaining experiments.

NO_2^- concentrations of SNP and SNAP treatments

From time 0, when the solutions were first applied to the seeds, to 48 h after application, NO_2^- concentrations increased to 0.4, 0.7, 2.2 and 41.7 µM in the 10 µM SNP, 100 µM SNP, 10 µM SNAP, and 100 µM SNAP solutions, respectively (Fig. 2). No detectable quantities of NO_2^- were found in the water, $NaNO_3$ or any of the smoke treatments. In previous publications, smoke-water samples and NO-treated water used to initiate germination in *E. penduliflora* contained 167–375 µM total nitrite/nitrate concentration, with pH values of 3.1–4.4 (Keeley and Fotheringham, 1997, 1998). Seeds were exposed to these solutions for very short time periods, 0.5–1 min, as longer exposures killed the seeds. Our solutions avoided the potential confounding effects of applying acidic solutions (Keeley and Fotheringham, 1998), and the NO levels applied in our experiment are similar, considering that some NO would have been converted to NO_3^-, which we did not measure, and the exposure time to these solutions was longer. Therefore, we conclude that there was sufficient NO available to the seeds in the solutions to test NO effects on the germination response.

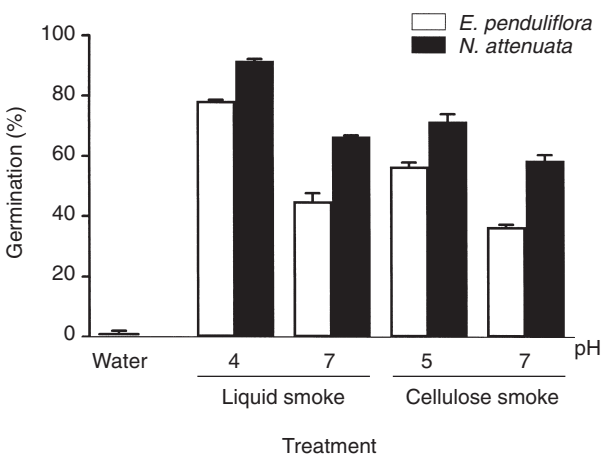

Figure 1. Mean (+1 SE) germination percentages for *Emmenanthe penduliflora* (white bars) and *Nicotiana attenuata* (black bars) seeds at day 7. Seeds were treated with water, liquid smoke at its initial pH of 4 and adjusted to pH 7, or cellulose smoke at its initial pH of 5 and adjusted to pH 7. Each treatment had three replicates of 20 seeds for each species. All seeds were soaked for 3–4 h prior to transfer into the bioassay containers.

NO effect on seed germination

The two different seed species were very similar in their responses to all the treatment solutions (Fig. 3). A repeated-measures ANOVA revealed significant differences among the treatments for *E. penduliflora* ($F_{8,36}$ = 31.2, $P < 0.0001$) and *N. attenuata* ($F_{8,72}$ = 44.4, $P < 0.0001$). Compared to the water controls, germination in the liquid smoke and the cellulose smoke controls differed significantly (P values < 0.0001), but not in the 100 µM nitrite and nitrate controls ($P \geqslant 0.44$). In subsequent experiments with *N. attenuata* seeds, only when seeds were exposed to very high nitrite or nitrate concentrations (10 mM or greater) did they germinate in a manner comparable to that of smoke-exposed seeds (data not shown). In *E. penduliflora* no concentration of SNP and SNAP stimulated germination significantly above that found in the water control ($P \geqslant 0.38$). In *N. attenuata* seeds, 100 µM SNP increased germination significantly ($P = 0.002$) over water controls, but the response was 5- to 18-fold less than that of the liquid and cellulose smoke treatments ($P < 0.0001$). In subsequent

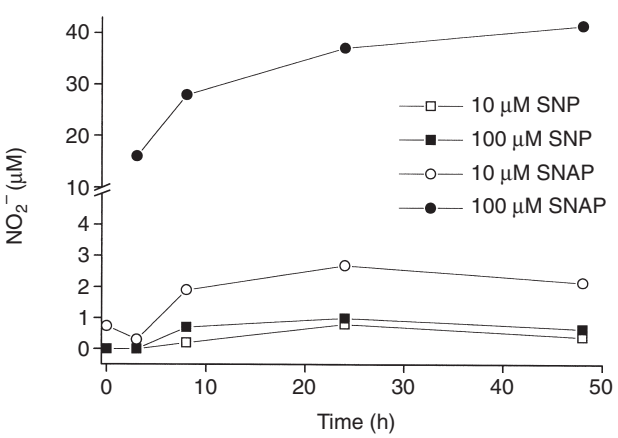

Figure 2. Nitrite (NO_2^-) concentration (µM), as determined spectrophotometrically, for the SNP and SNAP solutions used in the germination trial at time 0, 3, 8, 24 and 48 h after application to the seeds. NO_2^- could not be detected in the liquid smoke and cellulose smoke solutions.

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experiments, higher concentrations of SNP (1 mM and 10 mM) completely inhibited seed germination (data not shown).

To test the viability of the seeds that had not germinated on the final day of observation, GA_3 was added to each cup on day 8, and germinating seeds were re-counted 2 d later. GA_3 was used to elicit germination in *N. attenuata* seeds as a test for viability (Fig. 3C), but seeds of *E. penduliflora* do not respond to GA_3 (data not shown; Keeley and Fotheringham, 1998). For *N. attenuata*, all GA_3 treatments significantly increased germination ($t < -4.98$, $P < 0.038$), except for the liquid smoke treatment ($t = -3.24$, $P = 0.083$), which was already at a maximum. Seeds treated with SNP at concentrations greater than 100 µM SNP had significantly reduced germination even after exposure to GA_3, suggesting that SNP may be toxic at high concentrations.

Discussion

Both *E. penduliflora* and *N. attenuata* seeds germinate when exposed to smoke extracts, partially independent of the solution pH (Fig. 1). In our germination bioassay, which is optimized to study smoke-induced germination in *N. attenuata*, nitrogen oxides up to a concentration of 42 µM did not stimulate germination in either species (Fig. 3A,B) and were not detectable in the smoke extracts that stimulate germination. To determine whether NO is produced during the pyrolysis of a fuel more complex than α-cellulose, we also combusted juniper litter, a common fuel for lightning-initiated fires in the *N. attenuata* habitat. NO_2^- was not detected in the smoke solutions produced from juniper litter, which were also highly active in germinating *N. attenuata* seeds (data not shown). As NO_2^- would be an end product of NO_x release into water, we conclude that NO_x are not the components in smoke responsible for inducing germination in either of these smoke-stimulated species.

Our experiment enabled us to test the seed germination response to several forms of NO_x. When NO is released from SNP and SNAP into water, it is oxidized to form nitrogen dioxide, NO_2. Nitrogen dioxide reacts with water to produce nitrous acid, HNO_2, and nitric acid, HNO_3. Finally, the HNO_2 and HNO_3 disassociate to form nitrite, NO_2^-, and nitrate, NO_3^- (Cotton and Wilkinson, 1962). Although the conversion of NO to NO_2^- in water can occur quickly, we feel that there was sufficient NO available to the seeds in the solutions to test NO effects on the germination response. The 100 µM SNAP solution had increasing NO_2^- concentrations for the first 48 h of the experiment, confirming the release of NO into the aqueous solution (Fig. 2). From previous work, *N. attenuata* seeds become irrevocably committed to germination after exposure to smoke solutions for 48 h (Preston and Baldwin, 1999; Krock *et al.*, 2002);

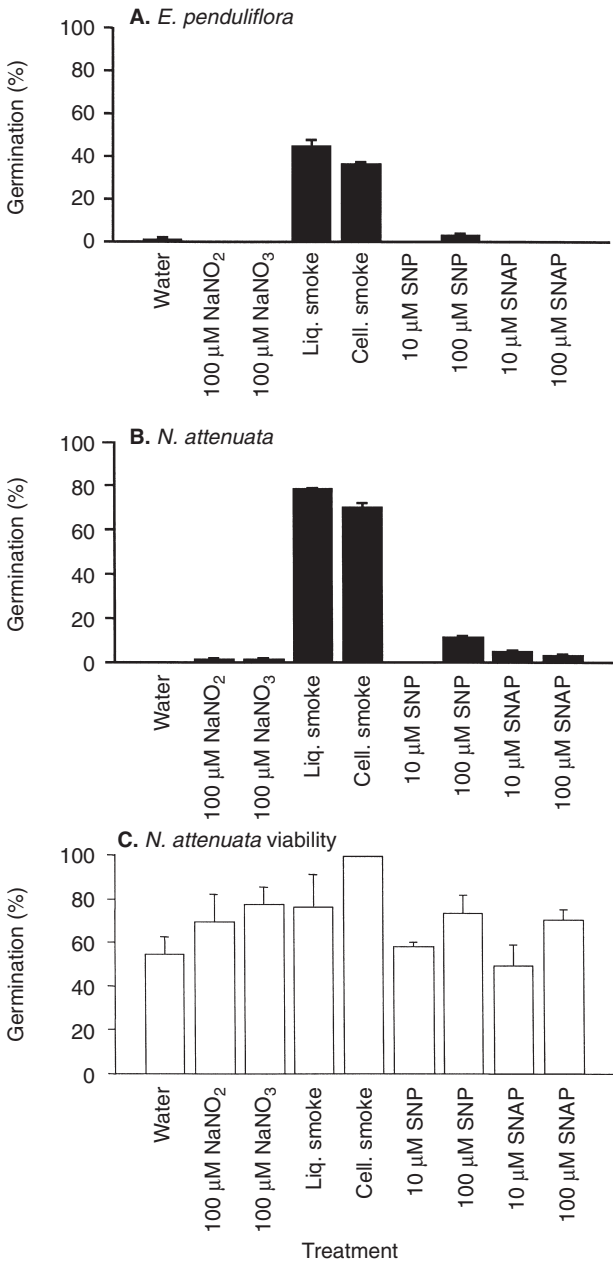

Figure 3. Mean (+1 SE) germination at day 7 for (A) *Emmenanthe penduliflora* and (B) *Nicotiana attenuata* seeds in bioassays treated with 1 ml of each treatment. Each bioassay consisted of 20 seeds in each of three replicate bioassay containers. For all bioassays, seeds were soaked for 3–4 h in their respective treatment solution before being transferred to the bioassay container. (C) Viability [mean (+1 SE) germination] of *N. attenuata* seeds that had not germinated for 7 d after initial treatments. Viability was assessed as germination within 2 d after application of 1 mM GA_3.

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hence, NO was present for an interval sufficient to initiate germination in the SNAP solution. Only at 100 µM SNP did germination increase significantly above that in the water controls (Fig. 3B), and subsequent experiments at higher concentrations (1 mM and 10 mM) inhibited germination. Our determination of NO_2^- concentrations revealed that SNP solutions produced lower NO_2^- concentrations than did SNAP (Fig. 2). This may be the result of a slower release of NO. Since neither species germinated in response to 100 µM nitrate (Fig. 3), the further oxidation of nitrite to nitrate in any of the treatments cannot explain the observed germination responses. In summary, the absence of NO_x in various smoke preparations indicates that exposure to NO_x is not responsible for the germination response elicited by smoke.

Seed dormancy allows plants to disperse in time, and the more habitat-specific the signals are that elicit germination, the more precisely a plant can locate a particular habitat. Seeds are known to utilize generalized environmental signals such as temperature, photoperiod, moisture, in addition to seed age, to time germination with environments that are compatible with vegetative growth (Mirov, 1936; Philippi, 1993), but little is known about the germination requirements of the vast majority of native plants that exhibit dormancy and are found only in specific habitats. Moreover, germinating in the 'wrong' habitat can have disastrous fitness consequences; lifetime seed production is 12 times greater for *N. attenuata* seedlings that germinate and grow in burned soil instead of unburned soil (Preston and Baldwin, 1999). For species that have stringent habitat requirements, such as *E. penduliflora* and *N. attenuata*, cues that stimulate germination should be equally specific. Seeds of *N. attenuata* time germination into the post-fire habitat by utilizing a combination of the presence of a positive cue(s) in smoke and the absence of an inhibitory germination signal(s) found ubiquitously in leaf litter of later successional species (Preston and Baldwin, 1999; Krock *et al.,* 2002). The negative signals are destroyed by the fire, but are able to inhibit germination in the adjacent unburned habitats, where the positive smoke-derived cue is deposited by wind or downslope water transport (Preston and Baldwin, 1999). Given the importance of accurate habitat location, the utilization of NO_x compounds as germination signals probably does not have sufficient habitat specificity, and, therefore, may result in improper germination for these fire-related species. While NO may be used as an indicator for soil nitrate content, season and climate status (Giba *et al*., 2003), the release of NO by microorganisms in the soil may promote germination too deep in the soil for successful seedling emergence. While nitrogen oxides can result from the combustion of litter and soil organic material (Keeley and Fotheringham, 1997) and nitrogen – as nitrogen dioxide, nitrite, nitrate and ammonium ions – may act to initiate germination in some species, albeit in a pH-dependent manner (Cohn *et al*., 1983; Cohn and Castle, 1984), there are apparently other germination signals found in smoke that are not NO-related. Dormant seeds should be under strong selection to utilize environmental signals that predict particular habitats reliably, and the challenge will be to identify what these signals are.

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