Seed Science Research (2005) **15**, 373–375 DOI: 10.1079/SSR2005228

LETTER TO THE EDITOR

Smoke and mirrors: reply to Fotheringham and Keeley

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Abstract

In their rebuttal, Fotheringham and Keeley (2005) (F&K, hereafter) assert that misinterpretations of previous research, errors in the presentation of the chemistry of nitrogen oxides and devious presentation of experimental results led to the conclusion of Preston *et al.* (2004). [These conclusions refute those of Keeley and Fotheringham's publication in *Science* (Keeley and Fotheringham, 1997).] We disagree and argue that the experimental evidence is consistent with the hypothesis that the ecologically relevant germination signals for the two post-fire annuals, *Emmenanthe penduliflora* and *Nicotiana attenuata*, are the specific pyrolysis products of cellulose rather than chemical scarification by nitrogen oxides (Keeley and Fotheringham, 1997).

While we agree that the chemistry of nitrogen oxides in the gaseous and water phases differs in minor details, neither the chemistry nor the methods that we used to estimate nitric oxide (NO) release from the NO donors [sodium nitroprusside (SNP) and S-nitroso-*N*-acetylpenicillamine (SNAP)] are wrong. The chemistry of nitrogen oxides is complex, and in biological tissues numerous species exist in equilibrium. Wherever NO is present, as either the radical ($NO^{\cdot}$), the nitrosonium cation (NO^{+}) or the nitroxyl radical (NO^{-}), numerous other species are present, many of them biologically active. However, a simple examination of the three equations that F&K present, after correcting for their errors in stoichiometry, can be reduced as follows:

$$2NO + O_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^{\oplus}$$

Nitrite enters the reaction cycle repeatedly before being oxidized to nitrate, which is the final reaction product. It has been shown that nitric acid is the final oxidation product of NO (Fitz *et al.*, 2003), which demonstrates that quantifying NO release from the NO donors by measuring NO_2^- levels is completely appropriate; moreover, this procedure is commonly reported in the literature (Neill *et al.*, 2003).

After a lengthy discussion about the chemistry of NO, and minor issues we address in the footnote, F&K finally arrive at a substantive point concerning the differences between our two studies: the concentrations of NO used to elicit germination. They argue that the amount of NO released from the highest concentration that we tested (100 μM) falls short of the levels that they used to trigger germination by gas-phase exposures, and that we did not present data on higher concentration exposures because 'they did not conform to the predetermined goal of the study, specifically to disprove any role for nitrogen oxides in the germination of *N. attenuata*'. While the former is true, the latter is not. As we state in our study, higher concentration treatments killed seeds; germination studies of dead seeds are not informative.

Numerous studies have examined the effect of SNP and SNAP on seed germination and, in these studies, dormancy release has consistently been found to occur at concentrations of NO donors within the range of concentrations that we tested. Moreover, these studies also report that higher concentrations kill seeds. For example, dormancy in *Arabidopsis* seeds can be broken by treatment with 25 μM SNP, and concentrations greater than 250 μM SNP impaired growth, inhibited root development and inhibited seed germination (Bethke *et al.*, 2004). While *Lactuca sativa* (Grand Rapids) seeds germinated in response to treatments with 100 μM SNP (Beligni and Lamattina, 2000), *Lupinus luteus* seeds required only 1 μM SNP (Kopyra and Gwozdz, 2003), while 0.1 μM SNAP germinated *Paulownia tomentosa* seeds (Giba *et al.*, 1998). These results demonstrate that a number of

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species, not known to be fire-associated, will respond to concentrations of NO releasers that the two fire-associated species, *N. attenuata* and *E. penduliflora*, do not respond to.

In our work, we demonstrate that commercially available smoke extracts prepared from the combustion of wood, as well as aqueous smoke extracts prepared from the combustion of cellulose, contain no detectable amounts of NO_2 and yet dramatically stimulate germination in both *E. penduliflora* and *N. attenuata* seeds. Our choice of cellulose was not 'frivolous' as F&K contend. Pyrolysis of the most abundant constituent of biomass that produces no nitrogen oxides provides a critical test of whether or not nitrogen oxides are required for the smoke-induced germination response. Clearly they are not. Other researchers working with red rice report similar results (Doherty and Cohn, 2000). More recently, Flematti *et al.* (2004) characterized a butenolide (3-methyl-2H-furo[2,3-c]pyran-2-one), which is produced by the pyrolysis of α-cellulose and found in smoke solutions that we used in our study; this compound elicited germination in several smoke-responsive plant species from Australia, South Africa and North America (including *N. attenuata* and *E. penduliflora*). Significantly, application of this butenolide increased germination in *E. penduliflora* seeds from 0% in water controls to 82% at an ecologically relevant level of 10 ppb (Flematti *et al.*, 2004). The small amounts of this non-reactive, water-soluble, organic metabolite contrast strongly with the quantities of NO required to elicit germination in the Keeley and Fotheringham (1997) study. These concentrations are worth considering in more detail, as herein lies the crux of our disagreement.

In the 'Research News' story that accompanied the *Science* paper (Keeley and Fotheringham, 1997), Keeley is quoted as saying, 'The germination response of these seeds to even small quantities of nitrogen dioxide was remarkable'. What exactly is meant by 'small'? In a departure from normal conventions of reporting concentrations of gases in ppm, K&F report that a 3-min exposure to 790 mg m^{-3} NO_2, or 20.8 g m^{-3} of $NO + NO_2$ for 30 s, stimulated 100% germination. These treatments are equivalent to 500 and 10,000 ppm (V) treatments and are misleadingly reported as representing 167–690 μM treatments in their rebuttal (F&K). Molarity is defined as the number of moles of a given substance dissolved in 1 litre of solvent, and should not be used for concentrations of gaseous systems, as illustrated by the following example. If 1 mmole of a compound is dissolved in 1 litre of solvent (e.g. a 1 mmolar solution), the ratio between compound and solvent is roughly 1:55,000 (1 litre = 1 kg, 1 mole H_2O = 18 g, 1 litre water = 55 moles). However, if 1 mmole of a compound is 'dissolved' in 1 litre of gas, the compound to solvent ratio is only 1:45 (1 mole of an ideal gas is 22.4 litres; 1 litre of gas is 0.045 mole), which means that a 790 μmol concentration of NO in a gaseous volume is roughly 1000-fold more concentrated than this value suggests. Hence, it is inappropriate to use molarity for gaseous systems because this term is defined for liquid solvents. Hence F&K are correct in noting that the concentrations of NO donors that we used in our study were below the concentrations that they used in their study. If we had used the same concentrations, the seeds would not have survived.

While such high concentrations of these reactive nitrogen oxides might occur in the smoke plume of a wildfire, living organisms can tolerate only brief exposures to such strong oxidants without suffering irreversible damage. Ask any fireman. An elegant microscopic analysis of intense chemical scarification caused by a 3-min exposure to smoke of *E. penduliflora* seeds has been described in detail (Egerton-Warburton, 1998). While we are less familiar with the behaviour of the seed bank responses of *E. penduliflora* after fires, the *N. attenuata* seed bank does not germinate immediately after fires, but instead remains dormant for 6–9 months before germinating. While this point will clearly require more study, we find it unlikely that chemically scarified *N. attenuata* seeds would not germinate during the multiple hydration events and warm spells, but rather wait 6–9 months after a fire. Scarification by heat, acid treatments, bleach, oxidizing gases and mechanical abrasion are some of the traditional procedures known to elicit germination. Once the integrity of the seed coat is violated, a seed has little choice other than to germinate; otherwise it risks being killed by invaders from the soil. Scarification-elicited seed germination requires very careful titration of the physical damage, very much like roasting a marshmallow. Too much kills the seeds, and too little doesn't allow the seed to overcome the dormancy control. We find it rather unlikely that the tremendous germination response of the seeds banks commonly observed after fires results from millions of seeds having found just the right 3-min exposure to nitrogen oxides. We wonder if seeds buried in the soil are even directly exposed to biomass smoke.

The concentrations of nitrogen dioxide required to chemically scarify *E. penduliflora* seeds makes a mockery of the suggestion F&K make in their 1997 *Science* paper and the accompanying 'Research News' story, namely that NO_x from smog might 'trick' seeds into maladaptive germination. Smog, even in the LA basin, has not attained concentrations above 1 ppm (Grosjean and Bytnerowicz, 1993; Fitz *et al.*, 2003).

While scarification might account for a small fraction of the germination response of the *N. attenuata* or *E. penduliflora* seed bank after a fire, we find it more probable that most seeds that germinate after fires are responding to specific pyrolysis products. In other words, chemical 'eavesdropping' on both the positive and negative environmental germination signals that

https://doi.org/10.1079/SSR2005228 Published online by Cambridge University Press

are altered by fires, as we have demonstrated for *N. attenuata* (Krock *et al.*, 2002), rather than scarification, is likely to explain the dramatic response of the seed bank.

Nitrogen oxides undoubtedly play many important roles in plants, and we look forward to understanding the mechanisms by which physiologically relevant doses of NO elicit germination. The nitrosation/nitroylation of regulatory proteins (Mikkelsen and Wardman, 2003) may be one such mechanism; it, in turn, may be elicited by the organic constituents of smoke, fuelling this debate for years to come. Clear falsification of the ecological relevance of the specific signalling versus scarification hypotheses will require the elucidation of the signal transduction cascades elicited by the smoke factors, and their genetic manipulation. Comparisons of the post-fire germination responses of seed banks that differed genetically in their ability to respond to the pyrolysis signals would resolve the debate once and for all. It is interesting to note that of the species that have been tested to respond to NO donors, only the two species that are known to respond to fires in their natural habitats did not respond. This may be an artefact of the difficulty of publishing negative results, or a hint that fire-chasing species are relatively insensitive to nitrogen oxides.

Footnote

F&K make a number of additional comments which distract from the substantive issues discussed above and these are addressed here.

They criticize our germination bioassay, which we clearly state was refined for *N. attenuata* germination and modified to accommodate the different germination characteristics of *E. penduliflora*. With regard to the bioassays, F&K make statements that are contrary to previous claims. Keeley and Fotheringham (1998) state that cold stratification 'only slightly stimulated' germination, increasing germination from approximately 80–86% in smoke-treated unstratified seeds to 99–100% in those seeds stratified for 7 or 30 d and subsequently treated with smoke. Additionally, *E. penduliflora* seeds are light insensitive (Keeley and Fotheringham, 1998). Nevertheless, we obtained significant responses between our standard negative control (water) and positive control (1:300 liquid smoke) in our germination conditions, thereby validating our method.

We are in complete agreement with F&K in that pH is a 'critical consideration in studies with nitrogen oxides and seed germination to understanding what species of nitrogen oxide is, or isn't, affecting germination'. A low pH is required to elicit germination by scarification with NO_x (Keeley and Fotheringham, 1998). However, we demonstrate that smoke-stimulated germination is independent of pH (Preston *et al.*, 2004). With the pH of post-fire soils slightly on the acidic side of neutral ($pH = 6.6 \pm 0.03$, $n = 90$ sites; J.E. Keeley, unpublished data cited in Keeley and Fotheringham, 1998), acid scarification seems an unlikely mechanism.

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https://doi.org/10.1079/SSR2005228 Published online by Cambridge University Press