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Vegetation-derived abscisic acid and four terpenes enforce dormancy in seeds of the post-fire annual, *Nicotiana attenuata*

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Abstract

The native tobacco, *Nicotiana attenuata*, synchronizes its germination with the immediate post-fire environment with a combination of germination stimulants found in wood smoke and inhibitors from the unburned litter of the dominant vegetation. The inhibitors override the stimulants and prevent seeds from germinating maladaptively in unburned habitats adjacent to burns. To understand the physiological basis of this environmental control of germination, we tested several previously isolated signals, phytohormones and their respective biosynthesis inhibitors. The germination inhibitors methyl jasmonate (MeJA, a constituent of sagebrush litter), bornane-2,5-dione (BD, a constituent of juniper litter extract, JLE) and JLE did not alter abscisic acid (ABA) content of imbibed seeds. Treatment with the ABA biosynthesis inhibitor, fluridone, inhibited the dormancy-inducing effects of BD, JLE and MeJA, but surprisingly did not affect endogenous ABA levels in treated seeds. However, ABA leached from litter of the species, which dominate the plant community before fires, plays an important role in germination control. We conclude that *N. attenuata* seeds, which can lie dormant in the soil for 150 years between fires, time their germination with the post-fire environment by responding to smoke, ABA and four terpenes (BD, 1,8-cineole, β-thujaplicin and camphor) leaching from the litter of the dominant vegetation.

Keywords: *Nicotiana attenuata*, secondary dormancy, smoke, abscisic acid, gibberellic acid, fluridone, methyl jasmonate, bornane-2,5-dione

Introduction

Dormancy is an undesired property in agricultural seeds, but a major adaptive response of native plants that allows them to cope with environmental variation (Harper, 1994; Bewley, 1997; Baskin and Baskin, 1998). As a consequence, both physiologists and ecologists have studied the phenomenon extensively, but with little cross-fertilization between the disciplines, particularly with regard to how the most prevalent form of dormancy in temperate seed banks, namely physiological dormancy (Baskin and Baskin, 1998), is broken by specific environmental signals (Vleeshouwers *et al*., 1995). Seeds of these plants are frequently shed from the plant with strong primary dormancy, which is subsequently broken or re-established by environmental signals. For these plants, dormancy is an adaptation that allows for survival through short periods of favourable conditions, during which germination would be decidedly maladaptive, as germination during benign periods in the autumn would be for a summer annual (Vleeshouwers *et al*., 1995).

Many seeds use cues as general as temperature, photoperiod, moisture, or seed age to trigger germination and initiate vegetative growth (Mirov, 1936; Philippi, 1993a). To cope with the lack of reliability of these proximate signals, some species may have evolved 'bet-hedging' strategies, whereby only a certain fraction of the dormant seed bank germinates under favourable conditions. This strategy provides a statistical solution to the problem of cueing germination with unreliable signals (Brown and Venable, 1986; Philippi, 1993b).

Substantial progress has been made in understanding how general environmental signals (light, temperature, etc.) are endogenously transduced to break primary dormancy (which is initiated during seed development and prevents precocious germination) and, to a lesser degree, secondary dormancy (which is initiated after primary dormancy is broken). In particular, the hormone balance hypothesis (Wareing and Saunders, 1971; Karssen and Laçka, 1985; Bewley and Black, 1994), which explains dormancy as resulting from the action of hormones (abscisic acid; ABA) that inhibit germination and those that stimulate it (gibberellins;

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GAs), has received substantial support *sensu lato*, although some details remain unresolved. The majority of the evidence suggests that the inhibitory effect of ABA must first be overcome before the role of GAs in promoting or maintaining germination can be discerned (Karssen and Laçka, 1985; Bewley, 1997, Yoshioka *et al*., 1998; Debeaujon and Koornneef, 2000; White and Rivin, 2000). However, GAs can directly or indirectly influence ABA production and sensitivity (Ni and Bradford, 1993; Grappin *et al*., 2000).

Nicotiana attenuata Torr. ex. Watson (*Solanaceae*) is native to the Great Basin desert of California, Nevada, Idaho, and Utah (USA) (Goodspeed, 1954; Wells, 1959; Brotherson *et al*., 1980) and primarily occurs in: (1) large ephemeral populations (typically less than three growing seasons) after fire in sagebrush and pinyon–juniper ecosystems, and (2) small persistent populations (for many growing seasons) in isolated washes, and as a roadside weed after new construction in a previously undisturbed area (Wells, 1959; Barney and Frischknecht, 1974; Young and Evans, 1978; Wright and Bailey, 1982; Koniak, 1985; Baldwin and Morse, 1994; Baldwin *et al*., 1994; Preston and Baldwin, 1999). Positive and negative control over germination from a long-lived seed bank (estimated to be at least 150 years; Preston and Baldwin, 1999) can account for its occurrence in both types of habitats.

N. attenuata seeds respond to environmental cues that stimulate germination (factors in wood smoke; Baldwin *et al*., 1994), as well as factors (leachates from the dominant vegetation; Preston and Baldwin, 1999) that inhibit smoke-induced germination and induce secondary dormancy. The distribution of the smoke germination cues did not completely coincide with the occurrence of germinating seeds in nature, prompting the search for additional controls over germination (Preston and Baldwin, 1999). Water-soluble germination inhibitors were found in the litter of unburned vegetation from the seven species that dominate the mature plant community of the habitat of *N. attenuata*. These inhibitors, which leach readily from intact litter, inhibit germination at concentrations that do not inhibit the growth of germinated seedlings or mature plants (Preston and Baldwin, 1999). In short, *N. attenuata* seeds appear to monitor their environment chemically to adaptively regulate their germination behaviour.

Genotypes of *N. attenuata* produce seeds that vary in their genetically determined primary dormancy (broad-sense heritability, estimated by parent–offspring regression to be 88%; Baldwin *et al*., 1994). Regardless of their degree of primary dormancy, seeds that are shed in unburned habitats with significant accumulations of litter develop strong secondary dormancy in response to the negative germination cues. If the seeds are shed into habitats without significant litter accumulations (e.g. in washes or roadside habitats), seeds without dormancy germinate. The addition of smoke extracts to these seed banks results in a dramatic increase in germination, demonstrating the presence of dormant seeds in these seed banks (Baldwin *et al*., 1994). *N. attenuata* seeds respond to these positive and negative cues and time their germination with the post-fire habitat, realizing a 12-fold increase in lifetime seed production as compared to plants germinating in unburned soils (Preston and Baldwin, 1999), due largely to the 40-fold differences in available nitrogen in these soils (Lynds and Baldwin, 1998). In summary, if seeds retain their dormancy during the brief periods each spring that are highly conducive for germination during the 10–115-year intervals between fires in the pinyon–juniper habitat (Wright and Bailey, 1982; Koniak, 1985), they will realize a large fitness advantage of germinating in the immediate post-fire environment. How they accomplish this physiologically is the focus of this paper.

Specifically, we test the predictions of the hormone-balance model for the germination behaviour of *N. attenuata* inbred genotypes with high and low dormancy exposed to the positive and negative germination signals, exogenous hormones and their respective biosynthesis inhibitors. While seeds germinated as expected when treated with GA and ABA biosynthesis inhibitors, ABA content in treated seeds was not consistent with the predictions of the hormone balance model. The ABA biosynthesis inhibitor, fluridone, efficiently broke dormancy, but had no effect on ABA content. Increased ABA in seeds treated with inhibitory litter extracts was not due to endogenous ABA production, but was traced to ABA derived from the litter. We show that this ABA derived from plant litter from the habitat of *N. attenuata* contributes to, but can not fully account for, the induction of secondary dormancy in *N. attenuata* seeds. However, when this exogenous source of ABA was combined with four terpenes identified in litter at natural concentrations, the inhibitory effect of the litter extract could be mimicked.

Materials and methods

Litter collections and extracts

The A_0 horizon was collected beneath holly oak (*Quercus* sp.), juniper (*Juniperus osteosperma*), sage brush (*Artemisia tridentata* var. *tridentata*), cliff rose (*Cowania mexicana* var. *stansburiana*), bitter brush (*Purshia tridentata*), sand sage (*Artemisia filofolia*), mormon tea (*Ephedra* sp.) and black brush (*Coleogyne ramosissima*) in July 2000 near Santa Clara, UT, USA

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and stored at –20°C until used. The litter extracts were prepared by adding 20 ml water at 95°C to 8 g of ground litter in a 50 ml centrifuge tube. The tubes were shaken for 12–16 h on a shaking table and centrifuged in an Avanti J-25 centrifuge (Beckman, Palo Alto, CA, USA) at 17,000 rpm for 20 min at room temperature. Supernatants were filtered through a paper filter (Whatman 2, Maidstone, UK) and stored at 4°C until used. These aqueous extracts, which were used in all bioassays, reflect the natural inhibitory potential of the A_0 horizon (Preston and Baldwin, 1999).

Bioassays

Since genotypes of *N. attenuata* are known to produce seeds that vary in their genetically determined dormancy (broad-sense heritability estimated by parent–offspring regression to be 88%; Baldwin *et al*., 1994), we used dormant seeds in bioassays and treatments that were from the third inbred greenhouse generation from a field collection on the DI Ranch (T40S R19W section 9 of Utah, USA) in 1988 and stored at room temperature. These seeds germinate only after smoke treatment. Non-dormant seeds belonged to the fourth inbred generation from a field collection on the DI Ranch in 1989. Non-dormant seeds do not require smoke and germinate readily after imbibition. Dormant seeds (10 seeds per seed cup) were exposed for 1 h to 100 µl liquid smoke (House of Herbs Inc., Passaic, NJ, USA):water (1:300, v/v) in 1 ml shell vials. Non-dormant seeds for the hormone treatments were treated with 100 µl of 1 mM KNO_3, when germination inhibition was expected, to prevent false negative results. One millilitre of the respective treatment was added, and seeds were transferred into a soufflé cup (P100, Solo Cup Company, Top Service, Neufahrn, Germany) containing 3 g analytical-grade sand (Merck, Darmstadt, Germany), which previously had been saturated with 0.5 ml water, so that the total volume in each seed cup was 1.5 ml. Seed cups were sealed with transparent lids (PL1, Solo Cup Company) and placed in a growth chamber (Economic deLuxe 432 L, Snijders Scientific, Tilburg, The Netherlands) under optimal germination conditions for *N. attenuata* germination (16 h/32°C day and 8 h/28°C night). Under these optimal conditions, a germination delay of 6 d represents a much longer delay under natural conditions (Preston and Baldwin, 1999). Between 3 and 8 d after sowing, seeds were examined daily for germination with a 10× microscope (Zeiss, Jena, Germany) and finally at day 12 to determine seed viability after GA_3 treatment. Germination percentages were calculated from the quotient of germinated and viable seeds. All bioassays were performed with water and smoke controls to monitor the seed behaviour (dormant/non-dormant).

Seed treatments

The GA biosynthesis inhibitor, paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentane-3-ol], was applied at 10 µM (Riedel-deHaën, Deisenhofen, Germany) and exogenous GA_3 at 250 µM and 500 µM for juniper litter extracts and paclobutrazol treatments, respectively (Duchefa, Haarlem, The Netherlands). Due to the low solubility of GA_3 and paclobutrazol in water, these compounds were first dissolved in ethanol and subsequently diluted with water to obtain a final ethanol concentration < 0.05% (v/v). Controls with 0.05% ethanol were performed in order to test whether ethanol at the applied concentration has an effect on seed germination. ABA was applied at 100 µM (mixed isomers, Sigma, Deisenhofen, Germany) and fluridone [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4-pyridone] at 10 µM (Duchefa, Haarlem, The Netherlands). The environmental signals, methyl jasmonate (MeJA; Aldrich, Deisenhofen, Germany) and bornane-2,5-dione (BD), were applied at 10 µM and 600 µM, respectively. The percentage of germinated seeds in each seed cup was arcsine-transformed to create data with normal distributions, which were evaluated with the *F*-test. Repeated-measurement ANOVAs were used to calculate main effects, and significant differences between single treatments were tested with Fisher's PLSD test. All statistical tests were performed with STATVIEW 5.0 (SAS Institute Inc., Cary, NC, USA).

ABA determination in seeds and aqueous litter extracts

Dormant seeds (100 mg per sample) were soaked in 100 µl smoke (as in the germination bioassay) for 1 h before being treated for 6 h with 100 µl of the following treatments: (1) 20 µM paclobutrazol; (2) 100 µM fluridone; (3) 600 µM bornane-2,5-dione; (4) 100 µM MeJA; (5) 2.5 mM GA_3; and (6) juniper litter extract (JLE, see section 'Litter collection and extracts') plus 2.5 mM GA_3. Each treatment was performed with three replicates.

Treated seeds were homogenized in 1.5 ml ice-cold acetonitrile and extracted at 4°C overnight. The extracts were centrifuged for 10 min at 16,000 *g*, and the pellets were re-extracted twice with the same volume of acetonitrile. To the pooled supernatants of each sample or aqueous litter extracts, 100 ng [$^2H_4$]ABA (Dr R. Baraldi, Istituto di Ecofisiologia delle Piante Arboree da Frutto, CNR, Bologna, Italy) was added as an internal standard before they were reduced to a small volume of aqueous phase on a rotorevaporator (130 mbar, 40°C). The residues were taken up in 1 ml water at pH 2.5, loaded on a 1 ml LC-

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18 cartridge (Supelco, Deisenhofen, Germany) and eluted with 40% (v/v) methanol. Eluates were dried under N_2 at 50°C before derivatization with freshly prepared diazomethane in ether. The solvent was evaporated, samples taken up in 1 ml 40% (v/v) methanol, loaded on a LC-18 cartridge, washed with 40% methanol, and eluted with 70% (v/v) methanol. Eluted fractions were dried under N_2 at 50°C and dissolved in 100 µl toluene for analysis by GC/MS (gas chromatography/mass spectrometry).

Fifty microlitres of each sample were injected by large-volume injection (LVI) into a Varian 3800 GC equipped with a 1079 injector and coupled to a Saturn 2000 ion trap (Varian, Darmstadt, Germany). GC conditions were as follows: carrier gas: helium at 1.0 ml min^{-1} constant flow; injector: initial: 80°C, 2 min, 20°C min^{-1}, 250°C, 32.15 min; split ratios: initial: 1:20, 0.01 min, 1:150, 2 min, off, 5.10 min 1:50; GC oven: initial: 80°C, 6 min, 30°C min^{-1}, 220°C, 3°C min^{-1}, 260°C, 40°C min^{-1}, 300°C, 10 min (total run time: 35 min); RTX-200 fused silica capillary (Restek, Bad Homburg, Germany, 30 m × 0.25 mm internal diameter (i.d.) × 0.25 µm film thickness).

High-performance liquid chromatography fractionation of JLE for ABA

Fifty millilitres of freshly prepared JLE were adjusted to pH 2.5 with hydrochloric acid and partitioned three times against diethyl ether. The combined organic phases were dried and taken up in 1.5 ml ethanol–water (1:1, v/v) and fractionated with a LC 1100 high-performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany). Chromatographic conditions were as follows: injection: 50 µl; flow: 1.0 ml min^{-1}; gradient: A: 0.25% phosphoric acid in water, B: acetonitrile; begin: 18% B, linear gradient to 29% B at 10 min, linear gradient to 90% B at 20 min; column: YMC ODS-A, 150 × 3 mm, 5 µm (YMC Europe, Schermbeck, Germany). Twenty 1-min fractions were collected, which were adjusted to pH 2.5 and partitioned three times against dichloromethane. The combined organic layers of each fraction were dried, taken up in 4 ml water and bioassayed.

Bioassay-driven fractionation of JLE

Prior to the fractionation of JLE, we conducted experiments to obtain more information about the active compound(s) in JLE, because little was known about the negative cue(s), except for the fact that the active constituents of JLE were sparingly water-soluble. Liquid–liquid extraction with organic solvents revealed that activity was easily extracted from aqueous JLE by ethyl acetate and dichloromethane. The molecular weight of the negative cue was determined to be smaller than 1 kDa by ultrafiltration. Filtrate and residue of ultrafiltrated JLE [MidGee cross flow filter with an UFP-1-C-1 membrane (Schleicher & Schüll, Dassel, Germany) with 1 kDa cut-off] were adjusted to the original volumes and tested in a bioassay; only the filtrate inhibited germination. Chromatographibility was confirmed by eluting the cue from silica [2.0 cm i.d. glass column with frit filled with 20 g activated silica (silica 60; 0.063–0.200 mesh, Merck, Darmstadt, Germany), 18 h, 120°C] with iso-propanol–hexane (1:4, v/v).

The bioassay-driven fractionation of juniper litter used the A_0 horizon collected underneath juniper (*Juniperus osteosperma*) from unburned areas in south-western Utah, USA (T4D5–R19W). Juniper litter was ground in an analytical mill (A-10 Tekmar, IKA, Staufen, Germany). Direct extraction of juniper litter with organic solvents resulted in very lipophilic extracts that could not be dissolved in water for the bioassays, a prerequisite for the bioassay-driven fractionation. Therefore, the ground litter was boiled with deionized water in a pressure cooker for 1 h. Extracts were centrifuged in an Avanti J-25 centrifuge (Beckman, Palo Alto, CA, USA) at 17,000 rpm for 20 min at room temperature. Supernatants were filtered through a paper filter (Whatman 2, Maidstone, UK) and stored at 4°C until use. Due to the formation of emulsions, which prevented the aqueous JLE from being extracted by organic solvents, the JLE was freeze-dried, and the freeze-dried extracts were extracted with ethyl acetate–cyclohexane (1:1, v/v) by microwave-assisted extraction (Soxwave 3.6 apparatus; Prolabo, Briare, France) with the following microwave radiation programme: 2 min at 30 W, 3 min at 45 W, and 10 min at 75 W. The first separation of JLE was performed on a straight phase silica column and resulted in a completely inactive hexane fraction, a very active 10% (v/v) iso-propanol in hexane fraction and a less active 20% (v/v) iso-propanol in hexane fraction. The 10% iso-propanol fraction was used for further fractionation by reversed-phase HPLC (µBondapak C_{18} column; 300 × 3.9 mm, Waters, Eschborn, Germany). The 10% iso-propanol fraction was evaporated to dryness, taken up in acetonitrile and separated by HPLC [Waters In-Line degasser, Waters 510 isocratic pump, Waters 717 plus autosampler, Waters 486 tuneable absorbance Detector (Waters Chromatography, Milford, MA, USA), HP 3395 integrator (Hewlett-Packard, Palo Alto, CA, USA) and a Foxy Jr. fraction collector (ISCO, Lincoln, NE, USA)]. Fractionation was performed during a 60-min run with acetonitrile–water (1:1, v/v) at a flow rate of 1 ml min^{-1} and detection at 254 nm. Thirty individual 1-min fractions and a single 30–60-min sample were collected. These fractions were extracted with dichloromethane, evaporated to dryness and taken up in water for the bioassay. Most of the inhibitory activity was found in the 4–5-min fraction,

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and less activity was found in the following two 1-min fractions. All other fractions did not show activity. The 4–5-min fraction was further fractionated with a less-polar eluent, acetonitrile–water (1:3, v/v). Again 30 1-min fractions were collected and activity was found in fractions 7 and 8.

Structure elucidation

A preparative gas chromatograph (GC) (HP 5890 series II gas chromatograph coupled to a HP 5971 mass selective detector) was used to determine whether the inhibitory activity could be eluted from a GC. Separation was performed on a DB-WAX column (30 m × 0.25 mm × 0.25 µm, J&W Scientific, Fisher Scientific, Nidderau, Germany) with a post-column splitter, which consisted of a borosilicate Y-junction (Restek) with a 0.1 mm i.d. transfer line connecting the column to the detector, and a 0.32 mm i.d. transfer line leading to a heated (250°C) outlet at atmospheric pressure. The lengths of the lines were adjusted for simultaneous detection and collection of eluting compounds. GC parameters were as follows: head pressure: 10 psi; injection: 1 min splitless; injector: 230°C; transfer line: 280°C; oven: 60°C, 1 min, 25°C min^{-1}, 250°C, 21.40 min (total run: 30 min). Several complete (5–30 min) collections of active HPLC fractions were subjected to a seed germination bioassay, which demonstrated that the cue could be chromatographed and collected. HPLC fractions at 7, 8, 9 and 10 min were further examined by GC–MS [3800 GC coupled to a Saturn 2000 ion trap] equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 µm, J&W Scientific, Fisher Scientific, Nidderau, Germany) at a constant helium flow of 1.0 ml min^{-1}, injector at 200°C, transfer line at 280°C, and column temperature programme: 60°C, 4 min, 10°C min^{-1}, 200°C, 20°C min^{-1}, 300°C, 7 min. Only one compound occurred in the active fraction 8, in lower amounts in the less active fraction 9, but not in the inactive fractions 7 or 10. This compound was identified as bornane-2,5-dione (BD) and was chemically synthesized according to the procedure of Bredt (1923).

High-resolution MS (EI) data were obtained with a Hewlett Packard HP6890 (Agilent, Waldbronn, Germany) gas chromatograph interfaced to a MasSpec 2 instrument (Micromass, UK), operated in positive ion mode using 70 eV ionization energy and perfluorokerosine mixture as an internal standard. Separations were performed on a J&W Scientific DB-5 capillary column, 30 m × 0.25 mm, 0.25 µm film thickness using helium (30 ml s^{-1}) as carrier gas.

Determination of terpenoids and MeJA

For the analysis of MeJA, 5 ml JLE were extracted three times with dichloromethane. The combined extracts were reduced to approximately 50 µl under a gentle nitrogen flux and injected into the GC/MS without further purification. No MeJA was detected (limit of detection: 50 pg ml^{-1} extract).

Ten millilitres of JLE were extracted three times each with 5 ml dichloromethane, 0.5 g silica were added to the combined organic layers and the solvent was evaporated to dryness in a rotary evaporator. The sample was placed on a 200 × 1 mm i.d. glass column filled with 7 g activated silica (24 h, 120°C) that was sequentially eluted with 75 ml *n*-hexane (fraction 1), 100 ml 10% 2-propanol in *n*-hexane (fraction 2) for camphor, BD and 1,8-cineole, and 100 ml 20% 2-propanol in *n*-hexane (fraction 3) for β-thujaplicin. Fraction 2 was mixed with 5 µg tetralin as an internal standard and 100 µl isooctane as a stopper before the solvent was removed in a rotary evaporator. Fraction 3 was evaporated to dryness and taken up in 100 µl MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, Macherey-Nagel, Düren, Germany) with 5 µg tetralin. The sample was derivatized to the trimethylsilylether in a closed vial at 70°C for 15 min prior to injection into the GC/MS. Quantitation was performed by external calibration using single standards.

Results

Hormone treatments

Phytohormones, their inhibitors and the germination cues of *N. attenuata* were tested in germination bioassays to examine their role in germination and dormancy (Fig. 1). Since germination rates vary slightly among experiments, only values within an experiment were compared. The two 0.05% ethanol controls confirmed that this solvent, which was used in the paclobutrazol stock solution due to the low solubility of paclobutrazol in water, had no effect on germination, and no significant differences were found in comparison with smoke and water treatments, respectively (treatments 18 versus 1, $P = 0.0695$; and 19 versus 2, $P = 0.2583$). Dormant seeds did not germinate after imbibition (treatment 20, 0%), but they germinated after exposure to smoke (treatment 2, 73%). In contrast, non-dormant seeds germinate after imbibition alone (treatment 1, 100%) (Fig. 1).

JLE clearly inhibited the germination of previously smoke-exposed dormant and untreated non-dormant seeds (treatment 3 versus 2, and 4 versus 1, Ps < 0.001). MeJA and BD (isolated from JLE), applied at 10 µM and 600 µM, respectively, both inhibited previously smoke-treated dormant seeds (treatments 14 and 15 versus 2, $P < 0.001$). The inhibitory effect of JLE, MeJA and BD on radicle emergence could be mimicked by ABA treatment (treatment 10 versus 2, P

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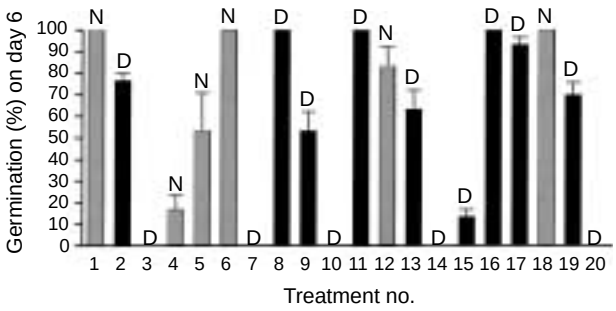

Figure 1. Mean (+ SEM) percentage seed germination on day 6 in the following treatments: (1) water; (2) smoke; (3) smoke + juniper litter extract (JLE); (4) JLE; (5) paclobutrazol; (6) paclobutrazol + GA_3; (7) smoke + paclobutrazol; (8) smoke + paclobutrazol + GA_3; (9) smoke + JLE + GA_3; (10) smoke + ABA; (11) smoke + fluridone; (12) fluridone + JLE; (13) smoke + fluridone + JLE; (14) smoke + MeJA; (15) smoke + BD; (16) smoke + fluridone + MeJA; (17) smoke + fluridone + BD; (18) 0.05% ethanol; (19) smoke + 0.05% ethanol; and (20) water. Seeds of treatments 3, 7, 10, 14 and 20 did not germinate. Treatments using non-dormant seeds are labelled 'N' (grey bars), and with 'D' for treatments using dormant seeds (black bars).

< 0.001). Application of the ABA biosynthesis inhibitor, fluridone, to smoke-treated dormant seeds resulted in 100% germination (treatment 11), whereas only 73% of smoke-treated dormant seeds had germinated on day 6 (treatment 2). Similarly, the inhibitory effect of JLE was completely reversed by fluridone treatment in both dormant and non-dormant seeds (treatment 13 versus 3, and 12 versus 4, *P*s < 0.001). Moreover, fluridone treatment also overcame the inhibitory effect of MeJA and BD (treatment 16 versus 14, and 17 versus 15, respectively, *P*s < 0.001).

Non-dormant seeds, which are likely to have low ABA levels, showed significantly reduced germination if treated with the GA biosynthesis inhibitor, paclobutrazol (treatment 5 versus 1, $P < 0.001$). This germination inhibition induced by paclobutrazol was completely reversed by addition of GA_3 (treatment 6 versus 5, $P < 0.001$). Similarly, paclobutrazol treatment of smoke-treated dormant seeds inhibited germination (treatment 7 versus 2, $P < 0.001$), and germination was restored if exogenous GA_3 was added to the paclobutrazol treatment (treatment 8 versus 7, $P < 0.001$). Moreover, exogenous GA_3 treatment reversed the germination inhibition induced by JLE (treatment 9 versus 3, $P < 0.001$).

ABA levels in seed treatments

To test the hypothesis that environmentally induced increases of endogenous ABA pools were responsible for secondary dormancy, ABA was measured in smoke- and JLE-treated seeds over a 16-h period. ABA increased rapidly from about 0.35 μg g^{-1} in dry seeds to 1.5 μg g^{-1} 1 h after imbibition and then varied over time. JLE-treated seeds always had the highest ABA content (Fig. 2A). However, when ABA was measured in smoke- and fluridone-treated seeds, there were no significant differences in ABA values between the treatments (Fig. 2B). ABA rapidly increased in both treatments from about 0.35 μg g^{-1} in dry seeds to 0.95 μg g^{-1} 1 h after imbibition. After 12 h ABA started to decrease and attained concentrations of 0.2 μg g^{-1} at 72 h (Fig. 2B). This lack of difference in ABA content was surprising, because fluridone-treated seeds germinated early (72 h), even before smoke-treated seeds. ABA varied somewhat between experiments with the same treatments and, for this reason, only values from the same experiment were statistically compared and all figures include only data from one experiment.

To further investigate the discrepancy between the predictions of the hormone-balance model for endogenous ABA contents and seed germination behaviour, ABA was determined in different seed treatments that all had dramatic effects on germination: JLE, fluridone, fluridone + JLE, GA, paclobutrazol, GA + JLE, MeJA and BD. Seeds in all treatments had ABA contents around 1.2 μg g^{-1}, except for the treatments with JLE, which had higher values (Fig. 3). Interestingly, of all the treatments that induced dormancy (solid bars: Fig. 3), only the JLE treatment exhibited elevated ABA. Germination behaviour did not correlate with ABA content. For example, JLE + fluridone treatments germinated the fastest, but ABA was in the same range of JLE-treated seeds that remained dormant (Fig. 3A). These results suggested that the increased ABA content in JLE treatments might not result from increased biosynthesis in the seed, but rather originated from the JLE itself. To test this hypothesis, ABA was measured in seeds treated with MeJA and BD with and without fluridone, JLE, water controls, and in JLE without seeds. ABA content of all seeds was in the range of 0.8 μg g^{-1} except for JLE-treated seeds, which had ABA contents of 1.05 μg g^{-1} (Fig. 3B). This difference in ABA (0.25 μg) corresponded to the amount of ABA measured in JLE: 0.27 μg (1 μM)(Fig. 3B, grey bar). The ratio of every treatment was 1 ml g^{-1} seeds and hence the values can be compared directly.

The presence of ABA in JLE and its inhibitory activity were confirmed by an HPLC fractionation of 20-fold concentrated JLE (Fig. 4A). Twenty 1-min fractions were tested in a seed germination bioassay and only fraction 9 (8–9 min) showed inhibitory activity in the bioassays. Injection of a synthetic ABA standard gave the retention times of 7.20 min (fraction 8) for the biologically inactive *trans-trans* isomer and

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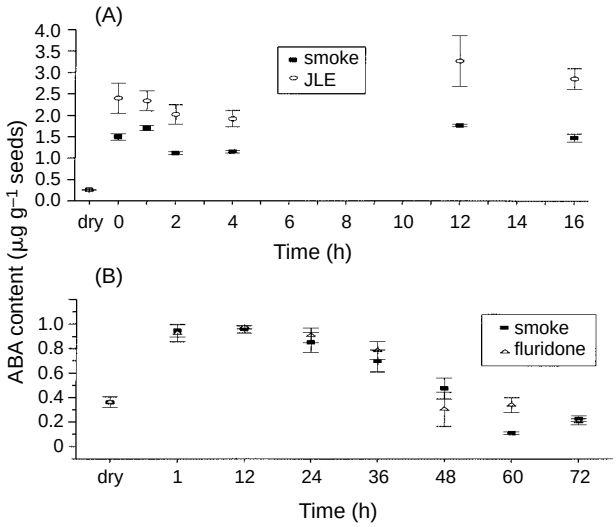

Figure 2. (A) ABA content of dry and hydrated seeds treated with smoke (filled squares) or JLE after smoke treatment (open circles) at time 0 and analysed at various intervals after hydration. (B) ABA content of dry seeds and hydrated seeds treated with smoke (filled squares) or fluridone after smoke treatment (10 µM, open triangles) for different time periods. Seeds treated with fluridone began germinating after 72 h, whereas smoke-treated seeds did not. Values ± SE.

8.73 min for the biologically active *cis-trans* isomer (fraction 9). ABA was identified in the bioactive fraction 9 of JLE by GC/MS. The fractionation was repeated with 3 ml JLE, the quantity of JLE used in the bioassays (1 ml treatment in each of three replicate trials of 20 seeds each). The results suggest that the inhibitory effect of ABA may not account solely for the inhibitory activity of JLE (Fig. 4B).

ABA content of different litter extracts and its effects on germination

After discovering physiologically significant quantities of ABA in JLE, we determined the ABA concentrations in aqueous extracts of the litter of other plant species in the habitat of *N. attenuata*, which were known to inhibit *N. attenuata* seed germination (Preston and Baldwin, 1999), specifically: holly oak, sage brush, cliff rose, bitter brush, sand sage, mormon tea and black brush. The ABA concentrations ranged from 0.6 µg ml^{-1} in holly oak to 0.15 µg ml^{-1} in black brush (Fig. 5) and are in the 1 µM (1 µM = 0.264 µg ml^{-1}) range of ABA's biological activity (Fig. 5 inset). Pure *cis-trans*-ABA applied to previously smoke-treated non-dormant seeds had no effect at concentrations below 0.1 µM, but delayed germination significantly when applied at 1 µM; concentrations of 10 µM and higher inhibited germination completely (Fig. 5 inset).

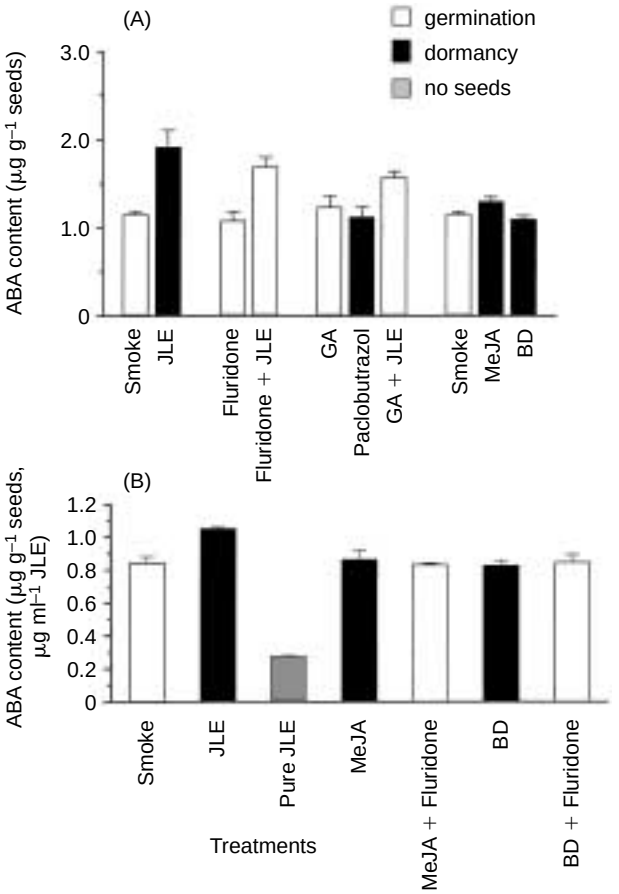

Figure 3. (A) Mean (± SEM) ABA contents of previously smoke-treated dormant *N. attenuata* seeds after 6 h in the following treatments: smoke, JLE (juniper litter extract), fluridone (10 µM), fluridone (10 µM) + JLE, GA_3 (250 µM), paclobutrazol (10 µM), GA_3 (500 µM) + JLE, MeJA (10 µM), and BD (600 µM). Treatments resulting in germination (white bars) or dormancy (black bars) by day 6 are indicated. (B) Mean (± SEM) ABA contents of previously smoke-treated dormant *N. attenuata* seeds after 6 h of the following treatments: smoke, juniper litter extract (JLE), MeJA (10 µM), MeJA + fluridone (10 µM each), BD (600 µM), BD + fluridone (600 µM and 10 µM, respectively) and the ABA content of the JLE (grey bar) used in the treatments. Each treatment consisted of 0.1 ml so that ABA values of seeds and JLE extract are directly comparable. Treatments resulting in germination (white bars) or dormancy (black bars) by day 6 are indicated.

We had operationally defined seed germination as the emergence of the radicle from the seed coat (Fig. 6D), and JLE inhibits germination before the seed coat splits (Fig. 6A). However, closer visual inspection of the seeds revealed that the effect of ABA in arresting seed germination occurred at a stage later in the germination process, after endosperm enlargement and seed-coat rupture (Fig. 6C).

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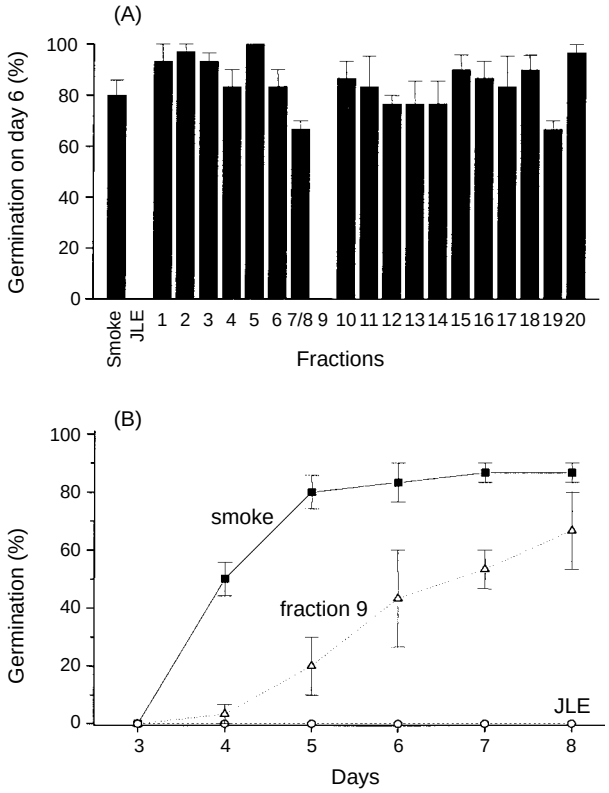

Figure 4. (A) *N. attenuata* seed germination assay of 1-min HPLC fractions of 20× concentrated JLE (juniper litter extract), where fraction number refers to the elution time (in min). Zero germination occurred in JLE and fraction 9 treatments. (B) Mean (± SEM) percentage germination of smoke-treated seeds (filled squares), seeds treated with the 9-min HPLC fraction of JLE at its original concentration (open triangles), and seeds treated with unfractionated JLE (open circles).

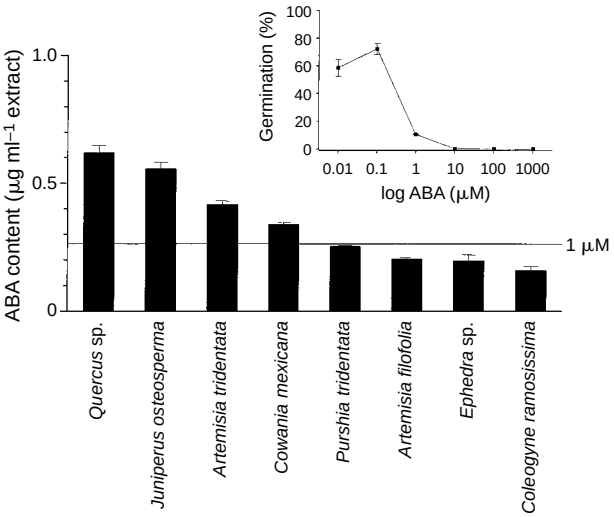

Figure 5. Mean (+ SEM) ABA contents of litter extracts of perennials in the habitat of *N. attenuata*: holly oak (*Quercus* sp.), juniper (*Juniperus osteosperma*), sage brush (*Artemisia tridentata*), cliff rose (*Cowania mexicana* var. *stansburiana*), bitter brush (*Purshia tridentata*), sand sage (*Artemisia filofolia* Torr.), mormon tea (*Ephedra* sp.) and black brush (*Coleogyne ramosissima*). Inset: mean (± SEM) percentage germination on day 6 when *N. attenuata* seeds were treated with ABA at concentrations from 10 nM to 1 mM.

Effect of fluridone on endogenous ABA levels in N. attenuata *seeds*

Fluridone has often been used as an inhibitor of ABA biosynthesis (Le Page-Degivry and Garello, 1992; Jullien and Bouinot, 1997; Yoshioka *et al*., 1998; Garello *et al*., 2000; Grappin *et al*., 2000). Even though fluridone has a strong promoting effect on seed germination (Fig. 1), no significant alterations in endogenous ABA content were observed in seeds treated with fluridone compared to all other treatments (Fig. 2B and 3).

Additional negative germination cues

Since the ABA content of various litter extracts was not sufficient to account for the inhibitory activity of JLE, particularly for the early stages of germination (Fig. 5), we searched for other inhibitory compounds by HPLC fractionation of JLE. MeJA, a potent germination inhibitor from *Artemisia tridentata* spp. *tridentata*, another dominant woody species in the habitat of *N. attenuata*, also inhibited seed germination of *N. attenuata* seeds at concentrations as low as 10 µM (C.A. Preston, unpublished results). However, we could not detect any MeJA in JLE (data not shown). We isolated an active compound from JLE by bioassay-driven fractionation, and accurate mass measurement gave the elemental composition $C_{10}H_{14}O_2$ (measured mass: 166.099396 Da; calculated mass: 166.099380 Da). We elucidated its structure by mass spectra comparison as bornane-2,5-dione (1,7,7-trimethylbicyclo[2.2.1]hepta-2,5-dione according to IUPAC). This structure was confirmed by the identical retention times and mass spectra of BD isolated from JLE and chemically synthesized BD. In bioassays, 300 µM solutions of BD were necessary to produce the same inhibitory activity as JLE. However, in JLE, BD concentrations were 2 µM. BD applied at this concentration to *N. attenuata* seeds did not inhibit germination, but rather appeared to stimulate germination (Fig. 7A). However, when 2 µM BD was applied together with 1 µM ABA, the combined treatment delayed seed germination significantly in comparison to seeds treated with 1 µM ABA alone (data not shown).

In addition to BD, we tested three additional

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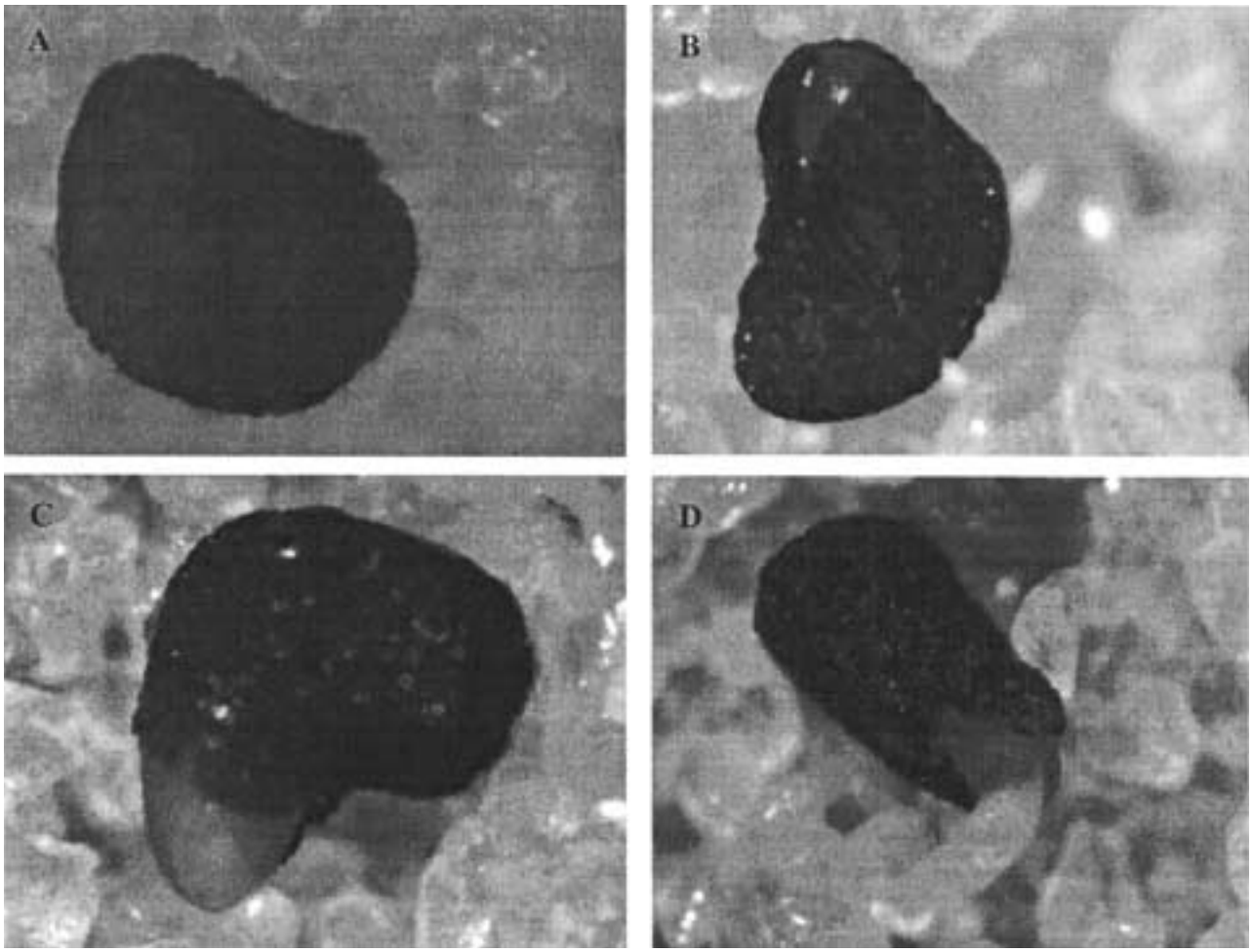

Figure 6. (A) *N. attenuata* seed after imbibition and treatment with 20 mM BD; (B) seed coat rupture shortly before radicle emergence (2 mM BD + 1 μM ABA treatment); (C) ABA-treated seed with emerged endosperm; (D) germinated seed with radicle fully emerged. Seeds were photographed 8 d after imbibition.

terpenoids found in *J. osteosperma* litter: camphor, the major terpenoid found in JLE and putative precursor of BD; 1,8-cineole, a known seed germination inhibitor (Romagni *et al*., 2000); and β-thujaplicin, the main tropolone of *J. osteosperma* (Hegenauer, 1973). These compounds showed inhibitory activity at the following concentrations: camphor, 6 mM; 1,8-cineole, 10 mM; and β-thujaplicin, 2.2 mM. However, the concentrations of these compounds in JLE were determined as follows: camphor, 800 μM; 1,8-cineole, 2 μM; and β-thujaplicin, 2 μM. When applied individually to seeds in the concentrations as determined in JLE, the compounds did not inhibit germination, except for camphor, which gave a slight germination delay (Fig. 7A). However, the combination of ABA, camphor, BD, 1,8-cineole and β-thujaplicin in the concentrations found in JLE mimicked the inhibitory effect of JLE (Fig. 7A), not only in terms of delay of germination, but also in seed behaviour: seeds treated with the five compounds in the concentrations found in JLE, showed no seed coat rupture and no endosperm emergence (Fig. 6A) as shown by seeds that were treated only with ABA (Fig. 6C).

Discussion

Ecological relevance of the bioassays

N. attenuata uses both positive and negative chemical signals from its environment to time its germination with the sporadic occurrence of favourable habitats for growth and reproduction. These chemical signals may alter endogenous ABA and GA levels, which, in turn, regulate secondary dormancy and prevent germination when conditions are favourable for

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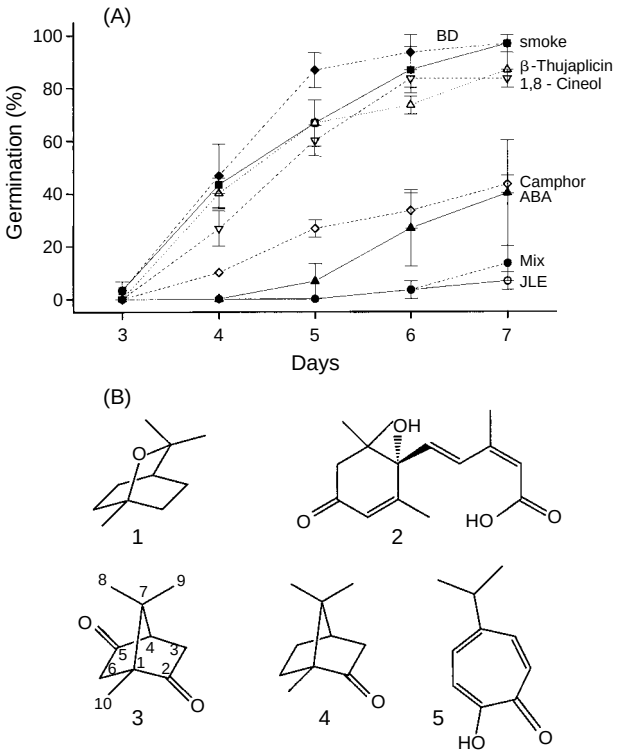

Figure 7. (A) Mean (± SEM) percentage germination of seeds treated with single and combined juniper litter extract (JLE) constituents at concentrations found in JLE: 1 μM ABA (filled upward triangles), 2 μM BD (filled diamonds), 2 μM 1,8-cineol (open downward triangles), 2 μM β-thujaplicin (open upward triangles), 800 μM camphor (open diamonds), combination of BD, 1,8-cineol, β-thujaplicin, camphor, ABA at the same concentrations as single treatments (Mix; filled circles), and smoke (filled squares) and JLE (open circles) controls. (B) Chemical structures of : 1, 1,8-cineole; 2, ABA; 3, bornane-2,5-dione; 4, camphor; and 5, β-thujaplicin.

germination, but unfavourable for growth and reproduction. Our 6-day bioassays were designed to optimize germination conditions and do not represent the duration of germination delays in the natural habitat of *N. attenuata*. In seed-bank experiments, conducted in a greenhouse with unlimited water and soil from the habitat of *N. attenuata* in Utah, USA, dormant *N. attenuata* seeds did not germinate for 14 d and remained dormant for more than a month if exposed to JLE (Preston and Baldwin, 1999). Preston and Baldwin (1999) also showed that JLE applied to a *N. attenuata* seed bank had the same effect as a 0.3 cm thick juniper litter A_0 layer. Hence aqueous extracts of litter conservatively mimic the natural exposure of seeds in the seed bank to constituents leached from the A_0 horizon, a soil horizon that varies in thickness from 0 to 20 cm. Only aqueous litter extracts were used in bioassays, and other extraction procedures were used exclusively for compound isolation and structure elucidation. Since *N. attenuata* seeds also respond to general germination cues such as photoperiod, temperature and moisture, and since the periods of favourable germination conditions in the Great Basin desert, the natural habitat of *N. attenuata*, are restricted to a few short intervals each spring, we conclude that the germination delays observed in our bioassays likely reflect long-term seed-bank behaviour in nature.

Hormone and inhibitor bioassays and hormone balance theory

We tested several environmental signal compounds that positively (smoke) and negatively (BD, MeJA and JLE) regulate germination in nature, hormones (GA_3, ABA) and their biosynthesis inhibitors (fluridone, paclobutrazol), to understand whether the regulation of germination by these environmental signals is consistent with the hormone balance hypothesis (Bewley and Black, 1994; Koornneef *et al*., 1998). Results from physiological studies with biosynthetic inhibitors of, and mutants defective in, ABA and GA biosynthesis are largely consistent with the predictions of the model. Measures of endogenous ABA flux, while few, are consistent with the model (Toyomasu *et al*., 1994; Grappin *et al*., 2000), although exceptions are known (Iglesias and Babiano, 1997). Over- and antisense-expression of the ABA biosynthetic gene, *ABA2*, in *Nicotiana plumbaginifolia* produces plants with seed phenotypes that are consistent with the theory that ABA plays an important role in maintaining dormancy (Frey *et al*., 1999). Moreover, general environmental cues, such as nitrate, temperature, moisture, oxygen availability and light were hypothesized to increase endogenous GA pools (Hilhorst *et al*., 1986), and exogenous GA application overrides the requirement for environmental cues (Bewley and Black, 1994). Finally, molecular analysis of GA-dependent regulatory elements has found many that respond strongly to ABA, suggesting that promotor sensitivity may provide a molecular mechanism for the hormone balance model (Ritchie and Gilroy, 1998). In summary, while the hormone balance model has received strong experimental support for mediating germination control in response to general environmental signals in model plant systems, its relevance in regulating germination in a native plant in response to specific environmental signals has not been established.

The results from our hormone bioassays are consistent with the hormone-balance model: secondary dormancy is induced by environmental signals that are thought to stimulate ABA biosynthesis, whereas germination is stimulated by

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signals that are thought to stimulate GA biosynthesis. MeJA, BD and JLE induce secondary dormancy comparable to that induced by an exogenous (1 μM) ABA treatment, and application of the ABA biosynthesis inhibitor, fluridone, reverses the inhibitory effects. These results are consistent with the findings of Grappin *et al*. (2000) with haplodiploidized *N. plumbaginifolia* seeds. However, elevated ABA levels in inhibitory treatments, when observed, resulted from exogenous rather than endogenous sources.

Although the precise chemical compounds in wood smoke (van de Venter and Esterhuizen, 1988; Brown, 1993; Baldwin *et al*., 1994; Baxter and van Staden, 1994; Pierce *et al*., 1995; Keeley and Fotheringham, 1997) and charred wood (Went *et al*., 1952; Stone and Juhren, 1953; Quick and Quick, 1961; Christensen and Muller, 1975a, b; Keeley *et al*., 1985; Keeley and Pizzorno, 1986; Emery, 1992) that act as positive germination signals are still unknown, our results suggest that these signals induce GA biosynthesis. Dormancy of all seeds can be broken by application of exogenous GA_3. Smoke also breaks dormancy, and the application of the GA_3 biosynthesis inhibitor, paclobutrazol, inhibits germination in non-dormant seeds as well as in previously smoke-treated dormant seeds (Fig. 1).

Effects of biosynthesis inhibitors on hormone levels

In contrast to our results from the bioassays with hormones and their putative biosynthetic inhibitors, our measures of endogenous ABA levels were not consistent with the hormone balance theory; germination behaviour did not correlate with endogenous ABA concentrations (Fig. 3A). The germination inhibitors, MeJA and BD, did not increase ABA content, and the increases in JLE-treated seeds could be attributed to ABA in the JLE, and not to endogenous ABA biosynthesis. Fluridone, which is reported to inhibit ABA biosynthesis from zeaxanthin pools downstream of carotenoid biosynthesis (Cutler and Krochko, 1999), had no effect on ABA content in treated seeds (Fig. 3). From this, we conclude that induced dormancy of *N. attenuata* seeds depends neither on the *de novo* ABA biosynthesis nor its release from storage pools, for example ABA-glycosides. However, fluridone-treated seeds germinate much faster than smoke-treated seeds, which clearly indicates that fluridone has a strong physiological effect on seeds other than interference with ABA biosynthesis.

The results from bioassays with the GA biosynthesis inhibitor, paclobutrazol, should also be interpreted with caution. Paclobutrazol and other triazole inhibitors bind to P450 monooxygenases and inhibit the oxidation of *ent*-kaurene to GA_{12}-aldehyde (Hedden and Kamiya, 1997). However, the triazole inhibitors also bind to the monooxygenases that hydroxylate ABA to form 8′-hydroxy-ABA, the first intermediate in ABA catabolism (Grossmann, 1990). Hence paclobutrazol may not only block GA biosynthesis, but may also inhibit ABA degradation. Clearly inhibitor studies should be accompanied by careful quantification of endogenous hormone flux (Grappin *et al*., 2000).

Unless seeds are altering their sensitivity to endogenous ABA content, or unless the physiologically relevant changes were below our detection limits, the endogenous ABA content measured in seeds does not support the hormone balance theory. Egerton-Warburton and Ghisalberti (2001) recently showed that endogenous sucrose linoleate content of fire-dependent *Emmenanthe penduliflora* and fire-independent *Phacelia tanacetifolia* seeds correlated with dormancy. These sucrose esters may reflect an ABA-independent signalling of environmentally induced dormancy.

Effect of environmental chemicals on dormancy of N. attenuata *seeds*

Even if the elicitation of endogenous ABA by environmental signals is not strongly correlated with dormancy in this species, exogenous ABA that leaches from leaf litter plays a significant role in regulating dormancy. However, ABA alone can not account for the dormancy-inducing activity of JLE (Fig. 4B), but when ABA is combined with four terpenes found in JLE (which alone are also not inhibitory at the concentrations found in JLE), the complete dormancy-inducing activity is mimicked (Fig. 7A). Alone, ABA inhibits seed germination after the endosperm has ruptured the seed coat (Fig. 6C), similar to what was initially observed in *Chenopodium album* (Karssen, 1976). This is probably due to inhibition of β-1,3-glucanases that prevents the radicle from penetrating the endosperm, as has been shown in *N. tabacum* seeds (Leubner-Metzger *et al*., 1995, Leubner-Metzger and Meins, 2000). Simultaneous treatment of *N. tabacum* seeds with 10 μM GA_4 and 10 μM ABA does not result in radicle penetration of the endosperm (Leubner-Metzger *et al*., 1996), but in seed coat rupture and endosperm emergence. This suggests that ABA does not inhibit the initial embryo growth inside the endosperm, but the subsequent events leading to penetration of the endosperm by the radicle. However, arresting germination after seed coat rupture is not an ecologically viable mechanism for long-term dormancy, for such seeds would not survive drought and pathogen attack. JLE-treated seeds clearly remain intact for a long time (Fig. 6A) and germinate rapidly once the seed coat has opened (Fig. 6B).

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BD was discovered by our bioassay-driven fractionation and found to be a potent inhibitor of germination, which unlike ABA, arrests germination before the rupture of the seed coat. However, for complete germination inhibition similar to that found after JLE exposure, concentrations of 300 µM were required, which is approximately 60× more than the concentration of BD in JLE (2 µM). A combined treatment of 2 µM BD and 1 µM ABA inhibited germination more than either compound alone, but still could only account for approximately half of the inhibitory activity of JLE (data not shown). However, when the next three most abundant terpenes in JLE (1,8-cineole, β-thujaplicin and camphor) were added at the concentrations found in JLE with BD and ABA at their native concentrations, the mixture completely mimicked the inhibitory activity of JLE (Fig. 7A). The fact that *N. attenuata* seeds do not respond to one single compound, but to a mixture of several terpenoids, suggests a general means of detecting intact vegetation. The dominant vegetation of most desert habitats characteristically produces an abundance of secondary metabolites, and hence monitoring the presence of several such metabolites would provide a reliable signal of intact vegetation. How these four terpenes synergize the effects of ABA in arresting seed germination remains to be explored. These results also highlight an important limitation of bioassay-driven fractionations: the assumption of non-additive effects. The inhibitory effect of litter extracts is clearly an additive effect of different compounds which, after separation and fractionation, are not sufficient to account for the inhibitory activity of the intact extract.

In summary, we demonstrate for the first time that ABA is present in litter in physiologically significant quantities and that ABA functions in combination with four other terpenes commonly found in leaf litter (camphor, BD, 1,8-cineole, β-thujaplicin) to establish durable secondary dormancy in the post-fire annual, *N. attenuata*. As such, these results provide a chemical ecological context for the physiological regulation of one of the most ecologically profound physiological capabilities of plants: to escape in time and select habitats by cueing germination from long-lived seed banks with ecologically informative environmental signals.

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