South African Journal of Botany 2001, 67: 636–640
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SOUTH AFRICAN JOURNAL
OF BOTANY
ISSN 0254-6299

Does smoke substitute for red light in the germination of light-sensitive lettuce seeds by affecting gibberellin metabolism?

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Received 9 January 2001, accepted in revised form 8 March 2001

Plant-derived smoke extracts are known to stimulate seed germination in a number of species, but the active compound(s) and mechanism remain unknown. The key to understanding the process underlying the induction of germination lies in the characterisation of the relationship between temporal patterns of physiological and developmental changes. Germination time-course studies revealed that the onset of germination in smoke-treated Grand Rapids lettuce seeds was delayed compared to seeds treated with red light or gibberellic acid. Both red light and smoke-induced germination can be reversed by far-red light, but the escape time is shorter for smoke-treated seeds. Paclobutrazol and AMO 1618, inhibitors of gibberellin biosynthesis, decreased the germination of smoke-treated seeds significantly at concentrations of 0.1 and 1mM, but did not substantially diminish germination of red light-treated seeds. Levels of endogenous gibberellins, estimated using the dwarf rice microdrop assay, were significantly higher in red light-treated seeds than in controls, and peaked after 100% germination was obtained. In contrast, the level of gibberellins in smoke-treated seeds remained low initially, but rose concurrent with the start of germination. The peak level of gibberellins in smoke-treated seeds was markedly higher than in red light-treated seeds. Gibberellin synthesis is thus considered to be a likely component of the mechanism underlying smoke-induced germination.

Introduction

A number of recent studies have detailed the ongoing search for clues to the physiology of the induction of germination by plant-derived smoke (Brown and Van Staden 1997, Van Staden *et al.* 2000). In its ability to stimulate seed germination (De Lange and Boucher 1990), somatic embryogenesis (Senaratna *et al.* 1999), flowering (Keeley 1993) and rooting (Taylor and Van Staden 1996), the active principle(s) in smoke solutions appears to behave in a similar manner to plant growth regulators (Senaratna *et al.* 1999). Van Staden *et al.* (1995) and Thomas and Van Staden (1995) suggested that the active component of smoke may act via the modulation of sensitivity to endogenous growth regulators, the activation of enzymes or the modification of receptor molecules. In contrast, some workers have suggested that the basis of the promotion of seed germination by smoke is a function of the ability of smoke solutions to break the integrity of the seed coat or endosperm membrane (Keeley and Fotheringham 1998, Egerton-Warburton 1998). This, in conjunction with the observed interactions of smoke and phytohormones (Van Staden *et al.* 1995), suggests that smoke may act via gibberellins (GAs) which demonstrably cancel the suppression of germination by the fruit wall (Toyomasu *et al.* 1993) and induce structural modifications of the endosperm prior to radicle emergence (Psaras and Georghiou 1983).

At least part of the system controlling dormancy break in photoblastic seeds is under phytochrome control (Bewley and Black 1982, Thomas 1992). Both smoke and gibberellins substitute, to varying degrees, for the red light requirement of *Lactuca sativa* L. cv. Grand Rapids seeds (De Greef and Fredricq 1983, Van Staden *et al.* 1995), suggesting that the production of bioactive gibberellins may be the primary physiological response of lettuce seeds to red light and smoke. Red light has been shown to induce a rapid accumulation of GAs (Inoue 1990) but the relevance of such increases *in vivo* has been subject to some debate (Inoue 1990). Similarly, findings that red light induces the production of mRNA for GA 3-hydroxlase (Toyomasu *et al.* 1998), the translation product of which controls the conversion of GA_{20} to the bioactive GA_1 (Yamaguchi and Kamiya 2000) are undermined by the existence of differential mRNA stability gradients and post-transcriptional regulation mechanisms which imply that temporal patterns of mRNA production may not correlate with the accumulation of biologically active GAs. As a result, it may well be preferable to examine the temporal pattern of GA *activity* in germinating seeds in order

to clarify the interactions of germination cues and phytohormones in the induction of germination.

In this study, an attempt to characterise the interaction between smoke, red light and endogenous gibberellins was made via an examination of the nature and sequence of the changes which these cues elicit and of their dependence on various conditions.

Materials and Methods

All manipulations of seeds were carried out in the dark or, where necessary, under a green 'safelight' (Drewes *et al.* 1995). Smoke solutions obtained by dilution of a smoke solution produced in 1994 from burnt *Themeda triandra* material as outlined by Baxter *et al.* (1994), which had been stored at 12°C, were used throughout. A 1:1 000 dilution of smoke was established to be optimal for germination of Grand Rapids lettuce seeds. The pH of a 1:1 000 dilution of smoke was 4.7. Mature achenes of *Lactuca sativa* L. cv. Grand Rapids were supplied by Peto Seed, Saticoy, USA and stored in the dark at 4°C.

Germination time-course analysis

An examination of the timing of the germination responses was conducted. Each treatment utilised 4 replicates of 25 seeds. All seeds, apart from smoke-treated seeds, were imbibed for 2h in 2ml distilled water in the dark at 25°C in 65mm plastic Petri dishes fitted with two sheets of 70mm Whatman No. 1 filter paper (Van Staden *et al.* 1995). Control seeds were left to germinate in the dark without any further treatment, while a set of seeds was treated with 10min exposures to red light of 660nm (PAR value = 26.4; $1.8 mol^{-2} s^{-1}$) as described by Van Staden *et al.* (1995). One set of seeds was transferred to new Petri dishes containing 2ml of 1mM GA_3 solution (Aldrich, USA). Smoke treatments involved exposing unimbibed seeds to 2ml of 1:1 000 aqueous dilutions of smoke for the duration of the experiment. All seeds were left to germinate in the dark at 25°C. Germinated seeds were removed and counted under safelight conditions every 2h for a total of 24h, and cumulative percentages of germination were plotted against time.

Far-red light escape times

The escape times from far-red light reversal of induced germination were determined for seeds treated with red light or smoke. Five replicates of 25 seeds were placed in plastic Petri dishes with 2ml of either distilled water or a 1:1 000 dilution of the smoke extract. Ten min after imbibition, seeds imbibed in water were treated with 10min red light. After 2, 3, 4, 5 and 6h of imbibition, the seeds were rinsed and transferred to new Petri dishes containing 2ml water and then exposed to 10min far-red light ($5.6 mol^{-2} s^{-1}$). Apart from the far-red light exposure, controls were similarly treated. Percentage germination was determined after 24h incubation at 25°C.

Effect of paclobutrazol and AMO 1618 on germination

Four replicates of 25 seeds, handled according to the methods outlined above, were treated with 0.1mM or 1mM solutions of paclobutrazol (ICI Agrochemicals, SA) or AMO 1618 (Calbiochem, USA) concurrently with treatments of red light, 1mM GA_3, smoke or water. Seeds were incubated in the dark at 25°C for 24h prior to determination of the numbers of germinated seeds.

Endogenous gibberellin activity

Seeds (1g) were imbibed in the dark in 12.5cm Petri dishes on two sheets of Whatman No. 1 filter paper moistened with 8ml distilled water or 1:1 000 smoke solution. After 1h, subsets of seeds imbibed in water were treated with a 10min red light pulse. Control seeds were harvested for GA determination after 0, 10, 12, 14, 16 and 20h, smoke-treated seeds after 6, 10, 14, 16, 18 and 20h and red light treated seeds 4, 6, 10, 12, 14, 16, 18 and 20h after treatment.

GAs were extracted according to a modified protocol based on those outlined by Endo *et al.* (1989) and Nakayama *et al.* (1989). Seeds were flash-frozen in liquid nitrogen and ground under safelight conditions with a mortar and pestle prior to extraction with 100ml methanol for 24h. Extracts were reduced to aqueous residues *in vacuo*, diluted with distilled water to 100ml and adjusted to pH 3 with 1N HCl. These were partitioned three times against aqueous ethyl acetate, and the combined ethyl acetate fractions partitioned a further three times against 0.1M $NaHCO_3$ (100ml). Combined aqueous fractions were adjusted to pH 3 with 10N HCl prior to triplicate extraction with ethyl acetate (100ml). The ethyl acetate was dried over anhydrous sodium sulfate and reduced to dryness *in vacuo* to yield an acidic ethyl acetate fraction (Endo *et al.* 1989).

Ethyl acetate fractions were resuspended in 2ml 80 % methanol, loaded into a Sep-Pak (ODS) cartridge and eluted with 6ml 80 % aqueous methanol (Nakayama *et al.* 1989). The eluates were reduced to dryness, and the resultant residues resuspended in 200µl methanol. Each fraction was assayed using the dwarf rice (*Oryza sativa* L. cv. Tanginbozu) microdrop method (Murakami 1970) at 30°C under continuous light. Each experiment consisted of 2 extractions and 5 bioassays involving 20 rice seedlings each. A standard curve was established for GA_3 and the level of gibberellins was expressed as GA_3 equivalents. The results of 2 replicate experiments were subjected to a multiple range test to establish significance.

Results

Germination time course

Red light treatments induced the most rapid germination response, with germination beginning after 6h. After 12h, 98% of red light-treated seeds had germinated. GA_3-treated seeds responded after 8h, with 88% germination recorded after 14h, 95% after 16h, and 100% only after 24h (Figure 1). A steady increase in the percentage germination of smoke-treated seeds from 12h onward was observed,

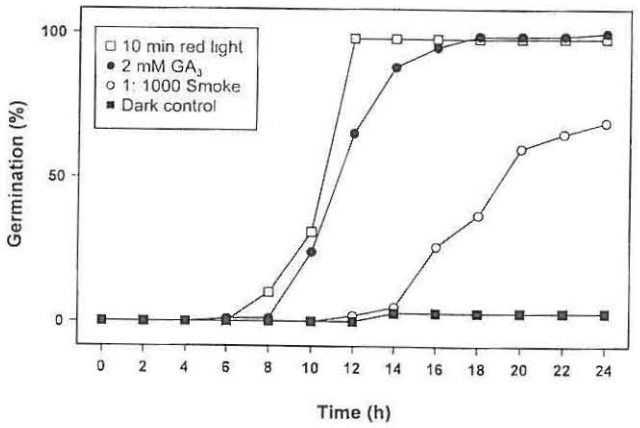

Figure 1: Germination time-course of Grand Rapids lettuce seeds exposed to red light, gibberellic acid and smoke

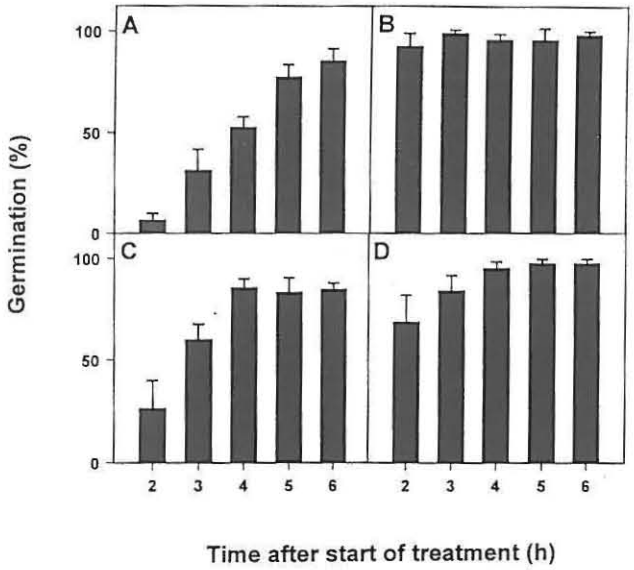

Figure 2: The escape times of Grand Rapids lettuce seeds. Figures reflect the percentage germination of lettuce seeds treated with (A) 10min red light followed by 10min far-red light, (B) 10min red light, (C) 1:1 000 smoke solution followed by 10min far-red light, (D) 1:1 000 smoke solution. Bars indicate maximum standard deviation, n=5

although only 69% of seeds had germinated after 24h. Control treatments elicited only 3% germination over a 24h period (Figure 1).

Far-red light treatments

Results clearly demonstrated the reversal of both red light and smoke-induced germination by far-red light (Figure 2A–D). Far-red light inhibited germination of seeds imbibed for 3h or less after red light treatment to a considerable extent below red light controls, but this reversion was nullified with increasing imbibition times (Figure 2A and B). Five hours after the red light treatment, far-red light could no longer inhibit germination entirely. In the case of the smoke-treated seeds the far-red light reversal was largely overcome within 4h after treatment (Figure 2C and D).

GA synthesis inhibitors

The GA synthesis inhibitor, paclobutrazol, applied at concentrations of 0.1mM reduced the germination of smoke-treated lettuce seeds by 54% and at concentrations of 1mM, by 63% compared to controls lacking the inhibitors (Figure 3A). However, red light-treated seeds were not subject to as great a reduction in the germination levels, displaying reduction of germination percentages by only 8% and 36% by 0.1mM and 1mM solutions of paclobutrazol, respectively. These trends were reflected to a much lesser extent in seeds treated with smoke and AMO 1618 (Figure 3B). Germination of GA_3-treated seeds was not significantly altered by paclobutrazol or AMO 1618 treatment. Seeds treated with smoke and inhibitors were still viable after 24h (results not shown).

Endogenous GA activity

A small peak (0.075ng/g seeds) in putative GA activity was observed in control seeds after 18h of imbibition in the dark (Figure 4A). The concentration of endogenous GAs in red light-treated seeds increased to 0.18ng/g dry seeds 4h after red light treatment. A maximum concentration of 0.9ng/g was recorded after 14h, 2h after 100% germination had been attained (Figure 4B). The GA concentration in smoke-treated seeds attained a maximum level of 7.4ng/g, but in contrast to other treatments, this peak occurred before the onset of germination (Figure 4C).

Discussion

The key to understanding the underlying mechanisms of germination lies in the characterisation of the relationship between temporal patterns of physiological and developmental changes. In accordance with this ideal, the results presented above provide insights into the features of the interaction between red light, smoke and GAs in the induction of germination of light-sensitive Grand Rapids lettuce seeds. The explicit anomaly of the timing of smoke-induced germination as compared with red light (Figure 1) implies that the physiological modes of action of the two cues differ in some respect.

The nature of the discrepancies in the responsiveness of seeds treated with smoke and red light to far-red light may indicate whether the active component in smoke acts via the phytochrome system. The results provide a clear indication that smoke-promoted germination can be effectively prevented by far-red light treatment (Figure 2), suggesting that at least part of the mechanism of smoke action is phytochrome-dependent. This finding is compatible with the proposed interaction of smoke with light and heat in the breaking of dormancy (Van Staden *et al.* 2000). However, it should be stressed that the reversal of smoke-induced germination by far-red light does not necessarily indicate that smoke acts *exclusively* through the phytochrome system, since a number of physiological mechanisms may be induced by the wide variety of components in plant-derived

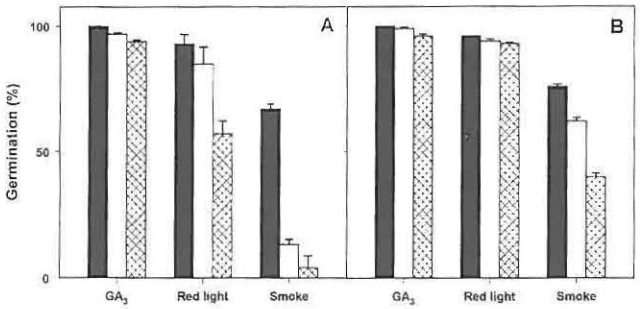

Figure 3: Effect of the gibberellin biosynthesis inhibitors (A) paclobutrazol and (B) AMO 1618 on induced germination of lettuce seeds treated with 1mM GA3, 10min red light or 1:1 000 dilution of smoke. Inhibitor concentrations: (■) 0mM, (□) 0.1mM and (☒) 1mM. Bars indicate maximum standard deviation, n=4

smoke (Maga 1988).

The GA synthesis inhibitors paclobutrazol and AMO 1618 interfere with the oxidative reactions involved in ent-kaurene and geranylgeranyl pyrophosphate conversion, respectively (Rademacher 1990). The pronounced effect of these compounds on germination of smoke-treated seeds, and its alleviation by exogenous GAs, indicates the dependence of the action of smoke on GA synthesis (Figure 3A and B), while the limited effect of these inhibitors on red light-stimulated germination implies that *de novo* GA synthesis is not central to the phytochrome-dependent mechanism of induced germination (Figure 3A and B), a result that is consistent with the evidence presented by Toyomasu *et al.* (1998) detailing the conversion of GAs during the onset of germination (Yamaguchi and Kamiya 2000). The rapid conversion of GAs in red light-treated seeds may also explain the rapidity of the action of this cue relative to the smoke cue. However, these findings do not conform to those of Inoue (1990) obtained in experiments utilising decoated lettuce seeds. It could be argued that this is due to the failure of the inhibitors to be taken up by the intact seed, and that uptake of inhibitors by smoke-treated seeds is a result of disruption of membrane systems (Keeley and Fotheringham 1998, Egerton-Warburton 1998). This in turn attests to the fact that smoke may influence the permeability of the endosperm via GAs (Psaras and Georghiou 1983).

Certainly, the accumulation of putative bioactive GAs provides striking evidence of the involvement of GAs in smoke-induced germination. At least partial causality for germination can be ascribed to this accumulation since the GA response precedes the onset of radicle emergence and since the levels of active GA attained are particularly high (Figure 4C). In contrast, the peak in GA bioactivity in red light-treated seeds was low and occurred co-incident with radicle emergence (Figure 4B). However, although it is conceded that the dwarf rice bioassay is not a definitive quantification of GAs, the increase in GA activity 4h after red light treatment is consistent with the data presented by Köhler (1966) and Inoue (1990) detailing a rapid increase in the levels of GAs following exposure to red light. It is thought that the peak in GA activity obtained for red light in this study reflects the involvement of these growth regulators in cell

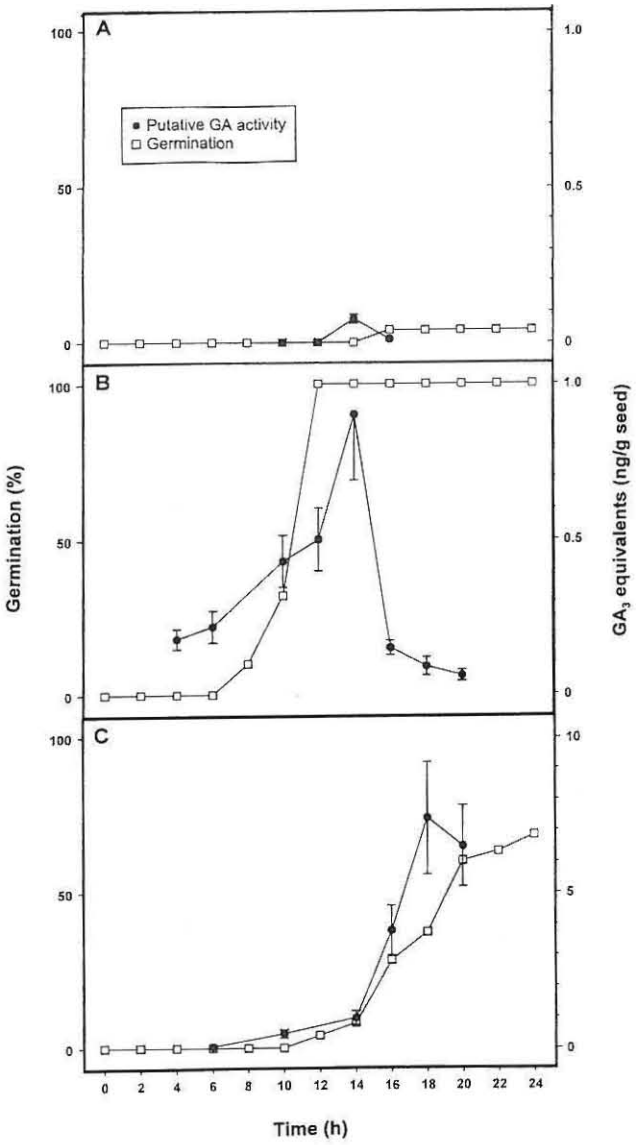

Figure 4: Levels of putative endogenous gibberellins in relation to germination of (A) control, (B) red light and (C) smoke-treated Grand Rapids lettuce seeds

elongation, which is essentially a post-germinative process (Bewley 1997).

The fact that putative GA activity in smoke-treated seeds was more than 8 times that in red light-treated seeds hints at a possible use for the active component(s) of smoke as a powerful plant growth regulator. It is probable that such a GA synthesis promoter may find widespread use in the control of innumerable processes. Indeed, evidence demonstrating the positive effects of smoke on flowering, rooting and embryogenesis has been presented (Keeley 1993, Taylor and Van Staden 1996, Senaratna *et al.* 1999) and commercially available smoke solutions are used as horticultural primers to ensure seed germination (Brown and Van Staden 1997, Van Staden *et al.* 2000). However, prior characterisation of the active component is essential.

Collectively these results provide evidence for the postu-

lated growth regulator-like nature of the active component in smoke and indicate that this principle acts, at least in part, via gibberellin synthesis, and thus, indirectly via modulation of the endosperm structure, and that this mechanism of germination differs distinctly from that under the control of red light.

Acknowledgements — The financial support of the University of Natal Research Fund and the National Research Foundation (Pretoria) is gratefully acknowledged.

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Edited by TH Thomas