Isolation of the major germination cue from plant-derived smoke

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Received 7 September 2004, accepted in revised form 21 September 2004

A highly active, heat stable, long lasting compound, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (1), that stimulates seed germination was isolated from plant-derived smoke water using bioactivity-guided fractionation. The identification of this natural molecule, the major germination cue from smoke, should now rapidly lead to a more comprehensive understanding of the role of smoke as a promoter of seed germination.

Introduction

Fire is well established as a major evolutionary driving force in seed biology and is clearly regulated and mediated via both physical and chemical cues involved in the germination process (Van Staden *et al.* 2000). The occurrence of wild fires during dry seasons in many parts of the world results in large volumes of volatile compounds being released into the atmosphere (Figure 1). These smoke clouds contain particulate matter, water vapour, gases and pyrolysis products which can drift over vast distances and may potentially impact on plants outside the immediate vicinity of the fire, either directly as aerosol smoke, or via aqueous media, be it moist soil or water runoff into streams, lakes and impoundments. For this reason, the burning of plant material is banned in many countries. Figure 1 shows the result of a grassland fire in a *Themeda triandra* climax community in KwaZulu-Natal, South Africa.

The fascinating discovery that cold aerosol smoke and aqueous smoke solutions stimulate seed germination in nature (De Lange and Boucher 1990) led to many attempts to understand the phenomenon of smoke-stimulated seed germination (Brown 1993, Baldwin *et al.* 1994, Baxter *et al.* 1994, Dixon and Roche 1995, Pierce *et al.* 1995, Roche *et al.* 1997a, 1997b). The production of volatile germination cues associated with natural fires has captured the imagination of plant scientists from a wide range of interdisciplinary fields and impacts heavily on our understanding of fire as a driving force in evolution. In particular, it highlights the fact that we must think well beyond the physical aspects of fire on plants in natural habitats. The smoke literature has been comprehensively reviewed (Brown and Van Staden 1997, Van Staden *et al.* 2000) and the potential of smoke in seed technology was recently highlighted (Light and Van Staden 2004). The topic received special attention in an editorial 'The Hot and the Classic' in Plant Physiology (Minorsky 2002).

The acrid smoke produced by combusting dry *T. triandra* in a controlled system (Figure 2) and bubbling it through water to produce smoke-saturated water, was just as active as that produced by burning fynbos vegetation (Baxter *et al.* 1994). The active principal(s) had the same chromatographic properties as did other material of plant origin such as cellulose, agar and food-flavouring extracts (Jäger *et al.* 1996a, 1996b). The slow combustion of different types of vegetation and organic products at temperatures between 180°C and 200°C produces heat stable, long lasting, water soluble compound(s) which stimulate seed germination (Jäger *et al.* 1996a, Brown and Van Staden 1997, Van Staden *et al.* 2000). The hitherto unknown active germination stimulant(s) is extremely active, remains active in soils for long periods (Roche *et al.* 1997b), and apparently adsorbs to charred wood from which it can be released (Keeley *et al.* 1985). Smoke-saturated water retains its activity after autoclaving and long periods of storage (Van Staden *et al.* 2000). Physiologically it sensitises seeds to many of the known plant hormones involved in seed germination (Strydom *et al.* 1996). Both wild (Pierce *et al.* 1995, Brown and Botha 2002) and cultivated plants (Drewes *et al.* 1995, Thomas and Van Staden 1995) respond to smoke and smoke solutions. It is of particular importance that the stimulant(s) substitutes for red light as a germination trigger in light-sensitive Grand Rapids lettuce seed. This resulted in this species being used as a rapid and efficient means to detect the biologically active

Figure 1: A grassland fire in the KwaZulu-Natal midlands, South Africa

Figure 2: Apparatus for producing smoke-saturated water

compound(s) during attempts at its isolation from smoke solutions (Drewes *et al.* 1995). Smoke contains several thousand compounds (Maga 1988). It was suggested by Keeley and Fotheringham (1997) that nitric oxide, which is an important messenger molecule in many biological systems (Lamattina *et al.* 2003), is the active principle in smoke. However, recent work has conclusively discounted this possibility (Light and Van Staden 2003, Preston *et al.* 2004). It is now generally accepted that the active principle is produced by the combustion of plant materials (Jäger *et al.* 1996a, 1996b) and smoke products are already widely used (Brown 1993, Dixon *et al.* 1995, Brown and Van Staden 1997, Roche *et al.* 1997b, Keeley and Fotheringham 1998, Enright and Kintrup 2001). Progress in our understanding of the function and mode of action of the germination stimulant(s) is hampered by the fact that the identity of the active principle remained unknown for a very long time (Van Staden *et al.* 1995a, 1995b, Preston *et al.* 2004).

Here we report the bioactivity-guided isolation and identification of the main active compound, 3-methyl-2*H*-furo[2,3*c*]pyran-2-one, from smoke-saturated water routinely used for the germination of South African wild species (Brown *et al.* 2004). This should advance our understanding of fire-related cues in evolution, the ecological regulation of plant succession and, in particular, seed and seedling physiology which impacts on agriculture, forestry and horticulture.

Materials and Methods

Isolation of germination stimulant

In this study a germination stimulant was isolated by bioactivity-guided fractionation using the lettuce seed bioassay (Drewes *et al.* 1995). Twenty litres of smoke-saturated water derived from burned *Passerina vulgaris* Thoday and *Themeda triandra* L. were concentrated under vacuum to 2l. This concentrate was exhaustively extracted with dichloromethane. The combined organic extracts were washed 6 times with 1% (w/v) aqueous NaOH, followed by 3 washings with H_2O to neutral pH. The extract (9.8g) was subjected to vacum liquid chromatography (VLC) using a 5 x 27cm column packed with 200g silica gel 60 (Merck, 0.040–0.063mm) and eluted with a hexane:ethyl acetate gradient (hexane proportions: 100%, 85%, 80%, 75%, 70%, 65%, 60%, 50%, 40%, 30%, 20%, 10%, 0% (v/v); 400ml aliquots of each mixture). Fractions with germination stimulating activity eluted in 70:30 and 65:35 hexane:ethyl acetate (v/v). These two fractions were combined and then fractionated on a Sephadex LH-20 column (90 x 2.5cm) eluted with 35% EtOH at 15ml h^{-1}. Activity eluted between 560-630ml. This active material was concentrated under vacuum and the aqueous residue extracted with dichloromethane to recover the active material. Further purification was achieved by repeated HPLC (60 runs) on a C_{18} reverse phase column (Haisil 300 C_{18}, 5µm, 250 x 10mm, Higgins Analytical) with 30% MeOH as mobile phase at 2ml min^{-1}. The active constituent eluted at 20–21min. The methanol was allowed to evaporate at room temperature and the active fractions combined and extracted with dichloromethane yielding 3.1mg of the target compound.

GC-MS analysis of active constituent

Aliquots of 1–2µl of the active HPLC fraction were subjected to preparative capillary gas chromatographic separation (Carlo Erba 400 series model HRGC) fitted with an on-column injector and a 40m x 0.3mm glass capillary column coated with 0.375µm of OV-1701 as stationary phase. A capillary effluent splitter, which allowed 10% of the column effluent to flow to the detector and 90% to the collection device, was made in-house. To avoid band broadening in space, expected to result from the injection of samples containing highly polar solvents, the column was connected to a 3m retention gap. Fractions were collected manually. Preparative capillary gas chromatography is problematic because some of the effluent organic material can be lost due to aerosol formation. To circumvent this problem, fractions were collected in methanol or dichloromethane in small conical sample vials. Another problem is the cross-contamination of fractions due to the condensation of organic material at the point where fractions from a hot capillary are col-

lected in a cold solvent. To solve this problem, each fraction was collected using a clean glass exit capillary, which was connected in a 'press-fit' fashion to the fused silica GC column. All fractions were tested for germination activity and the active fraction was subjected to GC-MS analysis on both more and less polar capillary columns to determine the purity of the isolated active material. The preparative separations were repeated 20 times with collection of the active fraction after which the preparative separations were repeated first on a 40m x 0.3mm glass capillary column coated with 2.5µm of the apolar phase PS-255 and finally on a 30m x 0.32mm fused silica column coated with 0.25µm Carbowax 20M. According to GC and GC-EIMS analyses (Carlo Erba QMD1000 quadrupole instrument, Carbowax 20M column), this procedure produced the active constituent in a highly pure state. The isolated compound had the same mass spectrum as the major constituent of the active material isolated by HPLC. This material was subjected to high resolution mass spectral measurements (MicroMass Autospec-TOF instrument; calculated for $C_8H_6O_3$ 150.0317; found 150.0316).

Structure elucidation

IR was recorded on a Perkin Elmer 1600 instrument, ($CHCl_3$):(C = O) 1 743cm^{-1}. NMR spectra were recorded on a Bruker Avance 600 or a Bruker Avance 400 spectrometer (proton frequency 600.13 and 400.13MHz, respectively) at 25°C, using $CDCl_3$ as solvent and TMS as internal standard. NOESY spectrum was obtained with mixing time of 300. The gHMBC and gHSQC spectra were optimized for $^nJ_{C,H}$ = 7.7 Hz and $^1J_{C,H}$ = 140 Hz, respectively. ^{1}H NMR (600.13 MHz, $CDCl_3$) δ 7.44 (1H, s, H-7), 7.32 (1H, d, *J* = 5.5 Hz, H-5), 6.51 (1H, d, *J* = 5.5 Hz, H-4), 1.93 (3H, s, H-8). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 7.70, 100.40, 103.46, 126.79, 139.73, 142.31, 147.97, 171.25.

Results and Discussion

Following liquid-liquid partitioning of the smoke-saturated water, the extract was fractionated using VLC. The VLC separation yielded 13 fractions, two of which showed germination stimulating activity (70:30 and 65:35 hexane:ethyl acetate, v/v). These two fractions were combined and subjected to column chromatography on Sephadex LH-20 followed by reverse phase HPLC. Gas chromatographic-mass spectrometric analysis (GC-MS) showed that the active fraction contained small quantities of impurities. It was, however, sufficiently pure for nuclear magnetic resonance (NMR) and infrared (IR) analysis. Further purification of the active material by preparative capillary gas chromatography on three columns coated with stationary phases having different polarities yielded a fraction containing a single compound. This compound had high germination stimulating activity and had the same mass spectrum (Figure 3) as the major constituent of the HPLC-isolated material mentioned above. Once again, the preparative gas chromatographic isolation of the germination stimulant rules out the possibility that nitric oxide could be the active smoke principal. It also rules out the possibility that an impurity could be responsible for the germination stimulating activity of the HPLC-isolated material.

Electrospray ionisation-mass spectrometry (ESI-MS) of the isolated (HPLC) active fraction gave mass spectra having an abundant pseudomolecular ion at m/z 151 (M + H$^+$). High resolution electron impact mass spectral measurements (HR-EIMS) of the gas chromatographically isolated active constituent showed that this compound has the molecular formula $C_6H_8O_3$. The possible presence of an α,β-unsaturated γ-lactone was indicated by a carbonyl stretch at 1 743 cm^{-1} in the IR spectrum and a signal at 171ppm in the ^{13}C NMR spectrum. A full assignment of data obtained from 1D ^{1}H and ^{13}C NMR, as well as 2D homonuclear and het-

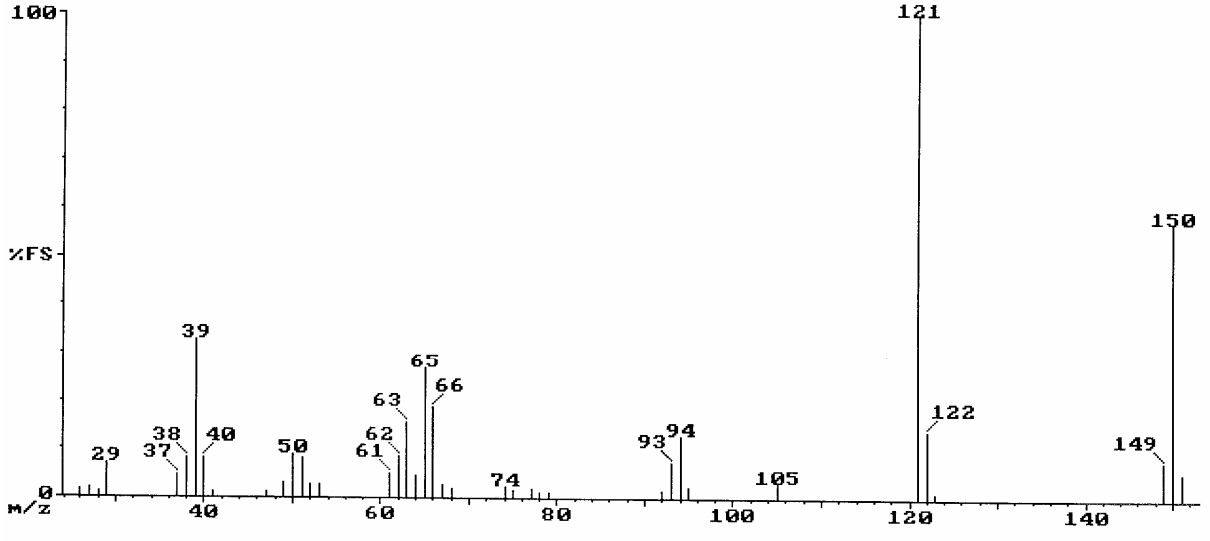

Figure 3: Mass spectrum of the germination stimulant **1**

eronuclear NMR experiments, showed the active constituent to be 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**). This is a previously unknown compound. Selected diagnostically relevant correlations observed in the heteronuclear multiple bond correlation spectrum are shown in Figure 4.

Compound **1** proved to be a highly active germination stimulant in the lettuce seed bioassay, where it was active at dilutions up to 10^{-9}M (Figure 5). The compound showed germination activity over a wide range of concentrations and does not inhibit seed germination at higher concentrations (Figure 6), as was observed with crude smoke extracts (Drewes *et al.* 1995). This indicates that the inhibitory effect observed with more concentrated and non-purified smoke water is due to inhibitory compounds present in the smoke water. A dual role for smoke, both stimulatory and inhibitory, in seed germination has been reported (Light *et al.* 2002).

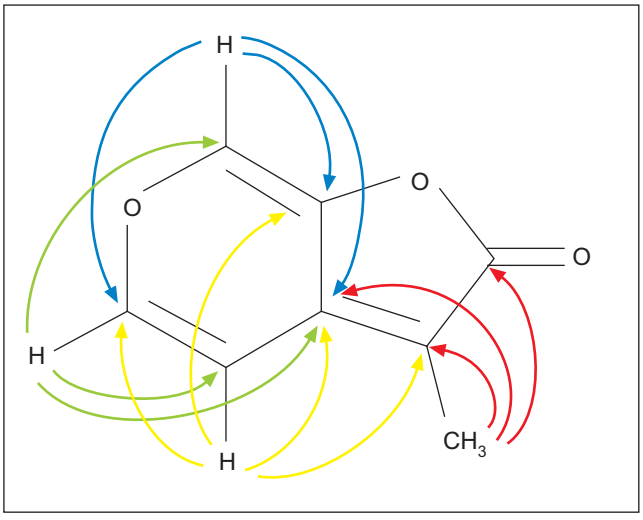

Figure 4: Selected heteronuclear multiple bond correlations used for structural characterisation of compound **1**

At the time this manuscript was being prepared, a paper appeared in Science in which the same compound as compound **1**, on which we report here, was isolated from smoke produced by burning cellulose (Flematti *et al.* 2004). Cellulose is a ubiquitously occurring plant compound which was previously reported to produce a germination stimulant (Jäger *et al*. 1996a). Using GC-MS, Flematti *et al.* (2004) also confirmed the presence of this compound in plant-derived smoke. They showed that this compound significantly improved the germination of Australian indigenous plants, and smoke responsive South African and North American species. The identification of this new germination cue from plant-derived smoke now opens the door to study its mode of action. Attempts can now be made to answer the question of how this molecule is capable of substituting for phytochrome effects in light-sensitive lettuce (Drewes *et al.* 1995) and celery seeds (Thomas and Van Staden 1995). While we have no answers at present, it is highly significant that smoke water and GA_3 both substitute for light in the germination of light-sensitive Grand Rapids lettuce seeds (Gardner *et al.* 2001).

Conclusion

Following many years of research in the area of smoke-stimulated seed germination, the present report opens a way forward to a greater understanding of the mechanism by which smoke acts to stimulate/regulate the germination of many wild plant species, particularly in ecosystems where fire plays an integral role. The use of compound **1** as a germination cue holds great potential in research fields where seed germination is of importance, for example agriculture, horticulture, forestry, conservation, ecosystem rehabilitation and weed science.

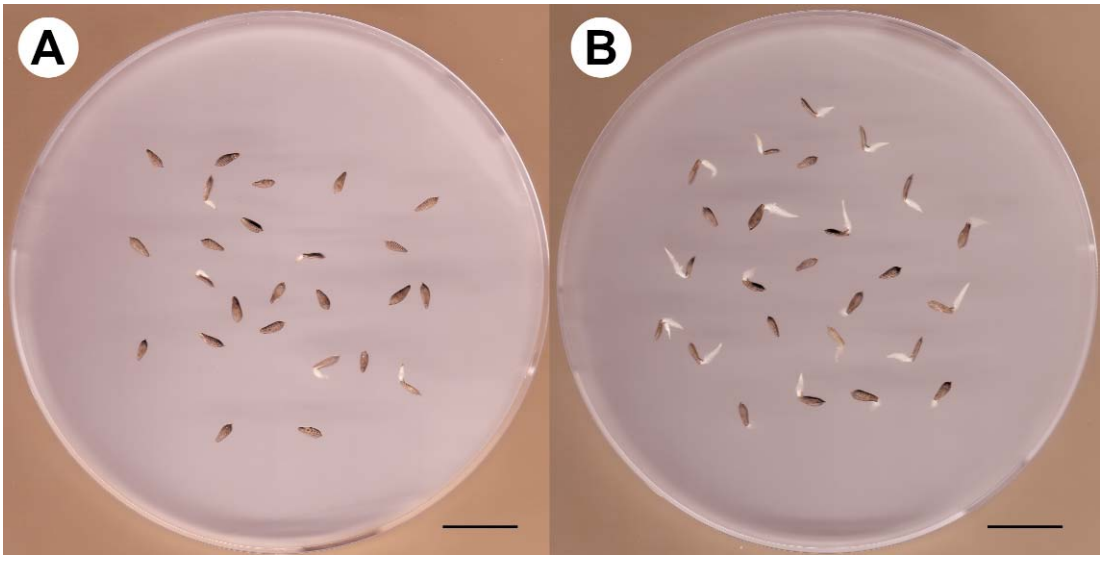

Figure 5: Germination response observed with light-sensitive Grand Rapids lettuce seeds after 24h in the dark at 25°C. (A) Distilled water control, (B) Treatment with compound **1** at 10^{-9}M. Scale bars, 10mm

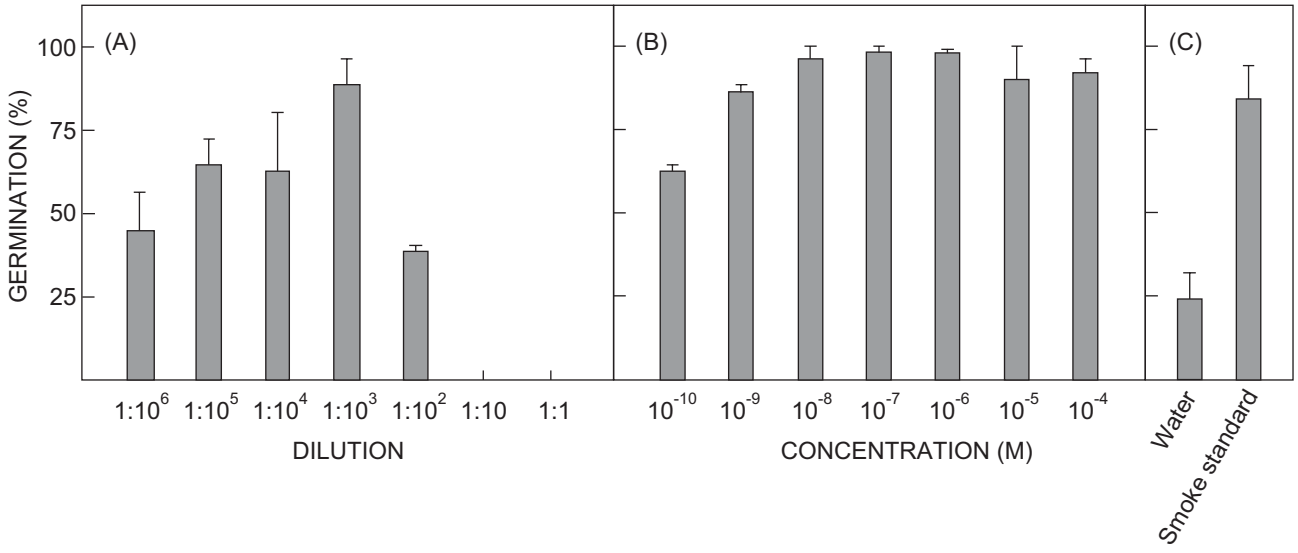

Figure 6: Germination (± SE) of light-sensitive Grand Rapids lettuce seeds after 24h in the dark at 25°C. (A) Dilution series of crude smoke water, (B) Dilutions of purified compound, following HPLC separation, (C) Distilled water control and standard crude smoke solution at a 1:1 000 dilution. Experiments were repeated three times

Acknowledgements — Kirstenbosch National Botanical Gardens are thanked for their generous contribution of smoke solutions. Financial support from the University of KwaZulu-Natal Research Fund and the National Research Foundation, Pretoria, is gratefully acknowledged. NMR equipment used in this work was purchased via grants from *Apotekerfonden af 1991*, Copenhagen, and the Danish University of Pharmaceutical Sciences. Dr D Stærk is thanked for recording the NMR spectra.

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