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Production of the Seed Germination Stimulant Karrikinolide from Combustion of Simple Carbohydrates

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ABSTRACT: The naturally occurring seed germination stimulant karrikinolide is formed from the combustion of plant material including cellulose. It has previously been reported that combustion of simple carbohydrates such as D-glucose does not produce extracts containing karrikinolide. Moreover, it was reported that extracts with germination-promoting ability could be obtained only by combustion of simple carbohydrates in the presence of amino acids such as L-glycine. By employing a ^{13}C-labeled karrikinolide to physically quantify natural karrikinolide, we now show that it is produced from combustion of simple carbohydrates in similar amounts regardless of whether L-glycine is present or not. The addition of L-glycine appears to be beneficial in reducing the inhibitory effect of smoke extracts and provides a greater concentration range for effective germination-promoting activity.

KEYWORDS: Karrikinolide, karrikin, germination stimulant, smoke, combustion, xylose

INTRODUCTION

The germination-promoting ability of smoke and smoke extracts derived from combustion of plant material has been recognized for two decades[1−8] since the discovery by De Lange and Boucher in 1990.[9] The major compound responsible for this effect was identified as 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one,[10] now commonly known as karrikinolide or KAR$_1$ (**1**, Figure 1). Closely related compounds have also been identified in smoke, and the name "karrikins" has been coined to describe this group of naturally occurring germination stimulants.[11]

The mechanism of formation of **1** from combustion of plant material has not yet been determined, but it is known to originate from cellulose.[12] This is supported by the fact that **1** was first isolated from smoke extracts derived from the combustion of filter paper,[10] which consists almost entirely of cellulose (>99%). Cellulose is a polymeric carbohydrate made up of β-1,4-linked glucose monomers, yet combustion of glucose has never been reported to produce germination stimulants. Surprisingly, recent work has suggested that **1** can be formed by combustion of D-glucose, but only in the presence of glycine or other amino acids.[13] In fact, a number of simple carbohydrates were reported to produce **1** when burnt in a 2:1 ratio with amino acids, yet combustion of the individual carbohydrates or the amino acids did not appear to form any **1** as determined using a seed germination bioassay. The authors went on to speculate that filter paper might be contaminated with traces of amino acids, which could explain the formation of **1** from this substrate.[13]

In this study, we investigate the combustion of simple carbohydrates in the presence and absence of the amino acid L-glycine. The volatile combustion products formed are tested for germination-promoting activity and examined for the presence of **1** using GC-MS. Using $^{13}C_5$-labeled **1** as an internal standard, we quantify the amount of **1** produced to determine whether amino acids are required for the formation of **1**.

MATERIALS AND METHODS

General Experimental. Cellulose was obtained from Sigma-Aldrich (St. Louis, MO), D-xylose from Carbosynth (Berkshire, U.K.), D-glucose from Ajax Finechem (Sydney, Australia), and L-glycine from Fluka (St. Louis, MO). Karrikinolide (**1**) was prepared as described by Flematti et al.,[14] and 3a,4,5,7,7a-$^{13}C_5$-labeled **1** was prepared from $^{13}C_5$-labeled D-xylose (Omicron Biochemicals, South Bend, IN) following the methods of Scaffidi et al.[15] Solvents used were freshly distilled before use except for HPLC grade acetonitrile. Milli-Q water was obtained by filtration of deionized (DI) water through a Milli-Q ultrapure water system (Millipore, Australia).

Combustion Experiment 1. In previous work, extracts were obtained by heating carbohydrates with or without amino acids at different temperatures, followed by the addition of water to the resulting pyrolysates to extract the germination active components.[13] Because the germination active component **1** is semivolatile, we preferred to heat the samples and pass the smoke produced through water to trap the resulting volatile combustion products in line with our previous studies.[10] Thus, D-glucose (2.6 g, 16 mmol), D-xylose (2.4 g, 16 mmol), and a mixture of D-xylose (2.4 g, 16 mmol) and L-glycine (0.6 g, 8 mmol) were combusted individually by heating the dry samples in a two-necked round-bottom flask (250 mL) with a flame for 15 min. The flask was purged with air, which was bubbled at a steady rate (30 mL/min) through deionized water (100 mL) to trap the smoke volatiles. The water became yellowish after 5 min with a distinctive smoky smell, and the combusted material was reduced to ash.

Aliquots (10 mL) from each of the aqueous smoke samples were tested in triplicate with *Solanum orbiculatum* as previously described.[16] The samples were tested at 0, 1/10, and 1/100 dilution levels. Milli-Q

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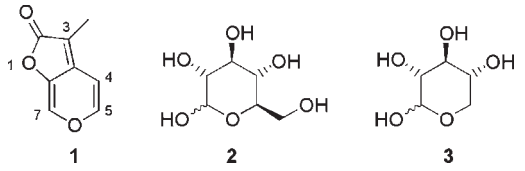

Figure 1. Chemical structures of karrikinolide (**1**), D-glucose (**2**), and D-xylose (**3**).

water and **1** (100 ppb) were both used as germination controls. A second aliquot (10 mL) from each of the smoke samples was taken for solid phase extraction (SPE).

The remaining smoke solutions (80 mL) were extracted with dichloromethane (3 × 20 mL). The organic layers were combined, dried ($MgSO_4$), filtered, and evaporated to dryness, and the residues were weighed (D-glucose = 56.7 mg; D-xylose = 80.4 mg; D-xylose + L-glycine = 16.4 mg). Samples were made up to approximately 5 mg/mL in HPLC grade acetonitrile for GC-MS analysis.

SPE of Smoke Samples. An SPE cartridge (1 g, Waters Sep-Pak) was conditioned with methanol (2 mL) and then equilibrated with Milli-Q water (10 mL). The smoke water (10 mL) was eluted through the SPE cartridge, followed by Milli-Q water (10 mL) and then 40% (v/v) methanol/water (10 mL) to elute the fraction containing **1**. Finally, 100% methanol was eluted to provide three fractions overall. The 40% (v/v) and 100% methanol fractions were evaporated under vacuum to remove the organic solvent (plus ~10% of the water for the 40% methanol fraction) and then reconstituted to 10 mL with Milli-Q water. The three fractions were tested in triplicate with *S. orbiculatum* as previously described.[16] The samples were tested at 0, 1/10, and 1/100 dilution levels. Milli-Q water and **1** (100 ppb) were both used as germination controls. Only the 40% (v/v) methanol fraction demonstrated germination activity.

Combustion Experiment 2. Cellulose (2.0 g), D-glucose (2.0 g, 11.1 mmol), D-xylose (2.0 g, 13.3 mmol), and a mixture of D-xylose (2.0 g, 13.3 mmol) and L-glycine (0.5 g, 6.6 mmol) were combusted individually by heating the dry samples in a two-necked round-bottom flask (250 mL) with a flame. The flask was purged with air, which was bubbled at a steady rate (30 mL/min) through two traps connected in series, both containing deionized water (150 mL) to trap the volatiles. After combustion, the two traps were combined, to which was added 1 μg of $^{13}C_5$-labeled **1** as internal standard, and then extracted with dichloromethane (3 × 40 mL). The combined dichloromethane extract was washed with 1 M NaOH solution (2 × 50 mL), followed by brine, and then dried ($MgSO_4$), filtered, and evaporated to dryness.

HPLC Separation. HPLC was conducted using a Hewlett-Packard 1050 HPLC system equipped with a multiple-wavelength detector (MWD). The dichloromethane neutral fraction from combustion experiment 2 was dissolved in 50% (v/v) methanol/water (1 mL) and separated by HPLC. Separation was achieved using a 250 × 10 mm i.d., 5 μm, Apollo C_{18} reversed phase column (Grace-Davison) with a 33 mm × 7 mm guard column of the same material. The column was eluted at 4 mL/min with 10% (v/v) acetonitrile/water increasing to 50% (v/v) acetonitrile/water over 30 min and then to 100% acetonitrile at 31 min and held for 9 min. UV absorbance was measured at wavelengths of 210, 254, and 325 nm. Fractions were collected every minute for 40 min. An authentic sample of **1** eluted at 19.1 min corresponding to fraction 20. Fractions 19, 20, and 21 were combined for each separation and evaporated to dryness under reduced pressure. The samples were resuspended in 20 μL of HPLC grade acetonitrile for GC-MS analysis.

GC-MS Analysis. GC-MS was performed using a Shimadzu GCMS-QP2010 instrument operating in the electron impact (EI, 70 eV) mode. Separation was achieved using a 30 m × 0.2 mm i.d., 0.33 μm, DB-35 ms (J&W Scientific) column with ultrahigh-purity (UHP) helium as the carrier gas (1 mL/min). The initial oven temperature was set to 40 °C and held for 1 min before increasing at 10 °C/min to 100 °C, then at 3 °C/min to 200 °C, then at 10 °C/min to 250 °C, and held for 10 min (inlet temperature, 250 °C; transfer line, 250 °C). The ion source was set at 200 °C, and the spectrometer was set to record from m/z 45 and 400. For the quantitation experiments, the spectrometer was set to record in selective ion monitoring (SIM) mode using ions m/z 121 and 126, which correspond to the base ions of natural **1** and $^{13}C_5$-labeled **1**, respectively.

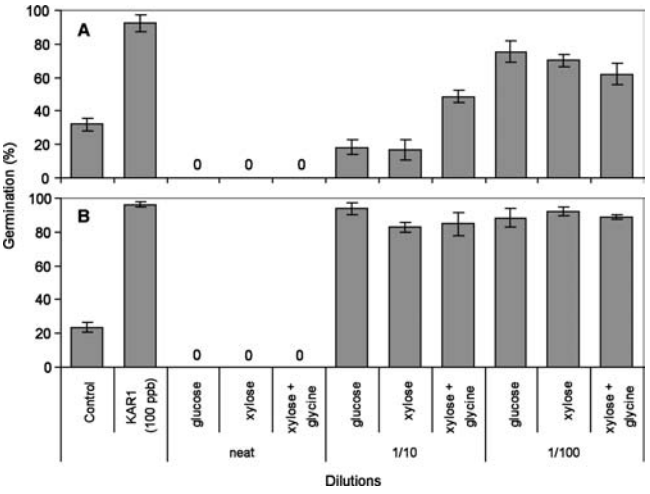

Figure 2. Germination of *S. orbiculatum* seeds tested with (A) different aqueous smoke extracts and (B) the SPE purified aqueous smoke extracts.

RESULTS AND DISCUSSION

D-Glucose (**2**), D-xylose (**3**), and a mixture of D-xylose and L-glycine were heated with a flame, and the smoke produced was bubbled through deionized (DI) water to trap the volatile compounds. The aqueous smoke solutions produced were tested for germination activity with the bioassay species *S. orbiculatum* Poir. (Solanaceae) (Figure 2A). *S. orbiculatum* has proved to be very sensitive to the germination promoting effects of **1**, with concentrations as low as 1 ppb known to be effective.[16] The results show that each of the undiluted extracts inhibited germination, whereas at the 1/100 dilution level all extracts stimulated germination (>60% germination compared to 31.9 ± 3.5% control germination). At the 1/10 dilution level, the mixture of D-xylose and L-glycine sample was found to give a higher germination response (48.6 ± 3.5%) compared to the D-xylose sample alone (16.7 ± 6.0%). In fact, the D-xylose and D-glucose samples both inhibited germination at 1/10 dilution compared with the control germination level.

To reduce the complexity of the smoke mixtures, the extracts were partly separated using SPE. Testing of the SPE purified samples gave improved germination results (Figure 2B) over the crude extracts (Figure 2A). All samples were shown to promote germination at the 1/10 and 1/100 dilution levels (>80% germination compared with 23.5 ± 3.0% control germination), whereas all undiluted extracts still inhibited germination.

These results show that heating either D-xylose or D-glucose produces germination stimulants, irrespective of whether an amino acid is present. The solutions derived from sugars alone have more of an inhibitory effect on germination at higher concentrations compared to the smoke solution derived from D-xylose and L-glycine. The activity of the D-glucose and D-xylose extracts has been detected at high dilution levels, which relies on a highly sensitive seed germination assay. This could explain why germination-promoting

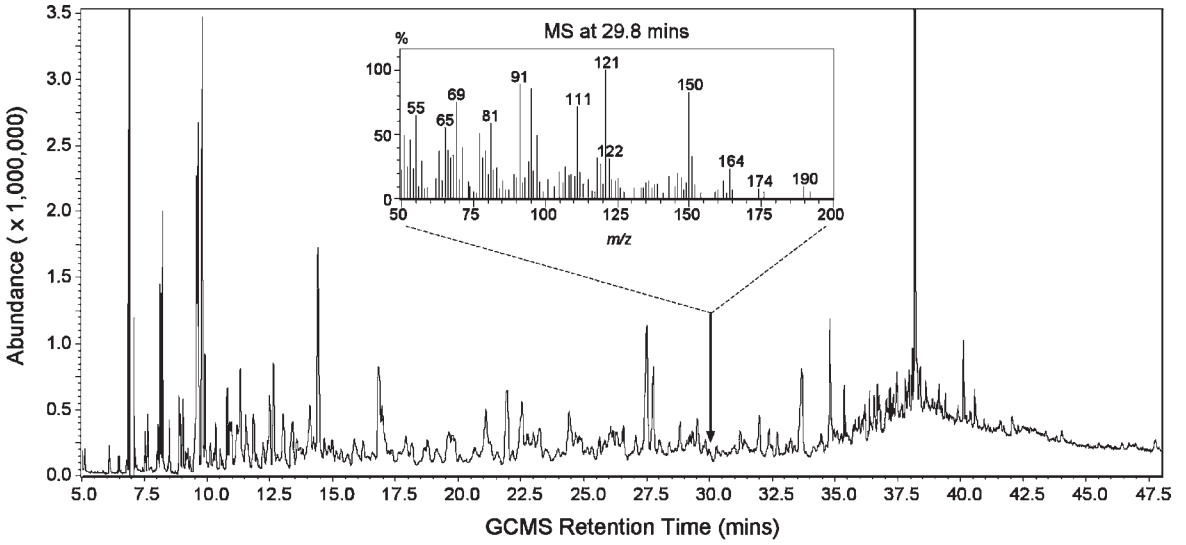

Figure 3. GC-MS chromatogram of the D-xylose neutral fraction showing the presence of **1** at 29.8 min. (Inset) Mass spectrum at 29.8 min and the presence of ions m/z 150 and 121, which represent the molecular ion and main fragment (base) ion, respectively, of **1**.[11].

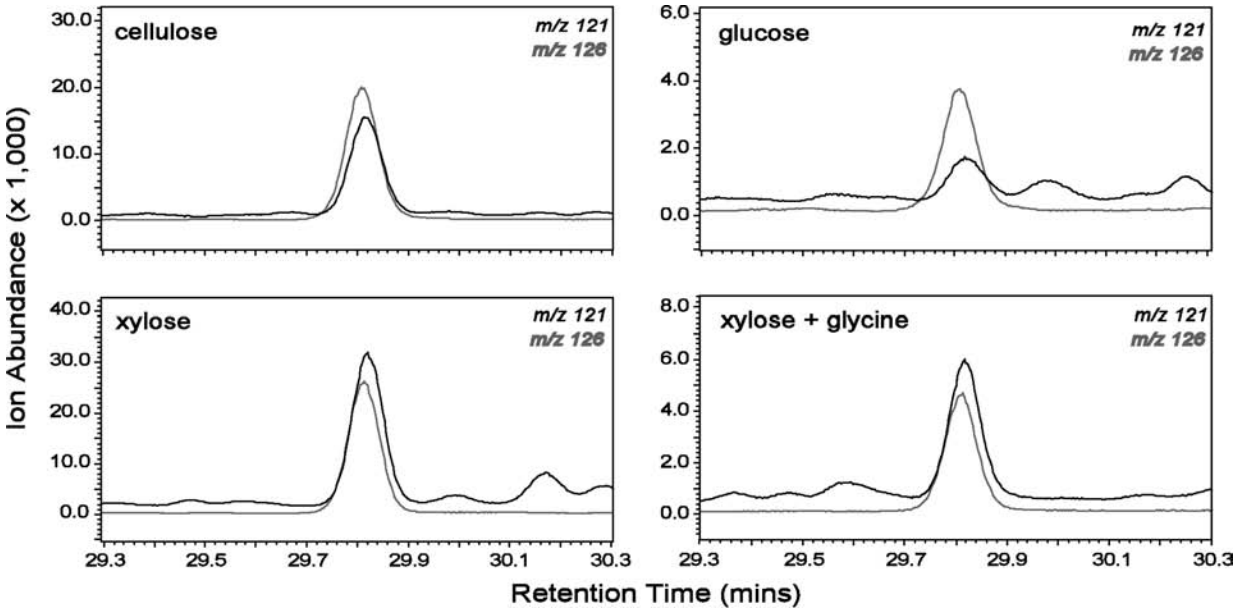

Figure 4. Expanded regions from the GC-MS separation of fractions from each smoke extract showing the presence of **1** (m/z 121) and the internal standard, $^{13}C_5$-labeled **1** (m/z 126).

compounds derived from combustion of these carbohydrates have not previously been reported.[13,17] It is also noteworthy that the mass returned from the D-xylose with L-glycine combustion extract was substantially lower than that obtained for D-xylose or D-glucose, indicating a lower concentration of combustion products in this smoke extract. A sample of the crude extract from D-xylose was dissolved in acetonitrile and analyzed by GC-MS for the presence of **1**. The extract was found to be very complex (Figure 3), but by comparison with an authentic sample, **1** was identified. Similarly, **1** was identified in extracts from D-glucose and D-xylose with L-glycine (data not shown). Thus, in each smoke extract, germination activity could be explained by the presence of **1**.

To investigate the presence of **1** in these mixtures further, triplicate samples of cellulose, D-glucose, D-xylose, and D-xylose with L-glycine were combusted, and the smoke produced was passed through two water traps in series to aid in the quantitative trapping of **1**. The two traps were combined for each experiment, and a known amount of $^{13}C_5$-labeled **1** (1 μg, $^{13}C > 99\%$) was added as internal standard. The aqueous smoke samples were extracted with dichloromethane and washed with aqueous sodium hydroxide to generate the neutral fraction.[18] The neutral fraction was separated by HPLC into 40 fractions, and the fraction that contained **1** and the fractions preceding and following it were combined and evaporated to dryness. The samples were analyzed for **1** using GC-MS. All of the extracts were shown to contain **1** (Figure 4), thus confirming that **1** is produced from combustion of D-glucose and D-xylose. The amount of **1** found was 740 ± 9 ng from 2 g of cellulose. D-Glucose produced a lower quantity of 302 ± 51 ng, whereas D-xylose gave a much higher

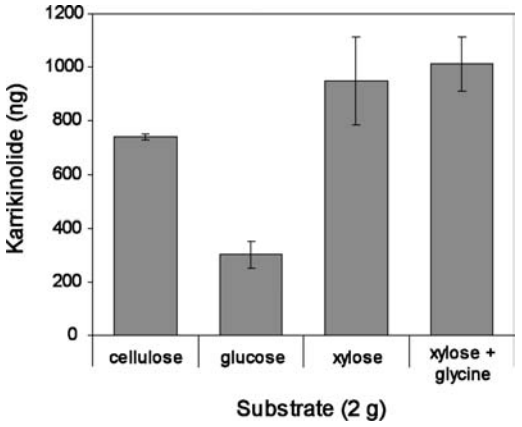

Figure 5. Amount of **1** produced from combustion of cellulose, D-glucose, D-xylose, and D-xylose with L-glycine.

amount of 948 ± 164 ng. The amount determined for D-xylose and L-glycine was 1012 ± 100 ng, which was not significantly different from the amount obtained from D-xylose (Figure 5). This result confirms that amino acids are not necessary for the formation of **1**.

The amount of **1** derived from D-xylose was found to be greater than that obtained from cellulose or D-glucose on a per gram basis. This might be due to the fact that D-glucose (**2**) has an extra hydroxymethyl group at C5 that presumably undergoes oxidation and decarboxylation under combustion conditions to produce **1**. As D-xylose (**3**) does not require decarboxylation to produce the pyran ring present in **1**, this could explain why more is formed from D-xylose (Figure 1). Furthermore, combustion of plant material often produces extracts with greater germination activity compared to those derived from cellulose alone. This can now be explained as plant cell walls are known to contain a high amount of D-xylose present in hemicellulose and xylans, with the latter previously reported to produce germination stimulants upon charring.[19] Amino acids, as demonstrated here, might also reduce the inhibitory effects of smoke extracts derived from combustion of plant material, which is interesting given the recent identification of a germination inhibitor in plant-derived smoke.[20]

In summary, we have shown that combustion of simple carbohydrates such as D-xylose and D-glucose produces the germination stimulant karrikinolide (**1**). Combustion of D-xylose produces greater quantities of **1** compared with combustion of D-glucose or cellulose, and heating D-xylose with the amino acid L-glycine gives a smoke extract that shows less inhibitory effect on germination compared to extracts derived from D-xylose alone. Thus, amino acids may be beneficial for providing smoke extracts with more effective germination-promoting ability, but are not required for the formation of **1**.

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