

Formation of a Seed Germination Promoter from Carbohydrates and Amino Acids

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The ability of plant-derived smoke to act as a germination cue in many species has led to widespread interest in this aspect of seed biology. Recently, 3-methyl-2H-furo[2,3-c]pyran-2-one was identified as the main germination cue from smoke. Here, we report on the formation of this compound from reactions of sugars with amino acids. Heating proteins or amino acids with sugars at 180 °C for 30 min produces water soluble extracts that promote germination. High-performance liquid chromatography indicated that the active compound(s) derived from these reactions coeluted with the active fraction from a smoke solution. Gas chromatography–mass spectroscopy showed that the active constituent is identical to the germination cue from plant-derived smoke. The results presented in this paper provide evidence for the formation of the major germination cue found in smoke from ubiquitously occurring organic compounds.

KEYWORDS: Amino acid; aminocarbonyl; carbohydrate; Maillard reaction; plant-derived smoke; seed germination; sugar

INTRODUCTION

Mediterranean type ecosystems, such as fynbos (South Africa), chaparral (southern California), kwongan (Australia) and areas of the Mediterranean, are dependent on fire to provide suitable germination cues for many species (1). The promotion of germination by fire may be as a result of a number of different effects, including the physical effect of dry heat on seed coat structure, the physiological effect of dry heat on seed embryos, and/or the dormancy-breaking effects of volatile compounds (1). In 1990, De Lange and Boucher made a highly significant discovery that cold smoke and aqueous smoke extracts could induce seed germination of dormant seeds of a threatened fynbos species (2). This observation indicated the presence of a chemical cue(s) in smoke and prompted many investigations of smoke-stimulated seed germination (3, 4). Surveys, mainly focused on species from fire-prone areas, have shown that smoke is effective on species from a wide range of families, which vary in ecology, reproductive strategy, seed size, and morphology (5, 6). Furthermore, the promotion of germination by smoke is not limited to species from fire-prone ecosystems. For example, germination can also be stimulated in lettuce (*Lactuca sativa* L. cv. Grand Rapids) (7) and red rice (*Oryza sativa* L.) (8). The use of smoke as a germination cue holds great potential in a variety of applications such as horticulture, agriculture, weed control, habitat restoration, ecological management, and con-

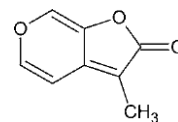


Figure 1. Chemical structure of 3-methyl-2H-furo[2,3-c]pyran-2-one.

servation (9, 10). The chemical identity of the germination cue has recently been characterized as 3-methyl-2H-furo[2,3-c]pyran-2-one (**Figure 1**) from burned cellulose (11) and plant-derived smoke (12). It can promote seed germination at concentrations as low as 10^{-9} M (11, 12), although the mode of action of this compound in promoting germination it still unknown and will require further research.

In some earlier studies on smoke-stimulated germination, the effect of different sources of smoke, including liquid food-flavoring smoke, on seed germination was investigated (13–15). Results from these studies suggest that the germination-promoting compound(s) is produced from commonly occurring plant constituents. Furthermore, chromatographic purification of aqueous smoke extracts from different sources produced results supporting the notion of a common active compound (15–17). The effect of germination stimulation is not limited to extracts produced from smoke only. The exposure of seeds directly to charred wood and aqueous extracts of charred wood and plant material also stimulates germination of a number of species (18–21). Furthermore, Keeley and Pizzorno (19) suggested that charring was not necessary and showed that the dry heating of *Adenostoma* wood at 175 °C for 30 min produced compounds, which promoted seed germination in chaparral species. In a study by Jäger et al. (14), heating dry *Themeda*

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triandra leaves at a range of temperatures showed that the active compound(s) was produced between 160 and 200 °C. At higher temperatures, the activity was no longer observed, probably because of volatilization of the active component(s).

It has been suggested that cellulose and hemicelluloses are the most likely common components for the production of active compound(s) in smoke (14, 19). Keeley and Pizzorno (19) observed increased germination of two chaparral species with an extract from crude lignin, although some purified lignin compounds did not increase germination. They tested a variety of extracts from heated cellulose and hemicelluloses and observed some increase in germination with extracts of heated xylan and glucuronic acid. However, these treatments were not as effective as the crude extracts from charred wood. Jäger et al. (14) did not observe any improved germination of light sensitive lettuce seeds with extracts of individually heated glucuronic acid, starch, glucose, and galactose. However, extracts from heated agar and cellulose did stimulate germination.

The aim of the present study was to investigate the possible role of Maillard reactions of amino acids and sugars in the formation of the compound responsible for the stimulation of germination by smoke and aqueous smoke solutions. We examined the effect of water extracts, from the products of reactions between a variety of amino- and carbonyl-containing compounds, on the germination of light sensitive lettuce seeds (7). Extracts showing high levels of germination stimulation were further analyzed using high-performance liquid chromatography (HPLC) and gas chromatography–mass spectroscopy (GC-MS).

MATERIALS AND METHODS

Instrumentation. HPLC was performed using a semipreparative reverse-phase HPLC column (Haisil, 300 C₁₈, 5 μm, 250 mm × 10 mm, Higgins Analytical) with 30% MeOH as the mobile phase at 2 mL/min. A Spectra System P4000 pump and UV6000LP photodiode array detector (Thermo Separation Products) were used.

Exploratory GC analyses were carried out with a Carlo Erba 5300 gas chromatograph equipped with a flame ionization detector, Grob split-splitless injector, and a glass capillary column (40 m × 0.3 mm) coated with PS-089-OH (DB-5 equivalent) at a film thickness of 0.25 μm. All analyses were done with hydrogen as a carrier gas at a linear velocity of 50.0 cm/s, determined by injecting methane at a column temperature of 40 °C. The injector was operated at 220 °C, and the flame ionization detector was operated at 280 °C. Samples were injected with a split ratio of 6:1, the analytes thermally focused on the column at ca. 27 °C, and analyzed using a temperature program of 2 °C/min from 40 to 250 °C (hold).

Electron impact (EI) mass spectra were recorded at 70 eV on a Carlo Erba QMD 1000 GC-MS system with VG Analytical Lab-Base software, using the column and conditions described above, except that helium was used as a carrier gas at a linear velocity of 31.4 cm/s, determined by injecting air at a column temperature of 40 °C. An interface temperature of 250 °C was used. The ion source temperature was set at 180 °C, and the pressure in the source housing was ca. 2 × 10⁻⁵ mmHg at a column temperature of 40 °C, decreasing to ca. 1 × 10⁻⁵ mmHg toward the end of the temperature program. A scan rate of 0.9 scan/s, with an interval of 0.1 s between scans, was employed.

Lettuce Seed Bioassay. All experimental solutions were tested for the germination promotion of light sensitive lettuce seeds. Mature achenes (referred to as seeds) of *L. sativa* L. cv. Grand Rapids (Peto Seed, Saticoy, United States) were stored in the dark at 4 °C until use. For each treatment, four replicates of 25 seeds were incubated in the dark at 25 °C for 24 h in a 65 mm plastic Petri dish fitted with two sheets of Whatman no. 1 filter paper moistened with 2 mL of test solution. All manipulations of seeds were carried out in the dark under a green “safelight” (7). A 1:1000 dilution of an aqueous smoke extract

produced from burnt *T. triandra* material, according to the method outlined by Baxter et al. (22), was used as a standard for germination stimulation. All experiments were repeated at least twice. Germination data were arcsine transformed and analyzed using a one-way analysis of variance (ANOVA) and a Tukey’s multiple range test, at a significance level of $p > 0.05$. MINITAB release 13.1 was used to conduct the analyses.

Chemicals. The chemicals used were as follows: DL-R-alanine (BDH), L-arginine (Sigma), L-asparagine (anhydrous) (Sigma), DL-aspartic acid (BDH), bovine albumin (Sigma), casein (Merck), L-cysteine (Sigma), D-(–)-erythrose (Sigma), D-fructose (BDH), D-glucose (BDH), L-glutamic acid (Ashe Laboratories), DL-glutamine (BDH), DL-glyceraldehyde (Sigma), glycine (Merck), L-histidine hydrochloride (Saarchem), L-isoleucine (Sigma), DL-leucine (BDH), DL-lysine monohydrochloride (BDH), maltose (Associated Chemical Enterprises), DL-methionine (Sigma), DL-β-phenylalanine (BDH), L-proline (Sigma), DL-serine (BDH), D-ribose (Sigma), sucrose (BDH), DL-threonine (Nutritional Biochemicals Corporation), L-tryptophan (Sigma), L-tyrosine (Sigma), DL-valine (Sigma), and D-xylose (Sigma).

Reactions of Proteins with Glucose. Bovine albumin or casein (0.075 g) was intimately mixed with equal amounts (w/w) of glucose in a glass vial. The dry mixtures were heated at 180 °C for 30 min in a muffle furnace. The temperature and duration of heating for these preliminary experiments were chosen in accordance with the experiments of Keeley and Pizzorno (19) and Jäger et al. (14) mentioned above. The reaction products were allowed to cool slightly before the addition of 15 mL of distilled water. The resulting suspensions were allowed to stand overnight and then vigorously agitated for 30 min prior to filtration through Whatman no. 1 filter paper. The extracts were tested in the lettuce seed bioassay at dilutions of 1:1 and 1:10, and the results were compared to those obtained with the protein and glucose (0.15 g) heated individually.

Reactions of Amino Acids with Glucose. Twenty commonly occurring amino acid compounds (3.75 × 10⁻⁴ mol) were mixed with glucose (7.5 × 10⁻⁴ mol) in a glass vial. The dry mixtures were heated at 180 °C for 30 min, after which 15 mL of distilled water was added to each. Each chemical (7.5 × 10⁻⁴ mol) was heated individually as a control. The extracts were prepared as above and tested in the lettuce seed bioassay at dilutions of 1:1, 1:10, and 1:100.

Variation of the Amino Acid-to-Glucose Ratio. Combinations of the aminocarbonyl compounds arginine, asparagine, or aspartic acid with glucose were prepared at the following molar ratios: 2:1, 3:2, 2:2, 2:3, and 1:2 (where 1 > 3.75 × 10⁻⁴ mol). The dry mixtures were heated at 180 °C for 30 min. The extracts were prepared as above and tested in the lettuce seed bioassay at dilutions of 1:1, 1:10, and 1:100.

Variation of Reaction Conditions. Mixtures of the aminocarbonyl compounds arginine, asparagine, or aspartic acid with glucose were prepared at a 1:2 molar ratio (where 1 > 3.75 × 10⁻⁴ mol). The dry mixtures were heated at 160, 180, 200, and 220 °C for 5, 15, or 30 min after which 15 mL of distilled water was added to each vial. The extracts were prepared as above and tested in the lettuce seed bioassay at dilutions of 1:1, 1:10, and 1:100.

Reaction with Different Sugars. The aminocarbonyl compounds arginine, asparagine, aspartic acid, glycine, serine, tyrosine, and valine were mixed with different sugars in a 1:2 molar ratio (where 1 > 3.75 × 10⁻⁴ mol). The sugars used were the monosaccharides DL-glyceraldehyde (triose), D-(–)-erythrose (tetrose), D-ribose (aldopentose), D-xylose (aldopentose), D-fructose (ketose), D-glucose (aldohexose), and the disaccharides maltose and sucrose. The dry mixtures were heated at 180 °C for 30 min. The extracts were prepared as above and tested in the lettuce seed bioassay at dilutions of 1:1, 1:10, and 1:100.

HPLC and GC-MS Analysis. Extracts were prepared in the manner described previously by heating different combinations of amino acids and sugars. The combinations used included the following: arginine and glucose, arginine and lactose, asparagine and lactose, aspartic acid and glucose, glycine and glucose, and glycine and xylose. The water extracts obtained from the reactions were extracted three times with equal volumes of dichloromethane. The dichloromethane phase was washed with 1% aqueous sodium hydroxide, followed by washings with water to a neutral pH. The extracts were fractionated using

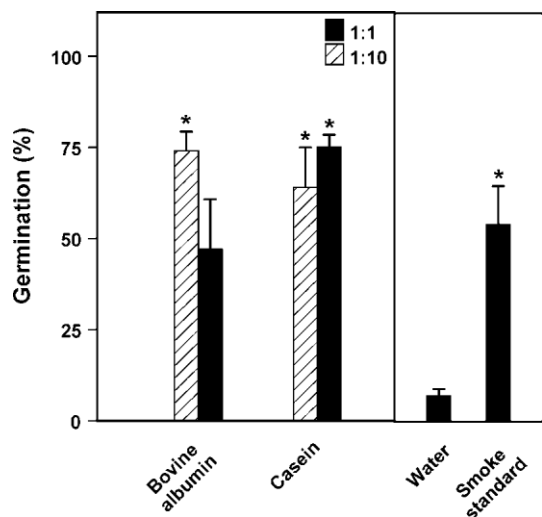


Figure 2. Effect of extracts prepared from heating proteins with glucose at 180 °C for 30 min on the germination of light sensitive Grand Rapids lettuce seeds in the dark. Asterisks denote a significant ($p < 0.05$) difference from the water control; bars indicate SE.

semipreparative HPLC as described above. The HPLC fractions (1 min) were collected and tested in the lettuce seed bioassay.

A mixture of larger quantities of glycine (3.75×10^{-3} mol) and xylose (7.5×10^{-3} mol) was heated at 220 °C in a custom-made apparatus. This apparatus allowed for the reaction of larger quantities of reactants, at a slightly higher temperature than that used in the examples described above, and for the collection of the volatile products in a 50 mL of water trap. The water containing the volatile compounds was extracted three times with 50 mL of dichloromethane. The dichloromethane phase was washed once with 1% aqueous sodium hydroxide, followed by a water wash. This dichloromethane extract was concentrated by allowing the dichloromethane to evaporate at room temperature. An aliquot of this extract was subjected to semipreparative HPLC separation as described above. The active fractions from the HPLC were verified using the lettuce seed bioassay and extracted with dichloromethane for further analysis using GC-MS. The 3-methyl-2H-furo[2,3-*c*]pyran-2-one previously isolated from a plant-derived smoke solution (12) was used in the GC-MS analysis as a standard for retention time and mass spectral comparison with the active fraction obtained from the glycine–xylose reaction.

RESULTS AND DISCUSSION

Heating proteins or amino acids with sugars at temperatures between 160 and 220 °C for 30 min resulted in the production of a dark brown insoluble “honeycomb” substance (40–50% of the starting material). Adding water to the products gave a solution containing the soluble components, although most of the product formed in the reaction was insoluble. Some solutions

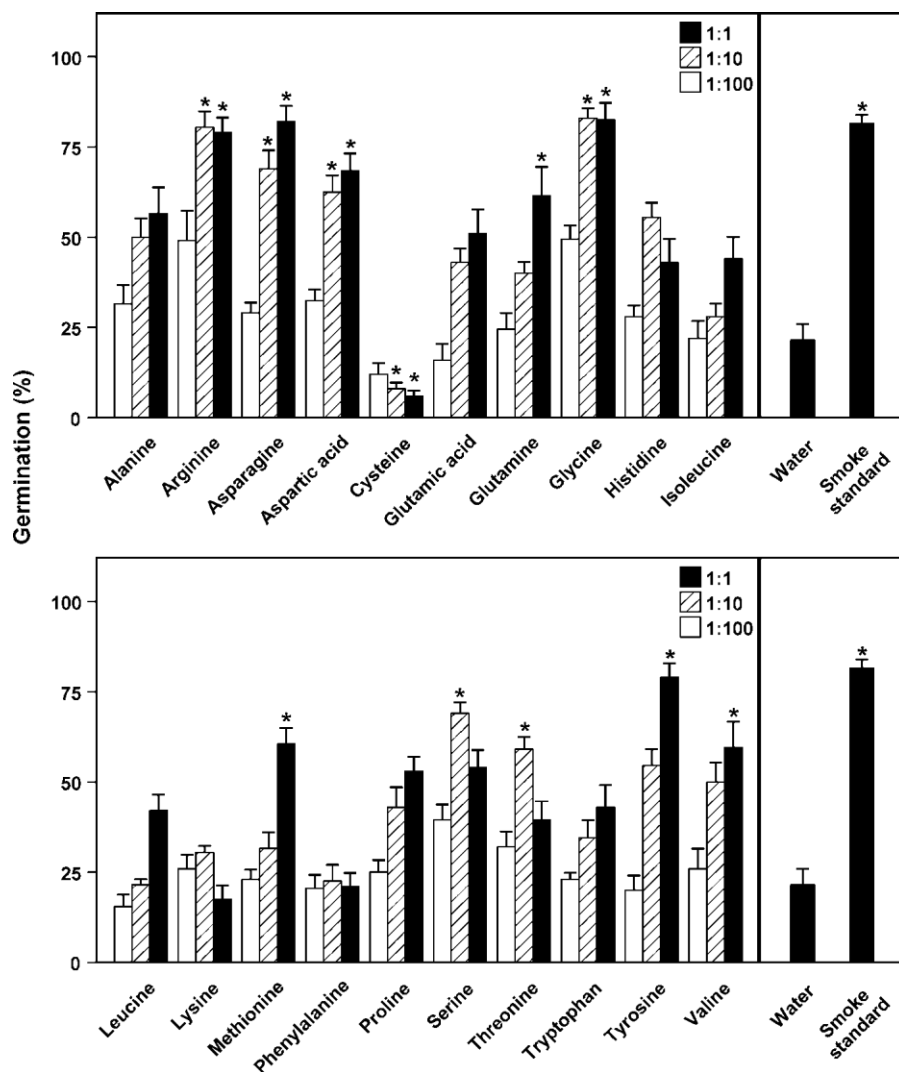


Figure 3. Effect of extracts prepared from heating amino acids with glucose at 180 °C for 30 min on the germination of light sensitive Grand Rapids lettuce seeds in the dark. Amino acids reacted with glucose in a molar ratio of 1:2, where 1 = 3.75×10^{-4} mol. Asterisks denote a significant ($p < 0.05$) difference from the water control; bars indicate SE.

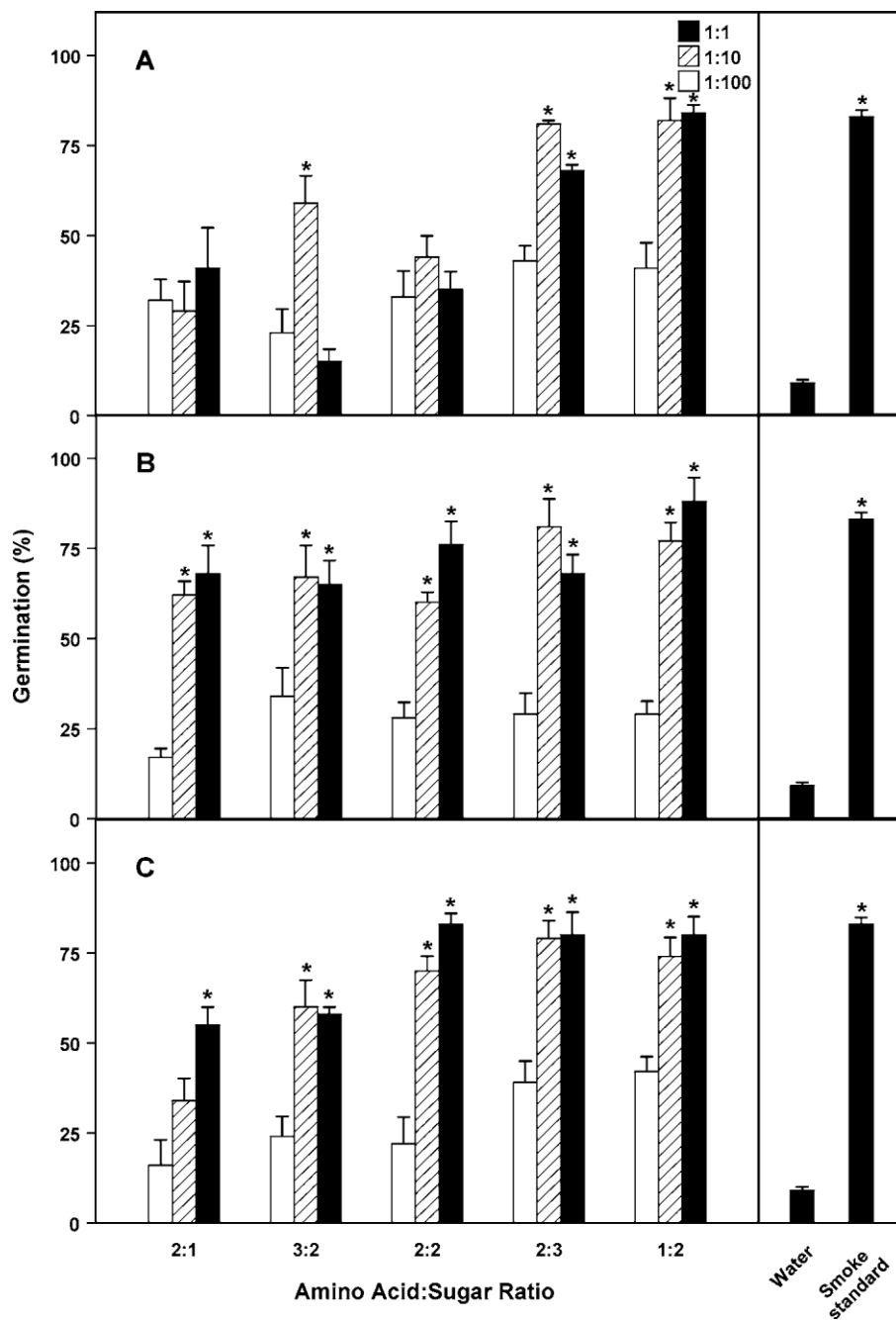


Figure 4. Effect of extracts prepared from heating different ratios of (A) arginine, (B) asparagine, and (C) aspartic acid with glucose at 180 °C for 30 min on the germination of light sensitive Grand Rapids lettuce seeds in the dark ($1 > 3.75 \times 10^{-4}$ mol). Asterisks denote a significant ($p < 0.05$) difference from the water control; bars indicate SE.

were clear, while others ranged from yellow or orange to dark brown in color. The color of the extract did not appear to correlate with germination activity. The germination activity was not related to the pH of the extracts, which ranged from 2.14 to 8.87. At lower concentrations (e.g., 1:100 dilution), the pH differences were negligible. The reactants used for the synthesis of the seed germination stimulant(s) were all heated individually in control experiments. The extracts obtained in these controls did not promote germination.

The extracts prepared from heating purified proteins with glucose resulted in a marked germination response of the light sensitive Grand Rapids lettuce seeds when germinated in the dark at 25 °C (Figure 2). Further experiments using common amino acids in combination with glucose were conducted. Germination of the lettuce seeds was enhanced by a number of

extracts prepared using a 1:2 molar ratio of amino acid and glucose (Figure 3). In particular, extracts prepared from arginine, asparagine, aspartic acid, glycine, and tyrosine gave high levels of germination. In contrast, an extract prepared from cysteine and glucose resulted in germination levels below that observed with the water control (i.e., inhibition of germination). These results indicate that the compound(s) responsible for germination stimulation can be formed from the amino-carbonyl reactions of a variety of amino-containing compounds with glucose. These “model systems”, however, are known to form a highly complex mixture of reaction products (23). It is possible that some of these reaction products may have an inhibitory effect (as observed with cysteine and glucose). As a result of the complex mixture of compounds formed during the reaction, the presence of compounds which promote germination may

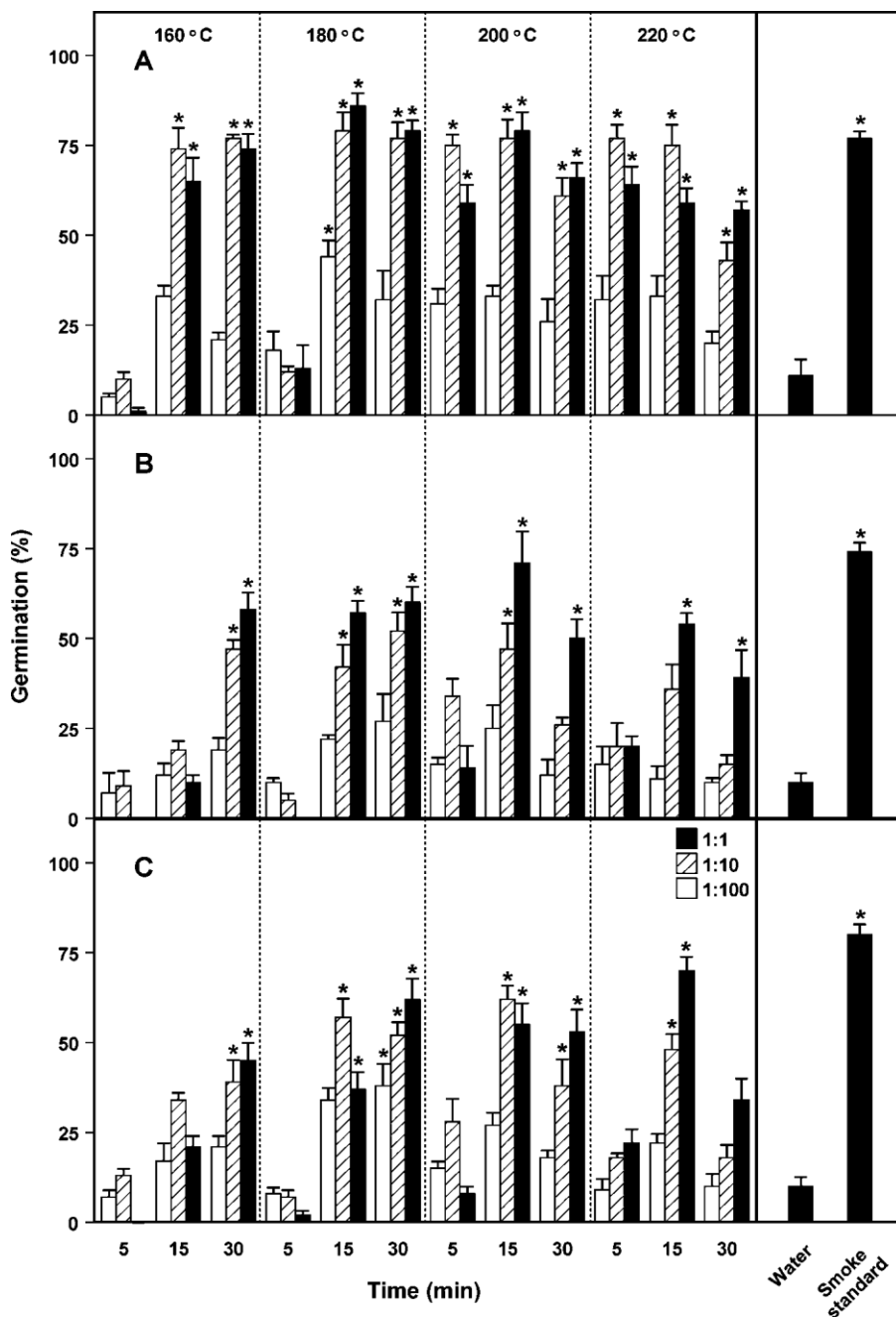


Figure 5. Effect of extracts prepared from heating (A) arginine, (B) asparagine, and (C) aspartic acid with glucose at different time and temperature combinations on the germination of light sensitive Grand Rapids lettuce seeds in the dark. Amino acids reacted with glucose in a molar ratio of 1:2, where 1 > 3.75 $\cdot 10^{-4}$ mol. Asterisks denote a significant ($p < 0.05$) difference from the water control; bars indicate SE.

be counteracted for by the presence of inhibitory compounds. It is thus difficult to fully evaluate the quantitative amount of the germination promoter from the observed germination results, although the lettuce seed bioassay is useful for demonstrating the presence of the germination promoter.

The results obtained with different amino acid and sugar ratios indicated that a higher proportion of glucose resulted in extracts, which gave higher levels of germination (Figure 4). Extracts prepared from a 2:1 molar ratio (i.e., higher amino acid) generally gave lower levels of germination. Following these results, all further experiments were conducted using a 1:2 amino acid-to-sugar ratio.

Reactions at different time and temperature combinations confirmed that the active products are formed between 160 and 220 °C (Figure 5). At 160 and 180 °C, a 5 min reaction time

was insufficient to allow for production of the germination stimulant. The 5 min reaction at 160 °C did not result in the formation of the brown insoluble “foam”, as observed for the other reaction conditions. The extracts from this reaction condition were much paler in comparison to extracts prepared at 160 °C for a longer reaction time or for 5 min at the higher temperatures. In general, however, an increase in reaction time, at each temperature, resulted in a decrease in coloration of the extract. As mentioned earlier, the color of the extract did not appear to relate to the germination activity. At 220 °C, a longer reaction time (30 min) resulted in lower levels of germination in comparison to extracts obtained following a 15 min reaction. Jäger et al. (14) observed a similar loss in germination activity from extracts prepared by heating dry leaves of *T. triandra* at this temperature. Following these results, experiments were

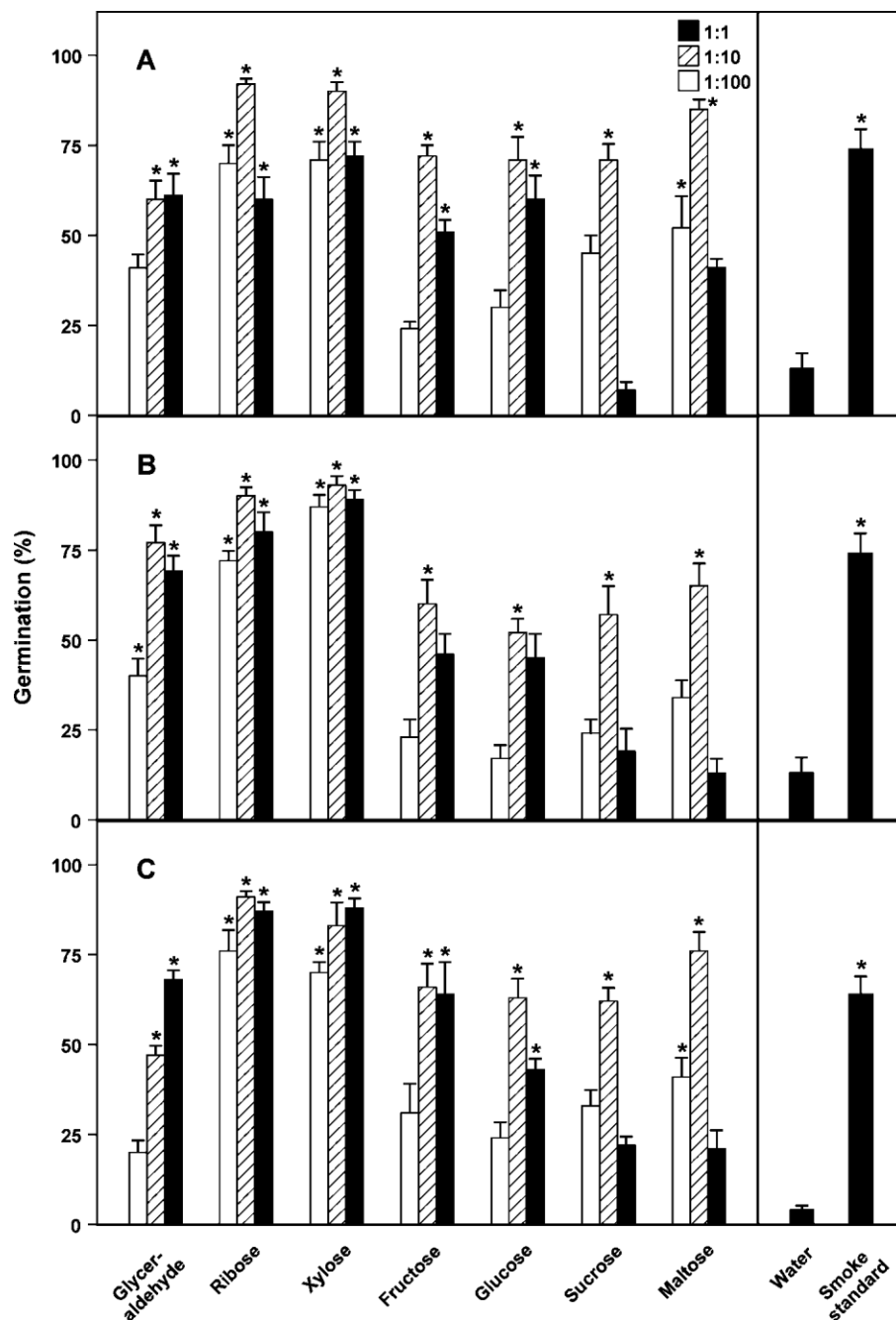


Figure 6. Effect of extracts prepared from heating (A) arginine, (B) asparagine, and (C) aspartic acid with different sugars on the germination of light sensitive Grand Rapids lettuce seeds in the dark. Amino acids reacted with sugars in a molar ratio of 1:2, where 1 \times 3.75 \times 10⁻⁴ mol. Asterisks denote a significant ($p < 0.05$) difference from the water control; bars indicate SE.

conducted at 180 °C for 30 min. For reactions conducted in the custom-made apparatus, a higher reaction temperature of 220 °C was found to be more suitable, and volatilized compounds were collected in a water trap.

The results of reactions of arginine, asparagine, and aspartic acid with different sugars are given in **Figure 6**. Results indicate that the active compound(s) can be formed from reactions between a number of amino acids and different sugars. All of the sugars tested, in combination with these amino acids, gave a germination response, although the extracts prepared from D-ribose and D-xylose (both aldopentose sugars) gave the greatest response. Similar trends were observed with glycine, serine, tyrosine, and valine (results not shown). The combination of the tetrose sugar D-(–)-erythrose with glycine, however, showed no significant increase of germination above that of the water control (results not shown). Extracts were prepared

from reactions of glycine or aspartic acid with D-(–)-mannitol or D-(–)-sorbitol. These combinations of amino acids and sugar alcohols as reactants did not result in the formation of the type of “brown foam” that was observed when amino acids were heated with sugars and did not show improved germination above that of the water control.

The extracts prepared for HPLC analysis all exhibited germination activity in fractions eluting at 20–21 min. This is the same retention time at which the active fraction of the purified smoke solution eluted (12). The bulk extract prepared from heating glycine and xylose at 220 °C in the modified oven and purified using HPLC also showed germination activity at the same retention time (20–21 min) as the active compound isolated from the plant-derived smoke solution (12). Although in the HPLC analysis, this fraction appeared to contain only one pure active principle, GC-MS analysis, in which a universal

and highly sensitive detection device is used, gave a total ion chromatogram (TIC) that revealed the presence of a large number of constituents. A sample of the germination cue from plant-derived smoke, 3-methyl-2H-furo[2,3-c]pyran-2-one (12), was available for GC-MS retention time comparison. Locating the active principle in the synthetic product under investigation was further simplified by plotting reconstructed single ion chromatograms for the two most abundant ions, m/z 121 and 150, in the spectrum of the plant-derived germination cue. The compound eluting at the same retention time as the plant-derived active principle has a mass spectrum containing the diagnostic ions m/z 150 (65), 149 (9), 122 (20), 121 (100), 105 (3), 94 (9), 93 (7), 75 (3), 74(3), 66 (18), 65 (26), 51 (8), 50 (7), 39 (35), 38 (8), 29 (8), and 27 (3%). Critical evaluation of the results of the present investigation against the background of our previously published results (12) reveals that the germination stimulant isolated from plant-derived smoke and the germination stimulant prepared from glycine and xylose elute at the same elution volume in HPLC, elute at the same retention time in GC when coinjected, and have identical mass spectra, proving without doubt that the compound prepared by heating a mixture of xylose and glycine is identical to the recently identified germination cue, 3-methyl-2H-furo[2,3-c]pyran-2-one. Furthermore, it shows that the germination stimulant in plant-derived smoke can also be synthesized from mixtures of certain amino acids and sugars. It is interesting to note that hemicelluloses, which are abundant in both primary and secondary cell walls of plants, are polymers of sugars such as arabinose, mannose, and xylose, the pentose sugar, which showed a good germination response when heated in combination with certain amino acids. The germination effect observed with heating cellulose and hemicelluloses (14, 19) may possibly be attributed to trace impurities in the starting materials.

During a wildfire, the soil, which contains some organic matter, would also be heated to high temperatures, possibly resulting in the production of germination-stimulating compounds. Soil temperatures during fires depend on the depth and type of fuel but can be as high as 600 °C at the surface and between 50 and 225 °C at depths of 2 cm (24, 25). Extracts from soil heated at 195 °C for 10 min were shown to stimulate the germination of seeds of two chaparral herbs (26).

The chemistry of Maillard reactions and pyrolysis reactions is certainly extremely complex. Although 3-methyl-2H-furo[2,3-c]pyran-2-one has not been previously identified in Maillard reaction products, it is of interest that we have observed germination stimulation in the lettuce seed bioassay with extracts of instant coffee and toasted bread. The results presented in this study provide further information on the germination cue found in smoke that is produced during wildfires from ubiquitously occurring organic compounds.

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