Aqueous smoke as a germination enhancer of *Arabidopsis thaliana* seeds

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Abstract

In this experiment, we investigated the effects of smoke-water extracts on the germination rate of *Arabidopsis thaliana* seeds of the Columbia (Col) ecotype. Smoke from burnt plant material contains chemicals called karrikins, a group of plant growth regulators found to potentially affect the germination rate of *Arabidopsis thaliana.* We applied smoke-water extracts to seeds as pre-germination treatments to determine if karrikins, presumed to be present in the smoke-water extract, could increase the rate of seed germination in *Arabidopsis thaliana.* The procedure consisted of generating a smoke-water extract by bubbling smoke (created by combusting xylose and glycine) through distilled water at a controlled flow rate, and then applying different dilutions of the smoke-water extracts to seeds before germination. Results suggested that there were significant differences in germination percentage (%) between the 1:10 smoke treatment and the water control after 5 days of incubation at 17°C with a 14 hour photoperiod. Our analyses revealed that *Arabidopsis thaliana* seeds can be forced to germinate faster when treated with specific dilutions of smoke-water extracts. The *Arabidopsis thaliana* seeds treated with the second highest concentration of smoke extract (1:10 dilution) used in this study demonstrated the fastest initiation rates of germination and photosynthesis.

Introduction

There have been few studies done on the stimulatory effects of smoke on seed germination and the processes which regulate this stimulatory effect. A variety of external stimuli (light, temperature and pH) affect seed germination. Seeds remain dormant until optimal environmental conditions occur, consequently giving them the best chance of survival. One of the main sources of smoke in the natural environment is forest fires. Forest fires are important natural disturbances which help keep the biodiversity of the ecosystems stable (Moretti *et al*. 2004).

Smoke generated from the burning of vegetation creates karrikins (KAR1, KAR2, and KAR3), which are plant growth regulators. The smoke derived from burnt plants has been shown to stimulate seed germination in over 1200 species from 80 genera. Smoke can be made from a wide variety of plant materials; straw, wood, or a mixture of dry and fresh plant material (Chiwocha *et al*. 2009).

The discovery of the germination effect of karrikins on *Arabidopsis thaliana* is considered a major breakthrough, because scientists are just discovering that *Arabidopsis thaliana* responds positively to smoke and this has never been realized before now (SeedQuest 2000). Furthermore, this area of research could bring economic benefits in the agricultural, environmental rehabilitation and horticultural industries. The karrikin compounds have been found to be more effective for stimulating seed germination than regular plant growth regulators like steroid and abscisic acid (Nelson *et al*. 2009). Using naturally occurring plant-growth stimulating compounds like karrikins to trigger germination may become an organic and possibly more cost effective method for encouraging uniform germination in many plant species. At this time, the molecular mechanism of karrikins in stimulating germination and the sensing mechanism of karrikins for *Arabidopsis thaliana* are not fully understood. However, the results could potentially be beneficial in terms of understanding and developing a similar process for other plant species. Therefore, we chose *Arabidopsis thaliana* for our study since much is already known about *A. thaliana* and studies have already proven some effects of karrikins on *A. thaliana* (Nelson *et al*. 2009).

Seed germination in *Arabidopsis thaliana* undergoes a process called 2 step seed germination.. The seed germination process starts with the uptake of water by a dry seed and ends with elongation of the embryo. During the process of seed germination, several metabolic processes, such as photosynthesis, are activated (Bewley 1997). First, the seed coat called the testa ruptures after 6 hours of incubation under continuous light. Second, 12 hours later, the advanced testa ruptures and the endosperm rupture is seen after 18 hours of incubation (Leubner 2000). The endosperm is the surrounding barrier of the embryo that prevents the emergence of radicle (the embryonic root which grows downwards into the soil) (Bewley 1997). The plant hormone Abscisic Acid (ABA) inhibits the endosperm rupture while Gibberellin Acid (GA) promotes rupturing (Leubner 2000).

In this study we asked the question: Is there a difference in germination response with wild-type *Arabidopsis thaliana* when seeds are treated with different concentrations (undiluted, high(1:10), medium(1:100) and low(1:1000) dilutions) of aqueous smoke-extract?

Ho: The seeds treated with low concentrations of aqueous smoke solution will germinate slower, or there will be no difference in germination response to the different concentrations of aqueous smoke treatments.

Ha: The seeds treated with low concentrations of smoke water will germinate faster than the seeds treated with highly concentrated, undiluted smoke water.

Methods

Water is the standard germination trigger in seeds, and therefore was used as a control. Three replicates of 10 seeds were included in each experimental and control treatment. The treatments consisted of low (1:1000), medium (1:100), high (1:10), and undiluted concentrations of aqueous smoke solution. We diluted the undiluted solution with distilled water using a dilution series method. Smoke was generated from the combustion of xylose and glycine as described by Flematti *et al*. (2011). All treatments were done using wild-type *Arabidopsis thaliana* seeds. For all treatments, seeds were exposed to the same duration of treatment, light intensity, moisture, and temperature.

The first day, we generated the aqueous smoke-extract over 3 hours. We weighed out 2.40g of xylose and 0.60g of glycine, then mixed them together using a mortar and pestle. This mixture was added into a 3 necked round-bottom flask that was attached to air tubing on both ends (Figure 1). One end led into the bottle of distilled water and the other was used for ventilation by a 1.2W aquarium pump, which was regulated by a simple valve.

The sand bath was raised to the flask once the temperature reached the range between 170-216°C. Air from the reaction flask was bubbled at an average flow rate of 61 mL/min through 150mL of distilled water. The white powder (of mixed xylose and glycine) started to caramelize and turn a liquid dark brown, almost black colour within a minute of contact with the heated sand bath which exceeded 220°C. The temperature was lowered and raised regularly until the sand bath stabilized below 180°C at 21 minutes into the reaction. There was moisture generated in the flask as combustion of the materials produced water. The combustion also produced carbon dioxide, which made the smoke combustion also produced carbon dioxide. This carbon dioxide made the smoke extract more acidic (pH=5) than distilled water (pH=6.5), as measured with pH paper (Figure 2).

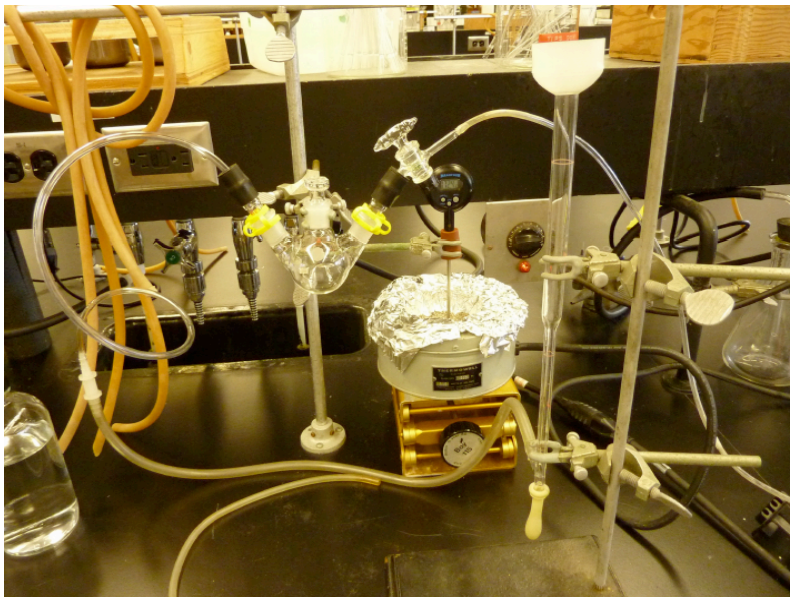


Figure 1: assembled combustion apparatus

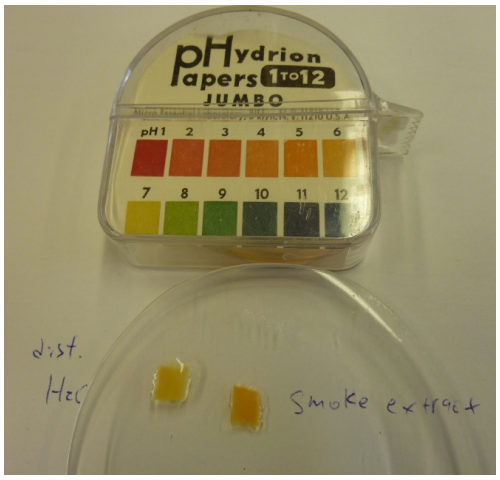


Figure 2: pH difference of 1.5 observed between distilled water control and our smoke extract.

Figure 3: smoke being bubbled through distilled water at ~61mL/min.

We let the xylose/glycine mixture melt and darken for 30 minutes. The distilled water which the smoke was bubbled into did not change colour (Figure 3), but it did have a unique smell (somewhat sweet and slightly smokey).

After the smoke extract was made, we did a dilution series to generate the appropriate dilutions (1:10, 1:100, 1:1000) .The resulting smoke water was considered our fully concentrated stock solution. For the 1:10 dilution, 3 mL of the stock solution was diluted with 27mL of distilled water to a final volume of 30 mL. Then, 3mL of the 1:10 dilution was diluted with 27 mL of distilled water to make the 1:100 dilution. To make the 1:1000 dilution, 2mL of the 1:100 dilution was diluted with 18 mL of distilled water to a final volume of 20 mL. All the solutions were sealed and put into the fridge for storage at 4°C over night.

The second day we treated the seeds with the smoke-extract solutions. Our Columbia type *A.*

thaliana seeds were harvested and dried late October of 2011 (less than a month old) and stored at 4 degrees Celsius. For each treatment and the control, we used 2mL of each treatment solution to moisten two sheets of Whatman no. 1 filter papers in separate 60 mm Petri dishes. Ten seeds in each Petri dish were then imbibed between the filter papers. We sealed the Petri dishes with parafilm and incubated them for 24 hours at 25 °C in the dark.

For germination, we used paintbrushes to transfer seeds into Petri dishes with two sheets of Whatman no.1 filter paper. The seeds were evenly distributed on top of the filter papers. Then 2mL of tap water was added to each Petri dish. The tap water added minerals the seeds need.

The Petri dishes containing all seed treatments were incubated at 17°C with 14 hours of light and 10 hours of dark to germinate. Seed germination was monitored regularly and the total number of seeds germinated. We moved the Petri dishes around each day to equalize the light exposure.

A seed was considered to have germinated if a radicle emerged. We considered emergence to be a radicle which was fully broken out of the seed coat, as seen through a dissecting microscope at X magnification. The entire germination process took a week to complete.

Green shoots started to emerge on day four (Figure 4), and we started to count them as well. The shoots were much easier to observe as they were usually larger. The radicle was always the first to emerge from the seed coat. Therefore if the seeds had a shoot, it was assumed they had a radicle as well. If only the shoot was seen without a visible radicle, we assumed the radicle was probably growing into the filter

Figure 4: Germination observed on day 4

paper and thus blocked from our view.

Results: Data points graphically joined to visually differentiate germination rates between treatments.

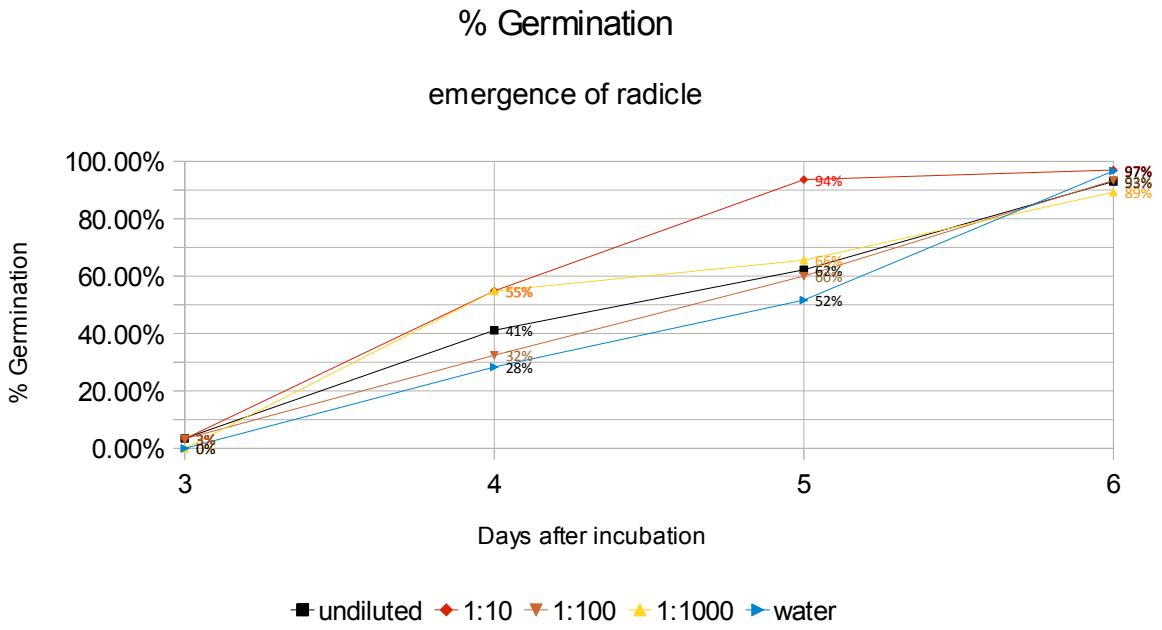


Figure 5: %Germination over time - An overall increase in the percent of germinated seeds as time increases from Day 3 to Day 6. No significant results were observed before Day 3. There is a drastic increase in the 1:10 smoke treatment from Day 4 to Day 5 (55% to 92% germination).

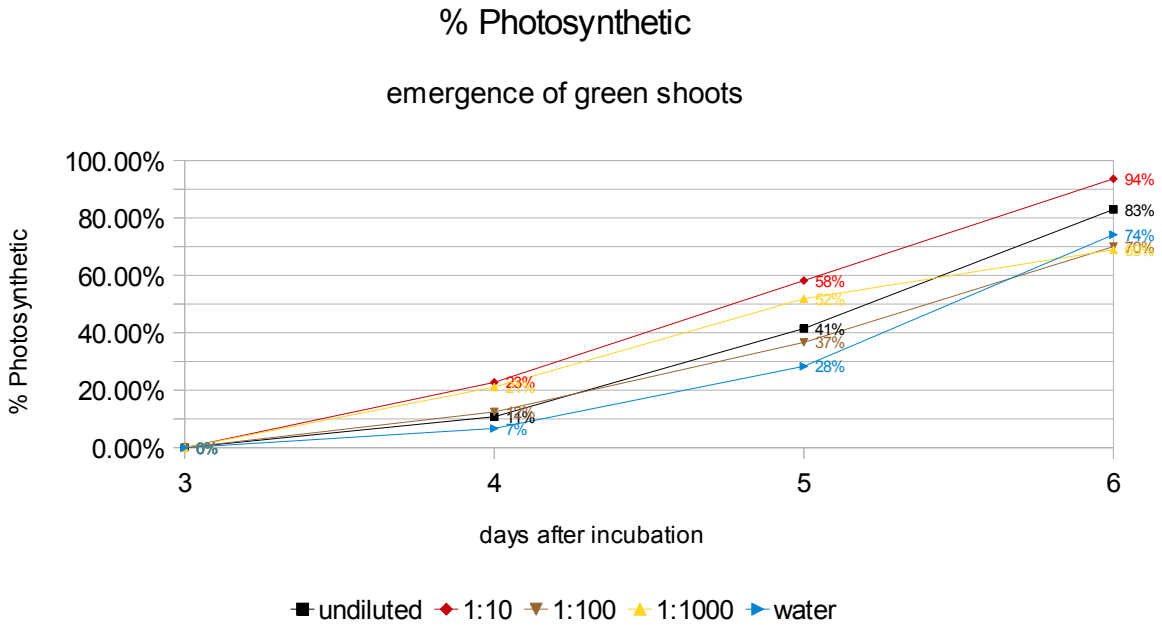
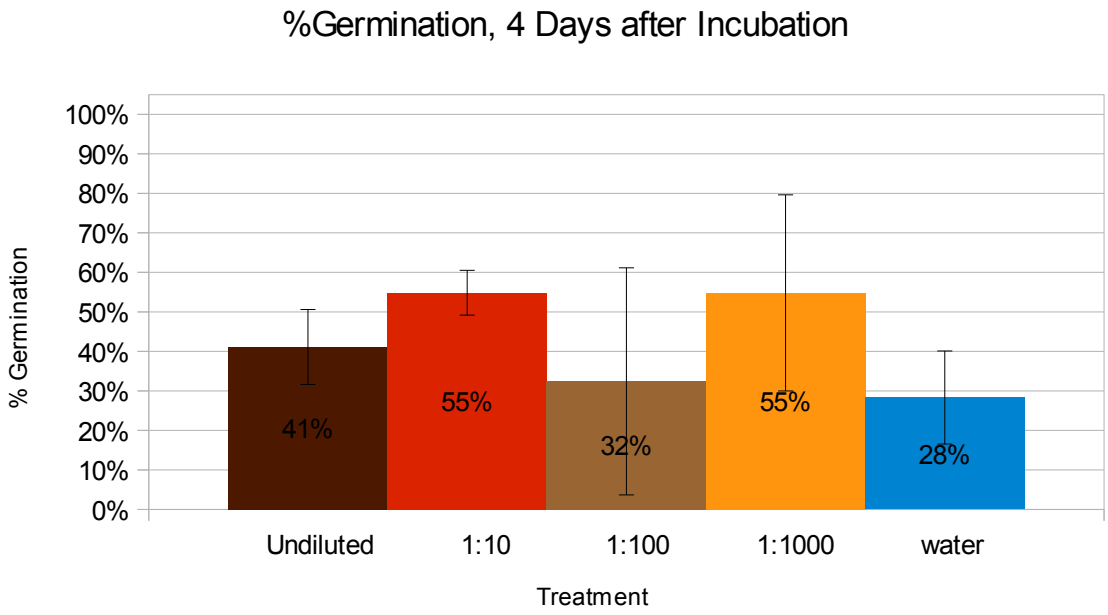


Figure 6: Emergence of green shoots over time - The data shows an almost linear positive relationship between the percent of green shoots appearance to the time. In other words, more green shoots started to emerge as we continued our experiment.

The error bars in all of the following charts indicate the 95% confidence intervals of the sampled means:



Figures 7: %Germination on Day 4 - There seems to be a significant difference in the 1:10 compared to the water control (as the 95% confidence error bars do not overlap) and the 1:10 and 1:100 are not significantly different in their average germination percentages.

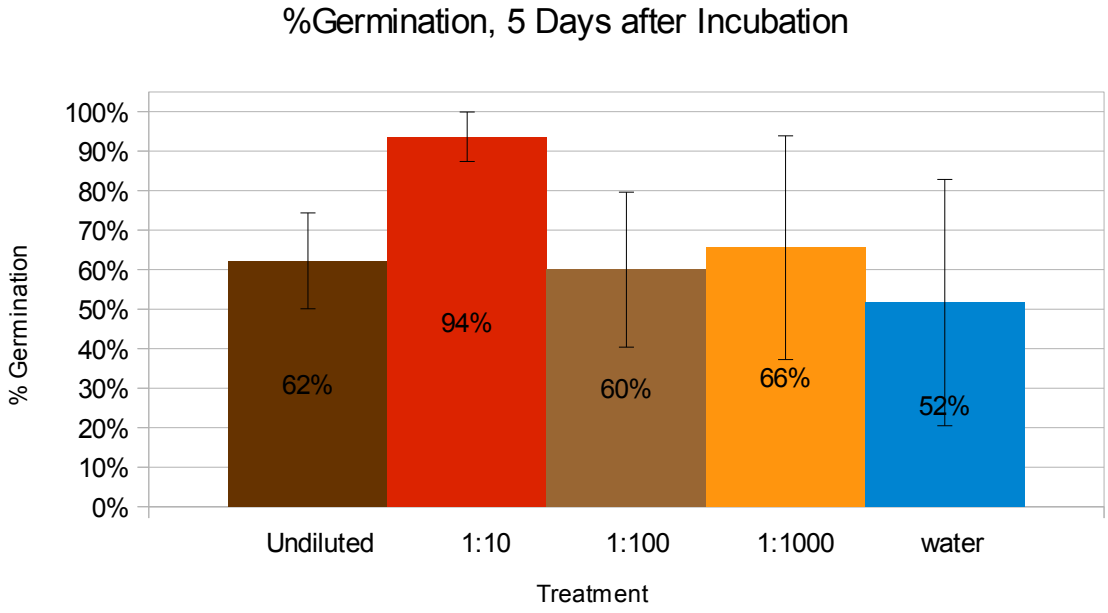


Figure 8: %Germination on Day 5 - The 1:10 is clearly leading in germination percentage. Again there is a significant difference in the 1:10 compared to the water. The water and 1:100 have almost doubled in %germination compared within the last 24 hours between days 4 and 5.

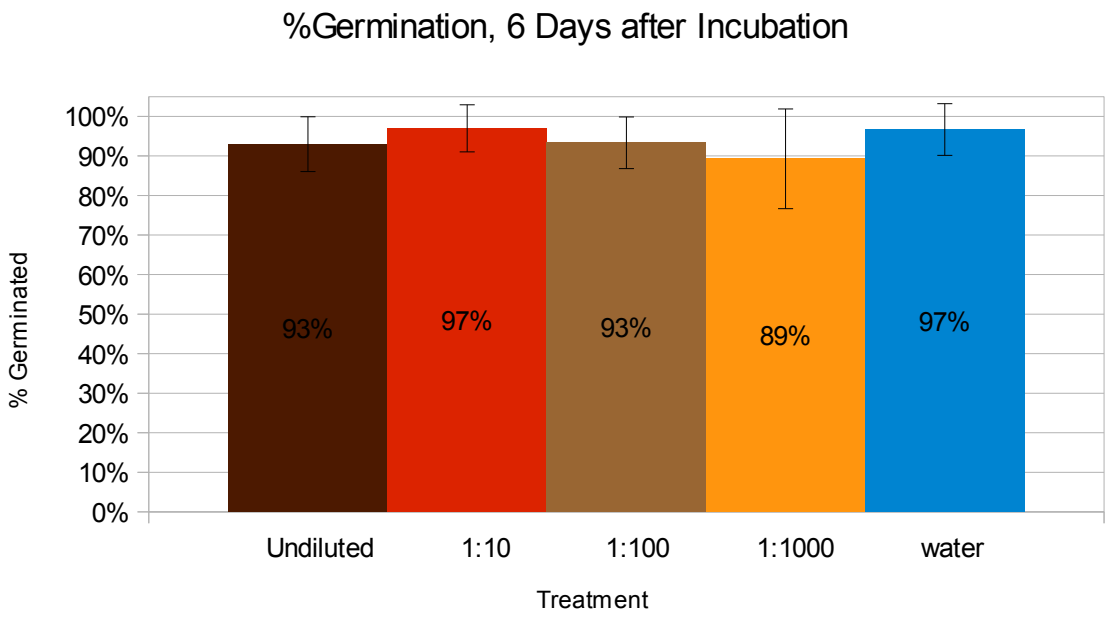


Figure 9: %Germination on Day 6 - There is no apparent significant difference between all of the treatments 6 days after the incubation. All treatment replicates have at least 80% germination.

As for the emergence of green shoots for Day 5 (Figure 10) and Day 6 (Figure 11) after incubation, the 1:10 smoke treatment had more % of seeds with green shoots emerging compared to the other treatments, with water having the least shoot emergence on Day 5 (Figure 10). This trend appears to parallel that of the % germinated by Day 5 (Figure 8) because green shoot emergence follows radicle emergence. There was also a statistical difference in % germination between the 1:10 smoke treatment and the distilled water control treatment.

Sample Calculations

$$\%germination = \frac{seeds\ germinated}{total\ seeds\ of\ treatment} \times 100$$

$$\%\text{germination replicate } 1 = \frac{6}{10} \times 100 = 60\%$$

$$\%\text{germination replicate } 2 = \frac{5}{10} \times 100 = 50\%$$

$$\%\text{germination replicate } 3 = \frac{6}{11} \times 100 = 54.5\%$$

$$\%\ average\ germination\ ,\ Day\ 4 = \frac{(50 + 60 + 54.5)}{3} \times 100 = 54.85\%$$

$$\text{standard deviation (S.D.), Day } 4 = \frac{\sqrt{(54.85-60)^2 + (54.85-50)^2 + (54.85-54.5)^2}}{3-1} = 0.0500688232$$

$$95\%\ \text{Confidence Interval, Day } 4 = 54.85 \pm 1.96 \times \left(\frac{(0.0500688232)}{\sqrt{3}}\right)\ 54.85 \pm 5.67\%$$

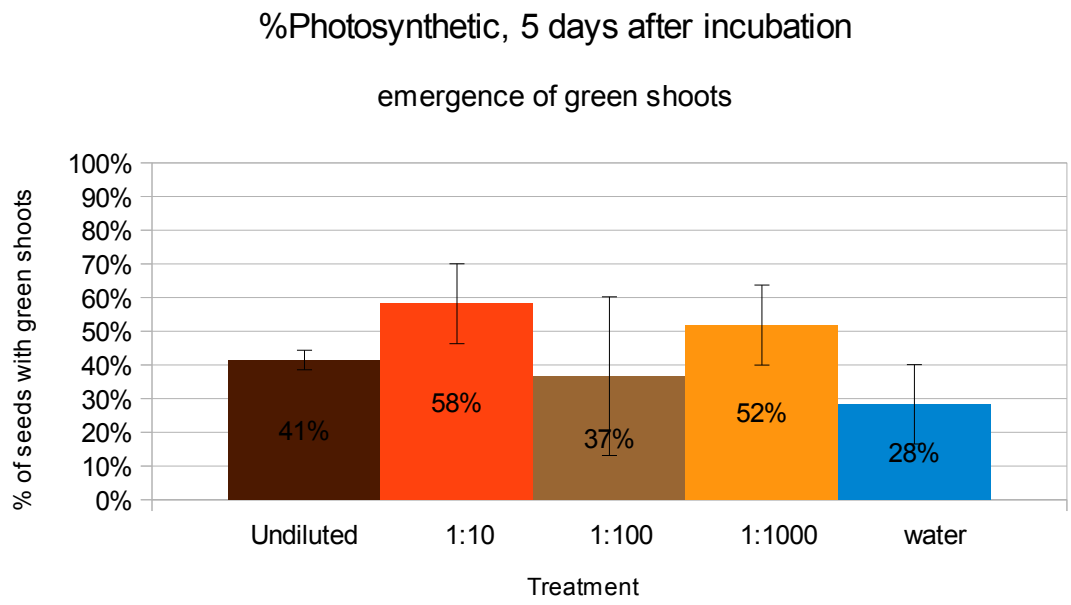


Figure 10: %Photosynthetic, Day 5

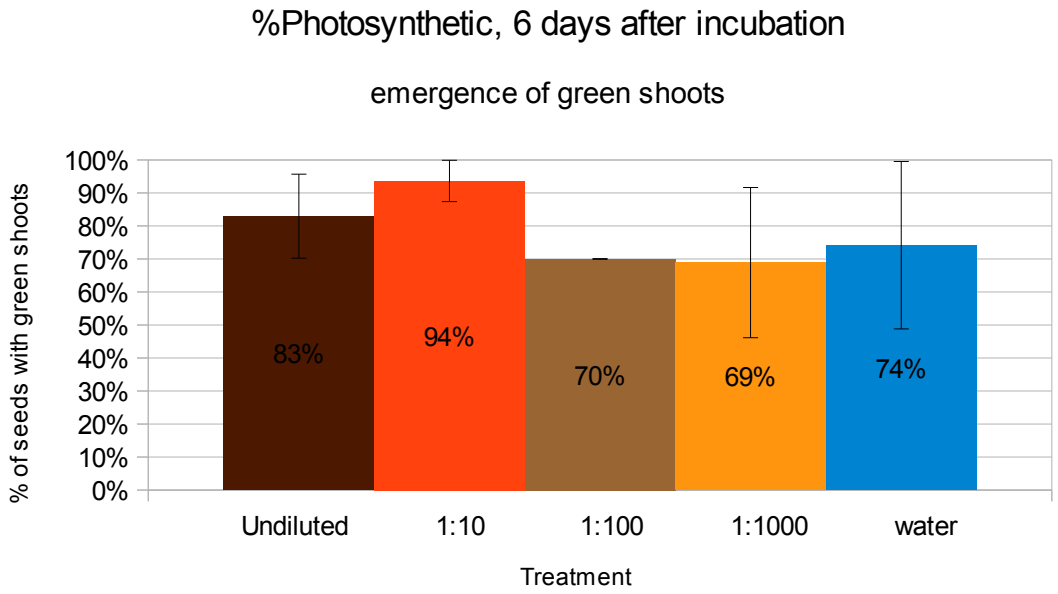


Figure 11: %Photosynthetic, Day 6

Discussion

Data collected on Day 5 for % Germination (Figure 8) and % with shoots on Day 5 (Figure 10) and Day 6 (Figure 11) indicated that the 1:10 dilution smoke-water treatment sped up early germination rates significantly when compared with the negative control (water treatment). These data provide support for the alternate hypothesis, based on the consistent trend of faster germination in seeds treated with 1:10 smoke water, compared to undiluted smoke treatments (Figure 8). The data supports the alternate hypothesis most noticeably when comparing the 1:10 and undiluted smoke treatments on Day 5.

Also observed was an exceptional consistency of average %Germination rates between replicates of the 1:10 smoke-extract treatment, as evident in Figure 7 (day 4), Figure 8 (day 5), and Figure 9 (day 6) by the smaller 95% confidence intervals of the 1:10 treatments. Eventually by the 6th day of incubation, all treatments had the same percentage of germination (Figure 5).

Data from days 4 and 5 of germination (Figures 7 and 8) indicate that the 1:10 smoke extract significantly increased germination when compared with water (control treatment). Exceptional consistency of %germination was observed between the replicates of the 1:10 smoke-extract treatment, evident by the much smaller 95% confidence intervals of the 1:10 treatment on day 4 (Figure 7), day 5 (Figure 8), and Day 6 (Figure 9).

Before day 6 some significant differences in germination and potential for photosynthesis were observed (Figures 7 and 8). This significant effect of the smoke extract treatment could be explained by the fact that *Arabidopsis thaliana* is known to respond positively to karrikins. The presence of karrikins was also found to induce the expression of minor genes like GAoX1, which serves as a signal for GA biosynthesis (Nelson *et al*. 2009).

The 1:10 smoke water treatment promoted early seed germination the most. This might be

explained by the dual regulatory of the karrikins, where they can induce but at higher concentrations can also inhibit the germination of seeds (Light *et al.* 2002).

A. thaliana seeds require light and the GA synthesis in order to germinate successfully. The Gibberellin (GA) signalling pathway controls the coat-dormancy release mechanism. Three Gibberellin Insensitive Dwarf1 (GID1) GA receptors have been found in *Arabidopsis thaliana* (GID1a, GID1b, and GID1c). The GA signalling from GID1 receptors is necessary for *A. thaliana* seed germination. The seed coat weakening is mediated in part by the GA- induced genes which encode for cell-wall modifying proteins. There are many genes that are turned on in this GA signalling pathway during early germination (Voegele *et al*. 2011), the same mechanism that is triggered by karrikins (Nelson *et al.* 2009). This could explain the early germination observed in the 1:10 smoke-water treatments.

Our results show a faster rate in early germination in the smoke water treatments. This correlates with Nelson et al. (2009) who exposed seeds to three kinds of karrikin molecules known for promoting germination (KAR1, KAR2 and KAR3). Gene expression occurred as a response to the exposure of KAR1 (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one), also known as karrikinolide. Recorded data showed that gene expression can occur as early as 6 hours after application (Nelson *et al.* 2009). Early gene expression induced genes involved in the GA biosynthesis pathway to be made earlier and thus speeding up germination. We can therefore support that the smoke extract generated by the methods described by Flematti *et al*. (2011) do contain karrikin-like chemicals that promote *Arabidopsis thaliana* seeds to germinate earlier than seeds treated with water. This phenomenon was also observed in the experiment performed by Nelson and colleagues (2009).

Overall, karrikins are effective at stimulating germination but it is not a direct cause and effect relationship between the exposure of seeds to karrikins and their increased germination rate (Flematti *et al*. 2004). Other factors need to be taken into consideration, like other potential sources

of karrikins in the environment. Some plants may already have a built-in karrikin signalling system; many seeds will recognize the presence of karrikin and may be triggered to germinate. Lastly, karrikins may be plant hormones already present in some plants that are just waiting for stimulation (Nelson *et al*. 2009). Light could have been a factor in influencing rates of germination and photosynthesis initiation. However, care was taken to ensure even and random light distribution over the Petri dishes containing the germinating seeds, which were moved daily before being replaced in the designated incubation location as a measure for negating the influence of differences in light exposure in our experiment. So we believe that the differences in seed germination were in fact due to differences in pre-germination treatments.

Another interesting experiment could look into how the presence of germination inhibitors in smoke-water affects net germination. In some experiments, seeds were rinsed before germination to get rid of those inhibitors (Light *et al.* 2002). Therefore diluting the smoke-extract reduces the active level of germination inhibitors present (Brown *et al*. 2000) which might explain why the 1:10 had a faster germination rate than the undiluted treatment. If that were true then it would mean that the optimum balance of germination promoting and inhibiting effects was somewhere between the undiluted and 10% concentration for the smoke-water extract generated in this study.

There were multiple sources of error while carrying out our experiment. The static of weigh boats made it so we could not transfer all of the xylose and glycine into the flask. Then more was lost during the dry mixing using mortar and pestle, and also when transferring the xylose and glycine into the flask with a funnel. Therefore, the amount of that we weighed did not all go into the experiment entirely. We also could not get the air flow to be completely steady and the temperature fluctuated over quite a large range while we were doing the smoke extraction. The fluctuations in the temperature and the air flow means that reproducing the exact procedure would be very difficult.

Transferring the seeds could have damaged the seat coat, which induces the germination through scarification. As a result, the germination rate could be higher than expected. Accidental loss or addition of seeds during their physical transfer after they were imbibed with the smoke extract could also have affected our results. Specifically, when seeds were misplaced into the wrong Petri dish as this could decrease or increase the total germinating seeds due to the fact that they were treated with a different smoke solution dilution.

The natural variability within our seed population should be taken into account when evaluating our results. Accounting for genetic variability means that the seed variety, in our case the Columbia ecotype, could affect how seeds respond to smoke treatments compared to other type of seeds. This particular ecotype offers a low dormancy and therefore more immediate germination compared to other wildtype *Arabidopsis thaliana* ecotypes. Seeds that exhibit more dormancy might show more differences between smoke-water and control treatments. Furthermore, seeds that are dormant due to age or storage conditions might be forced to germinate using smoke-extracts generated by similar methods. This seed pre-germination treatment can be utilized by the scientific community to speed scientific advancement, by lessening the time it takes to germinated particular species of plants.

Conclusion

The 1:10 promoted seed germination the most. This might be explained by the dual regulatory of the karrikins, where they can induce but at higher concentration can also inhibit the germination of seeds (Light *et al*. 2002).

We can conclude that there was an increased rate in the early stages of germination, but in the later stages all the seeds, including the water control, all treatments had achieved a similar rate of

germination. This might be due to the fact that the seeds did not have extreme dormancy behaviour. If another study was done using seeds of a species that possessed more dormancy, there might have been more differences in overall germination rates between the different treatments.

The varying rates of germination were most likely due to varying activity levels of germination inhibitors present in smoke extract. The 1:10 solution provided the optimal stimulation of the seeds to germinate compared with the other dilutions.

Acknowledgements

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