FastStart™ Final Report

A preliminary study on the effect of cold plasma treatment on cotton seed imbibition and germination

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Summary

Successful and further improved germination of cotton seeds in a range of environmental conditions is of high importance to the cotton industry. In this report we discuss the results of a pilot study investigating the germination of cotton seeds and other results following a plasma treatment using a laboratory-scale dielectric barrier atmospheric-pressure (or cold) plasma system.

In this study we use six different tests to assess the effect of plasma treatment on black cotton seeds. Plasma treatments are shown to statistically significantly (90% level) improve germination in the 4-day warm germination test and in the metabolic chilling test. While the other tests were not statistically significant, there were clear trends for the plasma treatments to also improve cold germination, seed imbibition of water, and the cool warm vigour index.

In the 4-day warm germination test the 27 minutes air plasma treatment improved germination from 75% for the control to 90%, and improvements persisted until 10 days of measurement. The metabolic chill test, a measurement of seeds to overcome cool

conditions over a long period, was poorer in the 3 minute air plasma treatment (down to 32% from 52% in the control). However, the 27 minutes air plasma and Argon treatments improved germination with both treatments exceeding 70% up from 52% in the control. Based on the cold tolerance classification by Deusterhaus [1] these two plasma treatments re-class the seeds from 'fair' for the control to the 'good' category.

This study particularly shows that cold plasma treatments of seed can be successfully applied without reducing seed germination properties. Importantly, there is strong evidence to suggest that across all germination tests in this study a 27 minutes air plasma treatment may improve both warm germination and cold tolerance.

Our measured improvements of the plasma treated seeds are of commercial significance to the Australian cotton industry through potential increases in plant establishment in a wider range of seedbed conditions

Seed germination is still favourably affected by plasma treatment despite a three months delay between the plasma treatment and the germination tests. This suggests that in future seed plasma treatments can be done at an industrial scale 'long' before planting and hence making the need for plasma treatment on the farm just before planting unnecessary.

This study does not aim to explain the specific mechanisms which cause the germination differences between plasma treatments.

Further assessment of the plasma treatment approach would benefit from:

- A larger/confirmation study using more seeds and more uniform seeds.
- An assessment of several air plasma treatments ranging from 3 to 27 minutes.
- An assessment against other existing seed treatments.
- A detailed assessment of the changes in seed coat properties that led to differences in germination outcomes.
- An assessment of seed decontamination when using cold plasma (from mould, fungi, and bacteria).

Introduction

Germination and seedling stages are critical for healthy plant growth. The presence of pathogens, water stress and other abiotic stresses (temperature and salt) at these stages can affect the successful establishment of crop. Plasma treatment of seeds is emerging as a new technology platform to address these issues.

Plasma is a discharge of gas molecules consisting of energetic ions, electrons and neutral species. Preliminary investigations so far have confirmed that the low-temperature plasma pre-treatment of seeds of important agricultural crops is an effective tool for improvement of germination, shoot and root growth. The plasma treatments provided good fungicidal and bactericidal effects, increased water permeability through surface coat etching and stimulation of germination and seedlings growth [2-7]. These desirable characteristics have resulted from surface modification by the charged particles and neutral radicals formed in

plasma. Such morphological changes on seed surfaces are safe and unlikely to have any genetic impact.

Reducing pathogens on seeds and improving germination, plant growth and crop yield, are becoming ever more important as the world needs more and more food and fibre crops. Planted seeds require the highest probability of survival and plants need to grow as efficiently as possible. Currently there are many approaches, such as treatment with chemicals that aim to help achieve this. Plasma treatment of cotton seeds is a new approach that is being proposed to assist germination and survival.

In a preliminary CSIRO funded pilot study (i.e. before the present studies I and II) we treated cotton seeds with a laboratory-scale dielectric barrier atmospheric-pressure (or cold) plasma system. The treatments were conducted using air for a range of treatment times. The treatments on cotton seeds show that with increased treatment time, the cotton seed surface becomes more hydrophilic (water attracting) and that the rate of water absorption of surface droplets increases. In another preliminary study plasma affected imbibition. This may have beneficial effects on seed germination under normal and stressed conditions.

A description of the plasma system is contained in the appendix of this report and a more general background information on plasma agriculture may be found in our recent literature survey[8]

Materials and Methods

The project examines the effect of cold plasma treatment on cotton seed germination and related tests. For this project we have conducted two separate cotton seed plasma treatment studies – Studies I and II. The plasma treatments were conducted in Lindfield and the germination and other tests were conducted in Narrabri. All treatments used black seed of the commercial cotton variety Sicot 74BRF taken from the same batch and all plasma treatments were replicated four times (7 seeds per rep in Study I and 15 seeds per rep in Study II).

Plasma Treatments (Studies I and II)

Study I

We commenced the first study using (dry) air as the feed gas, as air will be the easiest and cheapest gas to use for future commercial plasma applications. The air flow rate and voltage and frequency remained constant. We used three different treatment times and one control. For the first plasma treatment study, around Christmas 2014, we used a small plasma chamber that could only hold seven cotton seeds. Treatments were:

- **A** -control,
- **B**-20 seconds dry air,
- **C**- 1 minute dry air,
- **D**- 3 minutes dry air.

Study II

For the second plasma treatment study, around Christmas 2015, an upgraded chamber holding 350 seeds was developed. The first study needed to be repeated as the germination results were not statistically significant due to too low seed quantities. This much larger second study was with dry air with two different treatment times, and with Argon plasma with one treatment time. Both gasses are cheap with regards to possible future commercial scale application.

In preparation for this larger study the most suitable plasma treatments for this study needed to be determined. We examined the change in surface properties with plasma treatment of the cotton seed surface by measuring the water absorption times of 2 µl tap water drops and by measuring the water contact surface area of these drops on the seeds (Figure 1). The water contact area method was used instead of contact angle measurement, a well-established method for determining surface hydrophilicity and hydrophobicity. Contact angles are not used as they proved to be too unreliable to measure on the irregular curved surfaces of the seeds.

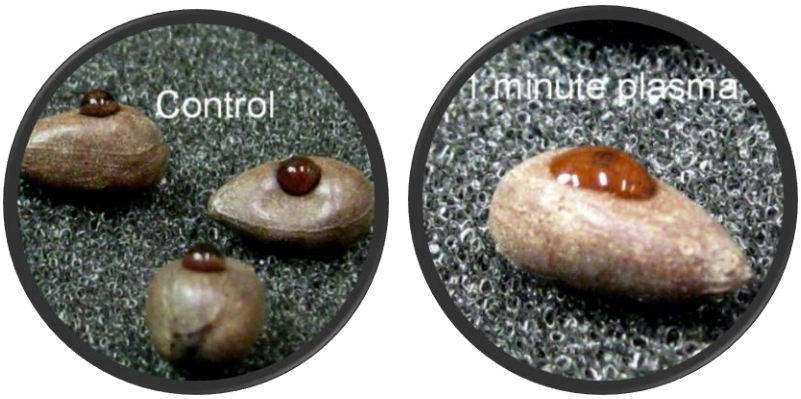

Figure 1. Water drops on plasma treated seeds (using a previous smaller plasma system, not from Study II). The plasma treated surface is more hydrophilic.

Twelve different plasma treatments, 6 in dry air and 6 in Argon, were investigated. From the 12 plasma treatments, 3 final treatments were selected in such a way that between these (as well as the control seeds) there was a maximum difference in water absorption time and in wet contact surface area. Assuming that these surface properties are related to germination outcomes, these 3 plasma treatment scenarios should give the highest probability of finding a (near) optimal plasma treatment for the cotton seeds.

The plasma treatments for this second study were:

- **V1** - control seeds,
- **V4** - 3 minutes dry air,
- **V6** - 27 minutes dry air,
- **W7** - 81 minutes Argon.

Each specific plasma treatment was repeated 5 times, so that for each specific treatment there were approximately 160 g (approximate 1600 cotton seeds) of cotton seed available for tests at CSIRO Narrabri. These seeds were plasma treated in December 2015 and the actual germination and other studies were conducted in March 2016, indicating that there was a three months period between the plasma treatment and seed planting/testing.

We believe there were no serious issues with too high seed surface temperatures during the plasma treatments.

Germination Studies

For each plasma treatment seed was subjected to the following tests:

1) Warm germination test – Seeds germinated at 30^{o}C in the incubator and germination percentage and seedling length are measured at 4, 7 and 10 days.
2) Cold germination tests –Seed germinated at 14^{o}C in the incubator and germination percentage and seedling length are measured at 4, 7 and 10 days.
3) Metabolic chill tests – Seeds are planted in sand in trays. They are germinated at 18^{o}C in the growth room. After 21 days germination percentages and above ground and total seedling height are measured [1].
4) Imbibitional chilling tests –Seeds are spread on a polyurethane foam pad and rolled up into a tube. This is filled with cool water and placed in the cold room at 5^{o}C for 6h. They are then germinated in trays of sand at approximately 30^{o}C in the glasshouse. After 14 days germination percentages and seedling height are measured.
5) Imbibition tests – Seeds are submerged in 5^{o}C water for 6h, air dried for 18h and weighed to determine weight gain percentages which may explain differences in imbibitional chilling tests [1].
6) Electrolyte leakage tests – Seeds are rinsed in deionised water and then placed in 50mL falcon tubes with 30mL of deionised water at 5^{o}C and allowed to imbibe for 24h. Conductivity measurements are then taken [1, 9]. Electrical conductivity measurements indicate the degree of chilling injury. A higher measurements means a higher degree of chilling effect
7) Cool Warm Vigour Index is calculated following Tuck *et al*. [10]. This index is the average of the warm (30^{o}C) seedling length on day 4 and cool (14^{o}C) seedling length on day 7.

Results and Discussion

As there were no statistically significant differences or any trends in the outcomes of Study I only results from Study II are presented.

Warm germination tests

4 day Warm Germination

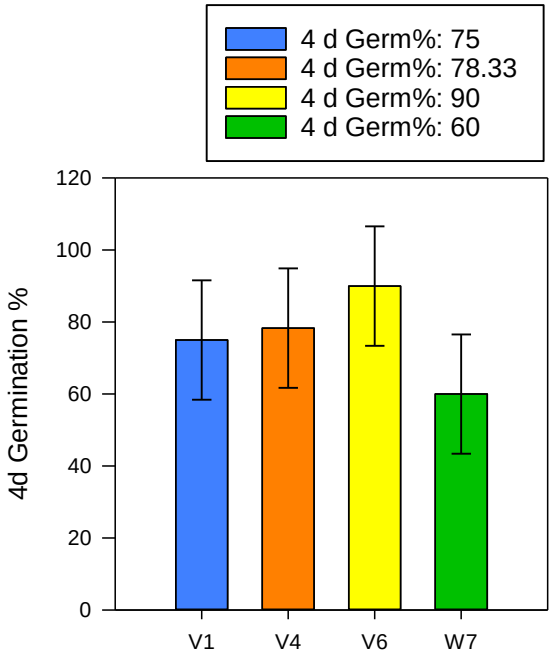

Figure 2: Results of warm germination studies measured at 4 days. Error bars are twice the LSD (least significant difference) at the 95% confidence interval for the mean. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

There were significant differences between treatments ($P < 0.1$) The Argon (W7) treatment had the lowest germination of all treatments. Across all treatments the air treatment at 27 minutes (V4) had the greatest germination at 90% compared to the control at 75% germination.

7 and 10 day Warm Germination

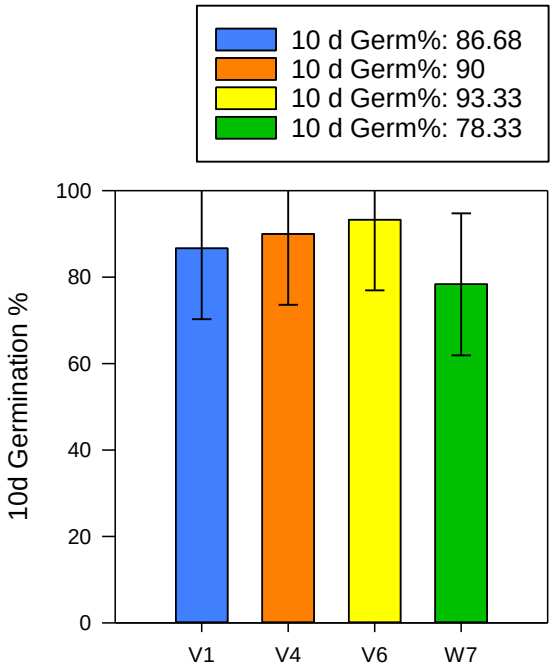

Figure 3: Results of warm germination studies measured at 10 days. Error bars are twice the LSD (least significant difference) at the 95% confidence interval for the mean. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

There were no statistically significant differences between treatments measured at both 7 and 10 days. However the numerical trends measured at 4 days persisted until 10 days (Figure 3).

Cold germination tests

4 day Cold Germination

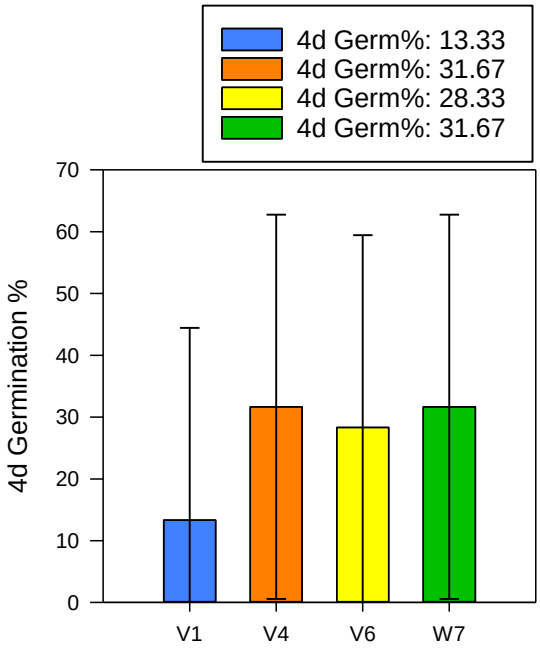

Figure 4: Results of cold germination studies measured at 4 days. Error bars are twice the LSD (least significant difference) at the 95% confidence interval for the mean. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

No statistically significant differences were measured at 4 days, but again there was a clear trend for the plasma treated seeds to have a higher germination rate (Figure 4). In fact the germination rate more than doubled for all plasma treated seeds.

7 and 10 day Cold Germination

No statistically significant differences were measured at both 7 and 10 days. Trends that were detected at 4 days persisted until 10 days but were considerably less numerically.

Metabolic chill test

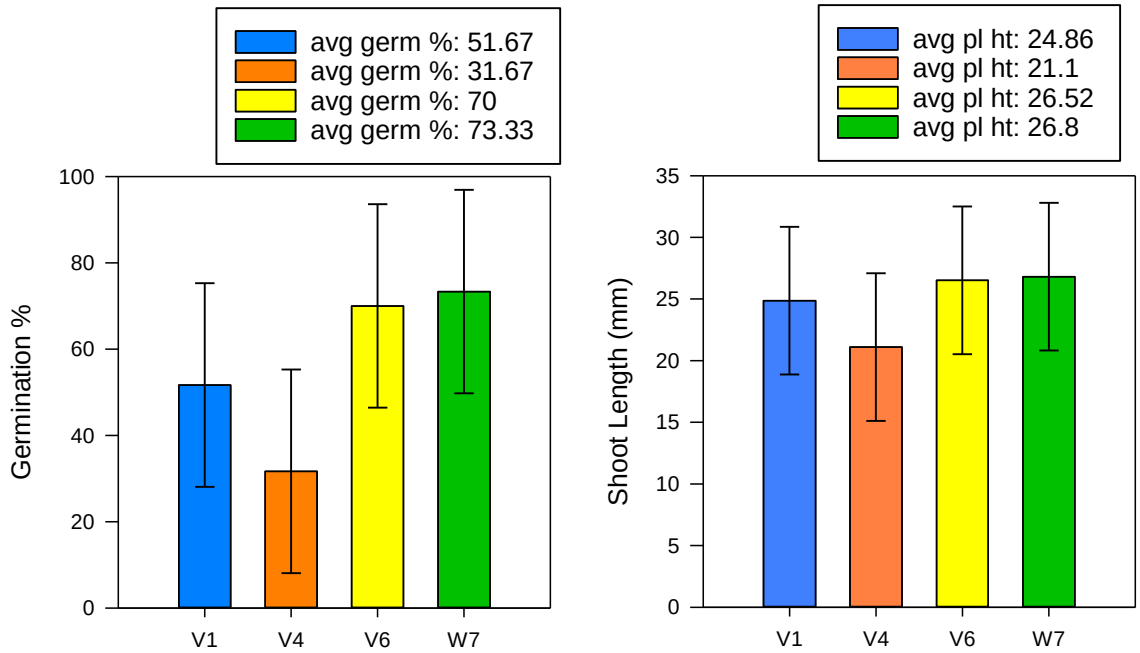

Figure 5: Results of the metabolic chill test in Study II. Error bars are twice the LSD (least significant difference) at the 95% confidence interval for the mean. (Germination left and plant height right.) (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

There were significant differences between the control and the treatments at the 90% confidence interval ($P<0.1$) for germination. The 3 minute air plasma treatment (V4) at 32% was less than the control (V1) at 52% while the 27 minute air plasma and Argon treatment were better than the control (above 70%). Although not statistically significantly the mean shoot length differences reflected the germination measurements (Figure 5).

Imbibitional chilling tests

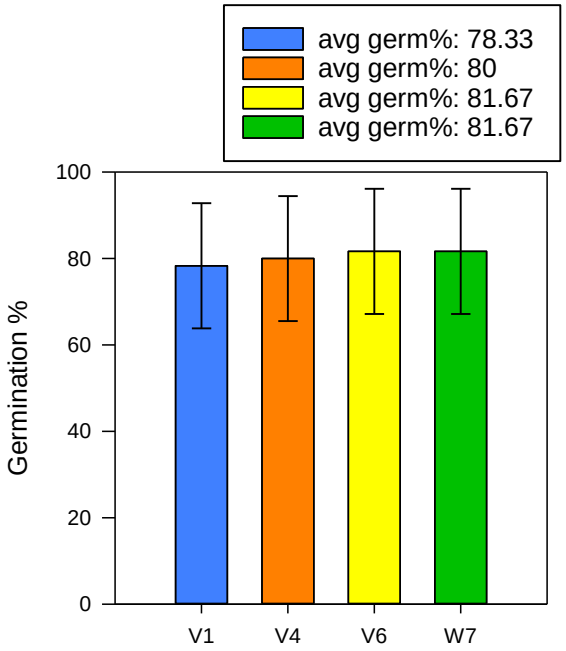

Figure 6: Results of the Imbibitional chill test in Study II. Error bars are twice the LSD (least significant difference) at the 95% confidence interval of the mean. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

The Imbibitional chilling tests revealed no statistically significant differences nor any other definitive trends for the plasma treatments to outperform the controls (Figure 6).

Imbibition tests

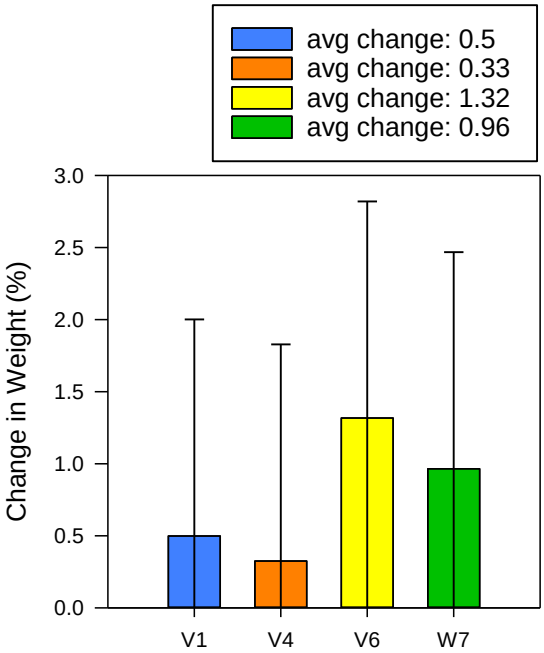

Figure 7: Results of the Imbibition of seed in Study II. Error bars are twice the LSD (least significant difference) at the 95% confidence interval of the mean. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

In study II there were no statistical differences in weight changes in the seeds but there was a trend for the plasma treatments to change, with the weight increase less in V4 and much more in V6 and W7. The imbibition test results are similar to the changes in the metabolic chill test.

Electrolyte leakage tests

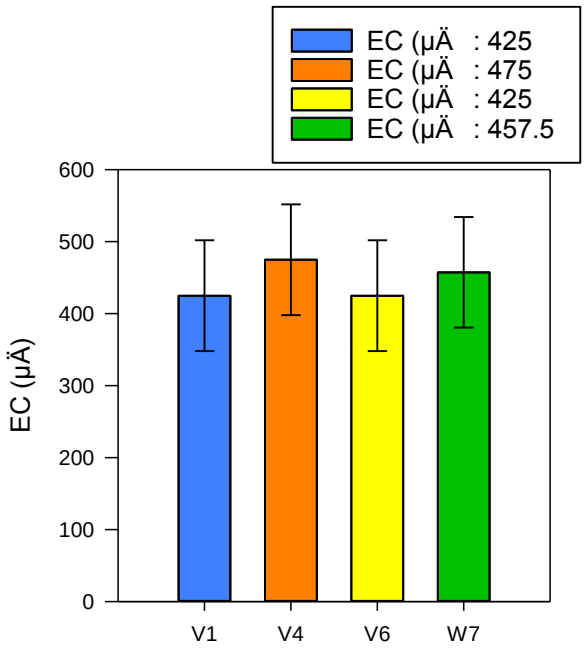

Figure 8: Results of the seed electrolyte leakage of seed in Study II. Error bars are twice the LSD (least significant difference) at the 95% confidence interval. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

No statistical differences seed electrolyte leakage were measured and there were no definitive treads for the plasma treatments to be any different from the control.

Cool warm seedling length test

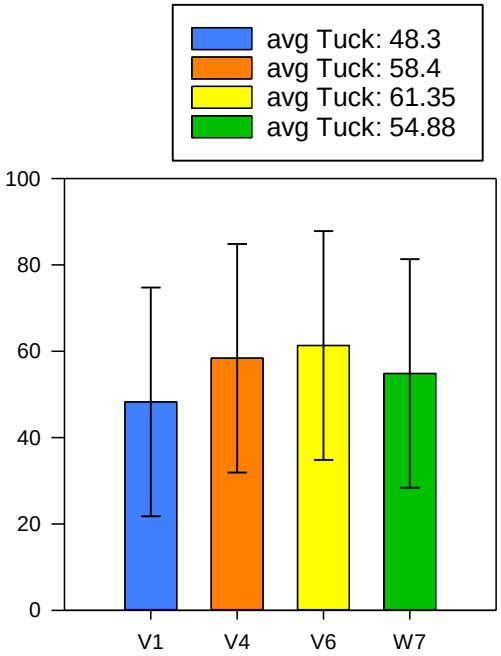

Figure 9: Results of the Cool Warm Vigour Index. Error bars are twice the LSD (least significant difference) at the 95% confidence interval. This index is the average of the warm (30^{o}C) seedling length on day 4 and cool (14^{o}C) seedling length on day 7. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

No statistically significant differences in the cool warm vigour index were measured, however there was a numerical trend for the plasma treatments to be better than the control. A true near 10% difference in 'cool warm vigour index' between the control and the 27 minute air is potentially of commercial interest.

Chilling Tolerance Assessment

A combined chilling tolerance assessment of the seed plasma treatments is derived from each of the two chilling tolerance tests [7]. A two-way scatter plot (Figure 10) shows the performance of these chilling tolerance tests.

The x-axis in the graph represent the imbibitional chilling tolerance test and the y-axis represents the metabolic chilling tolerance test. Higher values on each axis indicate higher levels of cold tolerance with respect to that chilling test. The overall cold tolerance for a treatment is determined by its performance in both tests. Treatments with tolerance percentages from both tests of 80% or above are classified as having **excellent** overall cold tolerance. Those with both tolerance percentages between 65% and 80% are ranked as having **good** overall cold tolerance. If both tolerance percentages are between 50% and 65%, a treatment has a **fair** overall cold tolerance. A **poor** ranking is given to treatments that have either one or both tolerance percentages 50%.

Based on these criteria the control treatment had **fair** cold tolerance, while V4 the 3 min air treatments had **poor** tolerance, and the 27 min air and Argon treatments with similar cold tolerances are identified as **good**.

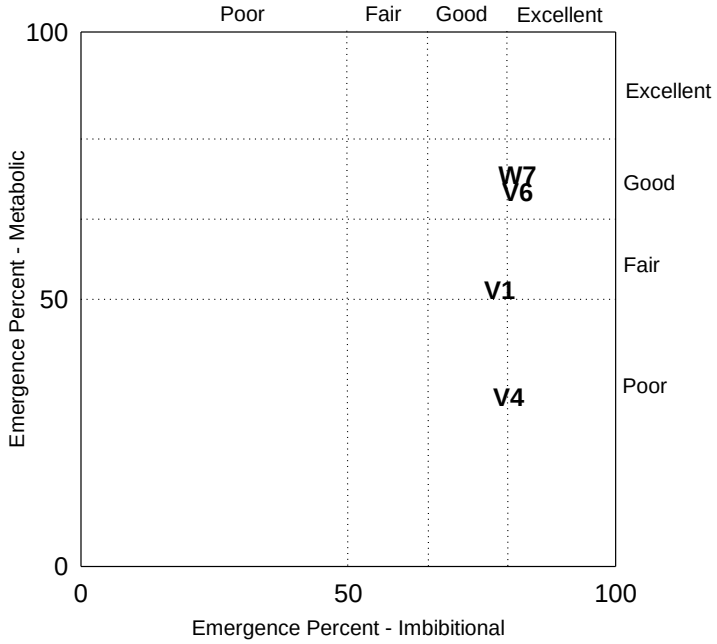

Figure 10: Scatter plot depicting plasma seed treatment chilling tolerances. Note that V1 is the control. (V1 is control, V4 is 3 min air, V6 is 27 min air, W7 is 81min Argon).

Presence of Visually Detectable Mould/Fungi

In both studies there were no treatment differences in the presence of mould or fungi. Note that this was not part of the original study plan, but it may be worth studying in a subsequent study.

Conclusions

The plasma treatments used on black seed in this study statistically affected (at 90% level) two germination measures used in this study, namely the four day warm germination and metabolic chilling tests. While other outcomes were not statistically significant, there were clear trends emerging for the plasma treatments to also affect cold germination, seed imbibition, and the cool warm vigour index.

In the warm germination test the 27 minute air plasma treatment improved germination compared to the control from 75% to 90% and this pattern continued until 10 days of measurement. The metabolic chill test - a measure of the seed to overcome cool conditions over a long period - was poorer in the 3 minute air plasma treatment (down to 32% from 52% in the control). However with the metabolic chill the 27 minutes air plasma and Argon treatments improved germination with both treatments exceeding 70% up from 52% in the control. Based on the cold tolerance classification by Deusterhaus [1] place these two plasma treatments in the 'good' category up from 'fair' for the control.

In general, differences in imbibitional chilling is often associated with the temperature of the water and/or the amount of seed imbibition. Higher cold water imbibition by the seeds is one cause of lower seed germination. However in this study plasma treatment did not affect imbibitional chilling (a chill caused by uptake of cold water) while the cold water uptake (imbibition tests) of the 27 min air plasma seeds in particular was much higher than that of the control. The large confidence intervals of the imbibition tests does not allow further conclusions on a possible contradiction against usual trends with these tests. These results needs further future detailed investigation.

This study showed that cold plasma treatments of seed can be successfully applied without reducing seed germination properties. Importantly there was evidence to suggest that across all germination tests in this study a 27 minutes air plasma treatment could be used to improve both warm germination and cold tolerance. This study did not intend to determine the specific mechanisms which cause the differences in test outcomes – possibly a subject of further detailed research.

Seed germination is still favourably affected by plasma treatment despite a three months delay between the plasma treatment and the germination tests. This suggests that in future seed plasma treatments can be done at an industrial scale 'long' before planting, hence making the need for plasma treatment on the farm just before planting unnecessary.

If we ignore the somewhat high uncertainties of the average values of our measurements and assuming that the average measured values in this report have negligible uncertainties, the plasma treated seeds have potentially commercial significance in improving plant stands and reducing in-field variability that can lead to improved yields and less need to replant.

Considerations for Future Work

This was a pilot study to assess if plasma treatments could affect the germination of commercially available black cotton seed. While the study was able to determine that there were affects generated there are opportunities to conduct some further assessments of the approach before establishing any potential investment in applied/commercial opportunity. Some of these assessments are listed below:

- Repeat germination studies with larger uniform seed batches to confirm the outcomes of this study (aim for at least 50 seeds per germination test replication).
- Assess air plasma treatments times between 3 and 27 minutes.
- Conduct an assessment of the seed coat between control and successful plasma treatments.
- Assess plasma treatments with existing seed coatings used in commercial practice.
- Assess plasma treatments on an alternative cultivar.
- Plant plasma treated seed in the field with an early and late planting time.
- An assessment of seed decontamination when using cold plasma (from mould, fungi, and bacteria)

Acknowledgements

- The CSIRO breeding team for provision of black seed.

- Chris Williams for his effort with the fabrication of the plasma chambers and assistance with the plasma processing of the cotton seeds.

- Alex Katsaros for his effort with the imaging and measurement of water drops on many plasma treated cotton seeds in preparation for this study.

- Lakshman Randeniya for useful discussions during the early stages of the first study.

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Appendix - Plasma Hardware

The plasma treatment chamber for the second study is shown in Figure 11. The power supply and the gas supplies are not shown. The CSIRO made chamber can hold 350 cotton seeds at a time. It is placed on a (seed) shaker to ensure that all seed surfaces are treated uniformly.

Figure 11. Plasma chamber for the second study. (Pictures taken on 17-Feb-2016.)

Cotton seeds being treated in the large plasma chamber can be seen in figure 12 below.

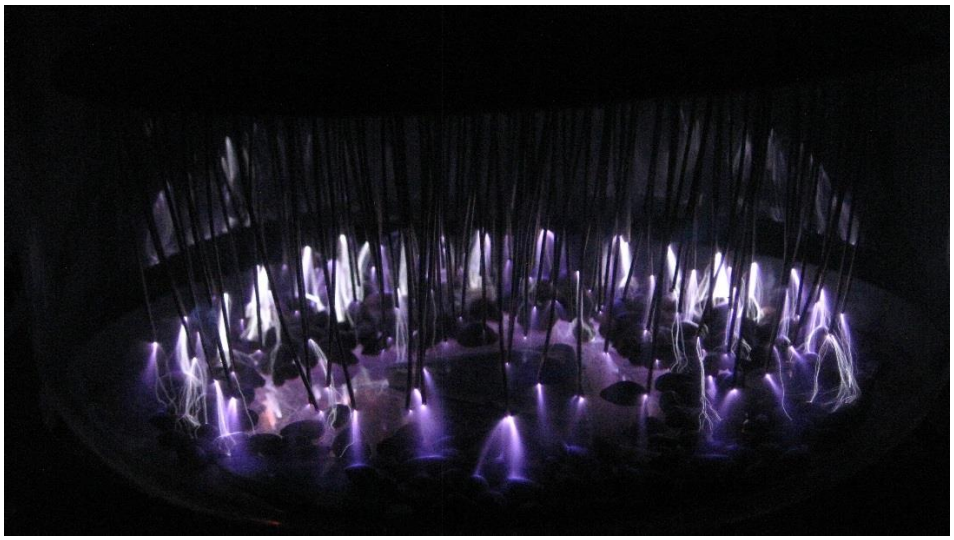

Figure 12. Cotton seeds undergoing plasma treatment

A diagram of the plasma chamber can be seen in figure 13 below.

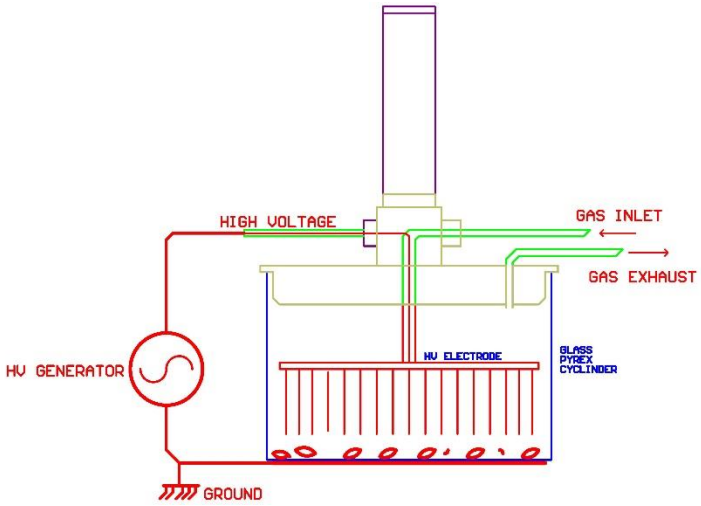

Figure 13. Diagram of plasma chamber used in the second study. Dielectric-barrier discharge treatment chamber, electrode and material exposed to plasma. (The chamber is placed on a shaker – not shown – to ensure rolling and mixing of the seeds during treatment.

For completeness HERE we also show the chamber used in the first study, see Figure 14

Figure 24. Left, the electrode of the small plasma chamber used in the first study. Right, plasma treatment of sesame seeds – an earlier study. (The chamber holds only seven cotton seeds.)