Final project report to the Australian Flora Foundation on Project

# PLASMA DISCHARGE TREATMENT FOR IMPROVED GERMINATION OF SEEDS AND KILLING OF FUNGAL SPORES ON SEED COATS

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**Summary**

A significant number of Australian plant species have adapted to an ecology in which periodic bush fires play a key role. Some species, particularly in the Fabaceae family, have evolved hard protective seed coats. Such hard seed coats, however, present challenges for the germination of such species. While a number of methods for their germination have been used, all have some disadvantage, which affect the use of such species in habitat restoration, ex-situ conservation, and horticulture. In this project, we have investigated a novel physico-chemical method for the treatment of seeds of Australian plants. The treatment may have two benefits: improved germination of seeds that do not germinate well without treatment (e.g., Fabaceae), as well as enhanced survival of seedlings via the effective killing of fungal spores on seed coats during treatment. Improved germination rates and decreased rates of fungal attacks will benefit the cultivation and conservation of various Australian native plant species, in particular those that traditionally have resisted high-yield germination by other methods and those whose seed numbers are so limited that effective usage of available seeds is essential. The approach we have used employs a gas plasma analogous to the etching of synthetic polymers in the semiconductor industry. Low pressure oxygen plasma exposure was used to treat seeds of a number of Australian plant species. Changes in the seed coat were investigated using swelling, measurement of seed coat thickness, and germination experiments. The treatment was effective for the germination of seeds of species such as *Kennedia rubicunda* (Vent.) whereas seeds of species such as *Banksia speciosa* (R. Br.) germinated equally with and without treatment. Microscopy showed no measurable changes in seed coat thickness; the effect of the treatment thus probably is an enhancement of water permeability through the seed coat. With the limited number of seeds, no effect could be observed of the treatment on possible destruction of damping-off fungal spores. A small number of plants were grown on in order to test for possible effects of the treatment on genes; no differences were observed in the morphology of the plants nor in their growth rates.

## Introduction

Difficulties in effective germination of seeds for can arise from hard seed coats or the presence of germination inhibitors. Seeds of many Australian plant species possess a hard coat that presents essentially an impermeable wall and protects the seed from damage and environmental influences; this is most pronounced in the family Fabaceae. Hard seed coats cause delayed germination: such seeds will usually only germinate after weathering for long periods of time under normal conditions, with weathering gradually breaking down the seed coat sufficiently to allow ingress of water to hydrate and swell the embryo and start germination. Germination can, however, occur rapidly after a bushfire or some other environmental cause that affects the hard seed coat. With the widespread control of bushfires and agricultural clearing, many such plants, such as the WA "poison peas," are becoming rare and endangered. Other Australian plants produce seeds with less hard and thick coats but the seeds contain germination inhibitors that delay germination and frustrate propagators. The inhibitors often appear to be located in the seed coat. While such mechanisms for delayed germination are an effective strategy for survival of seeds until optimal survival conditions for young seedlings have arisen, they present a serious challenge for the efficient propagation of such plant species and also reduce natural regeneration when humans alter ecosystems.

Reasons for wanting to propagate such species are horticultural, for conservation, or for revegetation purposes. For conservation efforts, survival depends on effective propagation and revegetation in the wild. Secondly, some plant species that are difficult to germinate often are pioneer species that are well adapted to harsh conditions and thus are an essential part of re-vegetation efforts where environmental damage has occurred. Thirdly, many "difficult to germinate" species have attractive flowers and foliage and would provide commercial opportunities in the nursery trade if the propagation problems can be solved. The sale of rare plants by nurseries for planting in home gardens would also contribute to ex-situ conservation.

Several methods can be used to germinate seeds that do not respond to simple planting in seedling mix, such as the treatment of seed with hot water, sulfuric acid, peeling (e.g., *Grevillea* seeds), or smoke, but they can be time-consuming and cumbersome for large volumes. Another disadvantage is that seeds treated with hot water or sulfuric acid swell during treatment and then need to be sown immediately; it would be much preferable to use a "dry" treatment that makes the seed coat permeable to water but allows storage of dry, non-swelled seeds after the treatment, yet when sown the seeds can swell and germinate efficiently. This would be particularly useful for plants from the Fabaceae family; many of the Western Australian Fabaceae, such as *Gompholobium*

(Sm.) sp., are difficult to propagate and hence are rarely available in horticulture and also are difficult to include in revegetation efforts.

Another challenge is that seedlings of some plant species are very vulnerable to fungal infections such as "damping off" and collar rot. Some rare and endangered species and also some species that would be very attractive for the horticultural trade (e.g. some *Banksia* species and other Proteaceae) are very susceptible to fungal attack particularly at the seedling stage, leading to the rapid death of young plants, and as a result, those plants are absent from nurseries and gardens except when grown by dedicated non-commercial collectors. Our experience is that even with thorough hygiene it can be difficult to avoid such infections, and at times most seeds of a particular lot suffer infections even when sown carefully in different mixes at different times; it can be surmised that such infections are caused by fungal spores "hitch-hiking" on the coat of seeds and that some seed lots are badly infected with dormant fungal spores. This causes problems in the effective propagation of many Australian plant species, for example within the WA Proteaceae. One possible solution is to disinfect seeds by the removal of fungal spores, thus increasing survival yields, but this can be tedious. It would be desirable to have available a method for rapidly disinfecting seed lots of dozens or hundreds at a time, to reduce processing costs and in particular to make horticulture viable.

Such difficulties in effective germination and/or survival of seedlings reduce the range of species offered commercially in nurseries; species that are "too hard" are not commercially viable in horticulture. In addition, these difficulties prevent the use of some species in revegetation projects and also are a major challenge in the propagation of rare and endangered species for which seed supply is very limited, such as *Banksia goodii*, *Dryandra ionthocarpa*, some WA "poison peas", and others that must be grown from seed.

This project was designed to explore the possibility of using a physico-chemical method for the treatment of seeds, a method which is rapid and has the potential to simultaneously improve germination yields and kill fungal spores carried on seed coats. Can a low temperature gas plasma (also known as a glow discharge) be applied to enhance the germination of seeds of Australian plants that are difficult and tedious to germinate ? Can seeds be treated in this way such as to avoid immediate sowing ? One aim is to achieve enhanced germination yields and perhaps shorter germination times by controlled erosion of seed coats. By improving the rate and yields of seedlings, the outcomes may contribute to the conservation of plants whose seeds are rare and valuable, and to the horticultural development of plant species that currently are not economical to produce by the nursery trade.

**Gas plasma treatment**

What is a gas plasma, and why is it of interest here ? A gas plasma is a gas in which some of the atoms or molecules have become ionised; in other words, electrons have become separated, and the gas plasma thus contains electrons, ions, and the original atoms or molecules. Chemical reactions can also occur in a gas plasma to produce entities that do not exist outside a plasma. For example, an oxygen plasma contains oxygen radicals that are chemically powerful, being able to attack and break down polymers (plastics). Thus, gas plasmas can perform chemical reactions that in some cases are unique. In this case, the ability of reactive oxygen species in an oxygen plasma to break down other chemicals is of interest; the intention is to use reactive oxygen species to soften the hard seed coat.

We used oxygen as the process gas to strike and apply a plasma, with the oxygen gas supplied from a gas bottle via a flow regulator, but an air plasma could equally well be used because an air plasma also generates reactive oxygen species.

Gas plasmas can possess a wide range of properties. So-called low temperature plasmas, operated at pressures of around 0.1 - 0.3 Torr, are best suited to the treatment of materials (and seeds) that could be damaged by elevated tremperatures. By reducing the intensity of the plasma, via the applied current, one can avoid thermal damage even to sensitive soft plastics. Thus, in this study we used low temperature plasmas, often also called glow discharges, to treat seeds, and we used air as the process gas for reasons of cost and the ability to create reactive oxygen species in the plasma glow.

In the semiconductor industry, plasmas are used to etch materials such as plastic insulation layers; for the controlled etching of polyimide and polyamide plastics, plasmas enable highly controlled, homogeneous, and reproducible thinning or ultimate removal of such plastics layers with nanometre precision. As hard seed coats and fungal spore coats resemble polyamide and polyimide plastics in their chemical composition and properties such as being a water barrier, we expected that a gas plasma likewise may be able to achieve the precise, controlled thinning, by reactive erosion by the action of reactive oxygen species, of seed coats and the concurrent etching of fungal spores.

A further advantage is the speed of the process, such erosion to relevant thicknesses can be done within time frames of a few minutes. The process is also scaleable, again based on semiconductor industry experience, and it can readily be envisaged that large volumes of seeds could be handled by an inexpensive custom treatment unit in order to treat seeds for customers in horticulture and revegetation.

For our treatment of hard-coated seeds, we intend for the reactive oxygen species to attack the seed coat and make it thinner and more permeable to water, so that water can get inside and

swell the embryo for germination. Some Fabaceae seeds also have a thin layer of lipids on their outer surface, which makes the seed surface water-repellent. Reactive oxygen species from an air plasma should be able to remove those lipids and make the seed more wettable. Finally, reactive oxygen species should also be able to destroy fungal spores.

Gas plasmas are, however, not a "natural" state of gases. They must be produced within a confined space that contains a suitable gas (in this case, oxygen) and a plasma is established by the application of an electric field across the gas. An example of a gas plasma in everyday life is the fluorescent tube; in that case, it is the light the plasma emits that is useful, whereas we are interested in the chemical effects of the plasma. In our laboratory, we create plasmas by putting an electric potential across a gas inside a glass chamber (Figure 1). When seeds are put onto the lower electrode (the round plate) and the drive voltage applied, the plasma starts to glow and the seeds are immersed in the glow and the reactive oxygen species it contains.

Figure 1. A gas plasma inside a glass chamber. The seeds are placed on the round table (diameter 90 mm).

Simply by controlling the length of time that the plasma is ignited, we can control the extent of treatment; as soon as the electric voltage is turned off, the plasma collapses and the treatment stops. One question was how long it would take to achive an effective treatment of hard-coated seeds. Treatment times of 5 to 60 minutes were used; these values were our best guesses based on previous research on plasma treatments to oxidise the surface or plastics.

Another important experimental variable is the pressure, as the chemical composition of the plasma depends on pressure, due to collisional processes that vary with the density of the gas phase, i.e., pressure. Very little is known how the relative percentages of various reactive oxygen species in an oxygen plasma vary with pressure. The operational pressure can also affect the spatial and temporal homogeneity of the plasma glow zone. In this study we performed some initial experiments at a pressure of 2 Torr but found that the plasma was not as stable as desired, and the treatment had limited effect, probably due to a limited density of reactive oxygen species. By lowering the pressure, such reactive molecules collide less frequently and the resultant longer lifetime can help produce greater activity (which, however, can be too strong for some polymer materials to be treated). As there is no way of predicting this, the pressure was then lowered to 0.3 Torr, and this was found to result in more efficient treatment (see below).

**Experimental methods**

A main aim of this series of investigation was to determine whether air plasma treatment would result in enhanced germination. Thus, we sought to establish what process conditions are required to erode part of the seed coat such that water could get inside. We also wanted to determine whether faster germination might result. We used scanning electron microscopy (SEM) and optical microscopy to measure the thickness of seed coats before and after air plasma treatment, so as to correlate germination with changes in seed coat thickness.

We also had to check that the air plasma treatment would not damage the embryo. Air plasmas do not produce heat (under our conditions in the above apparatus) and hence, we assumed, can be used for thinning seed coats without embryonal damage. However, we checked for this by growing on selected plants and recording their appearance in comparison with plants grown from seeds that had not been plasma treated. Resources permitted only very limited numbers of plants to be grown on and eventually be planted out in the garden of two of the authors for longer-term observation.

Seeds were purchased from the Western Australian commercial seed supplier Nindethana (http://www.nindethana.net.au). The plant species selected for the experiments were: *Kennedia*

*rubicunda, Banksia speciosa, Swainsona formosa, Acacia cardiophylla, Acacia decora, Grevillea excelsior, Grevillea superba (*Note: NOT the cultivar *Grevillea 'Superb'), Gompholobium hendersonii, Gompholobium scabrum,* and *Acacia denticulosa*. Information on the age and provenance of these seeds was not available.

For gemination studies, seeds were treated with air plasma for different time periods under various controlled gas pressures and then immersed and kept in cold water for 24 hours. For seeds of Fabaceae, two controls were used: for one, seeds were not subjected to plasma treatments but immersed in cold water, and for the other set of controls seeds were not plasma treated but covered with hot water which was then allowed to cool, with the seeds then removed from the water after 24 hours as in the usual scarification procedure used by many to germinate Fabaceae seeds. After 24 hours the number of swollen Fabaceae seeds were counted. All seeds (even those that had not swelled) were then planted in commercial fine seedling mix (Bunnings) and their germination recorded. Punnets were discarded after 3 months, assuming that any seed that had not germinated by then would not germinate. It is, of course, known that some seeds may germinate after longer incubation times, but such long germination times are not commercially viable. The aim of this study was to derive a method for efficient and timely germination.

For *Banksia speciosa,* seed swelling obviously did not apply and germination was used to measure outcomes. For control experiments, seeds that had not been plasma treated were sown and germinated. However, seeds (both treated and not plasma treated) were soaked in room temperature water for 24 hours to see if the plasma treatment would lead to water becoming discoloured due to part of the seed coat leaching out. Some propagators use such soaking for Banksia seeds, though it is the experience of one of the authors (HJG) with over 40 Banksia species that this is generally not necessary.

For Grevillea seeds likewise, swelling was not relevant and again the precentage of germination was measured. Two sets of controls were used: seeds that had not been plasma treated but had been peeled, and seeds that had been neither plasma treated nor peeled.

Seeds were put into plastic petri dishes or into wells of plastic multiwell plates so that constant volumes of water to cover the seeds were assured. The wells are covered with a transparent lid. Figure 2 shows a typical set of experiments with various seeds in the twelve wells of a 12-well multiwell plate.

Figure 2. Experimental setup for soaking plasma treated and control seeds for 24 hours.

A limited number of seedlings of *Kennedia rubicunda* (4) and *Banksia speciosa* (3) that were used for growing on were then potted up into pots with commercial potting mix for natives (Bunnings) and all seven plants were eventually planted out into a garden in loam soil. The location, near Gumeracha in the Adelaide Hills at an elevation of 360 m above sea level, had previously been found to be suitable for the cultivation of both species and one of the authors (HJG) had substantial previous experience with growing Banksia and Kennedia species. For both, plants were grown on that had come from plasma treated seeds and from untreated seeds, so that we could assess any differences in appearance, growth rate, etc that might arise.

For measuring the Seed Coat Thickness (SCT) before and after the plasma treatment, to investigate whether plasma treatment leads to any reduction in the thickness of the seed coat by plasma etching, seeds were cut in half with the help of a sharp scalpel blade and then were put onto a small dob of Blu-Tak on a microscope slide so as to be held in place for photography. The seed coat thickness was measured via a slide-mounted calibrated length scale supplied by the optical microscope manufacturer. Optical micrographs will be shown as SEM did not provide any additional information.

**Results**

1. Swelling Experiments

It is well known that seeds of Acacia species and many other hard-coated Fabaceae species will not germinate well if sown as extracted from seed capsules. The usual practice for growers is to pour hot water over the seeds and let them stand overnight or for 24 hours. This leads to some of the seeds swelling to 1.5-2 times their original length and width, and become much softer. Seeds that float usually are attacked by borers or do not contain fully formed embryos; these seeds are discarded as not viable. Seeds that did not swell usually germinate with a low precentage and after lengthy time intervals, whereas swollen seeds germinate well and usually within a few weeks. Hence, the percentage of swelled seeds was taken as an indicator of the success of the plasma treatment in the first instance.

Figure 3 shows a typical outcome. Seeds of *Kennedia rubicunda* treated in an oxygen plasma at a power of 40 W for 30 minutes followed by soaking in cold water for 24 hours showed high extents of swelling and softening, whereas seeds that were not plasma treated but soaked for 24 hrs in cold water showed much less swelling. Not shown is the conventional approach of hot water treatment, which also gave a high percentage of swelling. These results for seeds that had not been oxygen plasma treated are entirely consistent with much experience by many growers that hot water treatment is required for effective germination of many Fabaceae seeds. The swelling of plasma treated seeds suggests that this treatment may be an effective means of enhancing the swelling that is required for high germination yields within a reasonable time frame of ~ a month.

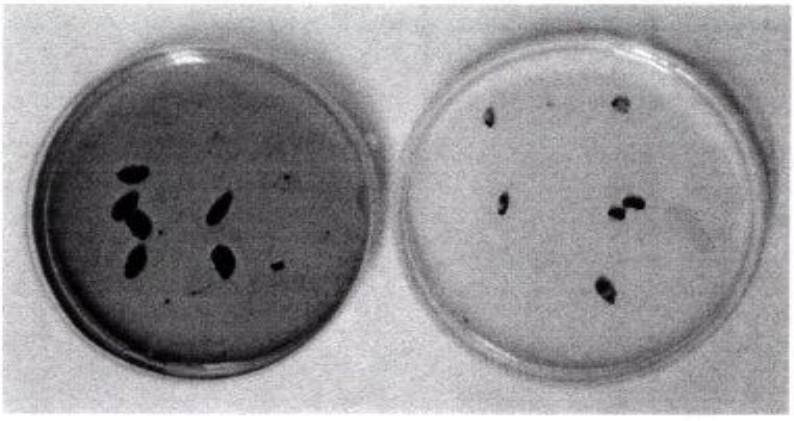


Figure 3. Seeds of *Kennedia rubicunda* that had been oxygen plasma treated (top) or not treated (bottom), after immersion in room temperature water for 24 hours.

Reducing the treatment time to 10 minutes gave a smaller percentage of swelled seeds, whereas reducing the applied electrical power to 20 W had no measurable effect within experimental accuracy.

Clearly, the plasma treatment gave a high precentage of swelled seeds, and this was repeated in successive experiments. However, the number of seeds in the untreated sample in Figure 3 is too small to assess reliably the extent of swelling, and also there seem to be one or two seeds with some lesser swelling. Using a larger number of seeds, it was again observed that some seeds swelled even without a treatment but the majority did not (Figure 4, bottom). Thus, it seems safe to conclude that the treatment had a beneficial effect.


Figure 4. Seeds of *Kennedia rubicunda* that had been oxygen plasma treated (top) or not treated (bottom), after immersion in room temperature water for 24 hours.

As expected, no analogous increase in the size of the seeds was expected for the seeds of *Banksia speciosa* and the two Grevillea species. There were no observable differences in the seeds

of these plants with and without oxygen plasma treatment followed by soaking for 24 hours. Note that soaking is usually not required for the germination of Banksia species seeds, but soaking was performed to test for any possible signs of changes. Grevillea seeds are soaked at times in order to enhance germination, but such soaking accelerates germination time but does not appear to change the size and morphology of the seeds.

Often, extended plasma treatment led to a yellowish-brownish discolouration of the water, (the left-hand petri dish in Figure 3 and some wells in Figure 5 show this clearly); such colouration was observed only for plasma-treated seeds, not for control seeds. The effect was not confined to Fabaceae seeds but also occurred with Banksia and Grevillea seeds. It suggests that leaching of molecular material from the seed coat had occurred, and that the plasma treatment had produced this material. The colouration suggests that this leachate comprised larger molecules, not low molecular weight compounds such as lipids. By analogy with known effects of oxygen plasma treatment on synthetic polymers, where plasmas often cleave polymer backbone chains to create lower molecular weight polymer chain fragments, it seems likely that the leachate comprises fragments of the biopolymers that constitute the seed coat. However, no attempt was made to chemically characterise the leachate.

Figure 5. Seeds after 30 minutes 40 W oxygen plasma treatment and 24 hours soaking in room temperature water.

It is interesting that the Banksia and Grevillea seeds produced leachates, but this did not affect the water uptake by these seeds.

Initial experiments used a plasma pressure of 2 Torr, but this gave plasmas that showed a degree of instability, with the visible sign being flickering. Results nevertheless showed promise; for example with *Kennedia Rubicunda* seeds, 6 out of 6 swelled after plasma treatment and water soaking, compared with 0 out of 6 for room temperature water soaking only, when using 60 minutes of treatment at 40 W.

On the other hand, with *Acacia decora* only 1 out of 6 seeds swelled, and with *Swainsona formosa* and *Acacia cardiophylla* it was 1 to 3 out of 6. However, control experiments using the hot water treatment also gave similar percentages of swelling (1 to 3 out of 6); perhaps the seed lots were a bit old. The seed supplier was contacted for information on the age of these seed lots but no answer was received.

Reducing the treatment time to 20 minutes, however, reduced the effectiveness with *Kennedia Rubicunda* seeds: 2 out of 6 swelled after plasma treatment. Yet, for *Acacia decora*, and *Swainsona formosa* and *Acacia cardiophylla* the reduction in treatment time did not make a difference to the swelling yield, which remained at 1 to 3 out of 6.

Using lower pressure (0.3 Torr) gave stable plasma glows. All further treatments were performed at that pressure, with two different power levels of 20 W and 40 W and treatment times between 10 and 30 minutes. *Acacia decora* and *Acacia cardiophylla* continued to give low yields of swelling not only after plasma treatment but also when using hot water soaking; perhaps more than half of those seeds were no longer capable of swelling the embryo. The swelling experiments gave better results with the other Fabaceae seeds. *Kennedia rubicunda* gave excellent results, as did seeds of *Gompholobium hendersonii*, whereas seeds of *Gompholobium scabrum* and *Acacia denticulosa* responded less well to the plasma treatments, with better results being obtained by hot water soaking.

Interestingly, when using the stable plasma at a pressure of 0.3 Torr, results were the same regardless of whether 20 W or 40 W of power were used. Likewise, 10 minutes and 30 minutes treatments gave the same results. We conclude that there are *more* reactive oxygen species in the 0.3 Torr plasma than in the 2 Torr plasma; while there are more molecules at 2 Torr, collisional deactivation at higher pressure seems to outweigh that factor.

Thus, oxygen plasma treatment at 0.3 Torr pressure and 20 W input power for 10 minutes appears to be sufficient for effective treatment of some Fabaceae seeds, but in some cases they might not respond well enough for high yields of swelling. It remains to be investigated with seeds of known age whether some of our data are due to seeds being too old.

2. Seed Coat Thickness

Our hypothesis was that the oxygen plasma would break down the biopolymers that make up the seed coat and thereby etch away the seed coat gradually, and that such thinning would facilitate the ingress of water for swelling of the embryo. Accordingly, the thickness of the seed coat was measured for seeds with and without the oxygen plasma treatment.

Figures 6 and 7 show typical photomicrographs of seed coats. The coat is clearly visible as a distinct stratum, usually of a darker colour. For each seed, the thickness of the coat was measured at several locations (away from near the apex of the seed), as it was evident that the thickness varied somewhat along its circumference.

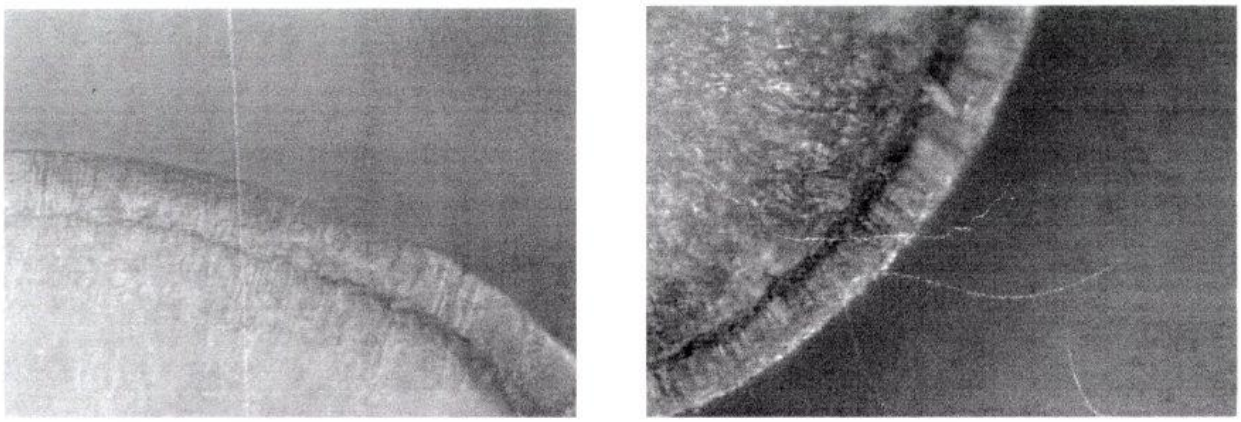



Figure 6. Photomicrographs of seed coats of untreated and plasma treated *Kennedia rubicunda* seeds.

The data recorded for seeds of *Kennedia rubicunda* are tabulated in Tables 1 and 2. The average SCT of untreated seeds was 20.8 μm with a standard deviation of 1.9 μm, and for oxygen plasma treated seeds it was 19.7 μm with a standard deviation of 1.5 μm. These results show that within the natural variability of seed coat thickness, no effect due to the plasma treatment can be observed; while some reduction in thickness might happen, it cannot be extricated from the current data due to the natural variations in seed coat thickness. In other words, contrary to our original hypothesis based on plasma etching of polyimide and polyamide plastics, the plasma treatment does not achieve a marked physical thinning of the seed coat. The changes that do occur seem to amount to *chemical* changes in the seed coat, apparently making it a lesser barrier to water transmission and thereby facilitating the observed swelling.

Table 1: Seed coat thickness of untreated Kennedia rubicunda

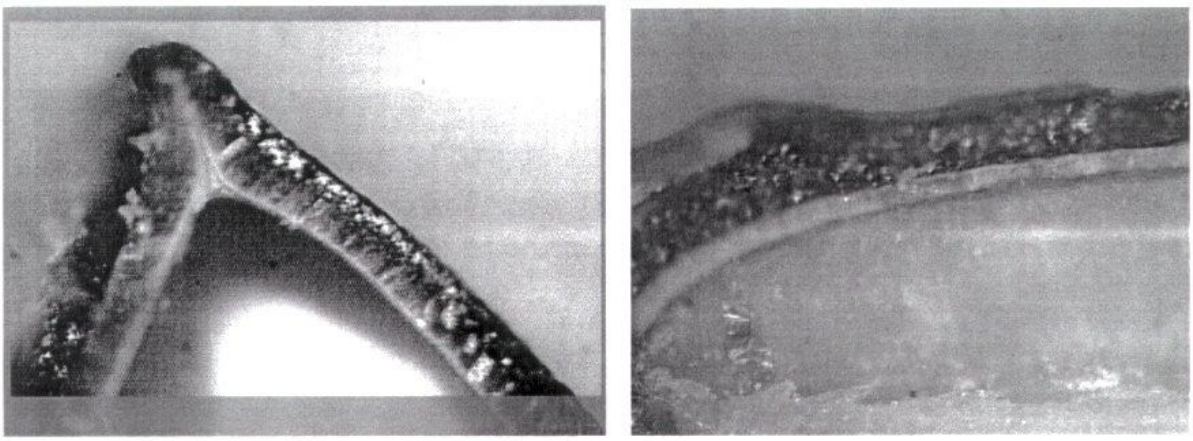
| Seed No. | Position 1 (μm) | 2 (μm) | 3 (μm) | 4 (μm) | 5 (μm) | 6 (μm) | Average (μm) |
|---|---|---|---|---|---|---|---|
| I | 25.2 | 30.7 | 26.5 | 22.3 | 21.7 | 23.4 | 25.0 |
| 2 | 22.7 | 18.2 | 17.1 | 17.3 | 22.2 | 27.4 | 20.8 |
| 3 | 21.1 | 20.3 | 19.8 | 20.1 | 23.1 | 17.5 | 20.3 |
| 4 | 19.8 | 17.6 | 19.8 | 18.5 | 22.7 | 20.4 | 19.8 |
| 5 | 18.7 | 18.4 | 16.2 | 19.3 | 23.5 | 20.7 | 19.5 |
| 6 | 18.8 | 18.6 | 19.7 | 17.1 | 20.5 | 23.0 | 19.6 |

Table 2: Seed coat thickness of oxygen plasma treated (60 min.) Kennedia rubicunda

| Seed No. | Position 1 (μm) | 2 (μm) | 3 (μm) | 4 (μm) | 5 (μm) | Average (μm) |
|---|---|---|---|---|---|---|
| 1 | 18.1 | 20.5 | 20.0 | 21.0 | 22.7 | 20.5 |
| 2 | 20.5 | 19.4 | 20.5 | 23.5 | 22.8 | 21.3 |
| 3 | 21.0 | 17.9 | 17.6 | 21.0 | 24.2 | 20.4 |
| 4 | 21.7 | 20.8 | 19.0 | 21.3 | 19.2 | 20.4 |
| 5 | 17.2 | 16.4 | 16.9 | 18.0 | 17.6 | 17.2 |
| 6 | 20.0 | 18.2 | 17.2 | 17.5 | 19.9 | 18.5 |

Seeds of Grevillea superba and Grevillea excelsior were studied analogously, taking photomicrographs on seed coat thickness with and without plasma treatment. Figure 7 shows representative photomicrographs. These species were chosen for these measurements because seeds of these species had shown above-average amounts of discolouration of the soak water for plasma-treated seeds (Figure 5), which led to the question of whether these leachates would lead to a measurable amount of thinning of the seed coat. However, as shown by the data in Tables 3 and 4 recorded for *Grevillea superba*, again no measurable thinning of the seed coat by the plasma treatment could be inferred. The average SCT of untreated seeds was 26.5 μm with a standard

deviation of 3.4 μm, and for oxygen plasma treated seeds it was 22.2 μm with a standard deviation of 1.3 μm. These data suggest some thinning but are too close to the confidence limit to make a definitive statement. Hence, while the plasma treatment creates some leaching compounds, the amount of material extracted is not a large proportion of the total seed coat, and we would not expect this small reduction in the barrier thickness to affect germination significantly by itself; increased water permeation (and permeation of other molecules ?) might be more relevant.



Untreated Grevillea Superba  Untreated Grevillea Excelsior

Figure 7. Photomicrographs of seed coats of untreated *Grevillea superba* and *Grevillea excelsior* seeds.

Table 3: Seed coat thickness of untreated *Grevillea superba*

| **Seed No.** | **SCT No. 1 (μm)** | **SCT No. 2 (μm)** | **SCT No. 3 (μm)** | **SCT No. 4 (μm)** |
|---|---|---|---|---|
| 1 | 22.1 | 19.8 | 20.6 | 20.2 |
| 2 | 21.6 | 21.1 | 29.3 | 23.8 |
| 3 | 33.3 | 25.2 | 27.5 | 28.2 |
| 4 | 30.7 | 25.9 | 23.3 | 30.8 |
| 5 | 23.9 | 33.0 | 29.4 | 29.4 |
| 6 | 36.0 | 30.6 | 34.9 | 28.1 |
| 7 | 21.5 | 27.0 | 23.9 | 22.2 |
| 8 | 24.7 | 27.9 | 28.2 | 23.5 |

Table 4: Seed coat thickness of oxygen plasma-treated *Grevillea superba*

| **Seed No.** | **Position 1 (μm)** | **2 (μm)** | **3 (μm)** | **4 (μm)** |
|---|---|---|---|---|
| 1 | 19.0 | 20.1 | 25.2 | 23.6 |
| 2 | 20.5 | 25.9 | 23.5 | 22.2 |
| 3 | 22.8 | 23.9 | 22.1 | 19.7 |
| 4 | 25.6 | 20.4 | 24.8 | 24.2 |
| 5 | 15.4 | 21.3 | 23.8 | 22.0 |
| 6 | 19.9 | 21.7 | 23.0 | 22.7 |

3. Germination

For Fabaceae seeds, the yields of germination corresponded well with the observed yields of swelling, in agreement with the general knowledge by growers that seeds need to be softened and swelled prior to sowing and that unswelled, hard seeds germinate poorly and slowly.

For *Banksia speciosa*, high yields of germination (75-100 %) were obtained regardless of whether or not they had been plasma treated, which at least showed that the plasma did not affect seeds adversely.

In contrast, not a single *Grevillea superba* seed germinated and only one seed of *Grevillea excelsior*, regardless of treatment (plasma plus water soak, peeling, or just water soak). We noticed on peeling that the seeds looked yellowish, and we suspected that they might be too old and had lost viability. The seed supplier was contacted for information on the age of these seed lots but no answer was received. These experiments should be repeated with reliable seeds of known provenance and age.

4. Extended growth of plants

Four seedlings of *Kennedia rubicunda* - 3 plasma treated and one untreated control - and three seedlings of *Banksia speciosa* - 2 plasma treated and one untreated control - were placed into pots of the size shown in Figure 8. All seven plants took well and made good growth, comparable with that of seedlings of other, related plant species. Care was taken to treat all seven plants in the same manner.

At that stage, no differences were observed between the plants grown from plasma treated seeds and the untreated control. Neither the shape of the leaves nor the growth rates differed significantly and all appeared to grow in the normal manner as per the previous experience of author HJG with the growing from seedlings of these plant species and other Banksia and Kennedia species.

Figure 8. A plant of Kennedia rubicunda grown from a plasma-treated seed.

The plants were grown in these pots for about 3 months and then potted up into 14 cm diameter pots, again using commercial potting mix designed for native plants. A small amount of fertiliser capsules (Osmocote for Natives) was added to the potting mix. Again all seven plants took well and grew at a good rate expected of healthy species of such plants (based on previous experience). At this stage also, there appeared to be no differences between plants from treated and untreated seeds.

After the plants had grown to a suitable size (about 25 cm tall for the Banksia species) in these pots, all plants were planted out in the garden of two of the authors near Gumeracha in the Adelaide Hills, at an elevation of 360 m above sea level and with an average annual rainfall of 815 mm. An area of the garden was selected in which other Banksia and Fabaceae plants had been established and grown successfully. The soil is brown loam, slightly acidic and well-drained, yet retaining moisture. The four Kennedia plants were grouped together, with a spacing of about 3 m

between plants, and allowed to climb up into young (3m tall) peppercorn trees. The three Banksia plants were also grouped together, separated by ~ 2 m. During the first summer, some additional water was provided, attempting to treat all plants equally.

No differences were observed at this stage either in the morphology of the plants and their growth rates. Plants from treated and untreated seeds were indistinguishable, with minor variations in growth rate all within the normal range.

Unfortunately, after ~ 20 months an unusually cold sequence of frosty winter nights killed all three *Banksia speciosa* plants (as well as many other Banksia plants and others; there was no reason to believe that the plasma treatment might have induced higher frost sensitivity). Of the four *Kennedia rubicunda* plants, the two more exposed plants were killed; as one of them was the control plant, it was then no longer possible to compare plants from treated and untreated seeds. However, despite the small numbers and the limited duration of the comparison, it appears reasonable to conclude that the plasma treatment did not induce any genetic changes that would have altered the morphology of the plants and their leaves, nor did it affect the growth rate.

5. Seed sterilisation by plasma

Gas plasmas are now being used to sterilise medical devices. Analogously, they might be able to destroy fungal spores "hitch-hiking" on seeds. However, in this study no problems were observed with root rot fungi or damping-off fungal attack, to which *Banksia speciosa* seedlings can be quite vulnerable. Hence, we cannot draw any conclusions on this aspect. Perhaps a study using deliberate spiking of seeds with fungal spores would be required to study this aspect.

**Conclusions**

The oxygen plasma treatment technique applied to hard-coated seeds has shown encouraging results with some Fabaceae seeds. With Grevillea and Banksia seeds, it made no difference, but the apparently old seeds of both Grevillea species may have prevented meaningful study. With the Banksia seeds and growing on several plants, it was shown that the plasma treatment does not cause any adverse genetic effects.

Study of the seed coat thicknesses showed that the plasma did not remove large fractions of the seed coat. The enhanced water uptake by (some) plasma-traeted seeds is probably due to a combination of factors: first, the plasma removes effectively the very thin lipid layer that makes

seeds water-repellent, as shown by much better wetting of seeds after treatment, and secondly the plasma probably reduces the length (and average molecular weight) of the biopolymer chains that make up the seed coat, thus enabling better water transport through the seed coat for swellign the embryo.

A key advantage of the plasma treatment is that it is a “dry” process. Seeds come out looking the same and can be stored until sowing is to be done. This may be a useful feature for use in large-scale revegetation projects.

It might be useful to extend this plasma approach to seeds that contain germination inhibitors in their seed coat. Perhaps the plasma could deactivate such inhibitors.