Controlling Leaf Blackening in Protea

A report for the Rural Industries Research and Development Corporation

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Foreword

Leaf blackening is a major problem for the cutflower Protea industry. Leaves often show symptoms of blackening as soon as 3 days after harvest. Presence of the disorder significantly decreases the vase life of the flowers as well as decreases returns to the grower.

This report is an extension of the work conducted by Crick and McConchie (1999) who found that stems treated with gaseous ethanol, had less than 20% of leaves blackened for a period of 18 days. In the present project a range ethanol and other alcohol concentrations were assessed, as well as various methods of applications such as gaseous application, the use of slow release sachets and addition of ethanol to vase water.

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Executive Summary

Leaf blackening is a serious postharvest disorder in many cut Protea species. Blackening symptoms can occur within 3-5 days after harvest and dramatically reduce the vase life of the stems. It can have a great effect on the marketability of the product. Stems showing blackening symptoms have been sold for less than 50% of the market value of those without blackening (Jones et al, 1995).

The cause of blackening is not well understood. Researchers have established the link between carbohydrate stress and the onset of blackening (McConchie et al, 1991) but as yet no preventative control methods have been developed. It was found by Ghahramani and Scott, (1999) that ethanol vapour successfully inhibited apple scald. Based on these results Crick and McConchie, (1999) conducted similar experiments and found that ethanol vapour applied at a concentration of 5.6g ethanol.kg⁻¹ stem weight effectively kept leaf blackening below 20% for two weeks.

The general aim of the project was to further investigate the use of ethanol to delay leaf blackening in cut Protea stems and to thus develop a commercial method of application for the industry.

Initial experiments were performed on single stems to ascertain the most effective concentration and duration of application. Further experiments were then conducted on larger quantities of stems. Several application methods were also assessed including use of a fumigation in a chamber, addition of ethanol to vase solutions and slow release sachets.

The most effective duration of application of ethanol tested was 24 hours. Blackening of P. ‘Pink Ice’ remained under 20% for 9 days when ethanol was applied at a rate of 5.6g ethanol.kg⁻¹.

The two experiments performed to refine the most effective concentration of ethanol showed that concentrations ranging from 6.5 - 7.0g ethanol. kg⁻¹ stem weight, was the most effective in delaying leaf blackening in P. ‘Pink Ice’.

Uptake of ethanol to the leaves increased linearly with concentration. The decline in effect of the ethanol at higher concentrations was not due to inadequate uptake but due to a toxicity effect. This toxicity effect was also found at higher concentrations by Crick and McConchie (1999). The range that gaseous ethanol application is effective at delaying leaf blackening is quite narrow, between 10-15ug.g⁻¹ FW. Leaves appear to be very sensitive and easily damaged if leaf uptake was too high and gaseous ethanol would therefore have to be carefully applied by growers in the commercial setting to avoid damage to stems.

The ability for ethanol to delay leaf blackening was quite variable. In one experiment blackening was at a level of 50% after 14 days in treated stems and in another was at a level of 50% after just 6 days. This variation under controlled laboratory conditions, casts some doubt on the adaptation of this method for commercial use.

Longer chain alcohols were found to be ineffective in delaying leaf blackening, tending to induce blackening, as opposed to delaying its onset. The premature browning observed in the Protea stems indicates the concentrations used may have been too high, however in other experiments lower concentrations were ineffective.

Results using sachets constructed of polyethylene and impermeable film showed that a steady rate of release can be achieved through this method of application. The amount of ethanol released was however extremely low with only 1.5 % being released in the first 24 hours. Although very consistent in the rate of release the amount released is far too low to be effective at delaying leaf blackening. Other sachets, such as those constructed of Tyvek® showed opposite results. The ethanol release rate
was very rapid with almost 100% being released in the first hour. These sachets were too unpredictable for commercial use. Antimold® sachets seemed promising as the ethanol was only released from the sachet when exposed to water vapour (Freund, 1986). This would enable easy storage of the sachets on farm before use. However the release rates of ethanol from the Antimold® sachets were too uneven for effective application.

Scaling up of gaseous ethanol application to enable multiple stem treatment in a fumigation chamber was successful. Results from the chamber experiment show that when using a concentration of 6.5g ethanol.kg⁻¹ stem weight, blackening was kept below 20% at day. These results again highlight the ability of ethanol to delay leaf blackening, but also draw attention to the problem of variable sensitivity. Application of ethanol at a lower concentration, say 70%, may avoid leaf damage, but still allow sufficient uptake of ethanol into the leaves to have an effect. These experiments were not complete at the time of reporting.

Ethanol applied through holding solutions showed promising results for delaying leaf blackening. However, there was a decline in quality of flowers treated via this method. This is therefore unsuitable for commercial use.

When ethanol was applied via pulsing with high concentrations of ethanol in the vase solution, blackening was also delayed significantly. However, a considerable decrease in the quality of the stems was observed prior to deterioration of the control stems.

After testing several methods of ethanol application at several concentrations, further work needs to be conducted to design a reliable method for commercial application. At high concentrations ethanol vapour is toxic to protea leaves. None of the application method tested to date can guarantee that leaves will not be damaged. Further work will continue with slow release sachets and understanding the mechanism of blackening inhibition by ethanol.
Introduction

The Australian wildflower industry is predominantly represented by the national organisation Australian Flora and Protea Growers Association as well as smaller other regional organisations. Production systems for both Australian flora and South African Proteaceae are often similar and consequently many growers produce Proteas as well as a range of Australian cut flowers. Proteas are grown successfully in every state and the industry doubled in size between 1980 and 1994 to reach approximately 1500 ha of production (Lawson, 1995). In 1997, Australian native flower production had an estimated wholesale value between $14-20 M. (Yencken, 1999). South African Proteaceae accounted for approximately 20% of the total native flower production, which suggests that in 1997, the Protea industry had a wholesale value between $2.8 and 4 M.

Export destinations for Proteas include Japan, USA, Germany and Holland. Between 1995-1997 there was a 40% increase in the number of stems reaching the Dutch auctions but Australia’s ranking dropped from 74 to 63 (Yencken, 1999). This indicates that Australian producers must continue to strive for top quality to retain and improve market share.

Leaf blackening is a serious postharvest disorder in many cut Protea species. Blackening symptoms can occur within 3-5 days after harvest and dramatically reduce the vase life of the stems. It can have a great effect on the marketability of the product. Stems showing blackening symptoms have being sold for less than 50% of the market value of those without blackening (Jones et al., 1995).

The cause of leaf blackening in Proteas is not well understood. Researchers have established the link between carbohydrate stress and the onset of leaf blackening (McConchie et al., 1991) but as yet, no preventative or control methods have been developed. Recently, ethanol vapour successfully inhibited apple scald (Ghahramani and Scott, 1998), a postharvest disorder which results in a similar discolouration to leaf blackening. On the basis of their success, similar experiments were conducted and found that ethanol vapour applied at a concentration of 5.6 g/kg stem weight effectively kept leaf blackening below 20% for two weeks (Crick and McConchie, 1999). These results indicate that, for the first time, there may be a way of controlling leaf blackening and improving the vase life and quality of those severely affected Protea species. Over 8 Protea species/cultivars are affected by this. The volume and value of exports of Australian wildflowers (which includes Proteas) has increased substantially in the past few years but to remain competitive, it is essential to improve quality in the blackening susceptible species.

The aim of the project was to further investigate the use of ethanol to delay leaf blackening in cut Protea stems and to thus develop a commercial method of application for the industry.

The major objectives of the project were;

1. Determine the most effective concentration and duration of application of ethanol.
2. To test the efficacy of other alcohols.
3. To develop and evaluate several application methods (including slow release and fumigation) for commercial application.
Materials and Methods

Plant Material

All stems were treated in the following manner before application of treatments, unless stated otherwise. Floral stems of *Protea* were obtained fresh from the grower and arrived at the Department of Crop Sciences, University of Sydney within 24 hours of harvest. On arrival at the department stems were recut to a length of approximately 50cm and the number of leaves reduced to a standard of 30 leaves per stem. Stems were stored with the base of stems in buckets of dH2O until required for treatment. All gaseous ethanol applications were made with 100% ethanol unless otherwise stated. Leaf blackening is hastened and induced by darkness. Consequently all experiments were conducted under darkness to ascertain the most effective treatment to prevent leaf blackening. The standard industry practice of addition of slow release chlorine (SDIC) and citric acid (0.03%) to the vase solution did not affect ethanol treatments and was omitted from experiments to reduce source of variability.

Determination of Ethanol Concentration and Duration for Vapour Application

a. Determination of Duration of Treatment for Vapour Application

Previous concentrations of absolute ethanol used by Crick and McConchie, (1999), were used as a guide to determine the most effective duration of application. Each stem of *Protea* ‘Pink Ice’ was weighed and the amount of ethanol to be applied was calculated on a fresh weight basis for each of the treatments. Twelve replicates were assigned to the following treatments: A. Stem in bag, no ethanol, B. Stem in bag, 4.0g ethanol.kg\(^{-1}\) stem weight, C. Stem in bag, 5.6g ethanol.kg\(^{-1}\) stem weight, D. Stem in bag, 7.2g ethanol.kg\(^{-1}\) stem weight, E. Stem without bag, no ethanol. Stems were individually placed into jars of dH2O and with the allocated amount of ethanol on a piece of filter paper, placed into a low density polyethylene plastic (LDPE) bag and inflated to a volume of 4L. Four replicates from these treatments were then assigned to one of the following durations of application -18 hours, 24 hours or 42 hours, at 20°C. After these times had elapsed the bags and the ethanol were removed from the stems and the stems placed uncovered in a darkened room (20°C) for the remainder of the experiment. The number of leaves with greater than 10% leaf area blackened were recorded every second day.

b. Determination of Effective Concentrations for Vapour Application

Methods to determine the most effective concentrations were similar to those used by Crick and McConchie, (1999). Stems of *Protea* ‘Pink Ice’ were weighed and the amount of ethanol to be applied was calculated for each of the treatments. The number of replicates used in each trial was dependant on the number of stems available but at least 5 replicates were used for each treatment. Each stem was placed into a jar of dH2O in a LDPE bag with dimensions of 60cm x 90cm and 68µm thick. The amount of ethanol to be applied was placed on a piece of filter paper in a 30mL medicine cup with the stem inside the bag. The bags were then inflated to a volume of 4L with air and sealed tightly with an elastic band. Stems remained in the bags for a period of 24 hours (as was determined by previous findings from the project) under dark conditions. After this period the stems were placed in the jars of dH2O under dark conditions (20°C) and the number of leaves with greater than 10% of the area blackened was recorded daily.
Evaluation of Efficacy of other Alcohols

Stems of Protea ‘Pink Ice’ were weighed and the amount of alcohol to be applied was calculated for each of the stems. The initial range of concentrations were based on results using ethanol. Treatments were as follows; A. Stem without bag, no alcohol, B. Stem with bag, 7.2g ethanol.kg⁻¹ stem, C. Stem with bag, 7.2g propanol.kg⁻¹ stem, D. Stem with bag, 7.2g butanol.kg⁻¹ stem. Each treatment was replicated 11 times. Three of these replicates were used to take samples for assay of alcohol concentration in the leaf tissue. Each stem was placed into a jar of dH₂O and treatments B-D were then placed inside a LDPE bag with the alcohol as described above. After 24 hours the bags and source of alcohol were removed and the stems placed in a darkened room (20°C) and monitored for leaf blackening.

Propanol and butanol were also tested using the equivalent molarity as was effective for ethanol the treatments were as follows; 1. Stem no bag no ethanol, 2. Stem with bag, 7.2g ethanol.kg⁻¹ stem, 3. with bag, 9.4g propanol.kg⁻¹ stem, 4. Stem with bag, 11.6g butanol.kg⁻¹ stem. The experiment was carried out as above.

Ethanol Application Methods

1. Slow Release Sachets

a. Sachets constructed of polyethylene and impermeable film.

An impermeable film, consisting of three layers, polyester, aluminium foil and polyethylene and polyethylene film was used to construct sachets of dimensions 12cm x 7cm. Squares of polyethylene plastic (38µm thickness) of differing areas were placed into the walls of the sachets, to create a window where gas could diffuse out. The areas of polyethylene film were as follows, 0 cm², 6.3 cm², 8.4 cm², 16.5 cm², 33 cm², 66 cm². Three additional treatments were also used which were constructed entirely out of the impermeable film with either one, two or three pin picks in the film to allow the ethanol to diffuse out.

Sachets were constructed by heat sealing three of the outer edges and placing a piece of Whatman No1 filter paper inside the sachet. The sachets were weighed and then 2mL of ethanol placed into each sachet. The fourth side was then heat-sealed. The sachets were reweighed at successive periods after the addition of the ethanol and the weight loss calculated.

b. Sachets Constructed of Tyvek®, Impermeable Film, Polyethylene (38µm), Polyethylene (65µm) and Gladwrap®.

Sachets were constructed from a combination of the following; Gladwrap®, polyethylene (38µm), polyethylene (68µm), Impermeable film, Tyvek® 1025D and Tyvek® 1085D. Sachets measured 7cm x 12cm and were constructed by heat sealing two equal sized sheets of the above materials. Treatments were as follows, 1. Tyvek® 1025D and impermeable film 2. Tyvek® 1025D, 3. Tyvek® 1085D 4. Tyvek® 1085D and Polyethylene film (68µm), 5. polyethylene film (38µm), 6. Gladwrap®, 7. Polyethylene film (68µm).

Sachets were constructed as described above with 2 mL ethanol applied to the filter paper inside the sachet. The sachets were reweighed at successive periods after the addition of the ethanol and the weight loss calculated.
c. Commercial Antimold® sachets

Antimold® 15 and Antimold® 20 sachets were obtained from Freund International Pty Ltd, Japan. Sachets were heat-sealed and constructed of a laminated sheet designed for the slow release of ethanol vapour. They contain microencapsulated food grade ethanol (at least 55% alcohol by weight) (Freund Ind. Co. Ltd, 1987). The encapsulated ethanol is released when in contact with water vapour. Three sachets of each Antimold® type were placed into individual glass jars of known volume (approx. 2.5L). The jars were fitted with a rubber septum to enable gas headspace sampling. A beaker containing 20mL of distilled water was also placed in each of the jars. One mL gas samples were taken from the jars and analysed using a Hewlett Packard Gas Chromatograph with Porapak T column and Nitrogen carrier gas at a flow rate of 35mL/min. Head space ethanol concentrations were then calculated from a standard curve.

2. Fumigation in Closed Chamber

Fumigation chambers were constructed from Qublok® aluminium framing and covered with a plasticised PVC film of 210 µm thickness. Chambers were air-tight and of dimensions 100 cm x 50 cm x 80 cm; i.e. a volume of 400L.

Protea ‘Pink Ice’ flowers were obtained fresh from the grower. Stems were weighed and placed into buckets of distilled water. Three buckets each with 5 stems were placed into each chamber. In one chamber stems were treated at a rate of 6.5g ethanol.kg⁻¹ total stem weight and stems in the other chamber were treated at a rate of 7.0g ethanol.kg⁻¹ total stem weight. Two sources of ethanol were placed in each of the chambers on either side of the stems. The stems were treated for 24 hours at 20°C. After this time the chambers were opened and the sources of ethanol removed. The experiment was repeated once. Flowers were monitored for blackening and floral quality was also recorded. Leaf samples were also taken for determination of ethanol content.


a. Holding Solutions.

Protea ‘Pink Ice’ flowers were obtained fresh from the grower. Stems were weighed and placed into solutions containing distilled water and various concentrations of ethanol. The treatments were: A. Control stems placed into distilled water. B. Stems placed into 0.5% solution of ethanol. C. Stems placed into solution of 1% ethanol. D. Stems placed into solution of 2% ethanol. The stems were placed under dark conditions at room temperature (20°C). To stop vaporisation of ethanol the opening of the jars were covered with Parafilm® around the stems. The number of leaves with greater than 10% leaf area blackened was recorded daily for 7 days. The stems were also given a score of floral quality (1-5) daily. Leaves were sampled from the stems for determination of ethanol content by enzyme assay.
b. Pulsed Solutions

Protea ‘Pink Ice’ flowers were obtained fresh from the grower. Stems were weighed and placed into buckets containing distilled water and various concentrations of ethanol. Nine stems were placed into each bucket. Five replicates were used for the recording of blackening and 4 for the sampling of leaves for determination of ethanol content. The treatments were as follows; A. Control - stems placed in bucket of distilled water, B. 0.03g/L citric acid (commercially recommended concentration (Jones and Moody, 1993)) and 20% ethanol in distilled water. C. 20% ethanol in distilled water. D. 30% ethanol in distilled water. E. 40% ethanol in distilled water. Stems remained in these solutions in the cool room (5°C) for 24 hours after which they were removed from the buckets and placed at room temperature (20°C) in individual jars containing distilled water. The number of leaves with at least 10% area blackened was recorded every second day. Floral quality scores (1-5) were also given to the stems every second day. Leaves were removed from some stems for determination of ethanol content.

Determination of Leaf Ethanol Content by Enzyme Assay

Samples of leaves were weighed and then homogenised with 10mL of distilled water. The slurry was then transferred to a capped tube and centrifuged for 10 minutes at 12000 rpm at 0°C. Two mL of the supernatant was transferred to another capped tube containing 2% Polycar. This solution was then centrifuged for 5 minutes at 5000rpm and 0°C. One mL of this supernatant was then added to 2mL Perchloric acid (1M) and centrifuged for 5 minutes at 5000rpm and 0°C. The solution was then neutralised with 1mL potassium hydroxide (2M). The white precipitate that formed was allowed to settle and the supernatant from this extract was used for enzymic assay.

The assay used for the determination of ethanol in the leaves was modified from Bergmeyer, (1986). The alcohol dehydrogenase was dissolved in dH2O and not ammonium sulfate as is suggested in Bergmeyer, (1986). The ethanol content was determined by use of a standard curve from 0 to 40ug ethanol/mL. Standards were made up with absolute ethanol in distilled water.

Statistics

All count data taken over time was analysed using a generalised linear model of binomial proportions with a logit transformation in Genstat for windows version 5. An individual analysis was performed for each day that data was recorded. Treatments were thus compared on a 5% level of significance.
Results

Determination of Ethanol Concentration and Duration for Vapour Application

a. Determination of Duration of Treatment for vapour application

Trials to determine the most effective duration of application showed that a period of 24 hours exposure to gaseous ethanol (5.6g ethanol.kg\(^{-1}\) stem weight) was the most effective in delaying leaf blackening (Figure 1). All three durations of exposure to ethanol were significantly more effective than the control, however the 24 hour treatment gave slightly better results, with blackening remaining under 20% for 9 days. In contrast, the control treatments had over 60% of leaves blackened by day 9. These results also show that increasing the period of exposure does not lead to a decrease in the amount of blackening over time. The 42 hour treatment showed slightly less control than the 24 hour treatment. Results from this trial were used in all future trials with 24 hours being the standard duration of treatment.

![Figure 1](image)

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened after exposure to 5.6g ethanol.kg\(^{-1}\) stem weight in a volume of 4L for 0, 18, 24 or 42 h. After treatment stems were removed from bags and source of ethanol and placed under darkness (24 h) for 14 days at 20°C. The control treatment had no ethanol application and was not bagged. n=4
b. Determination of Effective Concentrations for Vapour Application.

Placing cut stems inside polyethylene bags without ethanol did not have any effect on the delay of leaf blackening in *P. ‘Pink Ice’* (Figure 2). Within the range tested, there was decreased amount of leaf blackening with increasing concentrations of applied ethanol. The highest concentration used (7.2g ethanol.kg\(^{-1}\)) gave greater delay than all other treatments. On day 11, stems in this treatment had only 20% leaves blackened and after 14 days, had less than 50% of leaves black, whereas control stems had over 90% of leaves black.

Figure 2

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened after exposure to 0, 5.6, 6.4 or 7.2 g ethanol.kg\(^{-1}\) stem weight in a LDPE bag inflated to a volume of 4L for 24 h under darkness, at 20\(^{\circ}\)C. After treatment stems were removed from bags and source of ethanol and placed under darkness (24 h) for 14 days. The control treatment had no ethanol application and was not bagged. n=8

Optimum concentrations of applied ethanol were further determined by repeating the above experiment and applying a greater number of ethanol concentrations within the most effective range. The data in Figure 3 suggest that the effect of ethanol on leaf blackening diminishes above 7.0g ethanol.kg\(^{-1}\). The best control of leaf blackening was achieved with a concentration of 6.5g ethanol.kg\(^{-1}\) stem weight. On day 8 this treatment showed significantly less blackening than the controls (p=0.015). The 6.0 g ethanol.kg\(^{-1}\) and 7.50g ethanol.kg\(^{-1}\) treatments also resulted in significantly less blackening than the control (p=0.038 and p=0.044 respectively) and although not significantly different from the 6.5 g ethanol.kg\(^{-1}\) treatment a slightly greater amount of leaf blackening was observed.
Enzymic assay of ethanol concentration in the leaves after exposure to gaseous ethanol shows that there is a linear relationship between the amount of ethanol applied and that which is taken up by the leaves (Figure 4). As applied ethanol concentrations increased, the greater the amount of uptake by the leaves. In addition, this result demonstrates that applying ethanol as a vapour is an effective method of achieving ethanol uptake by the leaves. In the 6.5g.kg\(^{-1}\) stem weight treatment where leaf blackening was significantly reduced the internal leaf concentration of ethanol was approximately 10ug.g\(^{-1}\)FW.

**Figure 3**

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened after exposure to 0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, or 8.5 g ethanol.kg\(^{-1}\) stem weight in a LDPE bag inflated to a volume of 4L for 24 h under darkness, at 20°C. After treatment stems were removed from bags and source of ethanol and placed under darkness (24 h) for 8 days. n=5

Enzymic assay of ethanol concentration in the leaves after exposure to gaseous ethanol shows that there is a linear relationship between the amount of ethanol applied and that which is taken up by the leaves (Figure 4). As applied ethanol concentrations increased, the greater the amount of uptake by the leaves. In addition, this result demonstrates that applying ethanol as a vapour is an effective method of achieving ethanol uptake by the leaves. In the 6.5g.kg\(^{-1}\) stem weight treatment where leaf blackening was significantly reduced the internal leaf concentration of ethanol was approximately 10ug.g\(^{-1}\)FW.

**Figure 4**

Ethanol concentration (µg.g\(^{-1}\)) in leaves of *P. ‘Pink Ice’* after exposure to 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, or 8.5 g ethanol.kg\(^{-1}\) stem weight in a LDPE bag inflated to a volume of 4L, for 24 h under darkness at 20°C. n=3
Evaluation of Efficacy of other Alcohols

Application of the longer chain alcohols butanol and propanol at 7.2g.kg\(^{-1}\) stem weight was found to be ineffective in delaying leaf blackening (Figure 5). After as little as one day, blackening was observed in both the propanol and butanol treatments, whereas blackening was not observed until day 4 in the control treatment. Ethanol was again effective in delaying leaf blackening. In a separate experiment both propanol and butanol were applied at the molar concentration equivalent to 7.2g ethanol. kg\(^{-1}\) stem weight and caused up to 75% of leaves to blacken, indicating that these alcohols caused damage to the leaves (data not shown).

![Figure 5](image_url)

**Figure 5**

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened after exposure to ethanol, propanol and butanol vapour at a rate of 7.2g alcohol.kg\(^{-1}\) stem weight, in a LDPE bag inflated to a volume of 4L for 24 h under darkness at 20°C. After treatment stems were removed from bags and source of ethanol and placed under darkness (24 h) for 8 days. \(n=8\)

Ethanol Application Methods

1. Slow Release Sachets

   a. Sachets constructed of polyethylene and impermeable film.

Due to the risk of damage to leaves from high concentrations of ethanol vapour, slow release methods through permeable films was tested. Results from the trial of these sachets show that a very steady rate of release can be achieved through this method of application. Ethanol release from sachets with varying areas of permeable windows of polyethylene film, indicates that as the surface area of film was increased from zero to 66cm\(^2\) the amount of ethanol released over time also increased (Figure 6). The amount of ethanol released was however, quite low, with only 1.5% ethanol being released after 24 hours for the largest surface area of 66cm\(^2\).
Sealed sachets with pin pricks, released the ethanol at a much greater rate than those with permeable polyethylene film windows. Up to 10% ethanol was released in the first 24 hours (Figure 7). However there was no significant difference in the rate of release between those sachets having 1, 2 or 3 pin holes in the sachet. These sachets were also quite messy and considerable care had to be taken so that ethanol was not inadvertently squeezed out of the holes during handling.

Figure 6
Percent ethanol release over time from sachets constructed of impermeable film and various areas of permeable polyethylene. Areas of polyethylene are shown on graph. n=3

Figure 7
Percent ethanol release over time from sachets constructed of impermeable film with 1, 2, or 3 pin pricks in the wall for the diffusion of ethanol. n=3
b. Sachets Constructed of Tyvek®, Impermeable Film, Polyethylene (38 µm), Polyethylene (65 µm) and Gladwrap®.

The sachets constructed of Gladwrap® released ethanol vapour at approximately the same rate as those constructed of 38 µm thick polyethylene film. Less than 5% of the ethanol in the sachets was released in the 26 hour period they were monitored (Figure 8). However, sachets constructed either partly or entirely from Tyvek® released ethanol at an extremely fast rate. In all of these sachets over 90% of the ethanol was released within the first 3 hours. There was no significant difference in the rate of release between the five different Tyvek® containing sachets. In previous experiments where ethanol was applied to Protea stems via evaporation from filter paper, complete vaporisation was achieved with 2 hours after application (data not shown). Thus Tyvek® appears to be very porous and does not allow a uniform rate of release for the application of ethanol.

Figure 8
Percent ethanol release over time from sachets constructed of various materials designed to regulate the flow of ethanol into the atmosphere. n=3

The sachets constructed of Gladwrap® released ethanol vapour at approximately the same rate as those constructed of 38 µm thick polyethylene film. Less than 5% of the ethanol in the sachets was released in the 26 hour period they were monitored (Figure 8). However, sachets constructed either partly or entirely from Tyvek® released ethanol at an extremely fast rate. In all of these sachets over 90% of the ethanol was released within the first 3 hours. There was no significant difference in the rate of release between the five different Tyvek® containing sachets. In previous experiments where ethanol was applied to Protea stems via evaporation from filter paper, complete vaporisation was achieved with 2 hours after application (data not shown). Thus Tyvek® appears to be very porous and does not allow a uniform rate of release for the application of ethanol.
c. Commercial Antimold® sachets

Release rates of ethanol from the Antimold® sachets was not uniform (Figure 9). In both types of Antimold® sachets, ethanol was released at a reasonably steady rate for the first 24 hours. The concentration in the headspace then started to decrease. In previous experiments (data not shown) it was found that the level of ethanol providing the best control of leaf blackening was a headspace concentration of approximately 0.1%. is adequate in a 4 L container. Over ten times this amount was recorded for the Antimold® sachets, suggesting they may cause damage to foliage.

2. Fumigation in Closed Chamber

Application of ethanol vapour within a fumigation chamber demonstrated that the of vapour application method could be successfully scaled up to treat many stems at one time (Figure 10). When 10 stems were treated at a rate of 6.5g ethanol.kg⁻¹ stem weight, blackening was not observed until day 9. In the control stems and the higher ethanol treatment, blackening was observed on day 5. Furthermore, the ethanol concentration in the leaf tissue showed that more ethanol was taken up at the higher application rate within the 24 hour period of treatment than for the 6.5g ethanol.kg⁻¹ stem weight (Figure 11). The increased blackening in the higher treatment indicates that the uptake (14.95µ g.g⁻¹) was above the limit effective in delaying blackening.
Evaporation of ethanol in the chamber at 3°C was similar to the concentration in the headspace at 20°C (data not shown) indicating that treatment of stems could be conducted in the cool room. Uptake of ethanol by leaves has yet to be tested under coolroom conditions.

Figure 10
Number of leaves of *P. ‘Pink Ice’* with >10% leaf area blackened after exposure to 0, 6.5 or 7.0 g ethanol kg⁻¹ stem weight in an airtight fumigation chamber (400L) for 24 h, under darkness at 20°C. After treatment the chamber was ventilated with fresh air and the stems placed in fresh water under darkness (24 h) for 18 days. n=10

Figure 11
Ethanol concentration (µg g⁻¹) in leaves of *P. ‘Pink Ice’* after exposure to 0, 6.5, or 7.0 g ethanol kg⁻¹ stem weight applied in an airtight fumigation chamber (400L) for 24 h under darkness at 20°C. n=8

Evaporation of ethanol in the chamber at 3°C was similar to the concentration in the headspace at 20°C (data not shown) indicating that treatment of stems could be conducted in the cool room. Uptake of ethanol by leaves has yet to be tested under coolroom conditions.

a. Holding Solutions.

Ethanol via vase solutions provided a greater delay in leaf blackening than any of the gaseous applications trialed. At a concentration of 1% ethanol in distilled water blackening was kept below 20% for 7 days compared to controls which showed over 70% of leaves black over the same time period (Figure 12). Over the 7-day period stems continued uptake of ethanol from the vase water to the leaves (Figure 13). Ethanol was found in the leaves 24 hours after treatment began and after 1 week the concentration of ethanol had at least doubled. The 1% solution performed best in delaying blackening (Figure 12) and had comparable levels of leaf ethanol to the 6.5g ethanol.kg⁻¹ stem weight treatment applied gaseously (Figures 4 and 11). However, in the 2% treatment, although initial levels of ethanol were similar to the 1% treatment after 24 hours, ethanol appeared to accumulate over time and at 7 days was above the threshold at which ethanol is effective in delaying leaf blackening.

Despite the effectiveness of ethanol holding solutions in delaying leaf blackening, the floral quality of the stems declined more rapidly compared to control stems. Figure 14 shows that the control stems were still of saleable quality after 5 days however those stems treated with ethanol started to decline in flower quality on day 4 and remained in a poorer condition than the controls for the remainder of experiment.

![Figure 12](image)

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened placed in vase holding solutions containing 0, 0.5,1.0 or 2.0% ethanol under 24 h darkness over 7 days at 20°C. n=5
b. Pulsed Solutions

Pulsing flowers with 20% ethanol solutions with and without citric acid, for a period of 24 hours was found to be effective in delaying leaf blackening (Figure 15). In these two treatments leaf blackening was below 25% and 30% respectively on day 19. Leaves in the control (no ethanol treatment) had over 80% blackening. At the very high concentrations (30% and 40% ethanol) damage resulted in premature blackening of the leaves of these stems.
The ethanol uptake by leaves was much greater in this trial than in previous trials (eg. see Figure 16). In the 30% and 40% ethanol treatments where significant leaf damage occurred, leaf ethanol concentrations were above 40 $\mu$g.g$^{-1}$ and 60 $\mu$g.g$^{-1}$ respectively. This is over double and four times those concentrations which caused damage in the gaseous applications.

The ethanol uptake by leaves was much greater in this trial than in previous trials (eg. see Figure 16). In the 30% and 40% ethanol treatments where significant leaf damage occurred, leaf ethanol concentrations were above 40$\mu$g.g$^{-1}$ and 60$\mu$g.g$^{-1}$ respectively. This is over double and four times those concentrations which caused damage in the gaseous applications.

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**Figure 15**

Number of leaves of *P. ‘Pink Ice’* with > 10% leaf area blackened pulsed with 0, 20, 30 or 40% ethanol in the vase solution or 20% with 0.03% citric for 24 h under darkness, at 5°C. After treatment stems were removed the pulse solutions and placed in distilled water under darkness, at 20°C (24 h) for 20 days. n=5

**Figure 16**

Ethanol concentration (µg.g$^{-1}$) in leaves of *P. ‘Pink Ice’* pulsed with 0, 20, 30 or 40% ethanol in the vase solution or 20% and 0.03% citric for 24 h under darkness, at 5°C. After treatment stems were removed the pulse solutions and placed in distilled water under darkness (24 h) for 20 days, at 20°C. n=3
Although greater blackening control was achieved in the 20% pulsing treatments than the best gaseous application, higher concentrations of ethanol were found in the leaves. Ethanol concentration in the leaves were above $30\mu g.g^{-1}$ FW whereas in the gaseous application, damage was caused with as little as $14\mu g.g^{-1}$ FW.

Floral quality diminished more quickly in treated stems when compared to untreated stems. Quality began to decline in treated stems after 8 days compared to untreated stems which retained their quality for 2 weeks after the start of the experiment. (Figure 17).

The vase water in the pulsed treatments rapidly turned brown and became very viscous after the stems were removed from the ethanol solution. A brown exudate was also observed to weep from the petioles of attached leaves.

**Figure 17**

Floral quality scores (1-5) for *P. ‘Pink Ice’* stems pulsed with 0, 20, 30 or 40% ethanol in the vase solution or 20% and 0.03% citric for 24 h under darkness. After treatment stems were removed the pulse solutions and placed in distilled water under darkness (24 h) for 20 days. *n=5.*
Discussion

Determination of Ethanol Concentration and Duration for vapour application

a. Determination of Duration of Treatment for Vapour Application

The most effective duration of application of ethanol tested was found to be 24 hours (Figure 1). Leaf blackening of *P. ‘Pink Ice’ stems remained under 20% for 9 days when ethanol was applied at a rate of 5.6g ethanol.kg\(^{-1}\). A 24 hour treatment is more practical for use by growers and would fit in with their marketing schedule.

b. Determination of Effective Concentrations for Vapour Application

Two experiments were performed to define the most effective concentration of gaseous ethanol to apply to delay leaf blackening. An ethanol concentration ranging between 6.5 - 7.0g ethanol.kg\(^{-1}\) stem weight was optimal for delaying the occurrence of leaf blackening in *P. ‘Pink Ice’ (Figure 3). The uptake of gaseous ethanol to the leaves increased linearly with concentration (Figure 4). Therefore the decline in effect of the ethanol at higher concentrations is probably not due to inadequate uptake, but due to a toxicity effect. A similar toxicity effect was also found by Crick and McConchie (1999) when using higher gaseous concentrations. The internal leaf concentration range where gaseous ethanol application is effective at delaying leaf blackening is quite narrow, between 10-15ug.g\(^{-1}\) FW. Leaves appear to be very sensitive and easily damaged. Ethanol would therefore have to be carefully applied by growers in the commercial setting to minimise damage to stems.

The results from these experiments also demonstrate the variable nature of leaf blackening and the ability of ethanol vapour to effectively control the problem. The results in Figure 2, show that blackening was at a level of 50% after 14 days in treated stems whereas the results shown in Figure 3 show that a 50% level of blackening was reached by 6 days in treated stems. This variation in percent leaf blackening under controlled laboratory conditions, suggests that further work needs to be done before commercial use. The variability and differing sensitivity of leaves between experiments may be due to the extent of leaf schlerophylly and the thickness of cuticle, commonly observed in *P. ‘Pink Ice’ stems.

Evaluation of Efficacy of Other Alcohols

In contrast to results of Ghahramani and Scott (1999) who showed that longer chain alcohols such as propanol and butanol were more effective in controlling apple scald than ethanol, these alcohols were ineffective in delaying leaf blackening (Figure 5). The other alcohols actually induced blackening at the concentrations used, as opposed to delaying its onset. The damage observed in *Protea* stems treated with propanol and butanol may be similar to the browning of apple flesh observed by Ghahramani and Scott, (1999). They found that these alcohols controlled apple scald but some browning of the flesh was also observed at higher concentrations. The premature browning observed in the *Protea* stems indicates the concentrations used may have been too high. However, in other experiments using lower concentrations, no damage occurred, but the delay in blackening compared to controls was not significant (data not shown). Higher alcohols were thus found to be ineffective in delaying the onset of blackening.
Ethanol Application Methods

1. Slow Release Sachets

Due to the sensitivity of *Protea* leaves to damage by gaseous ethanol a slow release method was investigated. Results using sachets constructed of polyethylene and impermeable film showed that a steady rate of release can be achieved through this method of application (Figure 6). The amount of ethanol released however, was extremely low with only 1.5 % being released in the first 24 hours. Although very consistent in the rate of release the amount is far too low to be effective at delaying leaf blackening.

Other sachets, such as those constructed of Tyvek® showed opposite results (Figure 8). The ethanol release rate was too rapid, with almost 100% being released in the first hour. These sachets were too unpredictable to consider for commercial use.

Antimold® sachets seemed promising as the ethanol was only released from the sachet when exposed to water vapour (Freund, 1986). This would enable easy storage of the sachets on farm before use. However, the release rates of ethanol from the Antimold® sachets were not uniform and the amounts in the headspace were not as steady as would be required for this sensitive application (Figure 9).

2. Fumigation in Closed Chamber

Scaling up of gaseous ethanol application to enable multiple stem treatment in a fumigation chamber was successful. Results from the chamber experiment show that when using a concentration of 6.5g ethanol.kg⁻¹ stem weight, blackening was kept below 20% at day 13 (Figure 10). These results again highlight the ability of ethanol to delay leaf blackening, but also draw attention to the problem of variable sensitivity. For example, in contrast to results from previous experiments (Figure 2), applied concentrations of around 7.0g ethanol.kg⁻¹ stem weight, provided no improvement in the delay of leaf blackening. Application of ethanol at a lower concentration, say 70%, may avoid leaf damage, but still allow sufficient uptake of ethanol into the leaves to have an effect. These experiments were not complete at the time of reporting.

3. Application of Ethanol Through Vase Water Solutions

The tendency for at least some leaves to become damaged from gaseous ethanol application led us to revisit the work conducted by Jones (1995). Our work has shown that ethanol does inhibit leaf blackening but how to provide the most effective dose without causing damage is an issue. Jones (1995) tested ethanol dips of varying concentrations for 60 sec as well as application through the vase solutions, but did not obtain significant benefit from either treatment. In our results however, ethanol applied through holding solutions (1%) showed promising results for delaying leaf blackening (Figure 12). However, there was a decline in floral quality of stems treated via this method (Figure 14). This application method would therefore be unsuitable for commercial use.

When ethanol was applied via pulsing with high concentrations of ethanol in the vase solution, blackening was also significantly delayed (Figure 15). However, as was observed with holding solutions of ethanol, a considerable decrease in the floral quality of the stems was observed prior to deterioration of the flowers on control stems. A brown discharge from the stems after treatment with the ethanol indicated that ethanol may have extracted tannic substances from the stems. The deterioration of the flower head may have been due to damage caused to the vascular system and membrane breakdown in the stem which thus limited the transport of water to the flower head.
Recommendations and Outcomes

1. Clearly, when gaseous ethanol is applied to *Protea* stems at around 6.5 g kg\(^{-1}\) stem weight, it is taken up by the leaves and inhibits leaf blackening for between 7 and 18 days under darkness.

2. The method was successfully and simply scaled up to treat multiple stems at one time, in a low-cost fumigation chamber that would fit into a packing shed or small coolroom.

3. With gaseous application of 100% ethanol, the degree of inhibition from experiment to experiment was variable. While in each case the ethanol treatment was better than the control, the optimal concentration between 6.5 and 7.0 g ethanol kg\(^{-1}\) stem weight varied in the duration of effectiveness as well as the extent to which it is effective. Further work is required to determine an effective method of getting approximately 10 µg g\(^{-1}\) ethanol into the leaves without risk of damage.

4. Although some of the slow release methods tested were very controlled in the release of ethanol, the quantities released were far too small to be effective in delaying leaf blackening. The Antimold® sachets did release at a rate that was similar to that required for the delay of blackening but the sensitivity of this method of requires very controlled conditions of release and this was not observed with the Antimold® sachets. Trials are continuing with these sachets to determine optimum numbers of stems to treat per sachet.

5. The application of 1% ethanol through vase water seemed most promising in delaying leaf blackening. Unfortunately, although this method did decrease leaf blackening, an unacceptable decline in floral quality was observed. Work in this area will not continue.

6. The underlying physiological mechanism for the effect of ethanol is not known and its investigation was not the purpose of this project. However, an understanding of the mechanism may provide answers to some of the problems encountered.

7. After testing several methods of ethanol application at several concentrations, further work needs to be conducted to design a reliable method for commercial application. At high concentrations ethanol vapour is toxic to *Protea* leaves. None of the application method, tested to date can guarantee that leaves will not be damaged. Further work will continue with slow release sachets and understanding the mechanism of blackening inhibition by ethanol.
References


Jones, R. (1995), The postharvest control of leaf blackening in cut Protea blooms. RIRDC.


