Pollination and seed development in hybrid vegetable seed crops

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Final Report

Pollination and Seed Development in Hybrid Vegetable Seed Crops

Project Number: VG03084 (June, 2006)

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Tasmanian Institute of Agricultural Research

Project Number: VG03084 **Date:** June 2006

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The purpose of this report is to provide detailed information to the public about vegetable seed production and quality research conducted during this study

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Media Summary

Poor yields and low germination percentage and seed vigour are recurrent problems in Australian vegetable seed crops. Factors during pollination and seed development which contribute to these problems are not fully understood and the potential for their management largely unexplored. This project aimed to improve the understanding of the physiology of yield and quality limitations during pollination and seed development in hybrid cauliflower, carrot and onion crops produced in Australia to develop practical management practices to improve seed production. In particular the project addresses 3 key issues facing the Australian vegetable seed industry:

- Unreliable CMS hybrid cauliflower seed yields
- Rudimentary embryos in carrot seed lots.
- Expression of stunted root abnormality in onion seedlings

Major outcomes from this work include:

- Identification of pollination rate and temperature stress effects on pollen viability as constraints to CMS hybrid cauliflower seed yields. Management strategies based on time of transplanting and parent line arrangement were developed and in preliminary field evaluations in Tasmania were shown to substantially increase seed yields.
- Establishment of the relationship between seed maturity at cutting and occurrence of rudimentary embryos in carrot seed. In the absence of reliable tests to determine time of cutting, potential markers were evaluated. Seed coat chlorophyll fluorescence was developed as a simple, robust test of carrot seed maturity for use in determination of time of cutting. Subsequent experiments indicated significant potential to extend this test for use in determination of cutting time of *B. oleracea* crops.
- Development of seed pre-treatments based on hydropriming to minimise expression of stunted root abnormality in onion seed lots. This technique also benefited other onion seed lot quality attributes, including germination percentage and seed vigour. The finding that the benefits of hydropriming can be retained in storage is of particular importance for commercialisation of this technology.

The project outcomes provide significant opportunities for the Australian vegetable seed industry to improve hybrid cauliflower, carrot and onion seed production through an improved awareness of factors influencing seed yield and quality in these crops, identification of opportunities for improved crop management and adoption of technologies such as chlorophyll fluorescence testing and hydropriming.

Technical Summary

Approximately $20 million of vegetable seed is produced in Australia annually for export and domestic markets. Australia is seen as an important and reliable production location for 'out of season' seed crops by the major northern hemisphere vegetable breeders. In order to maintain this competitive advantage, it is important that Australian vegetable seed growers are able to consistently produce reliable yields of high quality seed. This challenge is greatest in hybrid seed crops grown from inbred parent lines. Difficulties in these crops are often attributed to impaired pollination or seed development although the specific factors involved and potential management options are poorly understood.

This project examined nature and management of three seed production issues of particular relevance to the Australian vegetable seed industry: unreliable seed set in CMS hybrid cauliflower seed production; occurrence of rudimentary embryos in carrot seed; and expression of stunted root abnormality in onion seedlings.

A series of pollen handling, storage and testing protocols were developed for trinucleate pollen of carrot and *Brassica oleracea* based on the FCR pollen viability test. It was demonstrated that a large proportion of the variation in seed yield in CMS hybrid cauliflower seed crops of late curding varieties grown in Tasmania during 3 seasons was caused by inadequate pollination and poor pollen viability at anthesis. Poor pollen viability was linked to high temperate stress. Under Tasmanian field conditions, management of flowering time through choice of transplanting time minimised loss of pollen viability due to high temperature stress and substantially increased seed yields in late flowering cultivars. Inadequate pollination was linked to incompatibilities between the conventional strip arrangement of hybrid seed production and the predominant pollinator insects, honey bees. Alternative parent line arrangements that promoted cross pollination were tested and shown to increase seed yields.

Pilot commercialisation of the research outcomes, including an evaluation of impacts on new management strategies on seed quality and adaptation to mechanised planting and parent line removal is currently underway in Tasmania. While the research has identified new approaches to management of cauliflower seed crops, it also highlights the importance of developing better understanding of the limitations within vegetable seed production systems. Adoption of these management practices into other cauliflower seed production systems, for example with different cultivars or production environments, should only be undertaken where there is a demonstrated need for improved management of pollination or temperature stress.

Following adoption of strategies to manage Rutherglen bug (*Nysius vinitor*) feeding damage (Spurr, 2003), the occurrence of seeds with rudimentary embryos was identified as the major quality constraint for carrot seed produced in south-eastern Australia. Rudimentary embryos were shown to relate to seed maturity at cutting time and their prevalence explained by the patterns of late embryo development in carrot seed crops produced in cool temperate conditions. Previous recommendations for time of harvest were based on studies in warmer climates or glasshouse production and were not relevant to the Australian production system. Screening and validation of

potential markers of carrot seed maturity led to the development of measurement of chlorophyll fluorescence of the seed coat as a robust test of carrot seed maturity / quality for use in determination of time of cutting of carrot seed crops. Preliminary experiments extending this work to *Brassica oleracea* seed production demonstrated that there is significant potential to use chlorophyll fluorescence as a marker of cutting time in these crops also.

It is recommended that the Australian vegetable seed industry consider commercialisation of this test to improve reliability of time of cutting in carrot seed crops and that its potential application in *B. oleracea* seed production also undergoes rigorous testing. The results of this work also highlight the potential value of a system of grading based on chlorophyll fluorescence for vegetable seed production in cool temperate climates.

Production of abnormal seedlings is an important quality limitation for onion seed lots grown in Australia. Although seed maturity at cutting has previously been implicated in this problem, no relationship between seed maturity, seed drying temperatures and expression of abnormal seedlings was observed in seed lots collected at a stage corresponding to current commercial harvest time. A range of seed pre-treatments were effective in reducing expression of stunted root abnormalities in seedlings. In particular, hydro-priming offers significant potential. The benefits of hydro-priming were shown to persist during seed storage, which would be a distinct advantage for commercialisation of this technology.

An important project outcome was the growth of ongoing research collaborations between researchers and Australian and international seed companies. The continued development of seed research expertise within the school of Agricultural Science and Tasmanian Institute of Agricultural Research represents an ongoing commitment of researchers, research providers and industry to investment in seed research.

SECTION 1

Pollen Viability and Pollination Research

Overview

Production of seed of the *Brassica oleracea* complex (cabbage, broccoli and cauliflower) in Australia is worth approximately $7.5 million annually and constitutes approximately 35% of the Australian vegetable seed industry (SIAA, 1999). With the global trend towards hybrid vegetable varieties, the majority of *B. oleracea* seed grown in Australia is hybrid seed. Although most of this is produced using self incompatible parent lines, there is an increasing demand for hybrid seed based on cytoplasmic male sterility (CMS). This demand is driven by the fact that CMS seed lots contain lower percentages of inbred seed than hybrid seed produced from self incompatible lines (Crisp and Tapsell, 1993) and are therefore of higher quality for vegetable growers. For seed producers, reliable production of CMS seed represents a significant challenge as seed yields from CMS crops are often poor or unreliable (George, 1999).

Poor CMS hybrid vegetable seed yields are often attributed to inadequate pollination (Erickson *et al.,* 1979; Rodet and Torre Grossa, 1991; Funari *et. al*., 1994). Whilst many studies have examined pollination of vegetable seed crops, few have looked specifically at pollination of CMS *B. oleracea* seed crops and, of the work that has been done in this area, little relates directly to the field based production system which predominates in the Australian industry. In addition, the role of pollen quality as a contributor to poor yields has largely been overlooked. This is mainly because pollen of *B. oleracea* is of the trinucleate type, which is comparatively difficult to handle, store, or test in *in-vitro* conditions (Shivanna and Rangaswamy, 1992).

This project studied the relationship between seed yield, pollination and pollen viability in field based CMS cauliflower seed production, with a view to developing practical strategies to improve seed yields. In order to achieve these objectives, it was first necessary to develop and validate methods for handling, storing and testing of cauliflower pollen. This work is outlined in Chapter 1. Chapter 2 describes a series of experiments that examine pollination and pollen quality as yield limiting factors in CMS cauliflower seed production in Tasmania. Chapters 3 and 4 build on the findings of Chapter 2 by evaluating practical strategies to increase CMS cauliflower seed yields by improving pollination and pollen quality.

As carrot seed production is also an important component of the Australian vegetable seed industry, with the same issue of unreliable yields from hybrid seed crops and trinucleate pollen similar to *B. oleracea*, the work in Chapter 1 was extended to include method development for carrot pollen handling, storage and viability testing as a basis for future yield studies in this crop.

CHAPTER 1

Development of Pollen Collection, Storage and Viability Testing Methods for Carrot (*Daucus carota*) and Cauliflower (*Brassica oleracea*)

Introduction

Brassicas and carrot both produce trinucleate pollen (Brewbacker, 1967; Spurr, 2003). In contrast to binucleate pollen, trinucleate pollen is generally difficult to germinate *in-vitro* (Shivanna and Rangaswamy, 1992) and easily loses viability, even in conditions routinely used for storage of binucleate pollen (Hoekstra, 1979). For many plants with trinucleate pollen, including carrot, methods for pollen handling, storage, *in-vitro* germination or other means of viability testing have not been developed or have not been validated against pollen performance *in-vivo* for use in pollen quality research. Storage, handling and *in-vitro* germination or other viability testing procedures have been published for many species of Brassica (for example see Mulchay and Mulchay, 1988; Rao*, et al.,* 1992; Jandurova and Pavlik, 1995; Shivanna and Swahney, 1995; Sato*, et al.,* 1998) but few studies have focussed specifically on *B. oleracea*. Whilst pollen of this species can be germinated *in-vitro* (Roberts*, et al.,* 1983) a comparison of the results of this method and *in-vivo* germination has not been published.

The work outlined in this chapter was undertaken to identify and validate suitable protocols for pollen collection, storage and viability testing that could be used in pollination research, and routine screening of pollen quality in seed parent lines for both carrot and *Brassica oleracea*.

Literature Review

Pollen Handling, Storage and Viability Testing Methods.

Introduction

Two forms of pollen are classified on the basis of the number of cells present in the pollen grain at anthesis, binucleate pollen and trinucleate pollen. Trinucleate pollen occurs in approximately one third of angiosperm species including both carrot (Spurr, 2003) and cauliflower (Brewbacker, 1967). In contrast to binucleate pollen, trinucleate pollen is generally difficult to germinate *in-vitro* (Shivanna and Rangaswamy, 1992) and easily loses viability, even in conditions routinely used for storage of binucleate pollen (Hoekstra, 1979). Consequently, there are relatively few trinucleate species for which storage and testing procedures have been developed and validated. The purposes of this review is to provide an overview of existing pollen viability testing procedures with respect to potential application in viability testing carrot and cauliflower pollen and to assimilate information that will form the basis of development of handling and storage protocols for pollen of these species.

Pollen Viability Tests

A range of pollen viability tests that have been used to test pollen of many plant species are outlined in the literature. These can be grouped into 3 main classes (Knox, 1984): 1) tests of fruit or seed set (*in-vivo* germination tests); 2) pollen culture (*in-vitro* germination tests) and; 3) histochemical methods.

1. In-vivo Germination Tests

Whilst artificial pollination of receptive flowers and assessment of seed set or pollen tube growth provides the most authentic test of pollen viability, it has several important limitations. Factors such as uncertainty about the amount of pollen deposited on the stigma (Young and Young, 1992), stigmatic receptivity (Stone*, et al.,* 1995), incompatibility reactions between the pollen and the pistil (Heslop Harrison*, et al.,* 1984) and abortion of the developing seed or fruit after fertilisation (Stephenson, 1981) can confound the assessment of pollen viability using this method. Waiting for seed maturation is time consuming and the seed counts that are obtained may be more qualitative than quantitative because, in many species, a small number of viable pollen grains may be all that is necessary for full seed set (Stone*, et al.,* 1995). Some of these limitations are overcome by studying pollen germination on the stigma surface, but this method is time consuming and generally not favoured for routine screening of pollen viability.

2. Pollen Culture

In-vitro germination tests circumvent some of the drawbacks of *in-vivo* germination tests. For many species they provide a direct and reliable assessment of pollen

germination capacity and correlate with fruit or seed set (Dafni and Firmage, 2000). The base of the germinating medium is a sucrose solution, typically of 10-40% concentration (Kearns and Inouye, 1993) into which other essential ions and nutrients are added. The pollen grains are suspended in the germinating medium in hanging drops, on microscope slides or on agar or gelatin media, and usually incubated for 1 to 12 hours before an assessment of germination is made (Shivanna and Rangaswamy, 1992). Under natural conditions, the stigma and style provide water, sugar and amino acids for nourishment and the appropriate osmotic conditions for germination and tube growth (Kearns and Inouye, 1993). The germination medium must therefore be formulated for each species to replicate these conditions. Failure to provide optimal conditions can result in a false negative assessment of pollen germination potential (Dafni and Firmage, 2000). For many pollen systems, only three elements, sucrose, boric acid and calcium nitrate are required for germination (Shivanna and Rangaswamy, 1992). The addition of magnesium sulfate and potassium nitrate to this basic medium produced a medium (known as Brewbacker and Kwack medium) that is suitable for the germination of pollen of a wide range of species including some trinucleate pollens (Brewbacker and Kwack, 1963). In addition to the effects of media composition, the results of *in-vitro* germination can be strongly influenced by factors such as incubation temperature and the concentration of pollen grains in the media (Brewbacker and Kwack, 1963).

Suitable *in-vitro* germination media have not been developed for carrot or other members of the Apiaceae family. The difficulty in developing media for these species appears to be caused by their reliance on exogenous sources for at least some metabolites from the start of germination, compared to binucleate pollen, which uses its own reserves during the first phase of germination (Mulchay and Mulchay, 1988).

3. Histochemical Tests

Histochemical tests can be divided into three types: the FCR test, enzyme tests and stainability. The histochemical tests have one common characteristic. In all cases, a positive result establishes the possibility, but not the certainty of effective function, whilst in a properly designed test a negative result should establish the certainty of non-function. The reliability of a histochemical test therefore depends on the closeness of the relationship between the property of the pollen grain assessed by the test and the reason for loss of germinability in the species under investigation.

The FCR test (Heslop Harison and Heslop Harrison, 1970) is principally based on the relationships between pollen viability and the integrity of the plasma membrane of the vegetative cell and the presence of active esterases within the pollen cytoplasm. Pollen samples are suspended in a sucrose solution of appropriate concentration containing the non-polar, non-fluorescent ester of fluorescin, fluorescin diacetate (FDA). FDA readily penetrates biological membranes. Cytoplasmic esterases cleave FDA to release fluorescin, which traverses intact membranes with greater difficulty and so accumulates intracellularly, where it can be detected by fluorescence microscopy. Thus, with the FCR test, viable pollen grains are indicated by their fluorescence under ultraviolet light. The results of the FCR tests are influenced by the concentration of sucrose in the FDA solution (Kearns and Inouye, 1993), through its effect on the osmotic potential of the solution. Optimal sucrose concentrations must

be determined for the species of pollen under investigation. These typically range from 2% to 40% (Kearns and Inouye, 1993).

The FCR test is a relatively simple procedure that provides a reliable estimate of pollen germinability and the capacity to effect fertilisation in a range of bi- and trinucleate species (Shivanna and Heslop-Harrison, 1981; Shivanna*, et al.,* 1991; Aronne*, et al.,* 2001). A number of studies have found close correlations between the results of FCR testing and *in-vitro* germination of freshly dehisced and aged or heat stressed pollen samples (Kearns and Inouye, 1993; Shivanna*, et al.,* 1991).

A major weakness with the FCR test is that it has an element of subjectivity associated with the scoring procedure, due to the continuum of intensity of fluorescence observed in some pollen samples (Kapyla, 1991 cited in Dafni and Firmage, 2000). Lipids are autofluorescent under the UV light used in the FCR test, so even dead, empty pollen grains will show some weak fluorescence (Shivanna and Heslop-Harrison, 1981).

Since the FCR test measures the potential for germination, rather than germination itself, it may overestimate viability in some cases, particularly if immature pollen is tested (Shivanna and Heslop-Harrison, 1981). As a positive result in the FCR test is reliant on a functional plasma membrane, the test can also yield false negative scores if dehydrated pollen is not properly pre-conditioned by re-hydration before testing (Shivanna and Rangaswamy 1992). Unlike *in-vivo* or *in-vitro* germination, the FCR test does not enable direct study of pollen germination or pollen tube growth (Shivanna*, et al.* 1991).

Tests of enzyme activity in pollen include the x-gal test (Trognitz, 1991 cited in Firmage and Dafni, 2001) and tetrazolium tests (MTT and Bakers solution tests) (Shivanna and Rangaswamy, 1992; Kearns and Inouye, 1993). The x-gal test is based on the hydrolysis of 5-bromo-4-chloro-3-indoyle-β-galactosidase to 5-bromo-4-chloro-indigo, a blue dye, by β-galactosidase (Trognitz, 1991 cited in Firmage and Dafni, 2001). Tetrazolium tests of pollen viability are based on the reduction of the tetrazolium salt by dehydrogenase enzymes to produce foramazan, which stains the pollen cytoplasm red. A positive stain is taken as an indication of a functioning respiratory system, an essential prerequisite for germination and tube growth. After staining, the pollen grains may vary from pale to deep red, making it difficult to establish a consistent cut-off point for viability (Shivanna and Rangaswamy, 1992).

For many species enzyme tests are a reliable indication of pollen viability (Stone*, et al.,* 1995); for others, they may yield false positive scores for viability when compared with the results from *in-vitro* germination tests (Heslop Harrison*, et al.,* 1984; Rodriguez-Riano and Dafni, 2000 cited in Dafni and Firmage, 2000). Therefore the reliability of enzyme tests needs to be established for each study species. Of a range of viability tests suitable for use in the field, MTT and Baker's solution provided the best estimates of *in-vitro* germination of pollen of 14 of 17 species including trinucleate species examined by Firmage and Dafni (2001).

Stains specific to pollen components can be used to test for viability. These include Alexanders stain and acetocarmine, which stain cytoplasm; and phloxin-green, which stains cytoplasm and cellulose (Kearns and Inouye, 1993). The reliability of such

stains is reduced in many systems because immature or non-viable pollen often contains enough of the target tissues to cause staining, and viable pollen of some species does not stain well (Kearns and Inouye, 1993). Stains often provide the least reliable means of estimating pollen viability (Heslop Harrison and Heslop Harison, 1984; Shivanna and Rangaswamy, 1991).

Pollen Testing of *Brassicas* and Carrot

There is anecdotal evidence of the use of acetocarmine staining by vegetable breeders as an indicator of pollen viability in carrot but the relationship between the results of this test and the capacity of pollen to germinate have not been published. The methods that are most appropriate for testing the viability of carrot pollen appear unknown, as no references to viability testing of pollen of carrot or other members of the Apiaceae could be found in the literature.

Several studies have evaluated the use of the FCR procedure as an indicator of the germination capacity of *Brassica* pollen. Sato, *et al*. (1998) rejected the FCR procedure as a reliable test of germination capacity of pollen of *B. rapa* on the bases that the results of this method were generally lower than those obtained in *in-vitro* germination tests and that pollen grains stained with FCR showed varying intensities of fluorescence, which made scoring of viability difficult. The original method of FCR testing (Heslop Harison and Heslop Harrison 1970) overestimated the *in-vitro* germination capacity of *B. juncea* pollen, but reliable estimates of *in-vitro* and *in-vivo* germination were obtained by allowing pollen samples to stand for 2 hours in the FDA solution prior to scoring (Rao*, et al.,* 1992). During this period, the membranes of some pollen grains began to leak resulting in a gradual reduction in the number of pollen grains fluorescing over time. It was concluded that this leakiness of membranes, which was not detected in the FCR test when samples were incubated for the usual period of 5 to 10 minutes, was linked to the main reason for pollen failing to germinate *in vitro*, or on the stigma.

A number of *in vitro* germination tests have been used to successfully germinate freshly collected pollen of Brassica species including *B. rapa* (Sato*, et al.,* 1998) *B. napus* (Shivanna and Swahney, 1995), *B. campestris* (Mulchay and Mulchay, 1988) *B. juncea* (Rao*, et al.,* 1992) and *B. oleraceae* (Roberts*, et al.,* 1983). Optimal germination media appear to be species specific (Jandurova and Pavlik, 1995), with failure to determine optimal media for the pollen being tested likely to yield false negative results. Despite this, most media developed for Brassica pollen share two common characteristics. Firstly, they are derivatives of Brewbacker and Kwack media (containing sucrose, boric acid, calcium nitrate, magnesium sulphate and potassium nitrate). Secondly, adjustment of the pH of the media to 8 to 9 is required for optimum germination rates. This is achieved with KOH (Sato*, et al.,* 1998), or buffers such as Tris and Taps (Roberts*, et al.,* 1983; Hodgkin and Lyon, 1985; Matsubera, *et al.*, 1999). In several studies, the addition of amino acids and other metabolites to the base media has been shown to improve germination rate or pollen tube growth of Brassica pollen (Roberts*, et al.* 1983; Mulchay and Mulchay 1988; Matsubera, *et al.*, 1999).

Pre-hydration, Storage and Re-hydration of Pollen

The pollen grain is a desiccated system. Equilibration of pollen in high humidity prior to germination or viability testing (pre-hydration) improves the test results for many species (Shivanna and Mohan Ran, 1993). Pre-hydration (>95% relative humidity (RH) for 30 minutes) improved the *in-vitro* germination response of freshly dehisced pollen of *B. juncea* and *B. oleraceae* (Chiang, 1974; Rao*, et al.,* 1992).

Pollen longevity in storage is greatly affected by the environmental conditions to which the pollen is exposed. For pollen shed in a partly dehydrated state, high or variable humidity and high temperature are most damaging to longevity (Johri and Vasil, 1961; Chang and Struckmeyer, 1975; Shivanna and Rangaswamy, 1992), but the combination of optimal preparation and storage conditions have to be determined for each species of pollen (Frankel and Gulan, 1977). Whilst binucleate pollens generally store well, under the same storage conditions, the longevity of trinucleate pollen is often short (Hoekstra, 1979). Preparation and storage conditions have been examined for a number of Brassica species (for example see Brown and Dyer, 1991; Sato*, et al.,* 1998) but no reports of suitable preparation and storage methods for carrot pollen were found in the literature.

Pollen of *B. rapa* germinated equally well *in-vitro* after 12 weeks storage at –20^{o}C and 15% RH as it did when freshly dehisced, but had lost approximately 50% of its viability after 12 months (Sato*, et al.,* 1998). At storage temperatures above 5^{o}C and high RH (66%) pollen viability was rapidly lost. Pollen of *B. oleracea*e and *B. napus* remained capable of fertilisation in test pollinations for 2 years following storage over silica gel at –20^{o}C (Brown and Dyer, 1991). Although confirming that some pollen remained viable under this storage regime for 2 years, neither study reports the extent of change in percentage of viable pollen over that time.

After low humidity storage, pollen generally shows some improvement of germination following exposure to a humid atmosphere (Hoekstra and Bruinsma, 1975; Shivanna and Heslop-Harrison, 1981; Sukhvibul and Considine, 1993). Optimal conditions for re-hydration vary according to the species involved and the degree of drying prior to storage but varying periods of exposure to 30°C and 97% RH have proven successful for several trinucleate species (Hoekstra and Bruinsma, 1975; Sukhvibul and Considine, 1993). Re-hydration at 25^{o}C and 66% RH for 5 hours was satisfactory for stored pollen of *B. rapa* (Sato, *et al.*, 1998).

Conclusion

The work reviewed clearly demonstrates that appropriate handling, storage and viability testing procedures for pollen should be determined for individual species. Currently, there appears to be no published information indicating suitable methods for use with carrot or other members of the Apiaceae family of plants. The information presented in this review provides a starting point for the experimental determination of such methods. Trinucleate pollens are generally shorter-lived than

binucleate pollens and require more extreme storage conditions to maintain longevity. Despite this, the principles of reduced temperature and humidity during storage, and the need to re-hydrate stored pollen before use apply to most species of both pollen types.

As there have been no published reports of appropriate pollen viability tests for use with carrot, potential tests must be identified and screened for suitability. There are anecdotal reports that acetocarmine staining has previously been used as an indicator of carrot pollen viability, but the information presented in this review suggests that it is unlikely to be the most suitable test. *In-vitro* germination has been successfully used for many species of pollen, but suitable media for some trinucleate families including the Apiaceae have not been identified or have not been reported. Difficulties establishing a reliable media may prevent the use of this method. Although it has several potential weaknesses and tends to overestimate viability in some species, the FCR test has been shown to produce reliable results for several bi- and trinucleate pollen systems, and should therefore be included in screening trials. Similarly, the reports of successful application of tetrazolium tests in the viability testing of a range of bi- and tri-nucleate pollen species (Firmage and Dafni, 2001) warrants an examination of their suitability for use with carrot pollen.

Pollen handling, storage and viability testing methods have been developed for research and practical application with a number of Brassica species. From these it can be concluded that the following are likely to be essential elements in a protocol for handling and viability testing cauliflower pollen samples: 1) Pre-hydration of freshly dehisced pollen samples prior to *in-vitro* germination or viability testing; 2) desiccation of pollen samples prior to storage; 3) a combination of low temperature (-20^{o}C) and low humidity storage conditions and; 4) re-hydration of desiccated pollen samples prior to germination or viability testing.

Most reports of viability testing of Brassica pollen involve either *in-vitro* germination or the FCR test. Across a range of studies and Brassica species, variable results have been achieved in terms of assessment of germination capacity using either test; due in part to the need to establish optimal *in-vitro* germination media for individual species or cultivars (Matsubera, *et al.*, 1999) and differences between FCR testing methodologies. Assessments of the use of other simpler procedures such as tetrazolium staining on pollen of *B. oleraceae* or other Brassicas were not found in the literature. Such tests may have merit in bulk screening of pollen samples, or in use with field sampling due to their minimal requirement for materials or specialised equipment and ease of preparation (Firmage and Dafni, 2001).

Materials and Methods

Plant Material

Pollen samples used in this work were from field or glasshouse grown plants of Nantes type carrot lines 22, ON44 and 8024 and temperate cauliflower lines, BL529, C, E and F. *In-vivo* germination tests used male sterile carrot and cauliflower grown in pots in glasshouse conditions.

Evaluation of Pollen Viability Tests

The pollen viability tests that were evaluated for use are described in Table 1. Specific methods for each test are given in Appendix 1. *In vitro* germination testing was not included in screening of tests for carrot pollen as preliminary attempts using Brewbacker and Kwack's medium (Brewbacker and Kwack, 1963) with a range of incubation temperatures between 15 and 30^{o}C, pHs between 5 and 9 and sucrose concentrations between 0 and 50% in both hanging drops and on agar failed to induce any carrot pollen to germinate.

Pollen samples used in the evaluation of the viability tests for each species were sourced from pollinator lines grown in glasshouse and field trials. After collection the samples were tested immediately, or exposed to a range of storage or stress treatments prior to testing. The origins and post anthesis histories of all pollen samples used in this work are given in Appendix 2. During the screening process, a representative fraction of each pollen sample was allocated to testing with each method. All tests were set up within 15 minutes of sample collection or retrieval from storage or stress treatments.

Relationships between individual tests and *in-vivo* germination were analysed using the linear regression function within SPSS 12.0. (SPSS Inc., Chicago)

Table 1.1 – Pollen viability tests evaluated for testing of carrot and cauliflower pollen.

Pollen Type	Viability Test	Properties Assessed
Carrot	*In-vivo* germination	Pollen germination on a receptive, compatible stigma under favourable conditions (plants housed in shadehouse at approx 25^{o}C and 55%RH)
	FCR test	Cell membrane integrity; competence of pollen esterase enzymes
	MTT test	Competence of pollen dehydrogenase enzymes
	Acetocarmine stain	Presence of cytoplasm in the pollen cells
Cauliflower	*In-vivo* germination	Pollen germination on a receptive, compatible stigma under favourable conditions (plants housed in shadehouse at approx 20^{o}C and 55%RH)
	In-vitro germination (Brewbacker and Kwack medium adjusted to 20% sucrose and pH8)	Pollen germination capacity in artificial media
	FCR test	As above
	MTT test	As above
	Bakers solution test	Competence of pollen dehydrogenase enzymes
	x-gal test	Competence of pollen β-galactosidase enzyme
	Acetocarmine stain	As above
	Alexander's stain	Presence of cellulose in pollen cell walls and cytoplasm in the pollen cells

The Effects of Pre-hydration, Desiccation and Re-hydration on Pollen Viability

The effects of pre-hydration of freshly dehisced pollen prior to testing, pollen desiccation prior to storage and re-hydration of stored pollen prior to viability testing were examined for both species (carrot lines 22 and 8024, cauliflower lines F and BL529). The treatments tested on each species are shown in Table 1.2. The effects of pre-hydration were tested in an ANOVA randomised complete block design, with treatments replicated through time. Desiccation and re-hydration treatments were

examined in a factorial ANOVA design replicated through time. All analysis was performed using SPSS V12.0 (SPSS Inc., Chicago).

Table 1.2 – Pre-hydration, desiccation and re-hydration treatments tested in this work

	Carrot	Cauliflower
Pre-hydration	0, 30 and 60 minutes @ 20°C and 97% RH	0, 30 and 60 minutes @ 20°C and 97% RH
Desiccation	0, 24 and 48 hours over silica gel @ 4°C and 20°C	0 and 48 hours over silica gel @ 20°C
Re-hydration	0, 15, 30 and 60 minutes at 30°C and ca.97% RH in a water bath	0, 15, 30 and 60 minutes at 30°C and ca.97% RH in a water bath

The Effect of Storage Temperature on Pollen Longevity

This work was undertaken primarily on carrot pollen. Pooled samples of freshly dehisced pollen were collected from glasshouse grown plants of lines 22 and 8024. A sub sample of pollen from each line was tested for viability using the FCR procedure and the remainder desiccated at 20°C for 48 hours. After desiccation, the pollen of each line was randomly divided between storage temperatures of 25, 4, -20 and –80°C. At time intervals of 3, 12, 30, 60, 120, 180 and 365 (8024) and 12, 60, 180 and 365 (22) days after desiccation, 4 replicate sub-samples of pollen of each line were removed from storage at each temperature, re-hydrated at 30°C and 97% RH for 30 minutes and tested for viability using the FCR procedure. Storage treatments were analysed using ANOVA analysis. Since temperature treatments could not be replicated, LSD values were calculated at a probability of 0.01. The analysis was performed with SPSS V12.0 (SPSS Inc., Chicago).

After 365 days storage a sub-sample of pollen of line 22 was removed from the -80°C treatment, re-hydrated as above and used to hand pollinate 20 receptive flowers on 4 corresponding male sterile line plants under glasshouse conditions. Seed set was scored 6 weeks after hand pollination.

Storage conditions determined to be suitable for carrot pollen were subsequently tested for on cauliflower pollen from line BL529. As above the suitability of the conditions was assessed on the basis of pollen viability (FCR test) and capacity to set seed after 1 year of storage.

Results and Discussion

The aim of this work was to identify suitable methods for the handling, storage and viability testing of pollen of carrot and cauliflower for use in research and routine screening of the pollen quality of seed parent lines that satisfied the following criteria: 1) retention of pollen viability during handling and storage; 2) reliability in prediction of *in-vivo* germination capacity under favourable conditions; 3) suitability for processing large numbers of samples and; 4) compatibility with transporting of samples over long distances from remote field sites.

Pollen Viability Tests

Staining responses of carrot and cauliflower pollen in the pollen viability tests are illustrated in Plates 1.1 and 1.2.

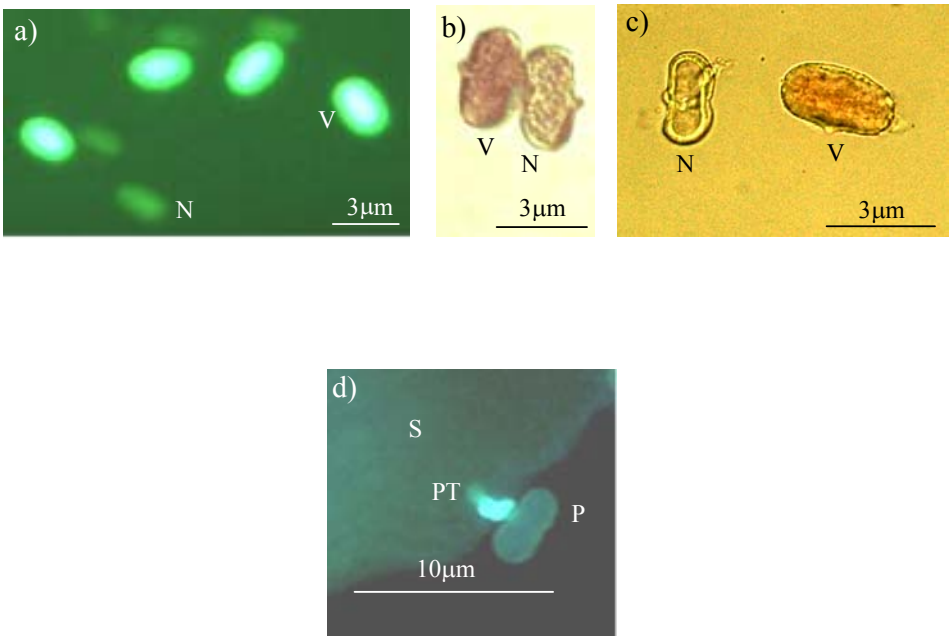


Plate 1.1 – Viable (V) and nonviable (N) carrot pollen as determined by a) the FCR test; b) MTT staining; and c) acetocarmine staining. d) – *In-vivo* germination of a carrot pollen grain (P) on the stigmatic surface (S) 24 hours after hand pollination as seen under a fluorescence microscope following staining with aniline blue. The grain has germinated and produced a pollen tube (PT). The pollen tube can be seen entering the stigmatic surface but is then obscured by the non-cleared stigmatic tissue.

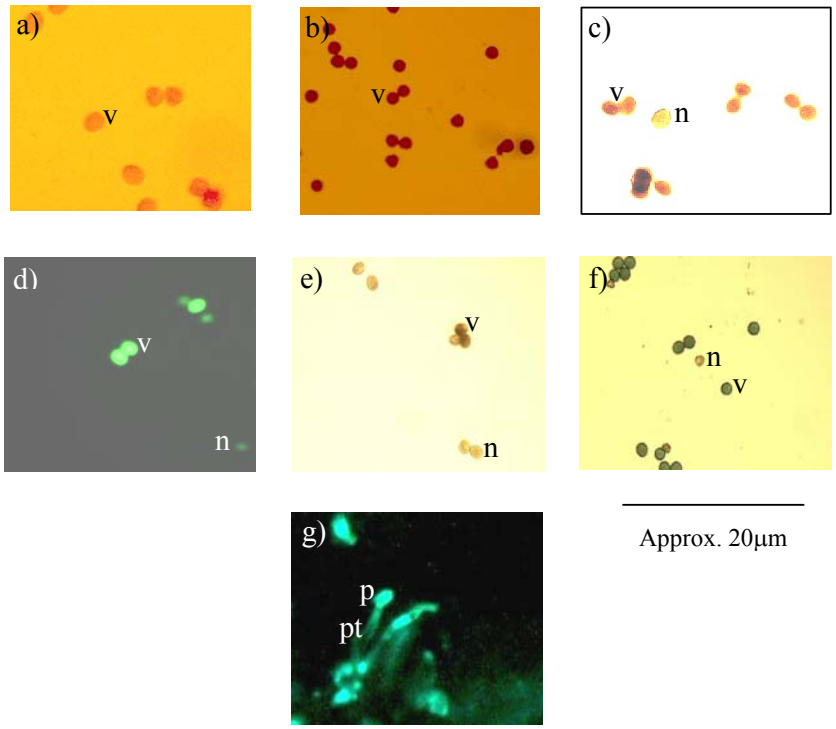


Plate 1.2 - Viable (V) and nonviable (N) cauliflower pollen as determined by a) alexanders stain; b) acetocarmine staining; c) bakers solution; d) the FCR test; e) MTT staining; and f) the x-gal test. g) Cauliflower pollen grains germinating *in vivo* on a section of stigmatic tissue (p=pollen grain, pt=pollen tube).

FCR testing has been shown to provide a reliable estimate of *in-vivo* germination in several species including *Nicotiana tabacum*, *Agave* sp., *Tradescantia virginiana* and *Iris* sp. (Shivanna *et al.,* 1991) and in this work accurately estimated the results of *in-vivo* germination of freshly dehisced pollen of carrot and cauliflower ($P< 0.01$ r^2(carrot) = 0.84; r^2(cauliflower) = 0.93) (Table 1.3; Figures 1.1 and 1.2). In both plants it was a much simpler and faster test to perform than *in-vivo* germination. Although the relationship was generally very close, FCR testing did overestimate *in-vivo* germination of some carrot pollen samples of poor viability by up to 14% (Figure 1.1). This trend was not isolated to a particular line of pollen or pollen history and is presumably a reflection of the fact that this method tests the potential for germination, rather than germination itself (Shivanna and Heslop Harrison, 1981). Thus whilst the FCR test result appears to be a good general measure of germination capacity, its accuracy may be reduced when testing poor quality pollen samples.

Previously reported difficulties associated with the continuum of intensities of fluorescence in FCR tests (Shivanna and Heslop Harrison, 1981; Kapyla, 1991 cited in Dafni and Firmage, 2000; Rao *et. al.,* 1992; Sato *et. al.,* 1998) were overcome in this work by use of a digital camera mounted to the fluorescence microscope (Leica 300F; Leica, Heerbrugg, Switzerland) to photograph the pollen samples for subsequent scoring using Adobe Photoshop® V7.0 (Adobe System Inc., USA) software with the Fovea Pro® plug in. This ensured that only pollen grains fluorescing above a set threshold of intensity were scored as viable, which markedly improved the repeatability of the test. This approach allowed for automated scoring of

large batches of images, which reduced the time required to process individual tests and increased the suitability of the FCR test for testing large numbers of pollen samples.

Table 1.3 – Comparison of *in-vivo* germination percentage of carrot pollen samples with the results of the acetocarmine, MTT and FCR viability tests. Note samples a and b refer to pollen collected on 2 separate days during the flowering period. The figures in italics indicate standard errors, n=4.

	Viability test							
Pollen source	*In-vivo*		Acetocarmine		MTT		FCR	
8024a	10.8	*1.4*	96.7	*2.3*	26.1	*7.6*	19.2	*3.0*
8024b	14.3	*7.1*	94.7	*1.5*	38.3	*6.0*	12.5	*3.9*
22	14.2	*4.8*	NA	NA	51.0	*15.9*	28.4	*5.1*
ON44a	10.4	*2.1*	97.7	*6.5*	40.7	*11.5*	14.6	*2.1*
ON44b	41.7	*11.8*	97.7	2.3	52.0	*4.8*	40.0	*4.9*

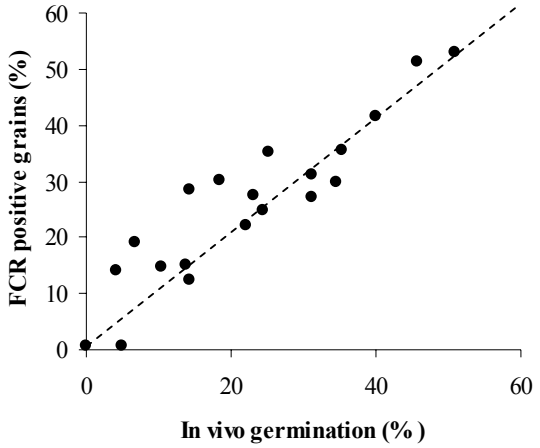


Figure 1.1 – Pollen viability estimated by the FCR procedure plotted against *in-vivo* germination capacity for pollen samples of the carrot lines 8024, 22 and ON44 with differing origins and histories. A significant linear relationship exists between the two variables ($P<0.001$; $r^2 = 0.84$). The broken line indicates a 1:1 relationship between the two variables. Each symbol is the mean of 4 replicate test samples.

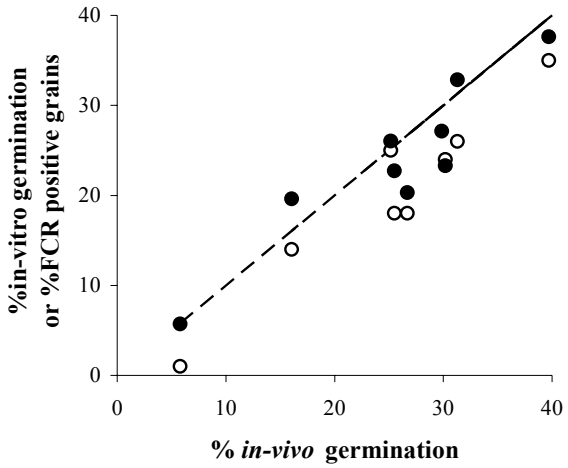


Figure 1.2 – Pollen viability estimated by *in-vitro* germination (open symbols) and the FCR procedure (filled symbols) plotted *in-vivo* germination capacity for cauliflower pollen samples from a range of lines with differing origins and histories (see Appendix 2). Significant linear relationships exist between the results of both test methods and *in-vivo* germination capacity ($P<0.01$ r^2 (*in-vitro*) = 0.93; r^2 (FCR) = 0.88). Each sample point is the mean of 4 replicate test samples. The broken black line indicates a 1:1 relationship between the x and y variables.

Although some studies have shown that enzymes tests and stains specific to certain components of the pollen cell provide a useful indication of pollen viability (Stone *et al.,* 1995; Firmage and Dafni, 2001), with the exception of *in-vitro* germination of freshly dehisced cauliflower pollen (Figure 1.2), the results of all other viability tests included in this work bore little relationship to *in-vivo* germination of carrot or cauliflower pollen and generally overestimated it by a large margin (Table 1.3; Figure 1.3).

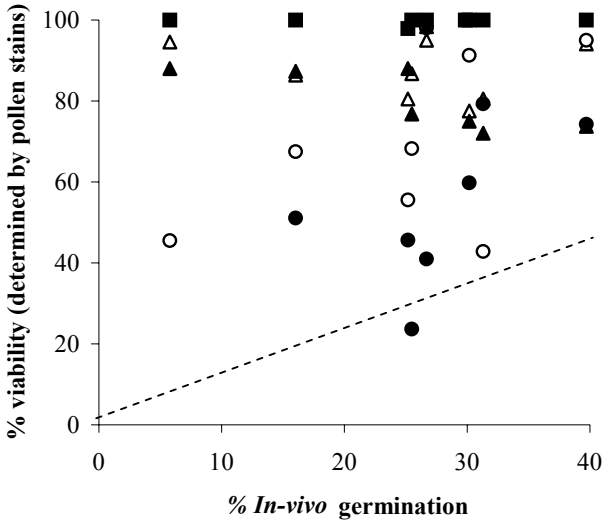


Figure 1.3 – The relationship between pollen viability estimated by *in-vivo* germination and the acetocarmine (Δ), alexanders stain (■), bakers solution (◊), x-gal (▲) and MTT (♦) pollen viability tests. The pollen samples were the same as those used for evaluation of the FCR procedure and *in-vitro* germination. Each sample point is the mean of 4 replicate test samples. The broken line represents a 1:1 relationship between the x and y variables.

Preliminary trials confirmed that freshly dehisced cauliflower pollen would germinate readily (within 6 hours) in Brewbacker and Kwack medium in hanging drops or on agar incubated at 15 to 20^{o}C with optimal sucrose concentration of 20% and pH of 8 (adjusted with KOH). Whilst this method provided a good estimate ($P<0.01$; r^2 =) of *in-vivo* germination of freshly dehisced pollen samples (Figure 1.2), pollen samples that had been stored and subsequently shown to be viable in FCR tests, *in-vivo* germination tests and by capacity to set seed did not germinate. Poor germination of stored pollen in media that were optimal for fresh pollen of the same species has been reported before (Shivanna and Rangaswamy, 1992) but an explanation for this phenomenon does not appear to have been published. A review of 9 other studies using *in-vitro* germination methods for Brassica pollen including relatively recent studies by Shivanna and Swahney (1995), Jandurova and Pavlik (1995), Sato *et al.* (1998) and Matsubera *et al.* (1998) confirmed that all used freshly dehisced pollen rather than stored samples. In the current work, varying the sucrose concentration of the media and duration and rate of re-hydration of stored pollen samples prior to testing failed to induce germination, or resulted in extremely low and erratic germination. The basis of this difference in germination behaviour of fresh and stored pollen remains unclear and requires further investigation.

An important limitation in the validation of testing procedures in this work is that no pollen samples of either plant with an *in-vivo* germination percentage above 55% were tested. Care should therefore be taken in extrapolating the findings of this work for use with higher quality pollen samples.

Pre-hydration, Desiccation and Re-hydration

While freshly dehisced samples of cauliflower pollen had higher *in-vitro* germination percentage or viability (FCR) after 30 minutes pre-hydration at 20^{o}C and 97% RH ($P<0.05$), freshly dehisced carrot pollen samples did not (Figure 1.4). The duration of pre-hydration treatment for cauliflower pollen was clearly important with longer periods of 60 to 120 minutes causing significant reductions in pollen quality.

As cauliflower pollen samples that had been stored did not germinate reliably *in-vitro*, the following discussion of storage and re-hydration conditions is based on the results of FCR testing only. Carrot and cauliflower pollen showed an absolute requirement for desiccation prior to storage, with failure to desiccate resulting in a complete loss of viability within 24 hours of anthesis, irrespective of storage temperature or re-hydration treatment. Such a response is typical of trinucleate pollens, which are characterised by short life spans following anthesis (Hoekstra, 1979) and has obvious implications for pollen handling prior to storage.

No loss of viability was observed during desiccation of freshly dehisced pollen over silica gel for up to 48 hours at 4^{o}C (carrot) or 20^{o}C (carrot and cauliflower) (Table 1.4). Following desiccation, re-hydration was required prior to FCR testing (Table 1.4) but as in pre-hydration the duration of the re-hydration stage was important, with a period of 30 minutes at 20^{o}C and 97% RH being optimal. The sensitivity of both pollen types to prolonged exposure to warm moist conditions is consistent with observations for other trinucleate pollens (Hoekstra and Bruinsma, 1975) and has been suggested to reflect relatively low levels of reserve metabolites (Frankel and Gulan, 1977) and high respiratory rates (Hoekstra and Bruinsma, 1979) in trinucleate pollen compared with binucleate pollen. In comparison, the two cauliflower lines studied showed marked differences in terms of reduction in pollen viability during prolonged exposure to warm humid conditions (Figures 1.4 and 1.5), which suggests that there could be substantial genetic variation in pollen longevity in cauliflower.

Overall, a combination of desiccation over silica for between 24 and 48 hours at 20^{o}C immediately following pollen collection and re-hydration at 20^{o}C and 97% RH after storage was shown to be satisfactory for preserving carrot and cauliflower pollen viability and optimising the FCR test results. The compatibility of these protocols with field based sampling and transport of pollen and that samples can be easily and rapidly re-hydrated in a water bath prior to testing are considerable advantages.

During 1 year of storage at -80^{o}C no significant loss of viability in 2 lines of carrot pollen was observed (Table 1.5). Over the same time period, the viability of pollen stored at 18, 4 and –20°C declined markedly ($P<0001$) to between 0 and 7.5%. 75% of male sterile carrot flowers hand pollinated with pollen stored at -80^{o}C for 1 year set seed. No significant loss of viability of pollen of cauliflower line BL529 (initial viability 44%) was observed during storage at -80^{o}C for 1 year. 100% of male sterile flowers hand pollinated with this pollen after 1 year of storage set pods with an average of 6 seeds pod^{-1}.

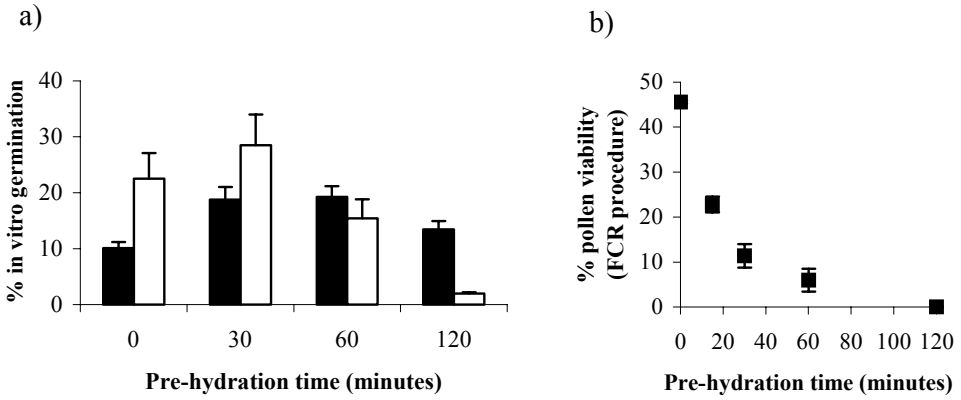


Figure 1.4 – The effect of different periods of pre-hydration at 97%RH and 20°C on the *in-vitro* germination of freshly dehisced pollen of: a) the cauliflower pollinator lines BL529 (■) and F (□); and b) the FCR test results for pollen of the carrot line WO8024. Bars on the graphs indicate standard errors (n=4). Differences between the viability of pollen samples after different periods of pre-hydration are statistically significant. LSD's (P<0.05): cauliflower line BL529 = 7.0, line F = 11.8; carrot line WO8024 = 5.13.

Table 1.4 – The effects of duration and temperature of desiccation over silica gel and duration of re-hydration at 30°C and 97% RH on viability (%) of pollen of the carrot pollinator lines WO030 and No. 22. Each data entry is the mean of four replicates.

Desiccation time (hours)	Desiccation temp (°C)	Re-hydration (minutes)	% Pollen viability (FCR test) WO8024	22
Freshly dehisced pollen (Control)			54.5	32.6
24	4	0	47.8	36.0
24	4	30	38.5	31.8
24	4	60	33.1	29.2
24	20	0	28.5	0.0
24	20	30	47.5	32.3
24	20	60	26.0	19.5
48	4	0	28.5	5.4
48	4	30	48.6	32.0
48	4	60	27.6	2.5
48	20	0	16.3	0.0
48	20	30	47.3	28.5
48	20	60	34.0	16.9
LSD (P<0.05)			9.24	8.51

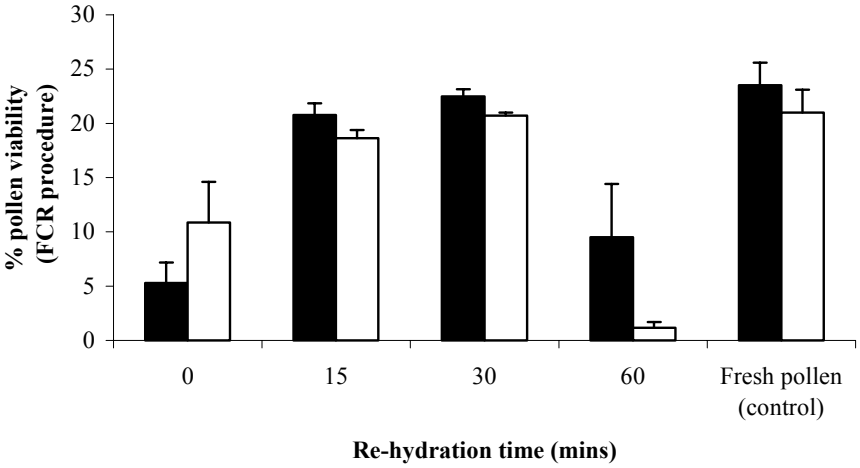


Figure 1.5 – The effects of duration of re-hydration at 30°C and 97% RH on the viability (as determined by the FCR Procedure) of pollen of the cauliflower pollinator lines BL529 (■) and F (□) previously desiccated for 48 hours over silica gel at 20°C and stored at –80°C. The bars indicate standard errors (n=4). Differences between pollen viability for different times of re-hydration are statistically significant: BL 529 LSD (P<0.05) = 6.7; F LSD (P<0.05) = 4.7.

Table 1.5 – The effect of storage temperature on the longevity of pollen of the carrot lines No. 22 and WO8024 (results as % viability). Due to the lack of randomisation between temperature treatments, significant differences between temperatures were determined at the 0.01 level of probability.

Line	Storage temperature (°C)	Time of storage (days)								LSD (P<0.05) (time)
		0	3	12	30	60	120	180	365	
WO8024	18	52.4	-	14.4	-	8.9	6.2	2.2	0.0	9.2
	4	52.4	37.5	32.0	28.5	21.3	18.6	14.7	4.2	13.2
	-20	52.4	-	51.1	36.8	37.4	-	19.7	7.5	9.0
	-80	52.4	52.4	53.0	50.5	53.2	51.5	48.4	43.9	11.4
	LSD (P<0.01) (temperature)		11.9	13.2	9.6	11.8	4.8	7.9	12.4	
No. 22	18	60.0		20.8		9.8		0.7	0.0	10.0
	4	60.0		55.0		34.4		24.1	0.0	8.0
	-20	60.0		56.9		46.0		26.5	0.0	6.5
	-80	60.0		59.4		55.9		53.5	50.0	17.9
	LSD (P<0.01) (temperature)			20.3		10.3		15.3	8.9	

In summary, the data presented in this chapter define a series of effective and relatively simple handling and storage procedures for pollen of carrot and cauliflower that are compatible with field sampling and transport of samples. The FCR test appears to be the most satisfactory pollen viability test currently available for routine screening of carrot pollen samples and was also shown to be satisfactory for routine screening of cauliflower pollen samples. Whilst an *in-vitro* germination test is available that appears to reliably indicate pollen quality for freshly dehisced samples, further work is required to develop a method that is suitable for use with stored pollen and to explain the discrepancy in performance of freshly dehisced and stored pollen in *in-vitro* media.

CHAPTER 2

Identification of Yield Limiting Factors in Cytoplasmic Male Sterile Hybrid Cauliflower Seed Production

Introduction

As noted in Chapter 1, production of hybrid seed of the *Brassica oleracea* complex (cabbage, cauliflower and broccoli) is a significant component of the Australian vegetable seed industry. It is a particularly important industry in Tasmania, where in excess of $6 million worth of seed is produced by around 130 farmers annually (SIAA, 1999; DPIWE, 1999).

Historically, hybrid Brassica seed crops have been based on the use of a self incompatibility (SI) reaction to ensure outcrossing between the two parent lines. A deficiency in this system is that self incompatibility can break down under certain conditions, resulting in unacceptably high levels of inbred (sib) seed within hybrid seed lots (Crisp and Tapsell, 1993). This limitation has led breeders to adopt an alternative system of hybrid seed production that uses cytoplasmic male sterility (CMS). CMS lines have cytoplasmic genes that prevent self pollination by blocking the late stages of anther development or pollen development. Whilst this system is reliable in preventing inbred seed production, seed yields from CMS crops are often lower than from SI crops, and in some instances are uneconomically low. This problem is compounded by the fact that in CMS crops only the male sterile line is harvested (seed set on the pollinator line is inbred).

Poor seed yields from CMS hybrids have often been associated with a low rate of flowers setting seeds indicating a link between poor yields and processes associated with pollination, fertilisation or early seed development (Erickson et al, 1979; Rodet and Torre Grossa, 1991; Hogarth, 1998). Inadequate pollination has been implicated as a yield limited factor in CMS crops including carrot (Erickson et al, 1979; Rodet and Torre Grossa, 1991), canola (Hogarth, 1998) and in self incompatible hybrid brussel sprout seed production (Faulkner, 1978). Funari *et. al (1994)* reported that in cauliflower CMS flowers were less attractive to pollinators that perfect flowers. This chapter reports on experiments that examine the link between pollination, pollen quality and seed yield in CMS cauliflower seed crops grown under Tasmanian field conditions.

Materials and Methods

Trial Sites

Field based trials were undertaken in a commercial hybrid seed crop at Sorell in southern Tasmania in 2003 – 04 and in trial plots of BL185 and BL529 at the Forthside Vegetable Research Station (FVRS) in north-west Tasmania in 2003 – 04 and 2004 – 05 (Figure 2.1). Field based screening of a range of pollinator lines was also undertaken at FVRS in 2003 - 04. Shade house based screening of pollinator lines was undertaken at the School of Agricultural Science glasshouse complex at the University of Tasmania in Hobart. Seasonal and long-term climatic data for the two field sites are given in Table 2.1. Cauliflower lines studied in this work flowered under temperature ranges of at 5.1 to 32.8^{o}C at Sorell, 7.5 to 25.8^{o}C at FVRS in 2003, at 6.5 and 26.5^{o}C at FVRS in 2004 and 8.8 to 29.8^{o}C in the shade house trial.

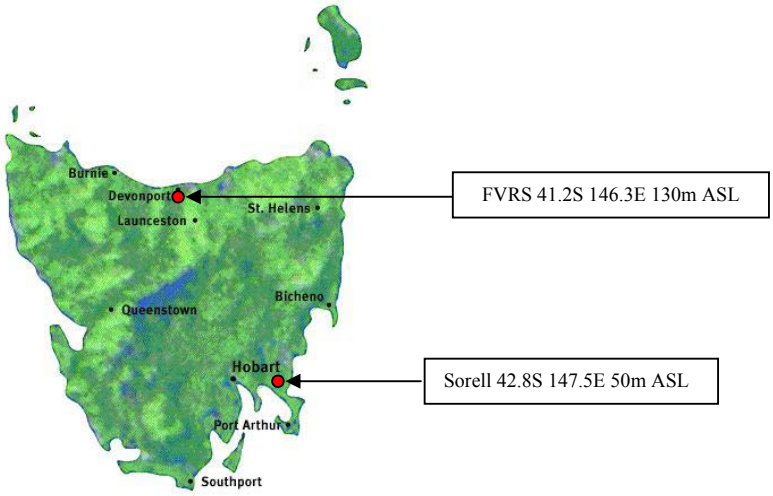


Figure 2.1 – Location of the Forthside Vegetable research Station (FVRS) and Sorell field trial sites in Tasmania, Australia.

Table 2.1 – Long term and season 2003 – 04 climatic data for Sorell and FVRS. Data were sourced from the Australian Bureau of Meteorology weather stations located at the Hobart Airport, 8km south west of the Sorell field site, and at FVRS, 1km from the field site.

Month	Long Term			Season 2003 - 04			Season 2004 - 05		
	Mean Max Temp °C	Mean Min Temp °C	Precipitation (mm)	Mean Max Temp °C	Mean Min Temp °C	Precipitation (mm)	Mean Max Temp °C	Mean Min Temp °C	Precipitation (mm)
Mar	19.8	10.4	55.5	21.2	10.2	56	20.4	9.4	24.8
Apr	16.9	8.2	74.3	18.7	8.5	96.6	17.1	8.4	69.4
May	14.4	6.2	89.1	15.9	7.8	70.8	14.6	5.0	70.6
Jun	12.2	4.1	103.2	13.7	5.4	152.4	12.5	5.4	159.6
Jul	11.5	3.6	125.3	12.1	3.7	95.8	11.6	3.2	123
Aug	12.1	4.2	116.9	NR	NR	NR	12.6	4.2	68.4
Sep	13.3	4.9	98.3	NR	NR	NR	14.5	5.1	32.2
Oct	15.4	6.2	84.9	15.2	4.7	28.6	16.5	6.6	30.8
Nov	17.1	8.1	69.5	18.5	8.5	21.2	18.1	8.7	2.9
Dec	18.9	9.6	67.5	21.0	10.8	73.2	21.2	10.4	1.4
Jan	20.6	11	51.5	20.7	9.6	149	21.9	11.4	16.2
Feb	21.1	11.6	46.7	21.7	11.5	17.4	21.4	10.5	35.6
Mar	20.6	10.7	37.2	19.5	11.5	112.4			
Apr	18.1	8.7	44.7	17	9.4	38			
May	15.1	6.5	35.9	15.9	8	38.8			
Jun	12.9	4.6	31.1	14.2	5.4	46			
Jul	12.4	4.0	45.0	12.9	4.2	25.2			
Aug	13.4	4.6	48.2	13.9	5.2	85.2			
Sep	15.2	5.9	40.3	14.1	5.1	64.4			
Oct	17.2	7.4	48.2	15.6	6.3	196			
Nov	18.9	9.0	45.0	20.3	9.5	1.8			
Dec	20.5	10.6	54.3	21.1	12.2	43.2			
Jan	22.4	11.9	41.6	21.5	11.6	76.6			
Feb	22.3	12.0	35.8	21.5	11.9	15.6			

Plant Material and Cultural Conditions

All trials used hybrid cauliflower parent seed lines. Field based pollination experiments used the CMS hybrid crosses BL529 and BL185. These were chosen on the basis of commercial importance, the fact that their seed yields are typical of many CMS hybrids and, in the case of BL529, previous yield difficulties experienced in Tasmania. Pollen viability was screened in 11 pollinator lines designated BL185, BL529, A and C to J under field (FVRS) and glasshouse (pot trial) conditions. Transplants were grown from stock seed in a commercial seedling nursery.

Field trials were grown on 1.2m wide raised beds, with 2 rows of plants, 800mm apart per bed. Within the hybrid crosses, the parent line arrangements were conventional strip designs (see diagram in Chapter 3) with 2:1 pollinator to male sterile row ratios. In these crosses the planting of the pollinator lines was split over two dates 1 to 2 weeks apart to ensure that the hybrid nicked well. All cultural practices used in field trials were based on current commercial practice.

The details of the shade house pot trial are as follows. Seedlings were transplanted into 20L plastic pots (1 seedling per pot) filled with a potting mix consisting of a 7:3:1

mixture of pine bark:sand:peat. Each 50L of potting mix contained 300g of Osmocote Plus® slow release fertiliser with micronutrients, 90g of dolomite, 25g of iron sulphate and 75g of lime. Irrigation was by Octadrippers® located in each pot at the potting mix surface. Daily irrigation rates were set to maintain non-stressed growing conditions and were gradually increased as the plants developed.

Full strength Hoagland's nutrient solution was applied to the potted plants from prior to curd initiation until the end of the trial at 14 day intervals. A 10 day fungicide program consisting of alternate applications of Kocide®, Mancozeb®, Sumisclex®, Foliar Phos® and Kocide®, Mancozeb®, Benlate® and Foliar Phos® mixes with Synetrol® oil was started prior to curd break and continued until just prior to flowering.

Pollination Studies

Supplemental Hand Pollination

Supplemental hand pollination experiments were undertaken in a 1Ha commercial crop of BL529 at Sorell and in 280m^2 trial plots of BL529 at FVRS in 2003 - 04. In 2004 - 05 the experiments were repeated in 280m^2 trial plots of BL529 and BL185 at FVRS. In each trial 12 male sterile plants of each line that flowered at peak bloom were selected for hand pollination in supplement to natural pollination. Each hand-pollinated plant was paired to a control plant of similar size, stage of development and position, which received natural pollination only. Due to the large number of flowers on individual plants, hand pollinations were performed on all flowers of 2 to 4 inflorescence branches. This resulted in hand pollination of between 15 and 30% of the total number of flowers on each plant. Hand pollinations were performed between 10am and 1pm on 4 occasions at each site, 3 to 4 days apart, to ensure that a large proportion of the target flowers were effectively pollinated. The hand pollination procedure was to collect freshly dehisced anthers from the pollinator line and brush these against the receptive stigmas of the male sterile line. On each day a sub-sample of the pollen used for hand pollination was collected, desiccated, stored and subsequently tested for viability (FCR procedure). The methods used were those recommended in Chapter 1 viz. desiccation for 48 hours over silica gel at ambient laboratory temperatures, storage at –80^{o}C, rehydration for 30 minutes at 30^{o}C and 90%RH, and testing in an FCR solution with 20% sucrose.

On each date of hand pollination 4 receptive flowers were selected at random, removed from each control and hand pollinated plant and pooled within the treatments to give two samples of 48 flowers. These samples were used to compare pollination rates between the two treatments. The method of assessment of pollination rate was adapted from Beattie (1971a; cited in Kearns and Inouye, 1993). The stigmas of the sampled flowers were excised with a scalpel and mounted on a microscope slide in a drop of melted basic fuschin gel. The prepared slides were examined under a light microscope at 100x magnification. Individual pollen grains present on the stigmatic surface stained red against a background of unstained stigmatic tissue. Pollination

levels were reported as the percentage of pollinated flowers and the mean number of pollen grains per stigma.

In 2004 pod set was assessed *in-situ* 14 days after the final hand pollination. Five randomly selected sub-branches were examined for each plant in each treatment and the % of flowers that had set pods determined.

At maturity all flowering branches of the hand pollinated and control plants were removed and counted. The branches from individual control plants were pooled to produce one sample per plant, which was bagged in hessian for drying. The branches of the hand pollinated plants were separated into two groups consisting of the 4 hand pollinated branches and the remaining non-hand pollinated branches, with each group bagged separately for drying. All samples were dried for 1 week at 25^{o}C in a commercial seed dryer. After drying, the samples were hand threshed. Seed cleaning was undertaken using laboratory scale air-screen, density and spiral separators to produce pure seed samples. Care was taken to ensure that all fully developed seeds were retained within the samples during the cleaning process. Clean dry seed lots were stored in sealed plastic bags at 4^{o}C.

Seed yields and 100 seed weights were determined on a dry weight basis using the low constant temperature method (ISTA 1993). Samples for drying were drawn using the method of repeated halving (ISTA 1993) and counted on an electronic seed counter.

Seed germination data were collected using an ISTA standard germination test for cauliflower (ISTA, 1993). Seeds were germinated on top of 2 layers of Advantec® No. 2 filter paper (Toyo Roshi Kaisha, Japan) in Petri dishes at 25^{o}C under continuous light in a germination cabinet (Contherm GPM). Germination tests were scored for normal seedlings at days 5 and 10.

The data generated from hand pollination experiments were analysed for each trial site using the paired t-test function of SPSS V12.0 (SPSS Inc., Chicago). Significant differences were determined at the 5% level of probability.

Pollination, Pollen Viability and Seed Yield Surveys.

Over 3 seasons (2004 to 2006) data on pollination rates, pollen viability and seed yield were collected from 15 naturally pollinated commercial and trial plots of lines BL185 and BL529 located at or near Sorell, FVRS and at Lauderdale, approximately 15km south east of Sorell. During peak bloom crops were sampled on 4 to 6 days to determine pollination rates (% pollinated flowers) and pollen viability. At maturity, representative samples of 10 to 15 plants were collected from each plot and seed yields per branch determined. All sampling methods were as outlined above. Seed yields from each plot were standardised to seed yield per major inflorescence branch (branches defined at the junction of the vegetative stem and inflorescence). Correlation of pollination rates, pollen viability and seed yield were undertaken using regression analysis function of SPSS V12.0 (SPSS Inc., Chicago).

Effect of Branch Position on Seed Yield

At FVRS in 2003, before anthesis, plants of BL529 lodged towards the east due to the prevailing westerly winds. This resulted in a spatial arrangement of the parent lines where western side of the male sterile inflorescence was closer to pollinator flowers than the eastern side, with branches in the centre furthest from the pollinator flowers. At harvest the branches of 12 randomly selected male sterile plants of BL529 were divided into three categories; 1) eastern side branches; 2) central (upright) branches and 3) western side branches. Two randomly selected branches were removed from each position on each plant and bagged separately. Prior to drying, the number of flowers that had formed on each branch was determined, so that yields could be standardised. After drying, each sample was individually threshed and the seed cleaned and weighed.

Pollen Viability Studies

Pollinator line screening trials were undertaken in 2003 – 04 using the methods of pollen handling; storage and testing developed in this project (see Chapter 1). Viability measurements were based on samples collected just prior to anthesis. Anthers at the same stage as those sampled dehisced *in-vivo* within several hours of sampling.

Shadehouse Trial

Six plants of each of the 6 lines (BL 529, BL185 and Pollinators C, F, H and J) were included in this trial. The plants were arranged on the shade house bench in 6 blocks, with each block containing 1 plant of each line. The arrangement of the plants in each block was randomised, giving a randomised complete block design. On 6 days during the peak flowering period for all lines, pollen samples were collected from each plant. Individual plant samples consisted of the pollen from 5 randomly selected flowers.

Field Trial

Ten lines (BL529, A and C to J) were screened for pollen viability under field conditions. Individual replicates of each line consisted of 10 plants. During the approximate 4 week flowering period of all lines, pollen samples were collected from each line on 5 separate days. On each day 3 replicate samples were collected for each line, with each sample consisting of the pollen of 20 flowers.

In both trials samples were collected between 10am and 1pm as this encompassed the peak period of pollen availability and pollinator activity.

Temperature Effects on Pollen Viability

Temperature and humidity loggers were placed within a Stevenson screen located at canopy level amongst the cauliflower plants in the shade house and field based screening trial. Daily germination results for all pollen samples collected were compared against the temperature and relative humidity data for the day of sampling and over the preceding week using regression analysis. Within this analysis pollen viability data for each line were converted to relative values. Relative values were calculated for each day of sampling as 100 x % viability of a line on a given day / the maximum % viability observed for that line during sampling. This standardisation enabled data from all lines to be included in the analysis.

The effects of temperature on pollen viability at anthesis were also examined in a series of 3 experiments conducted in a controlled environment glasshouse. The first two experiments examined the effects of fixed temperatures of 15, 20 and 25^{o}C and the effects of diurnal temperature cycles of 25/25, 25/15 and 25/5^{o}C on pollen viability at anthesis in lines BL529. The plants used in these experiments were raised in pots under shade house conditions (See Chapter 2 for details) and transferred into the controlled environment glasshouse approximately 3 days before first flower (6 plants per treatment). Samples of 2 flowers per plant were collected on 10 days spread over the full period of flowering in each treatment.

In the third experiment, plants of BL529 that had been grown at 15^{o}C were transferred to 20 and 25^{o}C at curd break, or left at 15^{o}C (control treatment) to complete flowering. There were 6 plants per treatment. The sampling schedule was the same as for experiments 1 and 2 above.

All pollen samples were desiccated and stored prior to viability testing using the FCR procedure using the procedures developed in Chapter 1.

Glasshouse based experiments were analysed as ANOVA designs using SPSS V12.0 (SPSS Inc., Chicago). As the temperature treatments could not be randomised, significant differences were determined at the 0.01 level of probability.

Results and Discussion

Identification of Yield Limiting Factors

Inadequate pollination has been reported to limit seed yields in several cytoplasmic male sterile (CMS) hybrid seed crops including sunflower (Singh *et al.,* 2000), carrot (Erickson *et. al.*, 1979) and canola (Hogarth, 1999). The experiments detailed in this chapter provide evidence that pollination is also a major constraint for seed yields from CMS cauliflower seed crops grown in Tasmania. In 2003 and 2004 at FVRS and Sorell, flowering branches of male sterile plants of BL529 and BL185 that were hand pollinated in supplement to natural pollination yielded between 30 and 340% (0.27 to 3.4g) more seed than corresponding flowering branches receiving natural pollination only (Figures 2.2 and 2.3). Yield increases due to hand pollination were not at the expense of seed set on the remainder of the inflorescence (Figure 2.2) and although seed from hand pollinated branches was slightly lighter, there was no evidence of an effect on seed germination (Table 2.2).

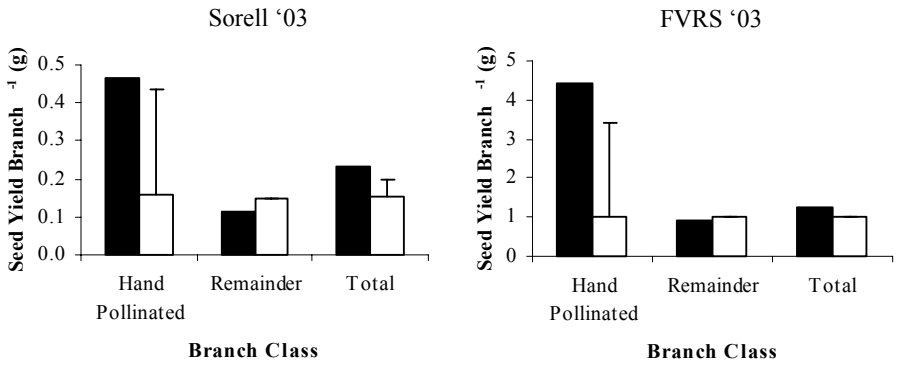


Figure 2.2 - Mean seed yields of hand pollinated branches (supplemental plus natural pollination); remaining (naturally pollinated) branches; and overall yields per branch (all branches) of hand pollinated plants (■) compared to the corresponding branches of control plants that received natural pollination only (□) at Sorell and FVRS in 2003. The error bars indicate LSD's ($P<0.05$), where significant differences were observed (n=12).

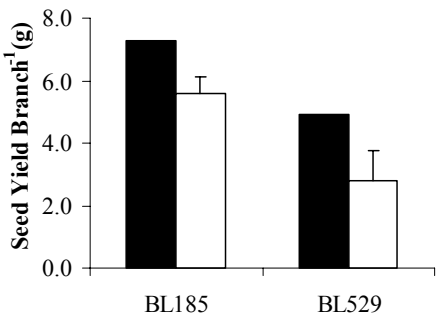


Figure 2.3 – Mean seed yields of hand pollinated (supplemental plus natural pollination) (■) and control (natural pollination only) (□) branches of the hybrids BL185 and BL529 grown at FVRS in 2004. The error bars indicate LSD's ($P<0.05$) (n=12).

Table 2.2 - Seed size and quality data from the 2004 and 2005 supplemental pollination experiments at FVRS and Sorell. Values presented are the means of samples from 12 plants.

Season	Location	Line	Treatment	1000 Seed Weight (g) +/-S.E.		% Germination			
						Day 5 +/- SE		Day 10+/- SE	
2004	FVRS	BL529	Supplemental Pollination	4.3	0.3	75.0	3.8	89.6	1.9
			Natural Pollination	4.7	0.2	74.3	5.0	85.3	1.9
	Sorell	BL529	Supplemental Pollination	5.5	0.3	74.6	4.5	88.4	2.4
			Natural pollination	5.8	0.2	68.4	2.3	88.0	3.9
2005	FVRS	BL529	Supplemental Pollination	3.4	0.2	83.7	1.2	86.6	1.7
			Natural Pollination	3.7	0.2	81.8	2.9	90.5	2.4
	FVRS	BL185	Supplemental Pollination	2.8	0.2	91.7	1.3	93.1	1.1
			Natural Pollination	3.1	0.1	92.3	1.4	94.0	1.1

Measurements of pollination rates of male sterile flowers and the viability of pollen in each hybrid suggest that both factors limited seed yield under the production conditions studied. Cauliflower flowers reportedly have up to 20 ovules and in one study up to 14 developed mature seeds following fertilisation (Gurusamy, 1999). In 2003 only 20% and 31% of flowers were naturally pollinated at Sorell and FVRS respectively with between 2.4 and 5.5 pollen grains per flower on average (Table 2.3) indicating a substantial deficiency in pollination. Although natural pollination rates were higher in 2004, (averages of 74 to 89% of flowers pollinated with 4.4 to 6.4 grains / flower across both hybrids) they remained inadequate for optimal seed yields (Figure 2.3). Across all experiments, supplemental pollination increased the percentage of flowers that were pollinated to between 69 and 89% and the average rate of pollen deposition to between 12 and 43 grains stigma^{-1}. Data collected in 2003 indicated that this resulted in a two to threefold increase in percentage of flowers setting pods and an extra 0.8 to 1.7 seeds per pod on average (Table 2.4; Plate 2.1).

Plate 2.1 – Pod set on naturally pollinated branches (left) and supplemental hand pollinated branches (right) of the male sterile line of BL529 at FVRS in 2003.

Table 2.3 – Daily pollination rates and daily pollen viability data for the 2003 supplemental pollination experiments for BL529 at FVRS and Sorell.

Location	Treatment	Date	% Pollinated Flowers	Pollen Grains / Stigma	% Pollen Viability (FCR Test)
Sorell	Supplemental Hand Pollination	5-Dec	50.0	10.6	19.7
		8-Dec	82.4	10.5	13.1
		11-Dec	61.5	15.5	19.9
		15-Dec	75.0	13.1	8.5
		Mean+/-SE	**67.2+/-7.17**	**12.4+/-1.18**	**15.3+/-2.76**
	Natural Pollination (Control)	5-Dec	22.2	2.5	
		8-Dec	18.8	1.0	
		11-Dec	10.5	2.0	
		15-Dec	28.6	4.0	
		Mean+/-SE	**20.0+/-3.8**	**2.4+/-0.6**	
Forthside	Supplemental Hand Pollination	19-Dec	91.7	28.0	41.5
		23-Dec	80.1	30.0	27.7
		24-Dec	66.7	57.0	26.0
		27-Dec	95.0	55.0	39.3
		Mean+/-SE	**83.4+/-6.4**	**42.5+/-7.8**	**33.6+/-3.95**
	Natural Pollination (Control)	19-Dec	34.8	4.5	
		23-Dec	30.2	4.6	
		24-Dec	29.4	5.0	
		27-Dec	29.6	7.7	
		Mean+/-SE	**31+/-1.27**	**5.45+/-0.75**	

Table 2.4 – Yield component analysis for 2004 supplemental pollination experiments on BL529 at FVRS and Sorell.

Site	Treatment	% of Flowers Setting Pods+/- se		No. of Seeds Pod^{-1}+/-se	
FVRS	Natural Pollination	28.3	2.6	3.9	0.3
	Supplemental Pollination	57.7	6.2	3.1	0.4
Sorell	Natural Pollination	4.4	2.4	3.0	0.4
	Supplemental Pollination	13.1	2.3	1.3	0.5

A gradient in seed yield across the inflorescence of male sterile plants of BL529 was observed and this appeared to be associated with distance from the pollinator line (Figure 2.4). The extent of yield variation was from 1.3g branch^{-1} for side branches closest to the pollinator line to 0.75g branch^{-1} for central upright branches with comparable numbers of flowers furthest from the pollinator line. Although explanations other than proximity to the pollinator line cannot be disregarded, other studies have also reported an impact of proximity to the pollinator line on seed set in hybrid seed crops over relatively short distances (0.5 – 3m) (Dudek and Woyke, 1990; Schittenhelm *et al.,* 1997; Lavinge *et al.,* 1998).

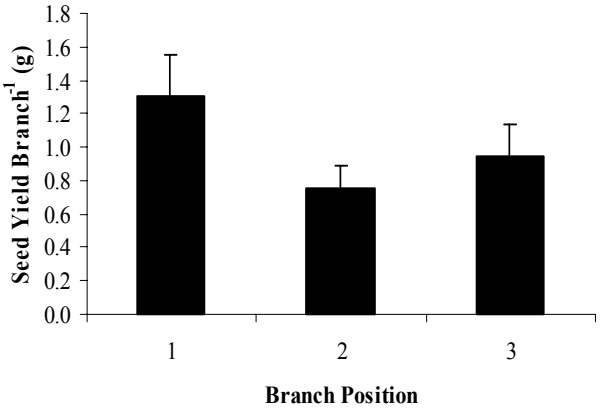


Figure 2.4 – The relationship of branch position to seed yield in male sterile plants of a lodged plot of BL529 grown at FVRS in 2003. Branch positions are as follows: 1 = side branches closest to flowers of the pollinator line, 3 = opposite side branches, 2 = branches in centre of canopy. Relative distances to the nearest pollinator flowers were 1<3<2. Standard error bars are shown for each branch position; n=12. Significantly higher seed yields occurred at position 1 compared with position 2 ($P<0.01$).

Whilst pollination rates observed in all trials were sub-optimal, honey bee stocking rates were above the levels considered adequate for pollination of vegetable seed crops (Delaplane and Mayer, 2000). Visual observations made between 10am and 2pm at peak flowering indicated high levels of honey bee activity in all trials. Studies of honey bee behaviour provide evidence that the inadequate pollination observed in this work may relate to apparent incompatibilities between the foraging patterns of honeybees and conventional hybrid seed production systems, rather than stocking rates or levels of activity. In particular, honey bees show a tendency to move short distances between flowers after visiting a rewarding flower and therefore tend to remain within a single row of plants (Cresswell *et al.,* 1995; Delaplane and Mayer, 2000), which limits opportunities for cross pollination in hybrid seed crops where the pollinator lines are planted in separate rows. Furthermore, differences in the floral morphology of the CMS line compared to the pollinator line have lead to reports of discriminatory foraging patterns in CMS hybrids (Faulkner, 1978; Erickson and Peterson, 1978; Funari *et al*., 1994; Singh et al., 2000) and side working of the male sterile line of CMS Brassica crops (Hogarth, 1998). The impact of parent line arrangement on seed yield is examined further in Chapter 3.

In comparison with reported *in-vitro* germination rates of between 70 and 90% for freshly dehisced glasshouse grown *Brassica oleracea* pollen (Hodgkin and Lyon, 1986) the mean viabilities of pollen samples collected in this work (determined by FCR test) were low, ranging from 15.3% (Sorell) to 33.6% (FVRS) for BL529 in 2003 (Table 2.3) and 38.4% to 44.5% for BL529 and BL185 at FVRS in 2004. Poor average pollen viabilities were also observed in both the field and shade house based screening trials in 2003, indicating a consistent problem with pollen viability across a range of inbred genotypes (Table 2.5). Although inbreeding depression is known to effect pollen viability (Aslam *et al.*, 1990), no other reports of pollen viabilities for inbred *B. oleracea* lines could be found for comparison.

Table 2.5 – Mean pollen viability for a range of inbred pollinator lines grown in field (FVRS) and shade house conditions in 2003. Means shown are the average of 5 (FVRS) or 6 days sampling (shade house).

Line	Mean Pollen Viability +/- se			
	FVRS		Shadehouse	
BL529	28.5	4.8	29.5	3.0
BL185			28.9	1.0
Pollinator A	10.7	5.8	-	-
Pollinator C	26.2	4.2	17.7	2.0
Pollinator D	28.1	8.9	-	-
Pollinator E	18.3	5.6	-	-
Pollinator F	26.0	10.3	25.1	2.0
Pollinator G	22.8	7.2	-	-
Pollinator H	23.1	8.4	20.2	7.0
Pollinator I	15.9	8.9	-	-
Pollinator J	25.2	6.1	22.0	4.0

In agreement with the findings of the supplemental hand pollination experiments, a survey of 15 plots and commercial crops of hybrids BL529 and BL185 grown near FVRS, Sorell and Lauderdale between 2003 and 2006 indicated a close linear relationship ($P<0.0001$; $r^2 = 0.78$) between yield and the rate of pollination with viable pollen (mean no of viable pollen grains deposited flower^{-1}) for both hybrids across all sites and seasons (Figure 2.5). This provides clear confirmation of the importance of pollination rate and pollen viability as a primary yield limiting factors for CMS cauliflower seed production in Tasmanian field conditions.

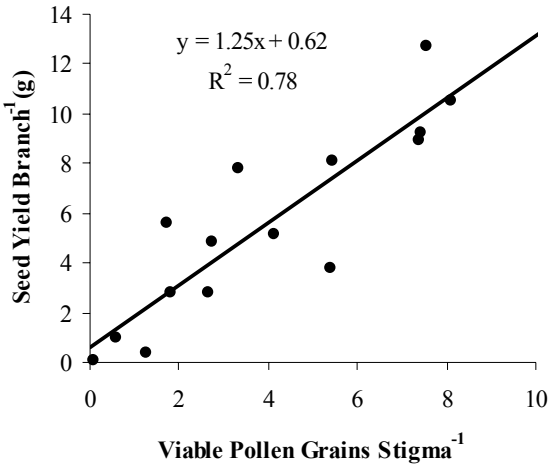


Figure 2.5 – The relationship between number of viable pollen grains deposited on individual stigmas and seed yield per branch in plots and commercial crops of hybrids BL529 and BL185 grown in southern and northern Tasmania between 2003 and 2005. The relationship is significant ($P<0.0001$; $r^2=0.78$).

Temperature Effects on Pollen Viability

Samples collected in this work showed a daily pattern of variation in viability that was consistent across lines, and appeared to relate to temperature conditions near the time of sampling (Figure 2.6). Correlation of mean temperature data over various periods of time preceding sampling with mean pollen viability values for each sample date in the field and shade house screening trials confirmed that much of the day to day variation in pollen viability could be explained by high temperature stress (Figure 2.7). The strongest relationship was observed for mean temperatures over the 24 hours preceding anthesis ($P<0.001$; r^2(shadehouse)=0.73; r^2(field)=0.61). In both environments, the relationships between temperature and pollen viability were similar, showing a substantial decline in pollen viability over mean daily temperatures ranging from 12.7 to 25.3^{o}C. Other than temperature, humidity is one of the most common climatic variables to affect pollen viability (Chang and Struckmeyer 1975; Hoekstra and Bruinsma, 1975; Shivanna and Rangaswamy 1992) but no effect of humidity on pollen viability prior to anthesis was observed. Given the requirement for desiccation before storage and sensitivity to prolonged periods of re-hydration after storage (see Chapter 1), it seems likely that longevity of cauliflower pollen after anthesis would be sensitive to high relative humidity

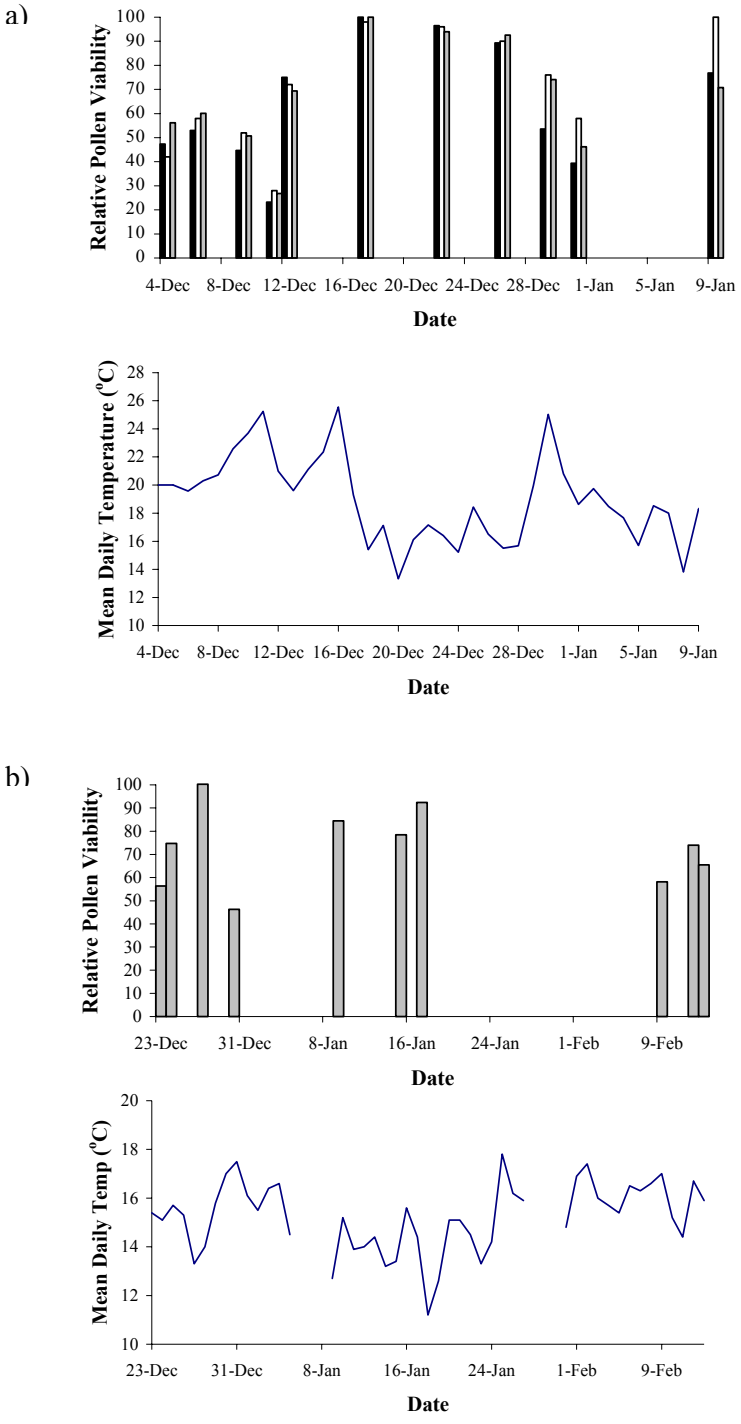


Figure 2.6 – Variation in the relative viability of pollen samples collected within a) the shade house and b) the FVRS field trial. Data presented in a) are for lines BL529 (■), F (□) and J (■). Data presented in b) are the average relative pollen viability for the 10 lines included in the FVRS field trial. Mean daily temperature data for the 24 hours preceding sampling are shown for each site.

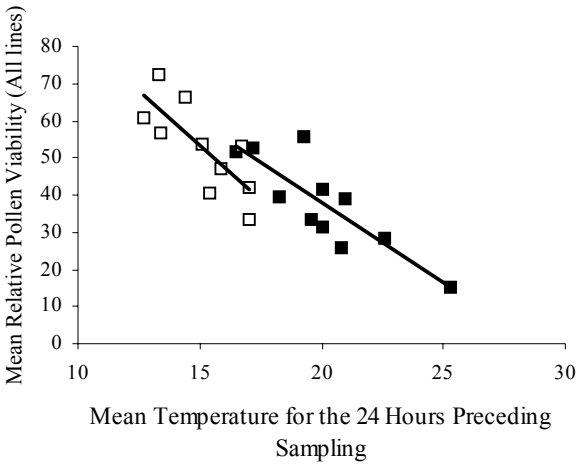


Figure 2.7 - The relationship between the mean temperature for the 24 hours preceding sampling and relative pollen viability for pollen samples collected from the shade house (■) and FVRS field site (□). Individual data points are the mean relative pollen viabilities of all lines sampled on each day. The relationships at both sites, indicated by the lines, are significant: Shade house $P<0.01$, $r^2=0.63$; FVRS $P<0.01$, $r^2=0.71$. Individual data points are the means of the relative pollen viabilities of all lines.

Field observations on the effects of temperature were confirmed in controlled environment trials where pollen from plants of BL529 held at a constant temperature of 25°C had significantly lower ($P<0.01$) viability (33.5%) compared with plants held at 15 or 20°C (55 to 53%) (Figure 2.8a). Under a diurnal temperature cycle, the effect of high daytime temperature (25°C) was partially, but not completely, reduced by cooler night time temperatures down to 5°C (Figure 2.8b). Pollen viability at 25°C was not improved in plants transferred to 25°C at curd break compared with plants transferred from 15°C to 25°C just prior to flowering.

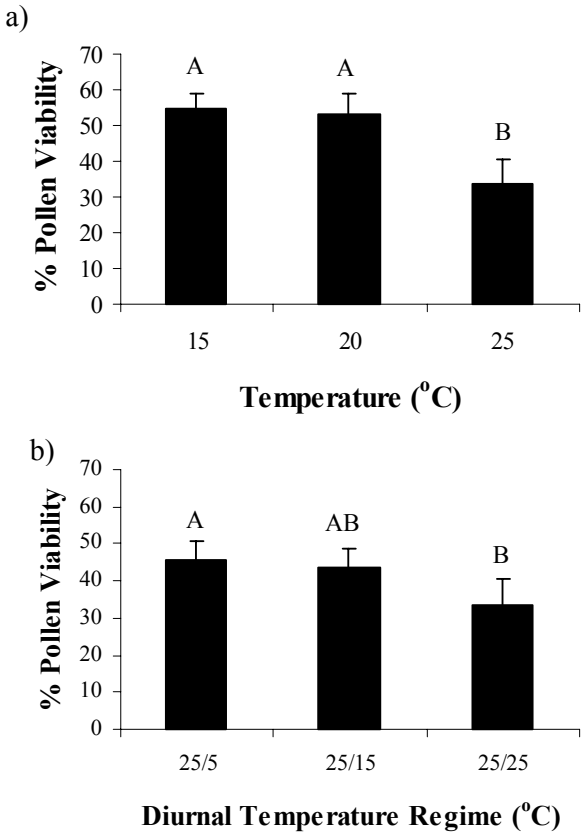


Figure 2.8 - The effect of constant temperatures of 15, 20 and 25°C (a) and diurnal temperature cycles of 25/25, 25/15 and 25/5 (b) on pollen viability at anthesis in line BL529. Data points are the means of 10 days of sampling. Standard errors are shown. Significantly different treatments ($P<0.01$) are denoted by the letters above the columns.

High temperature stress is a well documented cause of male sterility in a crop species (Johri and Vasil 1961; Shivanna and Rangaswamy 1992; Dafni and Firmage 2000) and within the Brassica genus there are reports of temperatures in excess of 25 to 30°C reducing pollen viability in canola (*B. napus*) (Young *et al.,* 2004). Consistent with the findings of the current work, Chinese kale plants held at 23°C had lower pollen viability than plants held at lower temperatures (Hossain *et. al.*, 1995). This suggests that *B. oleracea* may be more sensitive to high temperature induced male sterility than some other Brassica species and has important implications for management of field and glasshouse based production, particularly for late flowering cultivars such as the ones studied in this work. Strategies to minimise temperature stress in hybrid cauliflower seed crops are examined in Chapter 4.

CHAPTER 3

Alternative Parent Line Arrangements for Improved Pollination and Seed Yield in CMS Cauliflower Seed Crops

Introduction

Commercial Brassica seed crops are mainly pollinated by honey bees (*Apis mellifera*) due to the attractiveness of Brassica flowers to this species, their efficiency as pollinators and the ease with which producers can introduce and manage colonies (Delaplane and Mayer, 2000). While few problems have been encountered in pollinating open pollinated Brassica seed crops with honey bees, pollination limited seed yields have been reported in self incompatible cauliflower and brussel sprout seed crops (Faulkner, 1978; Dudek and Woyke, 1990) and in CMS canola (Hogarth, 1998). Funari *et al.* (1994) noted that perfect flowers of cauliflower were more attractive to pollinators than CMS flowers. In the previous chapter close correlations between pollination rate, pollen viability and seed yield were demonstrated for Tasmanian field based CMS cauliflower seed crops. In each instance cited, inadequate pollination was reported to be related more to honey bee foraging behaviour, than a lack of pollinators.

Optimal foraging theory predicts that pollinators will forage efficiently, minimising the amount of energy that is expended (Dafni, 1992). Based on this theory, three hypotheses have emerged which generally explain the foraging pattern of pollinators in the field (Delaplane and Mayer, 2000). These are; a) feeding rate maximisation, where foragers appear to maximise energy harvest per unit effort; b) minimal uncertainty foraging, in which the flower type least often empty of reward is visited; and c) individual constancy foraging, where individuals discriminately visit only one flower morph (Wells and Wells, 1986). For *A. mellifera*, individually constant foraging appears to provide a superstructure under which the sub-classifications of optimal diet and minimal uncertainty foraging may sometimes occur (Wells and Wells, 1986).

Generally, discriminatory behaviour by honey bees is thought to limit inter-specific foraging activity (Delaplane and Mayer, 2000) but, with the differences that are often apparent between hybrid parent lines, there is great potential for discrimination between parent lines within hybrid seed crops. Discriminatory foraging by honey bees between male fertile and CMS lines has been reported in sunflowers (Singh *et al.*, 2000), carrots (Erickson *et al*., 1979) and cauliflowers (Funari *et al.,* 1994). Effective pollination of CMS Brassica flowers may be further reduced by a corolla structure in certain lines that enables honey bees to ‘side work’ the flower for nectar without coming in to contact with the stigma (Hogarth, 1998).

Having encountered a patch of profitable flowers, honey bees tend to move short distances between flowers and forage in a more or less straight line (Pyke, 1978; Waddington, 1980 cited in Delaplane and Mayer, 2000; Cresswell *et al.,* 1995) and generally return to the same area on successive flights (Free, 1970; Rodet and Torre Grossa, 1991). This behaviour minimises the likelihood of leaving a profitable area whilst reducing the chance of revisiting a flower recently emptied of its rewards (Cresswell *et al.,* 1995) but in hybrid seed crops limits the potential for cross pollination between parent lines located in separate strips (Pierre *et al.,* 1999; Spurr, 2003). This behaviour may be further enhanced by a corridor effect resulting from height differences between hybrid parent lines (Pierre *et al.*, 1999; Erickson and Peterson, 1979). Whilst cross pollination may result from transfer of pollen between worker bees within the hive, movement of individuals between parent lines is also an important pollination vector (Free, 1970; Delaplane and Mayer, 2000).

Previous studies of parent line arrangement in relation to pollination generally confirm that cross pollination by honey bees is maximised when the plants of both parent lines are interspersed (Rodet and Torre Grossa, 1991). In this type of arrangement it is harder for pollinators to discriminate between the two parent lines and directional foraging favours cross pollination. Despite these advantages, such systems have not been widely adopted into commercial production due to the difficulties they present for identification and removal of pollinator line plants after flowering.

This chapter reports a series of experiments that examine the effects of alternative parent line arrangements on pollination and seed yield with a view to developing commercially feasible alternative system for improved seed yields. Initial experiments were conducted in field conditions but were subsequently extended to a glasshouse based production system.

Materials and Methods

The effect of parent line arrangement was examined in hybrids BL529 and BL185 in field trials at FVRS (2004) and Lauderdale (2005). Seasonal climatic conditions for each site are shown in Table 3.1. All trials were based on a 1:2 male sterile: pollinator parent line arrangement with split planting of the pollinator line (2 planting dates). All cultural practices were as outlined in Chapter 2.

Four parent line arrangements, as shown in Figure 3.1, were examined. These were: 1) the conventional strip arrangement for hybrid seed production (control); 2) a strip arrangement with the planting rectangularity altered so that plants of opposite gender in adjacent rows were closer together than adjacent plants of the same gender within a single row; 3) a checkerboard arrangement in which the two parent lines were offset within the rows by 200mm to facilitate planting and pollinator line removal; and 4) the conventional strip arrangement with the flower branches of the first and second pollinator plantings trellised between strings to draw them in close to overlap with the central row of male sterile flower branches. Each treatment plot was 2.4m wide, 8.4m long and consisted of 12 male sterile and 24 pollinator plants.

Start of peak bloom in each trial was as follows: FVRS 2004, BL185 – 15th December, BL529 – 20th December; Lauderdale 2005, BL529 – 15th October.

A second parent line arrangement trial was conducted in commercial glasshouse seed crops of hybrids BL047 and BL865. The treatments included in this trial were the conventional strip arrangement, the trellis arrangement, and an alternate strip arrangement in which adjacent male sterile and pollinator rows were planted on separate beds to facilitate separation of the two lines during removal of the pollinator line. In the latter, adjacent rows of the two parent lines were separated by 1.6 to 2.4m compared with 0.8m in the conventional strip arrangement.

In the 2005 trials, rates of pollination of the male sterile line (% pollinated flowers and number of pollen grains per pollinated flower) were determined for each parent line arrangement using the basic fuschin staining method outlined in Chapter 2.

At maturity, all male sterile plants within each treatment plot were individually harvested, dried and the seed threshed and cleaned for determination of seed yield. All methods were as outlined in Chapter 2.

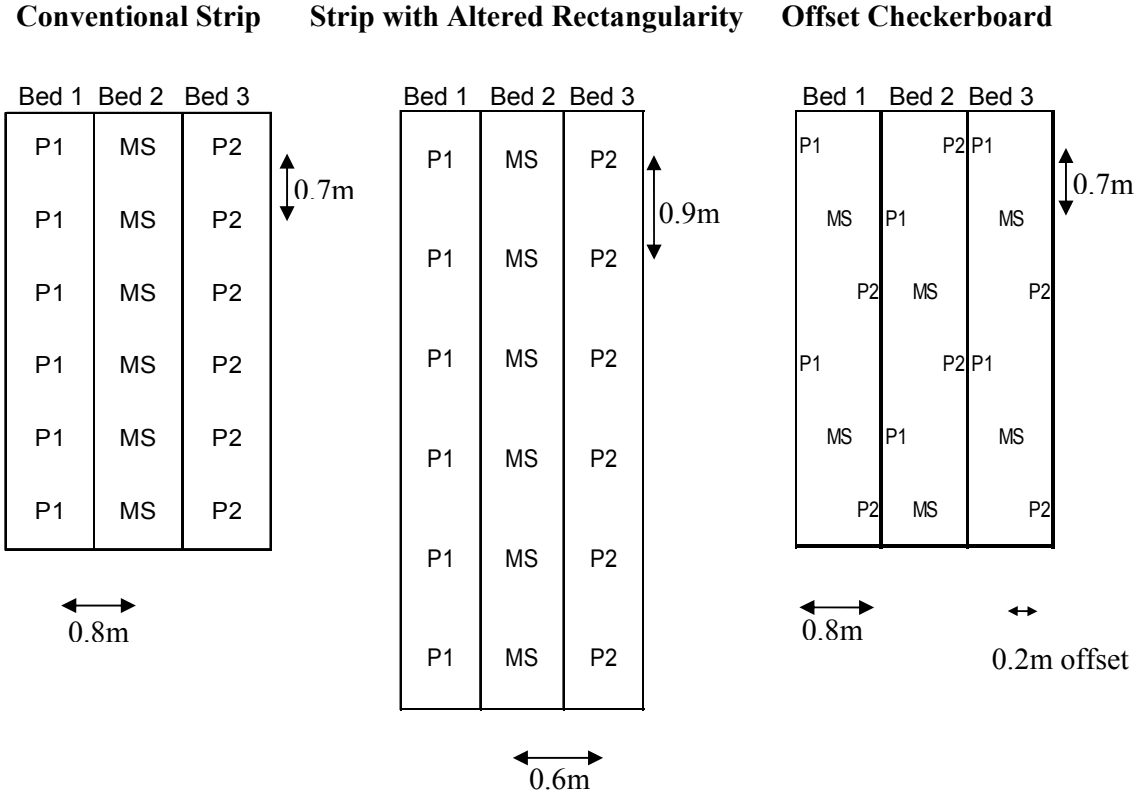


Figure 3.1 – The parent line arrangements used in this study. P1 = 1st planting, pollinator line , P2 = 2nd planting, pollinator line (later planting date) and MS = male sterile line. Conventional strip, strip with altered rectangularity and offset checkerboard arrangements depicted from above. Trellis arrangement viewed end on at canopy level.

Results and Discussion

Honey bees are the preferred pollinator of many seed crops because of their effectiveness as pollinators and ease of management. Despite these advantages, several aspects of honey bee foraging behaviour, outlined in the introduction to this chapter, may limit their effectiveness in CMS seed crops grown in a conventional strip arrangement. In particular, the arrangement of the parent lines in discrete strips throughout the field facilitates foraging discrimination between the two parent lines (Faulkner, 1978; Erickson *et al*., 1979; Singh *et al.*, 2000) and could minimise the chance of cross pollination given that honey bees tend to forage within a single row of plants in field crops (Pyke, 1978; Waddington, 1980 cited in Delaplane and Mayer, 2000; Cresswell *et al.,* 1995; Spurr 2003), make short flights between visits to rewarding flowers (Pyke, 1978; Waddington, 1980 cited in Delaplane and Mayer, 2000) and generally return to the same rewarding areas on successive flights (Free, 1970; Rodet and Torre Grossa, 1991). Despite these limitations, the strip system remains the preferred method of hybrid seed production because mechanical planting of the two parent lines and inclusion of multiple planting times is possible, and it is easy to separate the two parent lines during crop husbandry operations such as trimming and removal of the pollinator line after flowering.

The alternative parent line arrangements trialled in these experiments were designed to improve seed yields in CMS cauliflower seed crops by creating a reproductive canopy in which the requirement for cross pollination was more compatible with honey bee foraging behaviour whilst minimising the difficulty in separation of the two parent lines during planting and crop husbandry operations.

Initial field trials at FVRS in 2004 confirmed that seed yields could be increased in alternative planting arrangements (Table 3.1). In particular, arrangement of the parent lines in an offset checkerboard design, or trellis resulted in significant ($P<0.05$) yield increases of between 29 and 32%, or between 43% (BL529) and 100% (BL185) of the potential yield gain indicated by supplemental hand pollination experiments performed in plots of the same hybrids adjacent to this experiment (see Chapter 2). Increasing the inter-row space between plants within a strip arrangement did not significantly increase seed yield.

In 2005 the offset checkerboard arrangement also produced significantly ($P<0.01$) higher hybrid seed yields compared with the strip arrangement for BL529 (Table 3.1).

Table 3.1 – The effects of various parent line arrangements on seed yield and seed size in the hybrids BL529 and BL185 grown under field conditions in Tasmania during 2004 and 2005. Significant treatment effects (P<0.05) on pollination rates and seed yields are denoted by the letters.

Location	Line	Season	Arrangement	Pollination Rate (grains stigma^{-1})	Seed Yield Branch^{-1} (g)	1000 SW (g)
FVRS	BL529	2004	Conventional strip	-	2.8 a	3.1
			Offset Checkerboard	-	3.6 b	3.0
FVRS	BL185	2004	Conventional strip	-	5.6 a	3.6
			Increased inter-row	-	6.8ab	3.5
			Trellis	-	7.4 b	3.5
Lauderdale	BL529	2005	Conventional strip	8.1 a	8.1 a	3.0
			Trellis	11.0 a	8.9 a	2.9
			Increased inter-row	11.1 a	9.2ab	2.9
			Offset Checkerboard	12.4 b	10.5 b	2.8

Measurements of pollination rates (Table 3.1) and visual observations of honey bee movements on 4 days during peak bloom confirmed that yield gains in the offset checkerboard plots were due to increased rates of cross pollination. In this experiment the trellis arrangement did not significantly increase seed yields, most likely because a proportion of male sterile canopy was obscured due to trellising and was consequently poorly pollinated.

Following the initial results indicating yield improvements in field production, the effect of trellising the parent lines on seed yield was examined in 2 commercial glasshouse crops at Daylesford. Whilst trellising resulted in a significant increase in cross pollination in BL047, it had no effect on seed yield in either hybrid. High rates of pollination were observed in all treatments due to the enclosure of a relatively large population of pollinators in a confined space with a small number of plants (1 hive of honey bees per glasshouse) in this production system. In fact, in contrast to earlier reports for carrot (Rodet and Torre Grossa, 1991) turnip rape (Schittenhelm *et al.*, 1997), separation of male sterile and pollinator rows by up to 2.4m had no effect on seed yield in this system (Table 3.2).

Table 3.2 – The effect of different parent line arrangements on pollination and seed yield in commercial glasshouse crops of the hybrids BL865 and BL047. Significantly different means ($P<0.05$) are denoted by the letters. Treatment effects on yield were not significantly different.

Line	Arrangement	Pollen grains stigma^{-1}	Seed yield plant^{-1}
BL865	Strip	37.8 a	30.9
	Trellis	41.5 a	32.2
	Male sterile on own bed	20.8 b	28.1
BL047	Strip	13.7 a	17.4
	Trellis	20.6 b	17.2
	Male sterile on own bed	10.9 a	17.6

In summary, the findings of this work confirm earlier observations of improved pollination in hybrid seed crops when the pollinator line is interspersed with the male sterile line (Rodet and Torre Grossa, 1991). Whilst alternating pollinator and male sterile plants within the same row as suggested in this earlier study is impractical for cauliflower seed production, the offset checkerboard design tested in the current work may provide a workable compromise between increased seed yield, ease of crop management and mechanisation of the production system. In the glasshouse system studied in this work, varying the parent line arrangement did not impact on pollination rate to the extent that the parent lines could be separated onto adjacent beds without significant yield loss. The ability to arrange the parent lines in this way may offer a considerable advantage in terms of ease of crop management.

Chapter 4

Management of Temperature Stress Effects on Pollen Viability of Inbred Cauliflower Lines

Introduction

Research reported in Chapter 2 demonstrated that CMS hybrid cauliflower seed yields are limited under Tasmanian field conditions by inadequate pollination with viable pollen. High temperature stress was shown to be a significant contributor to poor pollen viability. Improved management of temperature conditions during flowering may therefore contribute to higher seed yields.

One way to manage this problem under field conditions is to ensure that flowering occurs before temperature conditions become too hot. Flower initiation in most cauliflower cultivars is dependant on low temperature stimulus (vernalisation) following a period of juvenility when the plants are insensitive to chilling (George, 1999). Duration of juvenility and extent of chilling required to induce curding varies widely between cultivars (Wien and Wurr, 1997), but after these requirements are met, genotype x environmental effects on curd maturation are generally small (Kesavan *et al., 1976* cited in Crisp and Tapsell, 1993). There may therefore be significant potential for manipulation of flowering time through time of transplanting. The possibility of promoting earlier flowering in late flowering genotypes, such as those studied in this project, and the effects of this on pollen quality and seed yield have not been reported. Potential benefits of earlier flowering time must be balanced against the risk of frost damage, less favourable conditions for pollinator activity and increased disease risk during cooler, wet weather.

In some cropping systems, such as glasshouse tomato and cucumber production, periodic low volume mist irrigation is used to manage temperature stress (Giacomelli *et al.,* 2005; Iglesias *et al.,* 2005). Such an approach may be suited to management of canopy temperature in Brassica seed crops, particularly in glasshouse based production. Use of mist irrigation would need to be balanced against potential effects on disease incidence, pollination and seed quality. No reports of use of overhead irrigation to improve pollen viability in Brassica seed crops were found in the literature.

In this chapter a series of experiments examining the potential application of both time of transplanting and use of low volume overhead mist irrigation to improve pollen viability and seed yield in CMS cauliflower seed crops are reported.

Materials and Methods

Time of Transplanting

The effects of time of transplanting on seed yield were examined in BL529 and BL185 in a field trial at Lauderdale in 2005. In each hybrid, 4 replicates of three planting dates were arranged in a randomised complete block design. Planting dates (for the male sterile line) commenced on the 12th of March, 2004 and continued at 14 day intervals until the 10th of April, 2004. Throughout the trial, temperature data were recorded using a Tiny Tag® logger (Gemini Data Loggers, West Sussex). During flowering (October and November), temperatures at the nearest weather station, located approximately 5km from the trial site, were slightly above the corresponding long term averages (Australian Bureau of Meteorology site no. 094008).

Timing of peak bloom was recorded by visual observation for each treatment. From commencement of flowering, pollen samples were collected at regular intervals for testing of pollen viability. Pollen samples were handled, stored and tested using the methods outlined in Chapter 1. At the same time, stigma samples were collected from the corresponding male sterile rows for determination of pollination rates, using the method outlined in Chapter 2. At maturity, all male sterile plants were harvested individually, for determination of seed yield using the methods outlined in Chapter 2.

Statistical analysis was by way of the ANOVA analysis functions within SPSS V12.0 (SPSS Inc, Chicago).

Overhead Mist Irrigation

The effect of overhead mist irrigation on cauliflower pollen viability was examined in a shade house trial at the School of Agricultural Science, University of Tasmania in 2004 - 05 and in 3 commercial glasshouse based crops at Rijk Zwaan, Daylesford in 2005 - 06. The earlier trial consisted of pot grown plants (see Chapter 2) of 3 pollinator lines BL185, F and J, and 2 treatments, misting and a non-misted control treatment. The lines and treatments were arranged in a split plot design with 3 replicate blocks. Within each block, the two treatments were separated by clear plastic sheets suspended from the shade house roof to the bench surface. During the experiment all plants were watered daily by hand.

Air temperatures were recorded at canopy level using a Tiny Tag® logger (Gemini Data Loggers, West Sussex) suspended in a Stevenson screen located within a control treatment plot.

The misting treatment used 2 Dan fan spray sprinklers (NaanDan Irrigation Systems, Dandenong) with a flow rate of 104LPH to irrigate 9 plants. Irrigation was provided for 1 minute each hour between 10am and 3pm. This regime was sufficient to ensure

the flowers retained surface moisture for the interval between misting events without being excessively wet. Treatments were applied daily between the 7th and 30th of January. Samples were collected on 7 to 11 days during the flowering period of each line. For individual lines, each sampling involved collecting 3 randomly selected flowers with freshly dehisced anthers from each treatment replicate. Due to variation in the flowering time of each line, not all lines were sampled on the same days.

In the commercial glasshouse trial in 2005 – 06, a misting treatment was applied to 3 hybrid cauliflower seed crosses, BL200, BL865 and BL047 between the 1st and 19th of October. In each cross, 3 replicate blocks consisting of a section of bed containing both the pollinator line and the male sterile line were misted for 2 minutes per hour between 10am and 3pm. Each block was paired to another similar control block nearby on the same bed that was not misted. All plants within the experiment were irrigated by drip tape at the soil surface in accord with commercial practice. Visible assessment during the trial indicated that the misting treatment had a negligible effect on soil moisture status. Air temperature records were collected at canopy level in cross BL865 using a Tiny Tag® logger (Gemini Data Loggers, West Sussex) suspended in a Stevenson screen at flower canopy level.

On 6 days during the experiment, samples of 12 flowers with freshly dehisced anthers were collected at random from each treatment replicate between 10am and 12noon. The samples were placed in 1.5ml Eppendorf® centrifuge tubes, desiccated over silica gel for 48 hours and subsequently stored at -18^{o}C. All pollen samples were transported to Tasmania in dry ice for testing. In both experiments, the pollen samples were viability tested using the FCR testing and image analysis system developed and validated for cauliflower pollen in this project (Chapter 1).

Several additional measurements were included in the commercial trial to determine the effects of misting on honey bee foraging activity, pollination rates, pollen performance *in-vivo* and seed yield. Honey bee visitation rates to pollinator and male sterile plants in the misted and control plots were measured on 3 days in hybrid BL865. On each day, a randomly selected plant from each parent line was observed in each plot for 5 minutes and the number of honeybees visits recorded. All counts were made between 10am and 2pm, as this was determined by visual observation to be peak daily period of foraging activity.

On 5 days during flowering, pollen deposition rates were measured in misted and control plots of each hybrid using the method outlined in Chapter 2. At maturity, all male sterile plants from the irrigation and control plots were harvested individually for determination of seed yield. Harvested plants were dried and hand threshed. The seed was cleaned using the methods outlined in Chapter 2.

Controlled bud pollinations were made on 4 days to test the effect of mist irrigation on pollen performance *in-vivo* in crosses BL200 and BL047. Pollen was hand crossed onto the male sterile line within the irrigation and control treatments and in reciprocal crosses between the two treatments. Buds at a stage of development 1 day from opening were used. Each day, 4 buds were selected on 2 plants in each plot and allocated to two treatments, pollination with pollen from the misting treatment or pollination with pollen from the control treatment. Pollination was effected by opening the bud with a pair of modified scissors and rubbing the anther on the stigma.

Preliminary trials demonstrated that this method resulted in deposition of greater than 100 pollen grains on each stigma. Following pollination each bud was tagged and covered with a cut down pipette tip to prevent insect visitation. At maturity, the pod from each pollination was collected and the seeds extracted by hand and counted.

In both experiments, data for individual lines were analysed separately using the paired t-test function within SPSS V12.0 (SPSS inc, Chicago). Data from the controlled pollination experiments in both hybrids were pooled and analysed as a factorial design.

Results and Discussion

Time of Planting

Time of planting is used to schedule harvest time for cauliflower curds, but the extent to which time of flowering in cauliflower seed crops can be managed by date of transplanting has not been reported. In this work, 28 days difference in time of transplanting (12th of March to 10th of April) resulted in similar differences in timing of peak bloom (30 to 33 days) for the male sterile lines of BL185 and BL 529 (Table 4.1). Earlier transplanting dates flowered earlier and also for a longer period (Table 4.1). Although there were minor differences in the relative flowering times of individual parent lines in each hybrid within different transplanting dates, these did not adversely effect nicking.

Table 4.1 – Flowering times and temperature and rainfall conditions during flowering for male sterile plants of BL529 and BL185 transplanted at different times. No. of rain days refers to days on which >1mm of rain was recorded. Rainfall data are from the Hobart Airport, located approximately 8km from the trial site.

Line	Transplanting Time	Peak Bloom	Climatic Conditions During Peak Bloom	
			Mean Daily Max / Min Temp ^{o}C	No. of Rain Days / Total Rainfall (mm)
BL529	12th March, 2005	2nd - 16th Oct	16.1 / 8.1	4 / 31.8
	26th March, 2005	20th - 31st Oct	18.9 / 11.7	6 / 40.6
	10th April, 2005	5th - 14th Nov	21.2 / 11.5	0 / 2.0
BL185	12th March, 2005	2nd - 18th Oct	16.5 / 8.0	4 / 31.8
	26th March, 2005	18th - 31st Oct	19.0 / 11.3	6 / 40.6
	10th April, 2005	2nd - 11th Nov	22.6 / 13.3	1 / 11.4

Planting time significantly affected seed yield, with optimal seed yields of 27.5g plant^{-1} and 47.2g plant^{-1} for BL185 and BL529 respectively observed for plants transplanted on the 26th of March. Seed yields were slightly lower for plants transplanted 14 days earlier, and markedly lower (57 to 68% less) for plants transplanted 14 days later. The same treatment differences were apparent when seed yields were standardised on the basis of number and size of inflorescence branches on individual plants from each transplanting treatment (data not shown), confirming that the difference in seed yield was largely attributable to level of seed set.

Seed yield differences observed between treatments were consistent with data collected on pollination rates and pollen viability across the spectrum of flowering times. Pollination rates increased with later flowering (Table 4.2), possibly due to increased pollinator activity under higher temperatures and reduced rainfall. In contrast, pollen viability at anthesis was high (>60%) in early flowering plots but declined sharply from late October, as daytime temperatures increased above 20^{o}C (Figure 4.1). This marked reduction in pollen viability in late flowering plots could largely explain the reduced seed yields caused by late transplanting.

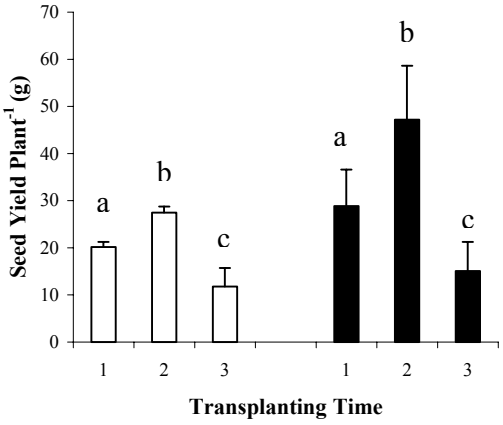


Figure 4.1 – Effect of time of transplanting on seed yield for hybrids BL185 (□) and BL529 (■). Standard errors are shown (n=4). For each hybrid significantly different yields are indicated by the letters above the columns. Transplanting times (for the male sterile line) were 1 = 12th March, 2005; 2= 26th March, 2005 and; 3 = 10th April, 2005.

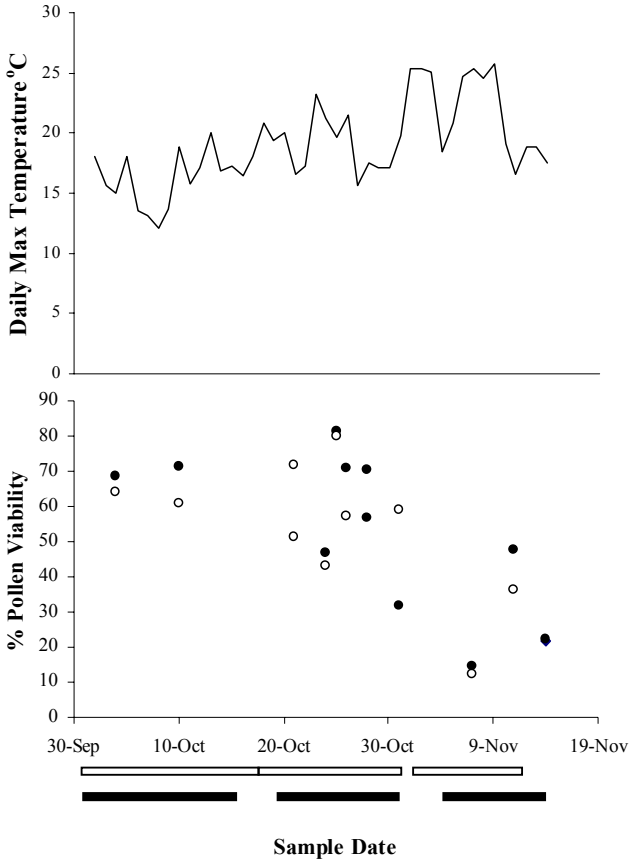


Figure 4.2 – Pollen viability at anthesis for samples from BL529 (●) and BL185 (○) collected at various stages of flowering during the time of transplanting trial. The bars under the x axis indicate the duration of peak bloom for each time of planting for BL529 (■) and Bl185 (□).

Table 4.2 – Pollination rates of male sterile plants in plots of hybrids BL529 and BL185 that flowered at different times in response to different times of transplanting.

Line	Transplanting Time	% Pollinated Stigmas ± SE		Mean Pollen Grains Stigma^{-1} ± SE	
BL 529	12th March	65.6	*2.0*	4.0	*0.6*
	26th March	66.6	*4.9*	16.8	*9.0*
	10th April	72.9	*4.7*	14.6	*5.5*
BL 185	12th March	67.5	*0.8*	6.7	*2.0*
	26th March	61.6	*0.7*	11.2	*4.9*
	10th April	86.8	*0.5*	24.8	*4.7*

The results from this experiment indicate that there is considerable scope for management of flowering time through choice of transplanting time and illustrate the importance of this variable as a determinant of seed yield. In line with the findings outlined in Chapter 2, the data collected in this work indicate that much of the negative effect of late flowering time on seed yield may be attributable to loss of pollen viability due to high temperature stress at temperatures above 20^{o}C. Whilst the experiment provides some indication of optimal time for flowering of cauliflower seed crops, care should be taken in its interpretation given that all data were generated for two late flowering genotypes at a single site in one season. Furthermore, the data presented consider the effects of time of transplanting or flowering only in terms of seed yield. The effects of these variables on seed quality should also be considered before the outcomes of this research are implemented in commercial production.

Mist Irrigation Trials

During the Tasmanian shade house trial, mean daily minimum and maximum air temperatures ranged from 6 to 27^{o}C. Under these conditions the three lines studied (BL185, F and J) had average pollen viabilities ranging from 35 to 57%. Compared to the non-misted control treatment, misting significantly ($P<0.05$) increased average pollen viability for each line by 9 to 22% (Figure 4.3). It was noted that the effect of misting was largest for sampling dates preceded by higher average temperatures, supporting the proposed role for overhead misting in mitigation of temperature stress.

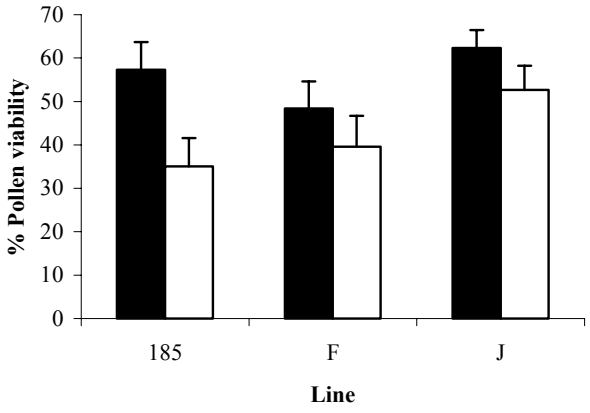


Figure 4.3 – The effect of overhead mist irrigation on mean pollen viability for pollinator lines 185, F and J in the Tasmanian shade house trial. (■) mist irrigated plants (□) control plants. Standard errors are indicated by the bars (n 185 = 8; n F = 7; n J = 11). Treatment means are significantly different ($P<0.05$) for each line.

Glasshouse temperature conditions during the commercial trial were lower than anticipated, with mean minimum and maximum daily temperatures during the trial period of 6.2 and 21.1^{o}C respectively. Comparison of air temperature records for

October 2005 with long term records for nearby Bureau of Meteorology weather stations indicates that conditions during the trial were only slightly cooler than average. Consistent with the cooler temperature conditions, pollen viability was on average higher than observed in the Tasmanian shade house trial and, overall, misting had little impact at this site (Table 4.3). Despite the generally higher pollen viability observed, there was substantial day to day variation in pollen viability that could not be explained in terms of high temperature stress (as in previous trials). The poorest pollen viability results for each cross (grown in 3 separate glasshouses) were recorded on the same days (3rd and 6th of October) and, on these days irrigated plants consistently produced pollen of higher viability (4 to 18%) than control plants. The current experiments do not provide sufficient information to explain these observations, although the data collected suggests an effect of either an environmental factor or management practice common in time in all glasshouses.

Table 4.3 – The effect of misting on pollen viability in crosses BL865, BL047 and BL200.

Line	Date	Control	+/-se	Mist Irrigation	+/-se
BL865	3-Oct	24.0	*6.4*	38.0	*6.9*
	6-Oct	30.0	*12.3*	38.0	*6.3*
	11-Oct	83.8	*1.0*	80.2	*1.7*
	13-Oct	85.0	*3.3*	85.3	*3.0*
	17-Oct	57.5	*5.1*	33.8	*8.3*
	19-Oct	41.3	*7.3*	34.2	*3.4*
	All dates	**53.6**		**51.6**	
BL047	3-Oct	12.0	*1.9*	17.0	*2.4*
	6-Oct	15.0	*1.4*	21.0	*4.2*
	11-Oct	48.7	*2.8*	47.8	*2.3*
	13-Oct	53.5	*1.0*	59.5	*4.4*
	17-Oct	45.6	*3.0*	54.5	*5.2*
	19-Oct	83.8	*5.2*	61.8	*5.2*
	All dates	**49.3**		**48.9**	
BL200	6-Oct	19.0	*3.8*	23.0	*4.6*
	11-Oct	-		-	
	13-Oct	74.0	*3.5*	73.0	*3.3*
	17-Oct	61.0	*1.9*	67.0	*3.1*
	19-Oct	78.0	*4.4*	49.0	*1.8*
	All dates	**71.0**		**63.0**	

Pollination rates in both the misted and non-misted plots were high in all hybrids, with 100% of stigmas pollinated with between 10.2 and 37.9 pollen grains on average. Although misting appeared to promote honey bee visitation (Table 4.4), rates of pollen deposition on male sterile stigmas in irrigated plots of each cross were 2.9 to 15.8% lower than in the control treatment (Table 4.5). It is unclear whether this was

due to lower rates of pollen transfer by the pollinators or displacement of pollen from the stigmas during misting.

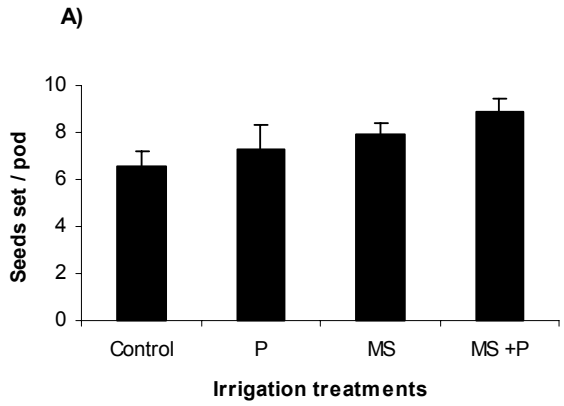
Table 4.4 – Honey bee visitation rates to the pollinator and male sterile lines of hybrid BL865 under the misting and control treatments. The data shown are the means of 3 days of observation.

Treatment	Bee visits plant^{-1} minute^{-1}	
	Pollinator	Male Sterile
Control	5.4	3.7
Misted	6.2	4.8
P value	<0.05	<0.01

Table 4.5 – The effect of misting on pollination of the male sterile line of hybrids BL200, BL865 and BL047. The data shown are the means for 5 days of sampling.

Treatment	Pollen grains stigma^{-1}		
	BL200	BL865	BL047
Control	13.7	37.9	13.9
Misted	10.8	22.1	10.2
P value	<0.05	<0.01	NS

In crosses BL 200 and BL047, reciprocal controlled pollinations were made between pollinator and male sterile lines in both the misted and control plots to test the impact of overhead misting on seed set. When both pollinator and male sterile plants from the misting treatment were used, seed set was significantly higher ($P<0.01$) in both hybrids (Figure 4.4). Interestingly, there were non-significant trends in both hybrids of increased seed set when either of the parent lines had been misted (Figure 4.4), but the effect was greatest when both were misted. The reason for this effect cannot be determined from the current experiments although based on the commercial drip irrigation regime used in all treatments and visual assessments of soil moisture status and the impact of the overhead mist irrigation treatment on this, it is unlikely that it could be explained in terms of soil moisture availability.



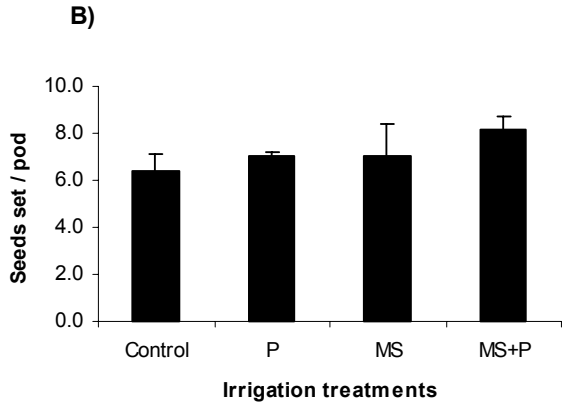


Figure 4.4 – The effect of misting on seed set in controlled pollinations in hybrids BL200 (A) and BL047 (B). The treatments are: no misting of either line (control); misting of the pollinator line only (P); misting of the male sterile line only (MS) and misting of both lines (MS+P). Each value is the mean of 48 controlled pollinations. The error bars indicate standard errors. ANOVA analysis of a combined data set for both lines showed a significant increase in seed set in the MS+P treatment ($P<0.01$).

Consistent with the observations from controlled hand pollinations, overhead irrigation significantly increased seed yields in BL200 by 5g per plant (26%) (Table 4.4) despite having little effect on pollen viability, as determined by FCR testing, and a negative impact on pollination rate. For BL865 and BL047 overhead irrigation did not significantly affect seed yield.

Table 4.4 – The effect of misting on seed yield in hybrids BL200, BL865 and BL047.

Line	Treatment	Seed yield (g plant$^{-1)}$
BL200	misted	25.2
	control	20.2
	significance	(P<0.05)
BL865	misted	35.0
	control	30.9
	significance	NS
BL047	misted	14.1
	control	17.4
	significance	NS

In overview, the data collected in the Tasmanian shade house trial supports the hypothesis that overhead mist irrigation may be beneficial in increasing cauliflower pollen viability under high temperature stress conditions. Similar observations of a positive effect of evaporative cooling have been reported for other crops with pollen that is sensitive to high temperature stress including tomato (Iglesias *et al.,* 2005). Although the mechanisms of temperature stress in pollen are not fully understood most authors propose a role for disruption to assimilate supply to the developing grains and / or reduced capacity to regulate the effects of desiccation (for example, see Sheoran and Saini 1996; Goetz*, et al.* 1998; Aloni*, et al.* 2001). The promotive effect of mist irrigation may therefore result from either evaporative cooling, or minimisation of desiccation rates.

Temperature conditions under the glasshouse based production system at Daylesford were lower than expected and, as a consequence, there was no evidence of high temperature stress or a positive effect of misting on pollen viability. Despite this, significant day to day variation in pollen viability was observed. The cause of this variability is unclear and warrants further investigation as a potential avenue for improving seed set. Although there was no benefit of misting in terms of pollen viability improvement and a slight negative impact on pollination rates at Daylesford, misting did promote seed set in controlled pollination crosses and resulted in significantly increased seed yield in BL200 and a similar, though non-significant yield gain in BL865. Further investigation is required to understand the basis of this yield gain and the potential opportunity it offers for managing for improved seed yield. Future work in this area should also consider the effects of overhead irrigation on seed quality, particularly in terms of disease incidence.

SECTION 2

Seed Development Research

Overview

Demand for high quality vegetable seed and expansion of vegetable seed production areas around the world have contributed to a substantial increase in the minimum acceptable vegetable seed germination and vigour standards for domestic and export markets. Although the Australian vegetable seed industry has a reputation for reliable production of quality seed, it must continue to seek improvement in order to remain competitive. This project examines two seed quality issues of particular importance to Australian seed producers, the occurrence of rudimentary embryos in carrot seed crops and stunted root abnormality in onion seedlings.

In many vegetable seed crops, a combination of variable, prolonged flowering and shattering of seed at maturity make time of harvest a compromise between seed quality and seed yield (Brocklehurst, 1985). In Chapter 5, a link is established between time of cutting and the occurrence of rudimentary embryos in carrot seed lots. Determination of time of cutting in carrot seed crops in Australia is currently based on recommendations from warmer production environments or glasshouse based studies. Recommended markers of maturity are either subjective, or don't apply to cooler production environments. A range of maturity indicators were evaluated and seed chlorophyll content was identified as a robust marker of seed maturity. A simple maturity test based on chlorophyll fluorescence was developed and validated using seedlines from a range of cultivars, production environments and seasons.

Based on the promising results obtained with carrot and reports of a seed quality grading system for Brassica based on chlorophyll fluorescence (Jalink, *et al.,* 1998), this work was extended to evaluate chlorophyll fluorescence as a marker of cutting time in *B. oleracea* seed crops. The outcomes of this work are described in CH 6.

Previous work has established a link between stunted root seedling abnormalities in onion and seed maturity at cutting, and has shown that seed pre-treatment may have potential to minimise the problem. In Ch7 the effect of interactions between seed maturity at cutting and seed drying conditions on expression of seedling abnormality is examined along with a more detailed study of the application of priming to minimise expression of stunted root abnormality.

Chapter 5

Embryo Development and Time of Harvest in Cool Temperate Carrot Seed Crops

Introduction

Poor seed germination is a well known problem in plants belonging to the *Apiaceae*, including carrot (*Daucus carota* L.) (Robinson, 1954; Dean *et al*., 1989). In the USA and Australia, feeding of insects of the *Lygus* and *Nysius* genera on the embryos of developing carrot seeds is a significant cause of loss of viability (Flemion, 1949; Spurr, 2003). Implementation of control strategies for these insects has significantly improved seed quality, but some seed lots still fail to meet the industry standard of 85% germination under International Seed Testing Association (ISTA) testing conditions (ISTA, 1999). One explanation for these crop failures is the occurrence of seeds with small, under-developed (i.e. rudimentary) embryos (Borthwick, 1931a; Dean *et al*., 1989; Spurr, 2003).

The causes of rudimentary embryos in carrot seed are not completely understood. Most evidence suggests that the underlying problem relates to the developmental pattern of carrot seed in which the endosperm grows first, with most embryo growth occurring later in seed maturation (Borthwick, 1931b; Gray *et al*., 1984). Given the asynchronous flowering pattern of the carrot plant and once over harvesting strategy employed by carrot seed producers (Rubatzky *et al*., 1999), seed immaturity may be a factor in the poor germination of commercial carrot seed. The germination percentage of carrot seed from higher order (i.e. later flowering) umbels is often less than the germination percentage of seed from lower order (i.e. earlier flowering) umbels when all seed is harvested at the same time (Borthwick, 1931a; Gray, 1979; Jacobsohn and Globerson, 1980; Steiner *et al*., 1990). Gray (1979) found that, where differences in germination between umbel orders existed, there was a positive correlation between germination percentage and embryo size. Later cutting times have been shown to improve the germination percentage of seed from higher order umbels in some studies (Gray and Steckel, 1982) but not others (Gray, 1979; Sandin, 1980). Sandin (1980) and Hawthorn *et al*. (1961) found that even with late cutting, carrot seed germination remained below 80%.

Determination of cutting time in Australian carrot seed crops is largely based on recommendations from northern hemisphere studies of time elapsed from peak bloom (50 to 79 days from first flower depending on climate and cultivar) or appearance of the seed heads (Hawthorn *et al*., 1961; Gray and Steckel, 1983; Gray *et al*., 1984; Steckel *et al*., 1989). Often these studies have been conducted on plants grown in polythene tunnels (Gray and Steckel, 1983; Gray *et al*., 1984; Steckel *et al*., 1989), or at field sites with hot summertime conditions (Hawthorn *et al*., 1961). Much of Australia's carrot seed production is field based in cool temperate climates, so the recommendations of these earlier studies may not apply. This work was undertaken to establish whether the occurrence of rudimentary embryos in carrot seed is linked to

time of cutting, and to identify appropriate indicators for determination of time of cutting of carrot seed crops grown under south-eastern Australian conditions.

Materials and Methods

Plant Material and Field Sites

Carrot seed lines studied in this work were open pollinated and hybrid 'Nantes', 'Amsterdam' and 'Kuroda' types. All seed lines were grown under field conditions during the 2004 and 2005 seasons in commercial crops or trial plot isolations near Cambridge (42.5°S, 147.3°E), Bushy Park (42.4°S, 146.5°E) and Whitemore (41.3°S, 146.5°E) in Tasmania, and Mt Gambier (37.7°S, 140.8°' E), in South Australia. Current management practices for seed to seed production were used. Temperature conditions during the study and long term averages are shown for each site in Table 5.1.

Examination of the Relationship between Embryo Size and Germination

The relationship between embryo size and seed germination was examined in 21 trial and commercial seed lines produced in Tasmania and South Australia between 2002 and 2004, using the methodology of Gray and Steckel (1983). Fifty seeds from each line were soaked in formalin acetic alcohol (FAA) (50% ethanol, 6.5% formalin and 2.5% glacial acetic acid) for 24 h. After soaking, each seed was cut to remove the caruncle and the embryo extruded by applying pressure to the back of the seed with a scalpel blade. Individual embryo lengths were recorded under a dissecting microscope. Three replicate samples of 100 seeds from each line were germination tested according to ISTA guidelines (ISTA, 1999).

Table 5.1 - Season 2003-04, 2004 - 05 and long term temperature data for the Cambridge, Bushy Park and Whitemore (Tasmania) and Mt. Gambier (South Australia) sites during carrot flowering and seed maturation (i.e. December to March). Long term averages are for a minimum of 47 years. Source: Australian Bureau of Meteorology.

Location	Month	Mean maximum temperature ^{o}C			Mean minimum temperature ^{o}C		
		03 - 04	04 - 05	Long term	03 - 04	04 - 05	Long term
Bushy Park	December	24.0	-	21.9	8.8	-	9.1
	January	22.5	-	23.7	9.1	-	10.0
	February	23.6	-	23.8	8.8	-	10.0
	March	21.4	-	21.7	7.2	-	8.5
Cambridge	December	22.4		20.5	11.9		10.6
	January	21.7		22.4	11.1		11.9
	February	21.9		22.3	11.5		12.0
	March	20.6		20.6	10.3		10.7
Whitemore	December	23.0	-	21.2	9.3	-	7.5
	January	21.7	-	23.2	9.0	-	8.2
	February	22.2	-	23.1	10.5	-	8.5
	March	21.0	-	21	7.0	-	7.2
Mt Gambier	December			-			-
	January			-			-
	February			-			-
	March			-			-

Embryo Growth Patterns

Embryo growth patterns were studied in the hybrid crosses 30 ('Nantes' × 'Amsterdam') and 68145 ('Nantes' × 'Imperator') grown near Cambridge. On 12 January 2004, 250 male sterile plants in peak bloom were tagged in each hybrid cross. At 3 day intervals from 24 days after peak bloom, the seeds of the primary and fourth secondary umbels of ten randomly selected plants were harvested. The embryos of a sample of 40 seeds from each umbel order were extracted and measured for length using the method outlined above. Dry weight data were obtained for a sample of 100 seeds from each umbel order using the low constant temperature method (ISTA, 1999).

Time of Harvest Studies

The relationships between time of harvest and seed quality and yield were examined in four hybrid carrot seed lines, 30, 68145 and 963 ('Nantes') grown near Cambridge and line I ('Kuroda') grown at Whitemore and an open pollinated line, Nantes 2 ('Nantes') grown at Bushy Park. Samples were collected from individual lines on 3 to 6 dates separated by 7 to 12 day intervals. Sampling dates were chosen so that the commercial cutting occurred in the middle of the sampling period. At each site the time of cutting treatments were arranged in a randomized complete block design with four replicates. Each replicate sample consisted of 20 consecutive plants from a row. The umbels of 18 plants from each plot were removed, separated into primary, secondary and higher order classes and dried in a forced air cabinet at 25°C for 2 weeks. These were threshed in a modified garden mulcher (Stihl, Virginia Beach, USA). The seed was de-bearded using a laboratory thresher (Winter Steiger, Salt Lake City, USA) and cleaned using air-screen (Blount Agri-Industrial, Indiana, USA) and density separators (Seedburo, Chicago, USA). One hundred seeds from each sample were germinated according to ISTA guidelines for carrot (ISTA, 1999). Seed yields and 100 seed weights were determined on a dry weight basis using the low constant temperature method (ISTA, 1999).

Evaluation of Seed Maturity Indicators

The relationship between germination percentage and three seed maturity indicators (i.e. thermal time from peak bloom, seed moisture content and seed chlorophyll content) were examined using samples collected from seven carrot seed lines grown in Tasmania. These included the five lines described previously plus the open pollinated lines Amsterdam 2 ('Amsterdam') grown near Bushy Park and Nantes OP ('Nantes') grown near Whitemore. Temperature data collected at canopy level at each trial site were used in the calculation of thermal time from peak bloom for each sample date using the equation thermal time (day degrees) = $\sum(T_{min}°C + T_{max}°C)/2) - 10$. Representative samples of seed from the remaining two plants collected from each time of harvest plot were used to determine seed moisture content and seed chlorophyll content. Seed moisture content was measured using the low constant temperature method (ISTA, 1999). 2 g samples of seed were immersed in 20 ml solutions of 90% methanol for 24 h in darkness. The methanol extract was decanted and absorbance (A) measured at 652 nm and 665 nm in a spectrophotometer (Shimadzu UV-160, Melbourne, Australia). Total chlorophyll concentration was calculated for each sample using the equation: total chlorophyll (ug/ml) = $0.28A_{665} + 27.64A_{652}$ (Lichtenthaler, 1987).

Following the promising results for chlorophyll content as a marker of seed maturity, chlorophyll fluorescence (Fo) was evaluated as a simpler, more rapid means of assessing crop maturity that could be readily adopted by industry. Chlorophyll fluorescence measurements were made for all samples used in the previous time of harvest studies (above) plus samples collected from time of harvest trials in 4 commercial carrot seed crops grown in South Australia in 2003 – 04 and 3 commercial crops grown near Cambridge (Tasmania) in 2004 – 05. Measurements

were made on 6 representative sub samples from each seed lot using a PAM fluorometer (Heinz Walz Instruments, Effeltrich) with the probe mounted on a retort stand, 15 mm above a 30mm x 10mm glass petri dish filled to level with seed (Plate 5.1).

Plate 5.1 – PAM fluorometer set up for chlorophyll fluorescence measurements of carrot seed

Statistical Analysis

Statistical analysis was performed with SPSS 11.5 (SPSS Inc., Chicago, USA). Examination of the relationship between embryo size and evaluation of maturity indicators was undertaken using regression analysis. The effects of harvest time on seed yield and quality were analysed using ANOVA for randomized complete block designs. Significantly different means were determined at the 5% level by Fischer's LSD.

Results and Discussion

Examination of the Relationship between Embryo Size and Germination

In 21 trial and commercial carrot seed lots from three seasons in south-eastern Australia, germination percentage under ISTA testing conditions (ISTA, 1999) was closely correlated ($P < 0.001$, $r^2 = 0.84$) to the proportion of seeds with embryos longer than 0.7 mm (Fig. 5.1). For embryos less than 0.7 mm in length, the cotyledons and radicle were typically incompletely differentiated. The occurrence of rudimentary embryos may therefore be the main reason for poor germination of carrot seed grown in Australia since the adoption of management strategies for *Nysius* insects (Spurr, 2003).

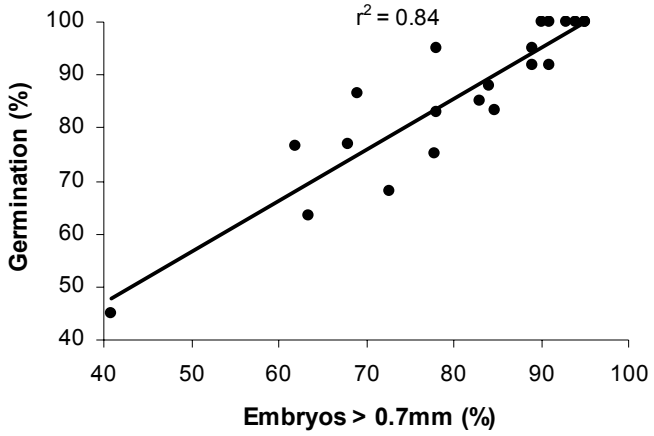


Figure 5.1. The relationship between the percentage of seeds with embryos > 0.7 mm in length and percentage germination for carrot seed lots grown in South Australia and Tasmania between 2002 and 2004. The relationship is significant ($P < 0.001$).

Embryo Growth Patterns

Embryo growth patterns were examined in seeds of the primary and secondary umbel orders, as these typically account for greater than 90% of total yield (Hawthorn *et al*., 1961; Hiller and Kelly, 1985). In lines 30 and 68145, the growth of embryos from the primary and fourth secondary umbel orders continued until 84 to 90 days after peak bloom (Fig. 5.2). There was a 7 to 12 day lag in development of embryos from the secondary umbel seed compared with the embryos of primary umbel seed, but these ultimately reached the same maximum size (i.e. 2.0 mm in line 30 and 1.8 mm in line 68145). Compared with previous reports of maximum embryo size at 60 to 75 days after first flower (Gray *et al*., 1984; Steckel *et al*., 1989), this highlights the

importance of later cutting dates for carrot seed crops grown in cool temperate conditions to avoid production of seeds with under developed embryos.

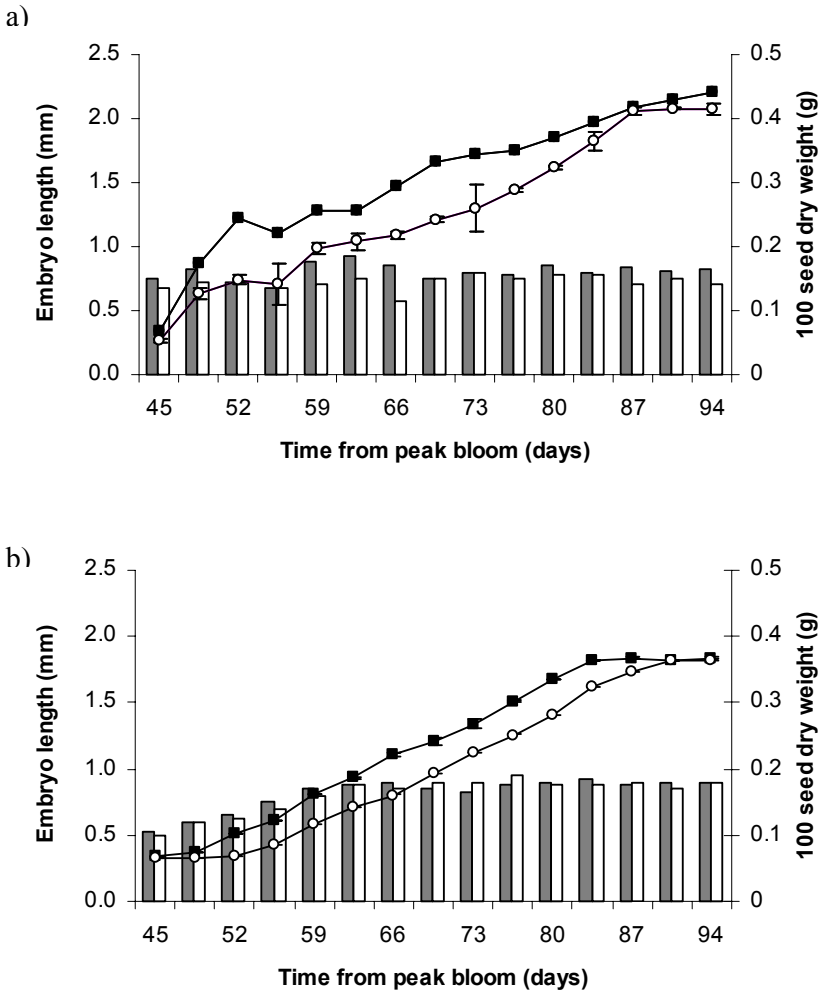


Figure 5.2. The mean lengths of embryos of seeds from the primary (■) and fourth secondary (○) umbels during seed maturation for line 68145 (a) and line 30 (b). Changes in seed dry weight for seed from the primary (■) and fourth secondary (□) umbels are shown in the columns. Error bars indicate standard errors, n = 4.

Time of Harvest Studies

For each of the five lines studied, germination percentages exceeding 85% were recorded when crops were cut later than 55 to 87 days from peak bloom (Figs. 5.3 & 5.4). Delaying harvest time to maximize seed quality must be balanced against potential yield losses due to shattering of mature umbels. In line 68145 a significant ($P < 0.001$) yield loss of 21% was recorded prior to achieving 85% germination.

Similar non-significant trends of yield loss were observed in other lines (Fig. 5.3). Under cool temperate conditions, yield losses may be inevitable in certain crops in order to achieve an acceptable germination percentage.

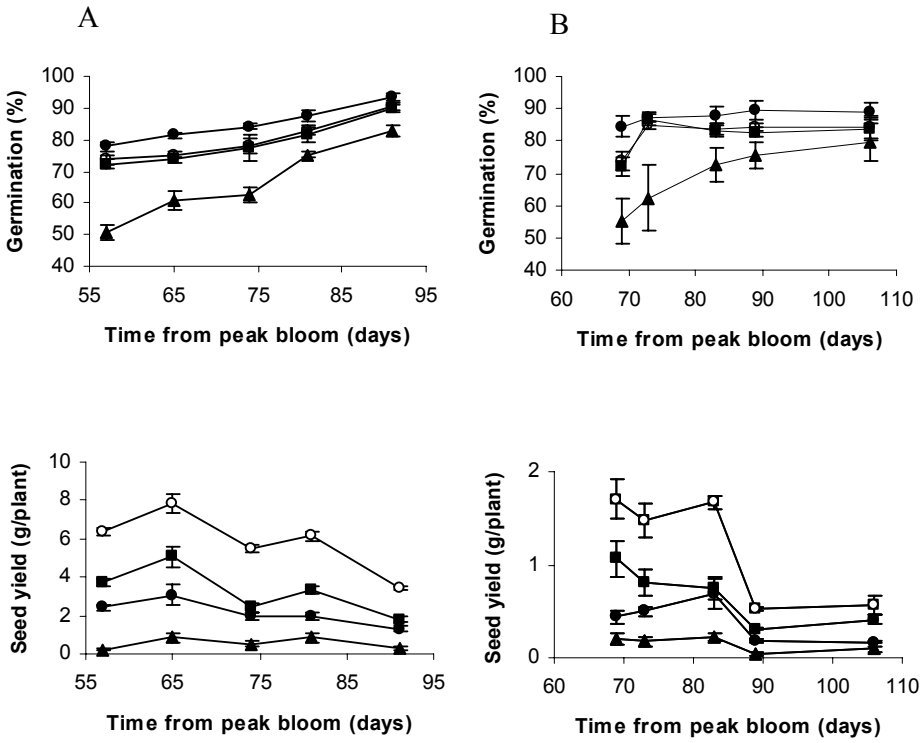


Figure 5.3. The effect of time of cutting on mean percentage germination and seed yield of from the primary (●), secondary (■) and tertiary umbel orders (▲) of line 68145 (A) and line 30 (B). The mean percentage germination and yield of the combined seed of all umbel orders is also shown (○). Error bars indicate standard errors, n = 4. LSD values ($P < 0.05$) combined seed line percentage germination: line 68145 = 1.2%; line 30 = 6.7%. LSD values ($P < 0.05$) combined seed line yield: line 68145 = 0.11 g; line 30 = 0.48 g.

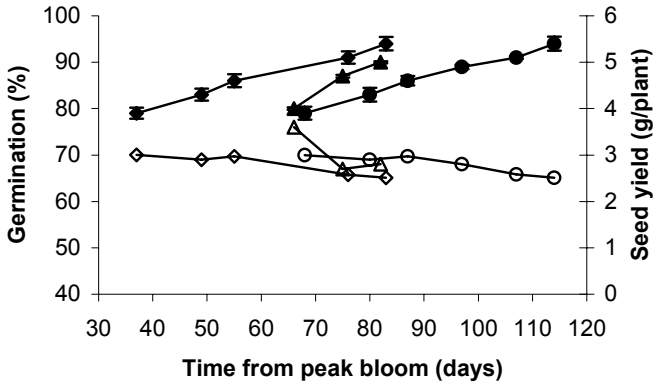


Figure 5.4. The effect of time of cutting on mean seed germination (filled symbols) and yield (open symbols) on seed of lines 963 (♦), I (●) and 'Nantes' 2 (▲). Error bars for germination data represent standard errors, n = 4. LSD ($P < 0.05$) line 963 = 6.1%; line I = 5.2%; 'Nantes' 2 = 4.8%. The effects of cutting time on seed yield were not significant in any of the lines.

Evaluation of Seed Maturity Indicators

Whilst most carrot seed producers judge time of cutting on the basis of time from peak bloom or onset of seed shattering, this study indicates the need for a more reliable method. Of the indicators we evaluated (Fig. 5.5), seed chlorophyll content provided a more accurate estimate of seed quality ($P < 0.001$, $r^2 = 0.81$) than either seed moisture content ($P < 0.001$, $r^2 = 0.62$) or thermal time from peak bloom ($P < 0.001$, $r^2 = 0.54$). Germination percentages above 85% were obtained for samples cut when the seed had a chlorophyll content less than 7 µg/ml (Fig. 5.5). In a previous study, carrot seed lots with chlorophyll contents less than 6 µg/ml had the highest germination percentage (Steckel *et al*., 1989). The agreement of these data sets supports the idea that a reliable and simple carrot seed maturity test based on chlorophyll content could be developed. One possibility is a test based on measurement of chlorophyll fluorescence levels in the seed coat. In this work, seed coat chlorophyll fluorescence level accurately estimated seed quality for seed lots grown in two different production environments (Mt Gambier and Tasmania) and in two seasons ($P< 0.001$ $r^2 = 0.73$ to 0.76) (Figure 5.5). Whilst the relationships between chlorophyll fluorescence and seed quality differed slightly between production environments and seasons, the relationship for the combined data sets (43 seed lots harvested at different stages in 11 crops from 2 different production environments and 2 seasons) remained strong ($P<0.001$, $r^2 = 0.65$), with a clear cut off in chlorophyll fluorescence level at which germination of all seed lots exceeded 85% of around 20mV. Based on these observations, there appears to be significant potential for use of chlorophyll fluorescence as a simple, robust marker of seed maturity and quality in carrot. As the level of amplification of the chlorophyll fluorescence signal is an individual property of a fluorometer, the absolute value (in mV) will vary according to the make and model of fluorometer used. Different fluorometers can be easily calibrated against each other using a common set of standards.

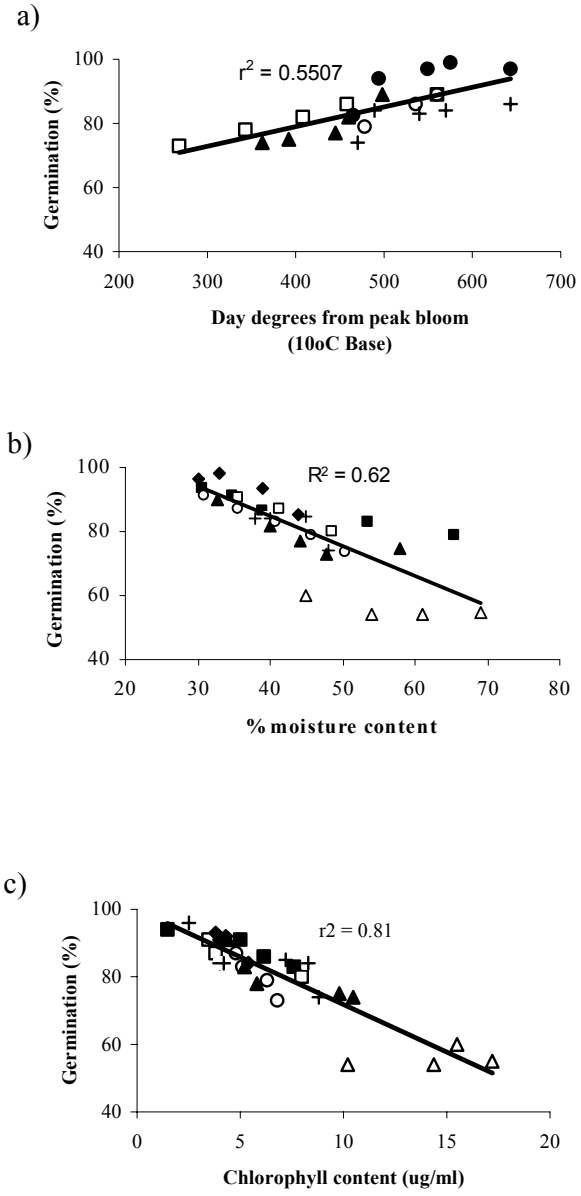


Figure 5.5. The relationship between the mean percentage germination for carrot seed lots harvested in Tasmania in 2004 and the maturity indicators thermal time from peak bloom (10°C base temperature) (a), seed moisture content (b), and seed chlorophyll content (c). The relationships between all maturity indicators and seed germination were significant ($P < 0.001$). r^2 values are shown on the graphs. Samples of different maturity from the same line are denoted by a common symbol: + = line 30; ♦ = line 963; ○ = line 68145; ▲ = Amsterdam 2; □ = Nantes 2; Δ = Nantes OP; and ■ = line I.

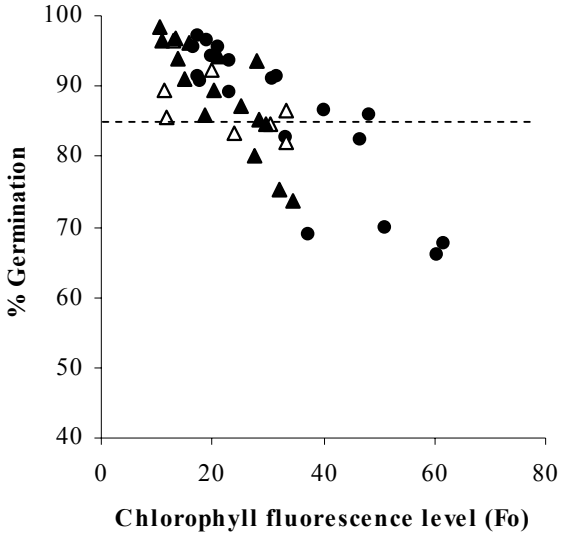


Figure 5.6 - The relationship between the mean percentage germination for carrot seed lots harvested in Tasmania in 2004 (▲) and 2005 (Δ) and in Mt Gambier in 2004 (●) and seed coat chlorophyll fluorescence level. The broken line indicates the 85% germination threshold for commercial seed lots.

Chapter 6

Chlorophyll Fluorescence as an Indicator of Cutting Time in *Brassica oleracea* Seed Crops

Introduction

The industry standard for germination of *Brassica oleracea* seed is around 90%, with premiums offered for seed lots of higher quality. The Australian industry has invested significantly in developing production systems that maximise seed quality and continues to seek improvement in this area. As shown in the previous chapter, choice of time of cutting of vegetable seed crop is a critical determinant of seed quality and seed yield. In Brassica, as in other vegetable crops, choice of time of cutting represents a balance between maximising seed quality and minimising seed losses through shattering of over-mature pods (George, 1999). This challenge is often complicated by plant to plant variability in flowering time and seed maturation within a crop (Brocklehurst, 1995).

Currently, prediction of time of cutting is based on physical appearance of the pods or seeds combined with the grower's experience. In the previous chapter, chlorophyll fluorescence was shown to provide a simple and reliable estimate of carrot seed maturity. Seed coat chlorophyll content has been correlated to seed quality in individual seed lots of oilseed and turnip rape (Ward *et al.,* 1992; 1995) and has been shown to be an effective basis for grading cabbage seed lots for improved quality (Jalink *et. al.,* 1998). This work aims to extend the outcomes of the previous chapter and earlier studies on chlorophyll and seed quality in Brassica by evaluating chlorophyll fluorescence as a potential marker for time of cutting of *B. oleracea* seed crops.

Materials and Methods

Samples were collected from 5 commercial field based seed crops of *B. oleracea* produced in the Coal River Valley, Tasmania during December 2005 and January 2006 (Table 6.1) Comparison of 2005 – 06 and long term minimum and maximum daily temperatures from the Hobart airport weather station (Australian Burueau of Meteorology site no 09004), located between 5 and 30km from all sites used in this work, indicate that temperature conditions during the study period were close to average.

In each crop, a representative 30 to 50m section of male sterile row was selected for sampling. Samples were collected on 4 to 6 occasions, separated by 3 to 7 day intervals. In each crop, commercial cutting occurred midway through the trial sampling period. Details of the crops used and sampling and timing are provided in Table 6.1.

Each sampling consisted of removal of single branches from 30 randomly selected plants within the sample area. Samples were placed in hessian bags and dried on a forced air dryer located within a glasshouse. After threshing by hand, and cleaning using an air-screen cleaner, the 30 samples from each sample date in each crop were pooled.

Table 6.1 – Details of *B. oleracea* seed crops sampled in this work

Type	First Sample	Last Sample
Summer/Autumn Cauliflower	14th Dec	27th Dec
Autumn Cauliflower	14th Dec	27th Dec
Autumn Cauliflower 2	19th Dec	13th Jan
Storage Cabbage	4th Jan	17th Jan
Japanese Sweet Cabbage	3rd Jan	17th Jan

Chlorophyll fluorescence measurements (Fo) were made on 8 representative sub-samples from each pooled seed lot. Measurements were made with a PAM fluorometer (Heinz Walz Instruments, Effeltrich) with the probe mounted on a retort stand, 15 mm above a 30mm x 10mm glass petri dish filled to level with seed (as shown in Chapter 5).

Eight representative samples of 50 seeds were drawn from each seed lot for germination testing. Seeds were germinated on two layers of filter paper in petri dishes at 25^{o}C with continuous light. Final assessment of the germination test was made at day 10.

On two harvest dates for the storage cabbage, 10th of January (immature) and 17th of January (mature harvest), chlorophyll fluorescence measurements and germination tests were made on each of the 30 individual samples as well as the pooled sample.

Results and Discussion

In each *Brassica oleracea* crop studied in this work significant gains in seed lot quality (percentage normal seedlings) were made during the late stages of crop maturation (Figure 6.1). This pattern of seed development highlights the value of a reliable indicator of cutting time for *B. oleracea* seed production. Although few references to seed dormancy in commercial varieties of *B. oleracea* were found in the literature, seed lots from three of the crops showed a partial dormancy in initial germination tests soon after harvest that was overcome during 3 weeks storage at 4°C (Figure 6.1).

Although they represent a diverse range of cultivars, there was a close relationship between chlorophyll fluorescence signal (Fo) and seed lot quality ($P < 0.001$, $r^2 = 0.94$) across the 5 crops studied in this work (Figure 6.2). This indicates that it may be possible to determine a common threshold level of chlorophyll fluorescence for cutting of *B. oleracea* seed crops.

While this work has shown that the relationship between chlorophyll fluorescence and germination percentage in *Brassica oleracea* is robust in small field plots, further work is required to determine the requirements in terms of intensity and pattern of sampling to obtain reliable estimates for whole crops. Furthermore, the relationship should be tested across seasons and in a broader range of production environments before it is commercially adopted.

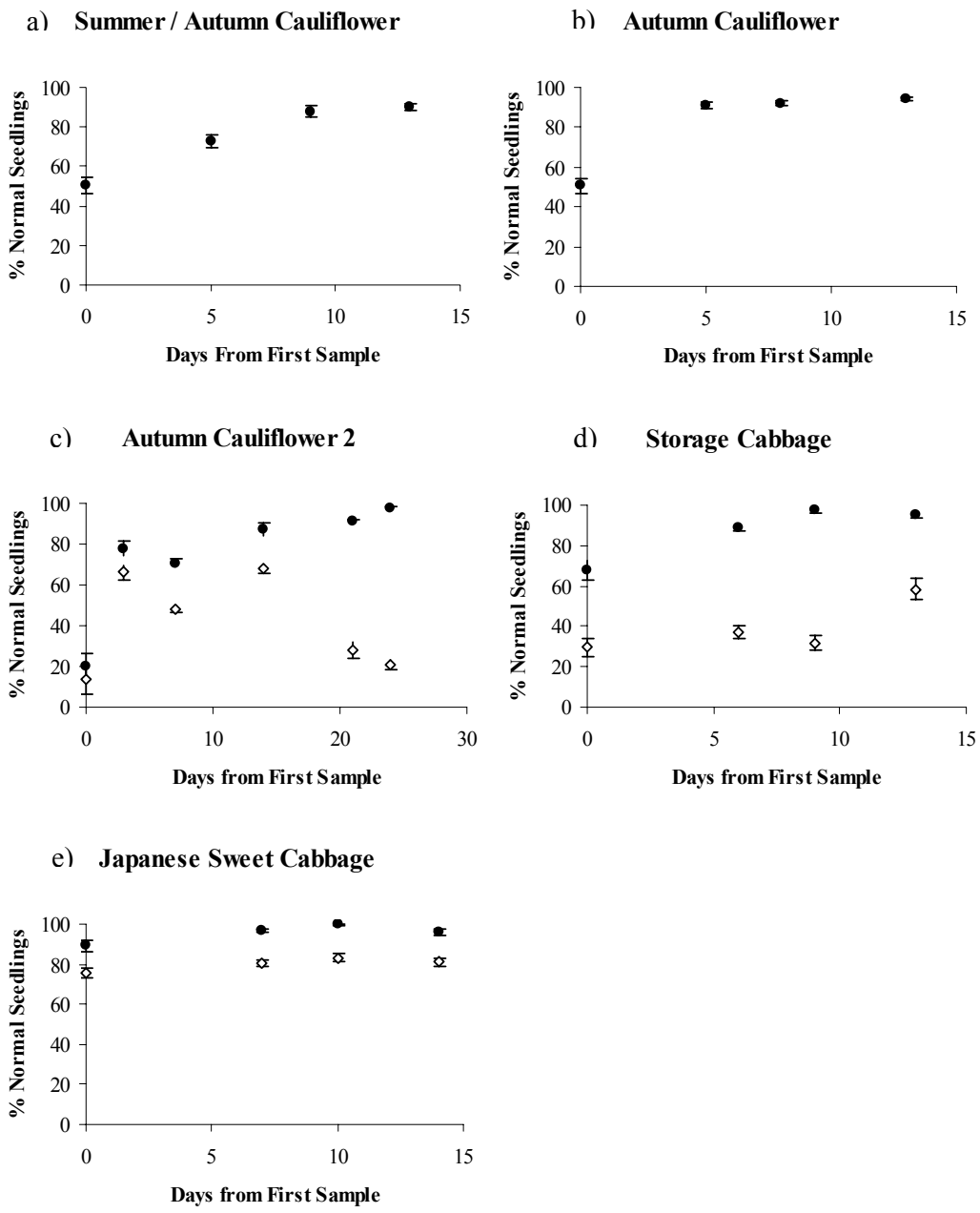


Figure 6.1 – Changes in percentage normal seedlings assessed at day 10 of the germination test (♦) during sampling for crops used in this work. Note, in c) to e) the open symbols are for germination tests performed 1 week after final sampling, filled symbols are for germination tests performed after 3 weeks of 4°C storage. Error bars indicate standard errors; n=8.

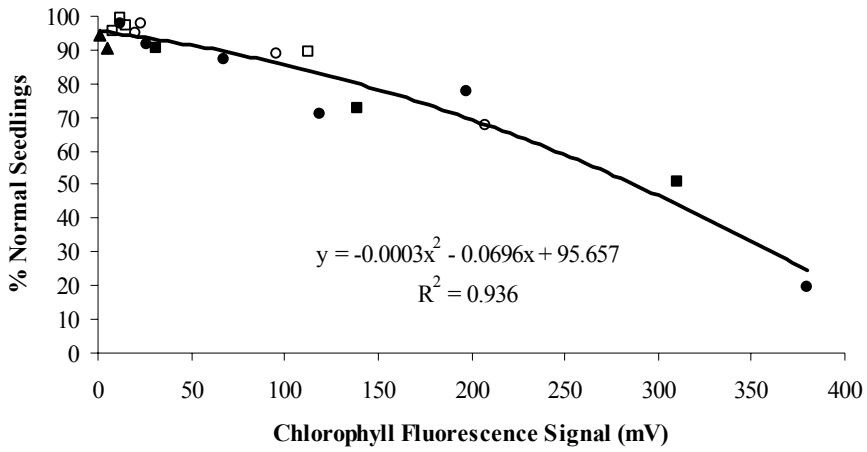


Figure 6.2 – The relationship between chlorophyll fluorescence signal and percentage normal seedlings for all seed lots collected in this work. Seed lots belonging to the same crop (different cutting time) are designated with a common symbol: Byrne cauliflower (■); Casimaty cauliflower (▲); SPS Gangel storage cabbage (◊); Gunn cauliflower (♦); Tapp Japanese cabbage (□). The relationship across all data points ($y = -0.003x^2 – 0.0694x + 95.64$) is significant ($P < 0.0001$; $r^2 = 0.94$).

The variation in chlorophyll fluorescence readings and germination percentage of seeds from different plants harvested approximately 3 days prior to the optimal harvest date within the storage cabbage crop (Figure 6.3) highlights the potential for quality improvement through a grading system based on chlorophyll fluorescence such as that developed by Jalink *et al.,* (1998). Even in the more mature seed lot, there was enough plant to plant variation in seed maturity and quality to suggest that quality improvement through grading may be possible.

In overview, this work demonstrates that the system developed for determination of time of cutting in carrot seed crops (Chapter 5) has significant potential for use in Brassica seed crops also. Further work across a range of seasons and production locations is required to validate the system for commercial use. The data collected also highlight the potential benefit of a commercial grading system for Brassica seed based on chlorophyll fluorescence.

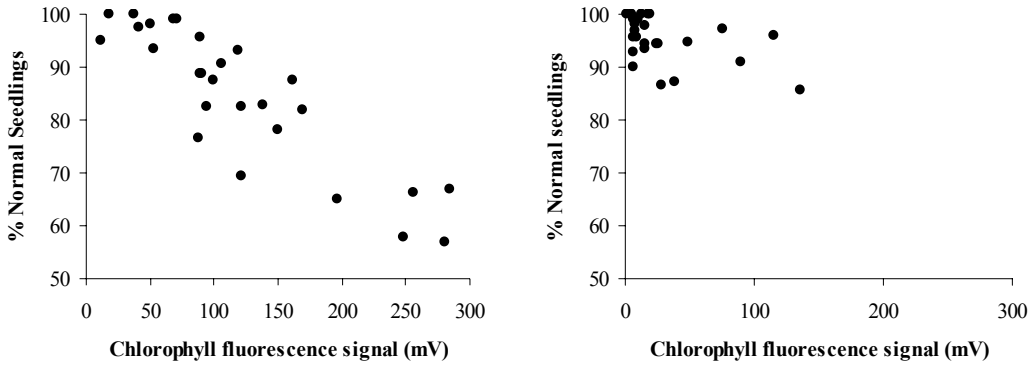


Figure 6.3 – The relationship between chlorophyll fluorescence signal and percentage normal seedlings for seed collected from individual plants in a) an immature seed crop and b) a mature seed crop. Data were collected for seed lots of the storage cabbage cut on the 10th and 17th of January.

Chapter 7

Stunted Root Abnormality in Onion

Introduction

The occurrence of abnormal seedlings in onion seed germination tests is an important quality issue for onion seed producers. A high incidence of abnormal seedlings reduces the value of seed lots and can threaten the viability of seed production in regions where the problem frequently occurs. Germination is defined by the International Seed Testing Association as the emergence and development from the seed embryo of those structures which, for the kind of seed in question, are indicative of the ability to produce a normal plant under favourable conditions (ISTA, 1979). Seeds that produce a seedling failing these requirements are termed abnormal. For onions, seedlings with a primary root less than one third of total seedling length 12 days after imbibition are commonly classified as a category of abnormal seedling. Seedlings with short primary roots constituted up to 35% of imbibed seeds and represented 87.8% of all seedling abnormalities in seed of the cultivar 'Creamgold' produced in Southern Tasmania (Spurr *et al.,* 2002). Despite the importance of seedling abnormalities as a quality criterion for seed producers, there is little information in the literature pertaining to the longer term growth and survival characteristics of onion seedlings with short primary roots.

In the sequence of quality acquisition during seed development, the capacity for germination, defined as radicle emergence after imbibition, is acquired prior to the ability to produce a normal seedling (McDonald, 1999). As a result seed immaturity at harvest, which in onions is exacerbated by asynchronous seed development (Currah, 1981), contributes to the incidence of seedling abnormalities in fresh seed lots (Spurr *et al.,* 2002; Globerson *et al.,* 1981). In addition to the effects of seed maturity at harvest, the incidence of abnormal seedlings can increase through seed deterioration in storage (McDonald, 1999).

The physiological basis of reduced root growth in seedlings is incompletely understood, with most information derived from seed deterioration studies. Radicle and primary root elongation during germination occurs through both new cell formation and cell expansion. Deterioration studies have demonstrated DNA degradation and impaired mitotic activity (Berjak *et al.,* 1986; McDonald, 1999) as well as impaired membrane functioning (Mohammed Yasseen and Splitstoesser, 1990) as likely causes of reduced seed performance. These changes would impact both cell division and expansion processes. In onion seedlings, mitotic activity was not detected in onion seedling radicle tips during germination until radicle length was 3mm or greater (Bitonti *et al.,* 1992). Therefore seedlings with radicle lengths up to 3mm may result from inhibition of cell division, but in radicles greater than 3mm, reduced rates of cell expansion and/or cell division must be occurring. The proportion of abnormal seedlings from a seed lot can be altered by varying the substrate used for germination testing (Taylor *et al.,* 2001), demonstrating that the seedling is not necessarily committed to a developmental pathway at the point of inhibition.

Few management options are available to reduce the incidence of stunted root abnormality in onion seed lots. Selection of appropriate production environments (Brewster, 1982) and harvesting dates (Spurr *et al.,* 2002) have been examined, but the effects of other pre- or post-harvest treatments have not been described.

Seed pre-treatments, including various forms of priming, are widely used by the seed industry to increase seed germination rate and uniformity of seedling emergence (Bujalski and Nienow, 1991) and benefits of various treatments have been demonstrated in onion (Drew *et al.,* 1997; Ali *et al.,* 1990; Gray *et al.,* 1990; Dearman *et al.,* 1986). Effects of the pre-treatments used in these studies on abnormality were not noted, but Rao *et al*. (1987) found that partial re-hydration of lettuce seeds increased the rate of seedling root growth and decreased the frequency of abnormal seedlings. The potential for using seed priming or other seed pre-treatments to overcome short roots in onion seedlings has not been reported.

In this study, the effects of a range of seed drying and post harvest seed pre-treatments on the incidence of abnormal seedlings arising from seed of the onion cultivar 'Creamgold' were investigated.

Materials and Methods

Harvest Maturity and Seed Drying Studies

The effects of 3 drying regimes on seed quality was tested using seed samples obtained from a trial plot of cv. Creamgold Regular grown in Southern Tasmania in 2005. The drying treatments were forced air drying at 40^{o}C, forced air drying at 30^{o}C and passive drying on racks in a glasshouse (mean air temperature ranged from 10 to 40^{o}C). In all treatments, samples were dried to 10% moisture content (w:w).

At the time of commercial harvest, umbels at two stages of maturity, without seed capsule dehiscence (immature) and with an area of capsule dehiscence of at least 20mm diameter (mature), were collected. Immature umbels were divided into two groups according to the length of scape left attached to the umbel, 150mm and less than 20mm, giving three umbel classes in total (mature, 20mm scape; immature, 20mm scape; and immature, 150mm scape).

Four replicates of each umbel class were dried in each temperature treatment. Seed quality was determined using standard germination tests. All germination tests were carried out in an incubator (Contherm GPM Scientific, Wellington, New Zealand) with forced air circulation and maintained at 20°C with 12/12 hour light/dark regime. Four replicates of 100 seeds were uniformly placed in 90 mm petri dishes on two layers of moistened Advantec Type 2 filter paper. Distilled water was added at intervals to keep the paper moist. Germination percentage was recorded 12 days after imbibition (DAI) and seedlings were classified using ISTA guidelines (ISTA, 1979).

Seed Quality and Pre-treatment Studies

Commercially available Tasmanian cv. Creamgold seed lots were screened for germination characteristics. Two seed lots with greater than 75% germination combined with a significant percentage of abnormal seedlings and a set of 9 seed lots with varying germination percentage and levels of abnormal seedlings were selected for the study. The seed lots, referred to as seed lot A and seed lot B in the remainder of the chapter were free from chemical treatment and had an initial moisture content of 7.4% (wet weight). Seed had been stored at 2°C prior to commencement of this work.

Seed Lot Characterisation

Seed lots were characterised using standard germination tests. All germination tests were carried out in an incubator (Contherm GPM Scientific, Wellington, New Zealand) with forced air circulation and maintained at 20°C with 12/12 hour light/dark regime. Four replicates of 100 seeds were uniformly placed in 90 mm petri dishes on two layers of moistened Advantec Type 2 filter paper. Distilled water was added at intervals to keep the paper moist. Germination percentage was recorded 12

days after imbibition (DAI) and seedlings were classified using ISTA guidelines (ISTA, 1979).

A second experiment was conducted to record the timing of abnormal seedling development within a population of germinating seeds. One thousand seeds of each seed lot were imbibed on filter paper (Advantec Type 2) in petri dishes. Seeds were examined daily and the number of germinants, determined by radicle emergence, recorded. Germinated seeds were carefully transferred to new petri dishes and labelled with the date of germination. Assessment and transfer were continued until 12 DAI. This procedure resulted in segregation of the seed lot populations into sub lots based on germination time. The proportion and classification of abnormal seedlings was recorded for each sub lot 8 days after germination as well as 12 DAI.

Growth of Seedlings with Stunted Roots

Survival and early development of normal seedlings and seedlings with stunted root abnormality were compared following transplanting to potting mixture. Four replicates of 24 seedlings were selected 12 DAI from the seed lot A population in the above experiment. Individual seedlings were planted in single cells (64 cm3) of seedling raising trays and grown under mist conditions in a poly tunnel. Sterilised potting mix was used and consisted of sand and peat (1:1). Lime was added to the mix to adjust the pH to 6.0. Seedlings were sown in a randomised complete block design. Seedling survival percentage and dry weights were recorded 22 days after transplanting.

Seed Pre-treatments

Five pre-germination treatments, previously demonstrated as effective in improving vegetable seed performance, were applied to samples from seed lots A and B. The five treatments were: 1) osmotic priming using -1.2 MPa NaCl solution; 2) osmotic priming using 1% KNO3 solution; 3) hydro-priming; 4) hardening; and 5) soaking in 50ppm gibberellic acid solution. Germination characteristics of treated sub lots were compared with untreated seed.

Methods for osmotic priming were adapted from those reported previously for onion seed (Suzuki *et al.,* 1989). Fifty seeds were placed on two 80 mm Advantec Type 2 filter papers saturated with -1.2 MPa NaCL, or 1% KNO3 solution. The plates were sealed with parafilm and incubated in the dark at 20°C for seven days. Seeds were then washed with distilled water and air-dried for two days.

The hydro-priming treatment was based on the method of Fujikura *et al.* (1993). Seeds were soaked in water at room temperature (22-24°C) for 5 hours, surface-dried and kept in a closed container at 100% relative humidity at room temperature for 3 days. Seeds were then air-dried for two days.

The hardening treatment involved placing seed on two layers of Advantec Type 2 filter papers moistened with 3 ml of distilled water. The dishes were sealed with parafilm and placed at room temperature for 16 hours. Seeds were then dried for two days to a constant seed weight.

Pretreatment with gibberellic acid involved soaking seeds in 50 ppm GA3 for 24 hours in darkness. Seeds were then dried for two days to a constant seed weight. Seed germination in the osmoticum during treatments with KNO3, GA and NaCl were 1.8, 1.5 and 1 percent respectively and zero in other treatments.

Germination characteristics of pre-treated and control (untreated) seed of seed lots A and B were compared using ISTA germination tests for onion (ISTA, 1979). One hundred seeds of each treatment were distributed uniformly on moistened Advantec Type 2 filter paper in 90 mm petri dishes and held at 20°C in a forced air incubator. Four replicates of each treatment were used in a complete randomised design within the incubator. Seeds were assessed daily and number of germinants, as evidenced by radicle emergence, was recorded. The data was used to calculate the mean time to complete germination (MTG) and the coefficient of uniformity of germination (CUG) for each seed lot using the following equations; $MTG = \Sigma(tx.nx)/\Sigma nx$ and $CUG = \Sigma n/[\Sigma(MGT- tx)2.nx]$, where nx is the number of seeds that germinated on day tx after imbibition and Σnx is the total number of germinants. Final germination percentage was recorded 12 DAI and seedlings were classified using ISTA guidelines (ISTA, 1979) with particular care taken when identifying stunted root abnormality. Radicle length of seedlings was measured under a binocular dissecting microscope and seedlings were defined as abnormal when the length of the primary root was less than one third the length of the entire seedling.

Storage Life of Hydro-primed Seed

Two onion seed lots of 'Creamgold Regular' of varying quality (initial germination percentages of 75 and 87%) were hydro-primed and dried back as described above. Following priming, each seed lot was divided into 4 replicate samples of approximately 1000 seeds and stored at 4^oC. A matching set of non-primed samples (control) were stored under the same conditions. At 0, 4, 8 and 12 months after priming, 100 seed samples were drawn from each replicate and germination tested. Each sample was uniformly distributed in a 90 mm petri dish on two layers of moistened Advantec Type 2 filter paper incubated at 20^oC. Distilled water was added at intervals to keep the paper moist. Germination percentage was recorded 12 days after imbibition (DAI) and seedlings were classified using ISTA guidelines (ISTA, 1979).

Statistical comparisons between treatments were undertaken through analysis of variance and regression analysis using Statistical Analysis Software (SAS version 6.12; SAS Institute Inc 1989). Fisher's LSD (least significant difference) was calculated where differences between means were significant at the 5% level.

Results and Discussion

In a previous study of onion seed quality, expression of stunted root abnormality was linked to seed maturity at cutting, but mature seed lots (approximately 30% moisture content at cutting) also expressed relatively high levels of abnormal seedlings, indicating the impact of other factors on this aspect of quality (Spurr *et al.,* 2002). The current work examined the effect of interactions between seed lot maturity and drying conditions on expression of seedling abnormality in seed samples collected at a stage of crop development equivalent to current commercial harvest time. Consistent with the earlier work, no effect of umbel maturity at cutting on seedling abnormality was observed at this stage of crop development. Previous studies have reported a negative effect of high temperatures during drying of leek seed quality (Gray *et al*, 1989). In this work the effects of the interaction between drying temperature and maturity on seed quality were significant ($P<0.01$). Whilst the germination percentage of seed for mature umbels (showing capsule dehiscence) was unaffected by drying temperatures of up to 40^{o}C, higher temperature conditions during drying significantly reduced the germination percentage of seed from less mature umbels (showing no capsule dehiscence) (Table 7.1). This was largely due to increased levels of dead seed. Despite the effect of seed germination, interactions between seed maturity and drying conditions did not alter the level of expression of seedling abnormality.

Table 7.1 – Effects of seed maturity at cutting and drying conditions on seed quality in onion cultivar 'Creamgold Regular'. Significant ($P<0.05$) differences in germination percentage are denoted by the letters after the values.

Maturity Class	Drying Conditions	% Germination	% Abnormal Seedlings	% Dormant seed	% Dead Seed
Mature	40^{o}C forced air	84.3 a	16.9	3.6	11.3
	30^{o}C forced air	82.5 a	15.8	6.2	10.6
	Ambient Glasshouse	87.0 a	14.2	3.0	9.7
Immature, Short Scape	40^{o}C forced air	58.1 c	14.2	10.0	30.4
	30^{o}C forced air	68.6 b	12.9	6.4	21.8
	Ambient Glasshouse	76.4 ab	13.7	5.5	17.4
Immature, Long Scape	30^{o}C forced air	62.3 bc	15.7	6.4	30.4
	40^{o}C forced air	66.9 b	14.2	10.5	20.6
	Ambient Glasshouse	77.8 ab	16.2	7.1	14.2

Priming of onion seed lots significantly reduced the percentage of abnormal seedlings recorded in standard germination tests (Table 7.2 and Figure 7.1). This benefit of priming onion seeds has not been described previously and may be of particular practical value to onion seed producers given the propensity of the crop to produce seed with unacceptably high percentages of abnormal seedlings. Seed priming also increased germination rate and promoted more uniform emergence, while in other studies germination under a broader range of environments and improved seedling vigour have been documented (McDonald, 1999).

Table 7.2 - Effect of seed priming treatments on germination characteristics. Priming treatments were hydropriming (hydro), osmopriming with –1.2MPa NaCl solution (NaCl) or 1% KNO3 solution (KNO3), soaked for 16 h then dried (Hardened) and 50ppm gibberellic acid solution (GA). Percentage of seeds germinating (Germ), mean time to complete germination (MTG), coefficient of uniformity of germination (CUG), percent normal (N) and abnormal seedlings (AB), and mean root length of normal seedlings were recorded. Values are means of four replications, and different letters indicate significant differences at $P \leq 0.05$. n.r. = not recorded.

	Treatment	Germ (%)	MTG (days)	CUG	N (%)	AB (%)	Root length (cm)
Seed lot A	Control	94.5^{bc}	4.91^{b}	0.204^{b}	71.5^{c}	23.0^{a}	3.41^{b}
	Hydro	99.2^{a}	3.98^{a}	0.251^{a}	87.7^{a}	11.5^{d}	3.77^{a}
	NaCl	91.5^{c}	4.78^{b}	0.210^{b}	79.2^{b}	12.2^{cd}	3.29^{bc}
	KNO_3	91.2^{c}	4.88^{b}	0.206^{b}	76.0^{bc}	15.2^{bc}	3.07^{c}
	Hardened	96.0^{ab}	4.61^{b}	0.211^{b}	79.0^{b}	17.0^{b}	3.55^{ab}
	GA	95.2^{bc}	4.62^{b}	0.217^{b}	78.2^{b}	17.0^{b}	3.80^{a}
Seed lot B	Control	77.0^{c}	6.11^{a}	0.164^{a}	54.5^{c}	22.50^{a}	2.13^{b}
	Hydro	86.3^{a}	6.02^{a}	0.166^{a}	73.5^{a}	12.75^{c}	2.61^{a}
	NaCl	80.0^{b}	6.81^{c}	0.147^{c}	66.7^{b}	13.25^{c}	n.r.
	KNO_3	80.5^{b}	6.78^{c}	0.147^{c}	65.2^{b}	15.25^{bc}	n.r.
	Hardened	82.0^{b}	6.34^{b}	0.158^{b}	64.0^{b}	18.00^{b}	n.r.
	GA	80.7^{b}	6.37^{b}	0.157^{b}	64.0^{b}	16.75^{b}	n.r.

Hydro-priming, osmotic priming, hardening and giberellic acid application all improved the performance of deteriorated onion seed lots (Table 7.2). Similar treatments used in the study had been reported as effective in vegetable seeds (Suzuki *et al*., 1989; Tewari *et al*., 2001; Fujikura *et al*., 1993). In this comparison between treatments hydro-priming resulted in the greatest improvement in seed lot quality. This contrasts with results reported for other species, for example the findings of Fujikura *et al*. (1993) where in cauliflower osmotic priming was more capable of repairing aged seeds than hydro-priming. The reason hydro-priming stimulated a higher proportion of seeds to develop as normal seedlings in this study is unclear. Adverse effects such as oxygen deprivation of seeds during the longer (7 day) osmotic

priming treatments was unlikely, as the seeds were never fully submerged, but cannot be ruled out. Further refinement of the treatments used (e.g. priming duration, water status, level of GA applied) in this study or the combination of treatments (e.g. hydro-priming plus GA application) may result in greater improvement in onion seed lot performance.

In contrast to previous studies that have shown more rapid deterioration of primed seed compared with non treated seed, the effects of hydro-priming persisted during 8 months of storage, with no evidence of accelerated deterioration (Figure 7.1). This finding has important implications in terms of ease of application of the technology within the vegetable seed and onion industries.

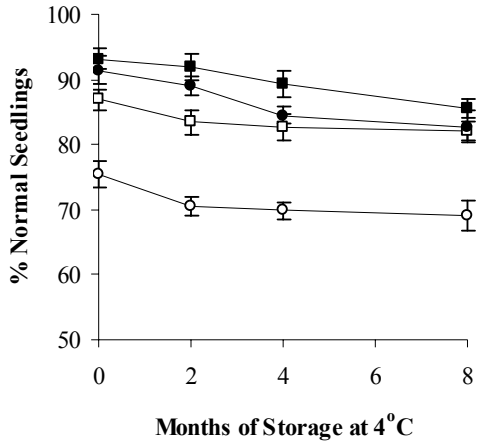


Figure 7.1 – Comparison of effects of storage at 4°C on % normal seedlings in germination on primed (□, ■) and non-primed seedlots (○, ●) of two 'Creamgold Regular' onion lines (denoted by filled and open sysmbols). Error bars indicate standard errors (n=4).

The proportion of abnormal seedlings within a seed lot has been regarded as an indicator of deterioration, with aged onion seed lots having a higher proportion of abnormal seedlings (Stanwood and Sowa, 1995). In this study, two seed lots of different ages and different degrees of deterioration, as evidenced by germination rates (Table 7.2), had similar, high levels of stunted root abnormality to that reported in freshly harvested onion seed of the same cultivar from the same production location (Spurr *et al.,* 2002). It was therefore concluded that while prolonged periods of storage may contribute to the incidence of unacceptable percentages of abnormal seedlings in seed lots, the physiological basis for the expression of abnormality is not only associated with storage deterioration reactions. This conclusion is consistent with the view that short term deterioration in the field is likely to be a different physiological event than long-term deterioration in storage (McDonald, 1999).

When seeds were segregated into sub lots based on time to radicle emergence following imbibition there was a trend towards increased levels of abnormality in slower germinating sub lots (Table 7.3). Although slow germination may result in an artificially high level of abnormality being recorded in 12 day ISTA germination tests

due to the small size of late germinating seedlings at the time of counting, assessment of abnormality levels independent of germination time (assessment undertaken 8 days after germination for each sub lot) revealed that levels of stunted root abnormality tended to increase with later germination dates for the seed lot A, but was high for all germination times for the more deteriorated seed lot B. For both seed lots the percentage of normal seedlings decreased with later germination dates due to an increase in the frequency of abnormalities other than stunted root with later germination times. The rate of germination has been demonstrated as the key facet of seed quality affecting onion seedling size (Wheeler and Ellis, 1994). The weak relationship between the time to complete germination and stunted root abnormality suggests that expression of the abnormality may not be associated with overall seedling development rate but rather may be more specific to root expansion.

Table 7.3 - Percentage of normal seedlings (N), abnormal seedlings with stunted primary roots (SR) and abnormal seedlings with other abnormalities (Other). Onion seeds were divided into sub lots based on time of radicle emergence after imbibition (RE-DAI). All sub lots were assessed at two times, 12 days after imbibition in accordance with ISTA test and 8 days from radicle emergence.

		12 days after imbibition			8 days after gemination		
	RE-DAI (Days)	N (%)	SR (%)	Other (%)	N (%)	SR (%)	Other (%)
Seed lot A	4	85.3	7.2	7.5	85.3	7.2	7.5
	5	79.2	7.5	13.3	81.0	9.5	9.5
	6	70.0	14.0	16.0	76.6	17.0	7.0
	7	66.7	13.3	20.0	58.5	20.0	21.5
	8	73.0	10.0	17.0	60.7	35.7	3.6
	11	*	*	*	63.6	30.3	6.1
	12	*	*	*	50.0	23.3	26.7
Seed lot B	4	52.6	26.7	20.7	52.6	26.7	20.7
	5	55.7	17.4	26.9	55.7	17.4	26.9
	6	46.4	20.0	33.6	42.1	30.8	27.1
	7	43.9	21.4	35.7	36.2	31.9	31.9
	8	54.5	9.1	36.4	41.4	27.6	31.0
	11	*	*	*	38.9	44.4	16.7
	12	*	*	*	21.7	30.4	47.9

* under developed

The conditions under which the onion seed germinates also influences the expression of stunted root disorder, with different substrates used in standard germination tests giving different abnormality levels for the same seed lot (Taylor *et al.,* 2001). In addition, the root growth of newly germinated onion seedlings is affected by the water potential under which it is grown. Exposing the seedlings to a water potential below the base water potential of expansive growth (-1.1 MPa) (Whalley *et al.,* 1999)

induced quiescence, with normal root growth of the water stressed seedlings resuming when seedlings were later placed on wet filter boards (Whalley *et al*., 2001). In contrast newly germinated onion seedlings exposed to a water potential of –0.66 MPa displayed reduced root growth that did not fully recover even when later placed on wet filter boards (Whalley *et al*., 2001).

These findings and the results reported in this study demonstrate that the processes involved in the elongation of the primary root can be influenced during pre- and post-radicle emergence. Further examination of the regulation of expansion processes in the germinating onion radicle may uncover further strategies for mitigating the expression of stunted root abnormality.

Chapter 8

Recommendations

In order to maintain a competitive advantage, it is important that Australian vegetable seed industry is able to consistently produce reliable yields of high quality seed. This project examined the nature and management of three seed production issues of particular relevance to this industry: unreliable seed set in CMS hybrid cauliflower seed production; occurrence of rudimentary embryos in carrot seed; and expression of stunted root abnormality in onion seedlings. Recommendations on adoption of research outcomes and further avenues of research identified in this work and maintenance of seed research activity at the School of Agricultural Science and Tasmanian Institute of Agricultural Research are outlined below.

Research Activity

The project has provided a basis for substantial growth in agricultural and forestry seed research activity which is responsive to industry identified problems and undertakes industry specific projects. During the course of this project a number of leading national and international seed companies have engaged in collaborative research projects with TIAR, opening a diverse range of opportunities for seed research and strengthening the relationship between the Australian and international industries. This growth in activity has resulted in a marked increase in postgraduate and honours students participating in seed research, leading to an accumulation of expertise and interest in this field. A recommendation of the project is that this activity should be maintained and supported into the future.

Yield Improvement in CMS Cauliflower Seed Crops

A series of pollen handling, storage and testing protocols were developed for trinucleate pollen of carrot and *Brassica oleracea* that will be applicable to future seed yield and quality research in these crops. One limitation is that a suitable *in-vitro* test of pollen viability for carrot or stored *B. oleracea* pollen was not identified. Suitable *in-vitro* methodologies for both species would be of considerable benefit for pollination and pollen quality research. Development and validation of these methods is considered a priority.

Under Tasmanian field conditions, poor yields from late flowering CMS cauliflower cultivars were shown to be mainly due to inadequate pollination and poor pollen viability at anthesis. Poor pollen viability was linked to high temperate stress. Under Tasmanian field conditions, management for earlier flowering time through choice of transplanting time minimised loss of pollen viability due to high temperature stress and substantially increased seed yields in late flowering cultivars. Inadequate

pollination was linked to incompatibilities between the conventional strip arrangement of hybrid seed production and the predominant pollinator insects, honey bees. Alternative parent line arrangements that promoted cross pollination were tested and shown to increase seed yields.

Pilot commercialisation of the research outcomes is currently being undertaken by industry in Tasmania. It is important that this work includes both an evaluation of impacts on new management strategies on seed quality.

While the research has identified new approaches to management of certain CMS cauliflower seed crops, it also highlights the importance of developing better understanding of the limitations within vegetable seed production systems. Adoption of these management practices into other cauliflower seed production systems, for example with different cultivars or production environments, should only be undertaken where there is a demonstrated need for improved management of pollination or temperature stress.

Rudimentary Embryos and Time of Cutting in Cool Temperate Carrot Seed Crops

Rudimentary embryos were shown to relate to seed maturity at cutting time and their prevalence explained by the patterns of late embryo development in carrot seed crops produced in cool temperate conditions. Previous recommendations for time of cutting of Australian carrot seed crops were based on studies in warmer climates or glasshouse production and typically overestimated seed maturity. Chlorophyll fluorescence of the seed coat was shown to be a robust test of carrot seed maturity / quality for use in determination of time of cutting of carrots in small plots. Preliminary experiments extending this work to *Brassica oleracea* seed production demonstrated that there is significant potential to use chlorophyll fluorescence as a marker of cutting time in these crops also.

It is recommended that the Australian vegetable seed industry consider commercialisation of this test to improve reliability of time of cutting in carrot seed crops and that its potential application in *B. oleracea* seed production also undergoes rigorous testing. The commercialisation process will need to involve development of protocols for sampling whole crops.

The results of this work also highlight the potential value of a system of grading based on chlorophyll fluorescence for vegetable seed production in cool temperate climates; an area that requires further investigation.

Stunted Root Abnormality in Onion Seedlings

No relationship between seed maturity, seed drying temperatures and expression of abnormal seedlings was observed in seed lots collected at a stage corresponding to

current commercial harvest time. A range of seed pre-treatments were effective in reducing expression of stunted root abnormalities in seedlings. In particular, hydro-priming offers significant potential. The benefits of hydro-priming were shown to persist during seed storage, which would be a distinct advantage for commercialisation of this technology. Whilst the technology has demonstrated benefits for reducing expression of seedling abnormality in germination, its effects on field emergence has not been thoroughly tested. Experiments to field test hydro-primed seed under Tasmanian conditions are currently being planned.

Chapter 9

Technology Transfer

This project has delivered a number of science and technology outcomes for the Australian seed industry, which have been extended to a diverse audience. The industry partners have been involved at all stages of the project and kept informed of research outcomes as they have arisen. In several instances, project outcomes have already been, or are being incorporated into commercial production. For example:

- Timing of cutting of Australian seed crops has been modified to accommodate the slower rates of embryo development observed in cool temperate climates. The benefit of this modification is already apparent in improved germination results for export carrot seed crops.

- Pilot scale commercialisation of modifications to cauliflower seed crop transplanting time and parent line arrangement are underway.

- Pollen handling, storage and testing protocols for carrot and cauliflower developed in this work have been adopted into commercial screening programs for seed parent lines and are currently in use in research projects in Australia, New Zealand and the Netherlands.

Additional evidence that the research outputs were valued by industry lies in the increased investment of Australian and international industry partners in seed research activity at the School of Agricultural Science / TIAR.

Specific Technology transfer activities in this project included:

Honour Theses

Geard, A. (2004) Embryo development and time of cutting in carrot seed crops. Honours thesis, University of Tasmania.

Book Chapter

Spurr, C. J. Geard, A. and Brown, P.H. Embryo Development and Time of Harvest in Cool Temperate Carrot Seed Crops. In: Adkins, S. W., Ashmore, S. E. and Navie, S. C. Seed Science Research; Advances and Applications. CABI, Wallingford, UK. (In Press).

Conference / Research Day Presentations

Spurr, C. J. Geard, A. and Brown, P.H. (2005) Embryo Development and Time of Harvest in Cool Temperate Carrot Seed Crops. 8th International Workshop on Seeds, Germinating New Ideas. Internationals Society for Seed Science Research. Brisbane, Australia.

Gracie, A., Spurr, C. J., Brown, P. H. and Clark, R. J. (2005) Factors affecting the incidence of root abnormalities in onion seedlings. 8th International Workshop on Seeds, Germinating New Ideas. Internationals Society for Seed Science Research. Brisbane, Australia.

Spurr, C. J. (2005) Vegetable Seed Research at TIAR. 8th Annual Potato and Vegetable Agricultural and Research Advisory Committees Research Development and Extension Day. Devonport, Tasmania.

Spurr, C. J. and Brown, P. H. (2005) Progress in hybrid onion, carrot and cauliflower seed research. South pacific Seeds Production Conference. Launceston, Tasmania.

Spurr, C. J. and Brown, P.H. (2004) Hybrid Vegetable Seed Research (Carrot, Onion and Cauliflower). South pacific Seeds Production Conference. Griffith, NSW.

Spurr, C. J., Wilson, S. and Clark, R. (2004) Low Seed Yield from ON019A. Enza / South Pacific Seeds Onion Seed Production Conference. Griffith, NSW.

Spurr, C. J. (2003). SPS / TIAR Seed Research. Research Outcomes and New Directions. South Pacific Seeds Production Conference, Melbourne, Victoria.

Other Presentations

Spurr, C. J., 'Rijk Zwaan / TIAR Research Collaboration', Invited presentation to Rijk Zwaan Departments of Seed Technology and Breeding, De Lier, Netherlands (2005)

Spurr, C.J., 'South Pacific Seeds/TIAR research in Carrot seed production', Invited presentation to South Pacific Seeds New Zealand, Methven, New Zealand (2005)

In 2005, Dr Spurr visited Rijk Zwaan's Headquarters in the Netherlands for 2 weeks to participate in seed research activities, present an overview of outcomes from this project and develop new research collaborations.

Visiting Students / Exchanges

Mr Etienne Leroy, final year student, Dronten University. Sponsored by Rijk Zwaan Netherlands to participate in 6 months research training in this project (2005 - 06) to satisfy the requirements his European Engineers Degree in Plant Production.

Other Output

Detailed annual progress reports were prepared and distributed to both industry partners.

The project has involved a large amount of informal communication between the researchers and the industry partners including visits to both industry partners in Australia by the researchers, discussion with growers, visitation of industry partners to TIAR, and demonstration of pollination and pollen viability and embryo testing procedures to industry production and research personnel.

This project has provided the platform for development of other seed research activities within the School of Agricultural Science. Currently there are 7 students undertaking postgraduate (PhD and Masters) research in seed production at the School of Agricultural Science, University of Tasmania across a range of industry and government funded projects. This activity has lead to over 20 book chapter, journal, conference and industry publications and presentations during the last 2 years.

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Appendix 1

Pollen Viability Test Methods for Carrot and Cauliflower

In-vivo Germination

A series of preliminary controlled pollinations were performed in both species to ensure that the parent lines used in *in-vivo* germination tests were compatible. Samples of pollen from the male fertile lines were sparingly hand pollinated onto 2 receptive flowers on each of 4 plants of the corresponding male sterile line. 24 hours after pollination, the pollinated flowers were removed from each plant, and the stylar and stigmatic tissue excised. The excised tissue was mounted on a microscope slide in aniline blue stain (0.05% water-soluble aniline blue in 0.1M K_3PO_4). The prepared mounts were observed at 100x magnification using a Leica Leitz DM RBE fluorescence microscope fitted with an HBO 50 Watt mercury vapour lamp, BP 340-380 excitation filter, RKP 400 dichromatic mirror and LP 430 suppression filter (Leica, Heerbrugg, Switzerland). All pollen grains present on the stigmatic surface were clearly visible, whilst the pollen tubes of germinated grains fluoresced brightly (Plates 1.1 and 1.2). Pollen grains located on the stylar tissue were scored for germination on the basis of the presence of a pollen tube. Typically, between 10 and 30 grains of pollen were scored on each stigma.

In-vitro Germination

The germination media was standard Brewbacker and Kwack mixture (Brewbacker and Kwack, 1963). Preliminary trials established that 20% sucrose and adjustment of the pH of the media to 8 gave optimal germination. In preliminary trials no difference in pollen germination rates was observed between preparations in hanging drops and on agar, so hanging drops were used in this work. Pollen samples were dispersed using a vortex mixer in 60µl droplets of germination media in 1.5ml Eppendorf® centrifuge tubes. After mixing, the pollen samples were suspended in the wells of inverted microplates (Greiner Bio One, Frickenhausen) and sealed in plastic containers lined with moistened filter paper. Pollen samples were incubated at 20^{o}C for 24 hours prior to examination under a light microscope. Four replicate tests were conducted for each pollen sample, with a minimum of 200 grains examined per test.

The FCR Test

The method of FCR testing was based on the original method of Heslop Harison and Heslop Harrison, 1970). Prior to conducting this test, the optimum sucrose content for the fluorescin diacetate (FDA) test solution was determined to be 20% for each species. The test solution consisted of 1ml of FDA dissolved in acetone (2mg/ml) added to 10mls of each sucrose solution. In each test four replicate sub-samples of

pollen were drawn at random from a pooled sample and dispersed into 60µl of FDA/sucrose solution in a 1.5ml Eppendorf® centrifuge tube using a vortex mixer. For each replicate, 30µl of pollen suspension was pipetted onto a microscope slide and covered with a cover slip. After 5 minutes standing under laboratory conditions the slides were examined at 100x magnification in random order using a Leica Leitz DM RBE fluorescence microscope fitted with a 50 Watt HBO mercury vapour lamp, BP 355-425 excitation filter, RKP 455 dichromatic mirror and LP 460 suppression filter (Leica, Heerbrugg, Switzerland). Six randomly selected fields of view (greater than 200 pollen grains in total) were photographed on each slide using a digital camera mounted to the microscope and connected to a computer running IM50 image management software (Leica, Heerbrugg, Switzerland). Pollen viability was initially scored by eye, but due to problems defining a threshold intensity of fluorescence for viable pollen, images were subsequently measured using Adobe Photoshop® V7.0 software (Adobe Systems Inc, USA), upgraded with the Fovea Pro® plug in. Batch automation of the image analysis system allowed rapid processing of large numbers of pollen samples (up to 150 samples per day).

The MTT and Bakers Solution Tests

The MTT staining procedure was adapted from Firmage and Dafni (2001). Staining solutions of MTT (1% MTT (w:v) in 20% (w:v) sucrose solution) and Baker's solution (10ml of 0.1M Phosphate buffer (pH 7.3 – 7.5) diluted 1:2 in distilled water to which is added 6mg NAD, 1ml of 95% ethanol and enough nitro-blue tetrazolium to give a slight yellow tint) were prepared. For each test, four replicate samples of pollen were brushed onto individual microscope slides, suspended in a drop of staining solution under a cover slip and incubated in darkness at 30°C for 30 minutes. Following incubation the slides were examined under a light microscope at 100x magnification. Pollen grains staining red were scored as viable whilst unstained pollen grains were scored as non-viable. Six fields of view were examined for each replicate.

The X-Gal Test

The x-gal medium was made up as follows: 1 mg of 5-bromo-4-chloro-3-indoyle-b-galactoside (x-gal) in 1ml of acetate buffer (50mmol pH 4.8). Four replicate samples of pollen were brushed onto individual microscope slides, suspended in a drop of staining solution under a cover slip and incubated in darkness at 30°C for 30 minutes. Following incubation the slides were examined under a light microscope at 100x magnification. Pollen grains staining blue were scored as viable whilst unstained pollen grains were scored as non-viable. Six fields of view were examined for each replicate.

Acetocarmine and Alexander's Stains

Four replicate samples of pollen were suspended in a drop of prepared acetocarmine stain solution (Southern Biological Services, Victoria, Australia) or Alexander's stain on individual microscope slides. After cover slips had been added, the slides were allowed to stand under laboratory conditions for 20 minutes. The slides were subsequently examined under a light microscope at 100x magnification. Pollen grains with cytoplasm stained pink were scored as viable. Six fields of view were examined for each replicate.

Composition of Alexander's stain: 20ml ethanol, 2mL 1% malachite green in ethanol; 50ml distilled water, 40ml glycerol, 10mL 1% acid fuschin (water soluble) to which 1g of phenol has been added, 5g phenol and 1ml of lactic acid. Allow stain to stand in dark bottle for 8 to 10 days before using.

Appendix 2

Source and History of Pollen Samples Used for Screening Testing Procedures for Carrot and Cauliflower.

Note: Aged pollen was exposed to ambient laboratory conditions after dehiscence for the time periods specified in the table.

Line	Field / glasshouse grown plants	History	No. of samples
Carrot			
ON44	Field	Tested freshly dehisced pollen	2
22	Field	Tested freshly dehisced pollen	1
22	Glasshouse	Tested freshly dehisced pollen	2
22	Glasshouse	Aged 1 hour	2
8024	Glasshouse	Tested freshly dehisced pollen	2
8024	Field	Tested freshly dehisced pollen	2
8024	Glasshouse	Aged 1 hour	2
8024	Glasshouse	Aged 2 hours	2
8024	Field	97% RH @ 30°C for 1 hour	1
8024	Field	97% RH @ 30°C for 4 hours	1
8024	Field	60°C for 1 hour	1
8024	Field	Desiccated over silica gel @ 18°C for 48 hours, stored 1 year @ -80°C, rehydrated for 30 minutes @ 97 %RH and 30°C	2
Cauliflower			
BL 529	Field	Tested freshly dehisced pollen	1
BL 529	Shadehouse	Tested freshly dehisced pollen	2
BL 529	Shadehouse	Aged for 1 hour	1
BL 529	Shadehouse	Aged for 2 hours	1
BL 529	Shadehouse	97% RH @ 25°C for 2 hours	1
Pollinator C	Field	Tested freshly dehisced pollen	1
Pollinator C	Shadehouse	Tested freshly dehisced pollen	1
Pollinator E	Field	Tested freshly dehisced pollen	1
Pollinator F	Field	Tested freshly dehisced pollen	1
Pollinator F	Shadehouse	Tested freshly dehisced pollen	1
Pollinator J	Shadehouse	Tested freshly dehisced pollen	1