A PREDICTION MODEL TO DETERMINE THE CROSS-POLLINATION ABILITY OF *Citrus* spp.

by

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ABSTRACT

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Due to a greater emphasis having been placed on seedlessness by citrus consumers, a need developed in the southern African citrus industry to avoid unwanted cross-pollination and hence seediness. There was a paucity of knowledge upon which to base sound decisions in order to ensure optimal orchard layout to achieve the objective of the production of seedless or "commercially seedless" fruit. *In vitro* pollen viability of all locally available citrus cultivars/selections was determined and pollen of cultivars with strong and weak pollination potential were used for *in vivo* pollen tube growth and seed content studies of 'Nules Clementine' mandarin and 'Delta Valencia' orange. Data from the *in vitro* assays were rated and categorized into 'pollen germination potential categories', while the relationship between these categories and seed content of 'Nules Clementine' was determined by regression analysis. The development of the 'pollen germination potential categories' provides an additional facet to the evaluation and development of new citrus cultivars, providing a means to determine the pollination potential at an early stage of that cultivar's development. Citrus cultivars with a pollen germination percentage exceeding 2 % are likely to set too many seeds for the export of 'Nules Clementine' mandarins according to South African export requirements. In addition to the two previously known pollen sterile cultivars, viz. 'Navel' orange and 'Satsuma' mandarin, 'Star Ruby' grapefruit and 'Delta Valencia' orange have been identified in this study as pollen sterile cultivars that can be used as buffers to avoid cross-pollination between two pollen fertile cultivars. Furthermore, 'Delta Valencia' is also ovule sterile. An initial model is presented whereby the seed content of cross-pollinated 'Nules Clementine' fruit can be predicted ($r^2 = 0.9192$) by determining the *in vitro* pollen viability of the cross-pollinating cultivar/selection and be calculated as follows: 2.619 x pollen germination category of compatible cultivars - 2.449.

DECLARATION

I, Graham Barry, declare
that this thesis is the result of my
own investigations except where the
work of others is acknowledged.

Signed: 

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Research of this nature and the writing of a thesis is not possible without the positive inputs, technical and otherwise, of numerous people. To all those who have stumbled on this path before me, I say thank you for your advice, encouragement and warnings of the many traps along the way. However, Linda's support during the tense writing period provided the necessary motivation to endure, even during self-destruct mode. Thanks Lind. My eldest brother, Kevin, also provided much needed encouragement over the years.

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My thanks also to the citrus producers and nurserymen where flowers were collected and trials conducted, and to the southern African citrus industry, in general, for providing such a stimulating and satisfying occupation, and the opportunity to conduct this research.

Finally, to those yet to walk this path, do not underestimate the wisdom of those who have been before you.

DEDICATION

This thesis is dedicated to
my parents, Jon and Jenifer Barry
of Hunters Road, Zimbabwe,
for their love and faith in me,
and without whom this study
would never have been possible;

and to the memory of
Mr. "Solly" Stevens, who inspired
the initial interest of botany in me.

CONTENTS

INTRODUCTION

The southern African citrus industry is ranked as the thirteenth largest in the world, and the fourth largest citrus exporter after the United States of America, Spain and Morocco (Davies & Albrigo, 1994). During 1995, Outspan International marketed 41.6 million 15 kg equivalent export cartons, compared with 39.6 million in 1994 (Anon., 1995a). In South Africa, horticulture is the next greatest earner of foreign exchange after mining, with an export value of R 4.879 billion in 1994, of which all deciduous fruit types contributed R 2 billion and citrus R 1.4 billion in 1994 (Thalwitzer, 1995). Thus, citrus is the single largest agricultural industry, or second largest after deciduous fruit, if all fruit types are pooled (Thalwitzer, 1995).

The importance of these industries to the South African and neighbouring countries' gross domestic product, foreign exchange income and socio-economic aspects cannot be overestimated. However, the requirements of the export market are demanding and competition with other citrus exporting countries in the southern hemisphere, as well as soft fruit producers in the northern hemisphere, mean that fruit quality characteristics must meet consumer preferences (Smith, pers. comm.[1]).

Since the overall objective in any commercial enterprise is to optimize profitability, it is imperative to maximize returns and contain costs. The latter can be achieved by using more cost effective means to produce the product. Returns, on the other hand, are dependant on price, yield, fruit size and packout percentage. In turn, packout percentage is a function of external and internal fruit quality factors.

One of the quality factors demanded by the citrus consumer is seedlessness (Saunt, 1990), which is becoming more important for the successful marketing of fresh citrus, especially for mandarin types (*Citrus reticulata* Blanco) in Western

[1]A.F.G. Smith, Capespan International, Farnham, England

Europe (Ollier, pers. comm.[2]). In southern Africa, seedlessness is a quality factor selected for in the development of citrus varieties, particularly of mandarin types (Barry, 1993) and forms a prominent part of the goals of the cultivar development programme (Burdette, 1994). Therefore, an active programme exists to develop and produce seedless citrus cultivars in order to achieve premiums on the export market by creating competitive advantage or maintaining market share, thereby optimizing profitability. "Commercial seedlessness" (an average of less than five seeds per fruit) is a characteristic of all major export cultivars, e.g. 'Valencia' orange, 'Marsh' grapefruit and 'Eureka' lemon, while 'Navel orange selections are usually completely seedless.

In order to achieve the objective of seedlessness, a knowledge of pollen fertility and pollination ability amongst the numerous cultivars in the citrus industry is essential to ensure proper orchard layout. In the planning of new orchards, cross-pollination is a critical factor affecting seediness in self-incompatible citrus cultivars. This has become important with the recent development of the mandarin industry in South Africa, many cultivars of which are self-incompatible but capable of significant seediness when cross-pollinated. However, there is a paucity of knowledge upon which to base sound decisions, although there is certain information on whether a particular cultivar is self-compatible, its pollination efficiency or ability, and effect on fertilization and seed development. Therefore, understanding how to effectively reduce seed content is of immense importance to the mandarin or soft citrus industry in southern Africa. With the many new mandarin hybrids becoming available to the citrus producer, decisions on which cultivar can be safely planted alongside another and the most appropriate layout for an orchard to avoid cross-pollination, and hence enhanced seediness, is integral to the successful production of marketable mandarins. In addition, if the potential cross-pollination ability and seedlessness of a new cultivar could be predicted during its early stages of evaluation and development, much time and expense could be saved.

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[2]K. Ollier, Capespan International, Farnham, England

In certain other crops, cross-pollination is desirable, and often essential to produce commercially acceptable yields, e.g. apples and stone fruits (Free, 1970; McGregor, 1976; Crane & Walker, 1984), kiwifruit (Hopping, 1988) and avocados (Sedgley, 1977). Due to the parthenocarpic tendency of certain citrus cultivars, pollination and fertilization are not always necessary to set acceptable crops in citrus. In South Africa, attempts are made to avoid cross-pollination in order to produce seedless fruit (Burdette & du Plooy, 1991). With strongly parthenocarpic cultivars fruit set is usually adequate, while certain fruit setting manipulations are required for weakly parthenocarpic cultivars (Jackson, 1986). However, in other citrus producing regions, e.g. Florida (Futch & Jackson, 1993b) and Australia (Vithanage, 1991), pollinators are planted to ensure fruit set in weakly parthenocarpic cultivars. In Florida, where seedlessness appears to be less important, producers are also advised to place beehives in their orchards (Futch & Jackson, 1993a; Anon., 1994; Adams, 1995a; 1995b), resulting in improved fruit set and high seed numbers per fruit (Jackson, 1986).

Previous citrus pollen research addressed the issues of (i) how pollination could improve fruit set and fruit size (Krezdorn, 1967; Hearn *et al.*, 1969; de Lange & Vincent, 1972a), and (ii) the determination of pollination requirements of a specific cultivar (Ton & Krezdorn, 1966; de Lange & Vincent, 1972b; 1979; de Lange *et al.*, 1974; de Lange *et al.*, 1979b). In the present study, the focus has been on how to prevent the production of fruit with high seed numbers, especially in mandarins and their hybrids. The aim of this research initially involved the *in vitro* testing of numerous pollen parents in order to determine their cross-pollination potential (Chapter 2). The scope of the research was extended to the development of a prediction model on the cross-pollination ability of new citrus cultivars (Chapter 4) and to identify suitable pollen sterile cultivars which can be used as buffers between two potentially strong, compatible pollen sources to prevent the production of fruit with high seed numbers (Chapter 2).

At present, the southern African citrus industry does not use many prediction models, although several have been developed over the past few years, viz.

maturity indexing (Bower & Turner, 1994), creasing prediction (Bower & Lovatt, 1994) and the prediction of *Alternaria citri* as a post-harvest decay organism (Schutte *et al.*, 1994). There is scope for the use of prediction models in terms of relating climatic parameters to fruit quality, fruit growth rate to final fruit size and crop estimates to yield. Such aspects help to optimize profitability by assisting with packing, logistics, market planning and marketing of the crop.

During the development of this prediction model, various results of practical importance were found. Essentially the model is based on determining *in vitro* pollen germination (Chapter 2), determining the effect of various pollen sources on *in vivo* pollen tube growth and seed content in 'Nules Clementine' mandarin (Chapter 3), and relating that to *in vivo* pollen tube growth and seed content (Chapter 4). The different cultivars were then ranked according to pollination potential, resulting in an initial prediction model on pollination ability (Chapter 4).

CHAPTER 1

CITRUS POLLEN BIOLOGY: A LITERATURE REVIEW

The production of seedless citrus fruit in commercial situations is often limited by the prevalence of cross-pollinating cultivars in the near vicinity (pers. obs.). Therefore, a knowledge of the pollen viability of a cultivar, its cross-pollination potential and sexual compatibility with other cultivars is required. This information will help to ensure that orchard layout can be optimized to avoid unwanted cross-pollination and seediness.

In this review, aspects relating to the current knowledge of *Citrus* pollen biology are discussed to gain a better knowledge required to optimize orchard layout. This review then forms the basis for the experiments that were carried out to further develop the known information on *Citrus* pollen biology.

1.1 Pollen and anther development

In *Citrus,* pollen development follows the usual course for angiosperm pollen as described by Frost & Soost (1968) and Stanley & Linskens (1974). *Citrus* pollen is usually binucleate (Brewbaker, unpublished, cited by Frost &Soost, 1968), though in grapefruit it has been reported to be trinucleate (Banerji, 1954). The majority of citrus cultivars are diploid, with a few exceptions (Frost & Soost, 1968), e.g. 'Oroblanco' and 'Melogold' grapefruit-pummelo hybrids which are triploids (Soost & Cameron, 1980). The monoploid number of chromosomes is nine (Frost & Soost, 1968).

Fertile anthers are bright yellow when mature, owing to their pollen load. In cultivars with defective pollen development, the anther's colour may be considerably lighter, whereas anthers containing no pollen are pale cream or white e.g. 'Navel' oranges, and usually do not dehisce (Schneider, 1968).

Anthesis is the stage of floral development when the flower opens and the anthers dehisce (Fig. 1.1). The opening of citrus flowers begins with the partial separation at the tips of petals. At about the same time anthers begin to dehisce (Zacharia, 1951). Petal opening has been shown to be a growth reaction controlled by the endogenous auxin, indole-3-acetic acid (IAA), and antagonized by an auxin antagonist, *p*-chlorophenoxy isobutyric acid (PCIB) (Goldshmidt, 1968). Anther dehiscence, and hence pollen release, is as a result of the partial and controlled dehydration of the anther walls. Each half of the anther dehisces by a longitudinal split between the lobes, at or shortly after the time of the separation of the petals (anthesis); the epidermis dries out and rolls back the anther wall, exposing the enclosed sticky, yellow pollen (Frost & Soost, 1968).

At anthesis in lemons, the stigma extends above the anthers, the stamens curve outwards exposing the receptacle, where nectar is secreted. Sepals, petals and stamens absciss a few days after anthesis, followed by the stigma a few days later (Moncur, 1988).

Fig. 1.1 Lemon flower at anthesis (Frost & Soost, 1968)

1.1.1 Environmental effects on pollen development and growth

No evidence exists concerning the effects of climate on *Citrus* pollen development *per se*. However, temperature affects pollen tube growth rate; pollen germination and tube growth are enhanced by high temperatures (25 to 30 °C) and reduced or totally inhibited at low temperatures (< 20 °C) (Frost & Soost, 1968).

Citrus pollen is not reported in the literature as being stress sensitive. There is less of a need for abundant pollen production in *Citrus* than other crops due to parthenocarpic fruit set, although pollination is often required as the stimulus to induce parthenocarpy (Frost & Soost, 1968). Furthermore, these effects are less important in *Citrus,* which usually flowers during a specific season and produces abundant flowers, than in ever-bearing fruit species (Monselise, 1985). However, the effects of temperature and relative humidity on pollen development and viability are important aspects that may influence pollen research results from season to season and region to region.

Reports concerning environmental effects on pollen development and growth in other crops showed that these effects cannot be ignored. For example, in walnut (*Juglans* spp.) temperature ranges and optima for pollen germination and pollen tube growth varied widely among species, and environmental conditions influenced competitive fitness of pollen. The optimum temperature for pollen germination increased with temperature prevailing during the period of pollen development (Polito *et al.*, 1991). Luza *et al.*, (1987) found that a variable response to temperature occurs amongst cultivars of *J. regia,* and that optimum temperatures for *in vitro* pollen germination correlated with the relative staminate bloom date for a given cultivar. A similar relationship was noted for almond (*Prunus dulicis*) and peach (*P. persica*) (Weinbaum *et al.*, 1984). However, in coffee (*Coffea arabica* L.), Morkel (1977) found that high temperatures at the time of flowering ($\pm$ 35 °C in October; southern hemisphere) caused a high percentage of pollen sterility and abortion.

In *Curcurbita pepo* L. and maize (*Zea mays* L.) loss of pollen viability occurred at less than 25% and 15% pollen moisture content, respectively (Digonnet-Kerhoas *et al.*, 1989). These researchers believe that biophysical methods, using ^{1}H-nuclear magnetic resonance, allows the determination of the quantity of 'vital water' in stress sensitive pollen types. Pollen naturally withstands a dehydration process in the anther before dehiscence. After release the fresh pollen grain may sustain further dehydration until it reaches a water content limit value characteristic of the

species. Once this limit is exceeded, pollen death and structural alterations occur. The threshold for *Citrus* is not known.

Low temperatures of 17°C day/12°C night were unsuitable for fruit set in avocado (*Persea americana* Mill.) due to, *inter alia*, retardation of pollen tube growth and resultant lack of embryo development (Sedgley, 1977). At high temperatures, 33°C day/28°C night, abnormal pollen tube growth occurred, while 25°C day/ 20°C night was satisfactory for pollen tube growth, embryo development and fruit set (Sedgley, 1977). However, no reference to conditions pertaining to pollen development prior to anthesis and their effects on pollen viability was given.

Allan (1963) attributed cold conditions as the main reason for poor pollen formation and development in papaya (*Carica papaya* L.). In addition, a rapid decline in pollen viability was demonstrated when exposed to high temperatures.

In muskmelon (*Cucumis melo* L.), three of four fungicides tested reduced the percentage of pollen germination and pollen tube kinetics (Abbott *et al.*, 1991). Similar results were achieved with mango pollen (Shu *et al.*, 1988).

Although environmental effects on *Citrus* pollen development and growth are apparently not well known, when reporting on pollen research, the conditions under which the work was conducted should be fully described, and extreme conditions avoided in preference to moderate conditions.

1.1.2 Pollen morphology

Citrus pollen grains are generally round (hydrated form) to prolate (Monselise, 1985). In early work by Reznick (1958) strong divergence from this shape was considered to indicate non-viable pollen. According to the definitions of Erdtman (1969), Faegri & Iversen (1975) and Moore & Webb (1978), ultrastructural observations indicate that 'Clementine' mandarin (*C. reticulata* Blanco) has tetracolporate pollen grains (at times pentacolporate), prolate and isopolaric with

foveolate exine (Crescimanno *et al.*, 1988a). Pollen size and shape varied between selections, with some ('Oroval' and 'Nules') being slightly longer than the rounder 'Monreal', 'Comune' and 'SRA 63' selections. Average pollen length of 30.1 μm and width of 18.7 μm were measured.

Lemon (*C. limon* L.) has pentacolporate pollen grains (sometimes tetracolporate), prolate with a reticulate exine (Crescimanno *et al.*, 1988b). Size and shape of the pollen grains of various cultivars were quite uniform; average length 30.5 μm and width 22.2 μm. Nakamura (1943) listed pollen diameters for seven lemon cultivars. All but 'Villafranca' lemon were within the range 34 to 36 μm, similar to that reported by Pereau-Leroy (1950) for lemons.

Similar in-depth studies of other *Citrus* spp. were not found. Generally, *Citrus* pollen is of the sticky, adherent type characteristic of entomophilous plants (insect pollinated) (Schneider, 1968). According to Erdtman's (1969) size categories, *Citrus* pollen was classified as medium. *Citrus* pollen assumes a round shape due to re-hydration when placed on a suitable germination medium (pers. obs.).

1.2 Pollination

1.2.1 Pollen transfer

Self-pollination involves the transfer of pollen from a flower to its own stigma, or the transfer of pollen from other flowers on the same tree. These two types of self-pollination may occur when the style elongates, extending the stigma to a point where it touches an open anther or when pollen is transferred by insects, respectively. Since commercial citrus orchards of a single cultivar comprise asexually propagated, genetically similar trees, pollination between such trees is self-pollination. Cross-pollination involves the transfer of pollen from one cultivar to another, usually by insect vectors (Frost & Soost, 1968).

As mentioned previously citrus pollen is of the sticky, adherent type characteristic

of entomophilous plants, and wind is therefore usually a minor factor in pollination. Four characteristics of the flowers make them attractive to insects: the conspicuous corolla (large, white petals), the strong fragrance, the pollen and the abundant nectar (Webber, 1930).

Insect vectors are the principal agents of pollen transfer from one flower to another. African honey bees (*Apis mellifera adansonii* and *Apis mellifera capensis*) play the leading role in pollen transfer in *Citrus*, although an array of mainly bees and wasps (Order Hymenoptera) and flies (Order Diptera) also forage in citrus flowers. Visitations by flying insects to citrus flowers result in pollen being accumulated on their body parts and transferred to other flowers whilst foraging (Sanford, 1986). Honey bees and other insects work the flowers for both nectar and pollen. Thrips and mites are often found in flowers and may occasionally inadvertently carry pollen (Uphof, 1934). Because of their fragrance and the abundance of nectar, *Citrus* flowers attract bees more than any other tree in bloom at the citrus blossom season, often to the detriment of other fruit crops, e.g. avocado (Monselise, 1985), and nectarines and apples (pers. obs.). *Citrus* flowers secrete nectar for two to five days after anthesis and can produce 25 to 80 mg of nectar per flower during this period (Monselise, 1985).

1.2.2 Germination and the stigmatic surface

When pollen grains are transferred by the vector from the anther to the stigma, they dehydrate after contact with the air, and the exine contracts (Rudall, 1987). Following pollination, the pollen is caught in the sticky stigmatic fluid on the stigma (Sanford, 1986). The stigma provides a physically and chemically suitable medium for pollen germination. On its surface substances produced within the stigmatic exudate, react with substances from the pollen grains to convey the stimulation to germinate (Martin & Brewbaker, 1970). On the stigmatic surface rehydration occurs, the exine expands and the proteins held in both the intine and exine are released (Rudall, 1987). These events signal the initiation of pollen tube growth and germination (Knox, 1979).

Stigmas are broadly divided into two types: 'dry', with little or no surface secretions, or 'wet', with surface secretions which assist in pollen germination (Heslop-Harrison & Shivanna, 1977; Cresti *et al.*, 1982). *Citrus* has a wet stigma (Ciampolini *et al.*, 1981). At the receptive stage the stigmatic exudate completely covers the numerous papillae (Cresti *et al.*, 1982). The stigma of *Citrus* is terminal, lobed and the internal part is formed by papillae (Cresti *et al.*, 1982). According to Dumas *et al.* (1974), the *Citrus* stigma can be divided in two zones: a glandular superficial or stigmatic zone formed by the papillae, and a non-glandular deeper region or stigmatoid tissue formed by parenchymatic cells (Fig. 1.2) (Ciampolini *et al.*, 1981):

- stigmatic or glandular zone: the papillae vary in size and structure (Fig. 1.2). Two regions have been distinguished; one is formed by the longer multicellular, papillae tip cells and the second consists of the short unicellular, basal papillae cells. The latter region consists of many layers of secretory cells;
- stigmatoid tissue: these are in continuity with the stylar canals. At the mature stage large intercellular spaces are present through which pollen tubes grow before entering the stylar canals (Cresti *et al.*, 1982).

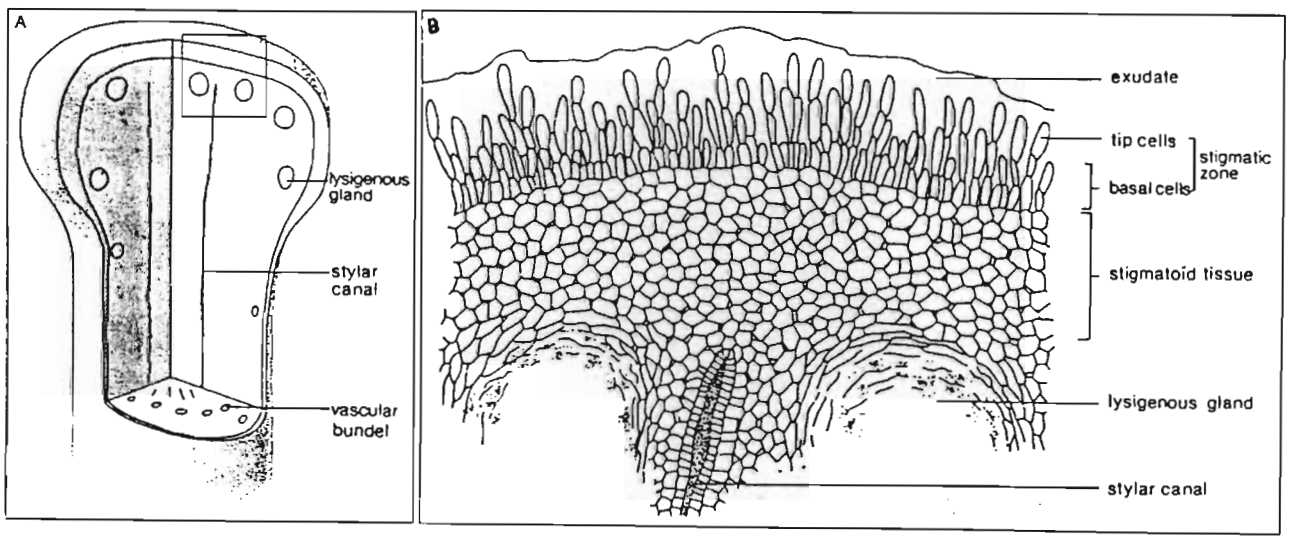

Fig. 1.2 Schematic representations of citrus stigma. A, stigma and upper portion of style; B, cellular organization of the stigma (Cresti *et al.*, 1982)

After the stigmatic papillae have reached their ultimate size, and following anthesis, exudate formation starts, completely covering the papillae and pollen grains after pollination. The stigmatic exudate not only serves to catch and hold pollen grains, but also provides suitable germination conditions (Frost & Soost, 1968).

Stigmas of *Citrus* spp. are receptive for one to three days prior to anthesis and retain receptivity for up to six to eight days after anthesis (Climenko, 1936; Singh & Dhuria, 1960; Randhawa *et al.*, 1961). In lemon, Jaganath (1981) showed that the stigma became receptive after anthesis and remained so for five days. The pollen grain absorbs moisture from the stigmatic exudate, and germinates 12 to 36 hours later (Frost & Soost, 1968). From the day of anthesis, the countdown begins which ends for each flower either with its abscission at the pedicel or peduncle abscission zone, or with its initial set (Zacharia, 1951).

Histochemical analysis has shown that the exudate of *Citrus* is composed of lipids, polysaccharides and proteins (Cresti *et al.*, 1982). The proteins possibly include certain enzymes. The lipids occur as free fatty acids, chiefly serving as protection from desiccation. These compounds, together with amino acids, sugars and water, provide a suitable substrate for pollen germination and pollen tube growth (Martin & Brewbaker, 1970).

Following germination, the pollen tube grows out and enters the stylar canal before growing down towards the ovules. Post-pollination stigma degeneration then occurs, involving stigmatic desiccation manifested as darkening of the stigmatic surface. About seven days from anthesis a brown abscission zone develops at the base of the style. After style abscission, the persisting ovary develops to become a mature fruit, unless shed later during fruit development (Zacharia, 1951; Monselise, 1985).

1.3 Pollen tube growth

1.3.1 Function of pollen tube and initial response

Since the initial discovery by Amici (1824), the pollen tube has been a subject of controversy (Johri, 1992). The main function of the pollen tube is to carry the male gametes for fertilization.

Citrus pollen tubes do not form bullae and are unbranched. The remnants of the pollen tube persist for a long time, even in the micropyle of mature seed, and are considered to be mere dead structures without any haustorial role (Johri, 1992).

Following germination, a second recognition event occurs; the activation of enzyme systems which enable the pollen to digest its way through the wall layers of the stigma and enter the stigma cell (Knox, 1979). Water uptake, and the activation or synthesis of enzymes, were presumed to be the basic initiating factors of the germination process (Rosen, 1970). In addition, oxygen is required which is accompanied by carbon dioxide production and the disappearance of starch during pollen tube growth (van Tiegham, 1869). The pollen grain usually contains food reserves to support germination. When the pollen tube grows through a hollow style, as in *Citrus*, it derives nourishment from the glandular epidermis of the stylar canal and the adjacent tissue becomes depleted of metabolites (Johri, 1992).

1.3.2 Growth in the style

Growth of pollen tubes is exclusively restricted to the tips, by elongation, when grown *in vitro* (Rosen, 1970). The direction of pollen tube growth is thought to be chemotropically controlled. This is particularly critical immediately after germination on the stigma surface and at the time of entry into the embryo sac (Welk *et al.*, 1965). Pollen tube growth appears to furnish or stimulate the production of growth regulators (Rosen, 1970).

In *Citrus*, the rate of pollen tube growth has been studied extensively in 'Orlando' tangelo (Ton & Krezdorn, 1966; Anderson, 1983). When selfed, 'Orlando' tangelo pollen germinated satisfactorily and the pollen tube grew into the base of the style in seven days. Pollen of 'Duncan' grapefruit, 'Parson Brown' sweet orange, and

'Minneola' and 'Orlando' tangelos germinated and penetrated the stigma of self-incompatible 'Orlando' tangelo styles within one day. All pollen tubes penetrated well into the style (approximately one-third of the length) within three days. Within six days, pollen tubes of the compatible cultivars 'Parson Brown' sweet orange and 'Duncan' grapefruit were present in the ovules of 'Orlando' tangelo, while pollen tubes from 'Minneola' and 'Orlando' tangelo pollen were only one-third of the way down the style. By seven days, the pollen tubes of 'Minneola' and 'Orlando' tangelos had grown to the base of the styles and style abscission began on the eighth day (Ton & Krezdorn, 1966; Anderson, 1983; Jackson, 1986).

Early research indicated that pollen tube growth was intercellular and not in the canals (Banerji, 1954). However, according to Webber (cited by Frost & Soost, 1968), *Citrus* pollen tubes grow in the stylar canals. De Lange (1973) showed that the growth of *Citrus* pollen tubes in the stigmas was both intercellular and through the stylar canals. In the remainder of the style, the growth of the tubes was confined mainly to the stylar canals.

Stylar canals start from just beneath the stigma and stretch to the ovary loculi. The pollen tubes pass through the stigmatic papillae, grow intercellularly between parenchyma cells and then between the canal cells (the cells lining the stylar canal), and eventually reach the stylar cavity through which they grow as far as the ovary. At maturity the canals are filled with the material secreted by the canal cells (Ciampolini *et al.*, 1981).

In plants with a hollow style, e.g. *C. limon* (Hanf, 1935), pollen tube growth is through the canal filled with mucilaginous material rich in polysaccharides (Rosen, 1970; Kroh, 1973). The mucilaginous material regulates pollen tube growth, acting as growth promoters, chemotropic agents, mediators of the incompatibility reaction, or perhaps some combination of these (Rosen, 1970). In addition to polysaccharides, the material in the stylar canal of lemon also consists of proteins and lipids; presumably to provide nutrients for the growing pollen tubes (Ciampolini *et al.*, 1981).

In certain plant species, e.g. *Petunia* and *Ornithogalum*, Ca plays an essential role in pollen germination and pollen tube growth (Brewbaker & Kwack, 1963). Calcium gradients are thought to encourage pollen tubes chemotropically toward the embryo sac (Mascarenhas & Machlis, 1962; 1964). In *Petunia*, Robbertse *et al.* (1990) showed that the pollen tubes consistently followed the shortest route to a B supply, clearly demonstrating directional growth, and possibly chemotropism. *In vivo* a B concentration gradient exists from stigma to ovary. Robbertse *et al.* (1990) suggested that there must be some unknown interaction between B and Ca in the pistil. The ovary probably acts as a B sink during flower maturation, creating a B gradient in the pistil. After pollination the growing pollen tubes form a new sink for B, and B moves from the ovary in the direction of the stigma causing directional growth of the pollen tubes.

Brewbaker & Kwack (1963) showed that *Citrus* pollen responded positively to Ca ions. Sahar & Spiegel-Roy (1980) included both B and Ca in their pollen germination medium, achieving 8 % germination for 'Valencia' and 75 % for 'Clementine'. However, de Lange (pers. comm.[1]) showed that B or Ca were not necessary for *in vitro* viability assays of *Citrus* pollen, and that a relatively simple medium was superior to that of Brewbaker & Kwack (1963). Recent *in vitro* assays of *Citrus* pollen appear to exclude Ca and B in the medium (Crescimanno *et al.*, 1988b; de Lange, 1989; Butt *et al.*, 1993; Gmitter, pers. comm.[2]).

1.3.3 Callose

Callose is a normal cell wall constituent in pollen tubes where its functional significance is not known for certain, although it is involved with intercellular movement. The greatest presence of callose in pollen tubes occurs as callose plugs or in the walls, with little or no callose in the tip (Currier, 1957). Therefore, callose lines and plugs pollen grains and tubes, but its amount and distribution are

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[1]J.H. de Lange, National Botanic Gardens, Kirstenbosch

[2]F. Gmitter, Citrus Research and Education Center, Lake Alfred, Florida, USA

highly variable. It sometimes nearly fills the tube making it visible for its entire length (when observed by fluorescence microscopy using the method described by Martin (1959)). In other cases, the callose is confined and localized by closely spaced plugs (Martin, 1959). In *Citrus*, pollen tubes are straight with thin-walled tips and regularly spaced callose plugs behind the growing tips (Kahn & DeMason, 1986).

1.3.4 Incomplete pollen tube growth

The interaction between the pollen grain or tube and the stigma, and between the pollen tube and the transmitting tissue in the style is determined by signal recognition (acceptance/rejection) which controls compatibility/incompatibility reactions (Shivanni & Johri, 1985). Frequently, no pollen germination takes place on the stigmatic surface or pollen tube growth may be arrested in the stigma, style or ovary. The site of inhibition is determined by the species concerned, prevailing temperature and other conditions (Lewis, 1949).

Incompatibility in *Citrus* is sometimes due to slow pollen tube growth, apparently caused by inhibitors in the style, possibly due to chemical changes within the ovary (Ton & Krezdorn, 1966). This results in abscission of the style before the pollen tube can enter the ovary (Krezdorn, 1986). Therefore, the incompatibility barrier may simply be one of distance and time. Whatever the precise nature of the barrier, pollen tube growth of sexually incompatible cultivars is retarded in the style (Ton & Krezdorn, 1966), indicating a gametophytic incompatibility system (Brewbaker, 1957), details of which are described in part 1.4.3 of this Chapter.

Pollen tube growth appears to stimulate the production of plant growth regulators that reduce ovary or fruit drop. Although incomplete pollen tube growth may occur due to incompatibility, the stimulation may be sufficient in certain cultivars to induce parthenocarpic fruit set (the production of fruit without the stimulus of sexual fertilization), i.e. stimulative parthenocarpy (Krezdorn, 1986).

1.3.5 Fertilization

The final stages of pollen tube growth are penetration of the micropyle, entry of the embryo sac, disorganization of one or both synergids and the discharge of the two sperm nuclei, followed by double fertilization (Bacchi, 1943; Banerji, 1954; Knox, 1979; Jackson, 1986). After discharge of the male gametes, the pollen tube collapses (Johri, 1992). Further details are described in part 1.5.1 of this Chapter.

1.4 Sterility and incompatibility

Fertility indicates the capacity for reproduction by means of fertilization of male and female gametes. Sexual sterility results in the complete inability to reproduce by means of seed. In plants having nucellar embryony, however, seedlessness requires not only sexual sterility, but also "nucellar sterility" (Frost & Soost, 1968). Therefore, seed production in *Citrus* may be affected by factors, either genetic or environmental, which affect gamete development, fertilization, nucellar embryo initiation, or embryo survival. A rough measure of sterility can be determined as the percentage of seeds developed per fruit compared with the total number of ovules per flower. There is a potential for 10 ovules per locule. In *Citrus*, varying degrees of sterility occur, involving pollen, the ovule, or both (Frost & Soost, 1968).

Sterility prior to fertilization is characterized by non-functional gametes, as described above. However, with gametic incompatibility the pollen and ovules are functional and failure to produce fruit with seeds results from a physiological hindrance to fertilization. This is usually manifested in citrus by slow growth of the pollen tube down the style due to sexual incompatibility (Jackson, 1986).

Sexual sterility may be divided into two main groups: gametic sterility and embryo (zygotic) abortion. Gametic sterility may be further divided into relative and absolute gametic sterility, involving the male component (pollen) or female component (egg cell) (Frost & Soost, 1968).

1.4.1 Gametic sterility

i) Relative gametic sterility: Self- or cross-sterility is associated with self- or cross-incompatibility, respectively. Therefore, self- or cross-sterility is the inability to form an embryo by self- or cross-pollination, although both the pollen and egg cells, respectively, are functional in suitable cross-pollinations (Frost & Soost, 1968). The pollination that occurs will not produce sexual embryos although the gametes are fully functional, e.g. 'Clementine' mandarin (self-sterile) and 'Minneola' and 'Orlando' tangelos (inter-cross-sterile) (Jackson, 1986).

ii) Absolute gametic sterility: Pollen sterility is the inability to produce functional pollen, e.g. 'Navel' oranges. Embryo sac sterility consists of the inability to produce embryo sacs with egg cells capable of development into embryos, e.g. 'Tahiti' lime (Frost & Soost, 1968). The origin of this sterility may be genetic, where the cultivar is sterile even under favourable conditions, or non-genetic, due to environmental conditions, such as low temperatures causing reduced pollen fertility in limes (*C. aurantifolia*) (Iwamasa & Iwasaki, 1963) or rootstock influence (Brieger & Gurgel, 1941).

In certain *Citrus* types, some of the flowers have abortive pistils resulting in functionally staminate flowers, e.g. lemon, lime and citron, while in others abortion of the pistil is much less common, e.g. sweet oranges, pummelo, grapefruit and mandarin (Frost & Soost, 1968).

The stamens, unlike the pistil, seldom fail in their development and normal anthers are usually produced. However, imperfect development of pollen is common. The ability of pollen to fertilize is better indicated in suitable culture media than by morphological appearance. However, germination *in vitro* is not an absolute measure of the ability to accomplish fertilization (Frost & Soost, 1968).

In 'Washington Navel' orange, pollen sterility is caused by certain genes preventing the development of pollen grains, since its pollen mother cells degenerate before mitosis (Frost & Soost, 1968). Many flowering plants are sterile due to chromosome aberrations and variations in chromosome number. Chromosome aberrations occur in microspore (pollen) mother cells of several citrus cultivars (Jackson, 1986) and induce gamete sterility (Iwamasa, 1969) Almost complete sterility in citrus triploids is due to irregular chromosome reduction, leading to the presence of extra chromosomes or lack of a complete set, causing gametic sterility or embryo abortion (Frost & Soost, 1968).

Male sterility (defective pollen development)

In certain cultivars of *Citrus* no pollen is shed and the mature anthers at flower opening are cream coloured, strikingly unlike the bright yellow produced by the pollen in fertile anthers. 'Washington Navel' orange is one such example. Remnants of pollen mother cells may sometimes be found in the mature anther but no mature pollen is produced (Nakamura, 1934; 1943; Moreira & Gurgel, 1941). 'Washington Navel' orange therefore is completely male sterile, and is incapable of sexual reproduction if used as the pollen parent. Similar behaviour occurs in 'Tahiti' lime (Uphof, 1931; Davenport, 1986). 'Satsuma' mandarins are often completely male sterile, producing very little or no well developed pollen. Most of the pollen degenerates at various stages of development and at best only a few pollen grains appear normal at anthesis (Osawa, 1912). 'Shamouti' sweet orange had 38 % of the pollen being pale and shrivelled (Zacharia, 1951); 40 to 60 % of 'Valencia' orange pollen was non-functional, while 'Marsh' grapefruit only had 5 to 15 % well developed pollen (Longley, 1925; Moreira & Gurgel, 1941). Nakamura (1943) found large proportions of defective pollen in several lemon cultivars. All of these cultivars or species mentioned above have low seed numbers (Frost & Soost, 1968), obviously associated with a lack of functional pollen.

Female sterility (defective embryo sac development)

In 'Navel' oranges and 'Satsuma' mandarins the embryo sac usually degenerates. A few embryo sacs develop fully and contain egg cells capable of fertilization. Degeneration is presumed to be due to the same general genetic cause as seen with pollen mother cells in the same cultivars. Both anther and ovule sterility is more extreme in 'Navel' oranges than in 'Satsuma' mandarins (Frost & Soost, 1968). However, when 'Navel' orange flowers are pollinated by highly fertile pollen, such as that of pummelo, fruit with high seed numbers are produced (de Lange *et al.*, 1979a). In 'Shamouti' sweet orange, the embryo sac is absent or abortive in a large number of ovules (Zacharia, 1951). Considerable female sterility is also indicated in 'Valencia' orange and 'Marsh' grapefruit by the low seed set obtained from cross-pollination (Cameron *et al.*, 1960; Frost & Soost, 1968).

1.4.2 Zygotic sterility (embryo abortion)

Embryo abortion is common in *Citrus*. Where functional gametes are produced, fertilization may occur, and yet the fertilized egg may be unable to develop into an embryo capable of germination. If no embryos are able to develop, complete zygotic sterility results. Like gametic sterility, zygotic sterility may be genetic in origin or due to environmental effects (Frost & Soost, 1968).

The occurrence of empty and nearly empty seed coats indicates embryo abortion. In seeds which produce only nucellar seedlings, there is failure or suppression of zygotic embryos. Gametic sterility usually necessitates nucellar sterility, while abortion of the zygotic embryo does not necessarily prevent the development of nucellar embryos (Frost & Soost, 1968).

Several cultivars which seem to be self-sterile occasionally produce fruit having imperfect seeds with poorly developed embryos, after self-pollination or apparently incompatible cross-pollination (Nagai & Tanikawa, 1928). The embryos of these

seeds may be nucellar embryos which result from pollen tube stimulus, without fertilization and, therefore, without normal endosperm development (Frost & Soost, 1968).

1.4.3 Incompatibility

Relative gametic sterility, described above, is often associated with incompatibility where self- and cross-sterility are equivalent to self- and cross-incompatibility, respectively.

Self-incompatibility was defined by Brewbaker (1957) as the inability of a plant, producing functional male and female gametes, to set seed when self-pollinated. According to the oppositional S-allele hypothesis proposed by East & Mangelsdorf (1925), pollen grains that possess S-alleles identical to one of those in the pistil will not be functional on that particular pistil. This condition holds only for the gametophytic homomorphic type of incompatibility (Townsend, 1971).

Three types of incompatibility are recognized (Lewis, 1949; Townsend, 1971).

i) gametophytic homomorphic,

ii) sporophytic homomorphic, and

iii) heteromorphic, associated with different flower forms on plants of the same species.

Angiosperm pollen grains are shed either at a binucleate or trinucleate stage. A variety of biological attributes of pollen relate to this cytological difference, and are briefly discussed (Lewis, 1949):

i) binucleate grains commonly germinate readily in culture, whereas trinucleate grains are difficult, at best, to grow *in vitro*;

ii) binucleate grains store readily, while trinucleate grains rarely retain viability more than a week; and

iii) with respect to self-incompatibility, binucleate grains commonly characterize plants in which S-allele action leads to inhibition of pollen tube

growth in the style, whereas trinucleate grains characterize species in which inhibition commonly arrests pollen germination itself. With trinucleate pollen grains a germination inhibitor must be overcome or removed by the stigma and self-incompatibility occurs at the stigmatic surface (Brewbaker, 1957; Brewbaker & Majumder, 1961).

Citrus pollen is binucleate (Brewbaker, 1957), insect pollinated and pollen tube growth occurs even in self-incompatible cultivars (Soost, 1965). Brewbaker (1957) associated these characteristics with gametophytically determined incompatibilities. In 1965, Soost hypothesized that incompatibility in *Citrus* was gametophytically determined by S-alleles.

In an independent study (of Soost), Ton & Krezdorn (1966) showed that incompatibility in 'Clementine' mandarin and 'Minneola' and 'Orlando' tangelos was not due to the pollen injuring the stigmatic surface, or to inhibition of pollen germination and growth as a result of a substance originating in the stigma. They proposed two possible explanations:

i) the ovary was a major area of inhibition, and

ii) an abscission layer at the base of the style prior to the penetration of pollen tubes beyond that point may have developed and presented a physical barrier.

Therefore, the pollen tube was retarded in the style, thus indicating a gametophytic self-incompatibility system in *Citrus* (Ton & Krezdorn, 1966).

The presence of a self-incompatibility system in several species or interspecific hybrids suggests that incompatibility is determined by a basic gene system of compatibility alleles as in many other plant genera (Soost, 1969). In the mid-1920's the genetic control of homomorphic incompatibility was discovered independently by East & Mangelsdorf (1925) working with *Nicotiana sanderae*, and by Filzer (1926) with *Veronica syriaca*. Homomorphic incompatibility in many species is controlled by a single gene with a large number of alleles, S_1, S_2, S_3,

....., S_n. These alleles act in such a way that pollen grains are unable to grow and effect fertilization in styles which have the same allele as the pollen (Fig.1.3). These alleles operate both in the pollen and the styles (Lewis, 1949). Soost (1969) showed that *Citrus* fits the postulation of a gametophytic S-allele system with a dominant self-compatibility allele.

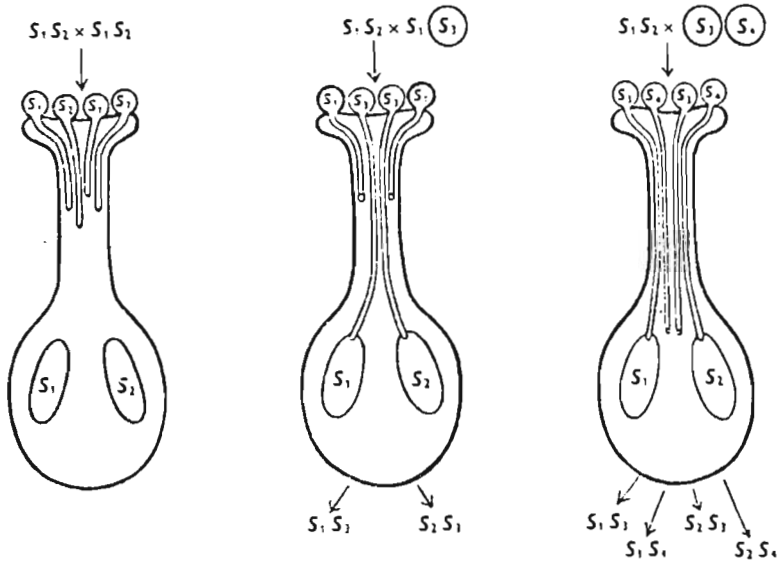

Fig 1.3 Pollen tube growth and types of progeny obtained from the three basic types of pollination illustrating the S-allele incompatibility system (Lewis, 1949)

Self-incompatibility in *Citrus* is fairly widespread in mandarins and their hybrids, e.g. 'Clementine' (Larcarelle & Miedzyrecki, 1936), 'Orlando' (Krezdorn & Robinson, 1958), 'Minneola' (Mustard *et al.*, 1956), 'Robinson' (Krezdorn, 1986), 'Page' (Reece & Register, 1961), 'Nova' (Hearn & Reece, 1967) and pummelos (Soost, 1964).

Cross-incompatibility is rare in *Citrus* and occurs between 'Orlando' and 'Minneola' tangelos (Reece & Register, 1961) and in crosses involving various interspecific mandarin hybrids, e.g. 'Robinson', 'Nova' and 'Page' with 'Orlando' and/or 'Minneola' tangelos (Hearn & Reece, 1967; Hearn *et al.*,1969).

Self-incompatible cultivars will set seedless fruit when self-pollinated, e.g. 'Clementine' mandarin, but fruit set by cross-pollination with compatible pollen will

be seedy. Cross-incompatible cultivars can set seedless fruit when pollinated by incompatible pollen, but will produce seedy fruit when compatible pollen was used (Frost & Soost, 1968).

1.4.4 Overcoming problems associated with self-incompatibility

Fruit set and/or fruit size, and resultant yield, may be poor in self-pollinated, self-incompatible cultivars. These problems associated with self-incompatibility can be overcome by cross-pollination with suitable compatible pollinators, induced parthenocarpy or crop manipulation using fruit setting sprays or girdling (Jackson, 1986). In certain citrus producing countries specific pollinators are planted in commercial citrus orchards to achieve this objective in weakly parthenocarpic, self-incompatible cultivars (Bukiya, 1985; Vithanage, 1991; Futch & Jackson, 1993a). However, excessive seed development usually takes place (Jackson, 1986). In South Africa, the aim is to produce seedless fruit and therefore this approach has not been followed.

1.5 Fertilization and embryo development

1.5.1 Fertilization

Following pollen tube growth through the style and ultimately into the ovule via the micropyle, the two pollen tube nuclei are discharged into the embryo sac. One of the pollen tube nuclei fuses with the egg nucleus in the embryo sac of the ovule to form the zygote. The zygote develops by cell division and differentiation into the sexual embryo. The other pollen tube nucleus fuses with the two polar nuclei in the embryo sac, and develops into the endosperm, a material used to nourish the developing embryo (Fig. 1.4) (Frost & Soost, 1968; Krezdorn, 1986; Jackson, 1986).

Fusion of the pollen cells with the female egg and polar nuclei cells respectively, is called double fertilization as opposed to single fertilization in animals. The

fertilized ovules subsequently develop into seeds. The period from pollination to fertilization is usually from two to four days in *Citrus*, occasionally taking longer (Bacchi, 1943; Furusato, 1951; de Lange & Vincent, 1979).

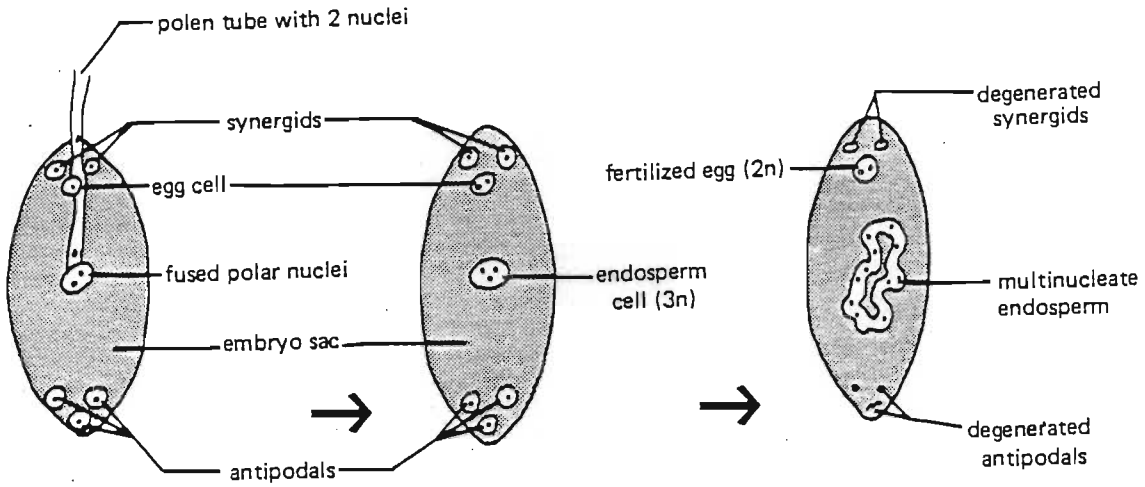

Fig. 1.4 Fertilization and endosperm development within the embryo sac of *Citrus* (Jackson, 1986)

The sexual process of fertilization is usually essential to fruit production, except where parthenocarpic fruit set occurs, as fertilization and subsequent seed development appear to play the most vital roles in fruit set and growth (Krezdorn, 1986). Monselise (1985) has divided *Citrus* into two groups with respect to the role of seeds in fruit development. Most recently developed *Citrus* cultivars have been selected for relative or complete seedlessness, and appear to be dependent on the rind for necessary plant growth substances. Many mandarins, mandarin hybrids and pummelos are, however, naturally seedy, and dependent on the seeds for both fruit set and growth.

1.5.2 Embryo development

The development of the *Citrus* zygotic embryo follows a regular order similar to that of most dicotyledons. Following fertilization, the zygote is dormant for several weeks (30 to 60 days) before dividing. Following the initial division, outgrowths give rise to the various parts of the complete embryo: hypocotyl, radicle, cotyledons and plumule (Frost & Soost, 1968; Jackson, 1986). Before the egg cell

divides, the embryo sac begins to enlarge rapidly, crowding and destroying many cells of the nucellus.

The endosperm nucleus divides successively many times to produce a multi-nucleate endosperm, and free nuclear endosperm can be seen up to 67 days after pollination. This endosperm material becomes cellular, with three layers of cells at the micropylar end and a single layer of cells further down the chalazal end. The endosperm is absorbed as the embryo develops, and is non-existent 100 days after pollination (Frost & Soost, 1968; Jackson, 1986). The nucellus shrinks, leaving only vestiges to contribute to the formation of the testa (seed coat) (Jackson, 1986). The function of the endosperm is to supply nutrients for the developing embryos (Frost & Soost, 1968).

Where sexual incompatibility occurs, for whatever reason, and parthenocarpy takes place, the ovule degenerates and aborted seeds remain, manifested as undeveloped seed coats (Jackson, 1986). Most "commercially seedless" orange, grapefruit and lemon fruits have numerous small to medium-sized seed remnants, depending on when abortion occurred.

1.5.3 Nucellar embryony

In most *Citrus* cultivars, additional embryos to the sexual embryos develop. These are derived from the somatic cells of the nucellus rather than cells of the embryo sac. They grow into the embryo sac and lie alongside the normal embryo. These extra, vegetative embryos are called nucellar embryos (Toxopeus, 1930).

Since nucellar embryos develop asexually by mitotic division of cells of the nucellus, no male cells contribute to their formation, and no meiosis occurs in the seed parent cells which give rise to them. Nucellar seedlings not only inherit from the seed parent alone, but are actually genetically identical to it, except for possible differences due to somatic variation and juvenility (Frost & Soost, 1968). This asexual reproduction has important consequences for evolution, breeding and citrus

culture. The only aspect relating to the present study is the effect of pollination on nucellar embryony and seediness in citrus.

1.6 Seed development

Following fertilization, the ovule develops into the seed. The endosperm enlarges at the expense of the nucellus. The enlarging embryos (zygotic and nucellar) gradually replace the endosperm, the latter being digested by the developing cotyledons. An inner seed coat (tegmen) is formed from the inner integument, the inner parts of the outer integument, and the remains of the nucellus and endosperm. The chalaza forms a cap over the distal end of the seed, seen as a brown-to-reddish coloured area adjacent to the nucellus. The testa (outer seed coat) is composed primarily of the outer epidermis of the ovular wall. The epidermal cells form a secondary wall which makes up a woody, cream coloured tough covering (Jackson, 1986).

Within the tegmen and testa are one or more embryos. They form a solid rounded mass in which the incipient radicles normally point towards the micropylar end. A seed with one embryo usually has two cotyledons of similar size and shape. Cotyledon size and shape varies if more than one embryo is present. The embryos in polyembryonic cultivars are usually crowded into the micropylar end. The cotyledons are white, cream or green and constitute most of the mature seed (Frost & Soost, 1968).

1.7 Export requirements

Citrus flowers have the potential of ten ovules per locule (Frost & Soost, 1968) and there are usually eight to twelve locules per ovary. However, varying degrees of sterility occur in *Citrus*, involving pollen, the ovule or both, resulting in less seeds than what could potentially develop (Frost & Soost, 1968). The standards for seed content for export citrus from Southern Africa are summarized in Table 1.1 (Anon., 1995b). These standards were set in accordance with export market requirements

and based on local research to determine the seed contents of the various cultivars (Burdette, pers. comm.[3]).

The more recently introduced marketing categories, such as "Midknights", "Delta Seedless", "Satsumas", "Clementines" and "Star Ruby", have relatively stringent maximum seed contents. Any newly developed cultivar would need to meet these requirements, while orchard layout must be such that cross-pollination is avoided or at least restricted to inefficient cross-pollinators.

Table 1.1 Standards for seed content for export citrus (Anon., 1995b)

Marketing category	Maximum average number of seeds per fruit in sample
Navels	Nil
Tomangos	Less than 7
Proteas	Less than 10
Valencias	Less than 10
Valentines	Less than 10
Midknights	2
Delta Seedless	Nil (see 1 below)
Minneolas	10
Tambors	10
Satsumas	Nil (see 1 below)
Clementines	3 (see 2 below)
Ellendales	3 (see 2 below)
Tangerines	10 (see 2 below)
Star Ruby	5

(1) Permitted deviation: 10 % of the fruit in the sample may contain 2 seeds per fruit on average.

(2) For "Clementines" and "Ellendales", not more than 10 % of the fruit sampled may contain more than 6 seeds per fruit. "Clementines" with more seeds than permitted can be exported as "Tangerines" provided that such fruit has no more than 10 seeds per fruit on average. In all other respects such fruit should comply with "Clementine" standards.

[3]S.A. Burdette, Outspan International, Centurion

1.8 Fruit set and development

1.8.1 Fruit set

Normal fruit set is the result of the stimulus of sexual fertilization and the resultant production of seeds. Parthenocarpic cultivars represent an exception to this and parthenocarpy is especially noticeable in incompatible or sterile cultivars. Non-parthenocarpic cultivars will set few if any fruit if fertilization and seed production do not occur. Therefore, enhancement of seed production is often important in maximizing fruit set and yield, and fruit size in naturally seedy cultivars (Jackson, 1986).

Initial fruit set, subsequent fruit drop and ultimately fruit yields are affected by several environmental and physiological factors (Davies & Albrigo, 1994). Most commercially important citrus cultivars bloom prolifically producing as many as 100 000 to 200 000 flowers on a mature tree; however, usually fewer than 1 to 2 % of these flowers produce harvestable fruit (Erickson & Brannaman, 1960).

Plant growth regulators are probably involved with the capacity of fruit to persist during initial fruit set based on circumstantial evidence (Davies & Albrigo, 1994). It is postulated that phytohormones which are produced by developing seeds result in mobilization of leaf photosynthates in the fruitlets which delay or prevent their abscission (Krezdorn, 1986).

Fruit set is not a limitation with oranges (excluding 'Navel' oranges), grapefruit and mandarin cultivars under optimal growing conditions (Krezdorn, 1986). Sexually incompatible cultivars, e.g. 'Robinson' and 'Sunburst', with weak parthenocarpic ability require cross-pollination with compatible pollen (Krezdorn, 1986; Futch & Jackson, 1993a; Davies & Albrigo, 1994). Sterile cultivars, e.g. 'Navel' oranges and 'Satsuma' mandarins, with strong vegetative parthenocarpic ability do not require pollination for fruit set. Self-incompatible cultivars with strong parthenocarpy, e.g. 'Clementine' mandarin and 'Nova' mandarin hybrid, set

adequate crops of seedless fruit under optimal conditions, but require some fruit setting enhancement under sub-optimal conditions (Jackson, 1986).

1.8.2 Parthenocarpy

Parthenocarpy is the capacity to produce fruit without the stimulus of sexual fertilization (Davies & Albrigo, 1994) and therefore without seeds. Thus, ovule abortion occurs at some stage. However, the precise time in which degeneration of citrus ovule tissues begins is unknown, and also whether ovules have some influence on setting and development of fruit before this phenomenon takes place (Tadeo & Primo-Millo, 1989). The level of parthenocarpy varies in different cultivars from strong in 'Tahiti' lime to weak in 'Wilking' mandarin (Frost & Soost, 1968; Jackson, 1986). Between the two extremes are moderately parthenocarpic cultivars.

The setting of seedless fruits without any external stimulation is autonomic or vegetative parthenocarpy, as seen in 'Tahiti' lime, 'Navel' oranges, 'Satsuma' mandarin, and certain other cultivars (Frost & Soost, 1968). In some instances, a stimulation caused by pollen tube growth and/or the initial stages of fertilization is necessary to induce parthenocarpic fruit set. This is known as stimulative parthenocarpy and usually results in increased fruit set, e.g. 'Star Ruby' grapefruit (Burger, 1982), and 'Oroblanco' and 'Melogold' pummelo-grapefruit hybrids (Soost & Cameron, 1980). Stenospermocarpy is where fertilization occurs followed by post-zygotic abortion, e.g. 'Temple' tangor (Vardi *et al.*, 1988).

Cross-pollination is often required with moderately and weakly parthenocarpic mandarin hybrids to achieve acceptable yields and/or fruit size (Jackson, 1986). However, seed content usually increases when this method is used, depending on the pollination potential of the pollinating cultivar. Fruit set may also be increased in weakly or moderately parthenocarpic cultivars through the use of girdling or plant growth regulators, e.g. gibberellic acid (Jackson, 1986).

1.9 Orchard layout

The following preliminary guidelines were provided to Southern African citrus producers to ensure that orchard layout does not allow unwanted cross-pollination, where potentially strong cross-pollinating cultivars are planted alongside one another (Burdette & du Plooy, 1991).

i) Pollen sterile cultivars to be used as buffers: 'Navel' orange and 'Satsuma' mandarin selections. It was not confirmed whether grapefruit, 'Delta Valencia' orange or lemons could be recommended as a buffer at that stage.
ii) At least 10 rows of a buffer cultivar must be planted between two cross-pollinating cultivars to prevent seediness or to decrease the potential seed content.
iii) The minimum distance between two cross-pollinating cultivars, where no buffer cultivars are planted, was estimated to be 500 m.
iv) The planting of 'Minneola' tangelo in the vicinity of other mandarin cultivars should be avoided.
v) The grower must also take his neighbours' situations into account when planning orchard layout.

In Florida, specific guidelines are given regarding what cultivars to use as pollinators where fruit set of weakly parthenocarpic cultivars is poor (Futch & Jackson, 1993a; Adams, 1995a; 1995b).

1.10 Conclusions

Citrus pollen development, pollination, fertilization, and seed and fruit development follow the usual course for angiosperms. *Citrus* pollen is characteristic of entomophilous plants and pollen transfer is principally by means of insect vectors, African honey bees playing the leading role locally. The receptive stigma is covered by a stigmatic exudate which provides suitable germination conditions for pollen, and is composed of proteins, lipids and polysaccharides in solution, the latter

providing a suitable substrate for pollen germination and pollen tube growth.

The main function of the pollen tube is to carry the male gametes for fertilization. In the stigma, pollen tube growth is intercellular, while in the style , the growth of the tubes is confined mainly to the stylar canals. When pollen tubes grow through the hollow style, nourishment is derived from the glandular epidermis of the stylar canal. Incomplete pollen tube growth may be due to an incompatibility barrier. Incompatibility in *Citrus* is gametophytically determined by S-alleles. Self-incompatible cultivars set seedless fruit when self-pollinated, but fruit set by cross-pollination with compatible pollen leads to seedy fruits.

In *Citrus*, varying degrees of sterility occur, involving pollen, the ovule, or both. In addition, embryo abortion is common. This results in the potential to produce seedless or virtually seedless fruit, the latter often referred to as "commercially seedless".

The export requirements concerning seed content have become stricter for the recently introduced marketing categories, stressing the importance of absolute or almost complete seedlessness in the citrus industry. Present guidelines concerning orchard layout are relatively limited, with few options available. Therefore, there is a need to develop further information on the pollination potential of the various citrus cultivars available to the producer.

This review of the current knowledge of *Citrus* pollen biology provides a platform from where the scope of the ensuing research may be identified, viz. the need to i) determine the pollination potential of numerous cultivars available, ii) provide additional options to the guidelines on orchard layout, and iii) elucidate the effects of cross-pollination on seed content.

CHAPTER 2

IN VITRO POLLEN GERMINATION

2.1 Introduction

In a population of plant species, and between cultivars within a species, pollen quality differs widely. In order to determine the relative quality, and therefore ability to pollinate and successfully fertilize compatible flowers, various viability tests may be conducted on the pollen.

Viability is defined as the capability for living or continuing to develop (Rieger *et al.*, 1976). More specifically, pollen viability is the competence of individuals of a given pollen population to deliver male gametes to the embryo sac (Stanley & Linskens, 1974). However, viability should not be confused with fertility which is a measure of an individual's ability to produce viable offspring, i.e. sexual embryos, whereas sterility is a measure of the proportion of abnormal gametes (Rieger *et al.*, 1976).

Viability studies of *Citrus* pollen have been used in breeding programmes to test the viability of stored pollen (Sahar & Spiegel-Roy, 1980; Carstens, 1990), or to determine the pollination potential of individual cultivars (de Lange & Vincent, 1972b; 1979). The need to determine relative levels of pollination potential arose from the requirements of the southern African citrus industry to be assured of the most appropriate orchard layout to prevent unwanted cross-pollination and seediness. Therefore, all cultivars needed to be tested and relative levels of pollination potential determined.

One method to assess a cultivar's pollination potential is to determine the viability of the pollen. Numerous techniques have been developed to do so and may involve *in vitro* germination, staining pollen tubes, *in vivo* assays, or most accurately by counting viable zygotic seeds produced (Stanley & Linskens, 1974; Heslop-Harrison *et al.*, 1984). In the present study, the *in vitro* pollen germination potential was determined over four seasons for all cultivar groups available in South Africa.

The pollen germination potential of a particular cultivar or selection of a cultivar (cultivar/selection) was determined by analysing the data from these assays within each season tested, and compared over all seasons. The pollen germination percentages for all cultivars tested were then summarized for the four seasons and categorized (Chapter 4). This information was then used to develop a prediction model for the pollination potential of citrus cultivars. In addition, functionally pollen sterile and very weakly pollen fertile cultivars were identified. These can be planted between two self-incompatible cultivars with fertile pollen as a 'buffer', so as to reduce the effects of cross-pollination on seediness in both cultivars.

2.2 *In vitro* pollen germination assays

2.2.1 Introduction

In most pollen viability tests a small sample of pollen is germinated and microscopically observed, and the percentage of pollen grains producing pollen tubes after a given time is determined. This percentage is considered an index of viability of the pollen sampled. Such tests assume that the optimum conditions have been established for the *in vitro* test so that germination approximates that *in vivo*. However, most pollen tubes cultured *in vitro* stop growing before they reach the size normally attained in the style, and the rate of pollen tube growth is seldom as rapid as *in vivo*. This suggests that optimum growth conditions are not always established in *in vitro* media (Stanley & Linskens, 1974).

Another assumption made in *in vitro* tests is that the micro-sample of pollen is representative of the cultivar tested. Stanley & Linskens (1974) propose that this assumption is permissable provided care is taken to mix the pollen batch before drawing the sample. Plant-to-plant variation must be considered when drawing a sample from bulk pollen for viability testing. However, where asexually propagated plant material is used, pollen should be sampled from numerous trees in order to have a representative sample for that clone or cultivar (Stanley & Linskens, 1974).

A biochemical analysis of the stigmatic exudate of a particular species will help to

determine what components should be incorporated into the germination medium of that species. Such studies have been done for *Citrullus lanatus* (Hawker *et al.*, 1983), tobacco (*Nicotiana tabacum)* (Cresti *et al.*, 1986) and *Citrus* (Martin & Brewbaker, 1970; Cresti *et al.*, 1982). Many chemical and physical factors are known to influence optimal pollen germination *in vitro*. Some chemicals stimulating germination, e.g. B (Schmuker, 1934), Ca (Brewbaker & Kwack, 1963), and Mg (Heslop-Harrison *et al.*, 1984), were initially noted as similar or identical factors found in the style tissue or stigmatic exudate in which the pollen naturally germinates (Stanley & Linskens, 1974).

Germination as determined by *in vitro* viability tests can be positive while, in fact, the pollen is unable to form normal pollen tubes *in vivo* to penetrate the female tissues, or to complete normal zygote formation. *In vitro* germination tests are generally sufficient to determine the effectiveness of pollen when the pollen is applied in field pollinations (Stanley & Linskens, 1974). Monselise (1985) claimed that one of the best methods to test citrus pollen viability is by germination assays. These tests seldom yielded more than 60 % germination, even in the best possible medium.

Other factors influencing pollen viability include: time of collection, maturity of anthers, method of collection and storage, and environmental conditions. The specific germination method used varies with pollen species and the accuracy or purpose for which the determination is made (Stanley & Linskens, 1974).

2.2.2 Techniques

In the literature on citrus pollen viability assays, and pollen biology in general, two techniques are commonly used for *in vitro* viability testing of *Citrus* pollen. The hanging drop technique is often used (Crescimanno *et al.*, 1988a; 1988b; Carstens, 1990), while the agar-based growth medium technique appeared to be used as often (de Lange, 1989). De Lange's early research on citrus pollen viability (unpublished), showed that the agar-based germination assay was a more reliable technique than the hanging drop method.

i) The hanging drop technique involves a drop of medium containing pollen inserted in a circular chamber from a coverslip suspended over water (Fig. 2.1A). This simple method is the most commonly used procedure for germinating micro-amounts of pollen to determine viability (Stanley & Linskens, 1974).

ii) Agar or gelatin-based method: Agar or gelatin are frequently used for germinating pollen. The agar supplies moisture at a constant relative humidity and various carbohydrates or other pollen growth stimulants can be readily incorporated. The thickness of the agar, as well as the agar and sugar concentrations affect moisture availability and germination. Use of a sugar, readily metabolised by pollen, can provide the required osmotic environment and minimize fungal growth. An additional advantage of agar slides (Fig. 2.1B) is the ease with which they can be handled. However, agar and gelatin tend to dry out unless a relatively high humidity is maintained. Moistened filter paper is usually placed in the near vicinity of the germination medium to raise the vapour pressure over the pollen and reduce evaporation of the medium. If germination tests are to be run for many days at above 28°C, then the filter paper should be remoistened frequently. Alternatively, silica gel can be used and will retain the moisture essential for germination. This technique has often been used in the citrus pollen literature and appears to be the most widely used method to determine the germination ability of pollen (Stanley & Linskens, 1974).

The inoculation of the germination medium is usually achieved by sprinkling the pollen with a camel's hair brush or tapped on from a spatula. Attempts to pipette pollen are generally not successful because of the tendency of the pollen to float or quickly sink and attach to the pipette walls (Stanley & Linskens, 1974).

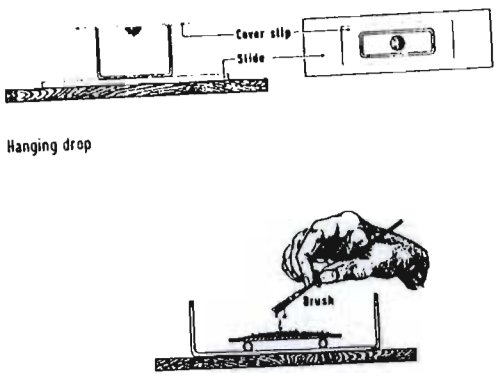

Fig. 2.1 Two methods of germinating pollen *in vitro* to determine viability (Stanley & Linskens, 1974)

2.2.3 Data recording

In viability counts one generally considers a pollen tube to have formed when it is equal to, or greater than, the pollen grain diameter (Stanley & Linskens, 1974). The total number of grains observed in a field is compared to the number of grains producing tubes.

A false germination count can result under certain conditions. The quantity of pollen in a spot can affect the capacity of pollen to grow. A 'mutual growth stimulation effect' occurs at a certain concentration of pollen (Savelli, 1940). According to Stanley & Linskens (1974), a certain minimum concentration of pollen grains must be placed in a given volume of solution for maximum germination. The mutual stimulation effect is not as strong in pollen spread evenly over agar. Conversely, in both the spot and agar tests high pollen concentration can inhibit germination. Viability tests are most valid when pollen is grown in an optimal concentration range. The addition of Ca ions (at ca. $2x10^{-3}$ M) to the germination medium tends to overcome the mutual stimulating effect (Kwack, 1965). *In vivo* tests can also be affected by too high a concentration of pollen. In such cases slow growing pollen will be inhibited or arrested in germination and the pollen tubes will not grow into the style (Stanley & Linskens, 1974). This has been termed the 'over-pollination effect' in kiwifruit (*Actinidia deliciosa* var. *deliciosa* (A. Chev.) C.F.

Lang *et* A.R. Ferguson) (Hopping & Simpson, 1982).

In *Citrus*, when excessively large numbers of pollen grains are employed in hand pollination studies, different pollen sources behave quite similarly, while clear differences are evident only when small numbers of pollen grains are used (Brown & Krezdorn, 1969). Therefore, to reach meaningful conclusions in such studies, it is essential to apply relatively low numbers of pollen grains to stigmas of the flowers to simulate the action of honey bees (de Lange *et al.*, 1979a).

In 1978, Sahar reported that the best possible medium for citrus pollen germination tests was Brewbaker's solution containing 15 % sucrose, boric acid, potassium and calcium nitrates, and magnesium sulphate, plus 1 % agar. Subsequent tests by de Lange (1989) have shown that 20 % sucrose in a 0.6 % agar based medium is sufficient, with over 80 % germination being possible with pollen from highly viable cultivars, whereas Gmitter (pers. comm.[1]) claimed that 15 % sucrose was optimal.

2.2.4 Conclusions

Often one technique may give poor results with a particular pollen which on retesting by another *in vitro* procedure shows a higher percentage of viable pollen (Stanley & Linskens, 1974). The best method for the particular species being examined should be chosen to meet the objectives of the research in question.

Non-germination pollen viability assays were considered unsuitable for a study of this nature as it was necessary to know what the germination ability, and hence fertilization potential, of the various cultivars/selections was. The various staining methods could only indicate pollen viability in general, and not the germination ability. Therefore, a relatively simple, yet effective, technique was selected to test the *in vitro* pollen viability of the different citrus cultivars using a semi-solid, agar-based growth medium poured into individual wells or vials.

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2.3 Materials and methods

2.3.1 Cultivars used

In vitro pollen viability assays involving 66 cultivars/selections of citrus were used to identify specific pollen parents with strong and weak pollination potential, for *in vivo* pollen germination and pollen tube growth studies (Chapter 3) (Table 2.1). The *in vitro* pollen viability assays were conducted over four seasons, viz. 1990, 1991, 1992 and 1994, with a different set of cultivars/selections being tested in each season. The year in which a specific cultivar/selection was studied is indicated in Table 2.1.

The criteria used to select the pollen parents to be assayed in a particular season were as follows:

i) *1990*: Since 1990 was the first year of assaying *in vitro* pollen viability, a wide range of cultivars/selections was used. Selections or cultivars from each cultivar group or citrus type, respectively, were included. In 1990, 52 cultivars/selections were tested. The results of 29 of these were considered valid for statistical analysis. Validity was determined by comparisons with reports of other researchers and comparisons between seasons tested. The author was also not fully *au fait* with the technique.

ii) *1991*: Initial analysis of the 1990 results showed (a) low pollen germination percentages of certain cultivars compared with reports in the literature and (b) wide variability between selections within a specific cultivar. Therefore, the pollen parents assayed in 1991 were selected with this in mind. For example, the complete range of 'Clementine' mandarin selections available were tested to confirm differences in pollen viability between selections. In 1991, 29 cultivars/selections were tested, of which 26 were considered valid for statistical analysis.

iii) *1992*: Twenty cultivars/selections were tested. The choice was based on the need to (a) confirm previous results, (b) determine viability of pollen to be used in *in vivo* pollen tube growth studies (see Chapter 3), and (c) test the viability of pollen of certain recently released grapefruit cultivars.

iv) *1994*: Nineteen cultivars/selections were selected according to their potential to be strong or weak pollinators, based on previous seasons' results. Determination of the pollen viability was necessary for use in *in vivo* pollen tube growth studies (see Chapter 3) and the development of a prediction model for pollination potential (see Chapter 4).

2.3.2 Blossom collection

Flowers of the various citrus cultivars or selections of cultivars given in Table 2.1 were collected during the spring flush at the so-called balloon stage of floral development, i.e. just before anthesis. Apical flowers in an inflorescence or individual flowers from leafy inflorescences were picked. De Lange & Vincent (1970) showed that there was no effect of flower position on fruit set. In the present study, it was assumed that flower position would not affect pollen viability. Flowers were collected from several trees of each selection at different positions on the tree in order to extract a random and representative sample of pollen for a specific cultivar/selection. Blossom collection took place before 12h00 so as to avoid the hottest part of the day and any potentially adverse effects on pollen viability.

Once picked, the blossoms were enclosed in moist cotton wool inside plastic bags. The bags were stored in a thermal cool box with ice bricks to keep the blossoms fresh and unwilted, until the anthers were extracted later that day.

Table 2.1 Citrus cultivars/selections used in *in vitro* pollen germination assays, *in vivo* pollen tube growth studies and fertility tests during 1990, 1991, 1992 and 1994

Citrus Type	Species Name	Cultivar	Selection	Year Studied
Lemon	*Citrus limon* L.	Eureka		1990; 1991; 1994
		Lisbon		1990;1994
		Fino		1991
		Verna		1991
Orange	*C. sinensis* L.	Navel	Navelina	1990
			Newhall	1990
			Painter Early	1990
			Palmer	1990
			Washington	1990
			Bahianinha	1990
			McClean	1990
			Nieuwoudt	1990
			Rautenbach	1990
			Neethling	1990
			Robyn	1990
		Clanor		1990; 1992
		Tomango		1990; 1994
		Shamouti		1990; 1992; 1994
		Salustiana		1994
		Valencia	Delta	1990; 1991; 1992; 1994
			Midknight	1990; 1991; 1992; 1994
			Late	1990; 1991; 1992; 1994
			Olinda	1990; 1992; 1994
			McClean	1990
			Du Roi	1990
			Excelsior	1990
			Margaret	1990
			Benny	1994
Pummelo	*C. grandis* L.	Java		1991
		Pomelit		1990; 1991; 1992; 1994
		Oroblanco		1992; 1994

Citrus Type	Species Name	Cultivar	Selection	Year Studied
Grapefruit	*C. paradisi* Macf.	Star Ruby		1990; 1992; 1994
		Marsh		1990; 1992; 1994
		Nartia		1990; 1994
		Redblush		1990; 1992; 1994
		Henderson		1992
		Nelruby		1992; 1994
		Ray Ruby		1992
		Rio Red		1992
Mandarin	*C. unshiu* Marc.	Satsuma	Owari	1990; 1991
			Miho Wase	1990; 1991
			Kuno	1990
			Imamura	1990
	C. reticulata Blanco	Clementine	SRA 63	1990; 1991; 1992
			SRA 70	1990; 1991
			SRA 84	1991
			SRA 85	1991
			SRA 88	1991
			SRA 89	1991
			SRA 92	1990; 1991
			Nules	1990; 1991; 1992; 1994
			Oroval	1990; 1991
			Clemlate	1990; 1991
Mandarin hybrid	*C. sinensis* x *C. reticulata*	Ellendale tangor	CSFRI	1990; 1991
			Koster	1990; 1991
			Leng	1990; 1991
			Herps	1990; 1991
			Nouvelle	1990; 1991
	C. reticulata x	Miscellaneous	Tambor	1990; 1991; 1992
			Sue Linda	1990; 1991
			Minneola	1990; 1991; 1992; 1994
			Fairchild	1990; 1992
			Nova	1990; 1991; 1992; 1994
			Fortune	1990
			Fremont	1990
			Page	1990

During 1990, blossoms of a specific cultivar/selection were collected from the Citrusdal Research Station (32°30′ S; 18°55′ E), Goede Hoop Citrus Co-operative Nursery cultivar variety block (32°35′ S; 19°00′ E), Weltevrede Experimental Farm, University of Stellenbosch (33°55′ S; 18°50′ E), and a local citrus farmer in the Klein Drakenstein area, S.W. Cape (33°45′ S; 19°05′ E), depending on availability.

The following season, 1991, blossoms were collected from citrus orchards of the same farmer in the Klein Drakenstein area, Delta Section of Pickstones Nursery, Groot Drakenstein (33°50′ S; 19°05′ E), Talana Nursery (orchards and nursery), Stellenbosch (33°55′ S; 18°50′ E), and Stellenrust Estates, Stellenbosch (33°55′ S; 18°50′ E).

During 1992, blossom collection took place at Malelane Kwekerye's citrus variety orchard, Malelane (25°30′ S; 31°30′ E), Tunzini Citrus Estate, IYSIS, Swaziland (25°55′ S; 31°45′ E), La Rochelle Estates, Malelane (25°30′ S; 31°25′ E), Crocodile Valley Estates, Nelspruit (25°30′ S; 31°05′ E), and Bakgat Boerdery, Schoemans-kloof (25°25′ S; 30°30′ E).

In 1994, blossoms were collected from the Institute of Tropical and Subtropical Crops' research stations at Nelspruit (25°25′ S; 30°55′ E) and Malelane (25°30′ S; 31°30′ E), Crocodile Valley Estates, and Tambankulu Estates, Swaziland (26°10′ S; 31°55′ E).

2.3.3 Anther removal and dehiscence

The anthers were stripped from the blossoms of each cultivar by combing them out with a piece of hair comb into labelled vials (de Lange, pers. comm.[2]). Each comb was washed in distilled water and 90 % ethanol prior to use. Care was taken throughout to ensure that no contamination of pollen occurred between cultivars/selections.

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[2]J.H. de Lange, National Botanic Gardens, Kirstenbosch

The vials were covered and kept overnight at ambient temperature, to allow the anthers to dry out and dehisce naturally, i.e. without being dissected. Twenty four hours after blossom collection, pollen of the respective cultivar/selection was available to conduct an *in vitro* pollen germination viability assay.

2.3.4 Composition and preparation of growth medium

A combination of the well and agar-based germination assays described by Stanley & Linskens (1974) was used.

During 1990 and 1991, 10 ml vials were used to contain the agar-based growth medium. In 1992 and 1994, a simple, triple container or well system was designed specifically for this study. Three perspex rings, 20 mm high with 18 mm inside diameter, were glued to a microscope slide using commercial silicon sealant. The three rings provided wells which could be used as three replications in the viability assays. Each well was approximately 5 mm^3 in volume.

Distilled water was adjusted to pH 7.0, to which Difco Bacto agar at 12 g ℓ^{-1} (1.2% w/v) was added. The solution was heated to 122°C for 15 min in an autoclave. After autoclaving, the agar medium was mixed with equal volumes of 40 % sucrose solution (400 g ℓ^{-1}) to give a final growth medium consisting of 20 % sucrose and 0.6 % agar at pH 7.0 (de Lange, 1989).

Although Brewbaker & Kwack (1963) maintain, and it is widely accepted, that B plays an essential role in pollen germination and tube growth, de Lange (1989) found no positive effects in citrus. Therefore, it is assumed that the addition of B does not augment citrus pollen germination, probably because of adequate pollen levels, and it was not included in the growth medium.

Pilot tests and research by de Lange (1989) showed that sucrose was a superior carbohydrate source for citrus pollen germination assays when compared with glucose, fructose, lactose, raffinose, maltose, melezitose, galactose, cellobiose and

trehalose. In the present study a 20 % sucrose concentration was used. In addition, 20 % glucose and 20 % sucrose + glucose sugar concentrations were used in the growth medium in 1991. The objective was to determine and cross-check whether sucrose was a good carbohydrate source for citrus pollen germination.

The carbohydrate concentrations were made up as follows:

i) 20 % sucrose = 200 g sucrose (MM = 342) ℓ^{-1} distilled water. Final molarity = 0.58 M,

ii) 20 % glucose = 200 g glucose (MM = 179.3) ℓ^{-1} distilled water. Final molarity = 1.12 M, and

iii) 20 % sucrose + glucose = 99.18 g sucrose + 52.00 g glucose ℓ^{-1} distilled water. Total molarity = 0.58 M.

The sucrose was not autoclaved as autoclaving of the carbohydrate results in decreased *in vitro* pollen germination in citrus (de Lange, 1989).

The medium was carefully poured into the vials or specially prepared wells, with three vials being prepared per cultivar/selection, i.e. three replicates, except in 1992 when four replicates were used. The wells were purposely overfilled until the meniscus was at its maximum and before its surface tension was broken. When the growth medium cooled and set, the meniscus became level with the top edge of the well. If the wells were underfilled, the top of the growth medium would set below the edge of the well with a concave surface, making it necessary to range up and down when viewing the pollen grains through the microscope.

2.3.5 Application of pollen onto growth medium

For each cultivar/selection, pollen grains were spread out evenly with a minimum of touching on the agar-based growth medium. Single grains are preferred to clumps of pollen as the 'growth stimulating effect' can lead to spurious results (Stanley & Linskens, 1974).

The dehisced anthers and pollen were first mixed with a fine bristled paint brush, whose tip had been cut off to make the following steps more effective. Excess pollen was removed by flicking the paint brush against the back of a scalpel. Pollen grains were then applied to the growth medium by flicking the paint brush against the back of a scalpel whilst moving the hands systematically across the medium surface. Pollen application was monitored using a dissection microscope. The brushes were cleaned in 90 % ethanol prior to use and examined under a binocular light microscope to ensure that they were clean. The scalpel was wiped clean with tissue paper moistened with 90 % ethanol, and air dried prior to use with the next cultivar/selection.

Attention was given to three important aspects at this stage, viz.

i) The pollen was thoroughly mixed in the vials with the paint brush before being applied. This helped to ensure that a representative pollen sample was taken for the cultivar/selection.

ii) Pollen grains were spread evenly with a minimum of touching and even distribution over the growth medium.

iii) Contamination between cultivars/selections was avoided.

2.3.6 Incubation, germination and scoring

The 10 ml vials (1990 and 1991) or microscope slides with perspex rings affixed (1992 and 1994) were placed in a tray containing a paper towel in a few millimetres depth of water to maintain a humid environment. The top of the tray was covered with another tray, without touching the top of the agar and allowing sufficient space for air exchange. This was placed in a growth chamber and allowed to incubate for 24 h at 25 °C.

Thereafter, individual vials were viewed through a binocular light microscope (2.5 or 10 x objective, 10 x ocular). Individual pollen grains were considered germinated when the length of the pollen tube exceeded the diameter of the pollen grain. For each replicate, 100 pollen grains were scored for germination and counted using a

hand counter.

Scoring of pollen grains was conducted systematically by moving from one side of the medium surface to the other. In order to ensure that there was no bias towards counting germinated or ungerminated pollen grains, all pollen grains in the field of view were scored before moving onto the next field of view. Where clumps of pollen grains occurred, these were ignored.

2.3.7 Statistical analysis

The pollen germination percentage data were analysed separately for each year. Based on comparisons with results reported in the literature and those from other seasons tested, data considered to be invalid for statistical analysis were excluded from the analysis but are presented in the tables below. Experimental error was considered to be the cause of invalid data. Pollen germination percentage data were transformed using the arcsine transformation. An analysis of variance (ANOVA) was conducted on the transformed data and the transformed means were separated by least significant differences (L.S.D.).

Where pollen was germinated on three different carbohydrate-containing media, viz. 20 % sucrose, 20 % glucose and 20 % sucrose + glucose, and allowed to incubate for 24 and 48 h in 1991, the data were compared using a paired 't' test.

2.4 Results

The mean *in vitro* pollen germination percentage and the standard error of individual means (back-transformed data) are presented, by year, in Tables 2.2.1 to 2.2.4. The data, summarized according to cultivar group for all four years of testing, are presented in Tables 2.3 to 2.10. The purpose is to show relative differences between cultivars, over all years tested.

Table 2.2.1 Mean *in vitro* pollen germination percentage ± SE for various citrus cultivars\selections assayed during 1990

Cultivar/selection	% germination ± SE
Navelina	0.0[a]
Newhall	0.0[a]
Painter Early	0.0[a]
Palmer	0.0[a]
Washington	0.0[a]
Bahianinha	0.0[a]
McClean	0.0[a]
Nieuwoudt	0.0[a]
Rautenbach	0.0[a]
Neethling	0.0[a]
Robyn	0.0[a]
Star Ruby	0.0[a]
Delta	0.0[a]
Miho Wase	0.0[a]
Imamura	0.0[a]
Owari	0.3 ± 0.33[a]
Kuno	0.3 ± 0.33[a]
Midknight	0.3 ± 0.33[a]
Shamouti	9.0 ± 0.58[b]
Minneola	10.0 ± 3.00[b]
SRA 63	13.0 ± 3.51[b]
Fortune	14.7 ± 0.88[b]
Fairchild	22.3 ± 2.73[c]
SRA 70	22.7 ± 1.20[cd]
Koster	24.0 ± 2.52[cd]
CSFRI	26.3 ± 2.91[cde]
Pomelit	29.7 ± 4.48[de]
Clemlate	32.3 ± 2.19[e]
SRA 92	59.3 ± 3.33[f]

Means not followed by the same letter or joined by a vertical line are significantly different at the 1% level of significance, using L.S.D. = 7.084

Table 2.2.2 Mean *in vitro* pollen germination percentage ± SE for various citrus cultivars\selections assayed during 1991

Cultivar/selection	% germination ± SE
Delta	0.0[a]
Owari	2.0 ± 0.58[ab]
Midknight	3.0 ± 0.58[ab]
Miho Wase	4.0 ± 0.58[abc]
Eureka	9.7 ± 0.88[abcd]
Fino	10.0 ± 2.08[abcd]
Tambor	10.3 ± 1.45[abcd]
Valencia Late	11.0 ± 1.53[abcde]
Verna	12.0 ± 1.00[abcde]
Leng	17.7 ± 3.76[bcdef]
Oroval	19.7 ± 1.45[cdef]
CSFRI	25.7 ± 1.67[defg]
Minneola	26.7 ± 3.76[efg]
SRA 88	29.3 ± 4.91[fgh]
Nova	31.0 ± 0.58[fghi]
SRA 92	40.7 ± 10.49[ghij]
Koster	43.0 ± 2.08[hij]
SRA 70	46.0 ± 5.13[ijk]
SRA 63	47.7 ± 1.86[jkl]
Herps	52.0 ± 1.15[jklm]
SRA 84	53.3 ± 1.76[jklm]
SRA 85	62.0 ± 4.36[klmn]
Pomelit	63.0 ± 4.58[lmn]
SRA 89	65.0 ± 3.06[mn]
Java	66.0 ± 2.00[mn]
Nouvelle	77.7 ± 5.24[n]

Means not followed by the same letter or joined by a vertical line are significantly different at the 1% level of significance, using L.S.D. = 16.150

Table 2.2.3 Mean *in vitro* pollen germination percentage ± SE for various citrus cultivars\selections assayed during 1992

Cultivar/selection	% germination ± SE
Star Ruby	0.5 ± 0.29[a]
Nelruby	0.8 ± 0.25[a]
Delta	1.3 ± 0.48[a]
Oroblanco	2.0 ± 0.71[ab]
Midknight	2.5 ± 0.87[abc]
Marsh	4.3 ± 1.11[abcd]
Olinda	4.8 ± 0.48[abcd]
Henderson	7.8 ± 0.63[abcde]
Valencia Late	8.8 ± 1.75[bcde]
Rio Red	9.8 ± 0.75[cdef]
Ray Ruby	10.3 ± 0.95[def]
Nova	11.3 ± 2.02[def]
Clanor	13.5 ± 1.04[ef]
Redblush	16.8 ± 4.53[f]
SRA 63	28.8 ± 2.32[g]
Shamouti	30.5 ± 1.44[g]
Pomelit	33.0 ± 1.29[gh]
Tambor	38.5 ± 0.65[h]
Fairchild	46.0 ± 1.47[i]
Nules	55.3 ± 1.65[j]
Minneola	58.5 ± 1.19[j]

Means not followed by the same letter or joined by a vertical line are significantly different at the 1% level of significance, using L.S.D. = 7.480

Table 2.2.4 Mean *in vitro* pollen germination percentage ± SE for various citrus cultivars\selections assayed during 1994

Cultivar/selection	% germination ± SE
Star Ruby	0.0[a]
Delta	1.3 ± 0.33[a]
Midknight	1.7 ± 0.88[ab]
Salustiana	6.0 ± 1.15[abc]
Nartia	9.7 ± 0.33[abc]
Oroblanco	10.0 ± 1.00[abc]
Redblush	10.3 ± 0.88[abc]
Valencia Late	12.0 ± 2.65[abc]
Nelruby	13.3 ± 2.03[abc]
Lisbon	15.0 ± 4.16[bc]
Marsh	15.3 ± 2.33[c]
Benny	15.3 ± 5.78[c]
Eureka	16.0 ± 2.52[c]
Tomango	17.7 ± 1.20[c]
Olinda	19.3 ± 1.67[c]
Nules	40.3 ± 1.86[d]
Nova	60.0 ± 7.94[e]
Pomelit	74.3 ± 2.03[f]
Minneola	76.3 ± 3.18[f]

Means not followed by the same letter or joined by a vertical line are significantly different at the 1% level of significance, using L.S.D. = 13.430

2.4.1 Lemons

In 1990, remarkably small amounts of pollen grains were extracted from the anthers of 'Eureka' and 'Lisbon' lemon blossoms. Blossoms were collected just prior to anthesis and very few pollen grains were found after the anthers had dehisced. The pollen grains applied to the growth medium were pale yellow, none were considered to have germinated, and all appeared to be abortive. The data were considered invalid for statistical analysis as it is known that lemon pollen does have a low viability (Crescimanno *et al.*, 1988b). Therefore, they were not included in the analysis of variance, where they could have skewed the results.

The following season, 1991, 'Eureka', 'Fino' and 'Verna' lemon blossoms were picked in the morning and anthers were stripped later the same day (approximately 6 h later). Blossoms which had not quite reached the balloon stage of development had less developed and paler anthers than those picked at a later stage. At the balloon stage, anthers were yellow and stodgy and none had dehisced. Those picked at a slightly later stage, when two petals had just opened, had some dehisced anthers. When flowers were picked at this stage of development care was taken to ensure that no bees had visited the flowers.

Within 2 h after stripping the anthers, those from the more developed blossoms had dehisced, and after 24 h, when pollen was applied to the growth medium, only the more advanced anthers had dehisced. When compared with other citrus types, lemon cultivars had relatively small amounts of pollen available for the viability assays conducted.

Pollen germination ranged from 9.7 ± 0.88 to 12.0 ± 1.00 % for the lemon cultivars tested during 1991 using the standard procedures (20 % sucrose; 24 h incubation period) (Table 2.3).

Following visual observation, where pollen grains were in clumps, germination percentage was higher than for single grains, confirming the 'mutual stimulation

effect' (Stanley & Linskens, 1974).

When 'Eureka' and 'Lisbon' lemons were tested in 1994, small amounts of pollen were available. Germination of 16.0 ± 2.52 and 15.0 ± 4.16 % for 'Eureka' and 'Lisbon' were achieved, respectively (Table 2.3).

Table 2.3 Mean percentage *in vitro* pollen germination ± SE of four lemon cultivars (*C. limon* L.) conducted in 1990, 1991 and 1994 using an agar based semi-solid growth medium (20 % sucrose; 24 h incubation period)

Cultivar	1990	1991	1994
Eureka	0.0	9.7 ± 0.88^{abcd}	16.0 ± 2.52^{c}
Lisbon	0.0	-	15.0 ± 4.16^{bc}
Fino	-	10.0 ± 2.08^{abcd}	-
Verna	-	12.0 ± 1.00^{abcde}	-

Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

2.4.2 'Navel' oranges

The anthers from the 'Navel' orange blossoms were pale green. Eight of the 'Navel' orange cultivars did not have any pollen, while 'Palmer', 'Washington' and 'Nieuwoudt Navel' oranges had small numbers of aborted pollen grains.

2.4.3 Midseason oranges

In 1990, pollen germination percentages of 'Clanor' and 'Tomango' were very low (Table 2.4), possibly indicating sterility. However, in subsequent tests (1992 and 1994) pollen germination for 'Clanor' and 'Tomango' was 13.5 ± 1.04 % and 17.7 ± 1.20 %, respectively. Germination percentage of 'Shamouti' pollen ranged from 9.0 ± 0.58 to 30.5 ± 1.44 % in 1990 and 1992, respectively. 'Salustiana'

pollen was tested in 1994, with 6.0 ± 1.15% germinating.

Table 2.4 Mean percentage *in vitro* pollen germination ± SE of four midseason sweet orange cultivars (*C. sinensis* L.) conducted in 1990, 1992 and 1994 using an agar based semi-solid growth medium (20 % sucrose; 24 h incubation period)

Cultivar	1990	1992	1994
Clanor	0.7 ± 0.33	13.5 ± 1.04^{ef}	-
Tomango	0.7 ± 0.67	-	17.7 ± 1.20^{c}
Shamouti	9.0 ± 0.58^{b}	30.5 ± 1.44^{a}	-
Salustiana	-	-	6.0 ± 1.15^{abc}

Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

2.4.4 'Valencia' oranges

i) 'Delta Valencia' orange: 'Delta' blossoms had relatively small anthers which were pale yellow at balloon stage and slightly darker at anthesis. Only the more developed anthers dehisced, with little pollen being available.

Over the four years tested 'Delta' pollen always had a very low germination percentage (0.0 to 1.3 ± 0.48 %) (Table 2.5), even when glucose or sucrose + glucose were used as alternative carbohydrate sources to sucrose and also when germinated for 48 h (Table 2.11).

ii) 'Midknight Valencia' orange: In 1990 few pollen grains were extracted from 'Midknight' blossoms. However, pollen was more freely available in 1991 when blossoms were selected at a later stage of development. Flowers with less advanced anthers dehisced poorly and had a low germination percentage (0.3 ± 0.33 %) in 1991 (Table 2.5). On the other hand, most anthers dehisced and plenty of pollen was available when flowers with well developed anthers were used. Pollen application was also quick and easy compared with 'Delta Valencia', and

germination percentage ranged from 1.7 ± 0.88 to 3.0 ± 0.58 %.

Table 2.5 Mean percentage *in vitro* pollen germination ± SE of nine 'Valencia' orange selections (*C. sinensis* L.) conducted in 1990, 1991, 1992 and 1994 using an agar based semi-solid growth medium (20 % sucrose [S]; 24 h incubation period)

Selection	1990	1991	1992	1994
Delta	0.0[a]	0.0[a]	1.3 ± 0.48[a]	1.3 ± 0.33[a]
Midknight	0.3 ± 0.33[a]	3.0 ± 0.58[ab]	2.5 ± 0.87[abc]	1.7 ± 0.88[ab]
Valencia Late	3.0 ± 0.00	11.0 ± 1.53[abcde]	8.8 ± 1.75[bcde]	12.0 ± 2.65[abc]
Olinda	3.0 ± 0.00	-	4.8 ± 0.48[abcd]	19.3 ± 1.67[c]
McClean	0.7 ± 0.67	-	-	-
Du Roi	1.3 ± 0.58	-	-	-
Excelsior	2.0 ± 1.00	-	-	-
Margaret	2.3 ± 1.45	-	-	-
Benny	-	-	-	15.3 ± 5.78[c]

[x]Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

iii) 'Valencia' orange selections: Of the seedy 'Valencia' selections tested, 'Valencia Late' and 'Olinda' are the most widely planted in South Africa (Burdette *et al.*, 1995; pers. obs.) and, therefore, are used as representative examples for 'Valencia'. The stage of anther development was not critically assessed in 1990 and, therefore, it is uncertain whether the low germination percentage of 1990 was characteristic of the cultivar or due to environmental conditions. In 1991, 1992 and 1994, virtually all the anthers dehisced and plenty of pollen was available.

Pollen germination percentage for old clone 'Valencia' selections was usually 11.0 % or less, with 'Benny' pollen giving 15.3 ± 5.78 % (Table 2.5).

2.4.5 Grapefruit

'Star Ruby' grapefruit pollen was virtually sterile with only one pollen grain germinating out of 1 000 counted in three different years (Table 2.6). The other grapefruit cultivars tested had pollen germination percentages ranging from 0.8 ± 0.25 to 16.8 ± 4.53 %, and from 8 to 13 % when conditions appeared ideal (Table 2.6). When tested in 1992, 'Henderson' pollen appeared to be more sticky than that of other grapefruit cultivars and tended to clump together.

Table 2.6 Mean percentage *in vitro* pollen germination ± SE of eight grapefruit cultivars (*C. paradisi* Macf.) conducted in 1990, 1992 and 1994 using an agar based semi-solid growth medium (20 % sucrose; 24 h incubation period)

Cultivar	1990	1992	1994
Star Ruby	0.0[a]	0.5 ± 0.29[a]	0.0[a]
Marsh	1.7 ± 0.33	4.3 ± 1.11[abcd]	15.3 ± 2.33[c]
Nartia	3.3 ± 0.67	-	9.7 ± 0.33[abc]
Red Blush	1.3 ± 0.67	16.8 ± 4.53[f]	10.3 ± 0.88[abc]
Henderson	-	7.8 ± 0.63[abcde]	-
Nelruby	-	0.8 ± 0.25[a]	13.3 ± 2.03[abc]
Ray Ruby	-	10.3 ± 0.95[def]	-
Rio Red	-	9.8 ± 0.75[cdef]	-

Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

2.4.6 Pummelos

Pummelo anthers were large and plump, dehisced readily and supplied large amounts of pollen. In many cases, pummelo anthers dehisced completely before petal opening. Pollen germination percentage was relatively high for 'Java' and 'Pomelit' pummelo cultivars, ranging from 29.7 ± 4.48 to 74.3 ± 2.03 % using

standard germination conditions (Table 2.7).

In 1992, an additional treatment was included, whereby 'Pomelit' pollen was left with the dehisced anthers for a further 24 h and then applied directly onto the growth medium, i.e. 48 h after stripping the anthers from the blossoms. Pollen germination percentage was 53.0 % as opposed to 33.0 % using the standard method.

The germination percentage of 'Oroblanco' pummelo hybrid pollen ranged from 2.0 ± 0.71 to 10.0 ± 1.00 % and was significantly lower than that of pummelo cultivars (Table 2.7), but not significantly different from 'Valencia' selections (Table 2.5) and grapefruit cultivars (Table 2.6).

Table 2.7 Mean percentage *in vitro* pollen germination ± SE of two pummelo cultivars (*C. grandis* L.) and two pummelo hybrids conducted in 1990, 1991, 1992 and 1994 using an agar based semi-solid growth medium (20% sucrose [S]; 24h incubation period)

Cultivar	1990	1991	1992	1994
Java	-	66.0 ± 2.00 ^{mn}	-	-
Pomelit	29.7 ± 4.48^{de}	63.0 ± 4.58 ^{lmn}	33.0 ± 1.29 ^{gh}	74.3 ± 2.03 ^{f}
Oroblanco	-	-	2.0 ± 0.71 ^{ab}	10.0 ± 1.00 ^{abc}

*Means not followed by the same letter are significantly different at P ≤ 0.01 level of significance, within a year for all cultivars

2.4.7 'Satsuma' mandarins

In 1990, extremely small amounts of pollen were available to inoculate the growth medium and very little pollen was applied. The anthers were very small and pale yellow, but not white, indicating that small amounts of pollen were present. Although more pollen was available in 1991 than 1990, very little pollen was

available for counting.

One out of 300 'Kuno' and 'Owari Satsuma' mandarin pollen grains germinated in 1990 and none of the 'Miho Wase' or 'Imamura'. However, in 1991 pollen germination was 2.0±0.58 and 4.0±0.58 % for 'Owari' and 'Miho Wase', respectively, after a 24 h incubation period (Table 2.8). When incubated for 48 h, germination percentage increased slightly to 3.7 and 4.7 %, respectively.

Table 2.8 Mean percentage *in vitro* pollen germination±SE of four 'Satsuma' mandarin cultivars (*C. unshiu* L.) conducted in 1990 and 1991 using an agar based semi-solid growth medium (20 % sucrose; 24 h incubation period)

Cultivar	1990	1991	
		24h	48h
Owari	0.3±0.33[a]	2.0±0.58[ab]	3.7±1.20
Miho Wase	0.0[a]	4.0±0.58[abc]	4.7±0.67
Kuno	0.3±0.33[a]	-	-
Imamura	0.0[a]	-	-

[x]Means not followed by the same letter are significantly different at P≤0.01 level of significance, within a year for all cultivars

2.4.8 'Clementine' mandarins

In 1990, a wide variation in pollen germination percentage of closely related 'Clementine' mandarin selections occurred, ranging from 0.3 % for 'Oroval' to 59.3 % for 'SRA 92' (Table 2.9). No cognizance was taken of stage of blossom development.

In 1991, blossoms from the SRA (Corsican) selections had fully developed anthers that dehisced adequately resulting in plenty of pale yellow pollen. Germination of SRA selections ranged from 40.7±10.49 % for 'SRA 92' to 65.0±3.06 % for

'SRA 89' (Table 2.9). Blossoms of the Spanish 'Clementine' selections, 'Nules', 'Oroval' and 'Clemlate' were less developed, with resultant poor anther dehiscence and pollen availability. Germination percentage of the available pollen was relatively low (5.3 to 19.7 %) (Table 2.9). In 1992 and 1994, germination for 'Nules' pollen was of the same order as that of the SRA selections during 1991 (Table 2.9).

Table 2.9 Mean percentage *in vitro* pollen germination ± SE of ten 'Clementine' mandarin selections (*C. reticulata* Blanco) conducted in 1990, 1991, 1992 and 1994 using an agar based semi-solid growth medium (20 % sucrose [S]; 24 h incubation period)

Selection	1990	1991	1992	1994
SRA 63	13.0 ± 3.51^{b}	47.7 ± 1.86^{jkl}	28.8 ± 2.32^{g}	-
SRA 70	22.7 ± 1.20^{cd}	46.0 ± 5.13^{ijk}	-	-
SRA 84	-	53.3 ± 1.76^{jklm}	-	-
SRA 85	-	62.0 ± 4.36^{klmn}	-	-
SRA 88	-	29.3 ± 4.91^{fgh}	-	-
SRA 89	-	65.0 ± 3.06^{mn}	-	-
SRA 92	59.3 ± 3.33^{f}	40.7 ± 10.49^{ghij}	-	-
Nules	9.7 ± 1.73	5.3 ± 1.20	55.3 ± 1.65^{i}	40.3 ± 1.86^{d}
Oroval	0.3 ± 0.33	19.7 ± 1.45^{cdef}	-	-
Clemlate	32.3 ± 2.19^{e}	8.3 ± 1.20	-	-

Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

In 1992 and 1994 additional tests were conducted on 'Nules' pollen. The effect of stage of flower development and type of flower on pollen viability were tested. Anthers taken from flowers at full bloom, instead of the balloon stage, produced pollen with a 54.8 % germination index, i.e. similar to pollen from the balloon stage (55.3 %). Staminate flowers appeared to have better developed anthers which carried more pollen than perfect flowers. Germination of pollen from staminate

'Nules' flowers was 70.0 % after 48 h incubation, compared with 55.3 % using the standard procedure described. In 1994, pollen from freshly picked flowers resulted in a 49 % germination after 24 h incubation compared with 40.3 ± 1.86 % for pollen from anthers which dehisced overnight.

2.4.9 Other mandarins and mandarin hybrids

2.4.9.1 Tangors

i) 'Ellendale' selections and hybrids: In 1990, pollen germination for 'Ellendale' selections and hybrids varied widely, ranging from 1.0 ± 0.57 % for 'Leng' to 26.3 ± 2.91 % for 'CSFRI' (Table 2.10). 'Herps' flowers did not produce pollen.

In 1991, when more attention was paid to stage of blossom development, pollen germination was considerably higher and ranged from 17.7 ± 3.76 % ('Leng') to 52.0 ± 1.15 % ('Herps') (Table 2.10). 'Nouvelle', an 'Ellendale' x 'Novelty' hybrid had a very high germination percentage of 77.7 ± 5.24 %.

ii) 'Tambor': The poor germination percentage in 1990 can be attributed to poor blossom selection. 'Tambor' pollen was shown to be capable of a moderately high germination percentage of 38.5 ± 0.65 % (Table 2.10). Only anthers from well developed blossoms dehisced.

iii) 'Temple': 'Sue Linda Temple' pollen showed no pollen germination in 1990 and very low germination percentage in 1991 (Table 2.10).

2.4.9.2 Tangelos

i) 'Minneola': 'Minneola' tangelo pollen had a 10.0 ± 3.00 and 26.7 ± 3.76 % germination in 1990 and 1991, respectively (Table 2.10). In 1991, most anthers dehisced and copious amounts of pollen were available. However, the pollen was pale yellow in colour. Nevertheless, a high viability was indicated. In 1992 and

1994, pollen viability was even higher, with 58.5 ± 1.19 and 76.3 ± 3.18 % of the pollen germinating in the two years, respectively.

Table 2.10 Mean percentage *in vitro* pollen germination ± SE for various mandarins and mandarin hybrids conducted in 1990, 1991, 1992 and 1994 using an agar based semi-solid growth medium (20 % sucrose; 24 h incubation period)

Cultivar	Selection	1990	1991	1992	1994
Ellendale	CSFRI	26.3 ± 2.91^{cde}	25.7 ± 1.67^{defg}	-	-
	Koster	24.0 ± 2.52^{cd}	43.0 ± 2.08^{hij}	-	-
	Leng	1.0 ± 0.57	17.7 ± 3.76^{bcdef}	-	-
	Herps	[x]	52.0 ± 1.15^{jklm}	-	-
Ellendale X	Nouvelle	5.0 ± 1.15	77.7 ± 5.24^{n}	-	-
Tambor		0.0 ± 0.00	10.3 ± 1.45^{abcd}	38.5 ± 0.65^{h}	-
Temple	Sue Linda	0.0 ± 0.00	0.3 ± 0.33	-	-
Minneola		10.0 ± 3.00^{b}	26.7 ± 3.76^{efg}	58.5 ± 1.19^{j}	76.3 ± 3.18^{f}
Fairchild		22.3 ± 2.73^{c}	-	46.0 ± 1.47^{i}	-
Nova		0.3 ± 0.33	31.0 ± 0.58^{fghi}	11.3 ± 2.02^{def}	60.0 ± 7.94^{e}
Fortune		14.7 ± 0.88^{b}	-	-	-
Fremont		2.7 ± 0.67	-	-	-
Page		1.0 ± 0.58	-	-	-

Means not followed by the same letter are significantly different at $P \leq 0.01$ level of significance, within a year for all cultivars

[x] no pollen in anthers

2.4.9.3 'Clementine' hybrids

i) 'Clementine' x 'Orlando' tangelo hybrids: 'Fairchild' pollen had a moderate to high germination of 22.3 ± 2.73 and 46.0 ± 1.47 % in 1990 and 1992, respectively (Table 2.10). Pollen of 'Nova' did not germinate well in certain years (1990 and 1992), while in 1991 and 1994 high germination percentages of 31.0 ± 0.58 and 60.0 ± 7.94 % were achieved (Table 2.10).

ii) 'Clementine' x 'Dancy' hybrid: In 1990, 14.7±0.88 % of 'Fortune' pollen germinated (Table 2.10). No subsequent tests of 'Fortune' pollen were conducted.

iii) 'Clementine' x 'Ponkan' hybrid: 'Fremont' was only tested in 1990 when 2.7±0.67 % of the pollen germinated (Table 2.10).

iv) 'Minneola' tangelo x 'Clementine' hybrid: Only 1.0±0.58 % of 'Page' pollen germinated when tested in 1990 (Table 2.10).

2.4.10 Reaction of pollen grains on growth medium

Immediately after pollen application on the growth medium, pollen grains of various cultivars were viewed through the microscope. The shape of these grains was typically prolate (Fig. 2.1) and within 40 s changed to almost perfectly round, due to hydration of the pollen grain. Within two hours of application, the first pollen grains germinated and started to grow, especially with the citrus types with high germination indices, e.g. pummelos and certain mandarins.

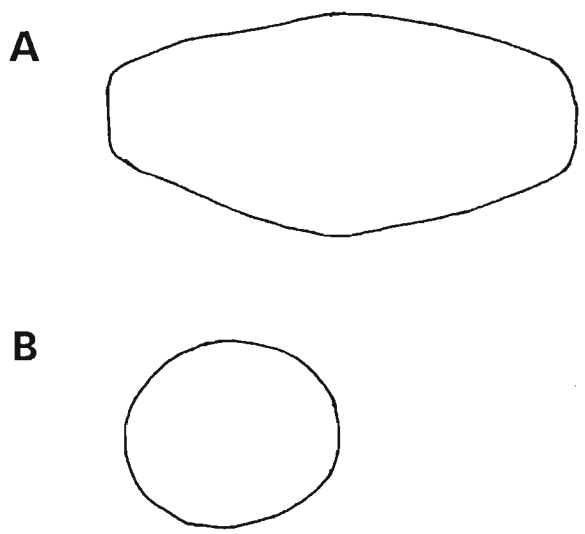

Fig. 2.1 Schematic representation of citrus pollen grains before (A) and after (B) rehydration on the *in vitro* growth medium (ca. x 1 000)

2.4.11 Effect of carbohydrate source and incubation period

Paired 't' tests showed that there were no significant differences in % germination between the carbohydrate sources used or for the two incubation periods tested (Table 2.11).

Table 2.11 Mean percentage *in vitro* pollen germination ± SE of sixteen cultivars/ selections conducted in 1991 using an agar based semi-solid growth medium as control (20 % sucrose [S]; 24 h incubation period) compared with an extended incubation period (48 h) and alternative carbohydrate sources (20 % glucose [G], and 20 % sucrose + glucose [S + G])

Cultivar	S		G		S + G	
	24h	48h	24h	48h	24h	48h
Eureka	9.7 ± 0.88	13.3 ± 1.45	8.3 ± 0.88	9.0 ± 0.00	10.0 ± 3.46	9.7 ± 3.28
Fino	10.0 ± 2.08	14.0 ± 0.58	15.7 ± 1.45	15.3 ± 0.88	20.3 ± 0.33	18.3 ± 4.48
Verna	12.0 ± 1.00	17.0 ± 0.58	3.3 ± 0.88	5.3 ± 0.33	6.3 ± 1.45	6.3 ± 0.33
Delta	0.0 ± 0.00	0.3 ± 0.33	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.33	1.0 ± 1.00
Midknight	3.0 ± 0.58	3.0 ± 1.00	-	-	-	-
Valencia Late	11.0 ± 1.53	7.3 ± 0.88	4.7 ± 1.20	4.3 ± 0.33	4.7 ± 0.67	2.3 ± 0.33
Java	66.0 ± 2.00	65.7 ± 0.33	*89.0	90.0	85.0	88.0
Pomelit	63.0 ± 4.58	56.7 ± 2.33	-	-	-	-
SRA 63	47.7 ± 1.86	67.3 ± 3.18	-	-	-	-
SRA 70	46.0 ± 5.13	46.3 ± 2.40	-	-	-	-
SRA 84	53.3 ± 1.76	53.3 ± 1.73	-	-	-	-
SRA 85	62.0 ± 4.36	67.2 ± 3.84	-	-	-	-
SRA 88	29.3 ± 4.91	36.7 ± 4.67	-	-	-	-
SRA 89	65.0 ± 3.06	65.0 ± 3.21	-	-	-	-
SRA 92	40.7 ± 10.49	35.7 ± 7.26	12.3 ± 2.40	6.7 ± 1.20	10.3 ± 0.88	14.3 ± 0.67
Nules	5.3 ± 1.20	6.3 ± 0.33	-	-	-	-

*Where no SE is given, only one replication was used

With lemons, variable results were achieved when glucose was included in the growth medium; decreasing for 'Verna' and increasing for 'Fino', while germination percentage for 'Eureka' remained similar to the sucrose containing medium (Table 2.11).

'Delta' pollen always had a very low germination percentage (0.0 to 1.3 ± 0.48 %) (Table 2.5 & 2.11), even when glucose or sucrose + glucose were used as alternative carbohydrate sources to sucrose and also when germinated for 48 h.

For 'Java' and 'Pomelit' pummelos, germination percentage did not increase substantially when the incubation period was extended to 48 h where alternative carbohydrate sources to sucrose alone were used. However, the use of glucose as a carbohydrate source, increased germination percentage from 66.0 to over 87.0 % on average (Table 2.11).

In 'Clementine', when incubated for an extra 24 h, germination percentage tended to increase slightly. Alternative carbohydrate sources to sucrose alone resulted in less germination with 'SRA 92' (Table 2.11).

2.5 Discussion

2.5.1 Procedures

Arising from the results, the following general aspects pertaining to the procedures used require further comment.

i) Effect of stage of blossom development: With virtually all the cultivars/selections tested, pollen germination percentage was lower when blossoms were picked at a stage of incomplete anther development. This resulted in poor *in vitro* anther dehiscence. Increased pollen germination percentage was achieved when blossoms were picked at a slightly later, more mature stage, except for pummelos. Therefore, it is proposed that blossoms should be picked rather at

a stage of development just after the balloon stage, i.e. when the first petal begins to fold back immediately prior to anthesis. *In situ*, anthesis occurs soon after this stage, before the third petal folds back (data not shown).

No account of environmental factors, such as temperature and relative humidity, has been given. By picking blossoms in the morning, adverse climatic conditions can be circumvented. Cultivar specific natural levels of pollen viability and fertility appear to be universal in citrus, since comparable levels are achieved in different seasons and in different geographic regions. Therefore, stage of blossom development appears to be an over-riding factor in successful pollen germination.

ii) Post-harvest handling of blossoms: Although pollen germination levels were adequate and comparable with the results of other researchers, e.g. Sahar & Spiegel-Roy (1980), Burger, (1985), de Lange (1989), and Crescimanno *et al.* (1988a; 1988b), adaptations to the post-harvest handling of blossoms could improve germination percentage.

Pilot trials should be conducted to test the effect of immediately stripping the anthers in the orchard and placing them in suitable receptacles to dehisce. These receptacles should be stored in a cool box in the shade. Pollen could then be applied onto the growth medium later the same day, i.e. within 6 h of picking the blossoms, resulting in fresher pollen being used for the germination test. However, practicalities associated with handling numerous cultivars in a given time period must not be overlooked.

iii) Carbohydrate source: Differences between different cultivars on sucrose or glucose-containing growth media indicated that, in most cases, sucrose alone was adequate, there being no significant differences between carbohydrate sources. However, 'Java' pummelo germinated slightly better on media containing glucose. Similar results were achieved by de Lange (1989). When de Lange (1989) took length of pollen tubes into account, sucrose proved to be a better option than glucose as the carbohydrate source for citrus pollen germination. Therefore, the

germination conditions were suitable for pollen viability assays, and a standard procedure should be used for reporting purposes and comparisons with other workers.

iv) Incubation time: In general, a longer incubation period resulted in slightly, but not significantly, higher germination percentage. Although pollen germination increased slightly, but not significantly, when calculated after 48 h, instead of 24 h, the shorter incubation period was sufficient to achieve comparable results, and it appears that 24 h is adequate for the purpose of the study. In addition, there is also the danger of fungal infection with a longer period, complicating counting.

v) Contamination: Great care was taken to avoid contamination of one pollen type by another during every step of the procedure. Contamination is especially important with pollen sterile cultivars/selections or those with very low germination percentages, as only a few viable pollen grains can result in spurious results.

2.5.2 Lemon

The lack of pollen and poor germination was considered strange as seed can be found in lemons planted in solid, isolated blocks, and pollen may be observed on naturally dehisced anthers after petal opening (pers. obs.) In addition, observations in solid blocks of lemons indicated that, although bees were present they carried relatively little pollen, but this pollen was not identified as being weed pollen or lemon pollen. Crescimanno *et al.* (1988b) achieved 19 % pollen germination with 'Eureka' lemon using the hanging drop technique. Earlier workers showed a high degree of pollen sterility in lemons (Frost & Soost, 1968). This indicated that the technique used needed to be improved to achieve results similar to those reported in the literature; blossom development, growth medium and time of incubation were considered.

Following the poor germination of lemon pollen in 1990 attempts were made to improve the technique in order to achieve similar results to other workers. This was

achieved in subsequent tests. Crescimanno *et al.* (1988b) demonstrated 19.0 % germination of 'Eureka' pollen grains using the hanging drop method, and 7.6 to 37.2 % for different 'Femminelli' selections. The majority of their results ranged from 6 to 20 % , i.e. similar to the 1991 and 1994 results in this study, thus indicating that germination conditions were suitable for lemon pollen.

Lemon pollen had a relatively low germination percentage (10 to 20 %), and relatively little pollen was available for germination tests and is available *in situ*. Frost & Soost (1968) stated that it was clear that a high degree of pollen sterility occurs in all lemon cultivars, possibly due to meiotic abnormalities. Therefore, lemons can be considered as relatively inefficient pollinators.

2.5.3 'Navel' oranges

The pale green anthers indicated a lack of pollen and that 'Navel' oranges are completely male sterile (Frost & Soost, 1968).

2.5.4 Midseason oranges

Pollen germination percentages of midseason oranges were generally low, usually ranging from 6 to 18 %. De Lange *et al.* (1974) described 'Tomango' as a poor pollen source for 'Tambor' tangor and 'Shamouti' was even less effective. Zacharia (1951) reported that, for 'Shamouti', 38 % of the pollen was shrivelled and pale, 23 % appeared normal and 39 % were large and normal. Germination results have been variable with usually less than 10 % of the pollen germinating, while Ozsan (1961) obtained a maximum of 27.9 %.

Midseason oranges are considered to be relatively poor pollinators.

2.5.5 'Valencia' oranges

i) 'Delta Valencia' orange: Pollen of 'Delta' was scarce and availability was

similar to that of 'Satsuma' mandarins. Germination percentage was very low, seldom more than 1 %. Similar results were achieved by de Lange (pers. comm[3].) who claimed a 1 % germination rate for 'Delta' pollen. Therefore, 'Delta' pollen is functionally sterile and 'Delta' can be rated as similar to 'Satsuma' mandarins in terms of pollination ability.

ii) 'Midknight Valencia' orange: The low germination percentage of 'Midknight' pollen makes it a weak pollinator.

iii) 'Valencia' orange selections: Germination percentages of seedy 'Valencia' selections were generally less than 11 %, in close agreement with Sahar & Spiegel-Roy (1980), Burger (1985), Mustard *et al.* (1956) and Krezdorn & Robinson (1958). High percentages (40 to 60 %) of empty pollen grains have been reported for 'Valencia' orange (Uphof, 1934). Cameron *et al.* (1960) indicated that low seed production was partly due to a lack of functional pollen. Therefore, old clone 'Valencia' oranges are considered to be relatively inefficient pollinators. However, de Lange (1980) noted that, in Morocco, 'Valencia' oranges were planted between 'Clementine' mandarins as pollinators to increase fruit set, with resultant seediness.

Iwamasa (1969) reported that chromosomal change was the main cause of gametic sterility and consequently low seediness in 'Valencia' orange.

2.5.6 Grapefruit

'Star Ruby' pollen had very low germination percentages. Burger (1985) achieved similar results with no pollen germination in 20 % sucrose and 1 % germination with 25 % sucrose after a 48 h incubation period. Therefore, 'Star Ruby' has no functional pollen and can be considered functionally pollen sterile.

Initially, the lack of pollen germination was surprising. It is speculated that this may

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[3]J.H. de Lange, National Botanic Gardens, Kirstenbosch

be attributed to the irradiation involved in the development of 'Star Ruby' (Hensz, 1971) which effectively 'sterilized' it. Although this is unsubstantiated, this concept may have far reaching implications and is worth pursuing regarding the development of self- and cross-incompatible mandarin cultivars through induced somatic mutations by irradiation. If this concept is true, then orchard layout may be simplified as adjacent cultivars will not be able to induce seediness in that cultivar. Irradiated cultivars are seedless due to pollen or ovule sterility or incompatibility due to chromosome aberrations. In 'Star Ruby', pollen sterility exists as described above; substantial ovule sterility also occurs as few seeds set when cross pollinated (Burger 1982; 1985; Barry, unpubl.) and numerous aborted, undeveloped seeds are present in 'Star Ruby' (pers. obs.).

Germination percentage of the other grapefruit cultivars tested was usually from 8 to 13 %. Burger (1985) managed to germinate 5 % of 'Ruby Red' pollen under similar conditions. Grapefruit generally have low percentages (5 to 15 %) of well developed pollen (Friend *et al.*, 1939; Ozsan, 1961; Louw, pers. comm[4].).

Grapefruit cultivars are considered to be weak pollinators.

2.5.7 Pummelo

Pummelo anthers dehisced before petal opening and readily produced large amounts of pollen. Very high germination percentages were achieved and results were of a similar order to those of de Lange *et al.* (1974) and de Lange & Vincent (1988). Pummelo pollen is reported to be highly viable, and when used as a pollen parent in pollination studies causes high fruit set percentages and seediness in the maternal parent.

Higher germination percentages were achieved when glucose was included in the growth medium. However, de Lange (1989) found that sucrose was generally

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superior to glucose for citrus pollen germination assays when pollen tube length is an additional index of viability. The present study shows that glucose is possibly more suitable than sucrose for pummelos. In order to standardize the procedures used, sucrose is suitable to show differences between high and low pollen viability.

There is no obvious explanation for why 'Pomelit' pollen had a higher germination percentage after being left in the anthers for an extra 24 h. Perhaps the pollen matured further whilst in the dehisced anthers. In future studies, this finding should be tested with other cultivars.

The low viability of 'Oroblanco' pollen is due to its triploid genetic make-up (Soost & Cameron, 1980). Irregular chromosome reduction leads to gametic sterility or embryo abortion and, therefore, almost complete sterility in triploids (Frost & Soost, 1968).

2.5.8 'Satsuma' mandarins

The pale yellow anthers are indicative of poor pollen availability. Very low germination percentages were achieved; comparable with that of 'Delta' and 'Midknight Valencia'oranges. 'Satsuma' mandarins produce very low percentages of well developed pollen (Frost & Soost, 1968). Therefore, the lack of functional pollen make 'Satsuma' mandarins practically pollen sterile.

2.5.9 'Clementine' mandarins

Following the 1990 results, it was speculated that the wide variability in germination percentages amongst 'Clementine' selections was due to the effect of climatic conditions on the different selections. In subsequent years, large seasonal differences were apparent. This could be attributed to numerous factors, but Allan (1963), Morkel (1977) and Sedgley (1977) have shown that the effects of climate, specifically high day temperatures and low relative humidity, play a major role in pollen viability. However, the stage of flower development also appears to play a

major role. The germination percentages achieved after 1990 (40 to 60 %) were of the same order as those achieved by Sahar & Spiegel-Roy (1980) and Crescimanno *et al.* (1988a).

Pollen germination did not change for pollen taken from 'Nules' anthers at full bloom. However, if picked too early, low germination percentages occurred. Staminate 'Nules' flowers had more viable pollen than perfect flowers.

Fresh pollen had a higher germination percentage. However, in the assay it is necessary for the anthers to dehisce before sufficient pollen is available for application to the growth medium, and hence a waiting period is required. Furthermore, the germination percentage of 'Clementine' pollen is sufficiently high using this technique and comparable with other researchers (Sahar & Spiegel-Roy, 1980; Crescimanno *et al.*, 1988a).

'Clementine' pollen has a high germination percentage and is a potential pollinator. Therefore, pollen sterile cultivars (buffers) planted alongside 'Clementine' orchards to prevent cross-pollination, should be carefully selected to prevent excessive seediness in the buffer cultivars that are compatible with 'Clementine' pollen.

2.5.10 Other mandarins and mandarin hybrids

Certain mandarins are known to be strong pollinators causing the set and development of high seed numbers in the fruit of adjacent cultivars. This is particularly true of compatible mandarin cultivars planted alongside one another.

The various 'Ellendale' selections had high pollen germination percentages, higher than midseason oranges and less than 'Clementine' selections. Therefore 'Ellendale' can be classified as a potentially strong pollinator.

'Tambor' pollen also had a high germination percentage and is, therefore, a potentially strong pollinator.

'Sue Linda Temple' pollen germination was very low, and of the same order as 'Delta Valencia'. It is presumed that germination conditions were not ideal as 'Temple' tangor fruit usually have high seed content (Saunt, 1990). However, 'Sue Linda' was selected in Florida for low seed content, with eight seeds per fruit, on average. Therefore, 'Sue Linda' pollen must be viable to be self-compatible. Further germination tests need to be conducted for 'Sue Linda'.

'Minneola' pollen is capable of very high germination percentages and is, therefore, classified as a potentially strong pollinator.

'Fairchild' and 'Nova' pollen germination was similar to that of one of their parents, 'Clementine', and these cultivars are therefore potentially strong pollinators.

Although 'Fortune' pollen only showed weak pollination potential, due to relatively low germination, it is known that 'Fortune' pollen causes unacceptably high seed numbers in Spanish 'Clementine' orchards (pers. obs.). Therefore, since this result is only from one season's viability test, further tests are required before these results can be used.

Both 'Fremont' and 'Page' had very low germination percentages, similar to those of 'Midknight Valencia' and 'Delta Valencia', respectively. Additional tests are required before further interpretations of these data can be made.

2.6 Conclusions

In vitro pollen germination assays provide a means to separate cultivars according to their pollination potential. A detailed analysis is given in Chapter 4 and will not be repeated here.

The technique used to determine germination percentage of citrus pollen appears to be adequate. However, great care should be taken to ensure that blossoms are picked at the optimal stage of development, i.e. just after initial petal opening,

immediately prior to anthesis.

It was known that 'Navel' oranges and 'Satsuma' mandarins are pollen sterile and could be used as buffer cultivars between two strongly fertile, compatible cultivars. This study has provided two additional cultivars, which are adapted to hot citrus production areas and are of commercial importance. The two additional cultivars are 'Delta Valencia' orange and 'Star Ruby' grapefruit. Therefore, there is now a wide choice of buffer cultivars of commercial importance that can be planted in all citrus production areas of southern Africa.

CHAPTER 3

IN VIVO POLLEN VIABILITY

3.1 Introduction

Pollen may not germinate *in vitro*, or may indicate a low viability, and yet give a high percentage of seeds (relative to the number of ovules) when used *in vivo* (Johri & Vasil, 1961). Since *in vitro* germination media may be deficient in growth factors when compared to those of the stigmatic surface or it may inhibit the pollen growth or may allow a pollen tube to grow only a short distance, it is often suggested that the only valid test is application of pollen to a compatible stigma and observation of pollen tube growth in the style or seed development. While *in vivo* growth criteria are not always valid, and the methods are slow and laborious, they do, in many instances, afford a more genuine measure of the quality of extracted or stored pollen than *in vitro* germination assays (Stanley & Linskens, 1974).

Assuming that the primary purpose of a viability assay, as distinct from a fertility assay, is to determine if the pollen can germinate, then an *in vivo* test is often the most valid. This test requires that pollen be placed on a stigma, the style be removed after a period and the number of tubes growing down the style be determined. However, certain assumptions made in this test may not always be valid. If the stigmatic surface or style inhibits pollen tube penetration, the test is invalid (Stanley & Linskens, 1974).

Once pollen tubes penetrate the style, the problem is how to view and assay them. Staining techniques which differentiate between the pollen tubes and the stylar tissue have been developed. Such preparations are made by gently crushing open the styles. Various preparation techniques are required for softening and mounting, depending on the species (Stanley & Linskens, 1974).

A sensitive and easily applied method of detecting germinated pollen tubes is based

on the fluorescence of callose in ultraviolet light (Martin, 1959; Kho & Baër, 1968; de Lange, 1973; Stanley & Linskens, 1974). Therefore, *in vivo* pollen growth tests depend on the occurrence of callose, which can be selectively stained with water-soluble aniline blue or fluorochrome dyes which fluoresce under ultra-violet light (Linskens & Esser, 1957; Stanley & Linskens, 1974). The dye links to the callose in germinating pollen tubes (Currier, 1957) and when viewed using a microscope with epilumination at 356 nm, callose appears light yellow-green.

Difficulties can occur in attempting to use the *in vivo* germination technique to assay pollen viability. Genetic incompatibility barriers to germination and growth of the pollen must not be present. The pollen must be applied to the stigma at a time when the stigma is mature and receptive to pollen growth. The application of excessive pollen may also distort the results (de Lange *et al.*, 1979a). A marked lowering of air temperature after pollination can drastically modify pollen germination *in vivo*, and lead to an apparently low viability (Stanley & Linskens, 1974).

The capacity of pollen to give rise to viable seed is often used as a criterion of pollen viability. This technique can be the most valid one, or conversely, because of interfering substances or incompatibility, it may be an unreliable index of viability. Where the time delay from pollination to harvest is not important, and potential sources of interference are recognized, this method may be preferred in determining pollen viability (Stanley & Linskens, 1974).

In this technique the stigmas of emasculated flowers are pollinated. The stigma must usually be protected from stray, non-controlled pollinations; cloth, plastic or paper bags are frequently used to cover the pollinated flowers (Stanley & Linskens, 1974). However, this is not always essential (Burger, 1982). When the fruit is mature the seeds must be removed and counted. A comparison of seed set to flowers naturally pollinated is generally made and a seed development index calculated. Occasionally, assays with dead pollen or pollen diluted with a large amount of inert substances, such as talc powder or aluminium oxide, are used (de Lange *et al.*, 1979a; Holcroft, 1988). Since parthenocarpic fruit set can be induced

by dead or poorly germinating pollen (Gustafson, 1939; Visser, 1955), this is an important control in this assay. However, seed set is usually the more important factor studied.

Since a relatively large quantity of pollen is applied to each stigma, the chances of a viable pollen grain fertilizing an ovule are quite high even with pollen which germinates poorly during *in vitro* assays. Brown & Krezdorn (1969) and de Lange *et al.* (1979a) developed techniques whereby known amounts of pollen could be applied to citrus stigmas. Visser (1955) compared *in vitro* germination assays in a study of fruit set in apples, pears and tomatoes. Tests with tomato pollen which did not germinate *in vitro* showed that the use of this pollen still resulted in fruit set and often in as many seeds as set by fresh pollen. The use of apple pollen which yielded only 35 % (considered low) germination *in vitro* resulted in good fruit set. This suggests that even though pollen may be relatively low in viability, i.e. 40 % or less, it will still form fruit normally. De Lange (pers. comm.[1]) observed that even when *in vitro* pollen germination in certain citrus cultivars was less than 5 %, numerous seeds still developed. Therefore, the amount of pollen being applied to the stigma must be controlled.

In the present study, *in vivo* pollen viability assays, including pollen tube growth and seed development, have been used to complement the *in vitro* viability assays. *In vitro* pollen germination thresholds can be determined and correlated to the effect of pollen on seed or fruit set to demonstrate some relationship between *in vitro* and *in vivo* pollen viability. This is discussed in more detail in Chapter 4. In the present chapter, the effect of strongly and weakly fertile pollen parents on pollen tube growth and seed set in 'Nules Clementine' mandarin and 'Delta Valencia' orange were investigated. The objective with the former cultivar was to study *in vivo* pollen tube growth, seed set and fruit development using various pollen sources. Ideally seed development should be limited in this cultivar. 'Delta Valencia' orange was used as a pollen parent to test its pollen compatibility reaction with cross-pollinating cultivars and to determine whether 'Delta Valencia'

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is ovule sterile, i.e. seedless under all conditions, even when pollen from strongly fertile cultivars is used as a pollinator. The results from this aspect of the study are then linked with those of the previous section in an attempt to develop a prediction model whereby the cross-pollination ability of a cultivar or selection can be determined.

3.2 Materials and methods

3.2.1 Pollen extraction and dilution

In order to apply known quantities of pollen to the stigmas of citrus flowers (de Lange *et al.*, 1979a), and not to apply copious amounts of pollen, by simply rubbing dehisced anthers of selected pollen parents against the stigma of the female parent, pollen was extracted and diluted with an inert substance. Anthers and pollen, collected in the same manner as for the *in vitro* viability assay (Chapter 2), were sieved over a 90 μm fine mesh sieve. Pollen was collected in a small bottle and weighed. The pollen was then mixed with 400 grit silicon carbide (approximately 30 μm) to give a 10 % (m/m) pollen-powder mix.

The germination ability of diluted pollen was also determined *in vitro* (as described in Chapter 2) to determine any effect of silicon carbide on pollen viability. Pollen germination was not statistically analysed since the objective was to determine whether the pollen still germinated after being mixed with silicon carbide and, due to the difficulties associated with distinguishing ungerminated pollen from silicon carbide crystals, there may have been a bias towards counting germinated pollen grains leading to an incorrect interpretation of the results.

The bottles containing the diluted pollen mix were placed in a cool box before being taken to the orchards for *in vivo* pollen viability studies.

3.2.2 Effect of selected pollen source on seediness

The emphasis of this aspect of the study was to develop a relationship between pollen germination and seediness in various citrus cultivars. Strong, intermediate and weak pollen parents (Table 3.1) were selected following *in vitro* pollen viability assays according to the percentage of pollen germination.

The female parents selected were 'Nules Clementine' mandarin and 'Delta Valencia' orange (Table 3.1). The objective with the former cultivar was to study *in vivo* pollen tube growth, seed set and fruit development using various pollen sources in order to identify potentially weak pollinators since seed development in 'Nules Clementine' should be limited. 'Delta Valencia' orange was used as a pollen parent to test its pollen compatibility reaction with cross-pollinating cultivars and to determine whether 'Delta Valencia' is ovule sterile, i.e. seedless under all conditions, even when pollen from strongly fertile cultivars is used as a pollinator.

For each treatment combination, 80 flowers were hand pollinated. Flowers at the balloon stage of development from leafy inflorescences were selected for the two female parents. They were carefully depetalled and emasculated, ensuring that no self-pollination occurred. The pollen-powder mix of selected cultivars (Table 3.1) was then dusted onto the stigmas of these flowers using the same method used when inoculating the growth medium. The 'Nules Clementine' hand pollinations were done at Bakgat Boerdery, Schoemanskloof (25°25′ S; 30°30′ E) in 1992/93 and 1994/95. In 1992/93, 'Delta Valencia' flowers were hand pollinated with selected pollen sources at Crocodile Valley Estates, Nelspruit (25°30′ S; 31°05′ E) and La Rochelle Estates, Malelane (25°30′ S; 31°25′ E), and at Crocodile Valley Estates in 1994/95 (Table 3.1).

The flowers were then labelled and, in all treatments except in the case of the open-pollinated treatment, the flowers were bagged to prevent cross pollination by insect vectors. This step is not absolutely necessary as it has been reported that bees do not readily forage in depetalled and emasculated flowers (Burger, 1985). However, it was considered prudent to bag the flowers to reduce any chance of foreign pollen pollinating the flowers.

Table 3.1 Combinations of pollinations conducted in 1992 and 1994 to determine the effect of selected pollen sources on seed content in 'Nules Clementine' mandarin and 'Delta Valencia' orange

Female parent	Pollen parent	Pollination potential	Year studied
Nules Clementine	Delta	weak	1992; 1994
	Valencia Late	intermediate	1992
	Pomelit	strong	1992; 1994
	Nules	self-incompatible	1992; 1994
	Minneola	strong	1992; 1994
	Nova	strong	1992; 1994
	Midknight	weak	1994
	Eureka	weak	1994
	self	self-incompatible	1992
	open	self-incompatible	1992; 1994
Delta Valencia	Pomelit	strong	1992; 1994
	Minneola	strong	1992; 1994
	Valencia Late	intermediate	1992
	Delta	weak	1992
	Nova	strong	1994
	Nules	strong	1994
	Marsh	weak	1994
	Valentine	weak	1994
	self	weak	1992
	open	weak	1994

After 1, 4 and 8 d in 1992 and 2,5 and 11 d in 1994, 20 fruitlets and pistils for each treatment were excised from the trees and fixed in 3 % gluteraldehyde (in 1992) or Carnoy's solution (Brooks *et al.*, 1950) (in 1994). The pistils and ovaries were fixed for 2 h in Carnoy's solution and then stored in 70 % ethanol. The excised flower parts, pistils and ovules, were later prepared for fluorescence microscopy when *in vivo* pollen tube growth was studied. The remaining 20 fruitlets were allowed to grow to maturity and were harvested 27 weeks after pollination, at which stage the seeds were counted.

3.2.3 Fluorescence microscopy and *in vivo* pollen tube growth preparation

The following steps were complied with during the preparation of specimens for fluorescence microscopy (Martin, 1959; Sedgley, 1976):

i) Series hydration of Carnoy fixed material: decant the 70 % ethanol in which the specimens were stored, hydrate for 10 min each with a 70:50:30 % ethanol series. The material fixed in gluteraldehyde was rinsed twice with distilled water for 10 min each.

ii) Softening: The samples were softened in 8 N NaOH at 60°C for 1 h. The material turned yellow then orange after 1 h; where the specimens were insufficiently soft to squash, the process was continued for up to 2 h, ensuring that the samples did not become too tender.

iii) Clearing: The orange-coloured samples were then cleared by bleaching with 100 % commercial sodium hypochlorite (Jik ® = 3.5 % sodium hypochlorite). After 10 min the stigmas turned white and clear; the ovaries cleared after 15 min and at removal after 20 min approximately two-thirds of the style remained pale brown. The samples were then rinsed with distilled water.

iv) Staining: The material was stained in 0.1 % aniline blue for 12h. The aniline blue was buffered in an 0.1 M K_3PO_4 solution at pH 12.4.

v) Mounting: One drop of aniline blue solution was placed on a microscope slide. The sample was placed on the slide, dissected longitudinally and then carefully squashed with a cover slip.

vii) Microscopy: The slides were studied under a fluorescence microscope with epilumination at 356 nm. Pollen tubes were observed as thin diaphanous, gossamer threads interspersed with dense plugs. Where possible, pollen

tubes were counted in the stigmatic area, style and in the ovaries.

3.2.4 Statistical analyses

An analysis of variance (ANOVA) of the number of pollen tubes detected for each treatment was conducted. Where significant differences occurred, the mean number of pollen tubes per style was separated by least significant differences (L.S.D.).

3.3 Results

3.3.1 Effect of silicon carbide on *in vitro* pollen viability

Where the dilution of citrus pollen was initially assessed to determine the effect of silicon carbide on pollen viability, it was difficult to distinguish the ungerminated pollen grains from the silicon carbide crystals using a compound microscope. However, after careful inspection at 100 x magnification, germinated and ungerminated pollen grains were scored.

For the first batch of pollen prepared in spring 1992, the presence of silicon carbide had the following effects:

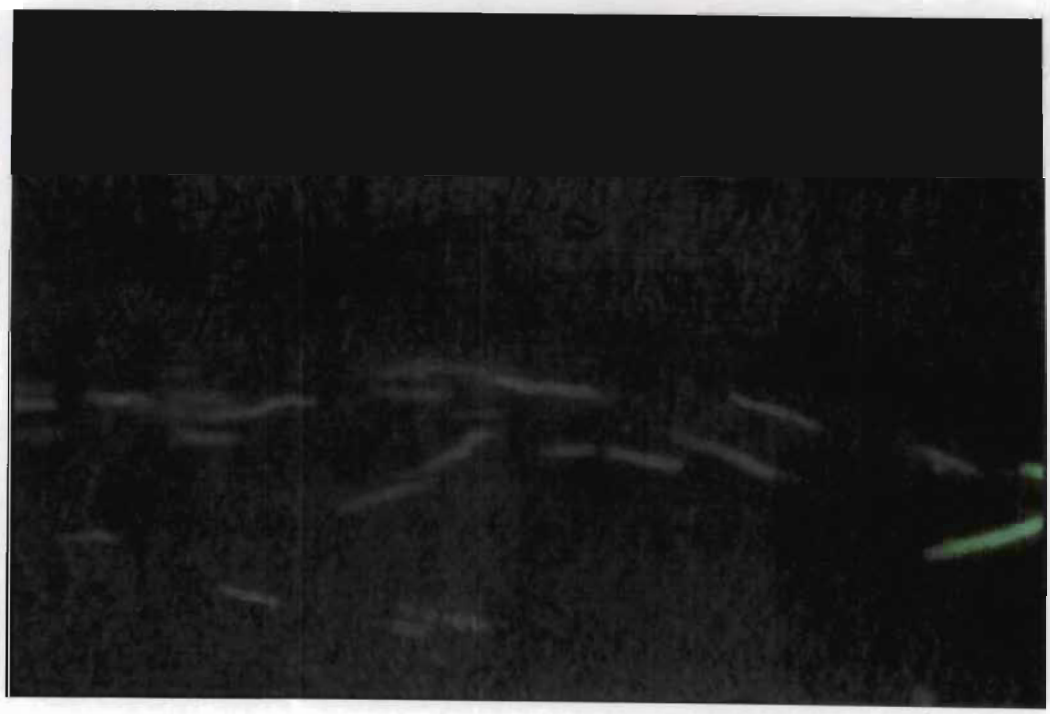
i) No germinated pollen grains were observed with 'Delta Valencia' orange, even after a thorough search.
ii) 'Nova' mandarin hybrid and 'Marsh' grapefruit had occasional germinated pollen grains.
iii) Numerous pollen grains of 'Nules Clementine' mandarin and 'Valencia Late' orange germinated.
iv) 'Minneola' tangelo and 'Pomelit' pummelo had more germinated pollen grains than 'Nules' and 'Valencia Late'.

When the second batch of pollen was prepared for *in vitro* viability determinations,

0.8 % of 'Delta Valencia' pollen germinated, 13.5 % of 'Valencia Late' pollen, 58.0 % of 'Minneola' and 53.0 % of 'Pomelit'. The results were comparable with those of Tables 2.5, 2.10 and 2.7, respectively, for the relevant cultivars.

3.3.2 *In vivo* pollen tube growth in 'Nules Clementine' mandarin

The pollen tubes were observed as thin diaphanous, gossamer strands interspersed with dense plugs (Photograph 3.1). In very few instances the pollen tubes apparently grew up and away from the stigmatic papillae, while most grew into the stigmatic surface towards the central stylar canal. The callose, observed as fluorescence, was not continuous. The pollen tubes ran parallel to the vessels alongside the stylar canal. They grew in a disorderly manner, often overlapping.



Photograph 3.1 Photomicrograph of fluorescing 'Pomelit' pollen tubes in 'Delta Valencia' styles (mag. x 160)

i) 1992/93: Hand pollinated 'Nules Clementine' styles removed after 1 d showed no fluorescence where 'Delta', 'Nova', 'Minneola' or 'Pomelit' pollen were used (Table 3.2). In addition, no pollen tubes were evident where flowers were self-pollinated. 'Valencia Late' and 'Marsh' pollen produced a mean of 0.3 and 0.5

pollen tubes per pistil (Table 3.2). Significantly more pollen tubes were observed where 'Nules' pollen was applied by hand to the stigmas and in open pollinated flowers (Table 3.2). Where observed, pollen tubes were only in the stigmatic area after 1 d.

Table 3.2 Mean number of pollen tubes observed per style using fluorescence microscopy to study *in vivo* pollen tube growth in 'Nules Clementine' mandarin (*C. reticulata* Blanco) styles following pollination with selected pollen sources (1992/93)

Pollen source	Mean no. of pollen tubes observed x days after pollination		
	1	4	8
Delta	0.0[a]	0.0[a]	0.0[a]
Valencia Late	0.3[a]	2.8[a]	5.3[ab]
Marsh	0.5[a]	4.7[ab]	0.0[a]
Nova	0.0[a]	29.0[d]	1.0[a]
Minneola	0.0[a]	17.7[cd]	1.8[a]
Pomelit	0.0[a]	24.2[cd]	9.2[b]
Nules	11.2[b]	20.7[cd]	3.8[ab]
Self[x]	0.0[a]	14.7[bc]	26.8[c]
Open[y]	20.3[c]	4.8[ab]	25.0[c]
LSD	$P \leq 0.01$	$P \leq 0.05$	$P \leq 0.05$

[x]self pollinated control

[y]open pollinated control

Means not followed by the same letter are significantly different at the specified level of significance

After 4 d, significant differences in pollen tube growth were discerned between the different pollen sources. 'Delta' did not show any pollen tube activity, while 'Valencia Late' (2.8 pollen tubes per stigma), 'Marsh' (4.7) and open pollinated 'Nules' flowers (4.8) were not statistically different to 'Delta' ($P \leq 0.05$), although a small number of pollen tubes were evident (Table 3.2). Self-pollinated flowers

(14.7) had significantly more pollen tubes in their styles than from 'Delta' and 'Valencia Late' pollen, while 'Minneola' (17.7), 'Nules' (20.7), 'Pomelit' (24.2) and 'Nova' (29.0) had significantly more pollen tubes per stigma (Table 3.2) than the other pollen sources. Where pollen tubes were observed, they were mainly in the stigma and entering the top of the style after 4 d.

After 8 d, no 'Delta' or 'Marsh' pollen tubes were observed, and very few 'Nova' (1.0) and 'Minneola' (1.8) pollen tubes were observed per style. Slightly, but not significantly, more were observed where 'Nules' (3.8) or 'Valencia Late' (5.3) pollen were used. 'Pomelit' pollen resulted in an average of 9.2 pollen tubes, while self (26.8) and open (25.0) pollinated flowers had significantly more pollen tubes than all other pollen sources (Table 3.2). At this stage the pollen tubes were observed through the entire length of the style.

ii) 1994/95: Two days after hand pollinating 'Nules' flowers with pollen from selected cultivars, pollen tubes could be detected in the stigmatic area, growing towards the base of the stigma, with all cultivars used, except 'Eureka' (Table 3.3).

There were significant differences between pollen cultivars ($P \leq 0.01$). According to the separation of the mean number of pollen tubes, the different cultivars could be separated into four groups; few to no pollen tubes were produced by 'Midknight' (4.3 pollen tubes detected per stigma) and 'Eureka' pollen (none); 'Pomelit' (17.0), 'Nules' (17.7) and open pollinated (16.8) treatments were in the second group (on average, approximately 17 tubes observed), 'Minneola' (33.7) (and 'Delta' [35.3]) in the third group, and 'Nova' (46.3) produced the most pollen tubes.

Pistils that were removed and fixed after 5 d showed considerably less tube growth than after 2 d, except for 'Nova'. 'Midknight' (0.5), 'Delta' (0.7) and 'Nules' (1.0) produced very few pollen tubes that could be detected. Open pollinated flowers (4.2) and 'Pomelit' (5.2) pollen produced significantly more pollen tubes ($P \leq 0.01$) (Table 3.3). However, 'Nova' pollen produced many pollen tubes (72.0) which had

grown the full length of the style and had reached the ovules where "bundling" of pollen tubes took place. 'Minneola' and 'Eureka' treatments were not used to assess pollen tube growth as the fruitlets were left on the trees to grow to maturity.

Table 3.3 Mean number of pollen tubes per style observed using fluorescence microscopy to study *in vivo* pollen tube growth in 'Nules Clementine' mandarin (*C. reticulata* Blanco) styles following pollination with selected pollen sources (1994/95)

Pollen source	Mean no. of pollen tubes observed x days after pollination		
	2	5	11
Delta	35.5^{c}	0.7^{a}	0.0^{a}
Midknight	4.3^{a}	0.5^{a}	0.0^{a}
Eureka^{x}	0.0^{a}	-	-
Nova	46.3^{d}	72.0^{d}	85.3^{b}
Minneola^{x}	33.7^{c}	-	-
Pomelit	17.0^{b}	5.2^{bc}	1.0^{a}
Nules	17.7^{b}	1.0^{a}	0.0^{a}
Open ^{y}	16.8^{b}	4.2^{b}	0.0^{a}
LSD	$P \leq 0.01$	$P \leq 0.05$	$P \leq 0.01$

^{x}pistils only removed after 2 d

^{y}open pollinated control

Means not followed by the same letter are significantly different at a specified level of significance

After 11 d, a trend similar to that after 5 d was observed (Table 3.3). No pollen tubes were detected for 'Delta', 'Midknight', 'Nules' or open pollinated flowers. Only 1.0 pollen tube was observed when 'Pomelit' pollen was used and 85.3 for 'Nova' ($P \leq 0.01$). Bundling of pollen tubes at the micropyle of the ovules was observed.

3.3.3 Fruit set and seed content in 'Nules Clementine' mandarin

i) 1992/93: When harvested 27 weeks after pollination, no fruit had set where pollen of 'Delta', 'Valencia Late', 'Nules' and self pollinated flowers were used. 'Nova' pollen set 17 % of flowers pollinated, 'Minneola' 33 %, 'Pomelit' 50 % and 'Marsh' 60 %. Of the fruit set, open pollinated 'Nules' flowers had no seeds, 'Nova' pollen resulted in 1.0 seed per fruit, on average, 'Marsh 8.0 seeds, 'Minneola' 16.0 seeds and 'Pomelit' 19.3 seeds.

ii) 1994/95: All pollen sources, except 'Midknight' and 'Eureka', set considerably more fruit than where 'Nules' pollen was used to pollinate itself (Table 3.4). Seed content was high where pollen with a high *in vitro* viability index was used, and *vice versa*, and zero when pollinated with its own self-incompatible pollen.

Table 3.4 Effect of various cultivars as pollen sources on fruit set and seed content of 'Nules Clementine' mandarin during 1994/95

Pollen source	% fruit set	Ave. no. of seeds/fruit
Delta	33.3	0.3
Midknight	2.3	0.8
Eureka	0	-
Nova	68.3	26.8
Minneola	65.5	14.5
Pomelit	26.7	18.7
Nules	4.9	0

3.3.4 *In vivo* pollen tube growth in 'Delta Valencia' orange

i) 1992/93 Crocodile Valley Estates: No fluorescence was detected in any of the styles removed after 1 d. After 4 d, a large number of 'Pomelit' pollen tubes were present in the stigmatic region and top portion of the style. Very few pollen

tubes of 'Minneola' were detected in the stigmatic region. No pollen tubes were detected for 'Valencia Late' and 'Delta' pollen. After 8 d, only 'Pomelit' pollen tubes were detected, by which stage they had reached the bottom half of the style.

ii) 1992/93 La Rochelle Estates: 'Delta' pistils removed 13 d after hand pollination had no detectable pollen tubes where 'Minneola' or 'Delta' pollen was used. However, 'Pomelit' pollen tubes had reached the ovary and were in the vicinity of the micropyle of the ovules. It was not possible to determine whether the pollen tubes had penetrated the micropyle.

3.3.5 Fruit set and seed content in 'Delta Valencia' orange

i) 1992/93 Crocodile Valley Estates: Between 5 and 20 % of the hand pollinated fruit set for the different pollen treatments. No fully developed seeds resulted, even when potentially strong pollinators, such as 'Pomelit' and 'Minneola' were used, nor with 'Valencia Late' pollen. However, two aborted, underdeveloped seeds were found in one fruit where 'Minneola' pollen was used.

ii) 1992/93 La Rochelle Estates: No fruits set where 'Minneola' or 'Pomelit' pollen was used, even though pollen tube growth was detected with 'Pomelit' pollen. Twenty per cent. of the flowers pollinated with 'Delta' pollen set fruit. No seeds were present.

iii) 1994/95 Crocodile Valley Estates: Good fruit set levels were achieved when pollen parents with strong pollination potential were used: 'Pomelit' 66.7 %, 'Minneola' 33.3 %, 'Nova' 41.7 %, 'Nules' 35.5 %, 'Valentine' 61.5 % and Marsh 73.3 %. None of the harvested fruit had any seeds.

3.4 Discussion

3.4.1 Effect of silicon carbide on *in vitro* pollen viability

Since the results were similar to previous tests without silicon carbide (Chapter 2), silicon carbide did not have an adverse effect on pollen viability when used as a diluent. De Lange *et al.* (1979) used aluminium oxide to dilute pollen grains and found no adverse effects on pollen. The objective of the present test was to determine any adverse effects of the inert carrier on pollen viability and not to precisely determine the percentage of pollen germination. Therefore, the assumption that silicon carbide does not materially reduce pollen viability is valid. In order to more easily distinguish pollen grains amongst the silicon carbide crystals, this technique could be adapted to include a specific stain/s which stains both viable and non-viable pollen grains.

3.4.2 *In vivo* pollen tube growth in 'Nules Clementine' mandarin

i) 1992/93: The lack of pollen tubes observed 1 d after pollination was considered to be due to the short time from pollination, for pollen tube growth to have occurred. Pollen tube growth of either applied 'Nules' pollen or open pollinated 'Nules' flowers occurred sooner than with other pollen sources, possibly due to their genetic affinities. Significant differences in pollen tube growth between pollen from different cultivars observed 4 d after hand pollinating 'Nules' flowers indicated that cultivars with high *in vitro* pollen viability (Chapter 2) displayed the most prolific pollen tube growth, and *vice versa*. This relationship is discussed more fully in Chapter 4.

The reason for the reduction in numbers of pollen tubes observed, after 8 d compared with 4 d, for all cultivars except self- and open-pollinated treatments, is not known. It is doubtful that the technique employed was not optimal as all specimens for the other treatments (1 d, 4 d and 8 d for all cultivars) were prepared simultaneously and adequate pollen tube growth was observed in those treatments. Perhaps the pollen tubes had collapsed (Johri, 1992) which could have resulted in chemical changes reducing fluorescence of pollen tubes.

ii) 1994/95: The large number of 'Delta' pollen tubes observed after 2 d is

inexplicable. Experimental error may have been a possible cause, although no plausible explanation can be given for this anomaly. For the other pollen cultivars tested, increasing pollen tube growth was observed with increasing *in vitro* pollen viability, a relationship which will be discussed in Chapter 4.

Five and 11 days after pollination, the amount of pollen tube growth observed was considered more typical for 'Delta', being comparable with the 1992/93 results and *in vitro* pollen viability data (Chapter 2). Where large numbers of 'Nova' pollen tubes were observed, from the stigma through to the base of the style, the concept that only one pollen tube enters the micropyle of an ovule became evident, as all other pollen tubes continued growing and formed bundles of pollen tubes.

3.4.3 Pollen tube penetration in 'Nules Clementine' mandarin

The growth and penetration of pollen tubes in 'Nules' styles is summarized in Fig. 3.3. Pollen tubes germinated and penetrated the stigmatic zone 1 to 2 d after pollen application. After 4 d they were in the style and approached the base of the style by 5 d. The pollen tubes of viable and compatible pollen had reached the base of the style by 8 and 11 d, by which time pollen of certain cultivars had completed its role in transferring the male nuclei to the ovules. Therefore, it appears that from 2 to 5 d after pollination is the ideal stage at which citrus pistils or fruitlets should be removed and fixed for *in vivo* pollen viability assays.

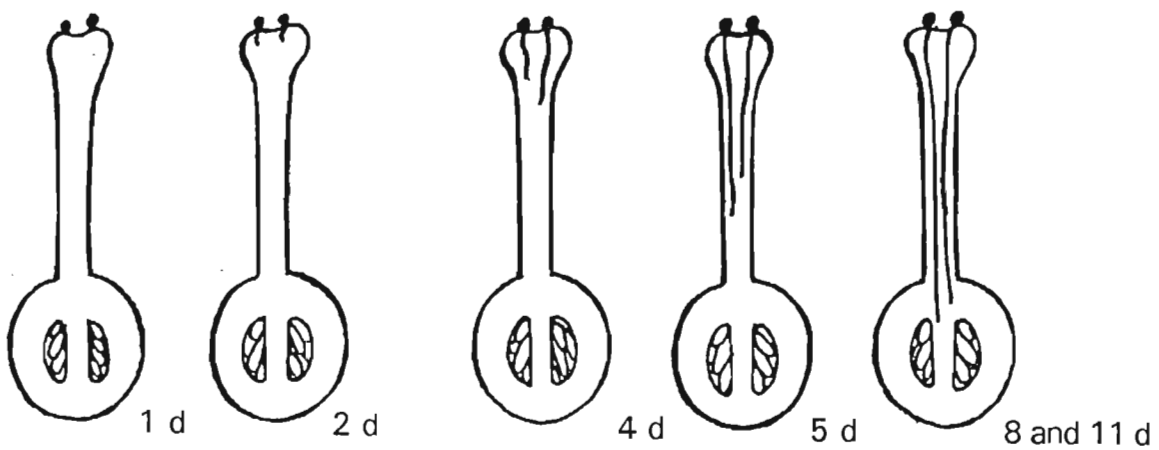


Fig. 3.3 Schematic representation of pollen tube growth in 'Nules Clementine' mandarin pistils as related to time from cross-pollination

3.4.4 Fruit set and seed content in 'Nules Clementine' mandarin

i) 1992/93: Pollen from cultivars which produced minimal to no pollen tube growth ('Valencia Late' and 'Delta', respectively) did not stimulate parthenocarpic fruit set. The lack of the stimulus of pollen tube growth was a presumed cause of poor fruit set. Where fruit set occurred, fruit from open pollinated 'Nules' flowers were seedless due to self-incompatibility, although pollen tube growth occurred. When pollen from other cultivars was used, seed content increased with increasing pollen tube growth, and hence *in vitro* pollen viability. The relationship between pollen tube growth and seed content is discussed more fully in Chapter 4.

ii) 1994/95: Fruit set increased with increasing pollen tube growth observed after 2 or 5 d, and was low for cultivars with little pollen tube growth, e.g. 'Eureka' and 'Midknight. More importantly, the cultivars which displayed very active pollen tube growth after 2 d, Nova', 'Minneola' and 'Pomelit', also set many seeds, indicating a positive relationship between *in vivo* pollen tube growth and seed content.

3.4.5 Pollen tube growth and seed content in 'Delta Valencia' orange

It was known that 'Delta' produces seedless fruit under most orchard situations (Rabe, 1992; Burdette *et al.*, 1995), but it was not certain whether 'Delta' was ovule sterile. Although cultivars with high *in vitro* pollen viability were used to pollinate 'Delta' flowers, and pollen tube growth was observed *in vivo*, no fully developed seeds were found. Therefore, 'Delta' is functionally ovule sterile and should produce seedless fruit irrespective of the cross-pollinating cultivar. The cause of ovule sterility is unknown and cytological work would be necessary to ascertain this. Where pollen tubes were observed, lack of ovule fertilization appeared to be related to slow pollen tube growth down the style, resulting in ovule degeneration. In addition, there was strong evidence for stimulative parthenocarpy in 'Delta'.

3.5 Conclusions

Cultivars with high *in vitro* pollen viability tended to display more pollen tube growth and set more seeds than cultivars with low pollen viability when applied to 'Nules Clementine' mandarin stigmas. The relationship between *in vitro* pollen viability, *in vivo* pollen tube growth and seed content is addressed in more detail in the following Chapter.

'Delta Valencia' orange did not set seed, even when pollinated by pollen with high *in vitro* viability, and is therefore ovule sterile. Besides ovule sterility, 'Delta' pollen is functionally sterile (Chapter 2). Therefore, 'Delta' is an additional cultivar of commercial importance that can be used as a buffer between two cultivars with strongly fertile pollen to avoid cross-pollination, and hence seediness. This is in addition to 'Navel' orange and 'Satsuma' mandarin selections, and 'Star Ruby' grapefruit, providing citrus producers with a broad range of buffer cultivars to plant, depending on climatic conditions and individual needs.

CHAPTER 4

DEVELOPMENT OF A PREDICTION MODEL

4.1 Introduction

Most commercially important *Citrus* spp. do not require cross-pollination to set and produce a crop (Davies & Albrigo, 1994). This is true for grapefruit, sweet oranges, lemons, limes and pummelos (McGregor, 1976). However, exceptions to this include certain mandarin hybrids, such as 'Orlando' tangelo and 'Robinson' mandarin (Krezdorn, 1986; Davies & Albrigo, 1994). In fact, many of the mandarin hybrids developed during the last few decades, especially those with 'Clementine' as one parent, have problems of self-incompatibility. In certain of these cultivars fertilization is required for fruit set as they are only weakly parthenocarpic (Futch & Jackson, 1993a). Providing opportunities for cross-pollination is the most common measure used to increase fruit set of self-incompatible cultivars in Florida (Krezdorn, 1986; Futch & Jackson, 1993b); however, this results in seedy fruit. Krezdorn (1986), Sanford (1986), Futch & Jackson (1993a; 1993b) and Adams (1995a; 1995b) describe the selection of suitable pollinators and orchard layout required to achieve optimal cross-pollination.

In the South African citrus industry, attempts are made to avoid cross-pollination, often at all costs (Barry, 1994). Fruit set of weakly parthenocarpic cultivars is improved by suitable phytohormone sprays (gibberellic acid), girdling and optimizing nutritional status. Since high seed numbers are undesirable for fresh fruit marketing (Saunt, 1990), attempts must be made to identify potentially strong cross-pollinators so that a greater source of knowledge exists as an aid for orchard layout. In addition, pollen sterile cultivars can be identified which can then be used as 'buffer' cultivars, whereby they are planted between two strong cross-pollinators to avoid high seed numbers. A third approach would be to identify relatively weak cross-pollinators which result in increased fruit set and yield, without the disadvantage of excessive seediness. Favourable effects on fruit size

in cultivars such as 'Clementine' would also an advantage.

In this chapter, the *in vitro* pollen viability results from Chapter 2 are ranked according to germination potential and rated according to pollination potential in terms of increased seediness. Germination percentage of pollen of selected cultivars is then related to pollen tube growth which in turn is related to seed content from Chapter 3. From this information a model is presented whereby pollination potential of cultivars can be determined by an *in vitro* pollen germination assay.

4.2 Rating of *in vitro* pollen germination potential

Following four seasons of *in vitro* pollen germination studies and their analysis (Chapter 2), sufficient data were available to develop a 'pollen germination potential rating system'. The development of this rating system involved the identification of cultivars with consistently low and high pollen germination percentages and fitting them to a suitable category. The categories were determined by ranking the *in vitro* pollen germination data and separating the means using least significant differences at the 5 and 1 % levels of significance (Tables 2.2.1 to 2.2.4). The letters given in the separation of the means, by using least significant differences, were utilized to determine the categories as defined in Table 4.1.

Table 4.1 Categories for the pollen germination potential rating system and their relative germination percentages

Category	% *in vitro* pollen germination
I	0 - 2
II	2.1 - 10
III	10.1 - 15
IV	15.1 - 35
V	> 35

Category I was subdivided into IA and IB, where IA had 0 to 1% germination and IB 1.1 to 2%. Category II was subdivided into IIA (2.1 to 5%) and IIB (5.1 to 10%). The pollen germination percentages of all the cultivars tested from 1990 to 1994 were then related to these categories, thereby developing a rating system for citrus pollen germination potential (Table 4.2).

Table 4.2 Summary of pollen germination potential as determined by *in vitro* viability assays

'Pollen germination potential' category	% germination	Cultivar/selection
1. **IA**	0 - 1	Navel oranges
		Delta Valencia
		Star Ruby grapefruit
		Satsuma mandarins
2. **IB**	1.1 - 2	Midknight Valencia
3. **IIA**	2.1 - 5	Marsh grapefruit
4. **IIB**	5.1 - 10	Nartia, Henderson and Rio Red grapefruit
		Salustiana sweet orange
5. **III**	10.1 - 15	Redblush, Ray Ruby and Nelruby grapefruit
		Oroblanco pummelo hybrid
		Olinda, Late and Benny Valencia oranges
		Eureka, Lisbon, Fino and Verna lemons
		Tomango, Clanor and Shamouti sweet orange
6. **IV**	15.1 - 35	Fairchild mandarin hybrid
		Tambor tangor
7. **V**	> 35	Nova mandarin hybrid
		Clementine mandarin selections
		Ellendale tangors
		Nouvelle mandarin hybrid
		Minneola tangelo
		Pomelit pummelo

The trend in pollination potential was as follows:

IA: Functionally pollen sterile; potential buffer cultivar, e.g. 'Navel' oranges, 'Satsuma' mandarins, 'Star Ruby' grapefruit and 'Delta Valencia' orange.

IB: Very low pollen viability; weak pollination ability, e.g. 'Midknight Valencia' orange.

II: Low pollen viability; relatively inefficient pollinator, e.g. certain grapefruit and midseason sweet oranges.

III: Moderately low pollen viability; intermediate strength pollinator, e.g. certain grapefruit and midseason sweet orange cultivars, 'Valencia' oranges and lemons.

IV: Moderately high pollen viability; potentially strong pollinator, e.g. certain mandarins and their hybrids.

V: Very high pollen viability; strong pollination ability, e.g. 'Clementine' mandarins, 'Ellendale' tangors, 'Minneola' tangelo, 'Nova' mandarin hybrid and pummelos.

4.3 Relationships between *in vitro* pollen germination, *in vivo* pollen tube growth, and seed content in 'Nules Clementine' mandarin

Simple linear regression analyses were conducted on relevant data where controlled hand pollinations of 'Nules Clementine' were conducted in 1992 and 1994. A multivariate analysis was not possible as there were differing numbers of replications for the different experiments conducted, since the concept of developing a prediction model was not the initial objective of this research, but was initiated at a later stage. Therefore, comparisons within a season were conducted in 1992/93 and 1994/95. In the first test, *in vitro* pollen germination percentage (independent variable) was compared with *in vivo* pollen tube growth (dependent

variable). In 1992, *in vivo* pollen tube growth results after 4 d were used, while in 1994 the results for 2 d were used. Secondly, seed content was compared with pollen tube growth; and finally germination percentage and seed content were compared. In addition, the pollen germination potential category was compared with tube growth and seed content for both years.

A summary of the r^2 value (regression co-efficient) for each of the comparisons is given in Tables 4.3 and 4.4. The most reliable relationship was then identified for both years, and relevant data from both 1992/93 and 1994/95 seasons were combined. A simple linear regression was conducted on these data to develop a regression equation. Hypothetical data were then applied to the regression equation to test the model.

i) 1992/93: *In vitro* pollen germination percentage was not closely related to resultant number of pollen tubes produced ($r^2 = 0.3342$), although the trend indicated increasing numbers of pollen tubes at higher pollen germination percentages. However, a stronger relationship was evident where the germination category was used ($r^2 = 0.7503$). A very strong relationship ($r^2 = 0.9959$) existed between pollen tube growth and seed content where sexually compatible pollen types were used, resulting in a strong relationship ($r^2 = 0.9226$) between pollen germination percentage and seed content, and germination category and seed content ($r^2 = 0.9194$) (Table 4.3). Therefore, if pollen germination percentage (and, hence category) is high, then pollen of compatible cultivars applied to 'Nules Clementine' stigmas will be expected to result in high number of seeds. However, even though germination percentage may have been relatively low, e.g. 'Marsh' grapefruit (category IIA), excessive seed numbers for export of 'Nules Clementine' were still produced. The extent of the problem was far less than with cultivars with high pollen germination potential and the problem of excessive seediness may be circumvented by planting a narrow buffer of a suitable buffer cultivar, i.e. category IA cultivars.

Table 4.3 Summary of regression analyses to determine the relationship between *in vitro* pollen germination, *in vivo* pollen tube growth, and seed content for 'Nules Clementine' mandarin in 1992/93

Regression analysis	r^2
% germination vs tube growth	0.3342
Germination category vs tube growth	0.7503
Tube growth vs seed content	0.9959
% germination vs seed content	0.9226
Germination category vs seed content	0.9194

ii) 1994/95: The relationships between pollen germination (or germination category) and number of pollen tubes were not very strong (Table 4.4), although the trend was similar to that of 1992/93. For the 1994/95 data, the relationship between pollen germination category and seed content was the strongest ($r^2 =$ 0.8446), and cultivars with very low *in vitro* pollen germination percentages, e.g. 'Delta' (category IA) and 'Midknight' (category IB) produced negligible amounts of seed.

Table 4.4 Summary of regression analyses to determine the relationship between *in vitro* pollen germination, *in vivo* pollen tube growth, and seed content for 'Nules Clementine' mandarin in 1994/95

Regression analysis	r^2
% germination vs tube growth	0.6210
Germination category vs tube growth	0.5103
Tube growth vs seed content	0.8073
% germination vs seed content	0.7216
Germination category vs seed content	0.8446

The most reliable parameters, over both seasons, to determine seed content in 'Nules Clementine' appeared to be pollen germination category. When the data for

1992/93 and 1994/95 were combined and a regression analysis conducted, a regression co-efficient of 0.9192 was calculated. Following on this analysis, a regression equation was determined where, seed content of 'Nules Clementine' =

2.619 x pollen germination category of compatible cultivars - 2.449.

Table 4.4 shows the expected number of seeds that would be produced in 'Nules Clementine' when pollinated by compatible pollen from cultivars with the specified pollen germination category (r^2 = 0.9192). Therefore, when evaluating new cultivars of citrus as potential cross-pollination threats to 'Nules Clementine', the pollen germination percentage under the standard conditions described earlier would need to be less than 2 % to ensure that the maximum seed content of three seeds per fruit, on average, for export Clementines is not exceeded. At present, this implies that only 'Navel' orange and 'Satsuma' mandarin selections, 'Delta Valencia' orange and 'Star Ruby' grapefruit would be safe to plant alongside 'Nules Clementine' without adverse affects on seed content, while 'Midknight Valencia' orange would pose a small threat, for which a narrow buffer would probably be required.

Table 4.4 Expected number of seeds in 'Nules Clementine' when pollinated by compatible pollen from cultivars with a specific pollen germination category (r^2 = 0.9192)

'Pollen germination potential' category	% germination	Expected average seed content
IA	0 -1	0.2
IB	1.1 - 2	2.8
IIA	2.1 - 5	5.4
IIB	5.1 - 10	8.0
III	10.1 - 15	10.7
IV	15.1 - 35	13.3
V	> 35	15.9

4.4 Conclusions

Numerous citrus cultivars and selections of cultivars have been rated according to their *in vitro* pollen germination potential and assigned to five major categories (Table 4.2). These categories help to determine the pollination potential of a cultivar and, therefore, its potential effects on seed content. In addition, new citrus cultivars can have their *in vitro* pollen germination percentage determined according to standard procedures, as part of their evaluation and development, for determination of pollen germination category, and hence their pollination potential of compatible cultivars from a seediness point of view.

The seed content of 'Nules Clementine' mandarin can be predicted to be (r^2 = 0.9192)

2.619 x pollen germination category of compatible cultivars - 2.449.

Therefore, by determining the *in vitro* pollen germination percentage, and hence pollen germination category, of new citrus varieties as part of their evaluation and development, their potential effect on seed content in 'Nules Clementine' can be predicted. However, where sexual incompatibility occurs, this prediction is not valid. Therefore, further research to determine the pollen compatibility of various cultivars still needs to be addressed, e.g. although pollen of 'Eureka' lemon results in an *in vitro* germination of 10 to 15 %, it apparently does not cause seed development in 'Nules Clementine' (Wahl, pers. comm.[1]). In addition, cultivars with an *in vitro* pollen germination greater than 2%, on average, are potentially able to set excessive amounts of seed in 'Nules Clementine', while low seed numbers may occur where 'Midknight Valencia' pollen is used. Therefore, a suitable buffer cultivar would be required between the two cultivars to prevent cross-pollination and rejections for export due to excessive seediness.

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OVERALL DISCUSSION AND CONCLUSIONS

The southern Africa citrus industry is export orientated and is ranked as the fourth largest citrus exporter in the world. Citrus consumers have become increasingly quality conscious with a strong emphasis having been placed on seedlessness. In southern Africa, the development of seedless or nearly seedless cultivars forms a prominent part of the objectives of the cultivar development programme, while aspects relating to optimal orchard layout are emphasized to producers in an attempt to avoid unwanted cross-pollination and seed. This is particularly relevant to the developing mandarin sector of the industry, where many cultivars/selections are self-incompatible, and potentially very seedy if cross-pollinated.

The overall objective of this study was to provide additional information and knowledge to the data base on *Citrus* pollen biology in order to optimize orchard layout, thereby reducing unwanted cross-pollination. The initial aim to achieve this involved the determination of *in vitro* pollen viability of all available citrus cultivars/selections. This was extended to categorise the cultivars/selections according to pollination potential and to identify suitable pollen sterile cultivars/selections to be used as buffers between two potential, strong, compatible pollen sources to prevent unwanted cross-pollination. In addition, the effect of pollen from various cultivars/selections on *in vivo* pollen tube growth and seed content in 'Nules Clementine' mandarin and 'Delta Valencia' orange was determined, in an attempt to elucidate the relationship between pollen viability and seed content with the former, and to determine the level of female sterility in the latter. Both cultivars are extensively planted in southern Africa; 'Nules Clementine' is a suitable cultivar for cool, coastal areas if seediness can be avoided, while 'Delta Valencia' is a widely adapted South African selection.

In an attempt to assist the citrus producers of southern Africa in their pursuit of the production of a product demanded by the consumer, various results of both practical and academic significance were obtained during the course of this study.

Therefore, this thesis served two main purposes, viz. i) to gain experience in horticultural research, and ii) to produce practical results for the industry, i.e. it has both academic interest, as well as practical significance to the citrus industry of southern Africa. The overall conclusions provide modifications to the *in vitro* and *in vivo* techniques used, as well as information that can be used to assist with orchard layout and cultivar development.

The *in vitro* pollen viability assay for *Citrus* described by de Lange (1989) provided a reliable technique, producing results similar to those of other researchers. However, refinements to the technique, relating to the stage of blossom development, are required. The following summarizes the technique proposed for the reliable testing of *Citrus* pollen viability using standardized *in vitro* pollen germination assays.

i) Blossom collection: Blossoms should be picked when the first petal begins to fold back, immediately prior to anthesis. However, care should be taken to ensure that the flowers have not been visited by bees or other insect vectors. Blossom collection should take place before 12h00, with flowers enclosed in cotton wool or tissue paper inside plastic bags, and stored in a thermal cool box with ice bricks.

ii) Anther removal and dehiscence: The anthers should be stripped from the blossoms within 6 h of picking and allowed to dehisce naturally at ambient temperature.

iii) Growth medium: The final growth medium must contain 20 % unautoclaved sucrose and 0.6% Difco Bacto agar at pH 7.0. The addition of Ca or B is apparently not necessary.

iv) Incubation: Pollen viability can be scored after incubation for 24 h at 25 °C by using a microscope at x 100 magnification.

v) Number of tests: At least two, but preferably three, season's results should be used.

An *in vitro* pollen germination assay provides a suitable means to determine the pollen viability of individual cultivars. In addition, and more importantly, a cultivar

can be assigned to a specific pollen germination potential category (Table 4.1) and the pollination potential from a fruit seediness viewpoint determined. The development of this technique provides an additional facet to the evaluation and development of new citrus cultivars. This provides a potential cost-saving method, whereby the pollination potential of a new cultivar can be determined at an early stage of its development, before extensive trials are planted. Using the *in vitro* pollen viability assay described, citrus cultivars with a germination percentage of more than 2 % are likely to set too many seeds for the export of 'Nules Clementine' mandarins.

In addition to the two pollen sterile cultivars, 'Navel' orange and 'Satsuma' mandarin, used as buffers between two strongly fertile, compatible cultivars, this study has provided two other functionally pollen sterile cultivars, viz. 'Star Ruby' grapefruit and 'Delta Valencia' orange. These two cultivars are adapted to the hot citrus production areas of southern Africa, thereby providing a greater choice of buffer cultivars of commercial importance that can be planted in all citrus production areas.

To corroborate the *in vitro* pollen viability of citrus cultivars, *in vivo* pollen tube growth with a compatible cultivar can also be studied. When such studies are conducted, the best time to observe optimal pollen tube growth is when the styles and ovaries are picked 4 to 5 d after hand pollination. Ten flowers per treatment appear to be sufficient to study *in vivo* pollen tube growth, while at least 20 flowers should be pollinated where seed counts are conducted in mature fruit. Furthermore, silicon carbide as an inert diluent had no adverse effect on *in vitro* pollen germination. However, the use of a specific stain to help distinguish pollen grains from silicon carbide would improve the technique used.

In addition to being pollen sterile, 'Delta Valencia' orange is also ovule sterile. Therefore, 'Delta Valencia' is an additional cultivar of commercial importance that can be used as a buffer between two cultivars with strongly fertile pollen to avoid cross-pollination, and hence seediness.

With 'Nules Clementine' mandarin, a strong relationship ($r^2=0.9192$) existed between pollen germination potential category and seed content where pollen from sexually compatible cultivars were applied. This provides a simple method to predict seed content of ' Nules Clementine' when pollinated by another cultivar without the use of a microscope fitted with epilumination.

The seed content in 'Nules Clementine' can be predicted ($r^2=0.9192$) by determining the *in vitro* pollen germination percentage, and hence pollen germination category, of new citrus cultivars as part of their evaluation and development by the equation

2.619 x pollen germination category of compatible cultivars - 2.449.

This technique should be extended to other cultivars to determine similar relationships, and hence prediction models, for those cultivars. It is anticipated that the relationship will be similar with other 'Clementine' mandarin selections, while the trend of increasing seed content of other compatible cultivars with increasing *in vitro* pollen germination is expected.

In this study the effect of cross-pollination on seediness has been addressed, while the effects of cross-pollination on other aspects have not been considered. However, it may be worthwhile pursuing these avenues in an attempt to identify weak pollinators that either have a positive influence on fruit set or size without increasing seed content above the prescribed levels for export. For example, determining the effect of 'Midknight Valencia' or 'Marsh' grapefruit pollen on fruit set, fruit size and seed content of 'Nules Clementine' mandarin.

Other future angles of research include:

i) The study of the effect of irradiation on pollen sterility. If irradiation is the cause of chromosomal aberrations, then it provides an effective mechanism to develop sterile cultivars from previously fertile cultivars, thereby improving a cultivar by producing a seedless clone and reducing the need for buffer

cultivars and concern about orchard layout.

ii) Cytological studies of 'Delta Valencia' orange pollen to determine the reason/s for pollen sterility and why seed abortion occurs.

iii) Compatibility studies with, for example, lemon pollen on 'Clementine' mandarins to elucidate the compatibility reactions between specific combinations and the effects of cross-pollination on aspects other than seediness, e.g. fruit set and size.

iv) Testing of the prediction model using pollen of cultivars from each pollen germination potential category.

v) To provide inputs to the potential pollination chart being developed by Rabe (1993) and the Spanish citrus industry (Anon., 1993).

vi) Conduct a basic study of pollen formation and development of *Citrus*.

SUMMARY

The citrus industry is the second largest South African agro-industry after deciduous fruit, being a major contributor to foreign exchange earnings (Thalwitzer, 1995). Southern Africa is the fourth largest citrus exporter in the world (Davies & Albrigo, 1994). In the competitive arena of fresh fruit marketing, producing what the consumer demands is the least that is required to maintain market share and is essential to create competitive advantage. Mandarin cultivars have become a rapidly expanding sector of the citrus fresh fruit market.

Seedlessness is one of the quality factors demanded by citrus consumers, especially for mandarin types in Western Europe (Ollier, pers. comm.[1]). Active programmes exist in the local citrus industry to develop and produce seedless or nearly seedless citrus cultivars to meet these demands.

Previous citrus pollen research addressed the issues of (i) how pollination could improve fruit set and fruit size (Krezdorn, 1967; Hearn *et al.*, 1969; de Lange & Vincent, 1972a), and (ii) the determination of pollination requirements of a specific cultivar (Ton & Krezdorn, 1966; de Lange & Vincent, 1972b; 1979; de Lange *et al.*, 1974; 1979b). In the planting of certain crops specific layouts are often followed to provide natural cross-pollination. In Florida (Futch & Jackson, 1993b) and Australia (Vithanage, 1991), for example, specific pollinators are planted in close proximity to weakly parthenocarpic cultivars to ensure fruit set. This, however, usually results in excessively seedy fruit.

Since information upon which to base sound decisions regarding orchard layout in an attempt to reduce unwanted cross-pollination and the resultant seediness was limited, a need developed to increase the data base on *Citrus* pollen biology and the pollination ability amongst the numerous cultivars in the citrus industry. To achieve

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this, *in vitro* pollen viability was determined using the method described by de Lange (1989) for all locally available citrus cultivars/selections. Thereafter, the relationship between pollen viability and seed content in 'Nules Clementine' mandarin was determined, and the effects of pollen from potentially strong pollinators on seed content of 'Delta Valencia' orange ascertained.

Following these studies, citrus cultivars/selections were categorised according to pollen germination potential (Tables 4.1 and 4.2). It has been demonstrated that pollinators with a pollen germination level exceeding 2 % are likely to induce too many seeds for the export of 'Nules Clementine' mandarin according to current export requirements.

In addition to the two pollen sterile cultivars, 'Navel' orange and 'Satsuma' mandarin, currently used as buffers between two strongly fertile, compatible cultivars, this study has provided two other functionally pollen sterile cultivars, viz. 'Star Ruby' grapefruit and 'Delta Valencia' orange. Besides pollen sterility, 'Delta Valencia' orange is also ovule sterile. These two cultivars are adapted to the hot citrus production areas of southern Africa, thereby providing a greater choice of buffer cultivars of commercial importance that can be planted in all citrus production areas.

An initial model is presented whereby the seed content of the sexually self-incompatible 'Nules Clementine' when cross-pollinated by new citrus cultivars can be predicted ($r^2 = 0.9192$) by determining their *in vitro* pollen germination percentage, and hence pollen germination category. This can now form part of the evaluation and development of these new cultivars. The equation used in the above-mentioned prediction is

2.619 x pollen germination category of compatible cultivars - 2.449.

This information is of importance to the citrus industry and can be used to optimize orchard layout, avoid unwanted cross-pollination and produce a superior product.

Refinements to the procedures used include (i) ensuring that blossoms are picked at the optimal stage of development for *in vitro* pollen viability studies, i.e. when the first petal begins to fold back, immediately prior to anthesis, and (ii) the optimal stage to observe *in vivo* pollen tube growth in citrus is 4 to 5 d after pollination.

Besides the actual results obtained in this study, the techniques used can form part of the cultivar development programme to determine the pollination potential of a new cultivar during the early stages of its development and evaluation, thereby saving costs associated with planting specific field trials. This research has also suggested new avenues whereby the seedlessness aspect can be addressed, most importantly, the effect of irradiation on pollen sterility and an understanding of the mechanism involved. A potentially useful extension would be to find pollinators which not only assure absolute or virtual seedlessness of cross-pollinated fruits, but also improve initial and final fruit set and yield, and possibly also fruit size where this is problematical.

LITERATURE CITED

ABBOTT, J.D., BRUTON, B.D. & PATTERSON, C.L., 1991. Fungicidal inhibition of pollen germination and germ-tube elongation in muskmelon. *HortScience* 26:529-530.

ADAMS, JANE T., 1995a. Pattern and placement of speciality citrus fruit pollinators. *Citrus & Vegetable Magazine* May:18-19.

ADAMS, JANE T., 1995b. Effective bee placement for maximum crop benefits. *Citrus & Vegetable Magazine* September:48-49, 62.

ALLAN, P., 1963. Pollen studies in *Carica papaya*. II. Germination and storage of pollen. *S. Afr. J. Agric. Sci.* 6:613-624.

AMICI, G.B., 1824. Observations microscopiques sur diverses especes de plantes. *Ann. Sci. Nat. Bot. Biol.* 2:41-70. (Cited by Johri, 1992).

ANDERSON, CATALINA M., 1983. Mandarina Ellendale determinacion del crecimiento del tubo polinico por fluorescencia. *Internal report, INTA, Argentina*.

ANON., 1993. Spanish cross pollination chart. Valencia, Spain.

ANON., 1994. New varieties require more bees. *Citrus & Vegetable Magazine* January:20.

ANON., 1995a. Outpsan International press release.

ANON., 1995b. Standards for seed content. In: *Extension services' packing guide for exporters.* Outspan International internal report. Pp 71.

BACCHI, O., 1943. Cytological observations in *Citrus*: III. Megasporogenesis, fertilization and polyembryony. *Bot. Gaz.* 105:221-225.

BANERJI, I., 1954. Morphological and cytological studies on *Citrus grandis* Osbeck. *Phytomorphology* 4:390-396.

BARRY, G.H., 1993. A study of pollen biology and seediness in citrus: preliminary results. *Proc. IVth Cong. ISCN, South Africa.* Pp 346-357.

BARRY, G.H., 1994. Influence of pollination and pollinators on yield and quality. III. Current research programme for citrus. *SASHS Working Group, Nelspruit.*

BARRY, G.H., unpublished. Effect of cross-pollination by selected pollen parents

on fruit set, fruit size and seediness in 'Star Ruby' grapefruit. *Outspan International internal report.*

BOWER, J.P. & LOVATT, CAROL J., 1994. A physiological prediction of citrus creasing. *XXIVth Int. Hort. Cong., Japan.*

BOWER, J.P. & TURNER, S.B., 1994. Maturity indexing. *Citrus Journal* 4:16-17.

BREWBAKER, J.L., 1957. Pollen cytology and self-incompatibility systems in plants. *J. Hered.* 48:271-277.

BREWBAKER, J.L. & KWACK, B.H., 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *Am. J. Bot.* 50:859-865.

BREWBAKER, J.L. & MAJUMDER, S.K., 1961. Cultural studies of the pollen population effect and the self-incompatibility inhibition. *Am. J. Bot.* 48:457-464.

BRIEGER, F.G. & GURGEL, J.T.A., 1941. Influencia do cavalo sobre a fertilidade do polen no cavaleiro, em citrus. *Bragantia* 1:713-757. (Cited by Frost & Soost, 1968).

BROOKS, R.M., BRADLEY, M.V. & ANDERSON, T.I., 1950. *Plant Microtechnique Manual.* Univ. Calif. Press, Davis.

BROWN, H.D. & KREZDORN, A.H., 1969. Hand pollination tests and field evaluation of pollinators for *Citrus*. *Proc. Fla State Hort. Soc.* 82:43-48.

BUKIYA, Z.M., 1985. Effect of pollinators on fruit and seed set in the dwarf mandarins Okitsuwase, Mihowase and Kowanowase. *Subtropicheskie Kul'tury* 3:116-122.

BURDETTE, S.A., 1994. Proposed breeding objectives per citrus climatic zone in southern Africa based on marketing demands. *Outspan International internal report.*

BURDETTE, S.A. & DU PLOOY, C.P., 1991. The current status of citrus cultivars. *Production Guidelines for Export Citrus* Vol. I, Ch. 5. Outspan International, Verwoerdburg.

BURDETTE, S.A., ALEXANDER, C.J., LEE, A.T.C. & RABE, E., 1995. Features of the main citrus cultivars of southern Africa: Navels, Midseasons and Valencias. *Citrus Journal* 5:15-21.

BURGER, D.W., 1982. Pollination and fruit set of Star Ruby grapefruit. *J. Rio Grande Valley Hort. Soc.* 35:163-166.

BURGER, D.W., 1985. Pollination effects on fruit production of 'Star Ruby' grapefruit (*Citrus paradisi* Macf.). *Scientia Hort.* 25:71-76.

BUTT, S.J., YUSUF, A., ALI, S. & KHAN, M.A., 1993. *In vitro* studies on viability and germination of pollen in various citrus species. *Pakistan J. Scientific and Industrial Res.* 36:10.

CAMERON, J.W., COLE, D. & NAUER, E.M., 1960. Fruit size in relation to seed number in the Valencia orange and some other citrus cultivars. *Proc. Amer. Soc. Hort. Sci.* 76:170-180.

CARSTENS, KAREN, 1990. Liquid nitrogen as preservative against aging of citrus pollen. *CSFRI Inf. Bull.* 220:8.

CIAMPOLINI, F., CRESTI, M., SARFATTI, G. & TIEZZA, A., 1981. Ultrastructure of the stylar canal cells of *Citrus limon* (Rutaceae). *Pl. Syst. Evol.* 138:263-274.

CLIMENKO, C.T., 1936. Period of the receptability of stigma in oranges. *Bul. Batum. Subtrop. Bot. Gard.* 1:127-129. (Cited by Frost & Soost, 1968).

CRANE, E. & WALKER, P., 1984. *Pollination Directory for World Crops*. Int. Bee Research Institute, London.

CRESCIMANNO, F.G., DE PASQUALE, F., GERMANA, M.A., CIAMPOLINI, F. & CRESTI, M., 1988a. Ultrastructual and physiological observations on pollen of five Clementine (*C. clementina* Hort. ex Tan.) cultivars. *Proc. VIth Int. Citrus Cong.*, pp 443-450.

CRESCIMANNO, F.G., DE PASQUALE, F., GERMANA, M.A., CIAMPOLINI, F. & CRESTI, M., 1988b. Ultrastructual and physiological observations on pollen of six lemon cultivars [*C. limon* (L.) Burm. f.]. *Proc. VIth Int. Citrus Cong.*, pp 451-457.

CRESTI, M., CIAMPOLINI, F. VAN WENT, J.L. & WILMS, H.J., 1982. Ultrastructure and histochemistry of *Citrus limon* (L.) stigma. *Planta* 156:1-9.

CRESTI, M., KEIJZER, C.J., TIEZZI, A., CIAMPOLINI, F. & FOCARDI, S., 1986. Stigma of *Nicotiana*: ultrastructural and biochemical studies. *Amer. J. Bot.*

12:1713-1722.

CURRIER, H.B., 1957. Callose substance in plant cells. *Amer. J. Bot.* 44:478-488.

DAVENPORT, T.L., 1986. Citrus flowering. *Hort Reviews* 12:349-408.

DAVIES, F.S. & ALBRIGO, L.G., 1994. *Citrus.* CAB International, United Kingdom.

DE LANGE, J.H., 1973. Pollen tube growth in citrus. *Agroplantae* 5:73-78.

DE LANGE, J.H., 1980. Besoek aan Europa en VSA i.v.m. sitrusverbouing en -bemarking. *CSFRI internal report, Nelspruit.*

DE LANGE, J.H., 1989. Significance of autoclaving-induced toxicity from and hydrolysis of carbohydrates in *in vitro* studies of pollen germination and tube growth. *S. Afr. J. Bot.* 55:1-5.

DE LANGE, J.H. & VINCENT, A.P., 1970. Cross-pollination studies on the Washington Navel orange. 1. Influence on set and growth of fruit. *Agroplantae* 2:121-126.

DE LANGE, J.H. & VINCENT, A.P., 1972a. A technique for studying the effect of seed content on fruit growth. *Agroplantae* 4:15-18.

DE LANGE, J.H. & VINCENT, A.P., 1972b. Pollination requirement of Ortanique tangor. *Agroplantae* 4:87-92.

DE LANGE, J.H. & VINCENT, A.P., 1979. Pollination requirements of Clementine. *Citrus & Subtrop. Fruit J.* 9-14.

DE LANGE, J.H. & VINCENT, A.P., 1988. Studies on *Citrus* pollination using gamma-irradiated pollen. *S. Afr. J. Bot.* 54:257-264.

DE LANGE, J.H., SKARUP, O. & VINCENT, A.P., 1974. The influence of cross pollination and girdling on fruit set and seed content of citrus 'Ortanique'. *Scientia Hort.* 2:285-292.

DE LANGE, J.H, VINCENT, A.P. & RHEEDERS, ELISABETH, 1979a. Evaluation of a technique for applying known quantities of pollen to citrus stigmas. *Agroplantae* 11:67-73.

DE LANGE, J.H., VINCENT, A.P., BRUWER, W.J., VON BROEMBSEN, L.A. & LEE, A.T.C., 1979b. Pollination and girdling studies on Clementine. *Citrus & Subtrop. Fruit J.* 6-10.

DIGONNET-KERHOAS, CATHERINE, GAY, G., DUPLAN, J.C. & DUMAS, C, 1989. Viability of *Curcurbita pepo* pollen: biophysical and structural data. *Planta* 179:165-170.

DUMAS,C., PERRIN, A., ROUGIER, M. & ZANDONELLA, P., 1974. Some ultrastructural aspects of different vegetal glandular tissues. *Proc. Int. Symp. Plant Cell Differentiation,* pp 501-520.

EAST, E.M. & MANGELSDORF, A.J., 1925. A new interpretation of the hereditary behaviour of self-sterile plants. *Proc. Nat. Acad. Sci. Wash.* 11:166. (Cited by Lewis, 1949).

ERDTMAN, G., 1969. *Handbook of Palynology: An Introduction to the Study of Pollen Grains and Spores.* Munksgaard, Copenhagen.

ERICKSON, L.C. & BRANNAMAN, B.L., 1960. Abscission of reproductive structures and leaves of orange trees. *Proc. Am. Soc. Hort. Sci.* 75:222-229.

FAEGRI, K. & IVERSEN, J., 1975. *Textbook of Pollen Analysis*. Blackwell, Oxford.

FILZER, P., 1926. Die selbststerilitat von *Veronica syriaca*. *Z. Indukt. Abstamm.- u. VererbLehre* 41:137. (Cited by Lewis, 1949).

FREE, J.B., 1970. *Insect Pollination of Crops*. Academic Press, London.

FRIEND, W.H., MORTENSEN, E., WOOD, J.F. & YARNELL, S.H., 1939. Citrus adapatability and breeding. *Texas Agr. Expt. Sta. 52nd Ann. Rep.* 1939:22-23. (Cited by Frost & Soost, 1968).

FROST, H.B. & SOOST, R.K., 1968. Seed production: development of gametes and embryos. In: *The Citrus Industry, Vol. II.* Ed. Reuther, W., Batchelor, L.D. & Webber, H.J., Univ. Calif., Davis. Pp 290-324.

FURUSATO, K., 1951. Genetics and breeding in *Citrus*. *Ann. Rep. Natl Inst. Genet., Japan* 2:41-42. (Cited by Frost & Soost, 1968).

FUTCH, S.H. & JACKSON, L.K., 1993a. Pollination of citrus hybrids. *Citrus Industry* December:8.

FUTCH, S.H. & JACKSON, L.K., 1993b. Cross-pollinating planting plans. *Citrus Industry* December:9.

GOLDSHMIDT, E.E., 1968. The auxin induced curvature of citrus petals. *Plant*

Physiol. 43:1973-1977.

GUSTAFSON, F.G., 1939. The cause of natural parthenocarpy. *Am. J. Bot.* 26:135-138.

HANF, M., 1935. Vergleichende und entwicklungsgeschichtliche untersuchungen uber morphologie und anatomie der griffel unf griffelaste. *Beih. Bot. Centralbl.* 54:99-141. (Cited by Ciampolini *et al.*, 1981).

HAWKER, J.S., SEDGLEY, MARGARET, LOVEYS, B.R., 1983. Composition of stigmatic exudate, nectar and pistil of watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, before and after pollination. *Aust. J. Plant Physiol.* 10:257-264.

HEARN, J.C. & REECE, P.C., 1967. Pollination needs of Page, Lee, Nova and Robinson citrus hybrids. *Citrus Industry* 48:9-23.

HEARN, J.C., REECE, P.C. & FENTON, R., 1969. Self-incompatibility and the effects of different pollen sources upon the fruit characteristics of four *Citrus* hybrids. *Proc. Ist Int. Citrus Symp.* 1:183-187.

HENSZ, R.A., 1971. 'Star Ruby', a new deep-red-fleshed grapefruit variety with distinct tree characteristics. *J. Rio Grande Valley Hort. Soc.* 25:54-58.

HESLOP-HARRISON, Y. & SHIVANNA, H.R., 1977. The receptive surface of the angiosperm stigma. *Ann. Bot.* 41:1233-1258.

HESLOP-HARRISON, J., HESLOP-HARRISON, Y. & SHIVANNA, H.R., 1984. The evaluation of pollen quality and a further appraisal of the fluorochromatic reaction (FCR) test procedure. *Theor. Appl. Genet.* 67:367-375.

HOLCROFT, DEIRDRE, M., 1989. Studies on potential low chill rootstocks, pollination and clonal identification of kiwifruit (*Actinidia deliciosa* var. *deliciosa* [A. Chev.] C.F. Liang *et* A.R. Ferguson). M.Sc. Agric. thesis, Dept Horticultural Science, University of Natal.

HOPPING, M.E., 1988. Irrigation, mechanical harvesting and spray pollination. In: *Proc. Kiwifruit Seminar, Tauranga.* Pp 24-25.

HOPPING, M.E. & SIMPSON, L.M., 1982. Supplementary pollination of tree fruits. III. Suspension media for kiwifruit pollen. *N.Z. J. Agric. Res.* 25:245-250.

IWAMASA, M., 1969. Chromosome aberrations in *Citrus* in relation to sterility

and seedlessness. *Proc. Ist Int. Citrus Symp.* 1:175-181.

IWAMASA, M. & IWASAKI, T., 1963. On the sterility phenomenon caused by low temperatures in the Mexican lime (*Citrus aurantifolia* Swing.). *Bull. Hort. Res. Sta. Japan* 2:25-45. (Cited by Frost & Soost, 1968).

JACKSON, L.K., 1986. Seed development in citrus. In: *Citrus Flowering, Fruit Set and Development (Citrus Short Course).* Ed. Ferguson, J.J., Fruit Crops Dept., IFAS, Univ. Florida. Pp 29-40.

JAGANATH, S., 1981. Studies on floral biology, fruit set and development changes in two cultivars of lemon (*Citrus limon* (L.) Burmann). *Thesis abstract: Univ. Agric. Sci., Bangalore, India.*

JOHRI, B.M., 1992. Haustorial role of pollen tubes. *Ann. Bot.* 70:471-475.

JOHRI, B.M. & VASIL, I.K., 1961. Physiology of pollen. *Bot. Rev.* 27:325-381.

KAHN, T.L. & DEMASON, D.A., 1986. A quantitative and structural comparison of *Citrus* pollen tube development in cross-compatible gynoecia. *Can. J. Bot.* 64:2548-2555.

KHO, Y.O. & BaëR, J., 1968. Observing pollen tubes by means of influorescence. *Euphytica* 17:298-302.

KNOX, R.B., 1979. *Pollen and Allergy*. The Institute of Biology's Studies in Biology no. 107. Edward Arnold, Australia. Pp 1-59.

KREZDORN, A.H., 1967. Influence of seeds on size of fruit of several *Citrus* varieties. *Proc. Fla State Hort. Soc.* 80:37-43.

KREZDORN, A.H., 1986. Flowering and fruit set of citrus. In: *Citrus Flowering, Fruit Set and Development (Citrus Short Course).* Ed. Ferguson, J.J., Fruit Crops Dept., IFAS, Univ. Florida. Pp 1-14.

KREZDORN, A.H. & ROBINSON, F.A., 1958. Unfruitfulness in the tangelo Orlando. *Proc. Fla State Hort. Soc.* 71:86-91.

KROH, M., 1973. Nature of the intercellular substance of the stylar transmitting tissue. In: *Biogenesis of Plant Cell Wall Polysaccharides*. Ed. Loewus, F., Academic Press, New York.

KWACK, B.H., 1965. Stylar culture of pollen and physiological studies of incompatibility in *Oenothera*. *Physiol. Plant.* 18:297-305.

LARCARELLE, F. & MIEDZYRECKI, Ch., 1936. Contribution a l'etude du

Clementinier au Maroc. *La Terre Marocaine* 192:19-24. (Cited by Soost, 1969).

LEWIS, D., 1949. Incompatibility in flowering plants. *Biol. Reviews* 24:472-496.

LINSKENS, H.F. & ESSER, K., 1957. Uber eine spezifische anfarbung der pollenschlauche und die zahl kallosepropfen nach selbstung und fremdung. *Natuurwissenschaften* 44:16. (Cited by Stanley & Linskens, 1974).

LONGLEY, A.E., 1925. Polycarpy, polyspory and polyploidy in citrus and citrus relatives. *J. Wash. Acad. Sci.* 15:347-351. (Cited by Frost & Soost, 1968).

LUZA, J.G., POLITO, V.S. & WEINBAUM, S.A., 1987. Staminate bloom date and temperature responses of pollen germination and tube growth in two walnut (*Juglans*) species. *Amer. J. Bot.* 74: 1898-1903.

MARTIN, F.W., 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technol.* 34:125-127.

MARTIN, F.W. & BREWBAKER, J.L., 1970. The nature of the stigmatic exudate and its role in pollen germination. In: *Pistil-Pollen Interactions*. Ed. Heslop-Harison, J. Pp 262-266.

MASCARENHAS, J.P. & MACHLIS, L., 1962. The pollen-tube chemotropic factor from *Antirrhinum majus*: bioassay, extraction and partial purification. *Am. J. Bot.* 49:482-489.

MASCARENHAS, J.P. & MACHLIS, L., 1964. Chemotropic response of the pollen of *Antirrhinum majus* to calcium. *Pl. Physiol.* 39:70-77.

McGREGOR, S.E., 1976. Insect pollination of cultivated crop plants. *USDA Agricultural Handbook.* 496 pp.

MONCUR, M.W., 1988. Floral development of tropical and subtropical fruit and nut species: an atlas of scanning electron micrographs. CSIRO, Australia. Pp 150-157.

MONSELISE, S.P., 1985. *Citrus* and related genera. In: *CRC Handbook of Flowering Vol. II.* Ed. Halevy, A.H. CRC Press, Florida. Pp 275-294.

MOORE, P.D. & WEBB, J.A., 1978. *An Illustrated Guide to Pollen Analysis.* Hodder and Stoughton, London.

MOREIRA, S. & GURGEL, J.T.A., 1941. A fertilidade do polen e numero de sementes, em especies e formas do genero *Citrus*. *Bragantia* 1:669-711. (Cited by Frost & Soost, 1968).

MORKEL, W., 1977. A feasibility study on growing coffee. Report on behalf of Caprivi Development Corporation.

MUSTARD, M.J., LYNCH, S.J. & NELSON, R.O., 1956. Pollination and floral studies of the Minneola tangelo. *Proc. Fla State Hort. Soc.* 69:277-281.

NAGAI, K. & TANIKAWA, T., 1928. On citrus pollination. *Proc. IIIrd Pan-Pacific Sci. Cong.* 2:2023-2029. (Cited by Frost & Soost, 1968).

NAKUMURA, M., 1934. Cytological studies in the genus *Citrus*. II. The chromosome number, pollen sterility and the formation of abnormal pollen tetrads. *Studia Citrol.* 6:162-178. (Cited by Frost & Soost, 1968).

NAKUMURA, M., 1943. Cytological and ecological studies on the genus *Citrus* with special reference to the occurrence of sterile pollen grains. *Mem. Fac. Sci. Agr. Taihoku Univ.* 27:53-159. (Cited by Frost & Soost, 1968).

OSAWA, I., 1912. Cytological and experimental studies in *Citrus*. *J. Coll. Agr. Univ. Tokyo* 4:83-116. (Cited by Frost & Soost, 1968).

OZSAN, M., 1961. Bazi onemli portakal mandarin, limon ve altintop cesitleri uzerinde sitolojik ve biyolojik arastirmalar. *Ankara Univ. Ziraat Fak. Yayiniari* 175:1-72. (Cited by Frost & Soost, 1968).

PEREAU-LEROY, R., 1950. Observations sur le pollen des agrumes. *Fruits Outre Mer* 5:290-293. (Cited by Frost & Soost, 1968).

POLITO, V.S., WEINBAUM, S.A. & MURAOKA, T.T., 1991. Adaptive responses of walnut pollen germination to temperature during pollen development. *J. Amer. Soc. Hort. Sci.* 116:552-554.

RABE, E., 1992. Comparative study of factors contributing to high export percentage of Delta Seedless Valencia [*Citrus sinensis* (L.)] Osbeck. *Appl. Plant Sci.* 6:40-44.

RABE, E., 1993. Clementine fruit set and fruit size strategy. *Proc. Soft Citrus Symp., W. Cape*. W. Cape Citrus Technical Association.

RANDHAWA, G.S., NATH, N. & CHOUDHURY, S.S., 1961. Flowering and pollination studies in citrus with special reference to lemon (*Citrus limon*

Burm.). *Ind. J. Hort.* 20:1-11.

REECE, P.C. & REGISTER, R.O., 1961. Influence of pollination on fruit set on Robinson and Osceola tangerines. *Proc. Fla State Hort. Soc.* 74:104-106.

REZNICK, M.E., 1958. Fisiologia y longevidad del polen en los citris. *Rev. Invest. Agr., Buenos Aires* 12:311-343. (Cited by Frost & Soost, 1968).

RIEGER, R., MICHAELIS, A. & GREEN, M.M., 1976. *Glossary of genetics and cytogenetics.* Springer-Verlag, Berlin.

ROBBERTSE, P.J., LOCK, JACKIE J., STOFFBERG, ELZABE & COETZER, L.A., 1990. Effect of boron on directionality of pollen tube growth in *Petunia* and *Agapanthus*. *S.Afr. J. Bot.* 56:487-492.

ROSEN, W.G., 1970. Pollen tube growth and fine structure. In: *Pollen: Development and Physiology*. Ed. Heslop-Harrison, J. Butterworths, London. Pp 177-185.

RUDALL, PAULA J., 1987. The flower. In: *Anatomy of Flowering Plants: an introduction to structure and development*. Royal Botanical Gardens, Kew, London. Pp 56-57.

SAHAR, N., 1978. Viability, storage and fertility of citrus pollen. *M.Sc. thesis, Hebrew University of Jerusalem, Rehovat*. (Cited by Monselise, 1985).

SAHAR, N. & SPIEGEL-ROY, P., 1980. Citrus pollen storage. *HortScience* 15:81-82.

SANFORD, M.T., 1986. Pollination of citrus by honey bees. In: *Citrus Flowering, Fruit Set and Development (Citrus Short Course).* Ed. Ferguson, J.J., Fruit Crops Dept., IFAS, Univ. Florida. Pp 21-28.

SAUNT, J., 1990. *Citrus Varieties of the World*. Sinclair International, England.

SAVELLI, R., 1940. Sur le mechanism de la stimulation mutuelle des grains de pollen germants en collectivite. *Compt. Rend. Acad. Sci., Paris* 210:546-548. (Cited by Brewbaker & Majumder, 1961).

SCHMUCKER, T., 1934. Uber den einfluss von borsaure auf pflanzen, insbesondere keimende pollenkorner. *Planta* 23:264-283. (Cited by Currier, 1957).

SCHNEIDER, H., 1968. The anatomy of citrus. In: *The Citrus Industry, Vol. II.* Ed. Reuther, W., Batchelor, L.D. & Webber, H.J., Univ. Calif., Davis. Pp

1-85.

SCHUTTE, G.C., BEETON, K.V., PELSER, P. du T. & LESAR, K., 1994. Post-harvest control of *Alternaria* navel-end rot with pre-harvest chemical sprays. *Citrus J.* 4:26-28.

SEDGLEY, MARGARET, 1976. Control by the embryosac over pollen tube growth in the style of the avocado (*Persea americana* Mill). *New Phytol.* 77:149-152.

SEDGLEY, MARGARET, 1977. The effect of temperature on floral behaviour, pollen tube growth and fruit set in the avocado. *J. Hort. Sci.* 52:135-141.

SHIVANNI, K.R. & JOHRI, B.M., 1985. *The Angiosperm Pollen: Structure and Function.* Wiley Eastern Ltd., New Delhi.

SHU, Z.H., SHEEN, T.F. & LEE, K.C., 1988. Current researches on the unfruitfulness of mango in Taiwan. *Acta Hort.* 231:68-72.

SINGH, J.P. & DHURIA, H.S., 1960. Studies on floral biology of sweet lime (*Citrus limettoides* Tanaka). *Ind. J. Hort.* 17:9-20.

SOOST, R.K., 1964. Self-incompatibility in *Citrus grandis* (Linn.) Osbeck. *Proc. Amer. Soc. Hort. Sci.* 84:137-140.

SOOST, R.K., 1965. Incompatibility alleles in the genus *Citrus*. *Proc. Amer. Soc. Hort. Sci.* 87:176-180.

SOOST, R.K., 1969. The incompatibility gene system in *Citrus*. *Proc. Ist Int. Citrus Symp.* 1:189-190.

SOOST, R.K. & CAMERON, J.W., 1980. Oroblanco: a new grapefruit hybrid. *Calif. Agric.* 6:16-17.

STANLEY, R.G. & LINSKENS, H.F., 1974. *Pollen: Biology, Biochemistry, Management.* Springer-Verlag, New York.

TADEO, F.R. & PRIMO-MILLO, E., 1989. An ultrastructural study on development and degeneration of unfertilized citrus ovules. *Proc. VIth Int. Citrus Cong.* 431-441.

THALWITZER, M., 1995. The South African citrus industry in perspective. *Citrus & Subtropical Fruit Conference, Nelspruit.*

TON, K.D. & KREZDORN, A.H., 1966. Growth of pollen tubes in three incompatible varieties of citrus. *Proc. Amer. Soc. Hort. Sci.* 89:211-215.

TOWNSEND, C.E., 1971. Advances in the study of incomaptibility. In: *Pollen: Development and Physiology.* Ed. Heslop-Harrison, J. Butterworth, London. Pp 281-309.

TOXOPEUS, H.J., 1930. De polyembryonie van citrus en haar beteckenis voor de cultur. *Veren. Landbouw Nederl.-Indie Landbouw Tijdschr.* 6:391-405. (Cited by Frost & Soost, 1968).

UPHOF, J.C.Th., 1931. Wissenschaftliche beobachtungen und versuche an agrumen. I. Ueber die blutenverhaltnisse der Tahitilimonelle. *Gartenbauwiss* 4:513-520. (Cited by Frost & Soost, 1968).

UPHOF, J.C.Th., 1934. Wissenschaftliche beobachtungen und versuche an agrumen. VI. Einiges ueber die blutenverhaltnisse (1. Teil). *Gartenbauwiss* 8:394-410. (Cited by Frost & Soost, 1968).

VAN TIEGHAM, P., 1869. Vegetation libre du pollen et de l'ovule et sur la fecondation directe des plantes. *Annals Sci. Nat. Bot.* 12:312-328. (Cited by Rosen, 1970).

VARDI, A., FRYDMAN-SHANI, A. & WEINBAUM, S.A., 1988. Assessment of parthenocarpic tendency in citrus using irradiated marker pollen. *Proc. VIth Int. Soc. Citriculture* 1:255-260.

VISSER, R., 1955. Germination and storage of pollen. *Meded. LandbHoogesch. Wageningen* 55:1-68. (Cited by Stanley & Linskens, 1974).

VITHANAGE, V., 1991. Effect of different pollen parents on seediness and quality of 'Ellendale' tangor. *Scientia Hort.* 48:253-260.

WEBBER, H.J., 1930. Influence of pollination on set of fruit in citrus. *Calif. Citrog.* 15:304, 322-323. (Cited by Frost & Soost, 1968).

WEINBAUM,S.A., PARFITT, D.E. & POLITO, V.S., 1984. Differential cold sensitivity of pollen grain germination in two *Prunus* species. *Euphytica* 33:419-426.

WELK, M., MILLINGTON, W.F. & ROSEN, W.G., 1965. Chemotropic activity and the pathway of the pollen tube in lily. *Am. J. Bot.* 52:774-780.

ZACHARIA, D.B., 1951. Flowering and fruit setting of Shamouti orange trees. *Palest. J. Bot.* 8:84-94. (Cited by Monselise, 1985).