DIFFERENTIATING APPLE SPORTS BY POLLEN ULTRASTRUCTURE

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ABSTRACT

Cultivars are plants that form distinct, uniform and stable phenotypes. New cultivars can be protected by Plant Variety Rights (PVR) which allow the owner exclusive rights to the propagation and sale of the plant material. Current PVR identification methods for apple cultivars require detailed records of tree, flower and fruit characteristics to differentiate the new cultivars from known cultivars. This method is slow, expensive and unable to cope with the increasing numbers of sports. Biochemical identification methods such as isozymes, restriction fragment length polymerisation (RFLP), random amplified polymorphism DNAs (RAPD), and minisatellite probes, can quickly and objectively differentiate cultivars, but cannot differentiate apple sports. Previous research suggested that pollen ultrastructure could be an alternative method for plant identification. This thesis is concerned with the development of a technique to differentiate apple sports using pollen exine patterns.

Scanning electron microscopy was used to capture images of the apple pollen grain and the exine surface. A digital image analysis algorithm was developed to extract quantitative data from the pollen grain dimensions and pore characteristics, and a Fast Fourier transform extracted quantitative data from the ridge patterns. Statistical methods were applied to the data to differentiate the sports.

Pollen harvested from apple flowers in the spring were wider than pollen harvested from flowers forced out of season under artificial conditions. Significant differences between trees were found for pollen grain length:width ratio, percent pore coverage, pore area and pore length but further research is required. However, apple cultivars types 'Red Delicious' and 'Gala' were successfully differentiated by pore and pollen grain variables, and 'Aversang' and 'Ultrared' sports of 'Red Delicious', and 'Splenola' and 'Galalea' sports of 'Gala' were successfully differentiated by exine ridge patterns and pollen grain measurements.

Differentiation of apple sports by pollen requires further development but may be one of the only quick, objective identification methods that can differentiate sports. Sport

differentiation would greatly aid PVR establishment and enforcement.

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1. INTRODUCTION

Cultivars are cultivated varieties of a crop and are the product of change to the genome, for example sexual reproduction or mutation. The cultivars that are the products of mutations are called sports and can either occur naturally or be induced with a mutagenic agent (for example radiation). In Brooks and Olmo's Fruit and Nut register there are approximately 2500 cultivars listed for the period between 1962 and 1991. Over 12% of these cultivars are sports. Sports form an even more significant proportion of apple cultivars; nearly 31% of the commercial cultivars in the Brooks and Olmo Register 1962-1991 are sports (Brooks and Olmo, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1978, 1982, 1983, 1984, 1991).

If it can be demonstrated that a cultivar is new, distinct, homogenous, and genetically stable then the developer may apply for a plant patent. In New Zealand new varieties have only been covered by plant patent laws since 1975 (Whitmore, 1992). The current laws dealing with plant patents are in the Plant Variety Rights (PVR) Act of 1987 which is based on the International Union for the Protection of New Varieties of Plants (UPOV) convention. The PVR office has to establish that each applicant for PVR status meets the criteria of distinctness, homogeneity and genetic stability. Applicant plants are planted in the field beside other known, similar cultivars and are observed over several seasons to remove the influence of environment. This can be quite a large undertaking. The Brooks and Olmo Register recorded 57 sports of Delicious between 1962 and 1991 (Brooks and Olmo, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1978, 1982, 1983, 1984, 1991). Once the PVR is established the developer usually licences nurseries to propagate the plant material and charges a royalty on each tree sold. The produce of the new cultivar can also be protected by marketing the fruit under a trade mark which can be licensed out as well (Selby, 1995). Over the years there has been a move away from government backed research for the public good and a move towards research for commercial returns which has meant an increase in interest in developing and patenting cultivars for financial returns.

In New Zealand, apples are an important crop. The annual return from fresh apple

exports was about $346.3 million in 1992/3, which represented more than 38% of the total export fruit sales (Fruit Research Council, 1994). Many cultivars of apple are used for export and each cultivar has minimum thresholds for colour, size and other quality factors. Mutations in the genome can enhance the quality of the fruit, and increase the value of the crop. The New Zealand Apple and Pear Marketing Board (NZAPMB) encourages the discovery of naturally occurring, new, improved sports. Recently there has been an increase in the numbers of sports as applicants for PVR status (Whitmore, 1992) and this may either be a reflection of the NZAPMB encouragement, the opportunity for financial gains from PVR, or the apple crop may be prone to mutation.

The numbers of cultivars submitted with little morphological or agronomic difference from established cultivars is a major concern for the PVR office (Whitmore, 1992). Plants can be identified or differentiated by differences in agronomy, morphology, or biochemistry. Field observation of morphology has been adequate for most apple cultivars but requires the use of a large area to grow the standards as well as the applicant plant, and an expert to record the large amounts of data (PVR evaluation form, appendix B). Although most of the measurements are objective, some like colour require judgement. Unfortunately biochemical markers do not, so far, offer appropriate solutions to differentiate sports. DNA techniques like polymerase chain reaction (PCR), restriction fragment length polymerisation (RFLP), and minisatellite probes do not show sufficient polymorphism to differentiate apple sports. Isozyme techniques can differentiate some sports, but not all, and isozymes are sensitive to the environment. Other simple biochemical tests can be applied in specific cases, for example phenol test for wheat, but cannot be applied to all crops. There is a need for a plant identification method for PVR that will easily and objectively differentiate sports.

The aim of this project was to develop a system that would differentiate apple sports using pollen grain ultrastructure. Scanning electron microscopy was used to capture the image and a new digital image analysis algorithm was developed to extract data from the image. Statistical methods were then applied to the data to differentiate the sports. This method could further be used to assist with the establishment of PVR status as well

as identifying cultivars and sports. Although the technique was developed on apples, it could also be applied to other crops.

2. LITERATURE REVIEW

2.1. New cultivars and plant variety rights

New cultivars are the product of sexual reproduction, gene transfer or mutation which change the genome and produce distinct, uniform and stable phenotypes. Mutants or sports are caused by spontaneous or induced changes in the DNA. Mutations may cause changes to only one gene so the new phenotype may differ from the original genotype in one character. Examples are 'Royal Gala', a sport of 'Gala' with increased red pigments, and 'Red Chief', a 'Red Delicious' sport with a compact vegetative habit. Propagating heterozygous plants such as apple trees without changing the genome requires vegetative propagation.

Development of new plant cultivars and varieties for commercial purpose is increasingly in the hands of private interests. Development of new cultivars is very costly in time and resources. Developers of new cultivars are keen to recover their costs and make a profit. Like any other 'inventions', plant cultivars can be protected and owners have the opportunity to exclusive rights to propagate and sell trees of their cultivars by applying for plant variety rights (PVR) (Calhoun, 1992; Selby, 1995; Whitmore 1992). The applicant plant is tested for distinctness, uniformity and stability over two years. The successful applicant gains the protection for 23 years. During that period compensation can be gained from violations of the PVR. Positive identification of the plant under PVR is needed to support any claims of violation of PVR.

Violations in PVR may result from lost identity of a plant due to mistakes in identification during propagation and distribution, for instance during the collection of budwood, in quarantine, in the nursery and during delivery. Mistakes can cost money. The grower loses income from growing the wrong cultivar or sport, incurs the cost of replacing the trees and loses income during the establishment years of the replacement trees. Positive identification early in propagation will prevent these errors from compounding.

The Plant Variety Rights Act (New Zealand Government, 1987) is the legislation that deals with PVR and states that a plant has to be demonstrated to be a new, distinct, homogenous and stable variety to achieve PVR status. A variety is distinct if it is "distinguishable by one or more characteristics from any other variety whose existence was common knowledge at the time of the application" (Section 10.4.b PVR Act 1987). In section 10.8 the Act further defines distinguishing characters as characters which may "be morphological, physiological, or of any other kind or description, so long as those characteristics are capable of precise description and recognition". In theory any method that identifies a cultivar could be used in establishing PVR, but in practice the judgement of the PVR office is applied to define whether a character is distinct or just different. Genotypes may have small differences in the genome which enable them to be distinguished at a biochemical level, but the phenotypes are not different enough to be classed as distinct.

The New Zealand PVR law is based on guidelines laid down by the International Union for the Protection of New Varieties of Plants (UPOV). Baltjes and Ghijsen (1992) discussed the problem of defining distinctness (the base for cultivar identification). The current practice is to define a distinct character as having a P value ≤ 0.01 in an analysis of variance for two consecutive years, or two out of three years. Statistical methods of defining distinctness have not been incorporated into the New Zealand Plant Varieties Act, but this measure could be used as a guide to determine if a new identification method showed sufficient differentiation.

2.2. Plant identification techniques

Plant macro morphology has been the main method of cultivar identification (Barnaby, 1992; Payne, 1987). Differences in morphology such as leaf shape or tree habit, and physiological characteristics such as fruit colour and fruit harvest dates have been used to differentiate cultivars. Unfortunately, in many crops, new cultivars and sports differ little in morphology (Amma, 1986; Hubbard *et al.,* 1992; Noiton and Shelbourne, 1992; Payne, 1987; Scorza *et al.,* 1985).

Biochemical techniques like isozyme and DNA finger-printing provide an objective method of identification. Isozymes are multiple molecular forms for enzymes sharing a catalytic activity (Simpson and Withers, 1986). These isozymes can be separated by electrophoresis at a rate dependent on the size and charge of the isozyme. Changes to the DNA that change the size or charge of the isozyme will produce a different pattern of isozyme separation. Isozyme analysis is a powerful tool for the genetic study of plants and can be used for cultivar identification. Samimy and Cummins (1992) differentiated 13 apple rootstocks by isozyme analysis but none of the rootstocks were sports. Stampar and Smole (1992) separated 17 out of 20 apple cultivars. However, the minute changes in the DNA between sports have not been sufficient to produce polymorphism in the isozyme analysis. Weeden and Lamb (1985) could not differentiate between sports of apple with isozyme markers.

Restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), minisatellite and simple sequence repeat DNA analyses are DNA techniques found to differentiate cultivars. The RFLP technique uses DNases to digest or cut up the genomic DNA at specific sites into fragments of different lengths (Walton, 1993). Point mutations can delete or create sites, and DNA rearrangements move sites about which can cause changes to the fragment lengths. Hubbard *et al.* (1992) claimed RFLPs had potential for sport differentiation and successfully applied RFLP to rose cultivar identification but, unfortunately, the authors did not say if any of the cultivars compared were sports.

Randomly Amplified Polymorphic DNA (RAPD) involves the multiplication of segments of the genome that hybridise with random primers. Polymorphism arises when one individual has a segment but the other does not (Walton, 1993; Williams *et al.*, 1990). Mulcahy *et al.* (1993) claimed that RAPD is a more reliable method than isozyme methods because it is not influenced by the environment, the tissue source, or the age of the plant. In addition RAPDs were more polymorphic than RFLPs. However, these authors were not able to differentiate apple sports by RAPD analysis.

Minisatellite regions of the DNA contain sequences of two to six base pairs repeated

many times. These repeat regions can show polymorphism between cultivars. Sharon *et al.* (1995) used minisatellite simple sequence repeats to differentiate species of onion, tomato, wheat, avocado, litchi, *Carica* and mango but did not compare cultivars or sports within species. Nybom (1990) analysed minisatellite DNA of 'Red Delicious' apple sports but found insufficient polymorphism to differentiate the sports.

Simple biochemical tests are widely used in other crops. Payne (1987) states that tests like the phenol test for wheat or the soyabean seed coat peroxidase test are objective, easy to perform and take a short time to provide results. However, they are needed to be used in conjunction with many other tests to differentiate all cultivars. Jay and Ferrero (1989) propose a method of identification of carnations that combines image analysis of the plant form, spectrocolorimetry and chromatography of the pigments in the flowers, as well as isozyme analysis.

Plant ultrastructure has been used to aid cultivar identification. Paunovic and Paunovic (1991) investigated apricot identification by the length and degree of pubescence on the pistils with light microscopy. Fujita and Uchikawa (1989) studied ultrastructure of mulberry idioblasts, trichomes and pollen to aid in cultivar identification. Many authors have applied pollen ultrastructure to the identification of cultivars (section 2.3).

An identical result from one test between two plants does not prove that samples are from the same cultivar, but a different result proves that they are different cultivars. No single test on its own can identify all cultivars, so many tests are required to confirm a plant's identity.

2.3. Pollen analysis and plant identification

Geographers have used the identification of fossil pollen to build up a picture of the flora of the past (Treloar, 1992). Plant scientists have used pollen to identify species and clarify taxonomy. Many authors have investigated the use of pollen to aid the identification of plant cultivars and species (Fogle, 1977a, b; Fujita and Uchikawa ,1986;

Hebda and Chinnappa, 1990; Kozaki and Hirai, 1986; Maas, 1977; Marcucci *et al.*, 1984; Martens and Fretz, 1980; Matsuta *et al.*, 1982,1986; Mulas *et al.*, 1988; Ueda and Okada, 1994; Vezey and Skvarla, 1990).

Pollen exine patterns were first used to differentiate tree fruit cultivars by Fogle (1977a, b). Fogle (1977 b) used pollen grain length, width and length:width ratio to differentiate peach, nectarine, apricot, plum, Japanese plum, cherry, apple, and pear pollen. This author showed that categories of exine patterns had potential to further differentiate between cultivars. He noted that for each species, within-cultivar pollen grain measurement variation was almost as large as between-cultivars, but that the exine pattern seemed constant regardless of pollen grain size. To limit the size variation the author suggested selecting only representative pollen grains. However, representativeness was not precisely defined and not all apple cultivars were separated.

Ridge width, ridge interval, perforation frequency and the presence of collared pores along the equatorial region of the pollen grain partially differentiated eight crabapple species (Martens and Fretz, 1980). The authors found exine topography and pore characteristics to be the most useful for differentiating crabapple species. A dichotomous key was proposed that could separate all species except *Malus x robusta* and *Malus sargentii*. These authors reported that the selection of mature representative pollen grains was necessary to avoid inconsistency. Representative grains were defined as mature, not distorted, generally tricolpate and broadly elliptical with flattened ends. In conclusion, the authors stated that pollen characteristics could be used with other morphological traits such as foliage characteristics to achieve full differentiation between the species.

Pollen from 31 apple cultivars and sports were investigated by Marcucci *et al.* (1984). These authors reported that pollen grain size and shape were quite uniform within a cultivar but that ridge patterns were sometimes variable within a cultivar. This conflicts with the observations of Fogle (1977 b). Categories of ridge patterns for the equatorial and polar regions, pit numbers, pollen grain length, width and length:width ratio, pollen viability and germination were used to differentiate between the cultivars. The most useful traits for differentiation of cultivars were the number and size of the pores. Pollen

size, exine pattern, germination and viability achieved the separation of spur and standard sports of 'Golden Delicious', 'Red Delicious' and 'Granny Smith'. These authors concluded that ridge patterns alone may not be adequate to differentiate cultivars and sports but pollen characters would be useful for cultivar identification if combined with other pomological characters.

Qualitative measurements were applied to the differentiation of blueberry, blackberry, raspberry, strawberry, *Duchesnea* and *Potentilla* (Maas, 1977); and oranges, kumquats, limes, microcitrus, lemons, pummelo, yuzu and manderins (Kozaki and Hirai, 1986). However, Crescimmano *et al.* (1988) could not differentiate lemon cultivars by pollen grain size and shape.

Mulas *et al.* (1988) measured almond cultivar pollen length, width and length:width ratio, pore number and diameter, and ridge frequency to evaluate the discriminating power of each variable. Pollen grain width was the most valuable variable in the differentiation of cultivar, followed by pollen grain length, number of pores, and the grain length:width ratio.

Ueda and Okada (1994) applied Principal Components Analysis (PCA) of quantitatively-measured rose pollen ridge and pore characteristics to partially differentiate rose cultivars. They confirmed that pollen surface ridge patterns and pore characteristics were important variables for differentiating cultivars. Vezey and Skvarla (1990) also applied PCA to successfully separate species within the Capparaceae sub-family Cleomoideae. These authors suggested that although their study was not statistically rigorous, it showed the potential of quantitative feature analysis.

Matsuta *et al.* (1982, 1986) took quantitative data from the pollen grain size, shape, pore and ridge characteristics of Japanese pears, and showed that although no single measurement could differentiate the cultivars, 76% of the pollen grains could be correctly identified by a multivariate technique called Discriminant Analysis. Total area of the pores was the most powerful differentiating variable.

These researchers have shown that pollen ultrastructure can be applied with varying success to the identification of many different fruit crops. By further investigating ridge patterning and pore characteristics, extracting quantitative rather than qualitative measurements, averaging the data from several pollen grains to reduce variation, and applying multivariate statistics, it may be possible to improve the discriminating power of pollen characteristics to enable the differentiation of not only cultivars but sports as well.

2.4. Image analysis

Much of the data gathered for the differentiation of species and cultivars by pollen ultrastructure has been qualitative. For example, pores were recorded as not visible, sparse, numerous, and very numerous by Marcucci *et al.* (1984). The use of categories makes it easier for the observer to make classifications without a lot of detailed measurement but the disadvantage is the loss of data. Quantitative data enables a greater number of details to be recorded but can be tedious and difficult to extract.

Recent advances in computer technology have meant that computers that are powerful enough to process images and extract quantitative data have become readily available. Computer image analysis holds great potential to make fast, objective, quantitative measurements to high resolution with little effort on the part of the observer (Cannon and Hunt, 1981). The benefits could include finer determination of surface details and the use of quantitative measurements to enhance the potential to discriminate between plant cultivars and their sports.

Image analysis can reduce a complex image into quantitative parameters. For instance the ridge pattern on the surface of the pollen grain is often wavy or curved, and the ridge widths vary. Extracting a measure of the average angle of the ridge curvature or the average width of the curvature can be time consuming by hand. Martens and Fretz (1980) averaged 50 ridge width measurements for crabapple species. However, image analysis can be used to extract these parameters from the whole image.

In the case of apple pollen the striking feature of the exine surface is the texture or pattern of the ridges (Martens and Fretz, 1980). Image analysis could be used to extract quantitative data on the ridge frequencies and angles. Texture is a measure of the spatial organisation of the tonal intensities in an image. Haralick (1979) grouped the texture analysis methods into eight categories: autocorrelation functions, optical transforms, digital transforms, textural edgeness, structural elements, spatial grey tone co-occurrence probabilities, grey tone run lengths and autoregressive models. This project applied a digital transform of the image called the Fast Fourier transform. The Fast Fourier transform converts a continuous waveform such as an image, a sound, or a beam of light into a spectrum of sinusoidal components. For example, a beam of white light can be described by the sum of the primary colours. Another example may be the breakdown of the tidal pattern into frequency components due to the sun and the moon. In images, information of the position, size and orientation of repetitive patterns can be extracted. Bailey (1993) applied the Fast Fourier transform to analyse the regular patterns in images of wasp nests. This analysis could be applied to the ridged patterning of the exine to extract quantitative data. The Fast Fourier transform converts the image of the exine ridge patterns into a Fourier image. Distance of a point from the origin is proportional to the frequency of the exine ridge variation. The angle between the point, the origin and the horizon is the angle of the exine ridge variation. The intensity of the point (0-255) in the Fourier image is proportional to the strength of the exine ridge pattern. Phase of the sine wave indicates position of the ridge pattern.

Image analysis applied by Vezey and Skvarla (1990) extracted pore characteristics and differentiated three Capparacea species (*Cleomella longipes*, *Oxystylis lutea*, and *Wislizenia refracta).* These authors defined five measurements to describe perforations in the exine: average percent coverage, average Waddel diameter, average longest dimension, average mean dimension perpendicular to the longest dimension, and average distance between the centres. Treloar (1992) investigated the automatic recognition of pollen grains from sediment for taxa of the Pacific islands. Differentiation of the taxa was achieved by measurements of the pollen grain shape and size.

2.5. Sample preparation

Scanning electron microscopy allowed high resolution and depth of field for viewing the surface of pollen grains. Samples needed to be dried to withstand the high vacuum environment of the scanning electron microscope without distortion (Marcucci *et al.*, 1984; Martens and Fretz, 1980). There are several methods of pollen preparation such as air drying, freeze drying, critical point drying, acetolysis, hot potassium hydroxide solution (10%) wash, fixation or combinations of these methods.

Acetolysis (Erdtman, 1960) removes the cell contents leaving only the exine layer. The advantages of this method is that it can be used on dried pollen and so does not depend on any critical timing. In addition it does not require expensive equipment and renders pollen in a comparable condition whether it is fresh, dried, or fossilized. The removal of the cell contents is an advantage in light microscopy as the contents of the cell can be observed through the exine and can confuse the surface sculpturing. However, this is not an issue for scanning electron microscopy as the specimen is coated with metal and only the surface is viewed. Hesse and Waha (1989) expressed concern that acetolysis removed too much of the pollen grain material but Adams and Morton (1972) reported that acetolysed pollen has less trouble with excessive contrast and glare in the scanning electron microscope.

Air drying can lead to the collapse of a specimen due to the high surface tension of water. Freeze drying and critical point drying fresh pollen removed the moisture in the sample without collapsing the cells (Adams and Morton, 1972; Falk, 1980; Large and Braggins, 1991). Freeze drying reduced the surface tension because at low pressure and temperature water sublimes from solid to gas. Critical point drying replaced water with liquid carbon dioxide at high pressure and temperature until the carbon dioxide reaches an equilibrium point where gas is formed at the same pressure, which reduced the surface tension of the drying process. Freeze and critical point drying methods were noted, but not favoured as they were demanding on resources and time.

Hot potassium hydroxide wash is a method described by Faegri and Deuse (1960),

Treloar (1992) and Large and Braggins (1990). Pollen is washed in 10% potassium hydroxide solution heated in a boiling water bath for ten minutes (Large and Braggins, 1990) to three hours (Faegri and Deuse, 1960) before rinsing with distilled water. Large and Braggins (1990) found hot potassium hydroxide had similar effects on the pollen as acetolysis. The method is relatively simple and is within this project's resources.

Plant tissue can be hardened by fixation process which enables specimens to be viewed under scanning electron microscope with little change to the ultrastructure. Formaldehyde-gluteraldehyde (Karnovsky, 1965) or osmium tetroxide vapour (Smith and Tiedt, 1991) can be used as fixatives. The pollen grains can be fixed fresh and not collapse under scanning electron microscopy.

According to Hesse and Waha (1989) more than 100 papers are published every year on pollen surface characteristics and three quarters of these used acetolysis. However, the literature on cultivar identification by pollen ultrastructure revealed that most authors simply used air dried pollen (Fogle, 1977a, b; Maas, 1977; Marcucci *et al.,* 1984; Martens and Fretz, 1980; Matsuta *et al.,* 1982, 1986; Mulas *et al.,* 1988; Pandey and Troughten, 1974).

2.6. Statistical analysis

Statistical analysis of the data to differentiate sports of cultivars could follow three steps. Firstly the data would need to be screened to select the variables that contributed the most to the differentiation of apple cultivars and sports. Secondly the differentiation of the cultivars and sports by the selected variables need to be maximised. Thirdly the discriminating power of the data would be tested on an unknown pollen sample.

Variable selection is the process of isolating the variables that contribute the most to the analysis. It is only necessary for multivariate analyses. Few authors have applied a multivariate approach to the differentiation of fruit cultivars (Langford *et al.,* 1990; Matsuta *et al.,* 1982, 1986; Ueda and Okada, 1994; Vezey and Skvarla, 1990). Langford

et al. (1990) used variable selection to find a smaller subset of variables that maximised the discriminating power of the data. These authors recorded 15 texture measurements from *Pinus sylvestris*, *Querrcus robur*, *Corylus avellana*, *Plantago lanceolata*, *Ulmus glabra* and applied Hotellings T^2 as a multivariate equivalent of the Students t-Test to select a smaller number of variables with the maximum discriminating power for separating two classes of pollen. These authors also found that the discriminating power was increased if the variables were selected for each pair of pollen class comparisons.

Canonical Variate Analysis (CVA) is a multivariate technique to differentiate groups of data and to aid in variable selection. CVA has not been applied in cultivar or sport differentiation by pollen ultrastructure. However, Cruz-Castillo *et al.* (1994) recommended that CVA should be used where the primary aim of the analysis was the separation of predefined groups such as apple sports. These authors stated that canonical variates were linear functions of the data that separated predefined groups (for example apple sport) by maximising the variation between groups and minimising the variation within groups. They also suggested that the scores for the canonical variates can be plotted to show the separation between the groups, and further interpretation of the canonical scores is possible with multiple comparisons of means. Ganeshanandam (unpublished) reported that Parallel Discriminant Ratio Coefficients (DRCs), a product of the canonical coefficients and the structure correlations, provided a measure of how much each variable was contributing to the canonical variates. High absolute values of DRCs could be used for variable selection.

Identification of unknown pollen can be done by plotting the canonical scores for the new observations and finding the group mean that is closest, but a more formal method of classifying unknown pollen is Discriminant Analysis (Cruz-Castillo *et al.*, 1994). Discriminant Analysis (DA) finds linear or quadratic functions that best separate the groups and apply these functions to unknown data to test the effectiveness of the classification (SAS Institute Inc, 1990). The author states that DA assumes multivariate normality which is partly dependent on the number of variables and the numbers of observations. Reducing the numbers of variables or increasing the numbers of observations increases the probability of multivariate normality. Data is needed to create

the discriminating functions but ideally not from the data that is used to test the functions. If the same data is used a bias is introduced. Langford *et al.* (1990) used Discriminant Analysis on image texture data extracted from the taxa *Pinus sylvestris*, *Querrcus robur*, *Corylus avellana*, *Plantago lanceolata*, *Ulmus glabra*, and *Lolium perenne*. The authors used a 'leave one out' strategy which calculated the discriminant function from all the data but one observation, and then classified the remaining observation with that function with a 94% success rate. The over all error rate tests the ability to classify unknown pollen.

Principal Components Analysis (PCA) can be used to investigate the over all variation in the data without regard to the predefined groups (Cruz-Castillo *et al.,* 1994). Broschat (1979) explained that PCA structured the data so that a factor, such as 'pollen ridge pattern', which was measured by 'p' variables can be expressed in terms of $n < p$ uncorrelated terms or principal components. So 'pollen ridge pattern' can have fewer principal components (usually two or three) to explain most of the variation observed in the data than the original variables. The resulting principal component scores can be plotted to show the variation of the observations, to spot outliers, or further analysed to find the important variables that contribute to the observed variation in the data. Vezey and Skvarla (1990) applied PCA to the differentiation of Capparaceae species and achieved separation but CVA might have been more appropriate.

3. MATERIALS AND METHODS

The present study on the differentiation of 'Red Delicious' and 'Gala' apple sports by pollen ultrastructure followed several steps including pollen collection, pollen preparation for scanning electron microscopy, scanning electron microscopy, image analysis and analysis of the variation from pollen collection, environment and genotype.

3.1. Pollen source

Two sports of the apple cultivar 'Red Delicious', 'Aversang' and 'Ultrared' , and two sports of the cultivar 'Gala', 'Galalea' and 'Splenola', were selected to study differentiation of apple sports by pollen ultrastructure.

'Aversang' is a bud mutation of Red Delicious imported from France in 1984.

'Ultrared' is a bud mutation of 'Starking Delicious' discovered in USA, 1964 (Brooks and Olmo, 1974).

'Galalea' is a sport of 'Gala' under protective direction in New Zealand in 1986 but the application for PVR was later withdrawn.

'Splenola' is a bud mutation of 'Gala' discovered in New Zealand but PVR status was refused in 1994 on the grounds that it was not distinct from existing 'Gala' sports 'Regal Prince' and 'Applewaites'.

Three trees per cultivar were selected. Two of these trees were at the Clyde Research Orchard (HortResearch), Central Otago, New Zealand (45°14'S 169°20'E) and the third was at the Havelock North Research Station (HortResearch), New Zealand (39°40'S 176°53'E). The trees were eight years old and grafted on MM106 rootstock.

3.2. Pollen collection

Spring pollen

Pollen was harvested by the method described by Galleta (1983). Flowers were collected from two year old wood just before the flowers opened in October. This ensured that the

pollen was mature and not contaminated by bee or wind-borne pollen. The anthers were removed using tweezers and dried on foil for 24 to 48 hours over silica gel. During the drying process the anthers progressively released the pollen grains. Pollen was stored on silica gel at room temperature in small vials loosely stoppered with cotton wool until it was used for light or scanning electron microscopy.

Forced pollen

For this project five to ten 30 cm sections of two year old shoots were collected after leaf fall in June and stored in plastic bags at 5°C for six weeks to break the dormancy. The base of the shoots were then trimmed under water and placed in small water-filled jars. Plastic bag covers maintained high humidity, and the shoots were kept at 20°C in continuous light provided by four Osram fluorescent tubes approximately 100 cm from the bench top. The base of the shoots were trimmed every two or three days and water was replaced every day. The flowers were harvested as described above for spring pollen.

3.3. Pollen preparation for light and scanning electron microscopy

Four preparation methods were compared for initial viewing under light microscopy. They were: air drying, acetolysis, hot potassium hydroxide solution (10%) wash and rehydration. The latter three methods were tested with pollen suspended in distilled and filtered water but subsequently all methods used pollen that was dried and sprinkled on double-sided tape. The wet mounts were tested to establish if the drying processes altered the size of the shape of the pollen grains. The two best methods were selected for viewing under scanning electron microscopy. Air dried pollen was selected as the best method and was used for the rest of the project.

3.3.1. Air drying

The vial of pollen was taken from storage and shaken. Pollen was collected from the sides of the vial with a cotton bud and sprinkled on double-sided tape mounted on a microscope slide for light microscopy.

3.3.2. Acetolysis

Air dried pollen was acetolysed (Erdtman, 1960) to remove the cell contents, rinsed three times in double distilled, filtered water, and fixed in gluteraldehyde fixative (Karnovsky, 1965) for 24 hours. The samples were rinsed three times in double distilled, filtered water. For the wet mount the pollen was suspended in a drop of water and covered with a coverslip. For the dry mount the suspension was dried overnight at 35-40°C in a drying oven. Dried pollen was sprinkled on the microscope slide prepared with double-sided tape.

3.3.3. Hot Potassium Hydroxide wash

Pollen was soaked in a potassium hydroxide solution (10%) in a boiling water bath for 15 minutes. The sample was rinsed three times in double distilled, filtered water and fixed in gluteraldehyde fixative (Karnovsky, 1965) for 24 hours. The sample was rinsed three times in double distilled, filtered water then wet and dry mounted as described above (section 3.3.2).

3.3.4. Rehydration

Pollen was washed in a 10% sucrose solution for 15 minutes before the sample was rinsed once in double distilled, filtered water and fixed in gluteraldehyde fixative (Karnovsky, 1965) for 24 hours. The sample was rinsed three times in double distilled, filtered water then wet and dry mounted as described above (section 3.3.2).

3.3.5. Scanning electron microscopy

For the scanning electron microscopy an aluminium Cambridge stub was coated with double-sided tape and pollen sprinkled on with a cotton bud. The surface of the stub was viewed through a binocular microscope to ensure that the maximum amount of pollen covered the stub without any grains overlapping. If the pollen was clumped then the cotton bud was used to lightly brush the surface and dry air was puffed over to dislodge any loose pollen grains. The stubs were stored on silica gel until they were sputter coated with gold and viewed under scanning electron microscopy.

3.4. Scanning electron microscopy

Pollen samples were examined and photographed using a Cambridge 250 Mark 3 scanning electron microscope with an accelerating voltage of 20 KV. Good resolution was obtained with a 45° stage tilt, however this introduced a foreshortening distortion. The tilt of the stage away from the observer meant that pollen grains orientated other than horizontally were rotated in three dimensions but viewed on the screen and on the photograph in two dimensions. This rotation caused pollen grains that were not horizontal through the longitudinal axis to appear shorter on the screen or in the micrograph than horizontal pollen grains (fig. 1).

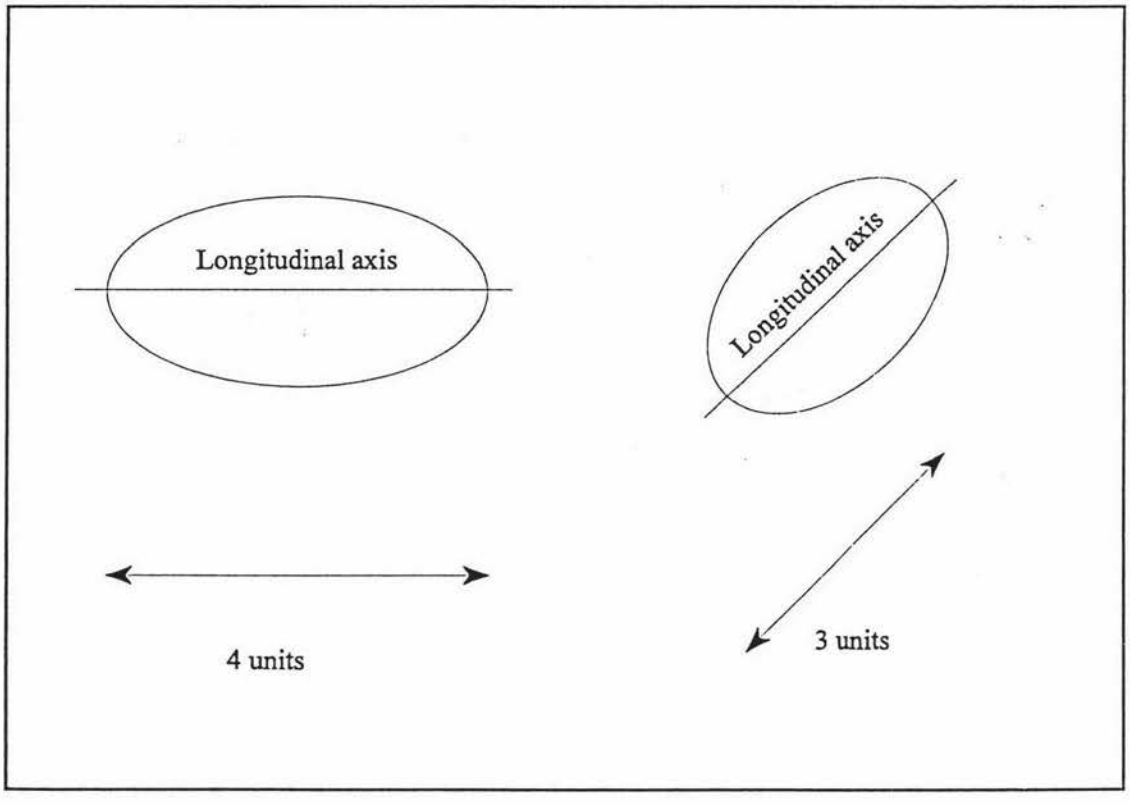


Fig. 1. A diagram demonstrating the distortion of measurements such as pollen grain length if the object is rotated in three dimensions and then viewed in two dimensions (as in a micrograph). Pollen grains not orientated horizontally appear to be shorter.

This distortion was minimised by selecting only horizontally aligned (± 10°) pollen

grains. Micrographs were taken at low magnification (approx. x 2000 to 2500) to view the entire pollen grain, and at high magnification (approx. x 8200 to 8700) to view the ridge details of the equatorial region between and excluding the germinal furrows.

3.5. Pollen characteristics and analysis

Terms for pollen morphology are complex and not standardised. Pollen has an inner cell wall made mainly of cellulose surrounding the living cytoplasm, the intine, and a hard chemically resistant outer layer, the exine, which is made from high molecular weight fatty acids called sporopollenins (Kapp, 1969). In scanning electron micrographs of apple pollen the major feature is the ridged pattern of the exine caused by columellae (fig. 2) joined together to form a striate pattern (more or less parallel) to a rugulate (irregular) pattern. The exine pattern was determined by the plant cells rather than by the individual pollen cells (Pandey and Troughten, 1974). If the pollen pattern is controlled by one genotype, then analysis of the pollen may reveal the identity of that genotype.

Apple pollen grains are described by Fogle (1977a) as elliptical and tricolpate. Colpi are the large germination slits extending longitudinally from the poles. The average pollen grain size was 46 x 26μm. The small perforations between the ridges have been termed pores (Fogle, 1977a, b), tectal perforations (Martens and Fretz, 1980; Vezey *et al.*, 1990), and pits (Marcucci *et al.*, 1984). This project refers to the tectal perforations as pores.

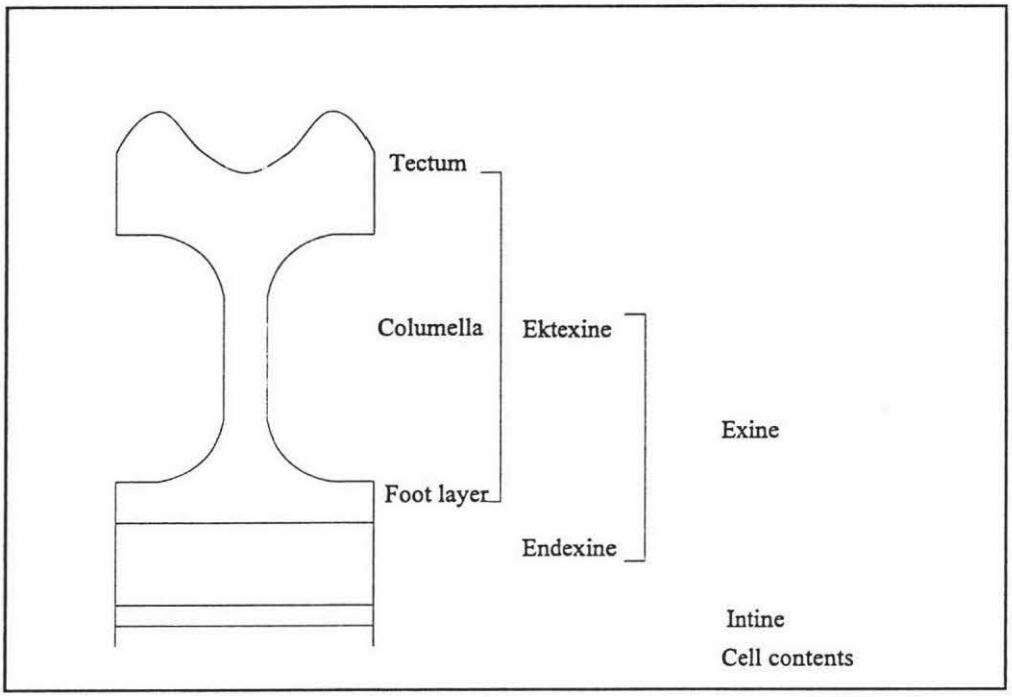


Fig. 2. A cross section of the pollen surface structure. The exine structures form patterns seen on the surface of the pollen grain.

3.6. Image analysis

Image analysis was used to extract quantitative data from the scanning electron micrographs. Hardware and software set-up that were established before this project are described below. The algorithm developed during the course of the project is closely connected to the results and so is described in the results (section 4.4)

3.6.1. Software and Hardware Systems

The software was the Visual Image Processing System version 5 (VIPS5) developed by Dr. Donald Bailey of the Image Analysis Unit, Massey University. VIPS5 was run on an IBM compatible 486 with Windows.

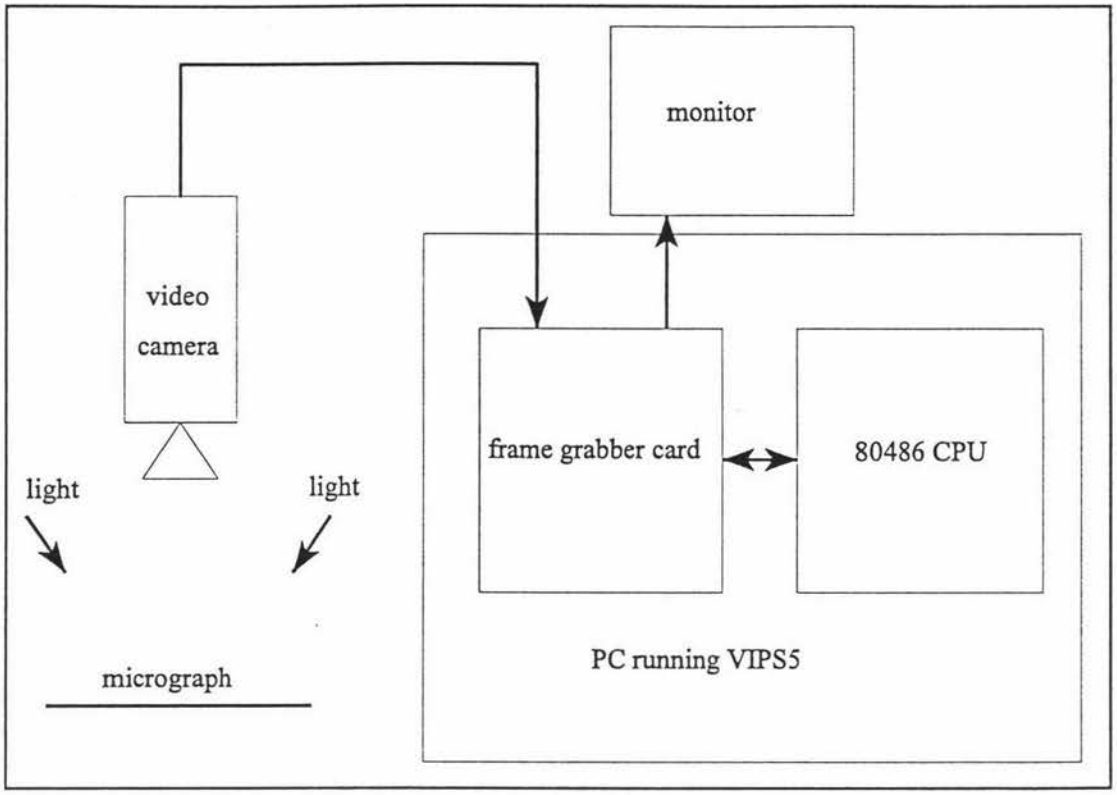


Fig. 3. A schematic diagram of the hardware set-up for image analysis. CPU = central processing unit. VIPS 5 = Visual Image Processing Software version 5.

The hardware set-up is shown in the diagram above (fig 3). The camera was a Sony colour video CCD camera model DXC-3000P linked to a PC Vision Plus frame grabber card manufactured by Imaging Technology connected to a multisync monitor.

3.6.2. Image capture

The low magnification micrograph was placed under the camera for image capturing. The operator adjusted the position of the micrograph to orientate the grain horizontally. The micrograph was then taped down in this position. Later, when the high magnification micrograph was analysed the high magnification micrograph was placed on top of the low magnification micrograph in the same orientation to correct for any

small deviation of the grain from horizontal. The image captured was 512 x 512 pixels and had 256 grey tone levels (0 = black, 255 = white).

3.6.3. VIPS Commands and Sub-routines

Definitions for the VIPS 5 commands used in the algorithm are extracted from the VIPS 5 manual and listed in the appendix A.

3.7. Comparison between spring flowers and forced flowers

Identification of 'Red Delicious' and 'Gala' apple sports using pollen is limited by the seasonality of flowering. The availability of pollen can be extended by forcing budwood to flower out of season (Layne, 1983). However, the suitability of pollen from forced flowers for fingerprinting is unknown. Forced flower and spring flower pollen production was compared on 'Harrold Red' apple. 'Harrold Red' was a bud mutation of 'Starking Delicious' discovered in USA, 1953 (Brooks and Olmo, 1962). Five trees of 'Harrold Red' apple were selected at the Fruit Crop Unit, Massey University, Palmerston North, New Zealand (40°23'S 175°37'E). Ten 30cm sections of two year old branches were labelled for spring flower observations and ten similar branches were harvested in July for forcing. The forcing conditions were described in section 3.2. The numbers of flower buds per total growing buds and the numbers of flowers per flower bud were measured and averaged for each 30 cm section of wood. The average fresh pollen weight per flower for a ten flower sample per 30 cm section of wood was also recorded.

3.8. Statistical methodology

3.8.1. Experimental design

The experimental design for the main scanning electron microscope study on dried 'Red Delicious' and 'Gala' cultivar pollen was a crossed, nested factorial design with two pollen collection methods (winter forced flowers and spring flowers), two cultivars ('Red Delicious' and 'Gala'), two sports nested within each cultivar ('Aversang' and 'Ultrared' within 'Red Delicious', and 'Galalea' and 'Splenola' within 'Gala'). Three replicate trees (two at Clyde and one at Havelock North) nested with each sport. A random sample of ten pollen grains without excessive debris or surface distortion, and in the correct orientation are selected, viewed with the scanning electron microscope, photographed at low magnification to view the whole grain and at higher magnification to view the central region of the pollen grain between the germination furrows.

The design for the comparison of spring flowers and forced flowers was a randomised block design. There were two treatments (winter forced flowers and spring flowers) applied to ten, 30cm sections of two year old wood replicated on five trees of 'Harrold Red'.

3.8.2. Data collected

The data collected from the scanning electron micrograph images fell into three groups. Measurements of the pollen grain, the ridges, and the pores in the exine. The measurements taken are listed below.

Pollen grain

Pollen grain length, width, length:width ratio and rectangular area were extracted.

Exine ridge pattern

Exine ridge run at 0 to 180° from the longitudinal axis of the pollen grain. The

proportion of the total angles and the ridge width were calculated for each of the 18 ten degree sections of the full 180°, generating 36 variables.

Pores

Pores per unit area, percentage area covered by pores, average area per pore, average longest pore diameter weighted by pore area (pore length), average pore diameter perpendicular to the length weighted by area (pore width), and length:width ratio weighted by pore area were extracted.

The exine ridge pattern data was not independent. For example an exine ridge pattern that had all ridges in the 90-100° angle range with an average ridge width of 0.3µm produces data with zeros for each 10° section apart from the 90-100° angle section. Average width in the 90-100° range = 0.3 and the frequency is 100%. To remove the possible bias, the lack of independence between the two variables a Principal Components Analysis was run separately for the average ridge width and for the percent occurrence of the ridge angle data. Then the principal component (PC) scores were then used instead of the raw data for further analysis. The PC scores for the ridge width and area were independent, which satisfies the assumptions for multivariate analysis of variance (MANOVA).

3.8.3. Analysis

Statistical analysis of the data to differentiate sports of apple cultivars followed four steps. Firstly, the data was tested for significant variation. Secondly, variables that contributed the most to the separation of apple sports were selected. Thirdly, the apple cultivars and sports were differentiated by the selected variables. Fourthly, the discriminating power of the data was tested on an unknown sample (section 2.6).

Multivariate and univariate analyses of variance were applied to know how the data changed with pollen collection method, environment and genotype. Canonical Variate Analysis (CVA) was applied to select variables that contributed the most to the

differentiation of apple sports. The CVA was then reapplied with the selected variables and the canonical scores plotted to visualise the separation of the apple sports. Canonical scores were further interpreted by Duncan's multiple range test to find significant separation of the apple sport means. Variation within the apple sports was reduced by aggregating observations within each replicate by three and finding the mean of each variable. A weight was assigned to each aggregate by applying a Principal Components Analysis (PCA) to the data, aggregating the first principal component scores and using the complement of the variance. So if an aggregate of three observations had a large variance then the weight factor would be small. CVA was reapplied with the averaged data and weights. Success of the method in identifying unknown pollen samples is tested by Discriminant Analysis (DA).

4. RESULTS

4.1. Pollen collection

The apple cultivar 'Ultrared' tree from Clyde did not produce pollen for either the forced pollen or the spring pollen treatments. At Havelock North 'Galalea' produced no forced pollen and 'Aversang' produced no spring pollen. Generally the forced 'Red Delicious' cultivars had low vigour.

4.2. Pollen preparation for scanning electron microscopy

Preliminary work was needed to select the appropriate methods for viewing pollen under the scanning electron microscope.

4.2.1. Air dried pollen

The initial air dried pollen (fig. 4) showed a high proportion of collapsed grains when viewed with the scanning electron microscope. However, the air dried method was perfected by minimising the time between drying and preparation for scanning electron microscopy. The air dried pollen viewed by light microscopy produced a relatively uniform elliptical shape which was elongated along the polar axis and slightly flattened at the poles (fig. 5).

4.2.2. Acetolysed pollen

The wet mounted acetolysis technique produced elliptical to spherical shapes which were elongated along the polar axis. The pollen grains were rounder and darker than the air dried pollen (fig. 6). The pollen from the dry mounted acetolysis technique tended to clump together and the exine shells shattered when manipulated to separate the grains. The whole grains were almost spherical and the orientation random when viewed under a light microscope (fig. 7).

4.2.3. Hot potassium hydroxide solution treated pollen

The wet mounted hot potassium hydroxide treatment produced variable shaped pollen grains when viewed with light microscopy (fig. 8). The grains either retained the elliptical shape of a dried grain or swelled into round or triangular shapes with the poles facing the observer. Various shapes between these two extremes were also observed. Pollen from the hot potassium hydroxide treatment congealed into a solid mass when dried. Individual pollen grains and features of the dried pollen grains could not be distinguished under the light microscope.

4.2.4. Rehydrated pollen

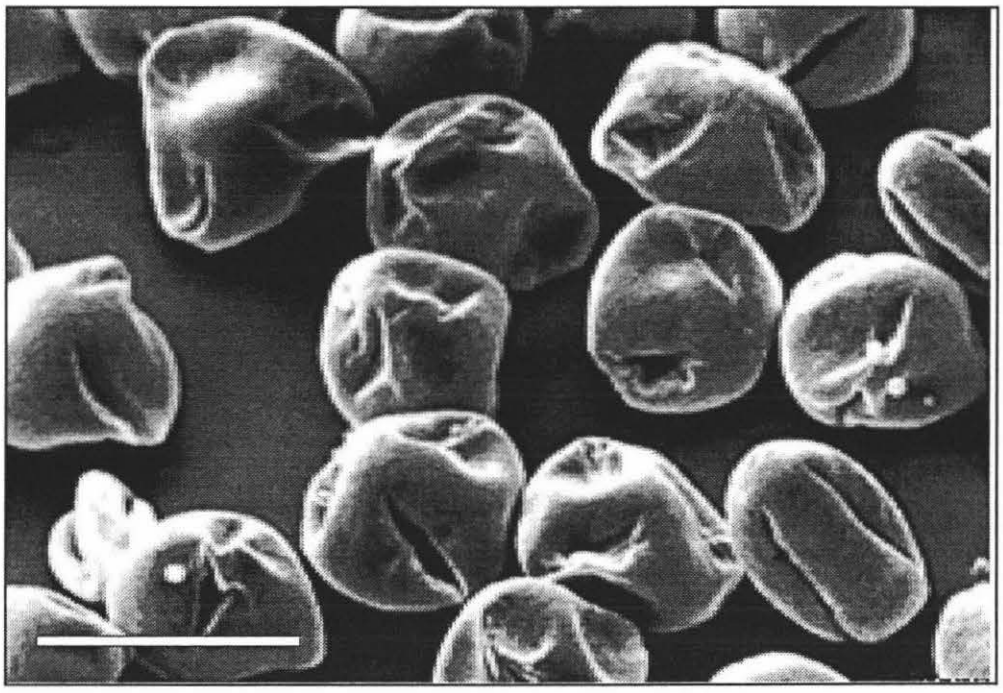
The wet mounted rehydrated pollen (fig. 9) was triangular rather than elliptical and was orientated to show the polar face. The germination furrows were pushed out to form the corners of the triangle. The dry mounted rehydrated pollen (fig. 10) produced consistent shapes which were not damaged by handling. This preparation was selected for further viewing under scanning electron microscopy.

4.3. Scanning electron microscopy

The scanning electron micrography confirmed the observations of the light microscopy. Air dried pollen produced elliptical shapes which were elongated along the longitudinal axis and slightly flattened at the poles (fig. 11). Rehydrated pollen had a triangular shape with the polar end facing the observer (fig. 12). The germinal furrows were pushed out to the corners of the triangle and expanded to reveal the intine.

Fig. 4. Scanning electron micrograph of air-dried 'Red Delicious' apple pollen which was stored for 12 months in a freezer. Most of the pollen grains are collapsed. Scale bar = 40μm.

Fig. 5. Light micrograph of freshly prepared air-dried 'Red Delicious' apple pollen. The pollen grains are not collapsed. Scale bar = 40μm.



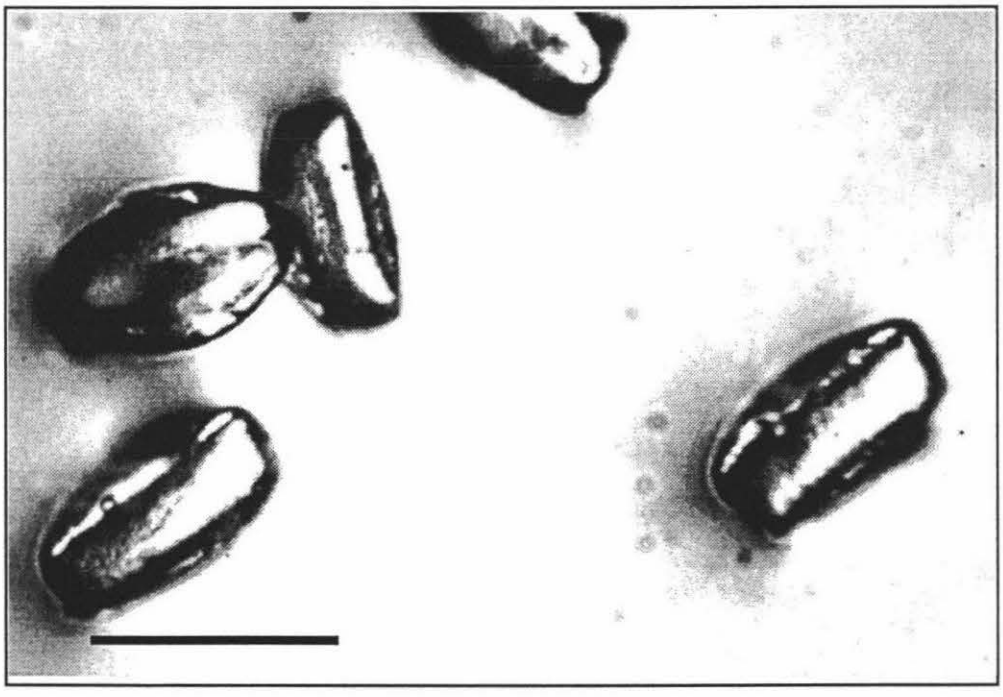
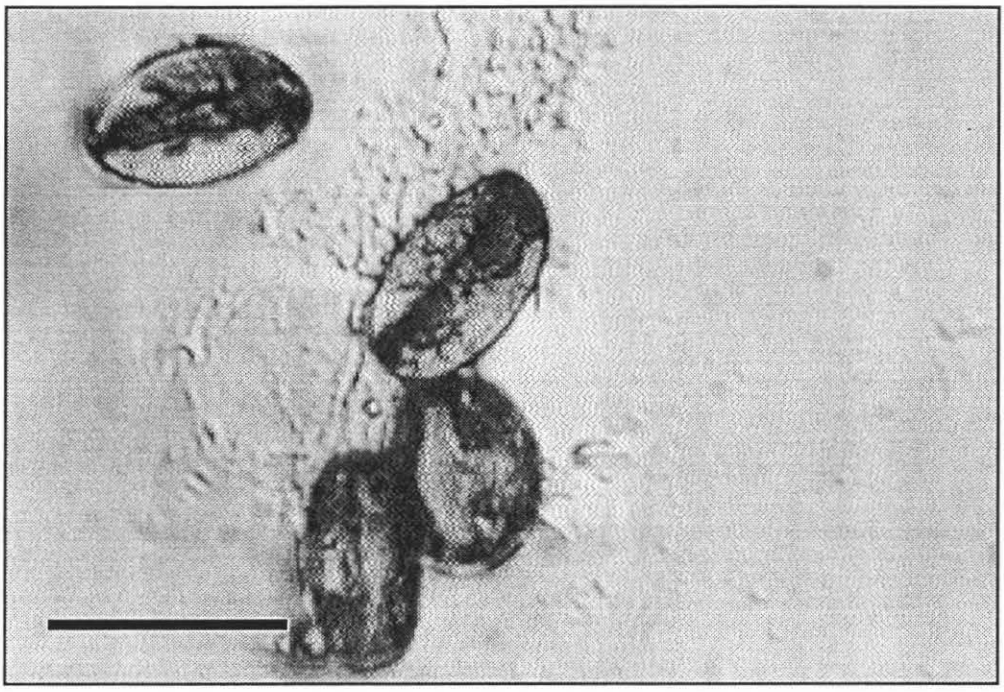


Fig. 6. Light micrograph of acetolysed 'Aversang' apple pollen suspended in water. Scale bar = 40μm.

Fig. 7. Light micrograph of acetolysed 'Aversang' apple pollen dried and sprinkled on double-sided tape. Scale bar = 40μm.



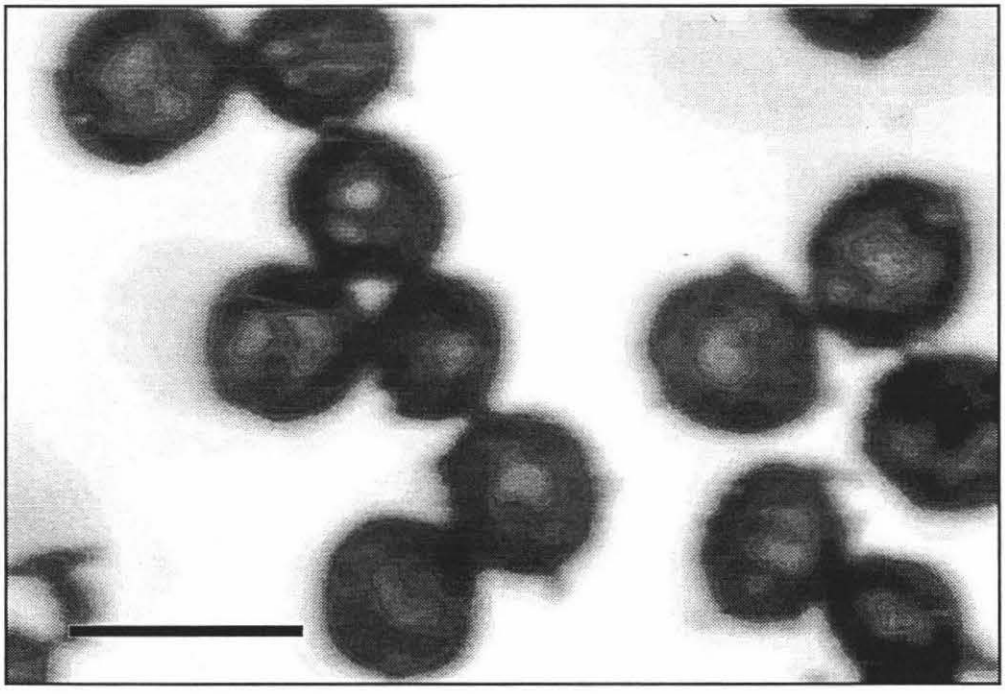


Fig. 8. Light micrograph of 'Aversang' apple pollen prepared with a hot potassium hydroxide (10%) wash and suspended in water. Scale bar = 40μm.

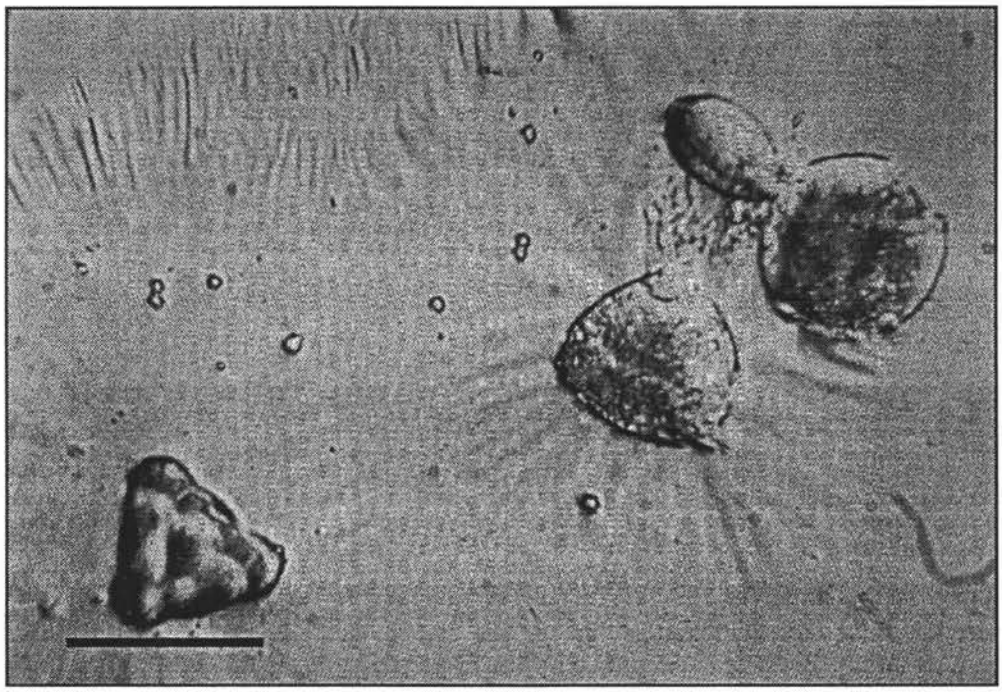
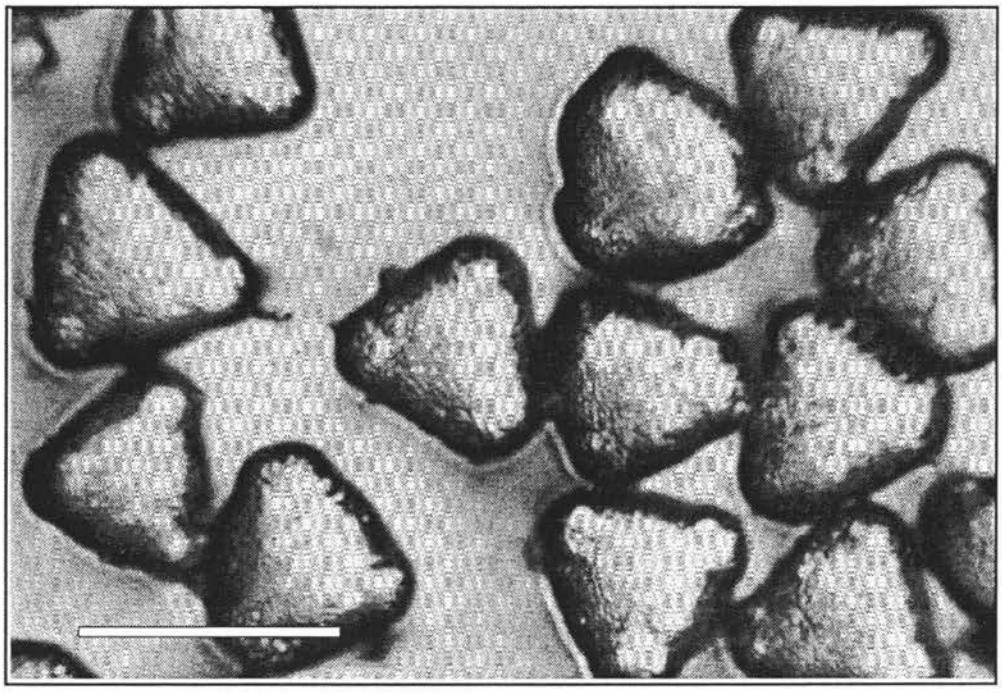


Fig. 9. Light micrograph of rehydrated and fixed 'Aversang' apple pollen suspended in water. Scale bar = 40μm.

Fig. 10. Light micrograph of rehydrated and fixed 'Aversang' apple pollen, dried and mounted on double-sided tape. Scale bar = 40μm.



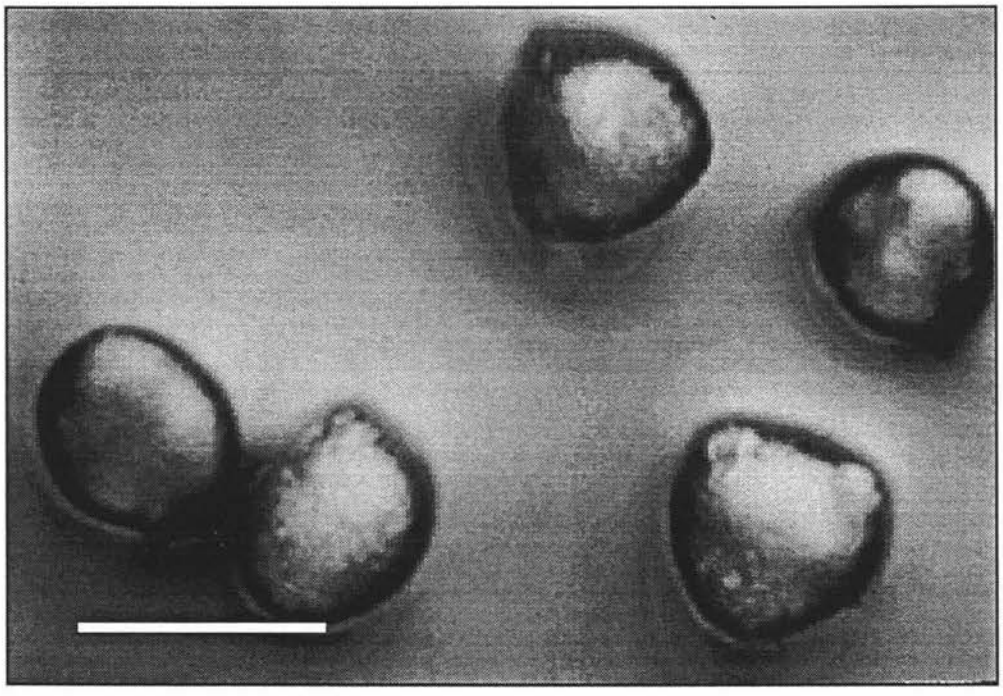
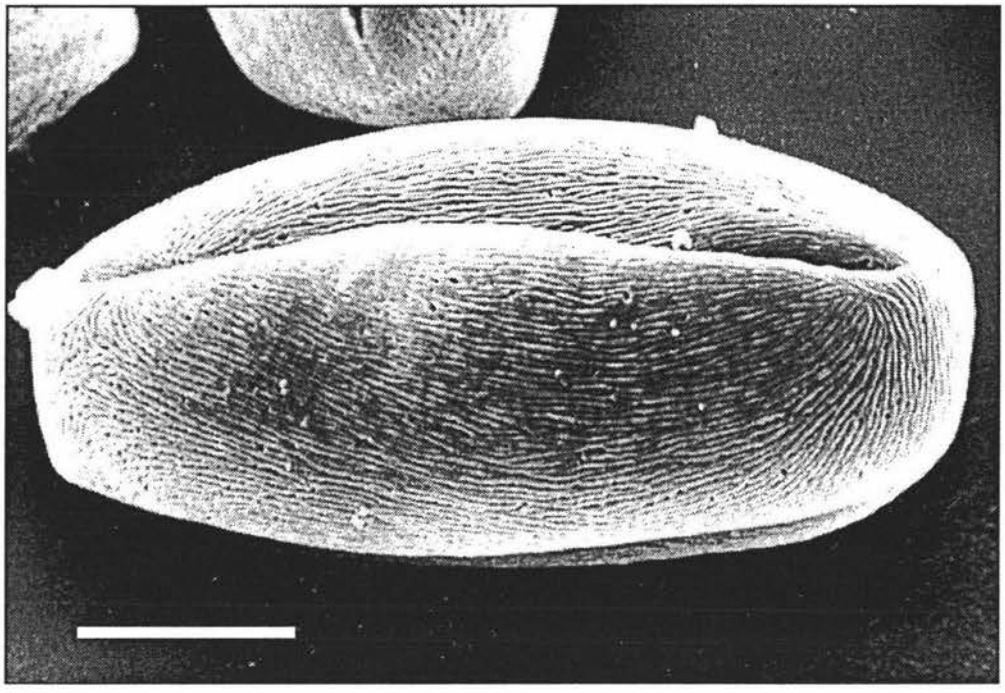
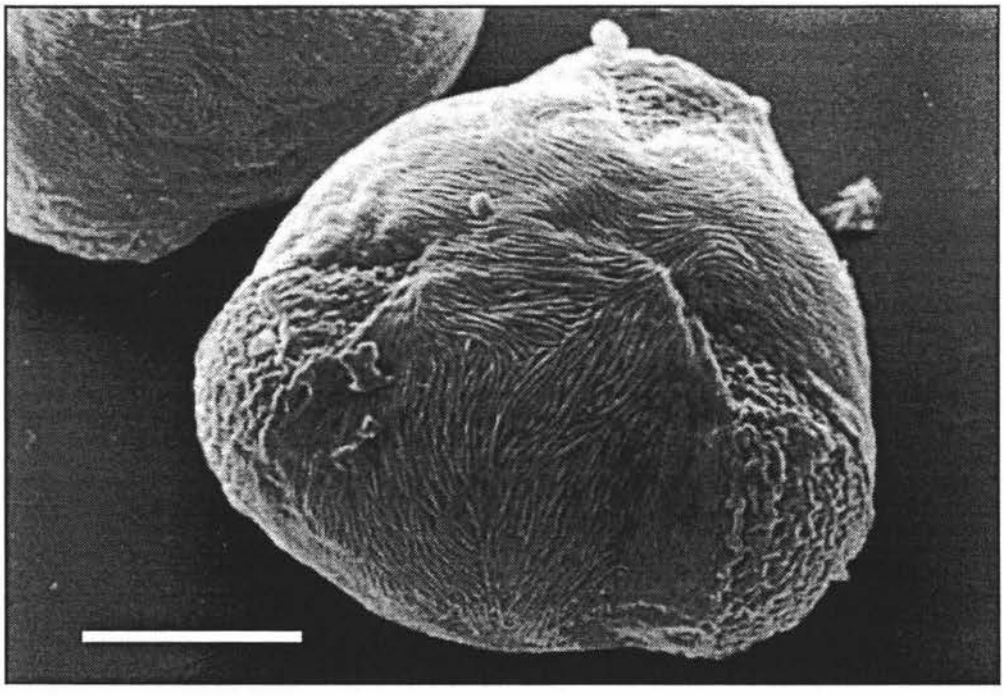


Fig. 11. Scanning electron micrograph of air-dried 'Aversang' apple pollen. Two of the three germinal furrows are visible and extend along the length of the pollen grain. Scale bar = 10μm.

Fig. 12. Scanning electron micrograph of rehydrated and fixed 'Aversang' apple pollen. The germinal furrows are split wide open on the corners of the pollen grain. Scale bar = 10μm.





4.4. Image analysis algorithm

The basic algorithm for the image analysis program is outlined in the flow chart in figure 13. The program included three sections. The first section extracted data about the pollen grain outline from the low magnification micrograph, the second and third ones used the high magnification micrograph to extract data from the ridge patterns and the pores on the surface of the pollen grain. Difficulties were encountered in the automatic selection of areas of interest for data extraction and operator interaction was required.

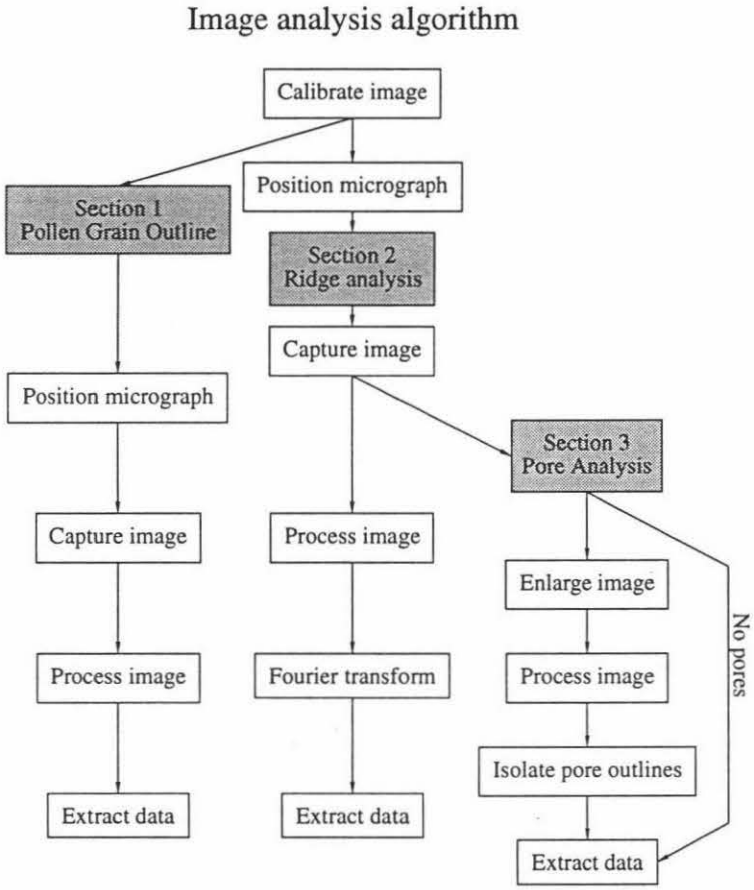


Fig. 13. Flow diagram outlining the three sections and general structure of the image analysis algorithm.

4.4.1. Whole pollen grain measurements

A low magnification image (2000-2500 x) of the whole pollen grain was captured (fig. 11). Pollen grain length, width, length:width ratio and rectangular area were extracted from the image. A series of steps was necessary to enhance the pollen grain outline, join gaps in the outline, remove noise and distortions, select the outline and extract the outline measurements.

Step one: Enhance the pollen grain outline.
Intensity variation in the micrograph was removed and the black regions enhanced by subtracting the average within a 35 x 35 pixel box. The boundary between the pollen grain and the background was enhanced by setting negative differences to zero and linearly expanding to fill the full 0-255 intensity range (fig 14 A). The intensities around the edges of the grain were not uniform, but the background area were mostly black. A threshold at intensity level 1 (0=black 255=white) was selected to transform all the pixels were not black to white (fig. 14 B). This threshold defined the boundary between the pollen grain and the background.

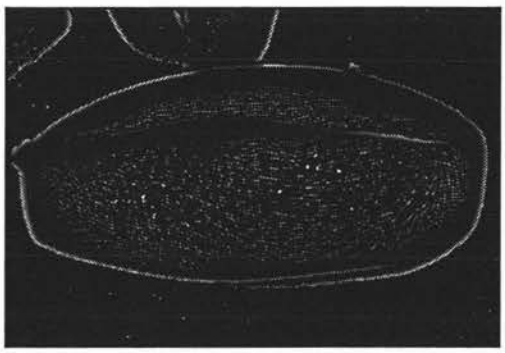
Step two: Join small gaps in the pollen grain outline.
Small gaps in the outline of the pollen grain must be joined to facilitate the isolation of the entire edge. A 5 x 5 pixel box average blurred the image slightly, joining small gaps. Then the maximum value of a 5 x 5 pixel box followed by the minimum value of a 5 x 5 pixel box enhanced the joins. A threshold was applied to the image at intensity value of 200 to get a binary image (fig. 14 C).

Step three: Remove noise and distortions.
Debris touching the pollen grain and distorting the outline of the image were removed and the segments of the outline were selected by the operator (fig. 14 D).

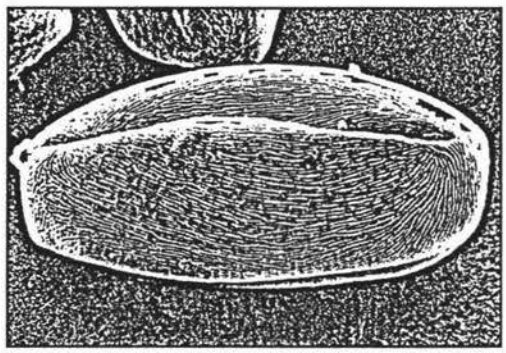
Step four: The outline segments were linked by lines to enable the outline to be selected as a whole (fig. 14 E).

Step five: Extracting the data.
The whole outline was 'chain coded'. A chain code is a list of pixels coding the boundaries of objects in an image. VIPS5 commands then extracted length and width measurements from the chain code. This chain code was filled with white and displayed (fig. 14 F).

Fig. 14. The processing sequence of a 512 x 512 pixel image from a scanning electron micrograph of air dried 'Aversang' pollen. **A.** The boundary between the pollen grain and the background enhanced. **B.** Theshold applied to the image to enhance the contrast. **C.** Small gaps in the pollen grain outline joined and noise in the image reduced. **D.** Debris or distortions in the pollen grain outline removed. **E.** Outline segments joined and isolated. **F.** The chain code redrawn and filled.

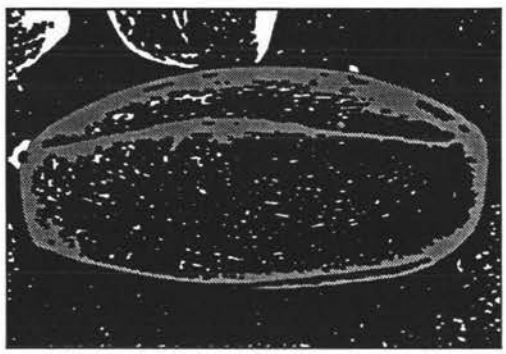


A.

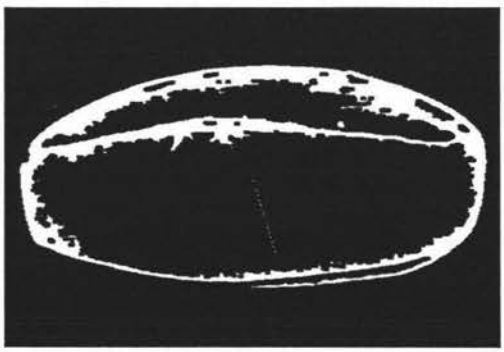


B.

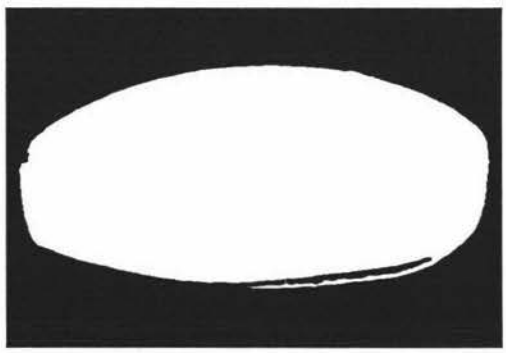
C.



D.



E.



F.

4.4.2. Fourier analysis of the ridge patterns

The high magnification image of the air dried 'Aversang' pollen grain was taken from the central region between the germinal furrows (fig. 15). This region has the largest area with few distortions due to folds or curves in the pollen grain surface. Curved surfaces will change the apparent width or angle of the ridges. Selection of pollen grains with the same orientation minimised the foreshortening distortion due to orientation of the surface (section 3.5). A Fast Fourier transform was used to extract information on the groups of ridge angles and ridge widths. The information that the Fast Fourier transform extracted is explained in section 2.4.

Several steps were required to enable exine ridge pattern data to be extracted. The first step was to equalise the image intensity range. Second, the image was 'windowed'. Third, the image was Fast Fourier transformed. Fourth, unwanted areas of information removed from the image, and in the fifth step the data was extracted.

Step one: Intensity range adjusted.
Differences in the intensity range across the micrograph of the exine pattern were due to variation in exposure to the scanning beam (fig. 15 A). These variations would be picked up by the Fourier analysis as unwanted low frequency components. Broad variations in intensity were removed without removing the fine detail by subtracting the average of a 35 x 35 pixel box, setting negative differences to zero and linearly expanding the grey scale as described in the low magnification image section (fig. 15 B).

Step two: Image was windowed.
The image of the exine ridge pattern was 'windowed' (fig. 15 C) prior to applying a two dimensional Fast Fourier transform. Windowing is a VIPS5 command that reduces the strength of the image near the edges to minimise artifacts from the Fast Fourier transform.

Step three: Fast Fourier transform applied.
The Fast Fourier transform was applied to the image of the exine ridge pattern and the results stored in a Fourier image (fig. 16). The centre of the Fourier image was the origin, the distance from the origin was proportional to the frequency of the sinusoidal variation, the angle corresponds to the angle of the sinusoidal variation and the intensity of the point was proportional to the strength of the variation.

Step four: Unwanted areas of information removed.
Since the frequency and angle were of primary interest, a polar to rectangular mapping was used to allow these variables to be viewed more conveniently. In this image (fig. 17) the horizontal axis represents the frequency of the variation across the image and the vertical axis represents the angle of the sinusoidal variation from the horizon. The pattern repeats because the 0-180° angle about the radial origin is a mirror image of the 180-360°. An average of the 0-180° and the 180-360° halves of the image was calculated and an 11 pixel Gaussian box average applied to smooth noise (fig. 18). Bright areas in the image correspond to frequency and angle of strong sinusoidal variation in the

original image. The isolation and collection of the information from these peaks gave information on the angle and width of the main exine ridge patterns (fig. 18 B).

Before information extraction, extra information like harmonics and low ridge frequencies needed to be removed. The intensity across the exine ridges was not purely sinusoidal and this resulted in a series of harmonically related peaks in the frequency domain (fig. 18 C). These additional harmonics did not contain any information of interest and had to be removed to prevent them from distorting the ridge width estimate. In addition very low ridge frequency information related to variation in the background shading (fig. 18 A). These areas of information were not desirable but were difficult to remove automatically without affecting the exine ridge dimension data. A threshold at intensity 128 removed information of low sinusoidal strength, most of the harmonics and the low frequency information (fig. 19). If there were still areas to be removed, the values of the peaks were checked by the operator against the original image of the ridges and the unwanted data deleted.

Step five: Data extracted.
After removal of the harmonics and the low frequency information the image was divided into 10° angle sections. In each section the average ridge width and the frequency of the ridge angles were recorded. An example of the data recorded could be that in the 0-10° angle section 100% of the ridges occurred with an average ridge width of 0.30 μm, and all the other sections recorded zero for ridge width and angle frequency.

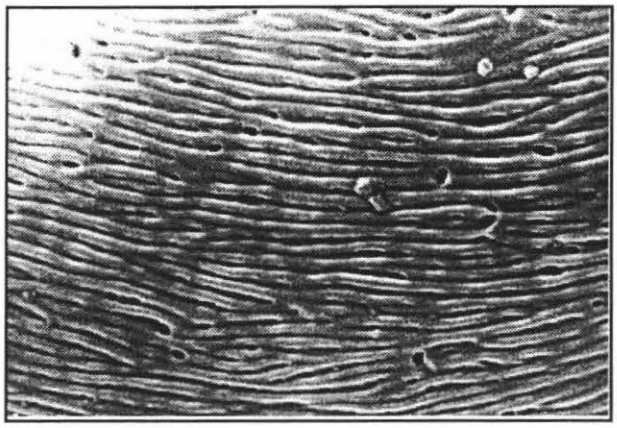
Fig. 15. Images of the central region of an 'Aversang' pollen grain between the germinal furrows prepared for the Fourier analysis.

A. The unprocessed image of the pollen exine surface captured from the high magnification scanning electron micrograph. The scanning intensity varies across the image.

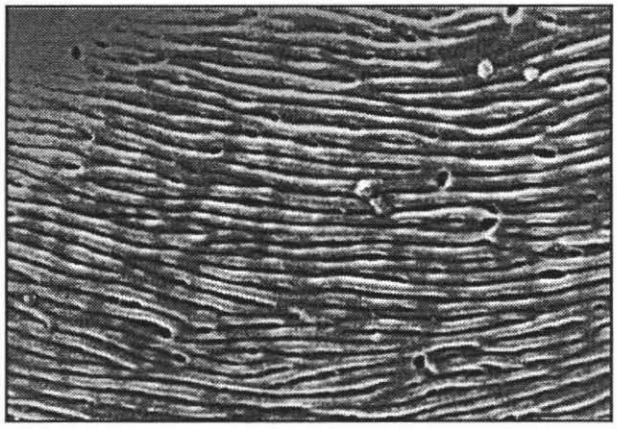
B. Broad variations in intensity were removed from the image by subtracting the average of a 35 x 35 pixel box, setting the negative differences to zero and linearly expanding the grey scale.

C. The image was processed to fade to grey at the edges (windowing). This reduced an artifact error during the Fast Fourier transform.

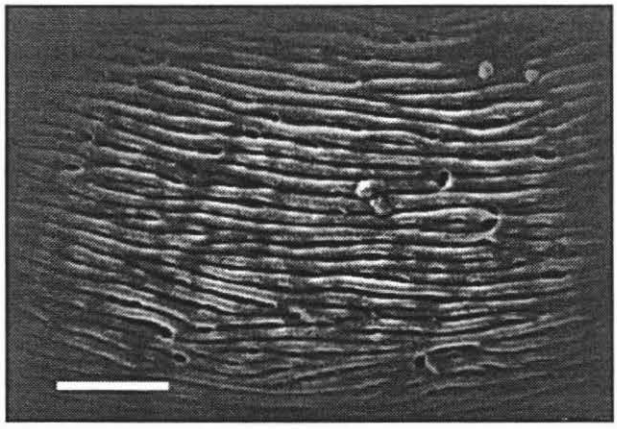
Scale bar = 2μm.



A.



B.



C.

Fig. 16. The Fast Fourier transform of the windowed exine pattern image. Angle about the origin represents the angle of the variation from light to dark (across the ridges) in the windowed image. Distance from the origin represents the frequency of the light-dark variation. The intensity of each point (colour coded) represents the strength of the light-dark variation.

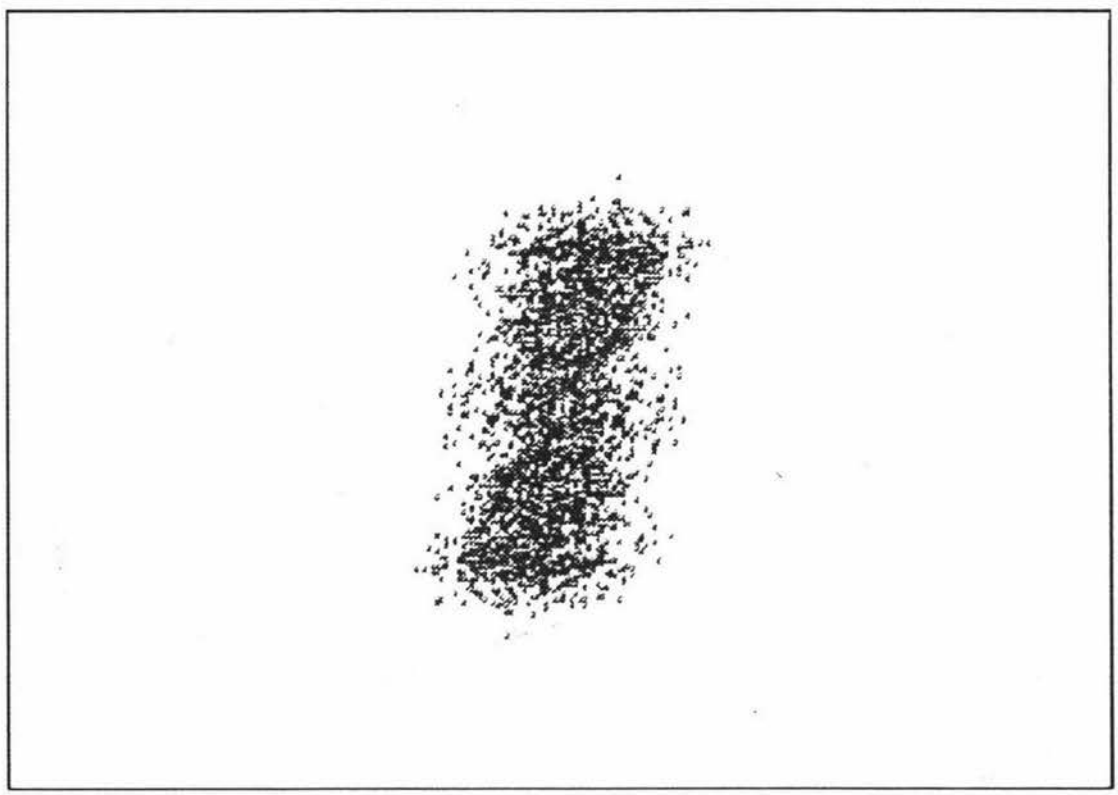


Fig. 17. The Fourier image after mapping from a radial plot to a rectangular plot. The y axis scale was the 0-360° angles of the sinusoidal variation and the x axis was the frequency (0-100) of the sinusoidal variation across the windowed image of the apple pollen exine pattern.

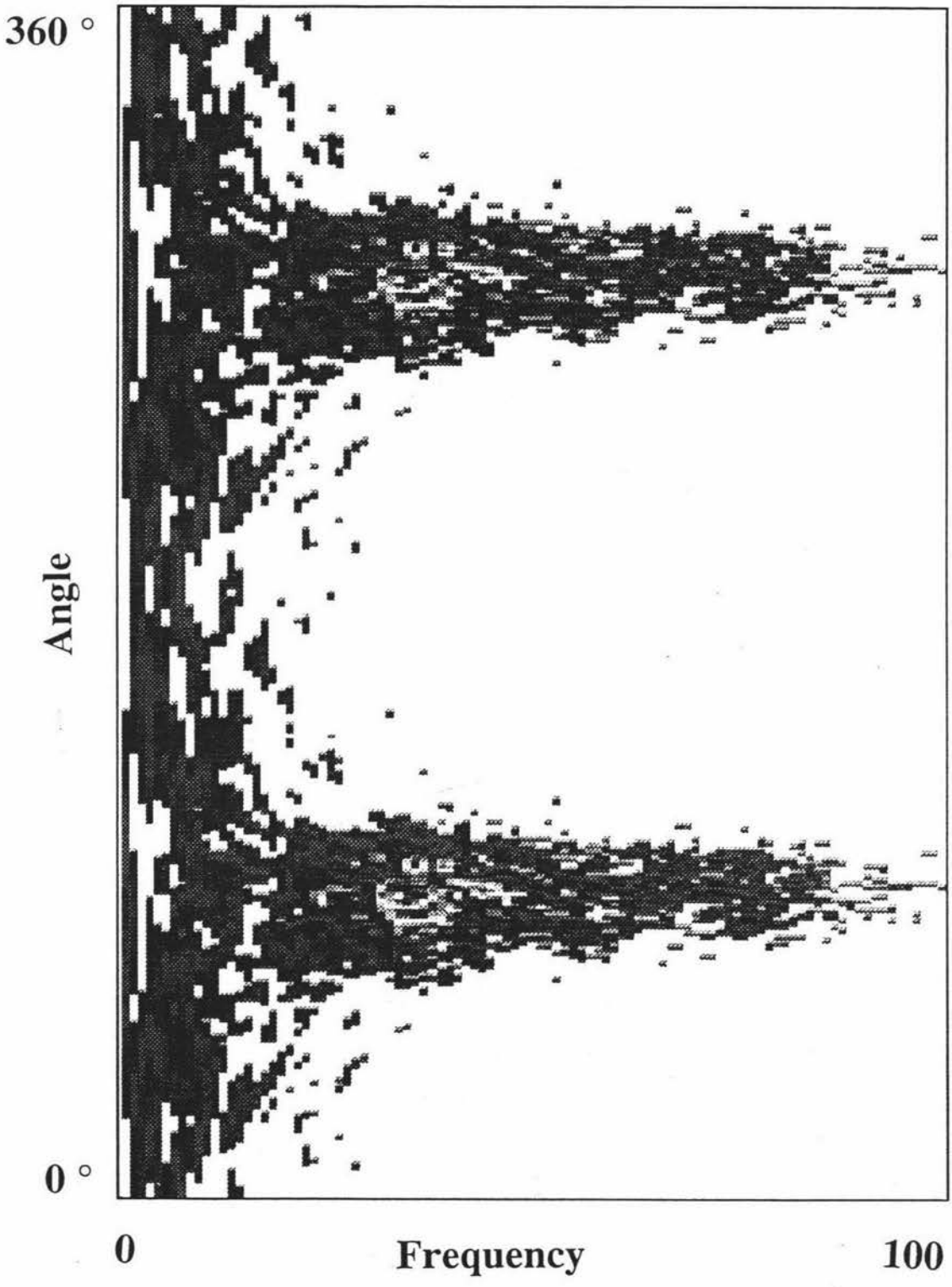
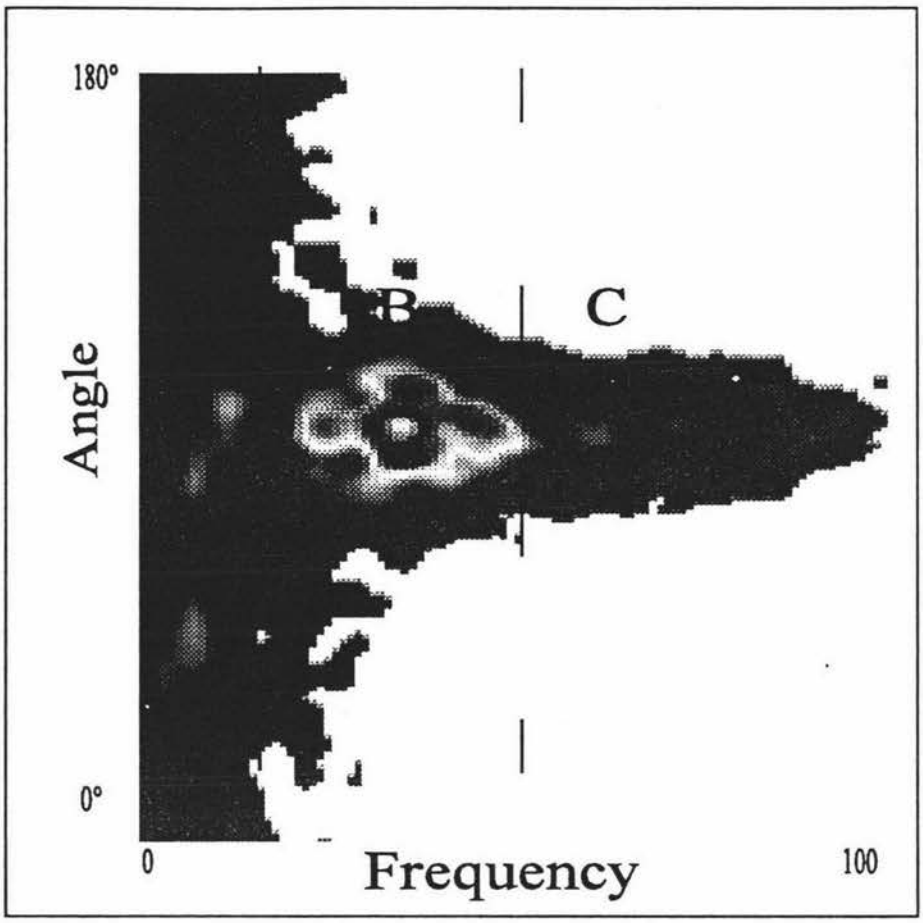
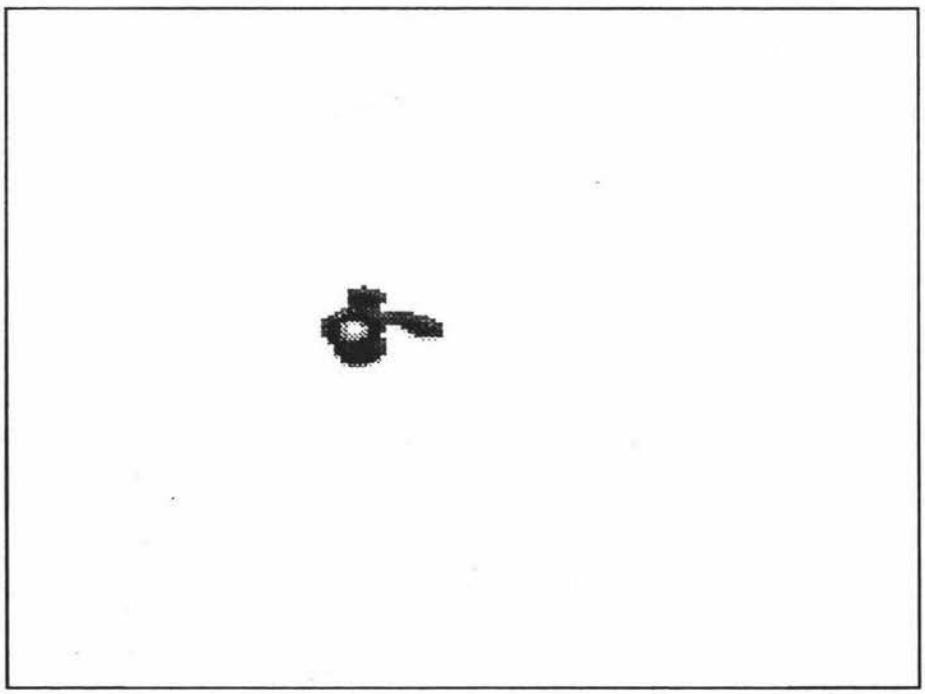

360 °
Angle
0 °
0
Frequency
100

Fig. 18. The Fast Fourier transform image of 'Aversang' exine ridge patterns processed and Gaussian smoothed, ready to extract ridge data. **A.** Low frequency information. **B.** Fundamental ridge frequency information. **C.** Harmonic frequency information.

Fig. 19. The processed Fourier image after applying a simple threshold to remove low frequency and harmonic frequency information. This isolated information on the strongest ridge patterns of the pollen exine.


180°
Angle
0°
B
C
0
Frequency
100



4.4.3. Analysis of pores in the exine

Pores in the exine varied in size, shape, and numbers from grain to grain and may be useful in differentiating the genotypes. This section describes the steps image analysis used to extract data from the pores in the exine surface. Isolation of pores automatically had the same limitations as the isolation of the pollen grain outline. There were many depressions in the exine surface. Variation in scanning beam illumination meant that these depressions could be difficult to distinguish from pores. Automatic selection of pores would require further development of the algorithm and was beyond the scope of this project. Pores were selected interactively by the operator. First the contrast was enhanced. Second, small gaps in the pore outline are filled. Third, noise is removed. Fourth, the edges are enhanced. Fifth, the pore are selected. Redefinition of the pore outline is the sixth step, and coding the pores for data extraction is the seventh step.

The operator was asked if there were any pores to analyse in the central equatorial region (fig. 20). If the operator indicated that there was no pores, the pore measurements were zero by default and no further processing was needed. If pores were present the sample area was enlarged (fig. 21) and a copy made. One copy for the operator to view was kept in the grey scale intensity range 1-254, leaving 0 and 255 free for overlaying the selected pore outlines. The other copy was used to calculate the dimensions of the pores.

To measure the characteristics of the pores it was necessary to isolate the pores from the rest of the image. Chain coding the pore outline was one way to isolate information on the pores. In order to chain code the pores the pore outlines needed to be complete and differentiated from the rest of the image.

Step one: Enhance the pore outline contrast.
The contrast of the pore edge was enhanced by dividing the image with a 35 pixel box average and then the image was linearly expanded to the full 0-255 grey scale (fig. 22 A).

Step two: Small gaps in the pore outline were filled.
The maximum value in a 3 x 3 pixel box then the minimum value in a 5 x 5 pixel box is taken to join small gaps in the pore outline and to remove some of the noise. The pores were slightly enlarged which resulted in a more accurate representation of the pore size after processing (fig. 22 B).

Step three: Noise removed.
A median filter took the median value of a 3 x 3 pixel box as the value for the centre pixel which smoothed noise and maintained edges (fig. 22 C).

Step four: Edges enhanced.
A Sobel filter enhanced the edges. The resulting image was expanded to a 0-255 grey scale intensity range then a threshold at intensity level 20 was applied (fig. 22 D). The resulting binary image was the result of steps one to four and represents the limit of automatic edge enhancement. The next steps required operator interaction.

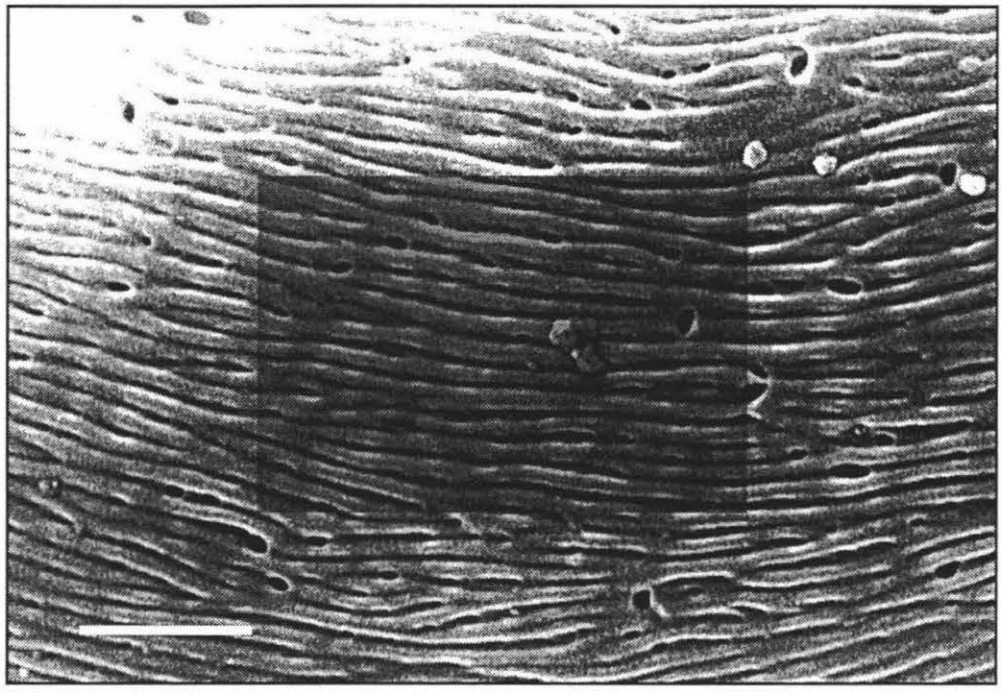
Step five: Pores selected.
The operator viewed a pseudo-colour image where 0 = blue and 255 = red and all other intensities were still on a grey scale. The operator clicked on all of the pores, using the micrograph as well as the image on screen to verify them. Each point on the screen identifying a pore was stored in a list of vectors and marked in blue for visual confirmation. The operator continued in the loop of selection until all the pores were selected. A copy of the binary image from the Sobel filter was made, then the operator-selected list of pore locations was used to fill the pores in the binary image with grey. The grey regions were selected by threshold (fig. 22 E).

Step six: Pore redefined.
The selected regions were chain coded and the chains were overlaid in red on the pseudo colour image the operator was viewing. If there was a break in the pore outline the chain coded outline could spill over into the non-pore region as the lower left pore does in fig. 22 E. The operator trimmed these pores by drawing lines in pseudo colour red (intensity = 255) on the screen. These lines were drawn at the same time in black (intensity = 0) on the binary image of the pores. The redefinition continued in a loop until the operator was satisfied with the pore outlines. When the operator exited the redefinition loop the vector list of selected pores was used to re-isolate the pores by filling them with grey and applying a threshold (fig. 22 F). The pore edges were chain coded again, redrawn, filled in on a blank image, reduced to the original size and then chain coded again for scaled measurements.

Step seven: Pore data extracted.
VIPS5 commands extracted information from the chain codes representing the pores. The number of pores per unit area, the percentage area covered by pores, the average area and the length, width and length:width ratio weighted by the proportion area of each pore were calculated and output for further analysis.

Fig. 20. The apple pollen exine surface in the central area of the pollen grain between the germinal furrows. The shaded area was selected for enlargement and pore dimensions analysis. Scale bar = 2μm.

Fig. 21. The central area of the pollen exine surface enlarged for pore analysis. Scale bar = 2μm.



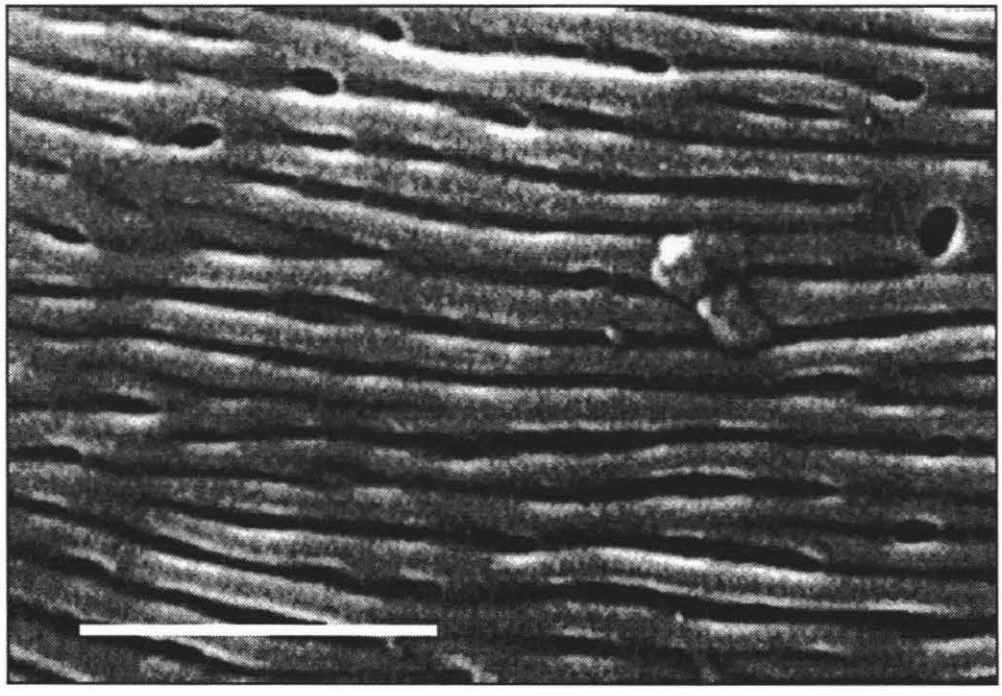
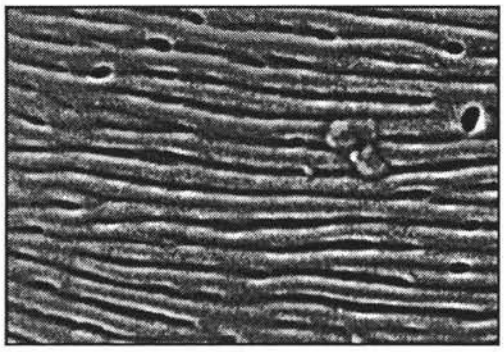
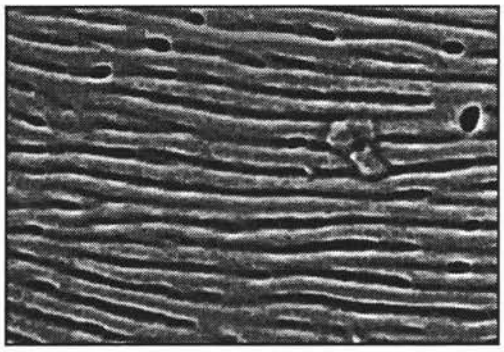


Fig. 22. Steps in the processing and measurement of pores in the exine of an apple pollen grain. **A.** The contrast across the image equalised by dividing the image by a 35 x 35 pixel box average. **B.** Noise removed, pores slightly enlarged, and small gaps in the pore outlines were joined. **C.** A median filter removed noise. **D.** A Sobel filter enhanced the edges, then a threshold converted the image into a binary image. **E.** The operator clicks on pores with the mouse then selected pores are filled and a threshold applied. The pore in the lower left corner needs redefining as the actual pore is not as long. **F.** Operator redefined pore boundaries are reselected by filling with grey before the application of a threshold.

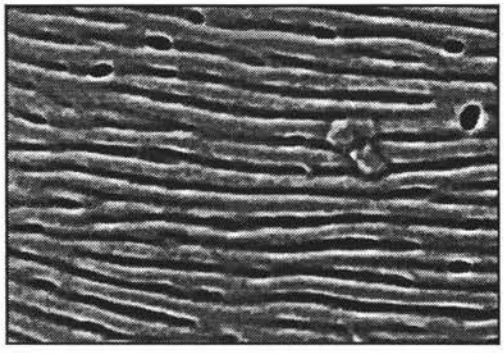
Scale bar = 2μm



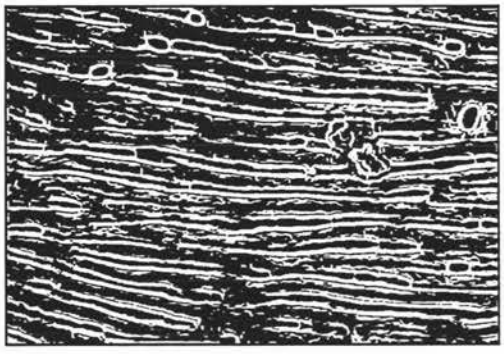
A.



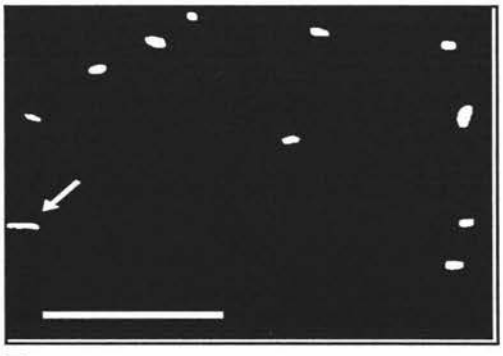
B.



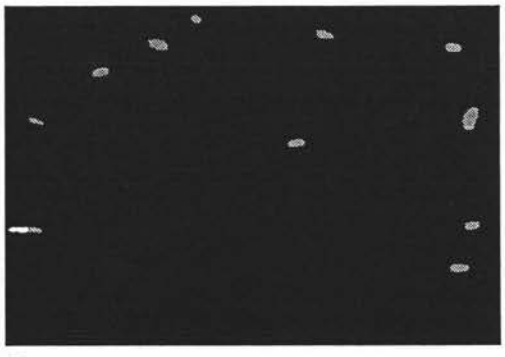
C.



D.



E.



F.

4.5. Comparison between spring flowers and forced flowers

The suitability of forced budwood as a pollen source was studied by comparing flowers of 'Harrold Red' collected from forced and spring budwood (section 3.7). The numbers of floral buds per growing bud, the flowers per floral bud and the fresh weight of pollen per flower was recorded for the apple cultivar 'Harrold Red'. The number of floral buds per growing buds, the numbers of flowers per floral bud and pollen yield per flower varied with pollen collection season ($P \leq 0.05$, 0.001 and 0.001 respectively). Spring flowers produced more flowering buds per growing buds, were more than twice as prolific as forced flowers and yielded more than three times the pollen per flower.

Table 1. Comparison between spring pollen and forced pollen yield components for apple cultivar 'Harrold Red'.

Flower harvest season	Flower buds / growing buds [y]	Flowers / floral bud [y]	Fresh pollen weight / flower (g) [z]
Spring flowers	0.80	5.0	0.0331
Forced flowers	0.56	2.2	0.0085
	*	***	***

*, *** Significance at $P \leq 0.05$, or 0.001 respectively (Duncan's multiple range test).
[y] Mean of buds on 30cm two year old apple shoots. Ten shoots from five replicates.
[z] Mean of ten flowers randomly selected per shoot.

4.6. Statistical analysis

Statistical analysis of apple pollen ultrastructure needs to follow several steps. The effect of pollen collection method, environment and genotype on the pollen ultrastructure was investigated with a multivariate analysis of variance. Individual variables were then examined to see how they were influenced by the pollen collection method, environment and genotype. Next Canonical Variate Analysis (CVA) was applied to the data to select variables that contributed the most to the differentiation of apple sports. The CVA was improved by averaging and weighting the data and analysing the canonical scores by multiple comparisons of means (Tukey's Honest Significant Difference test). Identification of an unknown sample is tested by Discriminant Analysis.

4.6.1. Nested factorial multivariate analysis of variance (MANOVA)

The multivariate statistic 'Wilks Lambda' was used as a multivariate approximation of the F distribution. The data showed strong evidence of differences between the pollen collection method, cultivar, sports and the interaction between pollen collection method by cultivar, and pollen collection method by sports. For the remainder of the analysis the 'pollen collection method' will be referred to as 'season'. The P values were 0.0001 for season and cultivar, P=0.0007 for the season by cultivar interaction, P=0.0181 for the sports and P=0.0013 for the season by sport interaction. The data did not show significant differences among the replicates (P=0.1397) (two trees at Clyde and one at Havelock North).

4.6.2. Univariate analysis of variance on non-ridge data

The effect of season was significant at the multivariate level so analysis of variance was carried out on each of the pollen grain variables and the pore variables to see what changes in morphology occur between the winter forced and the spring pollen. The ridge variables had been converted into principal component scores (PCs) and these were not analysed by ANOVA because the PCs were difficult to interpret.

There were no significant differences between the sports within a cultivar type for any of the univariate variables. The data showed differences between the cultivar types for pollen grain widths and length:width ratios ($p \leq 0.01$) and some evidence of differences for pollen grain area ($p \leq 0.05$). 'Red Delicious' pollen was wider, had a larger area and a smaller length:width ratio (table 2). Spring pollen grains were wider than forced pollen grains ($P \leq 0.05$) (table 2).

There was also evidence for differences between the replicates of pollen grain length:width ratio, pore area and pore length ($P \leq 0.05$) and total percent area ($P \leq 0.01$) (table 3). Contrasts were made between replicates within the same site (replicate 1 and 2 were from Clyde) and a contrast between the two sites (Clyde and Havelock North). Significant variation came from the contrast within the Clyde site for pollen grain length:width ratio and pore length, and percentage pore coverage was greater at Clyde

than at Havelock North ($P \leq 0.05$).

Table 2. Mean pollen grain dimensions from spring and forced flowers of the apple cultivars 'Red Delicious' and 'Gala'.

Pollen collection method	Cultivar type [x]	Grain character		
		width (μm)	area (μm^2)	length:width ratio
Forced flowers	'Red Delicious'	21.81	863.72	1.82
	'Gala'	20.74	843.89	1.97
Spring flowers	'Red Delicious'	22.77	980.89	1.89
	'Gala'	21.13	874.40	1.96
	Significance			
	Type	**	*	**
	Season	*	NS	NS

NS,*, ** Not significant, significant at $P \leq 0.05$ or 0.01 respectively

[x] Red Delicious = 'Aversang' and 'Ultrared' bulked, Gala = 'Splenola' and 'Galalea' bulked.

Table 3. Mean pollen grain and pore dimensions for bulked pollen from 'Red Delicious' and 'Gala' cultivar types.

Replicate [y]	Grain character	Pore character		
	length:width ratio	% area	area (μm^2)	length (μm)
1	1.90	0.24	0.007	0.106
2	1.94	0.56	0.012	0.137
3	1.89	0.24	0.008	0.116
Significance				
Combined replicates	*	**	*	*
Contrast between sites	NS	*	NS	NS
Contrast within a site (Clyde)	*	NS	NS	*

NS,*, ** Not significant, significant at $P \leq 0.05$, or 0.01 respectively

[y] Replicates 1 and 2 were collected from Clyde and replicate 3 was collected from Havelock North.

4.6.3. Canonical Variate Analysis (CVA)

A. Sport separation by CVA using all measurements of spring harvested pollen.
CVA is a multivariate technique that maximised the separation between the groups and minimises the variance within the groups. Significant differences between the spring and forced pollen meant that spring pollen was used for the remaining analysis to reduce the within class variation. The P values for the Mahalanobis distances were the probabilities that the squared distance between groups was zero (Table 4).

Table 4. P values of Mahalanobis Distance for Squared Distance to apple sports of 'Red Delicious' and 'Gala'.

	Aversang	Galalea	Splenola	Ultrared
Aversang	1.0000	0.0888	0.0002	0.0093
Galalea		1.0000	0.2616	0.0060
Splenola			1.0000	0.0006
Ultrared				1.0000

Members of the sport pairs 'Splenola'-'Aversang', 'Aversang'-'Ultrared', Ultrared'-'Galalea' and 'Ultrared'-'Splenola' had separations significantly different from zero ($P \leq 0.01$). Separation between 'Galalea' and 'Aversang' or 'Splenola' was not significantly different from zero.

The canonical variates are linear functions of the data that maximise the differentiation of the sports by maximising the variation between the sports and minimising the variation within the sports. The first two canonical correlations were reasonably high (0.84 and 0.79) indicating that the association between the first two canonical variables and the apple sports data was high. The proportion of the eigenvalue shows 50.9% of the variation differentiating the sports was explained by the first canonical variable and 86.8% by the first two canonical variates.

B. Variable selection
Parallel Discriminant Ratio Coefficient (DRCs) are the product of canonical coefficients and structure correlations from the CVA and were used as a measure of contribution to the canonical variable (section 3.8.3). The ten highest DRCs of 44 variables for the first two canonical variables are tabled below (tables 5 and 6). These were used to select the variables important in the separation of sports, and these variable were selected for the rest of the analyses. Variables associated with the first canonical variable (CAN1) are primarily pore and grain measurements (table 5). The variables associated with CAN2 are primarily ridge and grain variables (table 6). The CVA was re-run and the canonical

scores for the first two canonical variables were plotted to show the separation between the sports for the individual observations (fig. 23).

Table 5. Top ten DRCs for the first canonical variable (CAN1) used to select the most important variables for the separation of the apple sports groups in CAN1 using pollen grain characteristics.

Variable [z]	Canonical coefficients[x]	Canonical structure[y]	Absolute DRC
Pore length	-0.407	-2.309	0.940
Pore width	-0.334	1.139	0.380
Grain width	0.097	-2.648	0.257
Percent pore coverage	-0.329	-0.606	0.199
Ridge frequency (PC2)	0.131	1.444	0.189
Grain length	-0.081	-2.061	0.168
Grain area	0.035	4.087	0.143
Pore length:width ratio	-0.130	0.820	0.107
Average pore area	-0.420	0.228	0.096
Ridge frequency (PC14)	-0.130	-0.541	0.070

[x] Pooled within-class standardised canonical coefficients.
[y] Pooled within canonical structure.
[z] PC = Principal component

Table 6. Top ten DRCs for the second canonical variable (CAN2) to select the most important variables for the separation of the apple sports groups in CAN2 using pollen grain characteristics.

Variable [z]	Canonical Coefficient[x]	Canonical Structure[y]	Absolute DRC
Grain area	0.143	2.725	0.389
Ridge width (PC2)	0.187	1.949	0.364
Ridge frequency (PC2)	-0.137	-2.644	0.362
Ridge frequency (PC1)	-0.113	2.945	0.333
Grain length	0.127	-2.455	0.313
Ridge width (PC8)	0.110	2.638	0.290
Ridge width (PC16)	0.160	-1.395	0.223
Ridge frequency (PC16)	0.262	0.841	0.221
Ridge frequency (PC8)	0.150	-1.354	0.203
Ridge width (PC1)	0.100	-2.027	0.202

[x] Pooled within-class standardised canonical coefficients.
[y] Pooled within canonical structure.
[z] PC = Principal component

C. Improving the sport separation: CVA with weighted and averaged data.
A weighted CVA was run on the averaged data. The P values for the Mahalanobis distances were the probabilities that the squared distance between apple sports was zero (table 7).

Table 7. P values of Mahalanobis distance for squared distance to apple sports of 'Red Delicious' and 'Gala'.

	Aversang	Galalea	Splenola	Ultrared
Aversang	1.0000	0.0652	0.0029	0.0084
Galalea		1.0000	0.0435	0.0022
Splenola			1.0000	0.0016
Ultrared				1.0000

All the apple sports were significantly different from zero apart from the distance between 'Galalea' and 'Aversang' (P = 0.0652). This meant that there was significant differentiation between all sports apart from 'Galalea' and 'Aversang' in the CVA.

The first two canonical correlations were high (0.96 and 0.94 respectively) which indicated that the association between the canonical variables and the apple sports was high. The cumulative proportion of the eigenvalues was 93.2% which was the proportion of the variation between the sports groups that was explained by the first two canonical variates.

The first and second canonical variable scores for the averaged observations was plotted (fig. 24) to show the separation of the sports. Variation between 'Red Delicious' and 'Gala' sports was shown by the first canonical score which was mainly influenced by pore and grain variables (table 5). Variation between the sports 'Aversang' and 'Ultrared', and 'Galalea' and 'Splenola' was shown by the second canonical score, which was mainly influenced by ridge and grain variables (table 6).

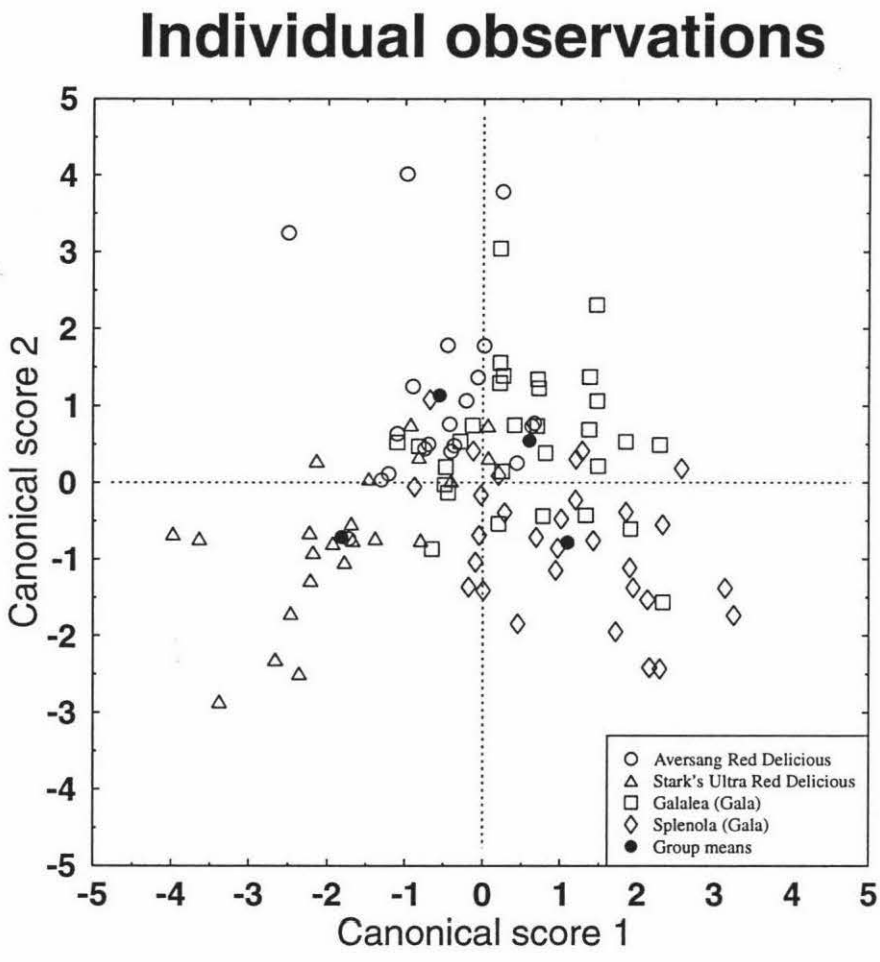


Fig. 23. Canonical scores for the first two canonical variables calculated from individual pollen grain data and plotted to show the separation achieved between the apple sports of 'Red Delicious' and 'Gala'.

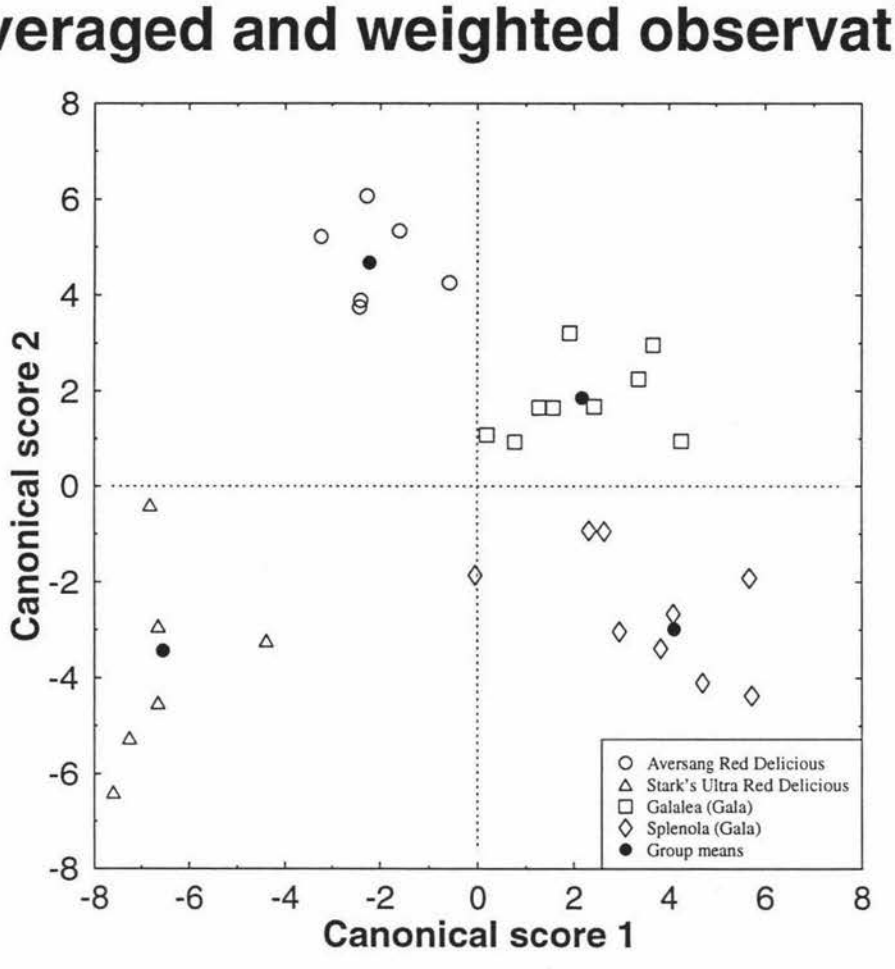


Fig. 24. The canonical scores for the first two canonical variables calculated from averaged and weighted data and plotted to show the separation between the sports of 'Red Delicious' and 'Gala'.

Although separation of all the sports was not achieved by the CVA further interpretation of the canonical scores was possible. Duncan's multiple range test for comparison of means was applied to the first and second canonical scores (CAN1 and CAN2). All the sports were successfully differentiated (P = 0.0001) (table 8).

Table 8. Mean canonical scores and significant groupings for sports of Red Delicious and Gala.

Sport	CAN1		CAN2	
	Canonical score	Tukey group [x]	Canonical score	Tukey group [x]
'Aversang' [y]	-2.09	b	4.75	a
'Ultrared' [y]	-6.56	c	-3.78	c
'Galalea' [z]	2.16	a	1.82	b
'Splenola' [z]	3.55	a	-2.58	c

[x] Multiple comparison of means using Tukey's test. Groups with the same letter are not significantly different.
[y] 'Red Delicious' sport.
[z] 'Gala' sport.

4.6.4. Discriminant Analysis for group classification

Differentiation of the sports was achieved but the next step was to identify an unknown pollen sample. Discriminant Analysis tested the identification error rate by a 'leave one out' approach. One observation was left out of the calculations for the discriminant function and then that function was used to classify that observation. The process was repeated for each pollen grain and the overall error rate was calculated.

The individual pollen grain observations were identified with a 67.46% error rate (33% success rate). The averaged data was reclassified with a 59.72% error rate (40% success rate).

5. DISCUSSION

The aim of this project was to develop a method of differentiating sports of apple by pollen ultrastructure. Pollen grains were viewed and photographed under a scanning electron microscope and a new digital image analysis algorithm developed to extract data from the image quickly and objectively. Statistical methods were then applied to determine the discriminating power of pollen characteristics for two 'Red Delicious' sports, 'Aversang' and 'Ultrared', and two 'Gala' sports, 'Splenola' and 'Galalea'. Preliminary work was needed to develop the technique, and then the methodology was applied to the four genotypes to determine the effectiveness of the fingerprinting. Differentiation of apple sports by pollen ultrastructure together with the development of a library of pollen ultrastructure of known apple pollen for comparison could be used to help establish plant variety rights status or to identify an unknown apple sport by its pollen ultrastructure. Past authors have achieved differentiation of species and cultivars by pollen ultrastructure and biochemical techniques with varying success, but a technique to differentiate sports has not been developed. Although the technique in this project was developed on apple sports, it could also be applied to other crops.

In New Zealand apple trees flower between October and November which limits the time when fresh pollen is available. Forcing flower and pollen development under artificial conditions extends the season but little is known about the effects of this technique on pollen production and pollen ultrastructure. The apple floral bud is a compound bud containing leaves and flowers. In this project two year old shoots from 'Harrold Red' forced under artificial conditions produced fewer floral buds per total growing buds and fewer flowers per floral bud. Pollen fresh weight per flower and pollen grain widths were also significantly reduced (table 1, table 2). Selected pollen ultrastructure variables that maximised the separation of the apple sports were analysed by multivariate analysis of variance. There was strong evidence in the data for differences between forced and spring pollen ($P = 0.0001$). Layne (1983) forced early production of viable apple and pear pollen by harvesting branches just as the buds started to swell and by placing them in vases at 23°C. Further investigation is needed to establish whether the effects of forcing on pollen size are due either to the season in

which budwood is collected for forcing, to differences in the forcing conditions such as light, media or temperature, or to the genotype itself. Crescimmano *et al.* (1988) confirms that environment may affect the morphology of the pollen grain. Citrus flower naturally several times a year. These authors compared spring, summer and autumn pollen of citrus and found the shape of spring pollen tended to be rounder than pollen in other seasons. For the purpose of fingerprinting, extending the availability of pollen through forcing may introduce a source of variation. The exine layer deposition is controlled by the parent tissue which is the reason that pollen has potential to differentiate the parent genotypes, but factors that influence the parent tissue may also affect pollen development. Future work on pollen for cultivar identification will either need to further investigate the effects of forcing on pollen ultrastructure, or to exclude this factor by comparing only spring pollen.

High resolution images of the pollen exine surface details are possible with the scanning electron microscope but the preparation method can change the size and shape of the pollen grains. Four pollen preparation methods were compared (air drying, acetolysis, hot potassium hydroxide solution wash and rehydration) but air dried pollen gave the most satisfactory results for this study (fig. 5). This agrees with Faegri and Deuse (1960) and Large and Braggins (1990) who stated it was common knowledge among palynologists that size of pollen grains vary with the previous treatment of the pollen. An initial sample of apple pollen air dried for use in the previous season's breeding program was viewed under the scanning electron microscope. This sample had a high proportion of collapsed pollen grains (fig. 4) but subsequent samples of pollen freshly prepared by this method did not. The reason for the high proportion of collapsed pollen grains observed by scanning electron microscopy for the first air dried apple pollen sample is not clear, but Marcucci *et al.* (1984) and Martens and Fretz (1980) observed that incompletely dried apple pollen collapsed in the scanning electron microscope. Many researchers in temperate fruit pollen morphology have also used air dried pollen with good results (Fogle, 1977 a, b; Maas, 1977; Marcucci *et al.*, 1984; Martens and Fretz, 1980; and Mulas *et al.*, 1988).

Acetolysed pollen has been used by palynologists but acetolysed pollen was not suitable

for the present analysis. Apple pollen grains were smaller, rounder, darker and more brittle than air dried pollen (section 4.2.2). Erdtman (1960) confirmed that acetolysis stained the exine of the pollen, especially large pollen grains such as *Geranium* and *Scabiosa*. Lynch and Webster (1975) reported that acetolysis can cause shrinkage of the exine which was confirmed by this project, and Smith and Tiedt (1991) generalised that acetolysis may cause bilaterally symmetrical pollen to appear radially symmetrical. In the present project the normally elliptical pollen appeared spherical. Spherical shaped pollen grains had variable orientation on the slide and observation of a specific area of each pollen grain was difficult. During the mounting of the pollen grains the brittleness of the exine shells made handling difficult without damaging the grains. Acetolysis has been a method applied by palynologists because pollen samples prepared with this method were comparable to fossilized pollen studied (Lynch and Webster, 1975). However, for scanning electron microscopy of fresh pollen, handling and viewing difficulties resulted in the acetolysis method being discarded.

Hot potassium hydroxide solution wash (10%) preparation method also did not produce satisfactory results. Individual pollen grains suspended in water were variable in size and shape (fig. 8), and during drying, congealed into a solid mass. The cytoplasm was still intact after a 15 minute treatment and the variable expansion of the cytoplasm may have been responsible for the range of sizes and shapes observed. This difference may result from the method which was not optimised for apple pollen. In particular, there are many variations in the time of treatment with the potassium hydroxide solution. Faegri and Deuse (1960) boiled *Betula* pollen for 3 hours in 10% potassium hydroxide, while Large and Braggins (1990) found 10 minutes adequate for fern spores. Neither of these researchers found the variation in size and shape observed in the present project. Future users of hot potassium hydroxide solution wash may need to increase the time from 15 minutes to several hours to ensure the effectiveness of the method. However, if the results are similar to acetolysis as suggested by Large and Braggins (1990) then the disadvantages of acetolysis in this present study hold for this treatment as well.

Rehydration treatment produced a triangular shaped pollen grain with the poles facing the observer and the germinal furrows at the apices. In addition the equatorial region

was expanded but the polar axis reduced (section 4.2.4). The change in shape, and the consequent change in orientation of the rehydrated pollen, was probably due to the expansion of the cytoplasm inside the pollen grain. Lynch and Webster (1975) confirmed that rehydration of pollen from the family Euphorbiaceae also produced expanded, non-collapsed, pollen grains with an altered shape. This method would be useful for future observations of the exine pattern at the pole, rather than from the equatorial region observed in the air dried preparation method.

Air dried apple pollen is viewed under the scanning electron microscope to extract data from the grain shape and the exine surface details. Past researchers have extracted data mostly by hand and this has meant that much of the data was categorised or qualitative. For example Fogle (1977a,b) classified exine ridge patterns as longitudinal, interwoven, branched or concentric, and ridge frequency as medium, narrow and very narrow. Qualitative data has the advantage of being quick and easy to extract but data precision is lost. Extraction of quantitative data such as pore areas or ridge patterns from images can be time consuming, tedious and difficult. Digital image analysis exploits the power of a computer to extract information from an image. Recent advances in image analysis and computer technology have meant that computers capable of performing image analysis are widely available. Many authors have used image analysis to aid the extraction of quantitative data from pollen micrographs (Langford *et al.*, 1990; Matsuta *et al.*, 1982, 1986; Treloar, 1992; Ueda and Okada, 1990; and Vezey and Skvarla, 1990).

In the present project an image analysis algorithm to extract quantitative data objectively and quickly with minimal input from the operator was developed. The main technique investigated in the image analysis system was focused on the isolation of the areas of interest in the image and then on the extraction of the data for further analysis. An image contains a large amount of information from which only a small region or object may be of interest. Isolation of objects from the rest of the image is called 'segmentation' (Haralick and Shapiro, 1985). In this project the aim was to fully automate both the isolation of areas of interest and the extraction of data, but this was only partly achieved (section 4.4). Haralick and Shapiro (1985) agreed that segmentation was difficult because not only were image boundaries poorly defined and regions of

interest were not homogenous, but image segmentation techniques were basically '*ad hoc*'. In this project isolating the pollen grain and pore outlines had similar problems. Since there was no established methodology available a suitable procedure had to be developed for apple pollen (fig 13). Several image enhancing techniques were applied to enhance and fill small gaps in the boundaries of the object of interest, but the resulting boundaries were not always continuous or distortion free. This meant that modification by the operator was required. Lack of homogeneity in the areas of interest was a problem in all three areas of the algorithm. Firstly the pixel values for the edge of the pollen grain were the same as other features on the micrograph (section 4.4.1). Secondly the pixel values in the regions of interest in the Fourier image were similar to values in the regions of low frequency information or harmonic frequency information (section 4.4.2). Thirdly the pixel values within the pores were similar to other dark regions such as depressions in the surface of the exine (section 4.4.3). A certain amount of automatic selection was achieved but interaction with the operator was still needed to isolate the areas of interest and to edit out unwanted data. However, the algorithm allowed the researcher to extract quantitative data quickly and objectively. One way to facilitate the isolation of objects is to increase the contrast between the background and the object of interest. Keefe and Draper (1988) isolated silhouettes of plants or seeds from a light background. Vezey and Skvarla (1990) isolated pores from the pollen exine image by overexposing the micrograph, then darkening the pores with a felt-tip pen before image analysis. Future work needs to be done on automatic boundary definition and isolation of the area of interest to increase the objectivity of the method and to reduce the operator input. Image analysis has enormous potential for further research on the extraction of numerical data.

In this project conflicting results were obtained on the effect of environment on pollen ultrastructure of 'Red Delicious' and 'Gala' apple sports. From the 44 measured variables, 18 were selected (section 4.6.3 B) and analysed together in a multivariate analysis of variance with no significant environment effect. Although pollen collected from three replicate trees were thought to be adequate for a pilot study, there may not be enough replicates to give a reliable result from the multivariate analysis of variance. However, when the pollen variables were analysed individually there was a significant environmental effect between the replicates (table 3). Pollen grain length:width ratio,

percentage pore area, average pore area and average pore length changed between the replicates. Contrasts were made within a site (replicates 1 and 2 at Clyde) and between sites (Clyde and replicate 3 at Havelock North). Pollen grain length:width ratio and pore length were significantly different within the Clyde site. Percent pore area varied significantly between the two sites. The transformed ridge data (principal component scores of the raw ridge data) was not analysed in the univariate ANOVA because the relation between the scores and the ridge data was difficult to interpret. Comparison of these results with previous research is difficult as little is known about the effects of environment on pollen ultrastructure. Past authors did not consider replication across different environments. Before this technique can be adopted on a national or international scale, further research is required to investigate environmental effects such as tree age, budwood age, as well as geographic location on pollen ultrastructure. Variables influenced by the environment could then be excluded to increase the robustness of this fingerprinting method.

The possible differentiation of apple sports is influenced by the collation of data and the choice of analysis. In this project the discriminating power of the data was increased by aggregating and averaging observations (fig. 24). The averaged data was weighted by the variation within each average to reduce the variation in the data and increased the differentiation between the groups. Several authors reported that the variation within the cultivars was reduced when 'representative' pollen was selected (Fogle, 1977a,b; Martens and Fretz, 1980; Mulas *et al.*, 1988). The problem with the selection of representative data is the difficulty in deciding what is representative and the possibility of non-random sampling. Averaging the data is an objective way to reduce the variation, but unfortunately it also reduces the number of observations for further analysis.

After separation between the sports of apples had been maximised by Canonical Variate Analysis (CVA), Discriminant Analysis (DA) was applied to test the identification of the apple sports by classifying a pollen grain into the known apple sport groups. This is done by a 'leave one out' process. DA functions are linear function of the data that maximise the separation between the apple sports. Each observation is left out of the analysis and reclassified using the resulting DA functions. This project achieved only

a best error rate for reclassification of 59.7% (section 4.6.4). DA assumes within-sport normality so the probable reason for the high error rate was non-normality induced by the small sample size (SAS Institute Inc., 1990). Cruz-Castillo *et al.* (1994) agreed that sample size plays an important part in the reliability of the results from the CVA and DA. Too few observations for each apple sport or too many variables leads to poor results. If there were too many variables then the results would be poor no matter how large the sample. The authors were not specific about the numbers required but suggested that the number of observations should be ten times the number of the variables. In this project 18 variables were selected from the 44 variables recorded on the basis of contribution to the separation of sport groups, and 30 observations were made for each apple sport. Applying Cruz-Castillo *et al.* (1994) suggestion of increasing the number of variables by ten, 180 pollen grains per sport rather than 30 would be needed for the individual observations; or 540 (180 x 3) pollen grains for the averaged observations. However, Matsuta *et al.* (1982, 1986) obtained good results from DA with 12 variables and sample sizes of 50 and 10 observations per Japanese pear cultivar respectively. The sample sizes of Matsuta *et al.* (1982, 1986) are considerably smaller than the sample sizes recommended by Cruz-Castillo *et al.* (1994) and confirms that good results are possible from DA with a modest reduction of variable numbers and increase in the numbers of observations. In this project the data extracted from the Fourier image accounted for 36 of the 44 original variables. Reducing the sensitivity of the data or extracting the information more efficiently from the Fourier image would reduce the numbers of variables and possibly reduce the error rate of the DA. Further investigation is needed to optimise the data extraction, number of variables and the sample sizes before DA can be applied in the identification of unknown pollen samples.

Changes in pollen ultrastructure due to differences in the cultivar or sport have to be identified because the changes will enable pollen ultrastructure to be used as an identification method. Current plant identification methods such as DNA, isozymes or field morphology can already differentiate cultivars of apples, but no method can differentiate sports of apples. Much information is available about the differentiation of cultivars by pollen ultrastructure, but little is known about the separation of sports. In the present study separation of the genotypes was successful between the cultivar types

'Red Delicious' and 'Gala', and also between the two sports each of 'Red Delicious' ('Aversang' and 'Ultrared') and 'Gala' ('Splenola' and 'Galalea') (section 4.6.3).

The most powerful variables for the separation of cultivar types 'Red Delicious' and 'Gala' were pore and grain measurements with some influence of ridge width (section 4.6.3 C). The variables found by other researchers to be important for cultivar separation were pore size and number for apple pollen (Marcucci *et al.*, 1984), total pore area for Japanese pear pollen (Matsuta *et al.*, 1982), grain width, length and number of pores for almond pollen (Mulas *et al.*, 1988), and ridge interval, total pore area and number of pores for rose pollen (Ueda and Okada, 1994). The predominance of pore and grain measures in the separation of cultivars confirmed the findings of this project. However, differentiation of cultivars by pollen ultrastructure would not be the preferred method because current isozyme and DNA fingerprinting technologies are already well developed for the differentiation and identification of cultivars (section 2.2) and have been successfully developed to differentiate cultivars. These methods gave objective, rapid results throughout the year for cultivar differentiation but they cannot separate sports.

Separation of the sports was successfully achieved in this project by information from ridge widths, ridge angles and with some influence of grain measurements (section 4.6.3 C). Little information is available about variables that separate sports of cultivars. Marcucci *et al.* (1984) confirmed that ridge patterns and grain size were important but germination and viability of the pollen were also needed to separate sports of apple cultivars. Biochemical markers applied to the fingerprinting of cultivars are not effective for differentiating apple sports. Weeden and Lamb (1985) successfully separated apple cultivars but were not able to differentiate sports with isozyme analysis, which is confirmed by unpublished data from isozyme analysis of 'Braeburn' apple sports at HortResearch, Havelock North. Random amplified polymorphic DNA (RAPD) techniques were successful in separating apple cultivars but not 'Delicious', 'Gala', 'Jonagold' or 'Golden Delicious' apple sports (Mulcahy *et al.*, 1993). These authors stated that this lack of differentiation at the sport level was an advantage because results would not be altered by mutational changes. Hubbard *et al.* (1992) separated rose cultivars by

restriction fragment length polymerisation (RFLP) techniques but did not specify if any of the cultivars compared were sports. Nybom (1990) applied a simple sequence repeat DNA technique to the differentiation of sports of 'Red Delicious' but found no polymorphism. Pollen ultrastructure has the potential to objectively differentiate apple sports, which none of the current plant fingerprinting techniques can do. Objective sport differentiation is needed in the establishment and protection of Plant Variety Rights (section 2.1). In the future new variables such as pollen exine pattern variables could be tested to see if further advances in discriminating power can be made.

6. CONCLUSION

Differentiation of 'Red Delicious' and 'Gala' apple sports by their pollen ultrastructure has been achieved in this project by the combined application of scanning electron micrography, image analysis, and multivariate analysis.

Extending the availability of pollen by forcing flower bud production under artificial conditions significantly reduced the pollen widths and caused a significant difference between the combined variables from spring pollen and forced pollen in the multivariate analysis of variance.

Environment may not have had a significant effect on the combined variables in the multivariate analysis of variance. However, when the variables from the three replicates across two sites were compared individually, significant differences were found within and between sites. Pollen grain length:width ratio and pore length varied within the Clyde site, percent pore coverage varied between the Clyde and Havelock North sites, and grain length:width ratio, percent pore coverage, average pore area and pore length varied across all three replicates.

Image analysis provided a quick and objective method of extracting quantitative data from the apple pollen ultrastructure. The image analysis algorithm was not fully automated because of the difficulties in isolating the objects of interest.

Freshly prepared air-dried pollen was found to be preferable for scanning electron microscopy to acetolysis, hot potassium hydroxide solution (10%) wash, and rehydration. It is suggested also that rehydration provides a useful tool to study exine patterns at the pole of the pollen grain rather than the equatorial region.

Separation of sports was successful but the classification or identification of unknown pollen was less than 41% successful. This was probably either because the assumption of within-sport normality of the data was not met, or there were too many variables. Normality of the data may be achieved by increasing the number of observations per

sport, but further research is required to find a smaller set of variables that can still differentiate apple sports. Extraction of the information from the Fourier analysis is a potential area for reducing the numbers of variables.

The introduction of Plant Variety Rights (PVR) law and the application of trade mark to fruit has provided a financial incentive to develop new cultivars and to protect their identity. The consequent increase in the numbers of sports as cultivars has placed a further strain on the abilities of the current plant differentiation and identification methods. Plant identification techniques such as DNA and isozyme analysis cannot differentiate sports, and PVR identification methods are subjective and slow. Phenotypic differentiation or identification is needed to establish that the new cultivar is distinct, as it is required for PVR to be granted. Differentiation of apple sports by pollen ultrastructure and the development of a library of known apple pollen for comparison and identification could be used to establish PVR status. An objective method for sport identification will ensure the financial incentive of PVR remains a possibility in the development of new, commercial sports. Although this technique was developed on apple sports it could be used for other crops.

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APPENDIX A: Summary of VIPS5 commands, functionals and utilities

This appendix is extracted from the VIPS5 manual and summarises the commands, functionals and utilities within the VIPS5 program. The commands have been grouped by function to make finding an appropriate command easier.

Point operators on two images:

ADD	Adds two images or two histograms together
AND	Logical AND, or minimum of two images
DIVIDE	Divides one image by another
MERGE	Combines two images by merging the edges of objects.
MULTIPLY	Multiplies two images together
OR	Logical OR, or maximum of two images
SUBTRACT	Subtracts one image from another
XOR	Logical exclusive OR of two images
ADD	Adds a constant to an image
CLIP	Clips the intensities of an image at specified limits
DIVIDE	Divides an image by a constant
EXPAND	Linearly expands the intensity range of an image
GAMMA	Applies a gamma transform to an image
HISTOGRAM EQUAL	Performs histogram equalisation on an image
HISTOGRAM SHAPE	Performs arbitrary histogram shaping on an image
INVERT	Finds the additive or multiplicative inverse of an image
LOOKUP	Translates the intensities of an image through a lookup table
MULTIPLY	Multiplies an image by a constant
SELECT	Selects the pixels of a specified intensity in an image
SUBTRACT	Subtracts a constant from an image
THRESHOLD	Thresholds an image at specified intensities

Filters:

AVERAGE	Obtains a row average or column average of an image
BOX AVERAGE	Filters an image using a moving average
BOX COUNT	Filters an image counting the non-zero pixels within a window
BOX ENHANCE	Applies rank based edge enhancement using an arbitrary window
BOX EXTREME	Detects the local extreme pixel values in an image
BOX MAXIMUM	Filters an image selecting the maximum within a window
BOX MINIMUM	Filters an image selecting the minimum within a window
BOX RANGE	Detects edges using the range within an arbitrary window
BOX STRETCH	Performs local contrast enhancement by within a window
FFT	Applies a Fast Fourier Transform to an image
FILTER ENHANCE	Applies rank based edge enhancement using a 3x3 window
FILTER LINEAR	Filters an image with e 3x3 convolutional filter
FILTER MOMENT	Uses moment based filtering for edge detection
FILTER RANGE	Uses a 3x3 range filter for detecting edges
FILTER RANK	Rank filters an image using a 3x3 window
FILTER SOBEL	Filters an image with a SOBEL type filter
FILTER TRIMMED	Applies a trimmed average filter to smooth noise in an image
SET MASK	Initialises a convolutional mask used by the linear filter
SHRINK	Shrinks or expands objects in a binary image

Geometrical manipulation:

FLIP	Flips an image horizontally, vertically or diagonally
RECTIFY	Warps an image to match one set of points to another set
ROLL	Rolls an image horizontally and vertically
ROTATE	Rotates an image by 90 degrees
TRANSPOSE	Transposes the rows and columns of an image
WARP	Performs an arbitrary perspective warp on an image
ZOOM	Expands or contracts the contents of an image

Image conversion or transformation:

COLOUR	Converts between colour an black and white images
CONVERT	Converts from one image type to another
DIFFUSE	Applies error diffusion to give a binary rendition of an image
DISTANCE	Codes each pixel with it distance from the nearest boundary
FFT	Applies a Fast Fourier Transform to an image
FOURIER	Converts between real and Fourier images
HOUGH	Obtains a circular Hough transform of an image
LEASTPATH	Finds the path with least weight going left to right in an image
THREED	Draws a 3D perspective plot of an image
WALSH	Applies a fast Walsh transform to an image

Drawing commands:

DRAW ARC	Draws a circular arc
DRAW BOX	Draws a box or polygonal shape
DRAW CIRCLE	Draws a circle or ellipse
DRAW LINE	Draws a line
DRAW POINT	Draws a single point or list of points
DRAW POLYNOMIAL	Draws a polynomial
DISPLAY	Displays a histogram onto the display or into an image
PROFILE	Draws the line profile of an image onto the display or an image

Display commands:

CAPTURE	Captures an image onto the display
CLEAR	Clears all or part of the display
DISPLAY	Displays an image or histogram onto the display
GET	Gets an image from the display into an image variable
MOVIE	Captures or runs a sequence of video frames
PROFILE	Draws the line profile of an image onto the display

PSEUDO	Selects a false colour mapping for the display
ROAM	Specifies the region of the display buffer that is visible
SET CURSOR	Sets the position and properties of the display cursor
SET DISPLAY	Selects the display device to use
SET LUT	Sets the hardware input or output lookup tables
SET ORIGIN	Specifies the position in the frame buffer represented by (0 0)
SLICE	Displays an image, interactively selecting a threshold level
TEXT	Writes text to the display or into an image

Image generation and retrieval:

BATCH	Saves variables for processing in batch mode
CAPTURE	Captures an image onto the display
GET	Gets an image from the display into an image variable
LOAD	Loads an image from a file into VIPS
NOISE	Generates a noise image with a specified intensity distribution
SAVE	Saves an image to a file
TEST	Provides a computer generated test image

Data extraction:

AREA	Calculates the area of an object in pixels
BLOB	Counts the number of independent blobs in an image
CHAIN BRANCHES	Counts the number of separate branches contained in a chain
CHAIN CONCAVE	Measures the distance of each point from the convex hull
CHAIN DISTANCE	Measures the distance of each point from a given point
CHAIN LENGTH	Counts the number of elements in a chain code
CHAIN MOMENT	Measures the specified moment of a chain loop
CHAIN PERIMETER	Measures the perimeter or length of a chain
CHAIN RECTANGLE	Determined the minimum area enclosing rectangle for a chain

CHAIN SIZE	Determines the row and column extents of a chain
COG	Finds the position of the centre of gravity of an image
EXTREME	Locates the extreme pixel values in an image
FIT	Fits a line or polynomial to a set of points
HISTOGRAM GET	Obtains the intensity histogram of an image
HULL	Finds the deepest concavity in an intensity histogram
LIST	Lists all of the non-zero points in an image
PROFILE	Draws the line profile of an image onto the display or an image
SLICE	Interactively determines a good threshold level
STATISTICS	Obtains intensity statistics from an image or histogram

Data manipulation:

CHULL	Performs a convex hull operation on white blobs in an image
COPY	Copies all or part of one image into another
EXTEND	Extends the edges of an image after filtering
FILL	Fills a region in an image within a specified boundary
HISTOGRAM SMOOTH	Low pass filters a histogram
HULL	Performs a convex hull of the intensities on the rows of an image
LET	Assigns one variable to another
PEAK	Finds and labels the blobs or intensity peaks in an image
SET VALUE	Sets a value in an image or histogram variable
THIN	Thins a binary image to a single pixel wide skeleton

Data input and output:

FILE	Opens an input or output data file
INQUIRE	Prompts for input from the user
READ	Reads data from the input data file
SET LOG	Opens a log file to record the commands in a VIPS session

WRITE	Writes text or data to the terminal or to the output data file

Chain coding:

AREA	Measures the area enclosed by a chain
CFIT	Segments a chain of several touching circles into separate circles
CHAIN BRANCHES	Counts the number of separate branches contained in a chain
CHAIN CODE	Obtains the chain code of all the objects in a binary image
CHAIN CONCAVE	Measures the distance of each point from the convex hull
CHAIN CURVATURE	Measures the curvature of a chain
CHAIN DISTANCE	Measures the distance of each point from a given point
CHAIN DRAW	Draws a chain into an image
CHAIN EDGE	Fills hollows in a chain which is touching the image boundaries
CHAIN EXTRACT	Extracts a single chain from a set
CHAIN FERRET	Measures the length of a projection of a chain
CHAIN JOIN	Replaces relative steps in a chain with a sequence of codes
CHAIN LENGTH	Counts the number of elements in a chain code
CHAIN LOOP	Determines if a chain is a closed loop or a line
CHAIN MOMENT	Measures the specified moment of a chain loop
CHAIN PERIMETER	Measures the perimeter or length of a chain
CHAIN RECTANGLE	Determined the minimum area enclosing rectangle for a chain
CHAIN SIZE	Determines the row and column extents of a chain
CHAIN SLOPE	Measures the slope at each point in a chain
CHAIN SORT	Sorts through a chain set keeping only selected chains
CHULL	Replaces a chain by its convex hull
COG	Returns the centre of gravity of a chain loop
ZOOM	Expands or contracts a chain by the specified zoom factor

Program commands:

ELSE	Starts the condition false part of an IF command
END	Indicates the end of a branch or loop construct, or a program
EXIT	Exits the program which is running
FOR	A loop command with an incrementing loop variable
IF	A branch command selecting one of two command sequences
ON ERROR	Specifies the action to take in the event of an error in a program
PROGRAM	Heads a program and specifies default parameters
REPEAT	Repeats a sequence of commands until a condition is true
RUN	Runs a VIPS program
SET OUTPUT	Controls whether output from commands is displayed
SET VERIFY	Controls whether program commands are displayed
UNTIL	The test command at the end of a REPEAT loop
WHILE	Executes a sequence of commands while a condition is true
WITH	Repeats a command sequence once for each value provided

Miscellaneous commands:

BEEP	Beeps to the terminal
CONTINUE	Delays for a specified time or until a key is pressed
DECLARE	Declares image processing variables
DEFINE	Defines a symbol or abbreviation to use as a command
DELETE	Deletes image processing variables, freeing their memory
EXIT	Exits the VIPS session
HELP	Provides user information from the help library
INFORMATION	Provides information on recent changes and updates to VIPS
LET	Assigns one variable to another

ON CHANGE	Specifies a command to execute when a variable us changed
SET ASPECT	Sets the pixel aspect ratio
SET ERROR	Sets the error handling characteristics of VIPS
SET TIME	Controls whether or not each command is timed
SHOW DEFINITION	Shows command definitions created using DEFINE
SHOW SYSTEM	Shows defaults various system parameters
SHOW TYPE	Shows variables that have been declared, by type
SHOW VARIABLE	Shows variables that have been declared, by name
SYNCHRONISE	Waits until a specified time before continuing
SYSTEM	Calls the host operating system to execute a system command

Functionals:

%	Returns an entity without using any parsing or case conversion
%ABSOLUTE	Returns the absolute value of a number
%ANGLE	Returns an angle, given x and y components
%COLUMN	Returns the column part of a vector
%COSINE	Returns the cosine of an angle
%DATE	Returns the current system date
%DISTANCE	Returns the length of a vector
%EXIST	Checks if the specified variable or file exists
%EXP	Returns the exponent of a number
%HISTOGRAM	Returns the count from a specified intensity in a histogram
%INDEX	Returns a value from a specified position in a list
%INTEGER	Converts a real number to an integer by rounding
%LENGTH	Returns the length of a string or a list
%LOG	Returns the natural logarithm of a number
%REAL	Converts an integer to a real number
%ROW	Returns the row part of a vector
%SINE	Returns the sine of an angle

%SIZE	Returns the size of an image
%SQRT	Returns the square root of a number
%STRING	Converts an entity into a string
%TIME	Returns the current system time
%TRANSPOSE	Returns the transpose of a vector
%TYPE	Returns the type of a variable
%VALUE	Returns the pixel value from the specified position in an image

APPENDIX B: Plant Variety Rights evaluation form

2/93

PLANT VARIETY RIGHTS OFFICE
NEW ZEALAND

OBJECTIVE DESCRIPTION OF VARIETY

APPLE
(*Malus* Mill.)
Fruit Varieties

Name of applicant:

Variety name or temporary denomination:

PVRO reference number (office use only):

Location test results presented from:

Year(s) of test:

Age of test plants:

Prepared by:

---

1. Place the appropriate number that describes the varietal character in the boxes below.
2. Measurements should be based on the mean of at least 20 typical plant parts from 5 trees in the second year of fruiting.
3. + see explanatory notes or diagrams.
4. Representative variety, as an example of the character, is in brackets.

TREE

☐ Vigour -

3 = weak (Akane) 5 = medium (Golden Delicious)
7 = strong (Bramley's Seedling)

☐ + Habit -

1 = columnar 3 = upright 5 = spreading
7 = drooping (Jonathan) 9 = weeping

☐ Predominance of bearing -

1 = on spur 2 = on shoots

DORMANT ONE YEAR OLD SHOOT **(Observe in winter on trees that have had at least one growing season)**

☐ Pubescence on upper half -

1 = absent or very weak 3 = weak (Golden Delicious)
5 = medium (Cox's Orange Pippin) 7 = strong
9 = very strong

☐ Thickness (centre diameter of middle internode) -

3 = thin 5 = medium 7 = thick

☐ Number of lenticels -

1 = absent or very weak 3 = few
5 = medium (Cox's Orange Pippin) 7 = many 9 = very many

FLOWER (at the start of anther dehiscence)

☐ Size (diameter with petals pressed flat) -

3 = small (Jonathan) 5 = medium (Cox's Orange Pippin) 7 = large

☐ Position of margins -

3 = free 5 = touching 7 = overlapping

Petal: Colour of upper side - RHS Colour Chart: _____

Petal: Colour of lower side - RHS Colour Chart: _____

☐ Time of beginning of flowering -

1 = very early 3 = early 5 = medium (Cox's Orange Pippin)
7 = late 9 = very late

LEAF (mature leaves from central third of an actively growing shoot on the outside of the tree in summer)

☐+ General pose -

3 = upwards 5 = outwards 7 = downwards (Granny Smith)

☐ Size -

1 = very small 3 = small (Akane) 5 = medium (Golden Delicious)
7 = large 9 = very large

☐ Ratio: Length/width (fourth to sixth fully expanded leaf) -

3 = small 5 = medium (Jonagold) 7 = large (Granny Smith)

☐ Glossiness of upper side -

3 = weak (Jonathan) 5 = medium (Granny Smith) 7 = strong

☐ Pubescence on lower side -

3 = weak 5 = medium 7 = strong

☐ Petiole length -

3 = short 5 = medium (Granny Smith) 7 = long

FRUIT (20 typical fruits selected out of a minimum of 50 fruits from 5 trees, excluding terminal fruits, at peak maturity)

☐ Size -

1 = very small
2 = very small to small
3 = small
4 = small to medium
5 = medium (Cox's Orange Pippin)
6 = medium to large (Gravenstein)
7 = large
8 = large to very large
9 = very large

☐ + Shape -

1 = globose
2 = globose conical (Cox's Orange Pippin)
3 = short globose conical
4 = flat
5 = flat globose (oblate)
6 = conical
7 = long conical
8 = trucate conical (Kidd's Orange Red)
9 = ellipsoid
10 = ellipsoid conical (ovate)
11 = oblong (Gravenstein)
12 = oblong conical
13 = oblong waisted (Delicious)

☐ Symmetry in side view -

1 = asymmetric
2 = symmetric

☐ Ribbing -

1 = absent (Cox's Orange Pippin)
9 = present

☐ Crowning at distil end -

1 = absent
9 = present

☐ Degree of crowning at distil end-

3 = weak (Cox's Orange Pippin)
5 = medium
7 = strong (Red Delicious)

☐ Aperture of eye -

3 = closed
5 = half open (Cox's Orange Pippin)
7 = open (Gravenstein)

☐ Size of eye -

3 = small
5 = medium (Cox's Orange Pippin)
7 = large

☐ Length of sepal -

3 = short
5 = medium
7 = long (Gala)

☐ Spacing of sepals at base -

1 = free
2 = touching (Gala)
3 = overlapping

☐ + Depth of eye basin -

1 = absent or very shallow 3 = shallow
5 = medium (Golden Delicious) 7 = deep 9 = very deep

☐ + Width of eye basin -

3 = narrow 5 = medium (Golden Delicious) 7 = broad

☐ Thickness of stalk -

3 = thin (Golden Delicious) 5 = medium (Cox's Orange Pippin) 7 = thick

☐ Length of stalk -

1 = very short 3 = short (Cox's Orange Pippin) 5 = medium
7 = long 9 = very long (Golden Delicious)

☐ + Depth of stalk cavity -

3 = shallow 5 = medium 7 = deep

☐ + Width of stalk cavity -

3 = narrow 5 = medium (Golden Delicious) 7 = broad

☐ Relief of surface -

1 = smooth 2 = hammered 3 = bumpy

☐ Bloom of skin -

1 = absent (Golden Delicious) 9 = present

☐ Greasiness of skin -

1 = absent 9 = present

☐ Cracking tendency of skin -

1 = absent (Golden Delicious) 9 = present (Cox's Orange Pippin)

☐ Thickness of skin -

3 = thin (Gala) 5 = medium 7 = thick

☐ Ground colour of skin -

1 = yellow (Golden Delicious) 2 = whitish yellow
3 = yellow green (Cox's Orange Pippin) 4 = whitish green
5 = green (Granny Smith) 6 = red

☐ Amount of over colour of skin -

1 = absent or very low (Granny Smith) 3 = low (Cox's Orange Pippin)
5 = medium (Gala) 7 = high
9 = very high

☐ Over colour of skin -

1 = orange
2 = red (Jonathan)
3 = purple
4 = brownish

☐ Type of over colour of skin -

1 = solid flush
2 = banded
3 = streaked
4 = washed out (faded)

☐ Amount of russet -

1 = absent or very low
3 = low
5 = medium
7 = high
9 = very high

☐ Position of russet -

1 = around eye basin (Cox's Orange Pippin)
2 = around stalk cavity
3 = on cheeks
4 = overall

☐ Size of lenticels -

3 = small
5 = medium (Cox's Orange Pippin)
7 = large

☐ Browning of the flesh (one hour after cutting with stainless steel knife) -

1 = absent or very weak
3 = weak
5 = medium
7 = strong
9 = very strong (Red Delicious)

☐ Firmness of the flesh (measured with penetrometer) -

1 = very soft
3 = soft (Jonagold)
5 = medium (Cox's Orange Pippin)
7 = firm
9 = very firm

☐ Colour of the flesh -

1 = white
2 = cream (Jonagold)
3 = greenish
4 = yellowish (Cox's Orange Pippin)
5 = pink

☐ Texture of the flesh -

3 = fine
5 = medium (Cox's Orange Pippin)
7 = coarse

☐ Juiciness -

3 = dry
5 = medium (Cox's Orange Pippin)
7 = juicy (Jonagold)

☐ + Fruit in cross-section: distinctness of core line (median through locules) -

1 = absent or very weak
3 = weak
5 = medium (Gala)
7 = strong
9 = very strong

☐ + Fruit in cross-section: aperture of locules -

1 = open (Gala)
2 = closed

☐ Time of fruit ripening for eating -

1 = very early 3 = early 5 = medium
7 = late (Golden Delicious) 9 = very late

PEST AND DISEASE

0 = not tested 1 = highly susceptible ---> 9 = highly resistant

☐ Black Spot (*Venturia inaequalis*)

☐ Powdery Mildew (*Podosphaera luecotricha*)

☐ Bitter Rot (*Glomerella cingulata*)

☐ Silver Leaf (*Chondrostereum purpureum*)

☐ Black Rot (*Botryosphaeria* spp.)

☐ European Canker (*Nectria galligena*)

☐ Scab (*Venturia* spp.)

☐ Blast (*Pseudomonas syringae*)

☐ Fireblight (*Erwinia amylovora*)

☐ Leaf rollers

☐ Codling moth

☐ Woolly aphid

☐ Mites

☐ Other specify: _____

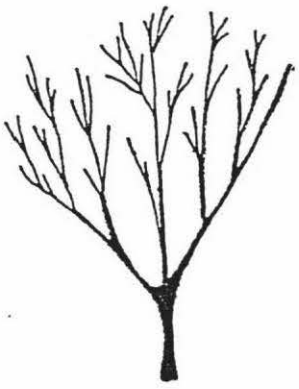
ADDITIONAL DESCRIPTION

Describe any characters that have not been described on the objective description form or whose description has not been accurately conveyed. Where possible compare with other known varieties and describe how it may be distinguished from these.

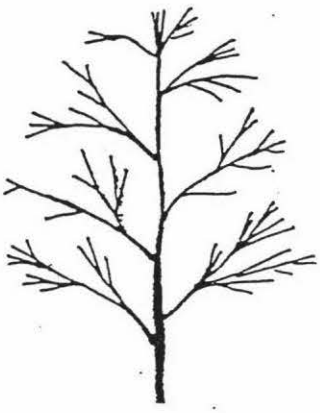
EXPLANATORY NOTES

Tree: Habit -

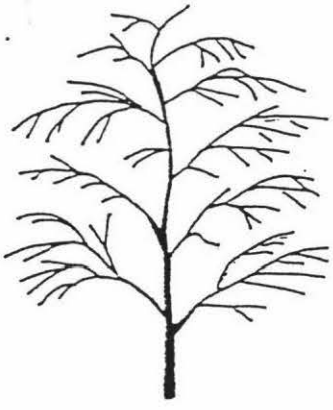
1
columnar



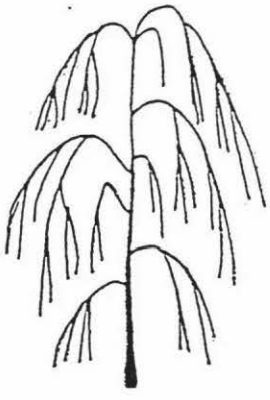
3
upright



5
spreading

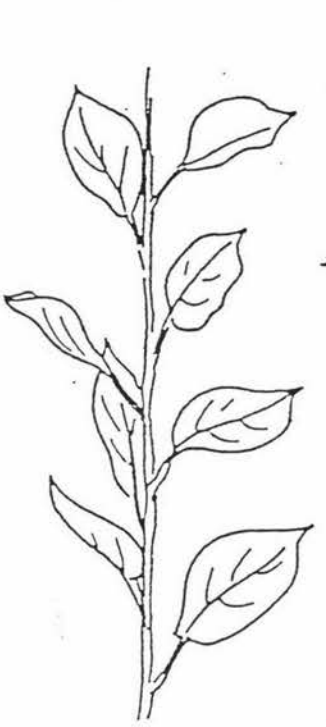


7
drooping



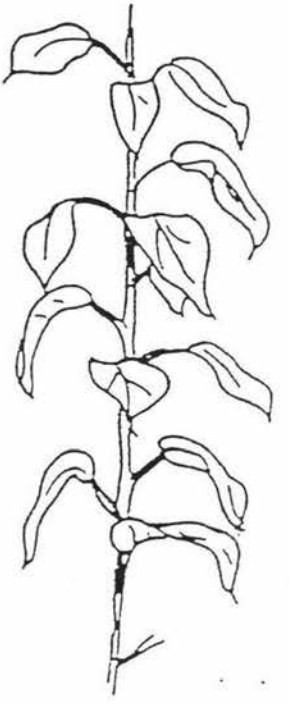
9
weeping

Leaf: General pose -



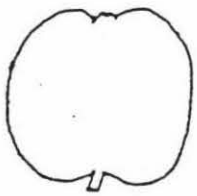
3
upwards

5
outwards



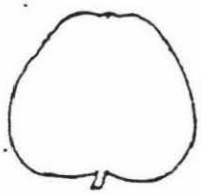
7
downwards

Fruit: Shape -



1

globose

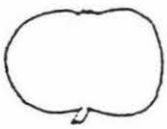


2

globose conical

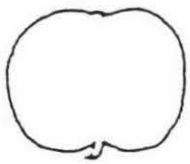
3

short globose conical



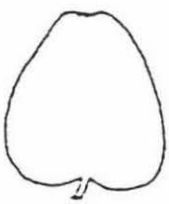
4

flat



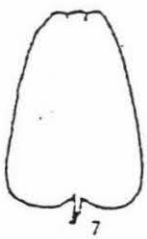
5

flat globose (oblate)



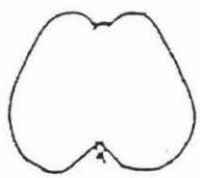
6

conical



7

long conical

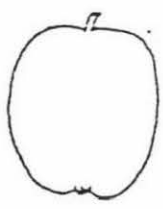


8

truncate conical

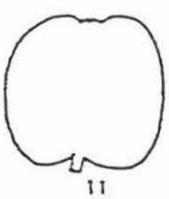
9

ellipsoid



10

ellipsoid conical (ovate)

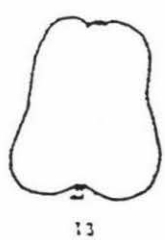


11

oblong

12

oblong conical



13

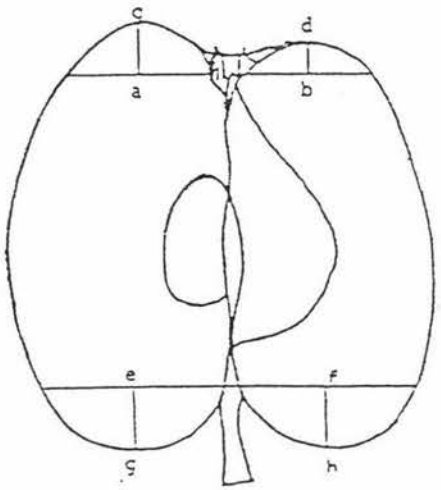
oblong waisted

Fruit: Depth of eye basin -

Depth is measured on the longitudinal section of the fruits, the lines shown in the diagram below being marked with a scalpel for easy measuring. A plastic protractor is useful to ensure that the axis of the fruit is at right angles to lines ab and ef. ab is marked at the base of the sepals.

Fruit: Longitudinal section -

Five of the ten fruits should be sectioned at one time as, if the variety "browns" rapidly, the internal characteristics will be quickly masked. Fruits should be cut through the central axis as accurately as possible. Cavity and basin depth and width and stalk length should be measured from the sectioned fruits. The following diagram indicates the areas of the fruit included in the description.



ab = width of eye basin

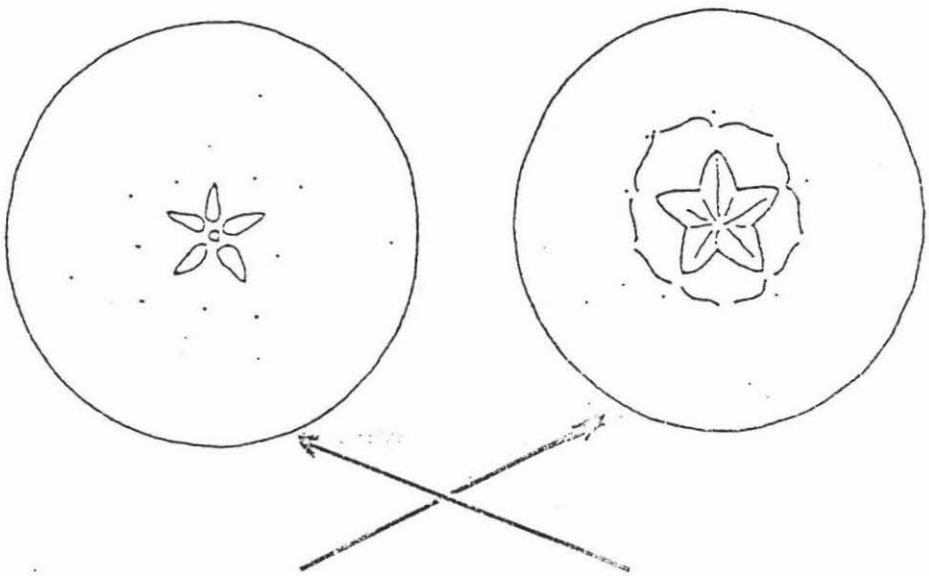
ac = depth of eye basin

ef = width of stalk cavity

fh = depth of stalk cavity

Fruit Cross-section:

Only about five fruits from the remainder of the sample should be sectioned transversely, the cut passing through the mid-position of the locules. If the locules are not in a median position in the fruit it will be seen in the L.S. The core line of Truelle's line is a line linking the vascular strands and in some varieties it is very distinct.



locules wide open
core line distinct

locules closed
core line absent