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Preliminary results of differentiating apple sports by pollen ultrastructure

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Summary

The pollen grain surface morphology of two Red Delicious apple (*Malus* × *domestica* Borkh.) sports, Aversang and Ultrared, and two Gala apple sports, Galalea and Splenola, were examined using scanning electron microscopy for genotype differentiation. Quantitative data on pollen dimensions, ridge patterns, and pore dimensions were extracted from micrographs using image analysis. Data were examined with analysis of variance, canonical variate analysis, and discriminant analysis in order to differentiate genotypes. The combination of image analysis to extract quantitative data and multivariate analysis was successful in differentiating apple sports with exine pore and pollen grain measurements.

Introduction

Positive identification of cultivars is essential to establish and protect plant variety rights, to confirm identity, and to test trueness-to-type. Identification methods should be positive, have a high resolution, ideally be perfectly predictive, be rapid and cost effective. Methods of identification need to address not only the differentiation between cultivars of similar phenotype, but also between sports arising from identical ancestors. Mutants or sports have a similar genotype to the cultivar they are derived from apart from mutations at a few loci coding for traits such as enhanced fruit colour, or full russet in apples. In most cases plant morphology is adequate for differentiating and identifying unique, phenotypically different cultivars. However, differentiation based on morphology has become difficult because of the increasing number of cultivars which have similar phenotypes (Whitmore, 1992).

Biochemical identification methods such as DNA or isozyme 'fingerprinting' have been applied successfully to differentiate cultivars, but have not differentiated sports derived from the same cultivar (Weeden & Lamb, 1985; Menendez et al., 1986; Nybom, 1990; Mulcahy et al., 1993; Tancred et al., 1994; Marquard & Chan, 1995; Matsumoto et al., 1995; Sharon et al., 1995). Pollen morphology also provides traits to distinguish species (Martens & Fretz, 1980; Kozaki & Hirai, 1986; Hebda & Chinnappa, 1990; Vezey & Skvarla, 1990) and cultivars (Fogle, 1977a,b; Maas, 1977; Matsuta et al., 1982, 1986; Fujita & Uchikawa, 1986; Mulas et al., 1988; Ueda & Okada, 1994). Marcucci et al. (1984) separated the apple cultivars Golden Delicious, Delicious, and Granny Smith from spurring (compact habit) sports with pollen dimensions, exine ridge patterns, pollen germination and viability traits. Their findings suggested that further study of pollen ultrastructure may provide a method that will differentiate apple sports.

The present study was a preliminary investigation of whether a combination of image analysis to extract quantitative data on pollen grain dimensions, exine pore and ridge patterns, and multivariate analysis would allow differentiation between genetically and phenotypically similar apple genotypes, in particular, sports derived from the same cultivar.

Materials and methods

Apple genotypes

Pollen was sourced from two Gala sports, Splenola and Galalea and from two Red Delicious sports, Aversang and Ultrared. Each of the four genotypes was sourced from three, eight-year-old trees on MM106 rootstock. Two trees were located at the Clyde Research Centre, Central Otago, New Zealand (45°14′S 169°20′E) and the third tree was located at the Havelock North Research Centre, Hawkes Bay, New Zealand (39°40′S 176°53′E). The Clyde site has a continental climate and the Havelock North site a maritime temperate climate. One Aversang tree and one Ultrared tree at Clyde did not yield pollen.

Pollen preparation

Pollen was collected in spring (October) from flowers harvested from two-year-old shoots using the method described by Galletta (1983). Flowers were collected just before opening to ensure the pollen was mature, but uncontaminated by foreign pollen. Anthers were removed using tweezers and dried on foil for 24 to 48 hours over silica gel to dehisce and release the pollen grains. Pollen was stored at room temperature over silica gel in vials loosely stopped with cotton wool. Previously Currie (1995) found firstly that pollen from winter-forced flowers was lower yielding and had a smaller pollen grain width from pollen collected in spring, and secondly that air-dried pollen gave the best preparation for viewing the equatorial region of pollen grains.

Air-dried pollen was lightly sprinkled on an aluminium Cambridge stub coated with double-sided sticky tape. The stub was inspected using a binocular microscope to ensure the pollen was evenly distributed. Pollen grains clumped together were separated by lightly brushing with a cotton bud and loose pollen grains were removed by puffing dry air over the stub. The stubs were sputter-coated with gold prior to viewing with a Cambridge 250 Mark 3 scanning electron microscope at an accelerating voltage of 20kV. Good resolution was obtained with a 45° stage tilt. However, this tilt introduced a foreshortening effect on pollen grains that were not longitudinally horizontal. For this reason, only pollen grains with the longitudinal axis close to the horizontal were selected for measurement.

Ten mature, fully-formed pollen grains were observed from each tree at low magnification (approx. 1800×) to record the pollen grain dimensions, and at high magnification (approx. 8700×) to study the ridge patterns and pores in the exine.

Image analysis

Images were captured using a Sony colour video CCD camera model DXC-3000P linked to a PC Vision Plus frame grabber card (Imaging Technology Inc., USA) and a multisync monitor. The image analysis software was the Visual Image Processing System version 5 (VIPS 5) developed by D. Bailey at Massey University and run on an IBM compatible 486 with Windows.

An image analysis algorithm was developed to extract measurements of pollen grain dimensions, exine ridge patterns, and pore dimensions. Pollen grain length and width were measured, and length:width ratio and rectangular area were calculated. Tectal perforations or pores in the exine surface were examined, pore number per unit area, area covered by pores, pore length (average longest pore diameter weighted by pore area), and pore width (average pore diameter perpendicular to the length weighted by pore area) were measured. The average pore area and average length:width ratio of the pores were then calculated. The ridged exine pattern was analysed by Fourier analysis (Bracewell, 1989; Bailey, 1993) and the mean and mode of the exine ridge angles and widths were extracted.

Statistical analysis

The 14 variables were tested for normality and homogeneity of variance and transformed if necessary for the analysis of variance (ANOVA).

Derived variables were removed from the data set and canonical variate analysis (CVA) was then applied to identify which of the ten remaining variables had the greatest influence in differentiating between the apple sports. CVA maximises the variation between the genotypes and minimises the variation within the genotypes (SAS Institute Inc., 1990). Thomas (1992) found that the discriminant ratio coefficient (DRC), calculated from the absolute value of the product between the canonical coefficient and the structure correlations from the CVA, was a good indicator of the relative importance of the measured variables in differentiating the classes. The variables with the five highest ranked DRCs for the first two canonical variates were selected and the other variables were removed before re-analysis by CVA. The canonical scores from

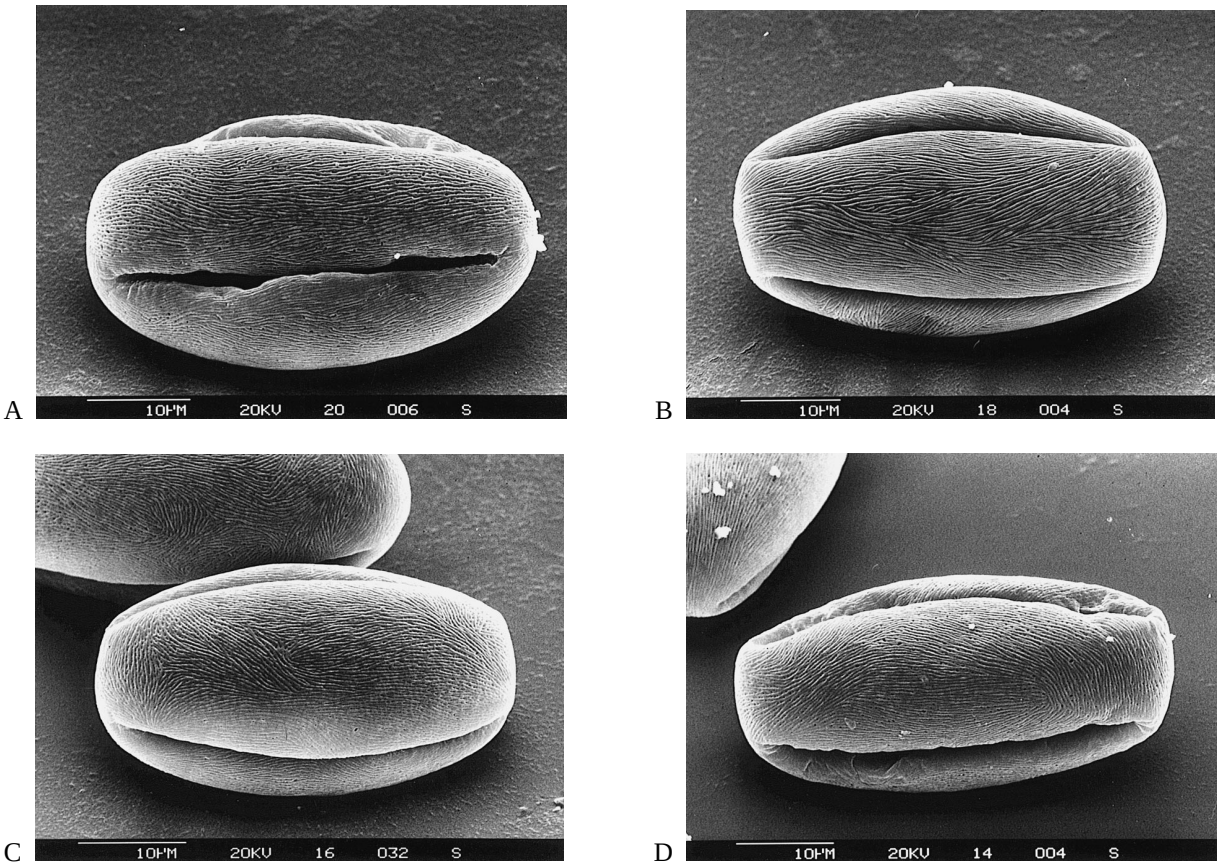

Figure 1. Air-dried apple pollen grains for the apple sports of Red Delicious (A=Aversang and B=Ultrared) and Gala (C=Galalea and D=Splendola) viewed under a scanning electron microscope. Scale bar = 10 μm.

the two canonical variates were plotted to depict the differences between the four genotypes and further analysed using Tukey's Honest Significance comparison of means (SAS Institute Inc., 1990; Cruz-Castillo et al., 1994).

Discriminant Analysis (DA) computes various discriminant functions to classify observations on the basis of one or more quantitative variables (SAS Institute Inc., 1990). The error rate in reallocating pollen grains to apple sports with the discriminant functions was used to test the accuracy of the DA method for identifying pollen grains.

Results and discussion

Morphology

The air-dried apple pollen grains were elliptical, tricolpate with three germinal furrows extending almost the full length of the grain (Figure 1). The average length was 42μm, average width 21μm, the exine surface had a striate pattern, and sometimes small pores in the exine surface were visible. This agrees with previous studies (Fogle, 1977a, b; Martens and Fretz, 1980; Marcucci et al., 1984).

In this study 10 fully-formed pollen grains were chosen at random from each tree. Several authors reported variation within genotypes was reduced when 'representative' pollen grains were selected for analysis (Fogle, 1977a, b; Martens & Fretz, 1980; Mulas et al., 1988). However selection of representative pollen may introduce a bias from the non-random sampling.

Table 1. Mean pollen grain and pore dimensions for the apple sports of Red Delicious (Aversang and Ultrared), and Gala (Galalea and Splenola)

Genotype	Pollen grain measurements				Exine ridge measurements				Pore measurements					
	length (μm)	width (μm)	area (μm^2)	length: width ratio	Mean ridge width^z	Mode ridge width^z	Mean ridge angle^z	Mode ridge angle^z	number^z	% coverage^z	area^z (μm^2)	length^z (μm)	width^z (μm)	length: width ratio
Aversang	42.1	22.3	939.3	1.89	11.3	10.5	9.6	9.7	4.1	-0.5	1.54	-1.62	0.31	1.68
					(0.39)	(0.40)	(94.9)	(96.0)	(843.9)	(1.61)	(0.027)	(0.210)	(0.122)	
Ultrared	43.8	23.2	1018.7	1.89	12.3	11.6	9.5	9.6	2.5	-2.3	1.71	-2.07	0.18	1.34
					(0.36)	(0.36)	(94.1)	(96.2)	(218.9)	(0.13)	(0.006)	(0.097)	(0.052)	
Galalea	40.1	20.9	842.4	1.94	14.3	13.1	9.4	9.3	3.5	-1.5	1.64	-1.90	0.26	1.76
					(0.34)	(0.35)	(89.6)	(87.4)	(448.8)	(0.41)	(0.012)	(0.153)	0.084)	
Splenola	42.3	21.4	906.4	1.98	16.3	15.5	9.4	9.3	2.6	-2.0	1.67	-1.869	0.2	1.72
					(0.33)	(0.32)	(89.2)	(87.9)	(221.1)	(0.14)	(0.008)	(0.123)	(0.058)	
Significance														
Sport	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS
Replicate	**	**	*	***	NS	NS	NS	NS	*	***	***	***	NS	NS
Between sites	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Within site (Clyde)	NS	**	NS	***	NS	NS	NS	NS	NS	*	*	*	NS	NS

^{z} Data transformed (untransformed in brackets)
***, **, *, NS = $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, Not significant respectively.

Another way of reducing the within-genotype variation without bias could be to take the mean of several observations. However, in the present study too few observations were made to allow the analysis of averaged data rather than the individual observations.

Univariate analysis

The restricted statistical design and the corresponding low degrees of freedom associated with the statistical analyses means that the following statistical analyses are to be taken as preliminary investigations of differentiating apple sports by pollen ultrastructure.

From the ANOVA, pore width was found to vary significantly between the Aversang and Ultrared, and the Galalea and Splenola sports ($p \leq 0.05$ Table 1). Vezey and Skvarla (1990) also measured pore features, including pore width, to differentiate Capparacceae species but did not discuss the comparative value of pore width. Marcucci et al. (1984) observed that size and number of pores were the most useful ultrastructural features of apple pollen to differentiate apple cultivars but were unable to differentiate sports using pore features.

The data showed significant variation ($p \leq 0.05$ Table 1) between the tree replicates for pollen grain measurements (length, width, rectangular area, and length:width ratio) and for some of the pore measurements (number, % coverage, average area, and length) but not for pore width nor the exine ridge measurements. Contrasts between the two sites and within the Clyde site showed that most of the variance between replicates was due to the tree effect within the Clyde site (Table 1). Crescimmano et al. (1988) and Currie (1995) found that citrus and apple pollen (respectively) varied in pollen grain width in different environments. No other researcher studied the effects of the environment on the pollen ultrastructure. However, our data suggests that the environment had a significant influence on some of the pollen measurements and that future work should take this into account by selecting measurements not influenced by the environment, such as exine ridge width and angle, pore width or pore length:width ratio, or by allowing for environmental effects in the experimental design.

Multiple analysis of variance

A multivariate approach was taken to see if the data could fully differentiate the apple genotypes. The multiple analysis of variance (MANOVA) for the four apple genotypes Galalea, Splenola, Ultrared and Aversang was highly significant ($p=0.0001$) These results indicate that a multivariate technique has the potential to differentiate the four genotypes. The data also showed

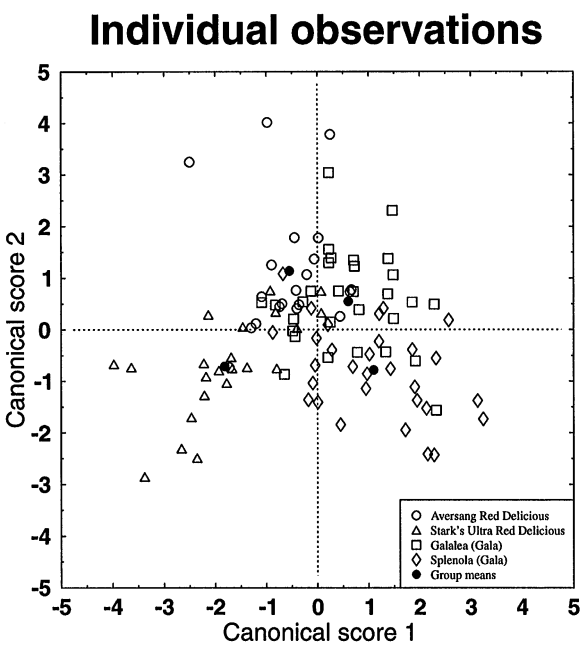

Figure 2. Canonical scores for the apple sports of Red Delicious (○=Aversang and △=Ultrared) and Gala (□=Galalea and ◇=Splenola). Solid symbols represent the mean.

Table 2. Classification of pollen from apple sports of Red Delicious (Aversang and Ultrared), and Gala (Galalea and Splenola) by discriminant analysis.

	Number of pollen grains assigned to:				
	Aversang	Ultrared	Galalea	Splenola	Error rate (%)
Aversang	8	5	2	4	57.9
Ultrared	5	7	3	5	65.0
Galelea	4	3	11	12	45.0
Splenola	2	6	7	15	50.0
Total				58.6	

significant differences between the tree replications (p=0.0002), supporting the findings of the ANOVA.

Canonical variate analysis

The variables with the five highest DRCs for the first two canonical variables were selected for the reanalysis by CVA. 'Average ridge angle' was the only variable dropped from the CVA based on this selection criteria. Removing variables that did not contribute significantly to the differentiation of the genotypes reduced the complexity of the analysis and may increase the reliability of the results. Cruz-Castillo et al. (1994) stated that the combination of too many variables and too few observations can cause unreliable results. According to these authors a sample size of ten times the number of variables produces a reliable result and a sample size less than the corresponding number of variables should be avoided. In this study the sample size of ten pollen grains for nine measured variables falls within this recommended range.

Table 3. Mean canonical scores and significant groupings for the apple sports of Red Delicious (Aversang and Ultrared), and Gala (Galalea and Splenola)

Genotype	Canonical variate 1		Canonical variate 2	
	Mean canonical score	Tukey group[x]	Mean canonical score	Tukey group[x]
Red Delicious cultivars				
Aversang	1.0917	a	0.6130	a
Ultrared	0.5586	a	-0.9996	c
Gala cultivars				
Galalea	-0.4077	b	0.5271	a
Splenola	-0.6561	b	-0.2489	b

[x]Multiple comparison of means using Tukey's Honest Significance test. Groups with the same letter are not significantly different at the 5% level.

For the second CVA with the nine selected variables, differences between each of the four genotypes were significant ($p \leq 0.05$) except for between Splenola and Galalea. Scores for the first and second canonical variable of the observations are plotted (Figure 2). Nearly all of the observed variation in the data was described by the first two canonical variables (50% and 40% respectively).

The DRCs were ranked for each canonical variable to see which measurement had the most influence. The first canonical variable, which separates the Red Delicious and Gala cultivars, was mainly influenced by the average exine ridge width. Ueda and Okada (1994) also found that ridge measurements (number of ridges along the polar axis and the ridge interval) were prominent in the separation of rose cultivars.

The DRC ranking showed that the second canonical variable is primarily influenced by the average pore width and separated the sports Aversang and Ultrared, and Galalea and Splenola. This supports the findings of the ANOVA.

Discriminant analysis

The DA function reclassified pollen grains into groups and could be used to identify unknown pollen grains. However, in this study DA produced a high error rate (59.1%) in reclassifying single pollen grains (Table 2). Ueda and Okada (1994) had error rates around 50% for the reclassification of rose pollen and Matsuta et al. (1982, 1986) obtained error rates of 20% for the classification of Japanese pear pollen. Error rates need to be considerably reduced before DA can reliably identify unknown pollen grains. The discriminatory power of this method could be improved in the future by increasing the number of observations and taking the means of multiple observations to reduce the within-genotype variation, or by trying additional measurements such as the exine pore and ridge characteristics at the poles of the pollen grain.

Further analysis of the canonical scores by multiple comparison of the means, as described by Cruz-Castillo et al. (1994), was able to fully differentiate the four apple genotypes (Table 3).

In this preliminary study, we have demonstrated that pollen ultrastructure has the potential resolution to differentiate between genetically and phenotypically similar genotypes. We have shown that the extraction of quantitative data was made possible with image analysis and that a multivariate rather than univariate analysis enhances the discriminatory power of the data. Canonical variate analysis was useful to reduce the dimensionality of the data from nine measurements to two canonical variables which maximise the between-genotype variance and minimise the within-genotype variance. Multiple comparison of the canonical score means for these two variables fully differentiated the apple genotypes, but further development is necessary before DA can be used to reliably classify unknown pollen samples to known genotypes.

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