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Identification of *Anthurium andraeanum* Cultivars by Gel Electrophoresis

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Abstract. **The use of gel electrophoresis of different isozyme systems was investigated as a possible tool to identify *Anthurium andraeanum* cultivars. Procedures were developed to extract active enzymes from anthurium leaves. Clear zymograms were obtained from only one of three extraction methods examined. This method consisted of grinding the leaf tissue in liquid nitrogen and then adding to the ground tissue a buffer containing a phenoloxidase inhibitor, reducing agents, and polyvinylpolypyrrolidone (PVPP). The identification of seven anthurium cultivars was undertaken using seven enzyme systems. Bands were observed in four of these systems; glutamate-oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), peroxidase (Px), and phosphoglucose isomerase (PGI). All seven cultivars were characterized by the combined data of Px, MDH, and PGI.**

Anthurium andraeanum is the most important cut flower crop in Hawaii. New anthurium cultivars are continually being introduced, and positive identification of cultivars is becoming more problematic. Thus far, anthurium cultivars have been identified mainly through morphological measurements and descriptions of leaves and flowers. Some cytological and biochemical studies of anthurium have been conducted, but biochemical evaluations have not included isozymes (4–6, 10). Isozyme electrophoresis has proven to be a useful tool for cultivar identification in a number of plant species (3, 8, 11, 14–16, 19). Isozyme banding patterns can be used as "fingerprints" to aid in the visual identification of cultivars (13).

The purpose of this study was to develop procedures to extract active enzymes from anthurium leaves and to examine isozyme polymorphisms for possible use in anthurium cultivar identification.

Materials and Methods

Seven *Anthurium andraeanum* cultivars were selected for this study, including three ('Kaumana', 'Kozohara', and 'Nitta') developed by growers, and four ('Manoa Mist', 'Marian Seefurth', 'Paradise Pink', and 'Uniwai') developed and released by the Univ. of Hawaii. The cultivars used in this study were grown under 78% shade at the Manoa Campus of the Univ. of Hawaii. Three extraction methods and seven enzyme systems were used to study these seven cultivars. The enzyme systems surveyed included alcohol dehydrogenase (ADH), esterase (EST), glutamate-oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), peroxidase (Px), phosphoglucose isomerase (PGI), and phosphoglucomutase (PGM).

Sample extraction. Mature leaf samples were thoroughly washed in cold water and blotted dry. Three extraction methods evaluated were: a) maceration of tissue with a mortar and pestle in 0.2 M phosphate buffer, pH 7.0, at room temperature; b) grinding of tissue frozen in liquid nitrogen (LN), followed by the addition of a 0.2 M phosphate buffer to make a coarse slurry; and c) method 2, using a 0.05 M Tris buffer containing 0.20 M sucrose, 0.002 M diethyl dithiocarbamic acid, 0.02% β-mercaptoethanol, and polyvinylpolypyrrolidone (PVPP). The pH was adjusted to 7.3 before adding the mercaptoethanol and PVPP.

Samples extracted by the first two methods were absorbed onto Beckman filter paper wicks. Lens paper was placed between the wick and the macerate to avoid uptake of fibrous residue.

Extracts produced by the 3rd method were centrifuged at 32,000 × *g* for 20 min. The clear supernatant was collected, and absorbed onto filter paper wicks for immediate use or frozen in LN and stored in vials at −10°C.

Gel preparation. Two types of gels were prepared for horizontal electrophoresis. Six percent polyacrylamide was used to evaluate the peroxidase system, and 12% starch was used for all other enzyme systems surveyed.

Polyacrylamide gels were prepared following methods described by Brewbaker et al. (2). Gels were immediately poured

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into 18 × 20 × 0.3 cm trays, covered with glass plates, and left to harden.

Twelve percent starch gels were prepared by adding 48 g of hydrolyzed potato starch (Sigma) to 400 ml of gel buffer in a one-liter Erlenmeyer vacuum flask. The gel buffer used for all enzyme systems except EST and Px was a 0.045 M Tris containing 0.001 M EDTA and 0.025 M Borate (TEB) buffer (pH 8.6). A 0.045 M Tris + 0.007 M citrate (pH 8.4) buffer combined with a 0.003 M lithium hydroxide + 0.019 M borate (pH 8.1) buffer was used for EST and Px. The starch mixture was heated with constant swirling to 76°C over a small open flame. The flask then was removed from the heat and evacuated until only large bubbles rose to the surface. The gel was poured immediately into a 18 × 20 × 0.9 cm tray. Remaining air bubbles, if any, were removed with a transfer pipette. The gel was allowed to cool for 30 min at room temperature, covered with plastic wrap, and refrigerated (4°C) for at least 1 hr before use.

Electrophoresis and enzyme staining. Horizontal electrophoresis was conducted following methods modified from Brewbaker et al. (2). The gels were loaded by inserting paper wicks into slots cut about 7 cm from the cathodal end. The electrode buffer for EST and Px systems consisted of 0.03 M lithium hydroxide and 0.19 M borate (pH 8.1). A 0.129 M Tris containing 0.003 M EDTA and 0.071 M borate buffer (pH 8.6) was used for all other enzyme systems studied. Initial voltages and associated buffers for the seven enzyme systems surveyed are listed in Table 1. Starch gels were run for 4–5 hr, while polyacrylamide gels were run for 6–7 hr. Electrophoresis was conducted at 4°C.

After electrophoresis, starch gels were sliced horizontally into three pieces, each about 2 mm thick. Staining procedure for each enzyme system was as follows.

Alcohol dehydrogenase (ADH). Thirty-nine milliliters of 0.128 M Tris·HCl (pH 8.0), 3 ml of 95% ethanol, 2 ml of 1% NAD, 5 ml of 0.2% nitro blue tetrazolium (NBT), and 1 ml of 0.2% phenazine methosulfate (PMS) were mixed together. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Esterase (EST). Ten milligrams of α-napthyl acetate was dissolved in 0.5 ml of acetone and 0.5 ml water and then added to 50 ml of 0.1 M phosphate buffer (pH 7.0). Fifty milligrams of Fast Blue RR salt was then dissolved in this solution. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Glutamate-oxaloacetate transaminase (GOT). Two hundred milligrams of aspartic acid, 100 mg α-ketoglutaric acid, 5 mg of pyridoxal 5′ phosphate, 50 ml of 0.1 M phosphate buffer (pH 7.0), and 1 ml of 0.1 M magnesium chloride were combined. Twenty milligrams of Black K salt dissolved in 2 ml of acetone was then mixed in. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Malate dehydrogenase (MDH). Twenty-five milliliters of 0.1 M Tris·HCl (pH 8.0), 25 ml of 0.5 M DL-malic acid (adjusted with NaOH to pH 7.0), 1 ml of 1% NAD, 1 ml of 1% MTT or thiazolyl blue, and 0.25 ml of 1% PMS were mixed. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Phosphoglucomutase (PGM). Forty milligrams of α-D-glucose-l-phosphate was dissolved in 35 ml of 0.2 M Tris·HCl (pH 8.0), 10 units of glucose-6-phosphate dehydrogenase, 0.5 ml of 1% NADP, 2 ml of 0.2% NBT, 1 ml of 0.2% PMS, and 10 ml of 0.1 M magnesium chloride. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Phosphoglucose isomerase (PGI). Twenty milligrams of fructose-6-phosphate was dissolved in 35 ml of Tris·HCl (pH 8.0), 10 units of glucose-6-phosphate dehydrogenase, 0.5 ml of 1% NADP, 2 ml of 0.2% NBT, 1 ml of 0.2% PMS, and 10 ml of 0.1 M magnesium chloride. The gel was incubated in the stain at 37°C in the dark for 2 hr.

Peroxidase (Px). Forty milliliters of 35% ethanol and 4.5 ml glacial acetic acid were slightly heated to dissolve 250 mg of benzidine dihydrochloride. Three milliliters of 3% hydrogen peroxide were placed in the developing tray. The gel was stained for 2 min at room temperature in the light, then rinsed thoroughly with water.

Stained starch gels were fixed overnight in a solution composed of methanol : glacial acetic acid : water (6:4:1) and stored at 4°C. Polyacrylamide gels were also stored at 4°.

Band migration was measured in millimeters and R_f values as described by Kuhns and Fretz (7) were calculated. The staining intensity of bands was also noted and visually categorized as either light, medium, or heavy.

Table 1. Buffer system and initial power used in electrophoresis of anthurium enzymes.

Enzyme	Buffer system	Initial power
Alcohol dehydrogenase	TEB[z]	30 mA
Esterase	LB[y]	35 mA
Glutamate-oxaloacetate transaminase	TEB	35 mA
Malate dehydrogenase	TEB	30 mA
Peroxidase	LB	40 mA
Phosphoglucose isomerase	TEB	30 mA
Phosphoglucomutase	TEB	30 mA

[z]Tris–EDTA–borate (pH 8.6).
[y]Lithium borate (pH 8.1).

Results and Discussion

Evaluation of different procedures for enzyme extraction. Leaf extracts obtained by the 3rd extraction method, which employed the buffer designed to reduce phenolic inactivation of enzymes, displayed no tissue browning. After centrifugation, a clear light yellow supernatant was collected. Zymograms obtained from these samples exhibited the least amount of streaking and the sharpest bands.

Brown macerates were obtained from the first two extraction methods, and browning was more intense in the first method than in the 2nd. Zymograms obtained from the electrophoresis of these samples were similar. Generally, light banding with streaking was observed, and, in a few instances, dark streaking made it difficult to distinguish any bands.

Poor zymogram quality resulting from dark streaking seems to be related to tissue browning noted during the grinding process. Kuhns and Fretz (7) reported that sample preparation for electrophoresis can be complicated by interactions occurring between proteins and other cell matter, such as carbohydrates, phenolic compounds, and hydrolytic and oxidative enzymes. These compounds are normally compartmentalized in vivo but become mixed during tissue maceration. Wendel (18) reported a buffer containing reducing agents such as ascorbic acid, mercaptoethanol, and metabisulfite, and a phenoloxidase inhibitor (diethyl dithiocarbamate) was effective in enzyme extraction from the tannin-rich *Camellia japonica*. The removal of the phenols was reported in some instances to be more effective than the mere prevention of the oxidative reaction (20). Phenol removal can be accomplished by the addition of excessive PVPP,

which binds the phenols that usually form complexes with proteins. The phenol–PVPP complexes can then be removed by centrifugation (7).

Cultivar identification. Isozyme bands were resolved in four of the seven enzyme systems surveyed: GOT, MDH, Px, and PGI. No staining was observed in gels tested for ADH and EST. In the esterase gels, unresolved red areas were observed after staining. MacDonald (9) reported EST bands as blue-black in color. The cause of the red staining is still unresolved. In gels stained for PGM, some staining was observed, but bands were not clearly resolved. Blue bands appeared in the GOT system, but were variable within cultivars, and the dark maroon background staining sometimes made it difficult to distinguish the bands.

In gels stained for PGI and MDH activity, only one banding pattern was observed in each system. Zymograms of PGI exhibited one violet-blue band at R_f = 0.03. Band presence was noted in 'Kaumana', 'Kozohara', 'Marian Seefurth', and 'Uniwai', but not in 'Nitta', 'Paradise Pink', and 'Manoa Mist'. Zymograms of MDH had a banding pattern with two bands, one at R_f = 0.15 and the other at R_f = 0.19. Violet-blue MDH bands were present in 'Kozohara', 'Nitta', and 'Uniwai', but were absent in the four other cultivars.

Five distinct banding patterns were resolved in Px (Fig. 1). 'Marian Seefurth' and 'Paradise Pink' showed similar triple banded patterns, while 'Manoa Mist' and 'Uniwai' each had a broad band around R_f = 0.54. 'Kaumana' also displayed one broad band, but it was located at about R_f = 0.57. 'Nitta' also exhibited a broad band of similar mobility, but its very heavy staining along with a unique band at R_f = 0.30, made it distinguishable from the other cultivars.

Employment of previously described electrophoretic techniques revealed that, out of the seven enzyme systems surveyed,

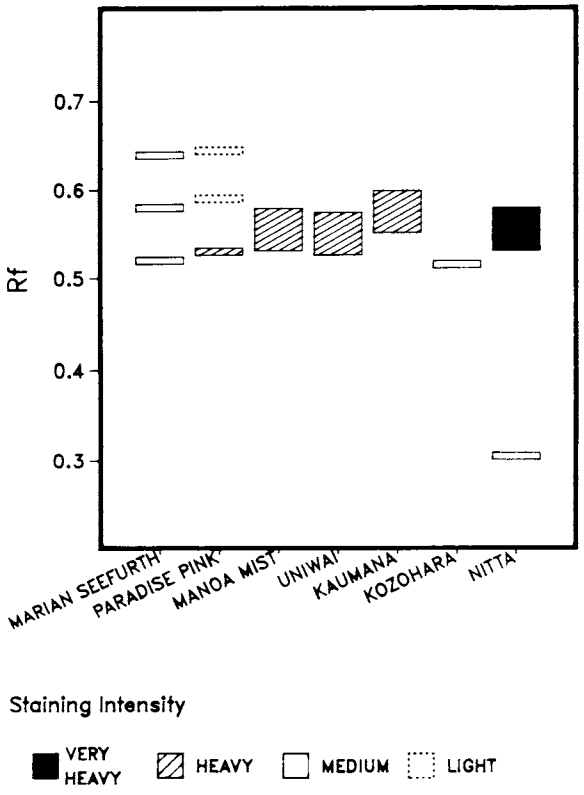

Fig. 1. Zymogram of peroxidase isozymes found in mature leaf tissue of seven anthurium cultivars.

Table 2. Identification of seven anthurium cultivars on the basis of zymogram differences in three enzyme systems.

Cultivar	Peroxidase[z]	Malate dehydrogenase[y]	Phosphoglucose isomerase[y]
Kaumana	C	−	+
Kozohara	D	+	+
Nitta	E	+	−
Marian Seefurth	A	−	+
Manoa Mist	B	−	−
Paradise Pink	A	−	−
Uniwai	B	+	+

[z]A–E represent different peroxidase banding patterns (see Fig. 1).
[y]+, presence of band(s); −, absense of band(s).

Px was the most useful for distinguishing anthurium cultivars. Peroxidase also was found to be the most stable. Leaf tissue could be stored frozen for weeks with no noticeable effect on enzyme activity or mobility.

The peroxidase data suggests that slight differences exist between the R_f values of 'Marian Seefurth' and 'Paradise Pink', and between 'Manoa Mist' and 'Uniwai'. The pairs, however, were not found to be significantly different. Also, in a combined sample analysis of 'Marian Seefurth' and 'Paradise Pink', the bands appeared to coincide, and the characteristic triple-banded pattern was observed. It is therefore believed that the differences are probably due to minor variations in buffer molarities or pH, and to rounding error during data compilation.

Based on Px alone, 'Kaumana', 'Kozohara', and 'Nitta' appear to be identifiable from the other cultivars. The combined results of Px, MDH, and PGI indicated that all seven cultivars can be characterized by these systems (Table 2). Genetic relationships also seem to be reflected in the Px zymograms. 'Marian Seefurth' and its offspring, 'Paradise Pink', have very familiar zymograms. 'Manoa Mist' also has a similar banding pattern to 'Uniwai', one of its parents. Px has been used in genetic studies of various species, such as avocado (17), corn (12), and petunia (1).

The potential of isozymes as a tool in genetic studies of anthuriums and in cultivar identification seems to be indicated by the data. Additional studies involving more enzyme systems and perhaps different techniques seem to be warranted.

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Inheritance of Ripening Uniformity and Relationship to Crop Load in Blueberry Progenies

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Abstract. **The intervals, in days, between 10%, 50%, and 90% ripened fruit, as well as crop load, were estimated over 2 years in progenies from a partial diallel cross among 17 blueberry (*Vaccinium corymbosum* L., *V. angustifolium* Ait., and *V. corymbosum* x *V. angustifolium* hybrids) parents. General combining ability (GCA) mean squares were highly significant for all ripening intervals and for crop load, while specific combining ability mean squares were nonsignificant, indicating a large proportion of additive genetic variance. Narrow-sense heritability estimates were about 0.50 for the three ripening intervals (10–50%, 50–90%, and 10–90%). Several parents had large positive GCA effects, indicating their contribution to a long ripening interval. Most progenies with large crop loads required >15 days between 10% and 90% ripened fruit. Despite the consistently positive relationship between ripening interval length and crop load, variation among families and the potential for within-family segregation suggest the possibility of obtaining genotypes with high yield potential and improved uniform ripening.**

A high degree of ripening uniformity could improve efficiency of hand or machine harvest of blueberry (*Vaccinium* spp.) fruit, although a grower also may be placed at greater risk from adverse environmental conditions.

Galletta (5) suggested that concentrated ripening may be achieved by selecting for a short bloom period, or a uniform ripening period, or for the tendency to maintain mature fruit on the bush in prime condition. He noted that certain highbush (*V. corymbosum* L.) cultivars like Croatan, Collins, Morrow, and Earliblue will mature >80% of their crop in a 7-day period with appropriate weather conditions. Likewise, Darrow and Scott (2) indicated that several highbush cultivars can mature 70–100% of their crop within a 2-week period.

Half-high (*V. corymbosum* x *V. angustifolium* Ait. derivatives) blueberry genotypes developed by the Univ. of Minnesota breeding program have a high yield potential (4–5 kg/bush), but may require 3–5 weeks to ripen their fruit, depending on environmental conditions and crop load (see ref. 11; unpublished data). The long ripening period may be due in part to use of parental genotypes only one or two generations removed from wild ancestors (11). While an extended ripening period may be an advantageous adaptation for wild genotypes to minimize risk from adverse environmental conditions, it is less desirable in commercial production where some environmental factors can be managed. Maintaining high yield potential with greater ripening uniformity would be desirable in future half-high cultivars.

Our objectives were to examine the inheritance of ripening uniformity and the relationship between crop load and ripening uniformity in a population of half-high blueberries. This information will provide guidance for effective selection and for planning future crosses to increase ripening uniformity.

Materials and Methods

Each of 17 parents (Table 1) was crossed with six other parents in a circulant partial diallel mating design (8). The parents were tetraploid (2n = 4x = 48) clones representing *V. corymbosum, V. angustifolium,* and hybrids between the species. The resulting progenies were planted in 1976 at Becker, Minn.

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