

# A Study of the Potential Use of Electrophoresis in Distinguishing Rose Cultivars

LARRY J. KUHNS

THOMAS A. FRETZ

**OHIO AGRICULTURAL RESEARCH AND DEVELOPMENT CENTER**  
U. S. 250 and Ohio 83 South  
Wooster, Ohio

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# A Study of the Potential Use of Electrophoresis in Distinguishing Rose Cultivars<sup>1</sup>

LARRY J. KUHNS and THOMAS A. FRETZ<sup>2</sup>

## ABSTRACT

Eight rose cultivars were grown under controlled conditions for variety identification studies. The cultivars included seven hybrid tea types (Peace and three of its sports, a hybrid of Peace and its sport, and Forever Yours) and one Grandiflora type (Sonia).

A technique was developed to extract active enzymes from rose leaves. Samples were run on 7% polyacrylamide gels, which were then stained for 16 enzyme systems and total protein. Systems which yielded clear, consistent results were peroxidase, esterase, malate dehydrogenase, cytochrome oxidase, phenoloxidase, polyphenoloxidase, and total protein.

Coefficient of similarity values based on all seven systems were calculated for all possible cultivar combinations. They proved useful for grouping cultivars and sharing relationships, but they were not useful for distinguishing closely related cultivars. Alone, some systems could not separate Peace cultivars from the distinctly related Forever Yours or Sonia. However, all cultivars could be distinguished by differences in their enzyme banding patterns if results from several systems were employed.

## INTRODUCTION

Identification of roses has traditionally been based on visual descriptions of growth and flowering habit. However, new cultivars which are very closely related to existing individuals are constantly being introduced through breeding and selection programs. Since a great deal of time and money is involved in the development and screening of new cultivars and the subsequent patenting process, a positive means for identification is essential for patent protection. Polyacrylamide gel electrophoresis (PAGE) is a relatively new taxonomic tool which has shown promise in the separation of varieties of several species (7, 17, 25, 33, 41, 42, 48, 54, 72) through enzyme characterization. Enzymes can be separated into different molecular forms, called isoenzymes, by PAGE

(18, 46, 48, 53). Since all the forms maintain similar or identical catalytic activity, patterns called zymograms are obtained by staining the gels with an enzyme specific stain (8, 53). Differences in these zymograms have been related to disease resistance (26), cold hardiness (1), photoperiod (70), tissue age or physiological condition (48, 49, 53, 76), dwarfism (16), and genetic differences (17, 41, 42, 53, 72).

By standardizing the environment and by sampling tissue of uniform age, all enzyme differences should be due to inherent genetic differences. Since each cultivar has a distinct genetic composition, the possibility exists that closely related cultivars may be distinguished by zymograms of one or more enzyme systems.

By applying PAGE techniques to several closely related rose cultivars, much information about enzymes in rose leaves was gathered. The feasibility of separating rose cultivars by using PAGE was evaluated.

## LITERATURE REVIEW

Proteins, as amphoteric compounds with variable electrophoretic mobilities, can be separated by zone electrophoresis. By performing the electrophoretic separation on a gel matrix which can impose a frictional resistance to the ions, further resolution is possible because separation is based on dimensional as well as charge differences (18, 46). Starch was the first matrix to be successfully used in gel electrophoresis of proteins (59). Starch gels are suitable for protein separation because their average pore size is in the range of the dimensions of proteins (60). Polyacrylamide was thoroughly tested as a gel matrix (18, 46) and was found to possess several advantages over starch. Pore size of acrylamide gels can be varied over a wider range; gels are mechanically stronger, more chemically inert, and transparent to visible radiation, making quantitation of bands by densitometry easier.

Polyacrylamide gel preparation techniques and operating conditions have with a few exceptions remained basically the same as those outlined by Davis (18). A sample gel is no longer considered necessary to prevent convection of the sample into the reservoir buffer. Instead, the same result is obtained simply by increasing the density of the sample with sucrose (13, 52). The spacer gel can also be eliminated if

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<sup>2</sup>Assistant Professor, Department of Horticulture, The Pennsylvania State University, State College, and Associate Professor, Department of Horticulture, The Ohio State University and Ohio Agricultural Research and Development Center, Columbus.

the protein concentration of the sample is high enough (24). Gel and reservoir buffers can be altered to facilitate the separation and staining of a specific enzyme system (35, 75). Review articles explaining the advantages and disadvantages of these options are available (9, 22, 24, 39).

Preparation of a protein sample for electrophoresis is complicated by the interactions which can occur among proteins and other cellular constituents that are rigidly compartmentalized *in vivo*, but are mixed during tissue maceration. Included are vacuole acids, carbohydrates, hydrolytic and oxidative enzymes, phytic acid, and phenolic compounds (49, 62). Adsorption of soluble proteins by cell wall fragments is also possible (43).

With some plants and enzymes, crude extracts can be used with no adverse effects on results (8, 14, 17, 29, 64, 72); with others, protective measures must be taken (20, 29, 30, 68). The major interaction to be controlled is that between proteins and phenols and their oxidation products (2, 20, 29, 38, 77). Phenols form complexes with proteins through strong hydrogen bonds and are readily oxidized to quinones which may in turn oxidize essential groups of proteins or form covalent bonds to the protein (38).

Many methods have been developed to prevent protein-phenol interaction during the extraction process, with the method of choice determined primarily by the particular requirements of the experiment. No single extraction technique can be expected to be effective for all enzymes of a species or for the extraction of a particular enzyme from all plant species (22). The choice of extraction technique and solution has been found to affect final results significantly (8).

Dimethyl sulfoxide (DMSO) is an organic solvent in which enzymes maintain activity for prolonged periods (12-20 hours) at moderate temperatures (37° C) in high solvent concentrations (35-60%) (50). It has been used as an extraction medium for soluble plant proteins and offers the advantages of simplicity, room temperature operation, and a storable product (3). Its major disadvantage is that it penetrates rubber and plastic gloves and skin, entering the body and imparting an oyster or garlic odor to the breath (51).

Extraction of cotton leaves with borate yielded samples with high total protein and enzyme activity, but was effective only in an extremely narrow pH range (34). Polyethylene glycol (PEG) has proven to be a useful additive to an extraction solution, apparently because of its protein solubilizing effect (11, 77), but is not widely used. Reducing agents such as ascorbate, potassium metabisulfite, cystine, mercaptoethanol, and dithiothreitol reverse the oxidation of

phenols and prevent the accumulation of quinones, lowering the probability they will react with proteins (26, 38). They also help maintain protein solubility by protecting sulfhydryl (SH) groups against oxidation to disulfide (SS) bonds (26, 65). Extraction with reducing agents alone in the extraction solution yields satisfactory samples in some cases (68), but much more rigorous procedures are required in others (20, 26, 67). Disadvantages of reducing agents are that they may cause changes which result in modified proteins (38) or create interference during electrophoresis (67).

In some studies, merely preventing the oxidation of phenols does little good; it is necessary to remove them (20, 29, 38). Techniques which separate proteins and phenols include dialysis (20, 38, 73), gel filtration (23, 38, 68, 73), the use of polyvinylpyrrolidone (PVP) (2, 11, 19, 20, 23, 29, 30, 38), and the preparation of acetone powders (5, 11, 21, 26). Dialysis and gel filtration work only if adequate precautions are taken to prevent oxidation, as oxidation products will not separate from the proteins (38). Enzyme activity is affected by the length of time a sample is dialysed, but the optimum length varies among enzyme systems (73). Therefore, the same dialysis procedure cannot be applied to prepare a sample for the assay of multiple enzymes. Also, if the phenolic content of the sample is sufficiently high, neither dialysis nor gel filtration will separate the proteins and phenols fast enough to prevent enzyme inactivation (20, 68).

Insoluble PVP contains groups similar to the peptide linkage of proteins. If excess PVP is added to the sample, those phenols which form H-bonded complexes with protein will instead bind to the PVP and can be removed by centrifugation (19, 38). Samples can also be passed through a column of PVP to remove phenols (23). These procedures are pH dependent and are not effective above pH 7.5. Optimum conditions for bonding of plant phenols to PVP can be found in review articles by Anderson and Sowers (2) and Loomis and Battaile (38). Pharmaceutical grade PVP is required, as the commercial grade contains impurities which make it ineffective (28). It should be hydrated for at least 24 hours before use as the hydrated form is much more effective than the dry powder, and thorough wetting of the polymer requires some time (38). Denaturing agents such as urea should not be included when extracting with PVP because they cause the PVP-phenol complex to dissociate (2). Again, if the phenolic concentration of the sample is sufficiently high, the use of PVP alone will not remove all the phenols and some enzyme inactivation may still occur (19, 20, 30).

Acetone can be used to precipitate protein with little loss of enzyme activity and at the same time extract most phenols (5). Alcohol would be a better solvent for phenols but causes considerable enzyme inactivation (58). Bendall and Gregory (5), Hare (26), and El-Basyouni and Neisch (21) present details on the preparation of acetone powders. Bendall and Gregory (5) recommended 20% water be included in the acetone except for a final drying of the precipitate with pure acetone. This is because phospholipids are soluble in aqueous but not dry acetone, and some enzymes are tightly bound as lipoprotein particles. Hare (26) recommended including 30% water to remove water soluble interfering substances such as chlorophyll. Dirr *et al.* (20) found the soluble fraction of acetone preparations to be inhibitory to enzyme activity while the precipitate contained no inhibitors. Although the preparation of acetone powders separates most phenols and enzyme inhibitors from the protein fraction, some combinations of PVP and/or reducing agents are still necessary when resolubilizing the protein to prevent enzyme inactivation (11, 26, 67).

Frequently used components of an extraction solution other than those for control of phenols include sucrose, urea, detergents, and salts for increasing ionic strength. Sucrose enhances chloroplast disruption by its influence on osmotic pressure, and minimizes the aggregation and precipitation of leaf proteins after they are extracted (6). Urea increases the solubility of certain classes of proteins and the number of detectable isoenzymes of some systems by causing disassociation of H-bonds (24, 26). Since removal of urea may lead to reaggregation, samples containing urea should be run on gels containing urea. Both sucrose and urea increase sample density sufficiently to preclude the need for a sample gel. Detergents such as triton X-100, tween-20, or tween-80 also increase the solubility of some proteins (6, 26, 36, 77). Salts used to increase ionic strength have been NaCl (8, 70), KCl (36), and CaCl<sub>2</sub> (12). A medium of high ionic strength was found to be essential for the quantitative extraction of peroxidase and indoleacetic acid oxidase (36).

Following extraction, samples may need to be concentrated prior to electrophoresis, because applying high amounts of sample to the gels may cause dark background staining and reduce the number of visible bands (26). Millipore filters (Millipore Corp.) can be used (67) or the sample may be placed in a dialysis bag which is then brought into contact with a hygroscopic compound which will not pass through the membrane. Sephadex (AB Pharmacia) (55), Lyphogel (Gelman Instrument Co., Gibson Electronics Co.) (10), Aquacide (Calbiochem)

(39), Carbowax 20M (Union Carbide) (39), and PVP (Calbiochem) (12) have all been used for this purpose. Precipitation by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and resolubilization in the desired volume is a suitable technique for some enzyme systems (11, 45, 66, 68, 78). Jakoby (32) describes these methods in detail. Samples can also be concentrated, within certain limits, by increasing the tissue to extraction solution ratio (47).

Techniques for preparing tissue for maceration vary considerably. The tissue may be frozen at -2° to -20° C (26, 70), in liquid nitrogen (LN) (10, 12, 42, 47), lyophilized (10, 30, 69), or simply ground fresh (64, 68). Bonner *et al.* (7) found no difference in peroxidase zymograms between tissues used directly and tissues frozen at -2° to -4° C prior to use. Betschart and Kinsella (6) observed that lyophilization did not impair protein extractability of alfalfa leaves, but it did reduce protein yield from soybean leaves. Freezing in LN facilitates tissue maceration and effectively stops all interactions between cell components.

Samples may be stored as frozen (7, 70) or lyophilized (30, 54, 69) tissue, acetone powders (26, 42), or frozen solutions (64). Length of storage and storage conditions are dependent on the enzymes to be analyzed. Freezing or lyophilization cause inactivation of catalase, and ascorbic acid oxidase is stable for only about 1 month (78). Many enzymes are stable as lyophilized powders at 5° C.

Using various combinations of these procedures, grape varieties (77), petunia species and cultivars (42), and Kentucky bluegrass cultivars (72) have been separated or classified. Wolfe (77) studied seven enzyme systems in extracts from ripe grape berries of 60 varieties and found three systems—esterase, peroxidase, and alcohol dehydrogenase—unsuitable for evaluation because of inconsistent results. With acid phosphatase and indophenol oxidase, some bands were consistent and useful for variety separation but others were not. Only the zymograms of leucine aminopeptidase and catechol oxidase were entirely consistent. Most, but not all of the varieties studied, could be separated on the basis of the zymograms of the four enzyme systems with consistent banding. Those that could not were distinguishable by distinctive morphological traits. This met the stated objective of the study which was not to replace, but to supplement, the morphological basis of identification with distinct, unvarying genetic markers.

Natarella and Sink (42) compared the protein and peroxidase zymograms obtained from leaf extracts of flowering plants of four species and 11 cultivars of petunia. Their results elucidated the classification of the four species and provided sufficient information to separate the 11 cultivars.

In the previous two studies, multiple systems were investigated and bands were recorded as present or absent. Wehner *et al.* (72) studied only peroxidase isoenzymes of Kentucky bluegrass cultivars but recorded band density in addition to presence or absence. With an elaborate system of comparing density ratios calculated from densitometer tracings, they were able to distinguish 11 of the 15 cultivars examined.

## METHODS AND MATERIALS

Six closely related rose cultivars were selected for the study: Peace and three of its sports—Chicago Peace, Flaming Peace, and Climbing Peace—and Pink Peace and its sport Candy Stripe. Plants were potted in two-gallon containers in a 2:1:1 soil, peat, perlite medium supplemented with 3 tablespoons of triple superphosphate (0-46-0) per container. One tablespoon of osmocote (18-6-12) per container was applied at 3-month intervals, and a fertilizer injector was used to apply nitrogen and potassium with each watering. Plants were placed in a completely randomized design, and standard disease and insect preventive procedures were followed.

Two other rose cultivars, Sonia and Forever Yours, were available for study. They were growing in ground beds in the same greenhouse as the other six cultivars and received the same cultural care. The descriptions and lineages of the eight cultivars are as follows:

- Peace — Hybrid Tea, yellow blend  
[(George Dickson x Souv. de Claudius Perret) x (Joanna Hill x Charles P. Kilham)]  
x Margaret McGredy
- Chicago Peace — Hybrid Tea, pink blend  
sport of Peace
- Flaming Peace — Hybrid Tea, red blend  
sport of Peace
- Climbing Peace — Climbing Hybrid Tea, yellow  
blend  
sport of Peace
- Pink Peace — Hybrid Tea, medium pink  
(Peace x Monique) x (Peace x Mrs. John  
Laing)
- Candy Stripe — Hybrid Tea, pink blend  
sport of Pink Peace
- Forever Yours — Hybrid Tea, cardinal red  
Yuletide x unnamed seedling
- Sonia — Grandiflora, pink blend  
Zambra x (Baccara x White Knight seed-  
ling)

Gels were prepared and run according to Davis (18) with two exceptions. No sample gel was used (sample density was increased with 15% sucrose or urea), and the stock reservoir buffer was diluted to

1/5 rather than 1/10 strength. This is because on dilution of the buffer in which proteins are dissolved, the probability of molecular association increases. Originally gels were run at 5mA/tube, but reduction to 3mA/tube resulted in more distinct bands. High currents cause excessive ohmic heating and may result in the formation of pattern artifacts (18, 24, 26).

Immediately after removal from the plant, leaves were prepared for extraction by thoroughly washing them in cold water containing a detergent, rinsing in distilled water, and blotting dry.

Several extraction techniques were evaluated to determine the simplest method which would yield clear, reproducible results. Because of the extremely high phenolic content of roses (29), all methods were directed toward the control of phenol-protein interactions. First the method of Ascher and Weinheimer (3) using DMSO was evaluated. Gels were stained for total protein and peroxidase, but no bands developed.

Acetone powders were prepared exactly as described by Hare (26). Temperature control was a problem, however, and peroxidase and protein bands were few and diffuse. Moving the extraction process to a 2° C refrigerated room solved the temperature problem and better results were obtained.

In an effort to avoid the discomfort of working at 2° C, techniques employing LN were evaluated. First, leaves were placed in LN, ground in an LN cooled mortar and pestle, then homogenized with extraction solution in a Potter-Elvehjem homogenizer. This system had two disadvantages. Samples were too dilute for direct application to the gels and had to be concentrated (dialysis bags placed in Aquacide or a Millipore filter system), and long term storage of the macerated tissue or extracted sample was not possible without considerable enzyme alteration.

The method which proved to be most satisfactory was a combination LN and acetone powder preparation. Prepared leaves were placed in LN and ground in an LN cooled mortar and pestle. The resultant powder was homogenized in a Potter-Elvehjem homogenizer with five parts 70% acetone (all acetone used in the extraction process was maintained at -20° C). The slurry was filtered under suction, and the residue was washed with 100% acetone until the filtrate was clear. Excess acetone was drawn off under suction, and the residue was lyophilized and stored at -20° C. Advantages of this system are simplicity, the removal of a major portion of the phenols, maintenance of enzyme activity, and the ability to store the resultant product for several months.

To extract the protein from the lyophilized acetone powder, three conditions had to be determined:

what to include in the extraction solution, the optimum tissue:extraction solution ratio, and the optimum extraction time. To determine the need for the components of extraction solutions used by others (26, 40), the following three solutions were prepared and used to extract the protein from rose acetone powders and commercially prepared bovine serum albumen (BSA) and horseradish peroxidase (HRP).

A. Dithiothreitol (DTT)	
(Clelands Reagent)	0.05 g
Potassium metabisulfite ( $K_2S_2O_5$ )	0.25 g
Ascorbic acid	0.50 g
PVP	0.75 g
Sucrose	7.50 g
Triton X-100	2 ml of 10%
Tris	pH to 7.3
Water	to 50 ml
B. PVP	
	0.75 g
Sucrose	7.50 g
Triton X-100	2 ml of 10%
0.1 M Na phosphate buffer, pH 7.3	to 50 ml
C. Sucrose	
	7.50 g
0.1 M Na phosphate buffer, pH 7.3	to 50 ml

Commercially prepared samples extracted with solutions B and C yielded sharper bands than samples extracted with the solution containing the reducing agents. With the rose acetone powders, however, no peroxidase bands developed, and protein bands were diffuse and hard to distinguish from a heavy background stain unless reducing agents were included in the extraction solution. So, despite their adverse effect on band clarity, reducing agents had to be included in the extraction solution to prevent enzyme inactivation and protein alteration, apparently caused by the oxidation products of phenolic compounds not extracted by the acetone.

Mercaptoethanol, cysteine, and DTT are all reducing agents used to maintain protein solubility by protecting SH groups against oxidation to SS bonds.

**TABLE 1.—Optimum Extraction Solution and Application Rates.**

Enzyme	Optimum Extraction Solution*	Optimum Application Rate (ml)
Cytochrome Oxidase	— C — U	0.30
Esterase	— C — U	0.05
Malate Dehydrogenase	+ C + U	0.30
Peroxidase	+ C + U	0.20
Phenoloxidase	— C — U	0.30
Polyphenoloxidase	— C — U	0.10
Total Protein	+ C — U	0.25

\*— C = no  $CaCl_2$  added; + C =  $CaCl_2$  added; — U = 15% sucrose added; + U = 15% urea added to extracting solution.

Their relative effects on peroxidase and protein banding were compared, and DTT was found to be slightly superior to cysteine, which was far superior to mercaptoethanol.

Insoluble PVP, included as a phenol scavenger, was initially used at a rate of 1.5 g/100 ml. This was determined to be too low and was raised to 1 ml of hydrated PVP added to 1.2 ml extraction solution. An extraction solution pH of 8.3 was originally used but was altered to 7.3 when it was discovered that PVP was ineffective above pH 7.5 (38). Triton X-100 (a non-ionic detergent) was included to help wet the lyophilized powder and increase the solubility of some proteins. The final extraction solution then contained:

DTT	0.05 g
$K_2S_2O_5$	0.25 g
Ascorbic acid	0.50 g
Triton X-100	2 ml of 10%
Tris	pH to 7.3
Water	to 50 ml

1 ml of hydrated PVP<sup>3</sup> per 1.2 ml extraction solution was added at the time of extraction.

Extracting with urea (24, 26) and/or a high ionic strength solution (8, 12, 36, 70) has been shown to improve results with some enzyme systems. Urea cannot be included in systems utilizing PVP as a phenol scavenger, however, because the PVP-phenol bond is an H-bond, which urea effectively disassociates. In this study, where urea was used, it was added to the supernate following separation of the PVP by centrifugation. To increase the ionic strength of the solution, 0.5 M  $CaCl_2$  was used. Its addition to the complete extraction solution caused precipitation of an undetermined compound. By stepwise addition of the individual components,  $K_2S_2O_5$  was found to be causing the precipitation and was eliminated whenever  $CaCl_2$  was used. A factorial experiment using all combinations of + or — 15% urea and + or — 0.5 M  $CaCl_2$  was run for each enzyme studied to determine which combination produced the best results for each particular enzyme. When urea was not included, solutions were made to 15% sucrose. Results of this experiment are presented in Table 1 and Figure 1.

Several tissue:extraction solution ratios were evaluated. If the ratio was too low, samples were dilute and needed to be concentrated prior to application to the gel. If the ratio was too high, much of the protein was not extracted. A ratio which proved to be satisfactory for all the enzymes in this study was 0.1 g acetone powder to 1.2 ml extraction solution to

<sup>3</sup>PVP is hydrated with demineralized double distilled water and stored at 4° C. Just prior to use, required volumes are rinsed with extraction solution.

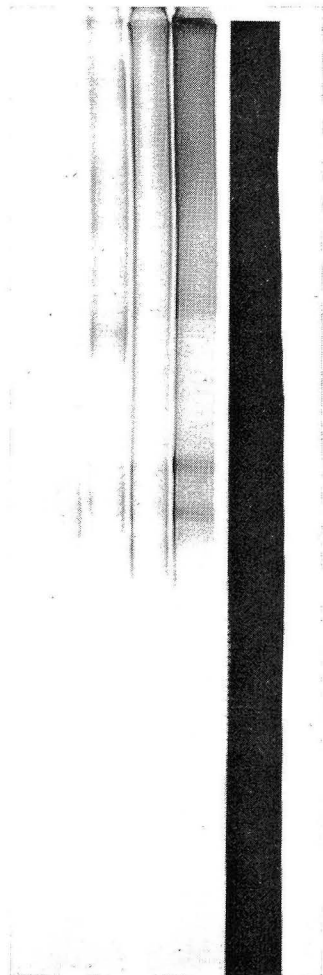


FIG. 1.—Inhibition of esterase by  $\text{CaCl}_2$  and/or urea. Sample extraction solutions were (left to right): +C+U, —C+U, +C—U, and —C—U with respect to  $\text{CaCl}_2$  and urea.

1 ml hydrated PVP. This ratio produced a sample which could be applied to the gels without concentration for all enzymes included in the study. Application levels found most suitable for each individual enzyme are given in Table 1.

Extraction times of 1 hour, 6 hours, and approximately 16 hours (overnight) were compared and found to produce similar results with peroxidase and total protein. For convenience, the 1-hour extraction was used throughout the experiment, and samples were used immediately after preparation.

The systems selected for investigation included total protein and 16 enzyme systems which had been used in other electrophoretic studies of plant tissue. Peroxidase and total protein were used while screening extraction techniques because they have been extensively studied and numerous references were available for both systems. All enzyme systems were evaluated on their ability to yield clear, reproducible banding patterns, with several staining techniques tried if the first failed (Table 2). Ten of the enzyme systems were eliminated from the study because of the absence of banding, or in the case of ascorbic acid oxidase, band instability. Of the seven remaining systems, cytochrome oxidase, phenol oxidase, and polyphenoloxidase were stained exactly as described by Hare (26). Since ascorbic acid strongly inhibits phenolases, it must be omitted from the extracting solution with these enzymes. Phenolases apparently are also inhibited or inactivated by 0.5 M  $\text{CaCl}_2$ , as there was no browning of sample precipitates extracted with  $\text{CaCl}_2$ , but rapid browning of precipitates extracted without  $\text{CaCl}_2$ .

TABLE 2.—Results of Staining Procedure Screenings.

Enzyme	Stain Reference	Results
Acid Phosphatase	8, 25, 53, 57	No staining; difficult to get substrate and stain into solution
Alcohol Dehydrogenase	26, 27, 35, 53	Enzyme present as a wide blur, no banding
Alkaline Phosphatase	8, 25, 53, 57	No staining
L-Amino Acid Oxidase	25, 26	No staining
Alpha Amylase	8, 25, 53	Enzyme present as a wide blur, no banding
Ascorbic Acid Oxidase	26	Good bands but fade too fast for consistent recording
Catalase	4, 8, 25, 26, 53	Enzyme present as a wide blur, no banding
Cytochrome Oxidase	26	Good banding
Esterase	26, 53	Good banding
Formazan Oxidase	26	No staining
Alpha Ketoglutaric Acid Dehydrogenase	26, 27	Enzyme present as a wide blur, no banding
Malate Dehydrogenase	26, 53	Good banding
Peroxidase	8, 25, 26, 36, 37, 44, 53, 63	Good banding
Phenoloxidase	26	Good banding with some background staining
Phosphorylase	25, 31	No staining
Polyphenoloxidase	25, 26	Good banding
Total Protein	18, 39, 56, 71	Good banding with considerable background staining



The final esterase and malate dehydrogenase staining methods were combinations of those developed by Scandalios (53) and Hare (26).

**Esterase:** After running gels were placed in a pH 6.2, 0.1 M Na phosphate buffer for 5 minutes. They were then placed in the substrate solution at 37° C until bands developed. The substrate solution contained 70 mg fast blue RR salt, dissolved in 100 ml buffer and filtered just prior to use, and 2 ml of alpha naphthyl acetate (1.0% in 1:1 acetone, water), which was added after filtration. Stained gels were washed and fixed in 10% trichloroacetic acid (TCA).

**Malate Dehydrogenase:** **Solution A:** 25 mg nitroblue tetrazolium (NBT) (dissolved in a minimum amount of ethanol), 80 mg MgSO<sub>4</sub> • 7H<sub>2</sub>O, and 2 mg KCN dissolved in 40 ml tris-HCl buffer pH 7.5, 0.1 M. **Solution B:** 2.5 mg phenazine methosulfate (PMS) dissolved in 40 ml buffer. Just before use, the solutions were mixed in dim light, 0.548 g dl-malic acid (0.05 M) was added, the pH was adjusted to 7.5 with NaOH and 56 mg nicotinamide adenine dinucleotide (NAD) was added (the pH must be adjusted before addition of the NAD). Gels were incubated in this solution in the dark at 37° C until bands developed, then washed and fixed in 2% acetic acid.

Peroxidase staining techniques are far more complex, mainly because peroxidase is not a specific enzyme but instead is a class of enzymes, which oxidize a wide range of substances (53). Peroxidase isoenzymes can have large differences in their reactivity with substrates, and isoenzymes specific for one substrate may go undetected if another is used in the staining process (37). In this study benzidine and o-dianisidine were compared, with benzidine proving to be superior based on the number and clarity of bands produced. The H<sub>2</sub>O<sub>2</sub> concentration also significantly affects results even more than enzyme concentration (44). Novacky and Hampton (44) found 0.0003% H<sub>2</sub>O<sub>2</sub> to be too low, 0.03% too high, and 0.003% optimum. Other recommendations are given by Scandalios (53), 1.5%; Lee (36) and Hare (26), 0.03%; and Hamaker and Snyder (25), 0.015% H<sub>2</sub>O<sub>2</sub>.

A series of concentrations ranging from 0.003% to 1.5% H<sub>2</sub>O<sub>2</sub> were evaluated, with 0.03% providing the best results. Stain solutions may be acidified with acetic acid (53) or buffered (25, 36). Lee (36) evaluated the effects of acetate, phosphate, and tris buffers on peroxidase staining and found the use of a pH 4.5 acetate buffer resulted in maximum peroxidase activity. Without proper treatment following staining, peroxidase bands will fade or disappear (8, 36, 63). Lee (36) increased peroxidase stain stability by immersing the gels in 7% acetic acid im-

mediately after staining and rinsing them in distilled water.

The final peroxidase staining method used in this study consisted of incubating gels in a 0.5% benzidine and 0.03% H<sub>2</sub>O<sub>2</sub> solution until bands were well developed, transferring them to 7% acetic acid for 5 minutes, and rinsing and holding in distilled water. The stain solution was prepared by dissolving 0.5 g benzidine in 4.5 ml of acetic acid, bringing it to 100 ml with demineralized double distilled water, and adding 0.1 ml 30% H<sub>2</sub>O<sub>2</sub> just prior to use.

Amido Schwarz and Coomassie Brilliant Blue were both used to stain gels for total protein. As previously reported (71), Coomassie Brilliant Blue again proved superior. The staining solution used contained 0.025% Coomassie Brilliant Blue, 10% acetic acid, and 25% methanol. After staining for 12 hours, gels were destained in 7.5% acetic acid and 50% methanol in a diffusion destainer, then rehydrated and stored in distilled water.

In recording results, each band was identified by its R<sub>f</sub> value and density.

$$R_f = \frac{\text{distance the enzyme band migrated}}{\text{distance the marker dye migrated}}$$

Density = light (L), medium (M), or heavy (H)

Natarella and Sink (42) found the marker dye completely disappeared in peroxidase stain solution, so gels were cut with a razor blade at the marker dye prior to staining. Photographs were taken of all gels, but as reported by Hare (26) and Clements (15), seldom showed all visible bands. Therefore, zymograms were drawn based on the average R<sub>f</sub> values and density recordings of at least four gels. Variation of R<sub>f</sub>'s was determined by running 12 subsamples from each of four Climbing Peace extractions and staining for peroxidase. Peroxidase and esterase gels were scanned for optical density on a Gilford 240 densitometer with linear transport at 410 and 360 nm respectively.

By applying these methods to the seven enzyme systems which produced good banding, four areas were investigated:

- *Effect of leaf position.* First and second, third and fourth, and fifth and sixth leaves below an unopened flower bud between 0.6 and 2 cm diameter were grouped and sampled. Climbing Peace did not form flower buds, so leaves below a vegetative bud had to be sampled.

- *Stability in storage.* Lyophilized acetone powders stored at -20° C were extracted and run after 0, 9, 12, and 16 weeks of storage.

- *Within cultivar variation.* Individual plants within cultivars were sampled and compared.

• *Cultivar variation.* Banding patterns of the eight cultivars were compared.

Coefficients of similarity of the eight rose cultivars were obtained by dividing the number of bands in common by the total number of bands present in the cultivars being compared and multiplying by 100.

## RESULTS AND DISCUSSION

### Variance

All  $R_f$  values are averages of the  $R_f$ 's representing a particular isoenzyme and cultivar. The overall variance of these values was 0.011, with the variance of specific bands ranging from 0.008 to 0.014. Where

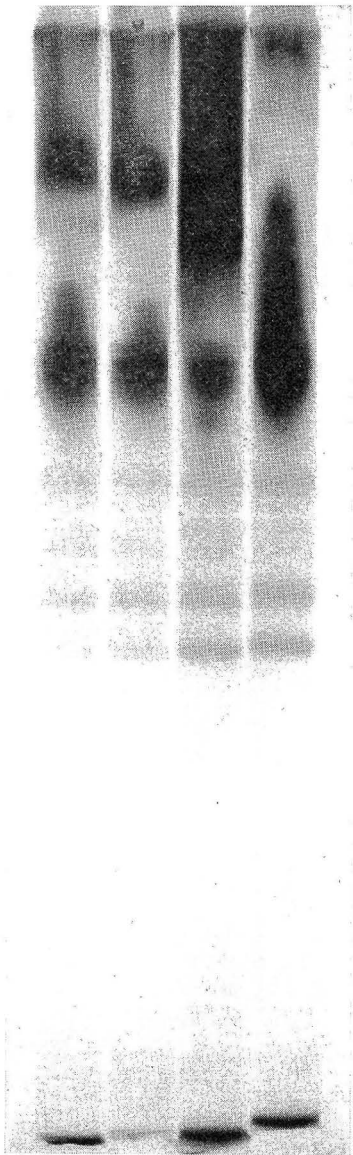


FIG. 2.—Peroxidase isoenzymes obtained from two separate samples applied to two gels each. Banding patterns on the duplicate gels were identical with one sample (left two gels), but blurred and inconsistent below  $R_f$  0.27 with the other (right two gels).

broad bands are present, averages were calculated for both the upper and lower boundaries. As in the study of Payne and Fairbrothers (47), if both the upper and lower mean  $R_f$  values of a band from one cultivar overlapped either the upper or lower mean  $R_f$  value of a band from another cultivar, the bands were judged to be identical.

The density value assigned was that which occurred most frequently within the four replications of the isoenzyme and cultivar. If two densities occurred with equal frequency, the isoenzyme was assigned the density of that isoenzyme in the majority of the other cultivars. Variance in band density was only from light to medium or medium to heavy. In no case did band density range from light to heavy within the replications of an isoenzyme of a cultivar.

Inconsistent banding (and blurring) occurred in the upper regions of gels stained for peroxidase (Fig. 2), cytochrome oxidase, and polyphenoloxidase. This problem was probably caused by aggregation of isoenzymes, resulting in larger molecules with lower mobility but still maintaining specific catalytic activity. For peroxidase, cytochrome oxidase, and polyphenoloxidase, all bands with mobilities less than 0.27, 0.24, and 0.13, respectively, were disregarded. Bands with higher mobilities were unaffected and remained consistent.

### Effects of Leaf Position

The eight cultivars studied can be divided into three groups by growth habit. Climbing Peace grows very fast and produces flower buds only after a per-

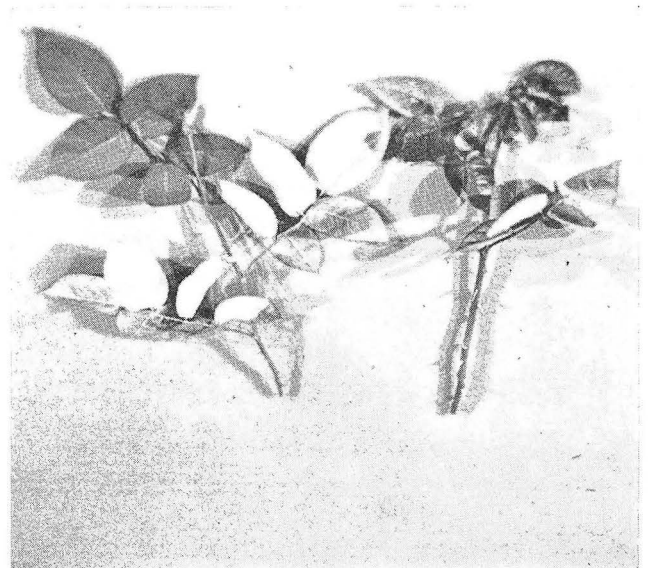


FIG. 3.—Stems of Candy Stripe (left) and Chicago Peace (right) containing flower buds 1 cm in diameter. Note the advanced stage of leaf development on Candy Stripe compared to Chicago Peace.

iod of dormancy. No flower buds formed during the course of the experiment. Pink Peace and Candy Stripe grow fast and flower regularly. All leaves on a stem of Pink Peace or Candy Stripe develop before the flower bud and are fully developed before the flower bud is 0.6 cm in diameter (Fig. 3). Plants of the other cultivars flower regularly but grow slower. Their leaves develop at the same time the flower bud is developing, and on these plants a 0.6 cm flower bud is surrounded by partially developed leaves (Fig. 3).

Because of these differences in growth habit, the first and second leaves below a flower bud 0.6 cm in diameter are much more physiologically and morphologically developed in Pink Peace and Candy Stripe

than on other cultivars. This means leaf position should not be the only criterion on which to base a sampling standard. For example, when staining for peroxidase, leaf position had no effect on results with Candy Stripe (Fig. 4), but with Peace results were greatly affected. Samples prepared from first and second leaves produced much lighter bands than samples prepared from third and fourth or fifth and sixth leaves (Fig. 5). Apparently, peroxidase synthesis increases as the leaves develop and levels off when the leaves become fully expanded. This is in agreement with the observations of Wise and Morrison (76),

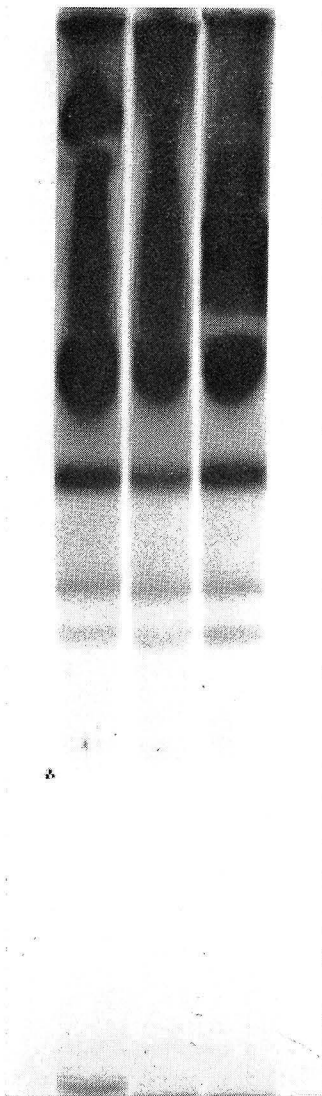


FIG. 4.—Effect of leaf position on peroxidase banding patterns. Samples were prepared from (left to right): first and second, third and fourth, and fifth and sixth leaves below an unopened flower bud on a Candy Stripe rose.

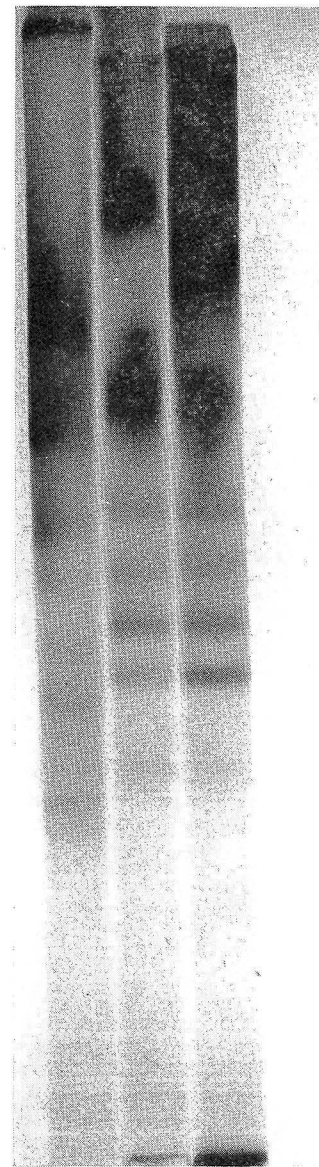
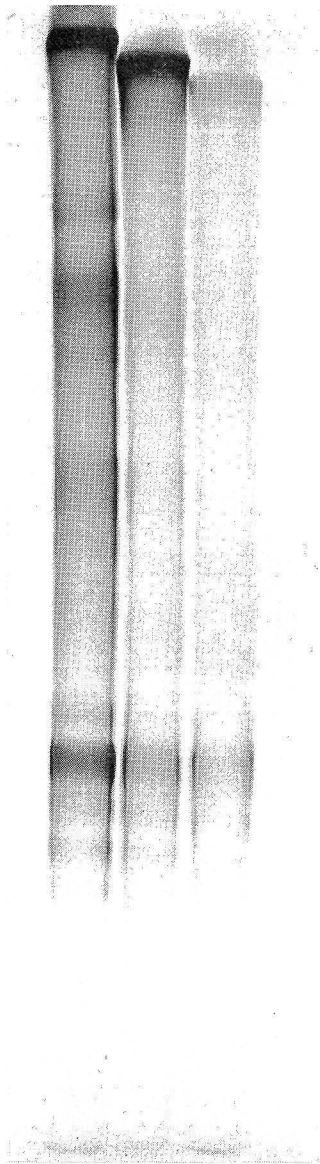
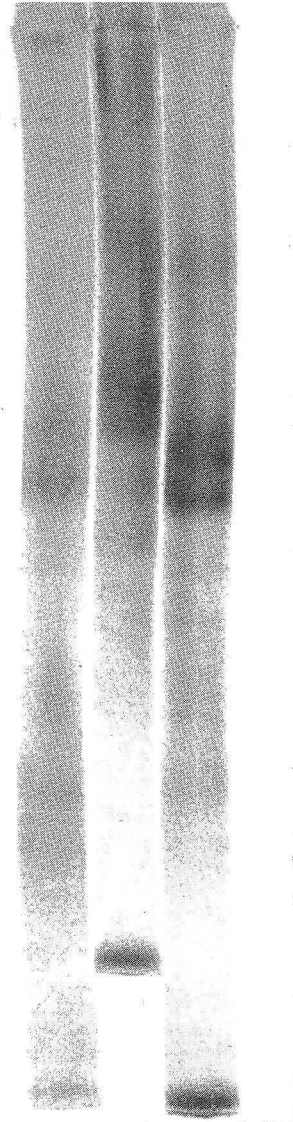


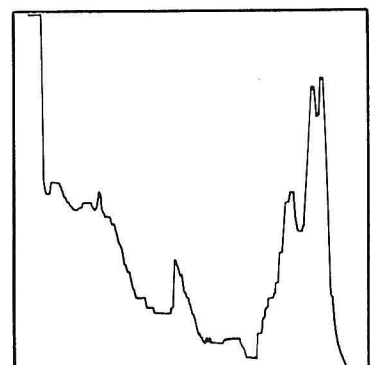
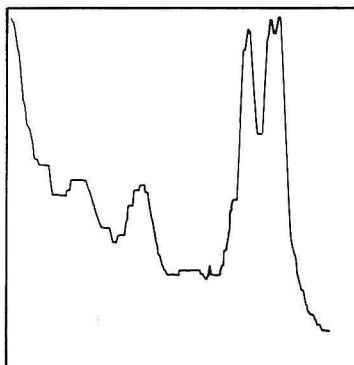
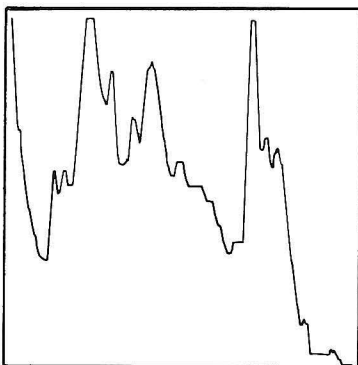
FIG. 5.—Effect of leaf position on peroxidase banding patterns. Samples were prepared from (left to right): first and second, third and fourth, and fifth and sixth leaves below an unopened flower bud on a Peace rose.



**FIG. 8.**—Effect of leaf position on cytochrome oxidase banding patterns. Samples were prepared from (left to right): first and second, third and fourth, and fifth and sixth leaves below an unopened flower bud on a Peace rose.



**FIG. 6.**—Effect of leaf position on esterase banding patterns. Samples were prepared from (left to right): first and second, third and fourth, and fifth and sixth leaves below an unopened flower bud on a Pink Peace rose.



**FIG. 7.**—Effect of leaf position on esterase banding patterns. Densitometer tracings are of gels on which were run samples prepared from (left to right): first and second, third and fourth, and fifth and sixth leaves below an unopened flower bud on a Peace rose.

who found an increase in peroxidase activity and the number of isoenzymes with an increase in the age of cotton leaves.

The exact opposite is true for esterase. The number and density of esterase isoenzymes decreases greatly from first and second to third and fourth leaves, and slightly from third and fourth to fifth and sixth leaves (Fig. 6). Esterase synthesis apparently decreases as the leaves mature, and the densitometer scans show that a change in the relative amounts of the different isoenzymes also occurred (Fig. 7). The change is from a high concentration of slow migrating isoenzymes to a high concentration of fast migrating isoenzymes. Two possible explanations are that the large, slow isoenzymes are segregating into smaller subunits which maintain catalytic activity; or since esterase is a non-specific stain (9), totally different isoenzymes may be present at the different leaf positions (55). Tissue specific isoenzymes of peroxidase and esterase have been recorded (22, 76), so it is not surprising to find this variation between different stages of development of the same tissue.

The other five systems evaluated fall into the same two categories but with less dramatic differences. First and second leaves yield slightly better results than the other positions when staining for phenoloxidase or polyphenoloxidase, while leaves in the third and fourth or fifth and sixth positions yield better results with total protein, malate dehydrogenase, and cytochrome oxidase (Fig. 8).

The complications of selecting a uniform sampling procedure are thus evident. Leaves at identical positions may be at different developmental stages on different plants, and different enzyme systems are

most active at different stages of development. To select the best sample, the enzyme to be studied and the growth habit of the plant being sampled must be considered. In no case were results from fifth and sixth leaves superior to those from third and fourth leaves, so in future studies with roses, samples can be limited to the youngest four leaves.

#### Stability in Storage

**Total Protein.** After 9 weeks' storage, one light band was lost, three medium bands faded to light bands, and the mobility of the fastest band decreased slightly (Fig. 9). After 12 weeks, another light band was lost. After 16 weeks, four more bands faded one density level and another changed from a sharp band to a blurred band.

Although there were few qualitative changes after 12 weeks' storage, samples to be stained for total protein should be compared only with others stored the same length of time under similar conditions because of the possibility of losing light bands during storage. If the relative density of individual bands is to be accurately measured for use in distinguishing cultivars, fresh material should be used because of the fading which occurs.

**Peroxidase.** Peroxidase is extremely stable in storage, and even after 16 weeks the only change recorded was one medium band faded to light (Fig. 10). This means fresh samples can be compared with stored samples and the density of isoenzymes of stored samples can be quantified and used in comparisons. Liu (37) has reported that the electrophoretic mobility of peroxidase isoenzymes can be altered considerably with no loss of activity by storage at 4° C at pH 7.0 or higher. Apparently, the

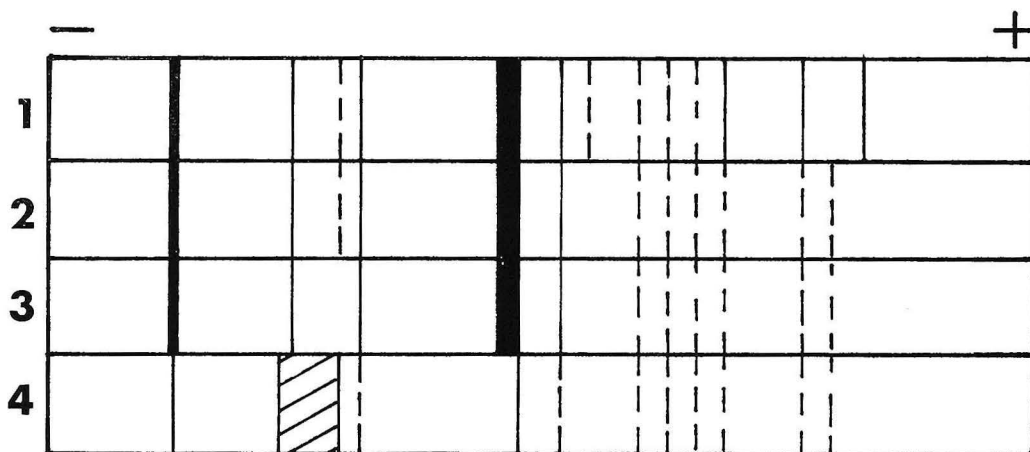


FIG. 9.—Effects of 0(1), 9(2), 12(3), and 16(4) weeks' storage as lyophilized acetone powders at  $-20^{\circ}$  C on the banding patterns of the anionic proteins of Candy Stripe rose leaves. Dashed lines and dotted areas indicate lightly stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

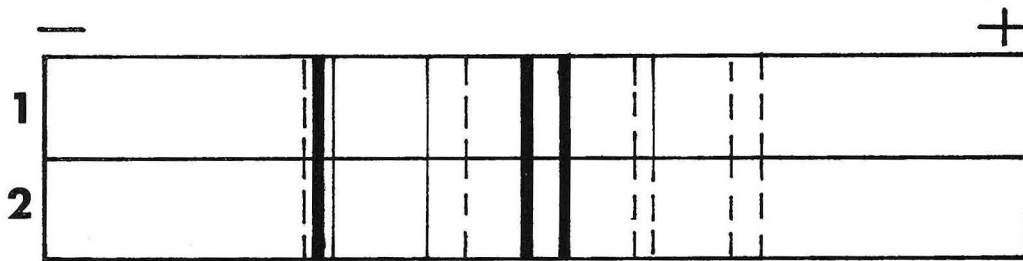


FIG. 10.—Effects of 0(1) and 16(2) weeks' storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the peroxidase isoenzymes of Peace rose leaves. Dashed lines indicate light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

lyophilized acetone powder is a more stable storage form for peroxidase.

**Esterase.** After 12 weeks' storage, one light band was lost and three medium bands faded to light (Fig. 11). Data for 9 and 16 weeks are unavailable because of sampling error. As with total protein, samples to be stained for esterase can be stored for 12 weeks, but stored samples should be compared with those stored an equal length of time, and fresh samples should be used if bands are to be quantified by densitometry.

**Malate Dehydrogenase.** Two light bands are lost during the first 9 weeks of storage, but all others are stable with respect to both mobility and density for at least 16 weeks (Fig. 12). The instability of the two lost bands relative to the stability of the others suggests they should not be used in cultivar comparisons. The other bands can be used freely, even when comparing fresh samples with samples stored 16 weeks.

**Cytochrome Oxidase.** After 9 weeks' storage, one light band is lost; after 12 weeks, two additional

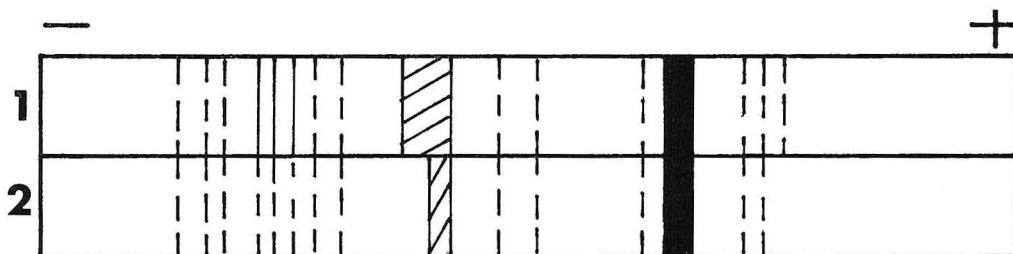


FIG. 11.—Effects of 0(1) and 12(2) weeks' storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the esterase isoenzymes of Candy Stripe rose leaves. Dashed lines indicate light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

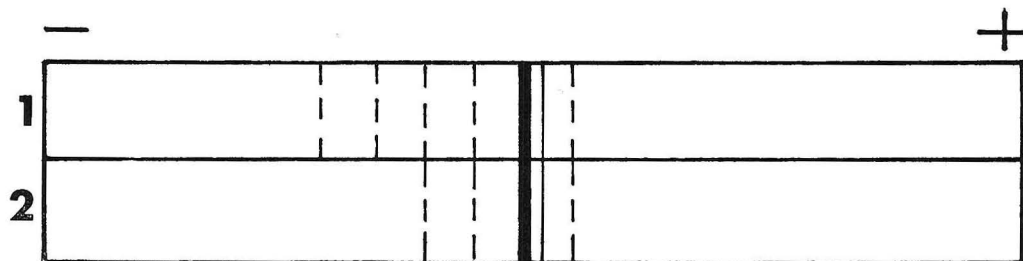


FIG. 12.—Effects of 0(1) and 16(2) weeks' storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the malate dehydrogenase isoenzymes of Candy Stripe rose leaves. Dashed lines indicate light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

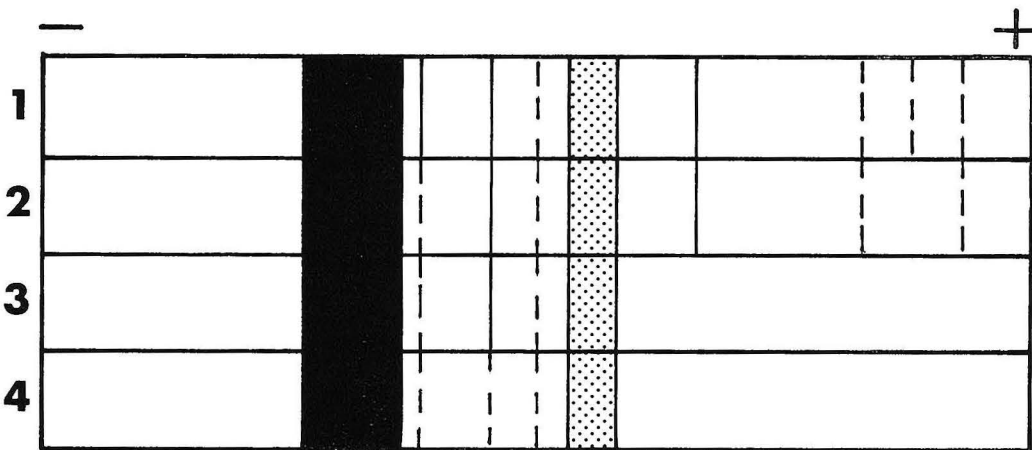


FIG. 13.—Effects of 0(1), 9(2), 12(3), and 16(4) weeks storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the cytochrome oxidase isoenzymes of Peace rose leaves. Dashed lines and dotted areas indicate light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

light bands and one medium band are lost (Fig. 13). The lost bands represent the fastest migrating isoenzymes while the slower isoenzymes maintain both their mobility and density for at least 16 weeks. Two possible explanations for this are the fast migrating isoenzymes are unstable and the slower migrating isoenzymes are subunits which aggregate during storage and form larger complexes which migrate at the same rate as the slower isoenzymes. The slower isoenzymes may in fact be aggregates of subunits, which with proper treatment could be split to yield more sharp individual bands. The proper treatment has not been developed in this study, but may include use of some denaturing agent, chelator, detergent, and/or adjustment of the ionic strength of the extraction solution. Samples to be stained for cytochrome oxidase can be stored for 16 weeks with no loss in density of bands with an  $R_f$  less than 0.60. To use all bands in a comparison, fresh material must be used.

**Phenoloxidase.** The only change after 9 weeks' storage was the change in density of one band from medium to light (Fig. 14). After 12 weeks the band is lost and one additional band changes from medium to light. There are no other changes after 16 weeks' storage. As with cytochrome oxidase, there are broad, heavy, slow migrating bands present which could possibly be split to yield additional bands.

The stability of all bands except the first, including light ones, suggests that samples to be stained for phenoloxidase can be stored for 16 weeks.

**Polyphenoloxidase.** There was a loss of one light band and a density decrease in five bands after 9 weeks' storage, but no more changes occurred up to 16 weeks (Fig. 15). Either changes to a stable state occur after a short period of storage, or the gels were overstained the first time. Whatever the reason, the same bands were present for 16 weeks with the exception of one light band, indicating that samples to be stained for polyphenoloxidase can be stored for 16

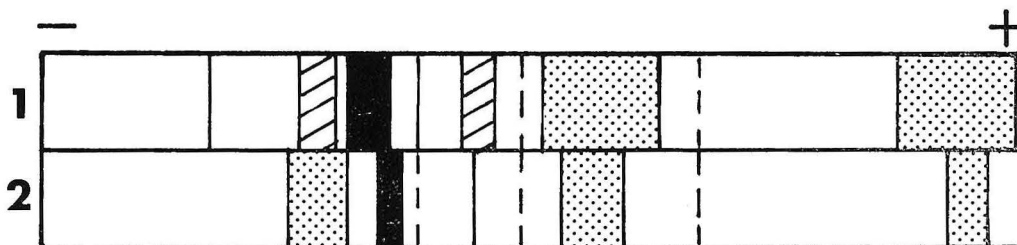


FIG. 14.—Effects of 0(1) and 16(2) weeks' storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the phenoloxidase isoenzymes of Peace rose leaves. Dashed lines and dotted areas indicate light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

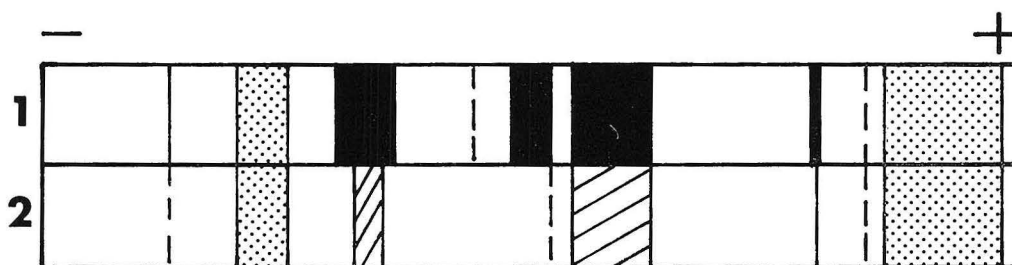


FIG. 15.—Effects of 0(1) and 16(2) weeks' storage as lyophilized acetone powders at  $-20^{\circ}\text{C}$  on the polyphenoloxidase isoenzymes of Chicago Peace rose leaves. All changes occurred during the first 9 weeks of storage. Dashed lines and dotted areas indicated light stained bands, light solid lines indicate medium stained bands, and heavy shading indicates dark stained bands.

weeks. However, if band densities are to be quantitatively compared, additional storage studies should be initiated.

According to Wilkinson (74), it is generally better to use fresh material for the study of the relative distribution of the various forms of an enzyme, since certain isoenzymes are frequently less stable than others. Also, some enzymes are stable at room temperature and unstable at  $4^{\circ}$  or  $-20^{\circ}\text{C}$ , while others are exactly the opposite. The enzymes included in this study may all be used after storage, but within the limitations described.

#### Within Cultivar Variation

No variation was found in the banding patterns of plants of the same cultivar. If differences had been found, new samplings, extraction, storage, and/or running conditions which produced results with no intra-cultivar variation would have had to be developed. Taxonomic characters must be consistent within the groups to be separated.

#### Cultivar Variation

Banding data for total protein and each enzyme system are presented in figures and used to prepare flow charts showing the cultivar separations which can be made based on the band differences. Separations will first be made on the basis of presence or absence of a band or bands. If all cultivars cannot be separated on this basis, differences in density of common bands will be compared. Density comparisons cannot be used as a means of identification without considerable refinement of the recording technique used in this study, but are included to illustrate their potential use. Wehner *et al.* (72) have successfully used relative densities of peroxidase bands to separate bluegrass cultivars. Sneath and Sokal (61) suggest weighting characters in numerical taxonomy to emphasize those characters which are most effective in distinguishing between previously established taxa. Weighting must take into account the ease of observ-

ing or measuring the given characters. In studies where bands and densities are to be compared, the presence or absence of a band could be weighted higher than density differences. To emphasize the difference between the quality of the comparisons, a dotted line will divide separations based on band presence from those based on band density.

**Total Protein.** The anionic protein bands of the eight cultivars are presented in Figure 16 and used for cultivar separation in a flow chart in Figure 17. Cultivars which cannot be distinguished from each other by differences in band presence are Peace, Chicago Peace, and Sonia; and Flaming Peace and Climbing Peace. Considering the taxonomic distance in relationship between Peace and Sonia and Chicago Peace and Sonia, they should have been distinguishable. That they were not suggests protein banding patterns may not be specific enough to be used alone in cultivar identification. A much wider sampling of rose cultivars is necessary to prove or disprove this hypothesis.

By including differences in band density in the comparisons, all cultivars could be separated except Peace and its sport Chicago Peace. However, separations based on density values are especially questionable when applied to the protein bands in this study because the considerable background staining present made density evaluations more difficult than with the other systems.

**Peroxidase.** The peroxidase banding patterns of the eight cultivars are presented in Figure 18 and used for cultivar separation in a flow chart in Figure 19. Peace, Chicago Peace, and Flaming Peace are indistinguishable and separated from Climbing Peace by only one light band. Pink Peace and Candy Stripe are easily separated from the other cultivars, but are distinguishable from each other only by comparing band densities. Forever Yours and Sonia each have unique banding patterns.

Peroxidase is an excellent enzyme for use in taxonomic studies because of its stability during extraction



Band #	R <sub>f</sub>	Density	CULTIVAR								
			1	2	3	4	5	6	7	8	
1	.13	H	+	+	+	+	+	+	+	+	
2	.25	M	+	+	+	+			+	+	+
3	.30	L				+	+	+	+	+	
4	.32	M	+	+	+	+	+	+	+	+	
5	.40	M								+	
6	.46-.48	H	+	+	+	+	+	+	+	+	
7	.52	M	+	+	+	+	+	+	+	+	
8	.55	L	+	+	+	+	+	+	+	+	
9	.60	L	+	+	+	+	+	+	+	+	
10	.63	L	+	+	+	+	+	+	+	+	
11	.66	L	+	+	+	+	+	+	+	M	
12	.69	M	+	+	L	+	+	+	+	+	
13	.77	M	+	+	+	+	+	+	+	+	
14	.83	M	+	+	+	+	+	+	H	H	
15	.87	L	+	+	+	+				+	+

FIG. 16.—Comparison of the protein bands of Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8). A + indicates the isoenzyme is present at the specified density, a blank space indicates it is absent, and a letter indicates it is present at a different density.

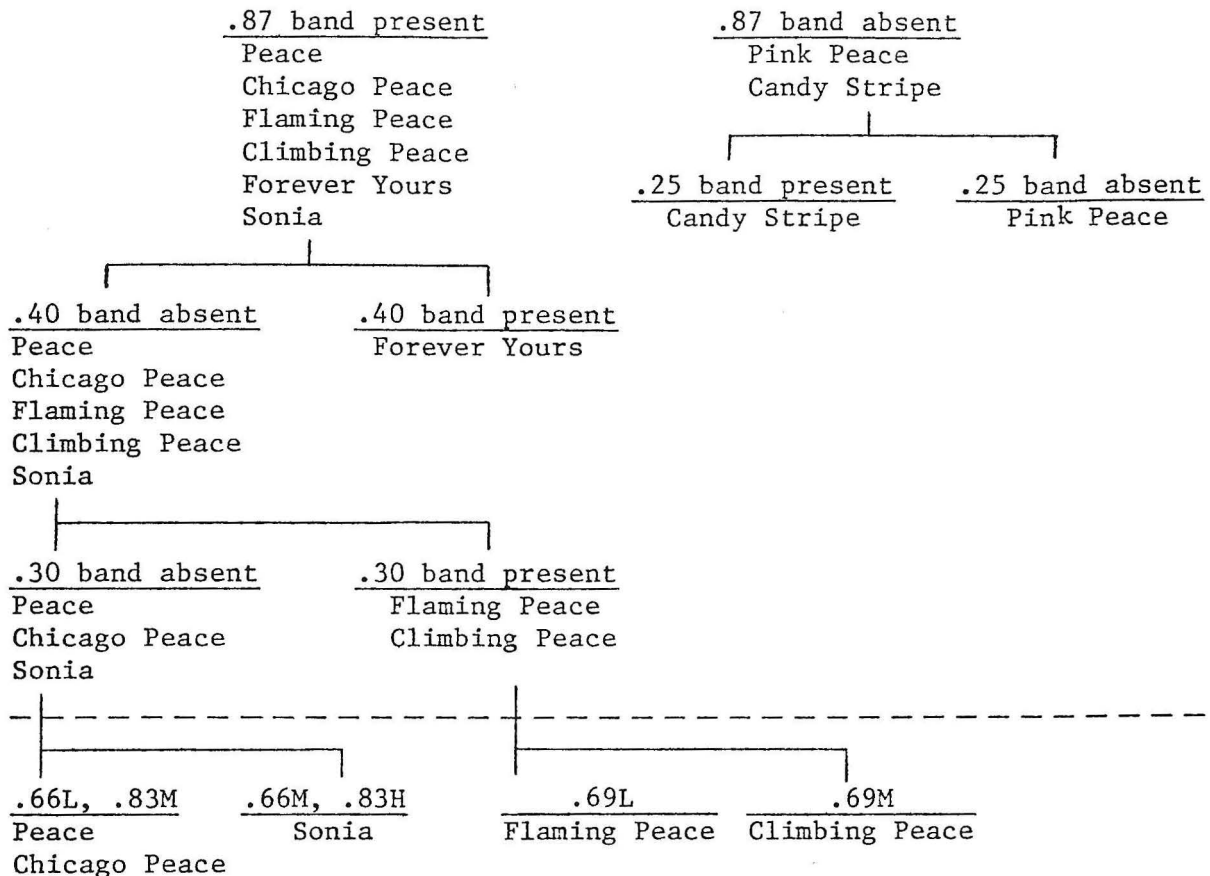


FIG. 17.—A flow chart separating eight rose cultivars on the basis of their anionic protein banding patterns. Separations above the dashed line are based on band presence or absence. Separations below the line are based on density differences of common bands.

Band #	R <sub>f</sub>	Density	CULTIVAR							
			1	2	3	4	5	6	7	8
1	.27	L	+	+	+	M	+	+	H	+
2	.28	H	+	+	+	+	+	+		+
3	.29	M	+	+	+	H	+	H		+
4	.39	M	+	+	+	H	+	H		
5	.44	L	+	+	+	+	+	+		
6	.49	H	+	+	+	+	+	+	L	
7	.53	H	+	+	+	+	+	+	L	
8	.56	L					+	+	+	+
9	.57	L								+
10	.60	L	+	+	+	+	+	+	+	+
11	.62	M	+	+	+	+	L	L		L
12	.64	L								+
13	.70	L	+	+	+					
14	.70-.76	M							+	+
15	.73	L	+	+	+	+				

CULTIVAR  
1 2 3 4 5 6

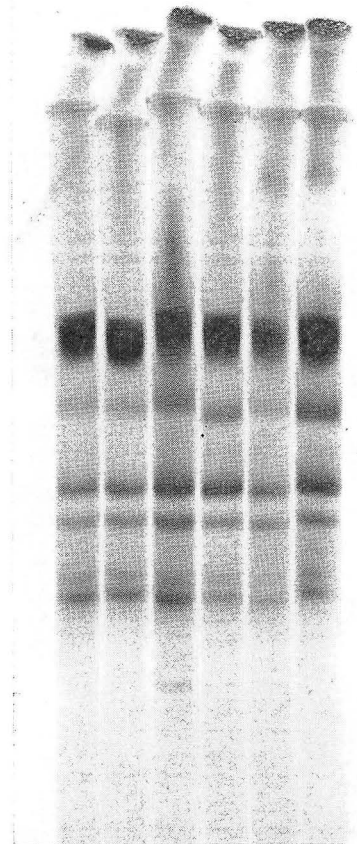
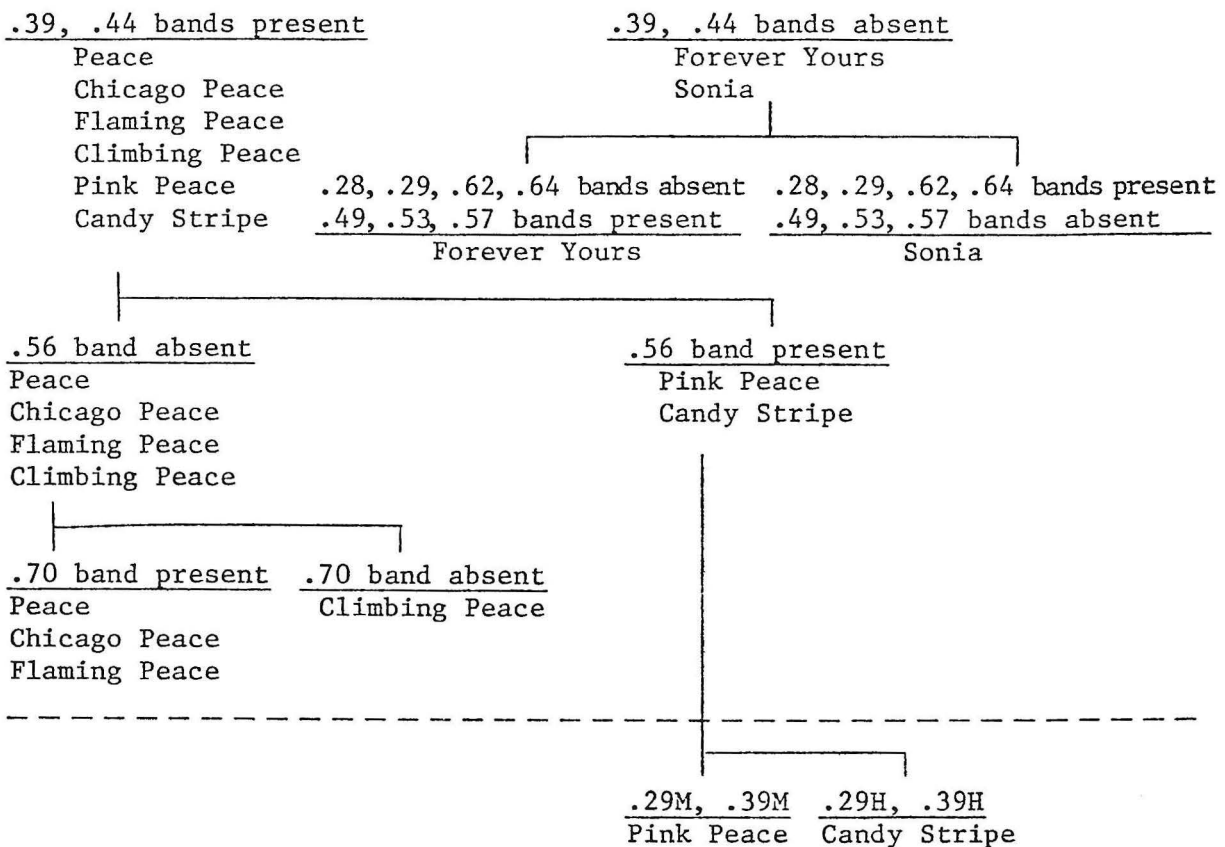


FIG. 18.—Comparison of the peroxidase isoenzymes (each band represents an isoenzyme) of Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8). A + indicates the isoenzyme is present at the specified density, a blank space indicates it is absent, and a letter indicates it is present at a different density. Not all bands can be seen in the photo, but obvious similarities and differences are evident.

FIG. 19.—A flow chart separating eight rose cultivars on the basis of their peroxidase banding patterns. Separations above the dashed lines are based on band presence or absence. Separations below the line are based on density differences of common bands.



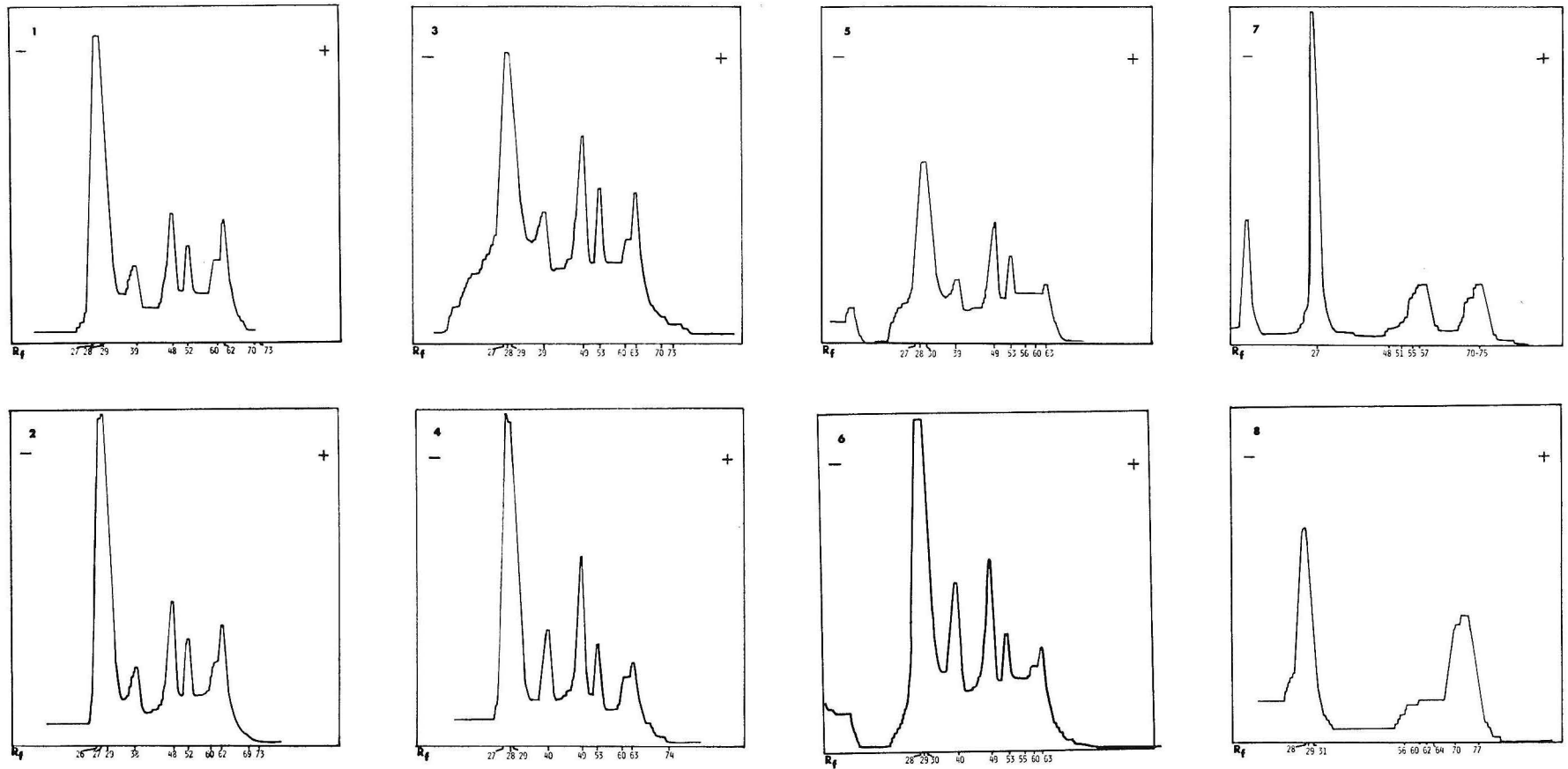


FIG. 20.—Densitometer tracings of gels stained for peroxidase and representing Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8).  $R_f$  values listed are those which were visually recorded.

and storage and the distinct, reproducible bands produced. Also, by adjusting the extraction procedure and/or staining solution, different banding patterns may be obtained (36, 37, 44), greatly increasing the amount of data on which cultivar comparisons can be made.

The densitometer scans did not prove to be useful in this study for, as in the work of van Loon and van Kammen (68), more bands could be detected by visual inspection of the gels because narrow bands lying side by side could not be resolved by the densitometer. Gordon (24) suggests that a densitometer with a very narrow beam and rapid response can make this separation. Scans obtained in this study (Fig. 20) did not separate close bands and did not distinguish the high mobility light bands from the background stain.

Although the relative densities indicated by the peak heights were very consistent for the run represented by Figure 20, variations were common in other runs. To use density comparisons in a study, sample protein content and enzyme activity must be strictly monitored and staining procedures must be perfectly uniform. Because of limited objectives, this was not done in this study.

**Esterase.** The esterase banding patterns of the eight cultivars are presented in Figure 21 and used for cultivar separation in a flow chart in Figure 22. Forever Yours and Sonia are readily distinguished from the Peace cultivars and each other. Pink Peace and Candy Stripe are separated from Peace and its sports by differences in four bands, but are virtually identical to each other with a slight density difference occurring in only one band. Peace, Chicago Peace, and Flaming Peace are identical and separated from Climbing Peace only by the density value of one band.

Esterase is another excellent enzyme for use in taxonomic studies. Although it is not as stable as peroxidase, it yielded many distinct bands with little background staining. Also, the bands are stable and can be analyzed on a densitometer up to several weeks after staining. Cultivar differences can be seen in the densitometer scans of gels stained for esterase (Fig. 23). As with peroxidase, however, visual recording was more effective in locating bands, and density comparisons can be made only if the previously mentioned procedures are followed prior to and during staining.

**Malate Dehydrogenase.** The malate dehydrogenase banding patterns of the eight cultivars are presented in Figure 24 and used for cultivar separation in a flow chart in Figure 25. Sonia, the Grandiflora type, can be distinguished from the Hybrid Teas by the absence of three bands present in all the Hybrid

Teas. Climbing Peace and Pink Peace can be separated from the other Hybrid Teas by the absence of one band and from each other in that they are not missing the same band. Peace, Chicago Peace, Flaming Peace, Candy Stripe, and Forever Yours are indistinguishable unless band densities are considered. Even then Peace, Chicago Peace, and Flaming Peace are identical, but separated from Candy Stripe and Forever Yours which are identical to each other. The only difference between these five cultivars is the density ratio of two successive bands at  $R_f$ 's .49 and .51. These bands had a high/medium density ratio for Candy Stripe and Forever Yours, and a medium/high ratio for the three remaining cultivars.

This is a prime example of how sensitive density comparisons can be and the importance of standardizing all operations in an electrophoretic study. Also, as with the total protein system, cultivars which are not closely related, like Candy Stripe and Forever Yours, could not be distinguished from each other. Therefore, a much wider selection of rose cultivars must be sampled before the usefulness of malate dehydrogenase in chemotaxonomic studies can be established.

**Cytochrome Oxidase.** The cytochrome oxidase banding patterns of the eight cultivars are presented in Figure 26 and used for cultivar separation in a flow chart in Figure 27. Comparisons within this system lead first to separation into their natural groups, then from each other. Forever Yours and Sonia were distinguished from the Peace cultivars by the absence of one band, then from each other by differences in three bands. Pink Peace and its sport, Candy Stripe, were separated from Peace and its sports by differences in two bands and from each other by one band. There were two band differences between Peace and Flaming Peace, and Chicago Peace and Climbing Peace. Peace and Flaming Peace could be distinguished by a band difference, but Climbing Peace and Chicago Peace could be separated only by density differences.

The fact that all eight cultivars could readily be divided into their natural groups on the basis of the banding differences of this one enzyme system suggests it may be useful in chemotaxonomic investigations.

**Phenoloxidase.** The phenoloxidase banding patterns of the eight cultivars are presented in Figure 28 and used for cultivar separation in a flow chart in Figure 29. Forever Yours and Sonia each have banding patterns which distinguish them from the Peace cultivars and each other. Pink Peace and its sport, Candy Stripe, possess three bands not present in Peace and its sports but cannot be separated from each other without relying on the density differences



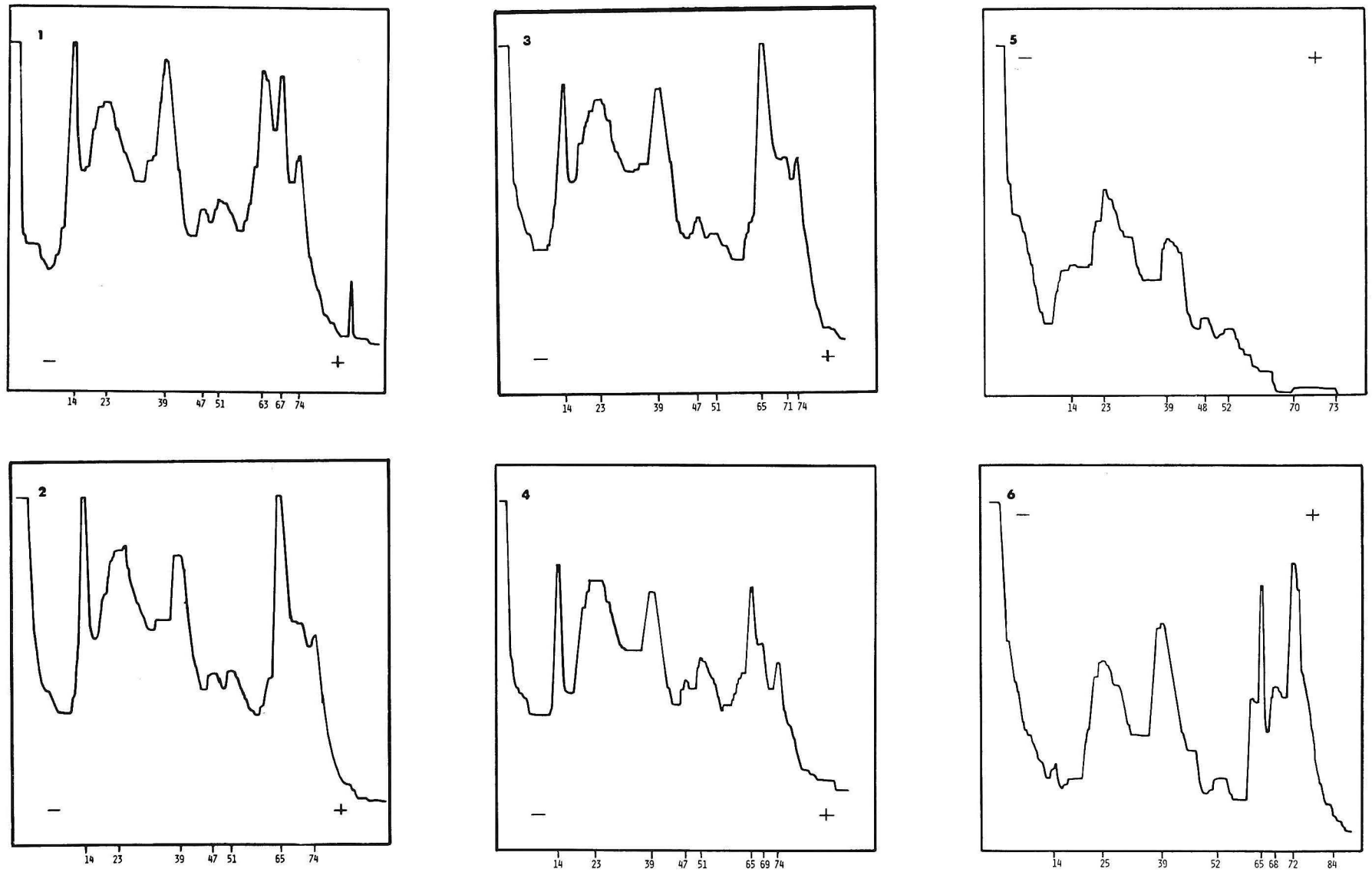


FIG. 23.—Densitometer tracings of gels stained for esterase and representing Peace (1), Chicago Peace (2), Climbing Peace (3), Pink Peace (4), Forever Yours (5), and Sonia (6).  $R_f$  values listed are those of the peaks which can be distinguished.



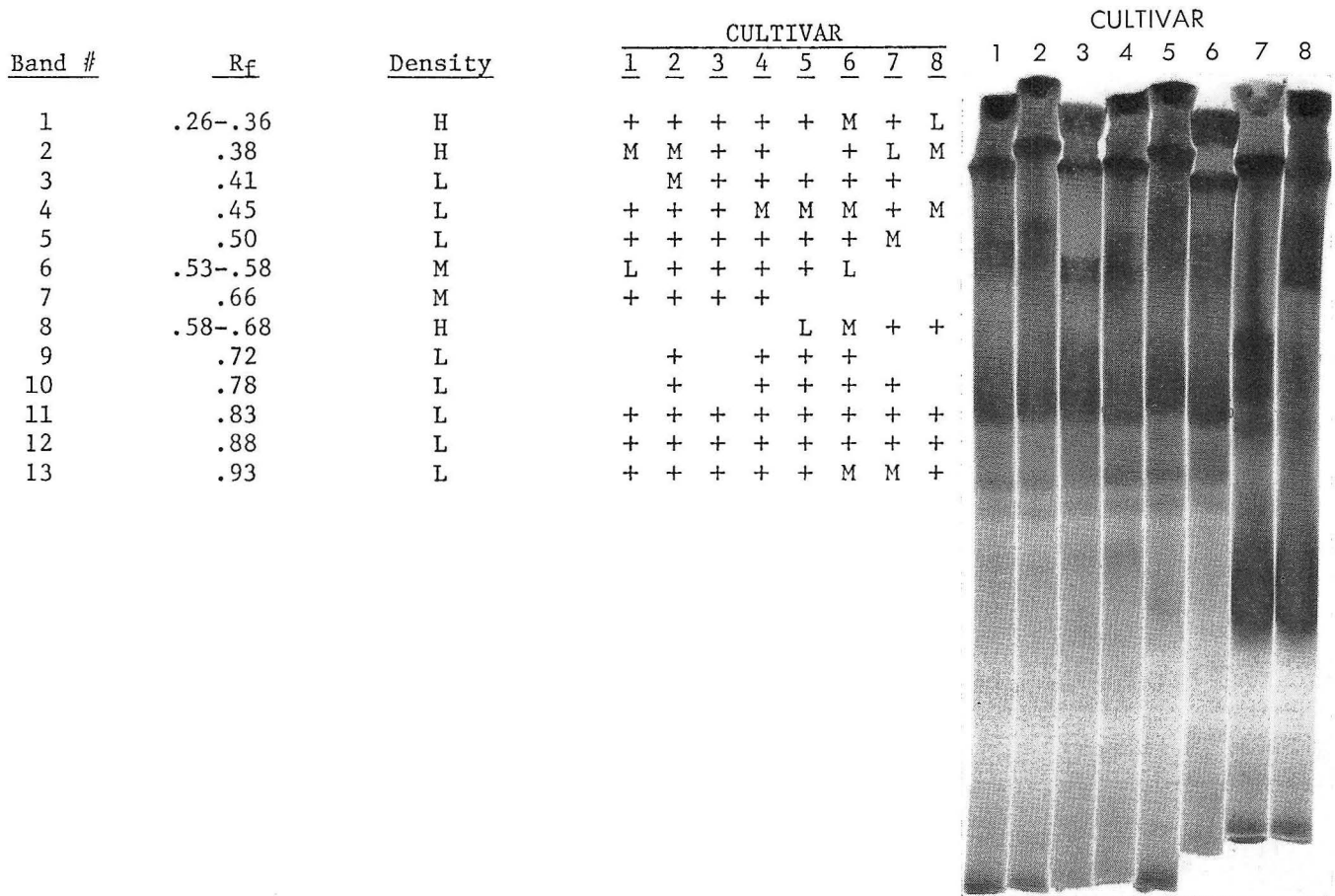


FIG. 26.—Comparison of the cytochrome oxidase isoenzymes (each band represents an isoenzyme) of Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8). A + indicates the isoenzyme is present at the specified density, a blank space indicates it is absent, and a letter indicates it is present at a different density. Not all bands can be seen in the photo, but obvious similarities and differences are evident.



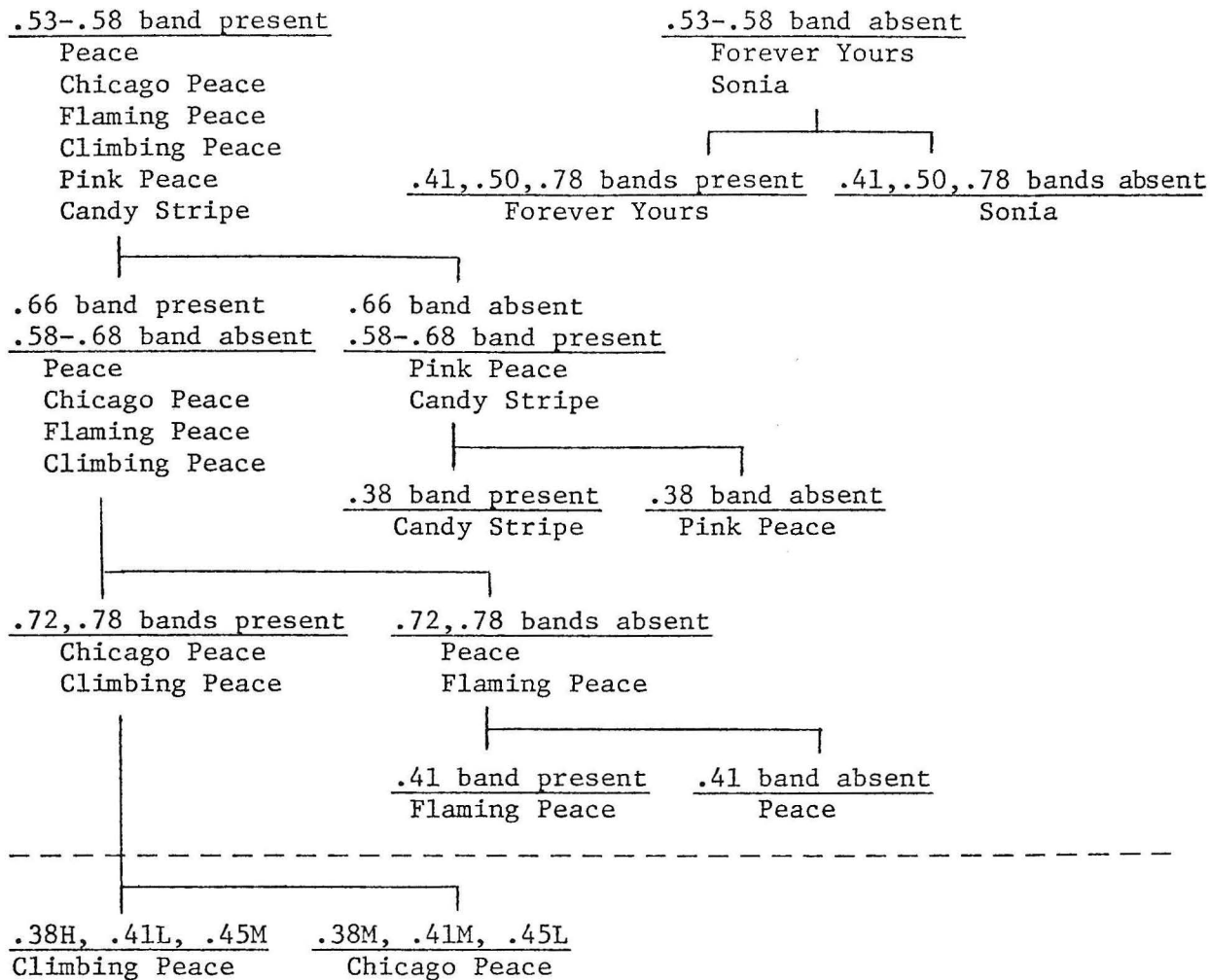


FIG. 27.—A flow chart representing eight rose cultivars on the basis of their cytochrome oxidase banding patterns. Separations above the dashed line are based on band presence or absence. Separations below the line are based on density differences of common bands.

Band #	$R_f$	Density	CULTIVAR							
			1	2	3	4	5	6	7	8
1	.16	M	+	+	+	+	+	+	H	H
2	.22-.25	H								+
3	.26-.30	M	+	H	+	+	+	+	H	+
4	.30-.35	H	+	+	+		+	+	+	+
5	.38	M	+			H	H	H	+	+
6	.41	L							+	M
7	.43-.46	M	+	+	+	+	+	+		
8	.49	L	+	+	+	+	+	+		
9	.51-.63	L	+	+	+	+	L	M	+	
10	.60-.70	H					L	L	+	+
11	.67	L	+	+	+	+				
12	.75	L					+	+		
13	.80	L					+	+		
14	.84	L		+	+	+	+	+	+	+
15	.87-.99	L	+	+	+	+	+	+	+	+

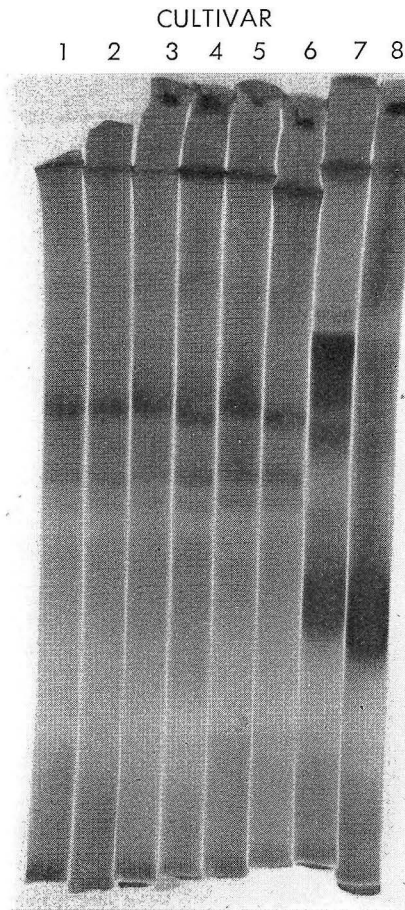


FIG. 28.—Comparison of the phenoloxidase isoenzymes (each band represents an isoenzyme) of Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8). A + indicates the isoenzyme is present at the specified density, a blank space indicates it is absent, and a letter indicates it is present at a different density. Not all bands can be seen in the photo, but obvious similarities and differences are evident.

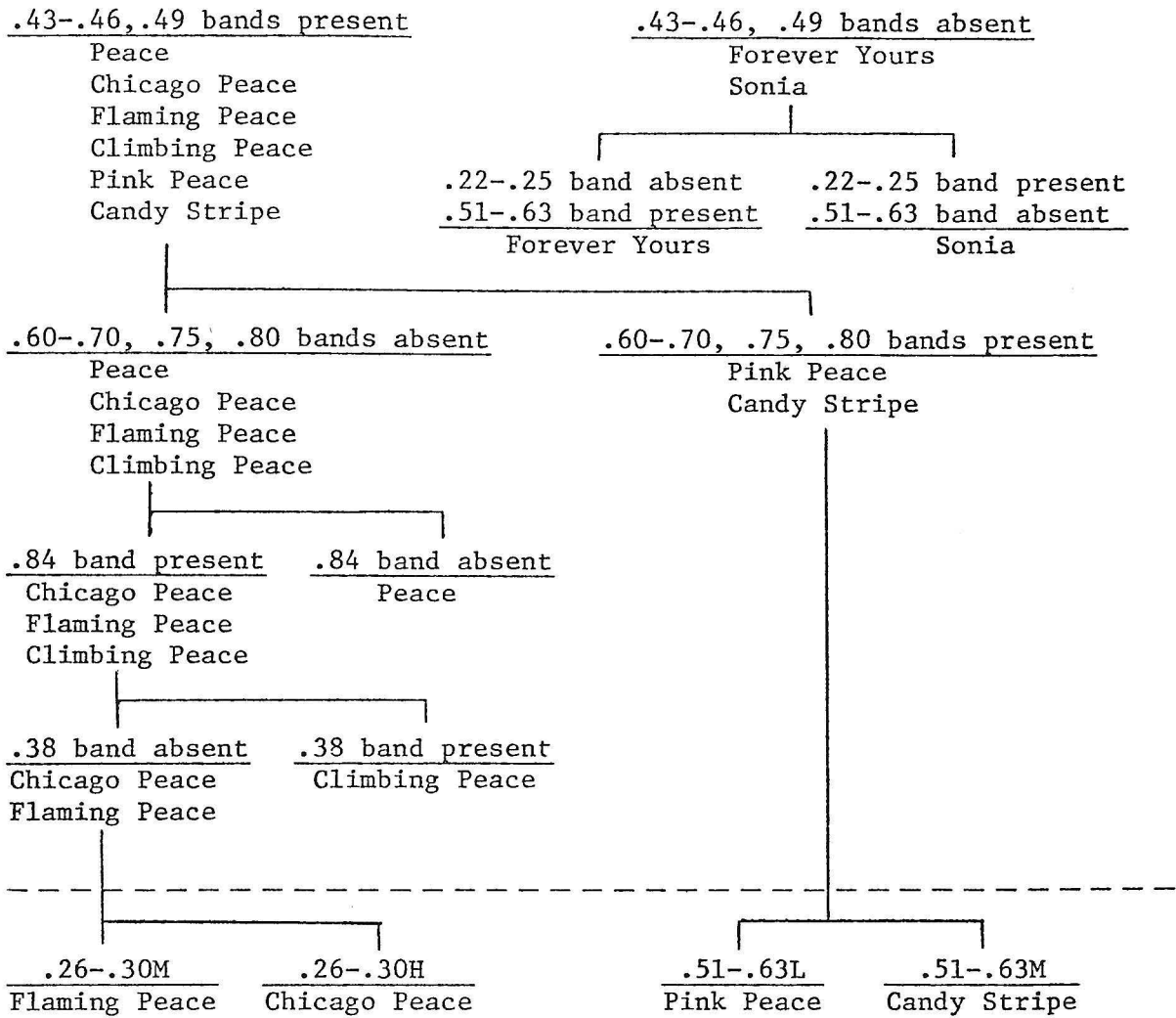


FIG. 29.—A flow chart separating eight rose cultivars on the basis of their phenoloxidase banding patterns. Separations above the dashed line are based on band presence or absence. Separations below the line are based on density differences of common bands.

Band #	R <sub>f</sub>	Density	CULTIVAR								CULTIVAR							
			1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	.13	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
2	.20-.25	L	+	+	+	+	+	+	M	+	+	+	+	+	+	+	+	
3	.30-.36	H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
4	.33	L														M	+	
5	.36	M														+	+	
6	.44	L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
7	.48-.52	H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
8	.49	L							+	+								
9	.52-.62	M	+	+	+	+	+	+	M	M	+	+	+	+	+	+	+	
10	.64-.70	L							+	+	H	H						
11	.76	L							+	+	+							
12	.79	M	H	H	H	H	H	+	+	+	+	+	+	+	+	+	+	
13	.84	L	+	+	+	+	+	+	M	M	+	+	+	+	+	+	+	
14	.85-.98	L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

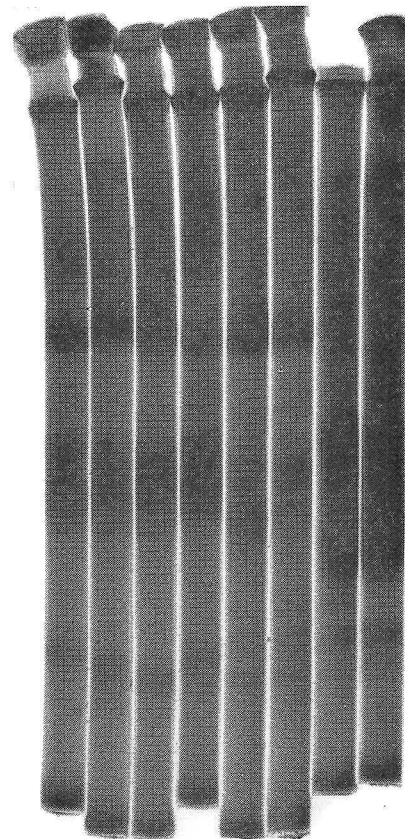


FIG. 30.—Comparison of the polyphenoloxidase isoenzymes (each band represents an isoenzyme) of Peace (1), Chicago Peace (2), Flaming Peace (3), Climbing Peace (4), Pink Peace (5), Candy Stripe (6), Forever Yours (7), and Sonia (8). A + indicates the isoenzyme is present at the specified density, a blank space indicates it is absent, and a letter indicates it is present at a different density. Not all bands can be seen in the photo, but obvious similarities and differences are evident.

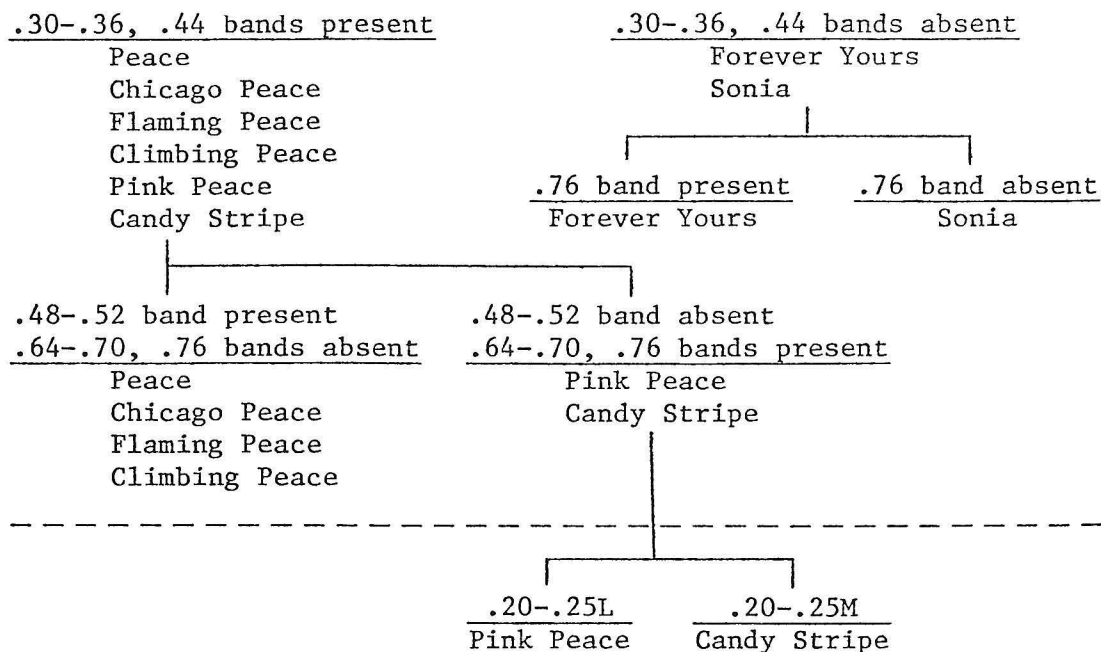


FIG. 31.—A flow chart separating eight rose cultivars on the basis of their polyphenoloxidase banding patterns. Separations above the dashed line are based on band presence or absence. Separations below the line are based on density differences of common bands.

of one band. Peace and Climbing Peace can be distinguished from Chicago Peace and Flaming Peace by a difference in one band and from each other in that the difference is not in the same band. Chicago Peace and Flaming Peace are identical except for the density value of one band.

The usefulness of this enzyme in chemical taxonomy is also demonstrated by the fact that all eight cultivars could be separated into their natural groups on the basis of major banding differences.

**Polyphenoloxidase.** Polyphenoloxidase banding patterns of the eight cultivars are presented in Figure 30 and used for cultivar separation in a flow chart in Figure 31. Based on differences within this one enzyme system, only the most natural separations can be made. Forever Yours and Sonia are missing three bands present in all the Peace cultivars, and are different from each other by one band. Pink Peace and Candy Stripe are distinct from Peace and its sports on the basis of differences in three bands, but cannot be separated from each other except by the density value of one band. Peace and its sports are indistinguishable. This information suggests polyphenoloxidase may not be useful in separating cultivars as closely related as sports, but could be useful where genetic hybrids are involved.

**Coefficients of Similarity**

The coefficients of similarity (c. s.), based on all seven systems investigated, were calculated for all possible cultivar combinations and are presented in Table 3. Cultivars as closely related as sports all had c.s. values of 90 or greater, while the Peace hybrid and its sport had c.s. values of 79 or less to Peace and its sports. Genetic variation is expected to be greater between hybrids than between sports, a fact substantiated by these c.s. values. These values also support the theory that isoenzyme bands are reflections of the genetic composition of an organism and that genetic changes will be seen in isoenzyme changes. Forever Yours and Sonia had average c.s. values of 64 and 55, respectively, to the Peace cultivars. This is as ex-

pected since Forever Yours is a Hybrid Tea, as are the Peace cultivars, and Sonia is a more distantly related Grandiflora type.

**SUMMARY AND CONCLUSIONS**

Roses grown in this country have a very high commercial value. However, a problem of identification by conventional means exists and is becoming greater with the continuing introduction of new cultivars. This study explored the possibility of applying chemical identification techniques to roses.

Polyacrylamide gel electrophoresis of tissue extracts and the subsequent staining for specific enzymes have been successfully used to classify other plant species. Rose plants, however, have been found to contain high levels of phenolic compounds which, along with their oxidation product, quinones, cause enzyme inactivation. Although compartmentalized *in vivo*, enzymes and phenols are mixed together when tissues are macerated during protein extraction. Until a suitable extraction technique was developed, either no enzyme bands were obtained or they were blurred and inconsistent.

The method which yielded clear, consistent enzyme bands included freezing the leaf tissues in liquid nitrogen, preparation of an acetone powder, and lyophilization. This removed most of the phenols and water from the tissue and resulted in a product suitable for storage. Protein was extracted from the lyophilized powder in an extraction solution containing a phenol scavenger and reducing agents to protect the enzymes from the remaining phenols. Also included were a detergent to increase protein solubility and some combination of salt to increase ionic strength and/or a denaturing agent to prevent enzyme aggregation. One extraction solution was not suitable for all of the systems studied, and various combinations of these ingredients were tested before the optimum solution for each was found.

Sixteen enzyme systems and total protein were screened to determine which would produce clear, consistent bands. Peroxidase, esterase, malate dehy-

**TABLE 3.—Coefficient of Similarity Values of Eight Rose Cultivars.\***

	Peace	Chicago Peace	Flaming Peace	Climbing Peace	Pink Peace	Candy Stripe	Forever Yours
Chicago Peace	94						
Flaming Peace	95	96					
Climbing Peace	90	94	93				
Pink Peace	72	74	74	75			
Candy Stripe	74	79	77	79	96		
Forever Yours	61	62	63	63	67	70	
Sonia	56	54	55	53	57	60	73

\*Values were obtained by dividing the number of bands in common by the total number of bands present in the cultivars being compared and multiplying by 100.

drogenase, cytochrome oxidase, phenoloxidase, polyphenoloxidase, and total protein were the only systems which proved useful. The extraction technique and solutions developed for this study can be applied to other plant material high in phenols when extracting for these systems. When extracting for other systems, modifications of this technique may be necessary. For instance, lyophilization inactivates catalase. To determine the optimum extraction solution for a system, various combinations of buffer, ionic strength, pH, chelators, and mild denaturing agents must be examined.

The standard Davis procedures (18) for gel preparation and running conditions were used throughout the experiment. Although suitable for screening, modification of these procedures may result in the discrimination of additional or more clearly defined bands. Different gel concentrations and buffers (gel and/or reservoir) need to be examined. Changing the pH of the whole system and separating cationic rather than anionic forms of the enzymes may produce additional useful data.

Recording of results is subjective because visual observation is the only method by which all bands can be determined. The ideal system would be to scan all gels with a densitometer and calculate  $R_f$  values and relative densities for all bands from the densitometer trace. There are several problems associated with this method. At the present time, densitometers cannot separate bands which are very close to each other or light bands from background stain. Also, with many enzyme systems, stains are unstable and bands may fade or background increase at rates which would prohibit use of a densitometer except under time limited conditions. Finally, if relative densities are to be used as distinguishing characteristics, extraction, running, and staining conditions must be rigidly controlled. In this study, band relative densities were inconsistent and unsuitable for use in cultivar comparisons.

Considering the present weaknesses of densitometry, a complete recording system for a thoroughly developed electrophoretic study should include visual recording of band location and density, location of the marker dye, a photograph, and densitometric scans of suitable gels. Comparisons can then be based on  $R_f$  values, band density, and relative density of major bands observed on the densitometry traces. Photographs cannot be used for cultivar separations because they do not show all bands, but they are helpful references when making  $R_f$  and density comparisons long after the results are recorded.

An attempt was made to set a standard sampling location. Tissue extracts were prepared from successive leaf positions below a flower bud, 0.6 to 2.0

cm in diameter. Optimum results were obtained at different leaf positions for different enzymes, and differences in growth habit caused variations in these results among the cultivars. Because of this variation, no sampling standard could be established.

Additional areas to be investigated as potential standards include root tissues from cuttings, vegetative buds, and flower buds. Based on the results of this study, however, it is unlikely that one standard sampling location can be established. It seems probable that different enzymes will be best extracted from different tissue or tissues of different ages. The possibility also exists that the isoenzymes of one enzyme system extracted from multiple locations can be used to make multiple comparisons between cultivars. Future studies will have to determine optimum sampling locations prior to extensive cultivar comparisons.

The effect of storage of the lyophilized acetone powders at  $-20^{\circ}$  C on the banding patterns of the six enzyme systems and total protein was evaluated. Some were almost unchanged after 16 weeks' storage and could be compared with fresh samples. Others were altered by 12 weeks' storage, but observed changes were minor and suggest that samples could be compared to others stored the same length of time but not to fresh samples. Storage changes will have to be determined for each additional system investigated.

No within cultivar variation was found, indicating all differences between cultivars can be used for cultivar separation.

Coefficient of similarity values were very useful for grouping cultivars and showing relationships, but they do not appear to be useful in distinguishing closely related cultivars.

The potential use of isoenzyme banding patterns as rose identification characters has been proven. All cultivars could be distinguished by the presence or absence of one or more bands in their enzyme banding patterns if several systems were employed. Alone, neither total protein nor malate dehydrogenase banding patterns could separate Peace cultivars from distantly related Forever Yours or Sonia; yet when combined with others, they became important ingredients of the overall taxonomic key.

Esterase and polyphenoloxidase banding patterns were effective in dividing the cultivars into their natural groups, but they could not be used to distinguish sports. Cytochrome oxidase patterns could be used to separate all the cultivars except Chicago Peace and Climbing Peace, suggesting that it may be the most useful enzyme system when trying to distinguish sports. Total protein, peroxidase, malate dehydrogenase, and phenoloxidase all separated

the cultivars differently, producing a unique characterization for all cultivars. The importance of basing similarities or differences on as many characters as possible is evident and should not be underestimated.

A cause for concern is that most of the cultivar separations were based on missing bands. There were few based on a unique band not present in the other cultivars. It is possible that refined techniques may "find" some of the missing bands.

The importance of this study was not only the discovery that rose cultivars can be distinguished by electrophoresis, as had been assumed by other workers as a result of studies of several other species, but that the groundwork for future studies has also been provided. An extraction technique has been developed and 17 enzyme systems have been screened, seven of which have been proven to yield consistent, clear results. The optimum extraction solution for each enzyme has been determined; a variety of staining methods have been tested, modified, and standardized; and the problems of developing a standard sampling location have been elucidated.

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# *The State Is the Campus for Agricultural Research and Development*



Ohio's major soil types and climatic conditions are represented at the Research Center's 12 locations.

Research is conducted by 15 departments on more than 7000 acres at Center headquarters in Wooster, seven branches, Green Springs Crops Research Unit, Pomerene Forest Laboratory, North Appalachian Experimental Watershed, and The Ohio State University.

Center Headquarters, Wooster, Wayne County: 1953 acres

Eastern Ohio Resource Development Center, Caldwell, Noble County: 2053 acres

Green Springs Crops Research Unit, Green Springs, Sandusky County: 26 acres

Jackson Branch, Jackson, Jackson County: 502 acres

Mahoning County Farm, Canfield: 275 acres

Muck Crops Branch, Willard, Huron County: 15 acres

North Appalachian Experimental Watershed, Coshocton, Coshocton County: 1047 acres (Cooperative with Agricultural Research Service, U. S. Dept. of Agriculture)

Northwestern Branch, Hoytville, Wood County: 247 acres

Pomerene Forest Laboratory, Coshocton County: 227 acres

Southern Branch, Ripley, Brown County: 275 acres

Western Branch, South Charleston, Clark County: 428 acres