IDENTIFICATION OF PETUNIA AND POINSETTIA CULTIVARS BY ELECTROPHORETIC AND CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT

IDENTIFICATION OF PETUNIA AND POINSETTIA CULTIVARS BY ELECTROPHORETIC AND CHROMATOGRAPHIC TECHNIQUES

By

Dennis James Werner

The feasibility of using biochemical characteristics as an aid in identifying cultivars of poinsettia, Euphorbia pulcherrima Willd. ex Klotzsch, and of petunia, Petunia hybrida Vilm., was determined. Disc-gel electrophoresis of proteins and peroxidases and two-dimensional chromatography of phenolic compounds of leaf extracts were used to ascertain biochemical differences between 18 cultivars of poinsettia, while seven red-flowered, grandiflora cultivars of petunia were biochemically examined by electrophoretically analyzing proteins and esterases extracted from seeds. Poinsettia leaf samples for the electrophoretic study were obtained from plants propagated from 10 cm rooted terminal cuttings and grown under controlled environmental conditions for 60 days, whereas leaf samples for phenolic analysis were obtained from stock plants maintained in a greenhouse. General protein was analyzed using both SDS and non-SDS gels. Peroxidases and esterases were analyzed on non-SDS gels.

Eighteen individual bands were resolved from poinsettia extracts on non-SDS gels, whereas on SDS gels, 24 protein bands were observed. All cultivars studied exhibited the same general protein banding pattern. A total of 11 different peroxidase bands, representing four different banding patterns, were obtained for the 18 cultivars. Commercially important poinsettia cultivars, with a single exception, possessed the same banding pattern. Fourteen cultivars exhibited the same peroxidase bands, and the remaining four cultivars of little commercial importance exhibited three different patterns; two cultivars had identical banding patterns.

Thirty-seven phenolic compounds were separated from poinsettia leaf extracts by two-dimensional chromatography. Of the eight cultivars analyzed in this study, six were commercially important and showed no qualitative differences among the phenolic compounds resolved. The other cultivars studied had profiles almost identical to the current commercial types, but minor qualitative differences were resolved that made biochemical characterization possible. Compared to the commercial cultivars, one cultivar differed in the presence or absence of three compounds, whereas another differed in two compounds.

The results of the electrophoretic and chromatographic investigations made indicated that the poinsettia cultivars studied possess a very narrow genetic base. The minute differences that characterize present cultivars

could not be biochemically resolved using these methods; those differences noted only occurred in those cultivars not closely related genetically to commercial types.

Eight individual bands were resolved from petunia seed extracts on non-SDS gels, whereas on SDS gels, 19 protein bands were observed. All the cultivars studied exhibited identical protein banding. A total of four esterase bands were resolved, with all cultivars exhibiting the same banding pattern. These results indicated that the petunia cultivars studied have a narrow genetic base, and that the techniques utilized did not aid in cultivar identification.

IDENTIFICATION OF PETUNIA AND POINSETTIA CULTIVARS BY ELECTROPHORETIC AND CHROMATOGRAPHIC TECHNIQUES

By

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LITERATURE REVIEW

Horticulturalists have long selected and propagated superior plant types to enhance mankind's work and recreational environment and to improve the quality of food which he consumes for nourishment. These plants generally have arisen from genetic manipulation by the plant breeder or as spontaneous mutants or sports. This continual selection of improved genotypes has resulted in the origination and release of innumerable plant types within a given species, the term cultivar being used to designate such plants. Most cultivars have been described and documented using only gross morphological characteristics, little use being made of biochemical characterization and micro-anatomical traits. A cultivar, as defined in the International Code of Nomenclature for Cultivated Plants (3), is:

> an assemblage of cultivated individuals which is distinguished by any characters (morphological, physiological, cytological, chemical, or others) significant for the purposes of agriculture, forestry, or horticulture, and which, when reproduced, retains its distinguishing characteristics.

As defined by the International Code, the terms "variety" and "cultivar" are equivalents, and may be used

interchangably. Within a particular species, numerous cultivars may exist with differences between them being very minute and often difficult to detect. Thus, cultivar identification is sometimes difficult, if not impossible, on a morphological basis alone.

The ability to establish cultivar identity has recently become increasingly important. The commercial grower needs assurance that a seedlot or clone of individuals ordered is the cultivar he intends to grow. A means of cultivar identification would be important to the seedsman in cases when seedlots become mixed or mislabeled. Since the adoption of the Plant Patent Act of 1930 and the Plant Variety Protection Act of 1970, which provide for legal protection of asexually and selected sexually propagated crops, the need for positive cultivar identification and characterization has become more important.

Since morphological characters are often not adequate to distinguish cultivars, other means of establishing cultivar status have been attempted. Investigations into using biochemical characteristics as a means of identifying and characterizing cultivars have received increasing attention. Larsen (30) states:

> All inherent morphological manifestations of varietal differences must have a biochemical difference, but not all biochemical differences are expressed morphologically. Thus, biochemical differences should outnumber morphological differences by several fold.

The biochemical characterization of cultivars, often referred to as "fingerprinting," is a relatively new

application of analytical techniques standardly used in chemotaxonomic studies. Chemotaxonomy, the concept of classifying plants on the basis of their chemical constituents, is not new, tracing back at least 169 years to DeCandolle (23). However, this area of study mainly has been applied to the analysis of relationships at or above the species level (33). Results of chemotaxonomic investigations are often used in conjunction with traditional taxonomic interpretations (6). Likewise, the use of biochemical techniques will probably supplement rather than replace other more conventional techniques used to distinguish cultivars (33). Also, in cases where the genetic diversity between cultivars is very narrow, a combination of several biochemical tests and techniques will probably be required to adequately characterize and identify cultivars (33).

Two widely used techniques of biochemical analysis for cultivar identification purposes have been disc-gel electrophoresis of proteins and enzymes and chromatography of phenolic compounds. Disc-gel electrophoresis has been successfully used in chemotaxonomic studies investigating relationships between species, hybrids, and interspecific hybrids (10,12,25,36,37,38,42,48). Its success in these investigations has led to its application as a means of identification of cultivars within a species. Larsen (29) electrophoretically analyzed seed proteins of 61 soybean cultivars, and found two protein components (termed A and B)

that separated the cultivars into two major groups. Component A was present in 13 cultivars, and component B in 48 cultivars. Since components A and B were never jointly observed in the same cultivar, Larsen concluded that they can be useful in cultivar identification. Natarella and Sink (36), studying proteins and peroxidases from leaf extacts of 11 cultivars of petunia, obtained specific protein banding patterns for each cultivar. After staining for peroxidase enzymes, seven banding patterns were observed for the 11 cultivars. Fedak (16), studying α-amylase, esterase, and acid phosphatase isozyme patterns from seed extracts of 55 cultivars of Canadian barley, observed that 12 cultivars had unique combinations of enzyme patterns that were not shared by the other cultivars. Although Canadian barley cultivars have a relatively narrow genetic base, which was reflected in a high degree of similarity in the isozyme patterns of the cultivars studied, Fedak concluded that progress could be made in cultivar identification through the use of isozyme patterns in conjunction with plant and seed morphological characteristics. Wilkinson and Beard (49), examining electrophoretic patterns of proteins from leaf extracts of cultivars of creeping bentgrass (Agrostis palustris Huds.) and Kentucky bluegrass (Poa pratensis L.), successfully obtained specific banding patterns for six cultivars of creeping bentgrass, and observed four banding patterns for eight cultivars of Kentucky bluegrass. They concluded that cultivar

identification is most successful if morphological characters are used in conjunction with electrophoretic patterns.

Successful taxonomic studies evaluating relationships between species (20,22,24,27,35,39,40,46) and investigating the nature of hybrid populations (21,24,32,35) by chromatographically analyzing phenolic compounds has stimulated interest in its use as a means of cultivar identification. Teas et al. (45), in studying the phenolic constituents of 21 cultivars of mango, found seven cultivars to have specific patterns, with the remaining cultivars separating into groups of two to five cultivars each. Singh (41) chromatographically studied the phenols of various vegetable cultivars. He successfully obtained specific chromatographic patterns for eight cultivars of cucumber, and was successful in separating 10 watermelon cultivars into four groups according to their chromatographic patterns. Harney (20), studying the phenols of selected Pelargonium species and cultivars of Pelargonium X hortorum Bailey, concluded that it was possible to identify each of the 14 cultivars on the basis of its chromatographic pattern alone. Brown et al. (7) analyzed the phenolic compounds from leaves of 43 snap bean cultivars. Although a high degree of similarity was noted among the chromatograms of the cultivars, the patterns allowed all but four cultivars to be classified into three major categories.

STATEMENT OF THE PROBLEM

In view of the success that has been attained in biochemically characterizing cultivars using electrophoretic and chromatographic techniques, investigations studying the feasibility of identifying and characterizing cultivars of other horticultural crops are necessary. Thus, this study was undertaken to determine if these analytical techniques would aid in cultivar identification of two important horticultural crops, the petunia, Petunia hybrida Vilm., and the poinsettia, Euphorbia pulcherrima Willd. ex Klotzsch.

IDENTIFICATION OF POINSETTIA CULTIVARS BY ELECTROPHORETIC ANALYSIS OF PROTEINS AND PEROXIDASES

INTRODUCTION

The poinsettia, Euphorbia pulcherrima Willd. ex Klotzsch, is an important greenhouse crop which was introduced into the United States about 1828. Early cultivars originated in Southern California, and the modern era of poinsettia culture began with the introduction of the seedling cultivar "Oak Leaf" in 1923 (14). From 1923 until the early 1960s new cultivars of commercial importance originated primarily as sports, most of which can be traced back to "Oak Leaf." Hybridization efforts started in the mid 1950s, and the first hybrids were released in the 1960s, but still many new commercial cultivars originate as sports. The poinsettia does not tolerate inbreeding, and thus most cultivars are highly heterogeneous, because they result from out-crossing. Due to this, and to insure uniformity in commercial production, it is asexually propagated. The majority of present day cultivars are patented; therefore the ability to identify cultivars is becoming increasingly important. Although many cultivars can be distinguished by morphological traits, the gross plant and flower morphology does not provide an adequate basis for cultivar identification.

Numerous biochemical and chemical analytical procedures have been used in chemotaxonomic studies. Often these procedures are used in conjunction with traditional taxonomic interpretations (6). Most of these biochemical and chemical applications have been applied to comparisons at and above the species level (33).

Electrophoretic separation of proteins and isozymes has been a widely used and a very valuable technique in the field of biochemical systematics. Many chemotaxonomic studies have made use of gel electrophoresis of proteins in the determination of relationships between species, hybrids, and interspecific hybrids (10,12,25,36,37,38,42,48). The success of disc electrophoresis in this field of study has led to its application as a means of identification of cultivars within a species, and numerous successful investigations have been made (1,16,32,36,37,49).

Proteins and isozymes are under genetic control, being direct consequences of the nucleotide sequence at the gene level. Thus, any morphological differences between cultivars not environmentally manifested or not caused by a histogenic rearrangement should have a biochemical basis that is reflected in differences in protein or enzyme structure.

This study was undertaken to determine whether the technique of disc-gel electrophoresis of soluble proteins and peroxidase isozymes would successfully aid in poinsettia cultivar identification.

MATERIALS AND METHODS

The plants used for this study originated as terminal cuttings from stock plants which were obtained from several sources (Table 1). These stock plants were maintained in a greenhouse and grown according to standard cultural practices. Uniform 10 cm terminal cuttings were removed from these stock plants, treated with Hormidin #2, and placed in a mist propagation bench for rooting. Subsequently, rooted cuttings were transplanted into freshly pasteurized soil mix (soil, peat moss, perlite, 1:1:1, v/v/v) in 12.7 cm clay pots. To minimize environmental effects, all plants were grown in controlled environmental chambers maintained at 25 $\pm$ 1 C day and 22 $\pm$ 1 C night with a 16 hr photoperiod. The light source consisted of high output cool white fluorescent lamps supplemented with incandescent light. Light intensity at pot level was 2,100 ft-c. Under these environmental conditions the plants remained in a vegetative state of growth. The plants were watered on alternate days with a full strength, modified Hoagland's solution (26).

Sample tissue, consisting of the third and fourth fully expanded leaves subtending the meristem, was harvested from each cultivar 60 days after placement of rooted plants in the growth chamber. This method of sampling minimized any variation due to age of the tissue, although a preliminary study performed in our laboratory indicated that

Table 1.--Sources of poinsettia cultivars.

Cultivar	Source
Paul Mikkelsen	Mikkelsens Inc.
Scandia	"
Mikkel Rochford	"
Mikkel Improved Rochford	"
Mikkel Super Rochford	"
706153	"
7006	"
Annette Hegg	Ecke Poinsettias
Eckespoint C-1 Red	"
Eckespoint C-1 Pink	"
Eckespoint C-1 White	"
Eckespoint C-1 Marble	"
Eckespoint New C-1 Pink	"
Ecke White	"
Scarlet Ribbons H-2	"
Henriette Ecke Supreme	"
Ecke Flaming Sphere	"
Truly Pink	U.S.D.A.

the soluble protein and peroxidase banding patterns did not change as the age of the tissue varied. Protein acetone pwoders were made according to the method of El-Basyouni and Neish (15). The powders remained stable up to three months at -20 C if stored in a moisture-free container.

Electrophoresis was performed by using standard gels (11) or SDS gels (17). This necessitated the use of two protein extraction procedures. Protein extracts for standard gels were prepared by adding 80 mg of the previously prepared acetone power to 5 ml of 0.2 M tris-citrate buffer, pH 8.3, containing 0.1% (w/v) L-cysteine, 10% (w/v) sucrose, and 1.5 g PVP (Polyclar AT), which had previously been cleared of fines and hydrated in tris-citrate

buffer. Extraction was carried out at 2 C for 12 hr with periodic stirring. The slurry was centrifuged at 2 C at 10,800 X g for 20 min, the supernatant decanted, and centrifuged again at 30,900 X g for 20 min. The extract was stored under N_2 at 2 C until use. Extracts were usually used immediately after preparation, but proved suitable for use up to 24 hr after preparation.

Standard 7% polyacrylamide gels, 4.5 x 0.5 cm, resolving gel pH 8.9, spacer gel pH 6.7, were prepared as described by Davis (11), but no sample gel was incorporated. Two hundred µl of the protein sample was loaded per gel. Electrophoresis was carried out at room temperature with a circulating cold water jacket used to provide cooling, since preliminary trials revealed no difference in the banding pattern or resolution when gels were run in a cold room at 4 C as compared to the room temperature setup. Gels were run at 1 ma per tube for the first 10 min, and adjusted to 2 ma per tube for the remainder of the run.

Protein extraction and gel preparation for SDS electrophoresis were carried out using a modified method of Flint et al. (17). Twenty mg of the acetone power was added to 2 ml of a solution containing 8 M urea, 2% (w/v) SDS (Pierce Chemical, Rockville, Md.), 5% (v/v) mercapto-ethanol, and .062 M Tris, pH 6.8. Extraction was carried out for 24 hr at 23 C with occasional stirring. The slurry was centrifuged at room temperature at 2120 X g for

10 min, the supernatant decanted and stored at room temperature until used. Extracts were suitable for use up to 24 hr after preparation.

Twelve percent (w/v) polyacrylamide gels, 10 cm x 0.5 cm, resolving gel pH 8.8, spacer gel pH 6.8, containing 0.1% (w/v) SDS were prepared. The discontinuous buffer system of Laemmli (28) was used for electrophoresis. The upper running buffer contained 0.1% SDS. The gel tubes had previously been acid cleaned and treated for 24 hr with a 5% (v/v) solution of dichlorodimethylsilane in toluene to facilitate gel removal from the tubes following electrophoresis. Twenty µl of the protein extract was loaded per gel. Electrophoresis was carried out under the same conditions as the 7% gels, and required 3.5 hr for completion.

Staining for general protein was carried out by immersing the gels for 45-60 min in a 0.05% (w/v) solution of Coomassie blue in 12.5% (w/v) trichloroacetic acid after previously fixing in a 12.5% solution of trichloroacetic acid (9). After staining, the gels were placed in a 10% solution of trichloroacetic acid. Bands became clear in 24-48 hr. Peroxidases were stained by using an ethanolic solution of o-dianisidine (3.3'-dimethoxybenzidine) as the hydrogen donor (42).

SDS gels were only stained for general protein. In this case, protein refers to polypeptides released by disulfide bond reduction as well as proteins not so derived. After electrophoresis was complete, the gels were fixed by

incubating them for at least 3 hr in a solution consisting of 10% (v/v) isopropanol, 7.5% (v/v) acetic acid, and 5% (v/v) methanol, followed by removal of SDS in an electrophoretic destainer. Gels were stained for 12 hr in fixing solution containing 0.15% (w/v) Coomassie blue and were destained electrically.

Bromothymol blue was used as a marker dye to facilitate the measurement of hR_f's. Since the dye front is difficult to observe after staining for total protein and disappears completely in the peroxidase stain solution, the gels were cut at the dye front after removal from the tubes. Relative migration values for each band were calculated from four independent electrophoretic runs from two separate extractions. These values have been used to construct the diagrammatic representations of the observed zymograms.

RESULTS AND DISCUSSION

All cultivars studied exhibited the same general protein banding pattern (Figures 1 and 2). A total of 18 individual bands were resolved using 7% gels, whereas on the SDS gels, 24 protein bands were observed.

Four different banding patterns were observed for the 18 cultivars after staining for peroxidase (Figure 3). A total of 11 different peroxidase bands were resolved (Table 2). Fourteen cultivars: "Paul Mikkelsen," "Scandia," "Annette Hegg," "Eckespoint C-1 Red," "Eckespoint C-1

Figure 1. Protein electrophoretic pattern exhibited by 18 cultivars of poinsettia using SDS gels.

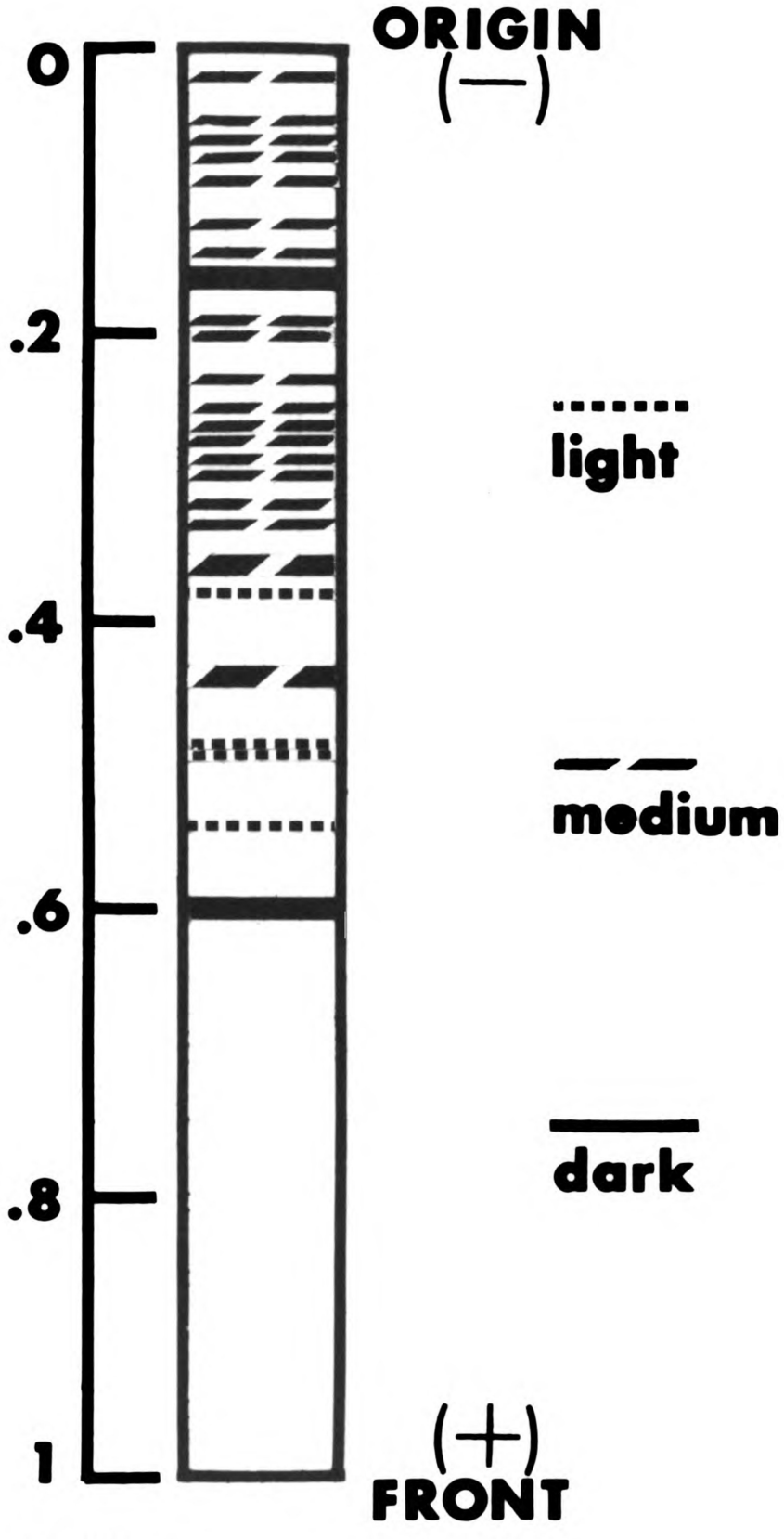

Figure 1

Figure 2. Protein electrophoretic pattern exhibited by 18 cultivars of poinsettia using 7% gels.

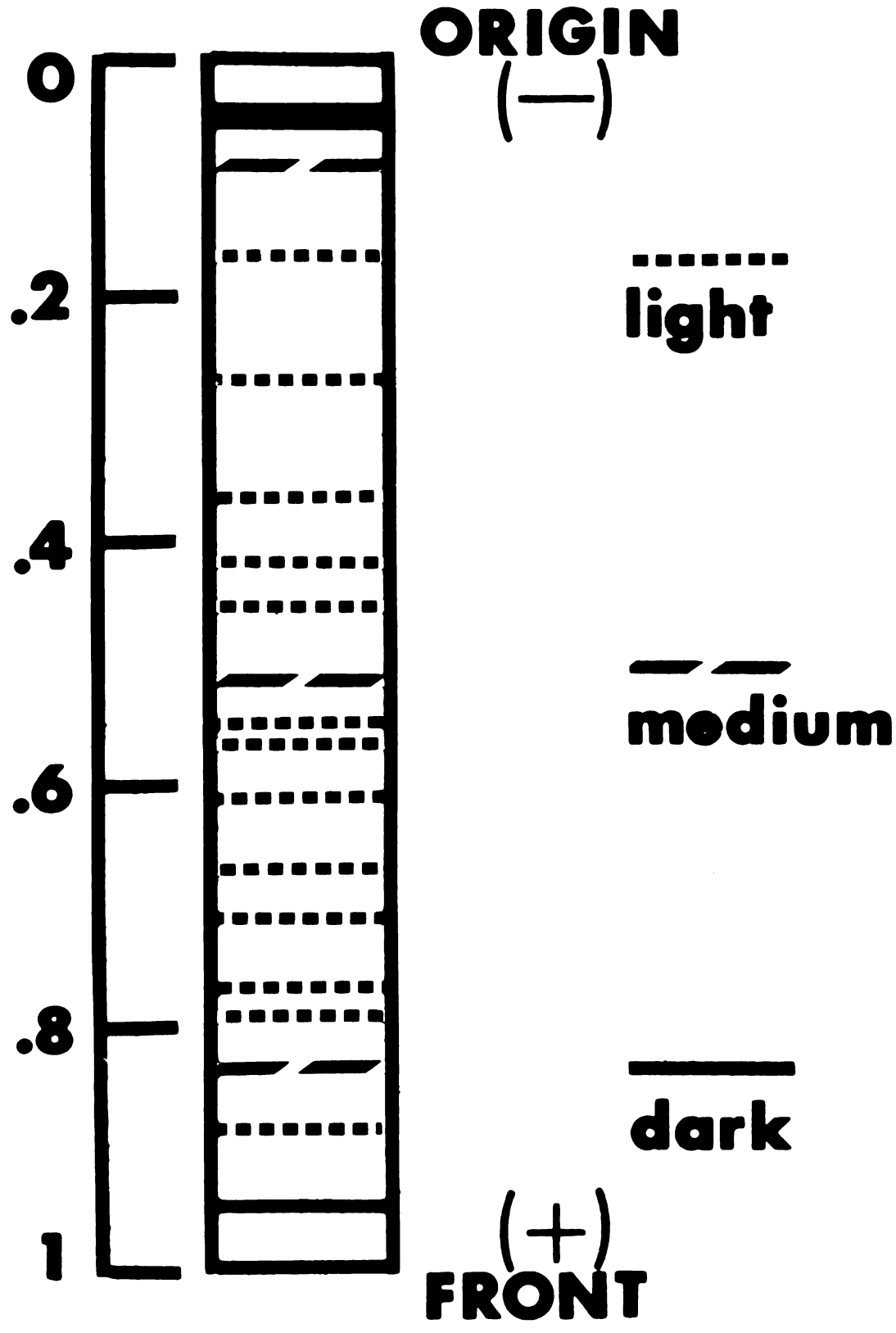

Figure 2

Figure 3. Peroxidase electrophoretic patterns exhibited by poinsettia cultivars. A. Pattern exhibited by "Paul Mikkelsen," "Scandia," "Annette Hegg," "Eckespoint C-1 Red," "Eckespoint C-1 White," "Eckespoint C-1 Marble," "Eckespoint New C-1 Pink," "Mikkel Rochford," "Mikkel Improved Rochford," "Mikkel Super Rochford," "7006," "Ecke White," "Scarlet Ribbons H-2," and "706153." B. Pattern exhibited by "Truly Pink." C. Pattern exhibited by "Henriette Ecke Supreme" and "Ecke Flaming Sphere." D. Pattern exhibited by "Eckespoint C-1 Pink."

Table 2. Disc-gel electrophoretic patterns of peroxidases from poinsettia cultivars.

Cultivar	Band										
	1	2	3	4	5	6	7	8	9	10	11
Paul Mikkelsen	x	x	x			x			x	x	x
Scandia	x	x	x			x			x	x	x
Mikkel Rochford	x	x	x			x			x	x	x
Mikkel Improved Rochford	x	x	x			x			x	x	x
Mikkel Super Rochford	x	x	x			x			x	x	x
706153	x	x	x			x			x	x	x
7006	x	x	x			x			x	x	x
Annette Hegg	x	x	x			x			x	x	x
Eckespoint C-1 Red	x	x	x			x			x	x	x
Eckespoint C-1 Pink	x	x	x			x			x	x	
Eckespoint C-1 White	x	x	x			x			x	x	x
Eckespoint C-1 Marble	x	x	x			x			x	x	x
Eckespoint New C-1 Pink	x	x	x			x			x	x	x
Ecke White	x	x	x			x			x	x	x
Scarlet Ribbons H-2	x	x	x			x			x	x	x
Henriette Ecke Supreme	x	x	x				x	x			x
Ecke Flaming Sphere	x	x	x				x	x			x
Truly Pink	x	x	x	x	x				x	x	x

White," "Eckespoint C-1 Marble," "Eckespoint New C-1 Pink," "Mikkel Rochford," "Mikkel Super Rochford," "Mikkel Improved Rochford," "7006," "Ecke White," "Scarlet Ribbons H-2," and "706153" showed identical patterns, exhibiting bands 1, 2, 3, 6, 9, 10, and 11 (Figure 3A). "Henriette Ecke Supreme" and "Ecke Flaming Sphere" showed the same pattern, exhibiting bands 1, 2, 3, 7, 8, and 11 (Figure 3C). "Truly Pink" had a unique banding pattern, exhibiting bands 1, 2, 3, 4, 5, 9, 10, and 11 (Figure 3B). "Eckespoint C-1 Pink" also had a unique pattern, exhibiting bands 1, 2, 3, 6, 9, and 10 (Figure 3D).

The origin and relationship between many of the poinsettia cultivars studied herein is unclear. The fact that the poinsettia has a tendency to sport readily has resulted in the commercial introduction of many cultivars whose genetic background is not well documented (Figure 4). Based on the protein and peroxidase banding patterns resolved, it appears that there is a very close relationship among many of the cultivars.

"Paul Mikkelsen," a hybrid whose background includes a cross between a "Barbara Ecke Supreme" seedling selection and "Ecke White," was introduced in 1963 (34). The cultivars "Scandia," "706153," "Mikkel Rochford," "Mikkel Improved Rochford," "Mikkel Super Rochford," and "7006" are all closely related to "Paul Mikkelsen," since they originated directly as sports from "Paul Mikkelsen" or indirectly from sport descendants of "Paul Mikkelsen" (34)

Figure 4. Relationship of poinsettia cultivars. Origin unknown refers to probable sport origin, whereas parentage unknown refers to probable hybrid or seedling origin.

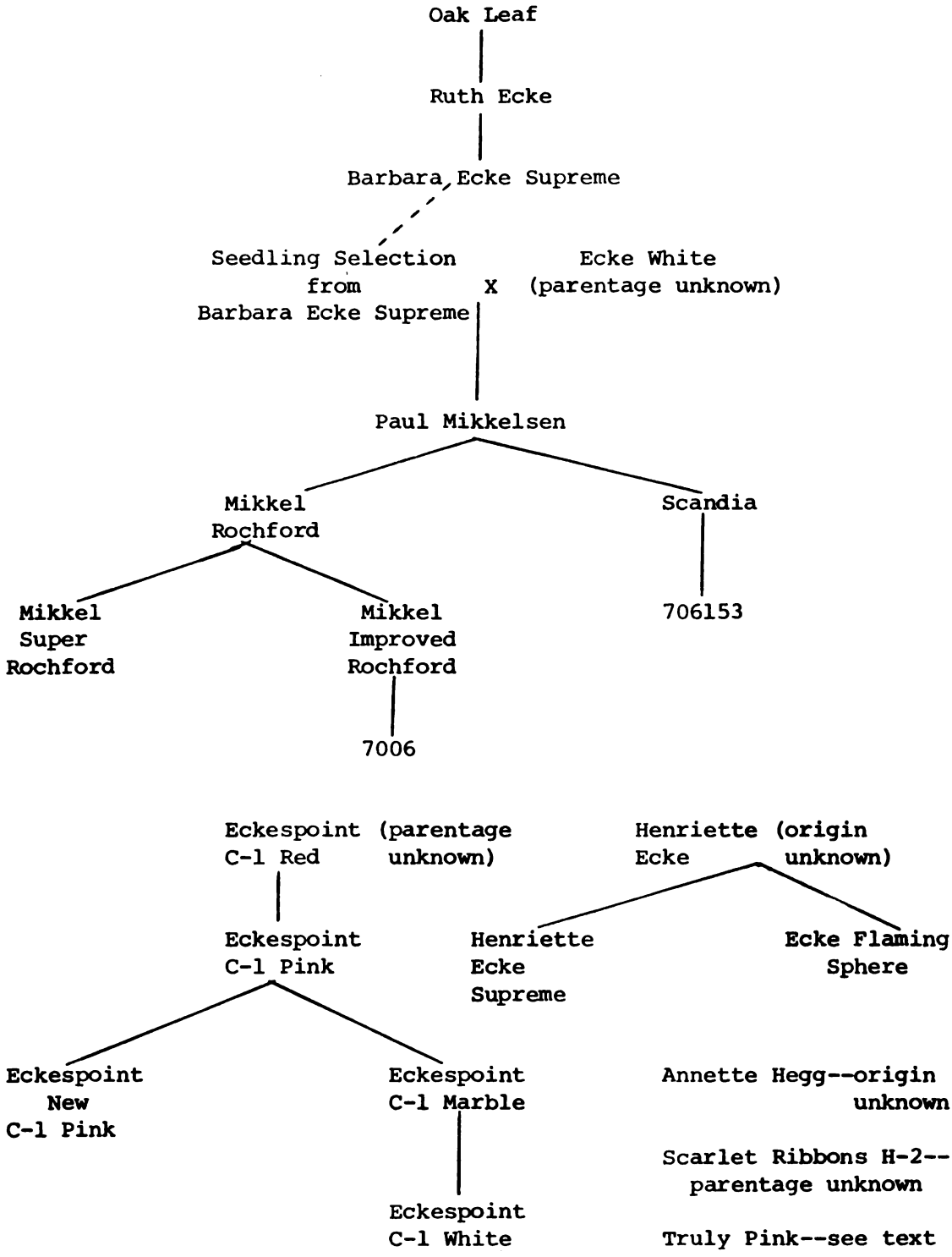
Oak Leaf
Ruth Ecke
Barbara Ecke Supreme
Seedling Selection from Barbara Ecke Supreme
X
Ecke White (parentage unknown)
Paul Mikkelsen
Mikkel Rochford
Scandia
Mikkel Super Rochford
Mikkel Improved Rochford
706153
7006
Eckespoint C-1 Red
(parentage unknown)
Henriette Ecke
(origin unknown)
Eckespoint C-1 Pink
Henriette Ecke Supreme
Ecke Flaming Sphere
Eckespoint New C-1 Pink
Eckespoint C-1 Marble
Annette Hegg--origin unknown
Scarlet Ribbons H-2--parentage unknown
Eckespoint C-1 White
Truly Pink--see text

Figure 4

(Figure 4). This close relationship is similarly reflected in their identical banding patterns.

"Eckespoint C-1 Red" was introduced in 1968, presumably as a hybrid of unknown parentage (13). The other members of the C-1 series have arisen directly as sports from "Eckespoint C-1 Red" or indirectly from sport descendants of "Eckespoint C-1 Red" (13) (Figure 4). Although it has not been investigated, one may confidently assume that the Eckespoint C-1 series of bract color sports have originated in the same manner as the Paul Mikkelsen series of color sports, as described by Stewart (44). Stewart concluded that "Mikkelpink," a pink sport of "Paul Mikkelsen," arose due to a somatic mutation from anthocyanin to anthocyaninless in a central apical cell of the shoot of "Paul Mikkelsen" resulting in a colorless epidermis. Thus, the anthocyanins in the internal cells of the bract appear diluted, resulting in the pink bract color of "Mikkelpink." "Mikkeldawn," which exhibits marble bract coloration (white edge and pink center), originated from "Mikkelpink" as a result of histogenic replacement by the colorless L-I, resulting in a colorless L-I and L-II over a colored L-III. "Mikkelwhite," a white bracted cultivar, originated due to histogenic replacement by the colorless L-I or L-II, resulting in three colorless histogenic layers (Figure 5). If the Eckespoint C-1 series of color sports originated in the same manner, one would assume the Eckespoint C-1 cultivars to be nearly genetically identical, differing

Figure 5. Origin of Paul Mikkelsen series of bract color sports.

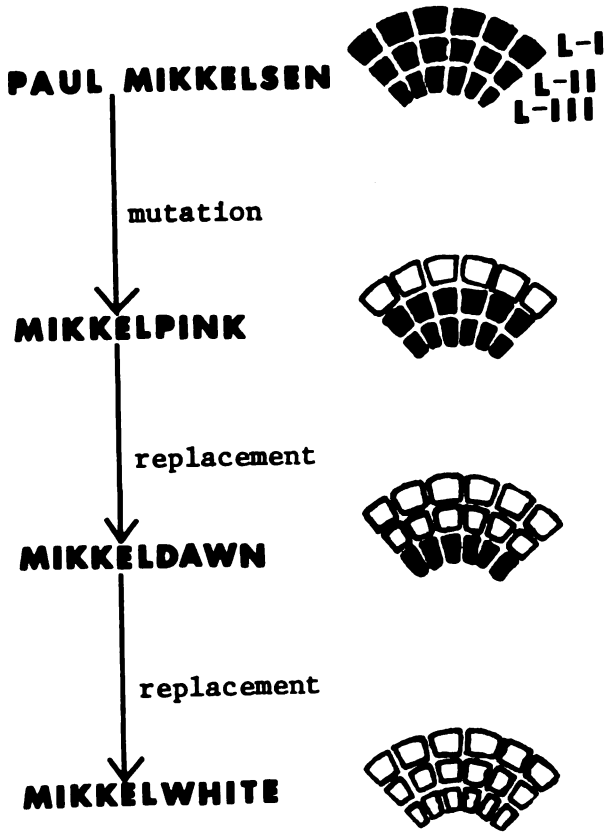
PAUL MIKKELSEN
L-I
L-II
L-III
mutation
MIKKELPINK
replacement
MIKKELDAWN
replacement
MIKKELWHITE

Figure 5

only in the number of histogenic layers capable of anthocyanin production. On this basis, the pattern exhibited by "Eckespoint C-1 Pink," which varies slightly from the rest of the series, was not anticipated. Actually, one would expect only a slight biochemical difference among all the Eckespoint C-1 cultivars, with the greatest difference expected between "Eckespoint C-1 Red" and "Eckespoint C-1 White." It was observed during the course of this study that the "Eckespoint C-1 Pink" plant appeared chlorotic and exhibited less vigor than the remainder of the cultivars. This may have been due to a physiological disorder caused by some unknown factor. Thus, it is possible that the banding pattern resolved may not be a true representation of the cultivar. Also, the occurrence of a banding pattern for "Eckespoint New C-1 Pink" identical to the remainder of the Eckespoint C-1 series further substantiates this explanation. That the Eckespoint C-1 series of sports and the Paul Mikkelsen series of sports exhibit the same banding pattern suggests a close relationship between these cultivars, the protein and peroxidase banding patterns failing to resolve the minute differences between them.

"Truly Pink," a cultivar with "Oak Leaf" and "Ecke White" in its ancestry, but with frequent backrosses to a wild pink (43), had a unique banding pattern, probably due to its diverse genetic background. In contrast to "Eckespoint C-1 Pink," which is a histogenic pink, "Truly Pink"

is a genetic pink, its bract coloration due to true pink pigmentation in all the bract cells.

"Annette Hegg," discovered in Norway in the early 1960s (13), exhibited the same banding pattern as the Paul Mikkelsen and the Eckespoint C-1 series of sports. The genetic origin of this cultivar is unclear, but the results indicated it may be closely related to the Paul Mikkelsen and Eckespoint C-1 series of sports. The banding pattern of "Scarlet Ribbons H-2" also suggests the same. Again, the difference in these cultivars could not be resolved electrophoretically.

"Henriette Ecke Supreme," a sport discovered in 1948 that originated from the cultivar "Henriette Ecke" (13), exhibited the same banding pattern as "Ecke Flaming Sphere," discovered in 1950. This can be expected, since "Ecke Flaming Sphere" also sported from "Henriette Ecke" (13) (Figure 4). That these two cultivars had a unique banding pattern different from the other cultivars studied is probably a reflection of their genetic constitution. Proven unsatisfactory as greenhouse pot plants, they are primarily used as outdoor ornamentals, and have not been used as a germ plasm source in the development of greenhouse cultivars (13).

The observations made herein indicated that protein and peroxidase banding patterns did not biochemically resolve all poinsettia cultivars. Using electrophoresis, other isozymes should be studied which may provide further

separation of cultivars on a biochemical basis. Using the extraction technique described earlier for 7% gels, no banding was attained after staining for esterases, leucine amino peptidases, and acid phosphatases, following the staining procedures described by Brewbaker et al. (8). Following the procedure described by Larsen (30), staining for oxidative enzymes was successful. However, this stain resolved the same patterns obtained by the peroxidase stain, so it was of not further use in cultivar delineation.

SUMMARY

Soluble leaf proteins and peroxidases from 18 cultivars of poinsettia were analyzed using disc-gel electrophoresis. All cultivars exhibited the same banding pattern after staining for soluble proteins. Four banding patterns were resolved after staining for peroxidase isozymes. With one exception, all cultivars of commercial importance displayed the same banding pattern. The results indicated that the close similarity in proteins and peroxidases among cultivars is due to the close genetic relationship between most poinsettia cultivars. Other techniques will have to be examined in attempting to biochemically identify specific cultivars of poinsettia, perhaps used in conjunction with the methods employed in this study.

IDENTIFICATION OF POINSETTIA CULTIVARS BY CHROMATOGRAPHIC ANALYSIS OF PHENOLIC COMPOUNDS

INTRODUCTION

The poinsettia is an important greenhouse cultivated pot plant. Many sports with new or desirable horticultural characteristics have been discovered and maintained asexually. Most of the present day commercial cultivars are patent protected; thus cultivar identity is very important. Since many cultivars exhibit a high degree of similarity, cultivar identity based solely on gross morphology is very difficult.

Numerous biochemical and chemical techniques have been used in attempting to identify cultivars (33). Chromatography of phenolic compounds has been very useful in taxonomic studies to evaluate relationships between species (20,21,39,40,46) and investigating the nature of hybrid populations and their origins (21,24,32). Chromatography of phenolic compounds has been successfully used in investigations identifying cultivars within a species (7,20,35,41,45).

The purpose of this study was to determine whether chromatography of phenols extracted from leaf tissue of selected poinsettia cultivars would aid in cultivar identification.

MATERIALS AND METHODS

Stock plants obtained from several sources (Table 3) were maintained in a greenhouse and grown using standard cultural procedures. The leaf samples used to obtain phenolic extracts were obtained from these plants.

Table 3.--Sources of poinsettia cultivars.

Cultivar	Source
Paul Mikkelsen	Mikkelsens Inc.
Mikkel Improved Rochford	"
7006	"
Annette Hegg	Ecke Poinsettias
Eckespoint C-1 Red	"
Eckespoint C-1 White	"
Ecke Flaming Sphere	"
Truly Pink	U.S.D.A.

Sample tissue was collected in August, 1975 from plants maintained in a vegetative state of growth. The sample tissue consisted of the second, third, and fourth fully expanded leaves subtending the terminal apex. This method of sampling was chosen to minimize any variation due to the age of the tissue, since previous studies have found that this factor can cause both quantitative and qualitative variation in phenolic compounds (18,50). Collected tissue was oven-dried at 50 C. After drying, the tissue was ground to a fine powder with a mortar and pestle, placed in tightly stoppered glass jars, and stored at room temperature.

A modified procedure according to Bate-Smith (4,5) was used for extraction of phenols. Two g of the dried tissue was added to 40 ml of 2N HCL in a test tube and heated for 20 min in boiling water, stirring occasionally. The cooled extract was filtered through a glass fiber filter (Millipore AP 2004200) under vacuum. The filtrate was then passed through moist Celite, transferred to a separatory funnel, and extracted twice with 15 ml portions of n-butanol. The n-butanol extract was evaporated to near-dryness and brought to 1 ml final volume.

Phenols were separated by two-dimensional descending chromatography. The extracts were applied with a micro-pipet as a 4-6 mm spot on the surface of 46 x 57 cm Whatman 3MM chromatographic paper 8.5 cm from each edge in the lower left hand corner. Two chromatograms were run for each extract, one loaded with 20 µl and the other with 40 µl of the extract. Sheets loaded with 20 µl were run to achieve satisfactory resolution of those compounds present in higher concentrations, while 40 µl runs were made to conclusively identify compounds present in lower concentrations that did not consistently appear on chromatograms loaded with 20 µl. The papers were folded on a line 5.5 cm from the edge and placed in a chromatographic chamber. The chamber was then equilibrated for 2 hr with the lower phase of a mixture of n-butanol, acetic acid, water (4:1:5). The extract was resolved by descending flow with the upper phase of the solvent and allowed to migrate until the solvent front

neared the bottom of the sheet (about 13 hr). After drying at room temperature, the chromatograms were developed in the second direction with a mixture of acetic acid, water (3:17), and required about 6 hr to run. Chromatograms were then dried at room temperature.

The dried chromatograms were examined prior to chemical treatment under both long-wavelength and short-wavelength ultraviolet illumination, then exposed for 30 min to the fumes of ammonium hydroxide (25%), and reexamined in the ultraviolet. They were then sprayed with a solution of equal amounts of 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide (19). The spots were outlined, R_f values calculated, and color reaction to each treatment recorded (Table 4). No attempt was made to determine chemical identity.

RESULTS AND DISCUSSION

A total of 37 compounds were resolved in the butanol fractions of the eight cultivars studied (Figure 6); of these, 34 (all but numbers 9, 11, and 20) occurred on every sheet. Most of the compounds appeared on chromatograms loaded with 20 µl of the extract. Spots 8, 9, 10, 11, and 12 were judged to be present in lower quantities, usually appearing only on those sheets loaded with 40 µl. Six cultivars: "Paul Mikkelsen," "Annette Hegg," "Mikkel Improved Rochford," "7006," "Eckespoint C-1 Red," and "Eckespoint C-1 White" showed the same compounds, exhibiting

Table 4.--R_f values and color reactions of the butanol-soluble phenolic compounds in poinsettia foliage.[a]

Compound Number	R_f		UV		Spray (See Text)
	n-Butanol, Acetic Acid, Water (4:1:5)	Acetic Acid, Water (3:17)	Untreated	NH_3	
1	.00	.00	Gr	-	-
2	.25	.00	Gr	-	-
3	.62	.00	Y	-	Bl
4	.81	.00	Pk	-	Bl
5	.33	.03	P	-	Bl
6	.33	.06	Gr	-	-
7	.68	.10	P	-	-
8	.32	.12	LBk	-	-
9	.33	.21	LGr	-	-
10	.48	.26	LBk	-	-
11	.61	.29	-	-	LBl
12	.49	.34	LBk	-	-
13	.63	.39	Bk	-	Bl
14	.70	.41	Bl	-	-
15	.76	.46	Bl	-	-
16	.49	.48	Or	-	-
17	.82	.52	-	P	-
18	.60	.55	P	-	DBl
19	.79	.57	Or	-	-
20	.53	.61	Gr	-	-
21	.60	.66	P	-	-
22	.80	.67	P	-	-
23	.29	.69	P	-	-
24	.52	.73	Bl	-	-

Table 4.--(Continued).

Compound Number	R_f n-Butanol, Acetic Acid, Water (4:1:5)	R_f Acetic Acid, Water (3:17)	UV Untreated	UV NH_3	Spray (See Text)
25	.71	.74	P	-	-
26	.68	.76	Gr	-	-
27	.44	.79	Bk	-	-
28	.84	.79	-	-	Bl
29	.98	.79	Gr	-	-
30	.84	.80	Gr	-	-
31	.81	.81	Bk	-	-
32	.79	.81	Gr	-	-
33	.46	.88	-	-	Bl
34	.37	.97	Gr	-	-
35	.45	.97	Gr	-	-
36	.49	.98	Gr	-	-
37	.15	.99	Bk	-	-

[a]Color abbreviations: P = purple; Bl = blue; Gr = green; Y = yellow; Pk = pink; Bk = black; Or = orange; L = light; D = dark.

Figure 6. **Composite chromatogram of butanol-soluble phenols extracted from poinsettia foliage.**

all spots except numbers 11 and 20. "Ecke Flaming Sphere" was very similar, exhibiting all compounds except number 9. "Truly Pink" was also very similar, exhibiting all compounds except numbers 9 and 20.

Previous biochemical studies and presumed ancestry have indicated that the cultivars "Paul Mikkelsen," "Annette Hegg," "7006," "Mikkel Improved Rochford," "Eckespoint C-1 Red," and "Eckespoint C-1 White" are genetically closely related (47). The occurrence of identical phenolic chromatographic patterns further supports this contention.

"Ecke Flaming Sphere" is primarily used as an outdoor ornamental and has not been used as a genetic source for the development of greenhouse cultivars (13). Thus, its genetic constitution probably differs from present day greenhouse cultivars. However, this genetic difference is only represented by a minor difference in the phenolics. Furthermore, "Truly Pink" which has a diverse genetic background, its ancestry including "Oak Leaf," "Ecke White," and a wild pink (43), showed only a minor difference in the phenolic constituents.

It was concluded that two-dimensional chromatography of phenols will not successfully aid in biochemical identification of poinsettias. Since most new cultivars arise as sports, new cultivars are very closely related genetically. The minute differences in the sports studied was not reflected in any differences in the phenolic compounds, those differences found being between cultivars known to be

genetically divergent from greenhouse cultivars. Other solvent systems should be attempted in future chromatographic studies, since all phenolic compounds are not successfully separated with only one particular solvent system.

SUMMARY

Phenolic compounds from leaf tissue of eight poinsettia cultivars were analyzed using two-dimensional paper chromatography in an attempt to determine if cultivars could be biochemically identified. Six of the eight cultivars studied, assumed to be closely related on the basis of presumed ancestry and previous biochemical studies, exhibited the same chromatographic pattern. The two other cultivars, each having a different genetic background and differing genetically from the other six cultivars studied, exhibited only minor differences in their phenolic constitution, but could be biochemically identified.

IDENTIFICATION OF PETUNIA CULTIVARS BY ELECTROPHORETIC ANALYSIS OF PROTEINS AND ESTERASES

INTRODUCTION

The need to develop methods to discriminate between cultivars of horticultural crops is of increasing importance. In addition to the use of morphological traits for identification of cultivars, biochemical characteristics are of increasing interest, especially in those cases where identification on a morphological basis alone is difficult or impossible. Positive cultivar identification would be of great importance to plant breeders and seedsmen in protection of priority rights on patented varieties. The use of biochemical identification of seeds would be helpful to seedsmen in the determination of cultivar purity or of the cultivar type of a seedlot.

Gel electrophoresis of proteins and isozymes has proved to be a useful tool in biochemical systematics. Its successful use in studies evaluating relationships between species, hybrids, and interspecific hybrids (10,12,25,36, 37,38,42,48) has led to its application as a means of identification of cultivars within a species, and numerous successful investigations have been made (16,29,36,49).

The objective of this study was to determine if cultivar specific general protein and/or esterase isozyme banding patterns could be obtained from seed extracts of selected cultivars of petunia.

MATERIALS AND METHODS

The seeds used for this study were obtained from several sources (Table 5). These seeds represent a group of red-flowered, grandiflora cultivars that exhibit a high degree of morphological similarity. Electrophoresis was performed using standard gels (11) or SDS gels (17). This necessitated the use of two protein extraction procedures. Active protein extracts for standard gels were prepared as follows. A 0.20 g seed sample was weighed from a seed lot that had previously been dried over silica gel. The sample was placed on moist filter paper and placed in a covered petri plate. The seeds were imbibed for 24 hr at 27 C and then finely ground with a mortar and pestle at 2 C. This slurry was added to 5 ml of 0.2 M tris-citrate buffer, pH 8.3, containing 10% (w/v) sucrose, and 1.5 g PVP (Polyclar AT), which had previously been cleared of fines and hydrated in tris-citrate buffer. Extraction was carried out at 2 C for 12 hr with periodic stirring. The slurry was centrifuged at 2 C at 10,800 X g for 20 min, the supernatant decanted, and stored under N_2 at 2 C until used. The extract was used within 1 hr of preparation.

Table 5.--Sources of petunia cultivars.

Cultivar	Source
Red Cascade	Geo. J. Ball Co.
Red Magic	Geo. J. Ball Co.
El Toro	Geo. J. Ball Co.
Tango	Geo. J. Ball Co.
Red Baron	Geo. J. Ball Co.
Candy Apple	Goldsmith Seeds Inc.
Bravo	Goldsmith Seeds Inc.

Standard 7% polyacrylamide gels, 4.5 cm x 0.5 cm, resolving gel pH 8.9, spacer gel pH 6.7, were prepared as described by Davis (11), but no sample gel was incorporated. The protein sample, 200 µl, was loaded on each gel. Electrophoresis was carried out at 2-4 C and the extracts were run at 1 ma per tube for the first 10 min, and adjusted to 2 ma per tube for the remainder of the run.

Active protein extracts and gel preparation for SDS electrophoresis were carried out using the method of Flint et al. (17). Twenty mg of homogenized dried seed was added to 1 ml of a solution containing 8 M urea, 2% (w/v) SDS (Pierce Chemical, Rockville, Md.), 5% (v/v) mercapto-ethanol, and .062 M Tris, pH 6.8. Extraction was carried out for 24 hr at 23 C with occasional stirring. The slurry was centrifuged at room temperature at 2120 X g for 10 min, the supernatant decanted and stored at room temperature until used.

Twelve percent (w/v) polyacrylamide gels, 10 cm x 0.5 cm, resolving gel pH 8.8, spacer gel pH 6.8, containing

0.1% (w/v) SDS were prepared. The discontinuous buffer system of Laemmli (28) was used for electrophoresis. The upper running buffer contained 0.1% SDS. The gel tubes had previously been acid cleaned and treated for 24 hr with a 5% (v/v) solution of dichlorodimethylsilane in toluene to facilitate gel removal from the tubes following electrophoresis. Fifteen µl of active protein extract was loaded per gel. Electrophoresis was carried out at room temperature with a circulating cold water jacket to provide cooling. Gels were run at 1 ma per tube for the first 10 min, and adjusted to 2 ma per tube for the remainder of the run, and required about 3.5 hr to run.

Staining for general protein was carried out by immersing the gels for 45-60 min in a 0.05% (w/v) trichloroacetic acid after previously fixing in a 12.5% solution of trichloroacetic acid. After staining, the gels were placed in a 10% solution of trichloroacetic acid. Bands became clear in 24-48 hr. Esterases were stained using the method of Smith et al. (42). One ml of 1% (w/v) alpha-naphthyl-acetate in 60% acetone was added to 25 ml of 0.11 M phosphate buffer, pH 5.9. Twenty mg of fast blue RR were added to 25 ml of the same buffer. These solutions were then mixed and filtered, the filtrate being used for esterase localization.

SDS gels were stained only for general protein. In this case, protein refers to polypeptides released by disulfide bond reduction as well as proteins not so derived.

After electrophoresis was complete, the gels were fixed by incubating them for at least 3 hr in a solution consisting of 10% (v/v) isopropanol, 7.5% (v/v) acetic acid, and 5% (v/v) methanol, followed by removal of SDS in an electrophoretic destainer. Gels were stained for 12 hr in fixing solution containing 0.15% (w/v) Coomassie blue and were destained electrically.

Bromothymol blue was used as a marker dye to facilitate the measurement of hR_f's. Since the dye front is difficult to observe after protein or enzyme staining, the gels were cut at the dye front after removal from the tubes. Relative migration values for each band were calculated from two independent electrophoretic runs from two separate extractions. These values were used to construct the disgrammatic representations of the observed zymograms.

RESULTS AND DISCUSSION

All cultivars studied exhibited the same general protein banding (Figures 7 and 8). A total of eight individual bands were resolved using 7% gels, whereas on SDS gels, 19 protein bands were observed.

Four bands were observed after staining for esterases, with all cultivars exhibiting the same banding pattern (Figure 9). The occurrence of a relatively low number of protein bands resolved using 7% gels is probably indicative of their function as storage proteins. Natarella

Figure 7. Protein electrophoretic pattern exhibited by seven cultivars of petunia using SDS gels.

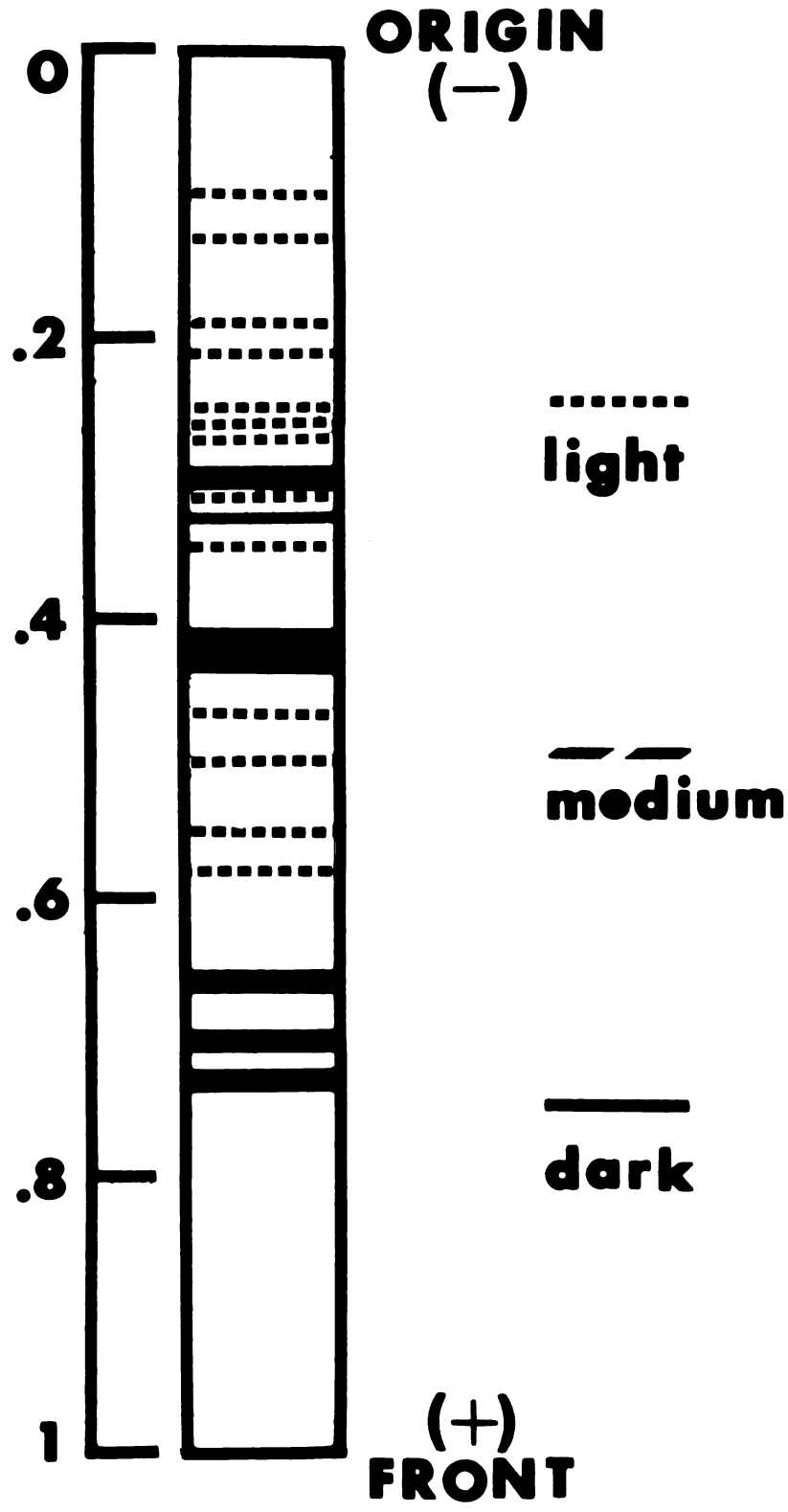

Figure 7

Figure 8. Protein electrophoretic pattern exhibited by seven cultivars of petunia using 7% gels.

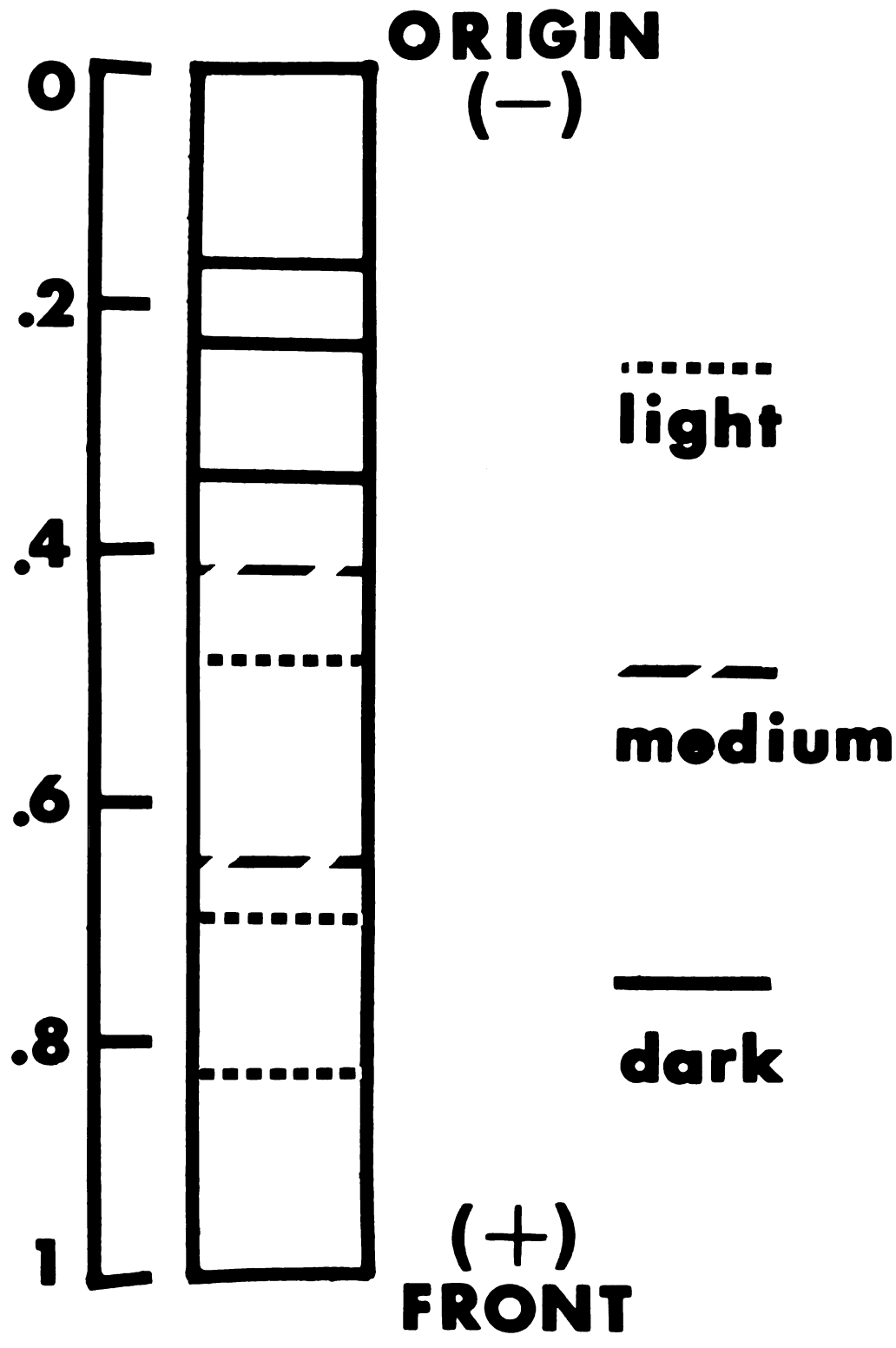

Figure 8

Figure 9. Esterase electrophoretic pattern exhibited by seven cultivars of petunia.

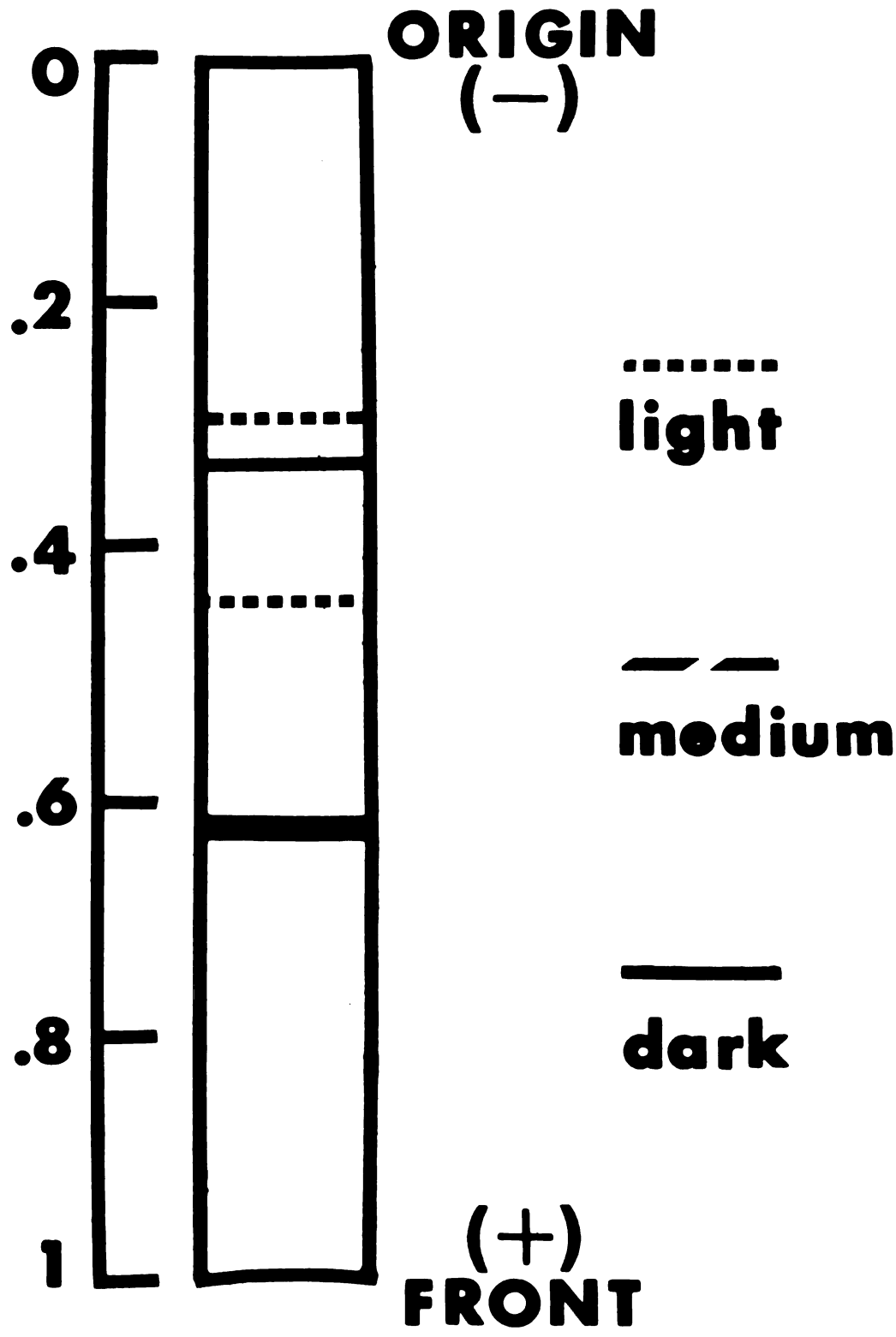

Figure 9

and Sink (36), using leaf extracts from selected petunia cultivars, resolved an average of 27 general protein bands. Although the same cultivars were not used in this study, this highly suggests that one may expect more protein diversity in extracts prepared from leaf tissue as compared to seed. These authors also found that the cultivars studied could be identified electrophoretically. Thus, leaf tissue may be more suitable than seed in studies attempting to electrophoretically identify cultivars, although identification of cultivars using seed rather than foliage samples presents certain advantages.

Since proteins and isozymes are under genetic control, the occurrence of identical protein and isozyme patterns suggests that these cultivars are closely related genetically. Because electrophoresis failed to resolve the differences between these cultivars, it provided no basis for biochemical identification. Other isozymes should be studied in attempting to identify these cultivars electrophoretically. Using the extraction procedure described for 7% gels, no banding could be obtained after staining for peroxidases, following the procedure described by Smith et al. (42). Staining for leucine amino peptidases and acid phosphatases following the procedure described by Brewbaker et al. (8) was also unsuccessful.

SUMMARY

Soluble proteins and esterases from seed of seven cultivars of petunia were analyzed using disc-gel electrophoresis. All cultivars exhibited the same banding pattern after staining for soluble protein. All cultivars also exhibited the same banding pattern after staining for esterase isozymes. This suggests that the cultivars studied are very closely related. Other isozymes should be examined in future studies attempting to biochemically identify these cultivars.

CONCLUSIONS AND RECOMMENDATIONS

The results of this study indicate that the genetic base of the petunia and poinsettia cultivars studied is very narrow, and that the analytical techniques employed were not successful in biochemically separating the cultivars. Future attempts in trying to biochemically separate these cultivars should focus upon the separation and staining of other isozymes. The failure to obtain a staining reaction for leucine amino peptidases, acid phosphatases, and esterases from poinsettia leaf extracts were disappointing, but may in part be due to the high amounts of phenolics present in the foliage, as indicated by the chromatographic analysis. More elaborate techniques will probably be necessary to successfully extract these and other enzymes so that phenolics and other interfering substances are removed.

The petunia electrophoretic study suggests that leaf extracts may be more suitable than seed extracts in attempting biochemical cultivar identification. Future studies should examine the protein and enzyme banding patterns obtained from leaf tissue of these cultivars in

order that positive conclusions may be reached concerning this point.

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