Peroxidase Isozymes in Various Tissues for Discrimination of Two Tuberous Solanum Species.

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Peroxidase isozyme activities were examined to compare the usefulness of various tissues under different environments for estimating interspecific differences between two potato species, Solanum pinnatisectum and S. multidissectum. A total of sixteen peroxidase isozyme bands were distinguished in the two species. The expression of these bands changed depending on species and tissue sources as well as environmental conditions. The environmental effect was larger in leaf tissues than in tuber tissues, and larger in S. multidissectum than in S. pinnatisectum. S. pinnatisectum and S. multidissectum had four and two species-specific bands, respectively, of which four were unique either to leaves or tubers and the remaining two were common to both tissues. These species-specific bands are useful to distinguish between the two species. Based on total band activities including non-specific ones, a mean Euclidean distance was utilized to estimate an interspecific difference between them. Both tuber and leaf tissues gave significant interspecific differences under the controlled environment. Our study thus indicates that leaf tissues, like tubers, can provide a useful material in peroxidase isozyme analysis in Solanum species.

KEY WORDS: Solanum pinnatisectum, Solanum multidissectum, peroxidase isozyme, environmental variation, tissue-specificity, electrophoresis.

Introduction

In the potato, electrophoretic comparisons of tuber proteins and enzymes have been made in cultivar identification (Zwartz 1966, Desborough and Pelouquin 1968, Stegemann and Loeschcke 1976, Seibles 1979, Stegemann 1979, and Oliver and Martinez-Zapater 1985) and in study for interspecific differences among wild relatives (Desborough and Pelouquin 1966, 1967, 1969, Rickeman and Desborough 1978b, and Hosaka and Matsubayashi 1983). Using tuber samples, genetic studies have been conducted on three protein bands (Rickeman and Desborough 1978a) and several isozymes including alkaline phosphatase and glucos phosphatase isomerase (May et al. 1982) and peroxidase and malate dehydrogenase (Quiros and McHale 1982). The stability of proteins and isozymes in tuber storage tissues has also been critically investigated (Zacharias et al. 1971, Stegemann et al. 1973, and Staub et al. 1982). Compared with the usefulness of tuber tissues as demonstrated in these studies, little information is available on the other tissue sources, particularly leaf.

We here examine peroxidase isozymes of various tissues in two wild potatoes grown under three different environmental conditions, and discuss the usefulness of leaf tissues in peroxidase isozyme analysis to identify species and to estimate interspecific differences.
Materials and Methods

Seeds of Mexican diploid species *Solanum pinnatisectum* Dun. (P. I. 186554) and South American diploid species *S. multidiscissectum* Hawkes (P. I. 230506) were supplied from Potato Introduction Station, Sturgeon Bay, Wisconsin, U. S. A. Plants were grown in a glass-house in autumn, 1981, to provide tubers with the same genotype (clone), which were stored at 4°C until use. On the 18th of September, 1982, tubers were planted in 3-inch pots and grown under the following three different environments; A) growth chamber with 12 hr day-length at 12,500 lux provided by fluorescent lamps and at constant day-night temperature of 23–14°C, B) the same as A except for uncontrolled natural day-light, and C) natural condition in glass-house.

At about 70 days after planting, six plants of *S. pinnatisectum* from each of the three environments were harvested and divided into various sections including leaf, stem, tuber and root, which were immediately frozen and stored at −28°C until use. As to *S. multidiscissectum*, three plants (80-day-old) from each of A and C environments were harvested and treated in the same way.

After thawing at room temperature for about 20 min, samples were centrifuged at 15,000 rpm for 15 min at 4°C, and clear sample solutions obtained in outer tubes of dual-structured centrifugation tubes were used for the following electrophoretic analysis.

Electrophoresis was performed by means of a discontinuous buffer system using a vertical-type slab polyacrylamide gel. A separating gel (138×100×1 mm) was made of 7.5% acrylamide, 0.2% N,N′-methylene-bis acrylamide (BIS) and 0.0288% N,N,N′,N′-tetramethyl ethylenediamine (TEMED) in 0.378 M Tris-HCl buffer (pH 8.9), and polymerized by 0.07% ammonium persulfate at 25°C for 3 hr. A stacking gel (138×25×1 mm) having 20 sample wells with 15 mm depth, was made of 3.75% acrylamide, 0.625% BIS and 0.0575% TEMED in 0.0617 M Tris-HCl buffer (pH 6.7) and photo-polymerized by 0.0005 % riboflavin for at least 30 min. Five µl sample solution was added into each well. Tris-glycine (pH 8.6, 0.6 g Tris, 2.88 g glycine/1000 ml) was used as anodic and cathodic electrode buffer, and bromophenol blue was added as a front marker at the concentration of 1.25×10⁻⁴ % in the cathodic buffer. Running was made under constant 200 V at 4°C and terminated when the front marker reached 95 mm from the origin of the separating gel.

Peroxidase staining method was according to *Yamamoto* and *Momoita* (1971) with minor modifications. A staining solution contained 100 mg o-dianisidine which was dissolved with 0.84 ml acetic acid and diluted to 70 ml with cold distilled water. The solution, while being stirred, was gently filled up to 100 ml with cold 0.2 M acetate buffer (pH 4.9), to which was added two drops of 30% hydrogen peroxide. The gel, after immersing in this solution for 15 min at 4°C while shaking, was transferred into cold 100 ml 1/15 M phosphate buffer (pH 7.0) containing 0.5 ml 30% hydrogen peroxide, and incubated at room temperature for 5–20 min until clear pattern became visible. The reaction was stopped by dipping the gel into 7% acetic acid. The stained gel was wrapped between wet cellophane sheets and dried by a hot-air drier. All solutions used were made immediately before staining.
Each band-intensity was estimated by the naked eyes and the following five degrees were assigned: 5=very strong and wide, 4=strong and sharp, 3=medium, 2=weak, and 1=very weak. To the absence of bands was assigned zero. A comparison of band expression was made based on mean band intensities among plants in the respective environments. Differences of isozyme pattern between individual plants were estimated by the Euclidean distance, \( d_{ij} \), which was calculated by the formula:

\[
d_{ij} = \left( \sum_{a=1}^{t} (X_{ia} - X_{ja})^2 \right)^{1/2},
\]

where \( t \) represents the number of isozymic bands compared, and \( X_{ia} \) and \( X_{ja} \) the respective intensities of band No. \( a \) of plants \( i \) and \( j \). The mean Euclidean distance among \( n \) plants grown under the same environmental condition was calculated as \( \left( \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} d_{ij}}{n^2-n} \right) \), while that between \( n_1 \) and \( n_2 \) plants under different environmental conditions as \( \left( \frac{\sum_{i=1}^{n_1} \sum_{j=1}^{n_2} d_{ij}}{n_1 n_2} \right) \).

Results and Discussion

Environmental variation and organ-specificity in \( S. \) pinnatisectum

\( S. \) pinnatisectum had 14 distinctive peroxidase isozyme bands (Fig.1(A)). Environmental and tissue-specific differences in these 14 bands are shown in Fig.2. In addition to these bands, some others with lower mobility were observed (Fig.1(A)), but excluded from the analysis because of uncertainty and unstability due to smeared background.

Environmental conditions did not greatly affect the expression of all bands, except for a band p12 in leaf tissues which expressed significantly lower activity under condition C.

Fig.1. Peroxidase isozyme patterns in various plant tissues in \( S. \) pinnatisectum (A) and \( S. \) multidosissectum (B). Plant parts examined are designated as follows; UL (=upper leaf), ML (=middle leaf), LL (=lower leaf), US (=upper stem), MS (=middle stem), LS (=lower stem), UgS (=underground stem), St (=stolon), BSt (=basal stolon), MSSt (=middle stolon, not shown here), TSt (=tip stolon), PT (=parental tuber), NT (=newly formed tuber), and R (=root).
Fig. 2. Peroxidase isozyme distribution in various plant tissues in *S. pinnatisectum* grown under conditions A (○), B (△) and C (●). Location of the respective bands from p1 to p14 are shown in Fig. 1(A). The axes of ordinate indicate band intensity. For designation of plant tissues see a legend of Fig. 1. Because of short stolon size, the whole stolon (MSt) was analyzed in A and B conditions.

(Fig. 2). On the other hand, considerable differences were observed in band intensity among various tissue sources including leaf, stem, tuber and root. Bands p2 and p3 expressed higher activity in the upper and younger leaf and stem, but very weak in underground tissues. Bands p4, p10 and p12 did not appear in most of the underground tissues, but expressed higher activity in the lower leaf and stem than in upper ones. The expression of bands p5, p7 and p9 was high in leaf and stem but low in parental tuber and root. Bands p6 and p8 appeared only in aboveground parts of the plant. Bands p11 and p13 expressed medium activity in all tissues except for root. It is particularly interesting that the presence and the intensity of each set of two or three bands, namely, bands p2 and p3, p4, p10 and p12, p5, p7 and p9, p6 and p8, and p11 and p13, seemed to depend on given tissue sources and be closely related to their physiological characteristics. A unique band, p14, was limited to the vicinity of the basal section of stem.

The intensity of bands p1, p5, p7 and p9 differed depending on tuber ages, and parental tubers showed lower activity than newly formed ones. The differential expression of these band intensities may be related to the physiological differences in these tissues.

**Environmental variation and organ-specificity in *S. multidissectum***

*S. multidissectum* had 12 distinctive peroxidase isozyme bands (Fig. 1(B)). Environ-
Peroxidase Isozymes in Various Tissues for Discrimination

Fig. 3. Peroxidase isozyme distribution in various plant tissues in *S. multidissectum* grown under conditions A (○) and C (●). Location of the respective bands from m1 to m12 are shown in Fig. 1(B). Refer to a legend of Fig. 1 for explanation.

Mental and tissue-specific differences in these 12 bands are shown in Fig. 3. Several minor bands with lower mobility (Fig. 1(B)) were excluded from the analysis because of uncertainty due to faint band expression.

Environmental conditions affected the band expression in this species. The differences in band intensity between conditions A and C were larger in leaf and stem than in underground tissues. Effects of different environments on the activity differed in each band: the activity of band m9 was higher in condition C than in condition A, whereas that of bands m1, m2 and m6 was weaker in the former condition.

Four sets of bands consisting of bands m1 and m2, m4, m6 and m8, m5 and m7, and m9, m10 and m11 seemed to behave in a similar manner in various tissues studied. A band m3 was present only in underground tissues.

**Interspecific differences revealed by species-specific isozyme bands**

The isozyme patterns observed in the two species and their intra- and inter-relationships are shown in a schematic representation (Fig. 4). Ten out of 16 bands were of similar mobility in *S. pinnatisectum* and *S. multidissectum*, among which pairs of bands p5 and m6, p7 and m8, and p6 and m7 seemed to show the same manner of expression even in different tissues (Fig. 2 and 3). Other four bands (p2, p10, p12 and p14) were specific for *S. pinnatisectum* and two bands (m1 and m3) for *S. multidissectum*. Of these six species-specific bands four were unique either to leaf tissue (p10 and p12) or to tuber tissue (p14 and m3) and the remaining two (p2 and m1) were common to both tissues (Fig. 2 and 3). It is thus apparent that leaf tissues provide a useful material as tuber tissues in species identification. A band m1 which was previously designated
as 'Band 10', has already been proved useful to distinguish the Mexican from the South American diploid species (Hosaka and Matsubayashi 1983).

Interspecific differences estimated by mean Euclidean distances

We examined the possibility to identify species and to estimate quantitative differences between species by means of a divided value, a mean Euclidean distance. Such values obtained using two major tissues, leaf and tuber, are shown in Table 1, which represent differences in the isozyme activities within and between species under different environmental conditions. The interspecific differences estimated based on the total bands amounted to 7.4~9.1 in leaf tissues and 7.4~10.5 in tuber tissues, which well exceeded the intraspecific differences in both species (1.5~4.2), except for those (3.7~6.5) obtained using leaf tissues of S. multidissectum.

This large variation in S. multidissectum may be due to a limited sample size and/or to a large response particularly

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Table 1. The mean Euclidean distance and its standard deviation within and between two diploid species grown under three different environments, estimated from total peroxidase isozyme bands

<table>
<thead>
<tr>
<th>Tissues&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Species</th>
<th>S. pinnatisectum</th>
<th>S. multidissectum</th>
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<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>Leaf</td>
<td>S. pin-</td>
<td>2.2±0.58</td>
<td>2.5±0.51</td>
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<td></td>
<td></td>
<td>(1.8±0.51)</td>
<td>(2.0±0.44)</td>
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<td></td>
<td>S. multi-</td>
<td>1.5±0.57</td>
<td>3.1±0.57</td>
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<td></td>
<td></td>
<td>(1.2±0.54)</td>
<td>(2.2±0.49)</td>
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<td></td>
<td></td>
<td>2.5±0.74</td>
<td>7.4±0.78</td>
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<td>(2.3±0.77)</td>
<td>(4.9±0.84)</td>
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<td></td>
<td>3.7±1.44</td>
<td>6.5±1.66</td>
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<td></td>
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<td>(3.6±1.37)</td>
<td>(5.9±1.54)</td>
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<td>6.2±1.81</td>
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<td></td>
<td></td>
<td>(6.2±1.77)</td>
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<tr>
<td>Tuber</td>
<td>S. pin-</td>
<td>1.7±0.86</td>
<td>3.1±0.93</td>
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<td></td>
<td></td>
<td>(1.6±0.88)</td>
<td>(2.9±1.00)</td>
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<td>S. multi-</td>
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<td>(3.1±0.98)</td>
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<td></td>
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<td>(2.4±0.89)</td>
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<td>3.3±1.69</td>
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<td>(3.1±1.71)</td>
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Note: Parenthesized figures indicate values obtained using common bands. Figures in Italic indicate values among plants under the same environmental condition.

1<sup>st</sup>: Samples used were leaves from middle part of the plant and newly formed tubers.
2<sup>nd</sup>: See explanation in Materials and Methods.
3<sup>rd</sup>: Data not obtained, because the number of newly formed tuber in this plot was only one.
of common bands to different environments in this species. Our results therefore suggest that mean Euclidean distances obtained based on isozyme activities of total bands provide a useful means to estimate interspecific differences in *Solanum* species.

**The usefulness of leaf tissues in peroxidase isozyme analysis**

In isozyme analysis of tuberous *Solanum* species, tuber tissues have been used most extensively. However, there are some species in which tuberization does not occur under any conditions, in the series *Conicibaccata* (Ross and Rowe 1969, and Hosaka unpublished). Thus, one has to look for other alternative tissues to find interspecific differences in these species as well as in non-tuberous species.

Leaf tissues of *S. pinnatisectum* and *S. multidiscsectum* had a total of thirteen and eleven bands, respectively, of which four were species-specific as already described. Tuber tissues provided six such unique bands. In addition to these species-specific bands, the divided values (mean Euclidean distances) gave large enough interspecific differences based on even common bands excluding species-specific ones (Table 1). Although the importance of tuber tissues cannot be denied, leaf tissues also provide another useful source material in isozymic studies in *Solanum* species. Moreover, the availability of leaf tissues should enable a more rapid determination of genotypes than using tuber tissues. It is, however, emphasized that because of somewhat larger environmental effects on isozyme activities in leaf tissues than in tubers donor plants should be grown under controlled environments and comparisons be made at the same growth stage.

**Acknowledgement**

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**Literature Cited**


パレインショ近縁種２種における諸機能中のバーオンキサーゼアイソサイム
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パレインショ近縁野生種２種について、まず、バーオキサーゼアイソサイムパターンに及ぼす種々の組織による差異、及び植物体の生育環境による差異を明らかにした。次に、各アイソサイムバンドの発現程度に基づいて得られた種間差異を表す推定値の有効性、及び分析材料としての薫の有効性について検討した。

メキシコ産原種二倍体 Solanum pinnatisectum 及び南米原産二倍体 S. multifidissimum を 3 つの異なる環境条件下（A、12,500 lux で 12 時間日長、昼温 23°C、夜温 14°C；B、秋期の自然日長で温度条件は A と同じ；C、ガラス室で秋作普通栽培）で栽培し、十分生育した植物体から個体毎に、種々の組織から試料を抽出した。平板型ポリアクリルアミドゲルを用いた不連続緩衝液系電気泳動法により、バーオキサーゼアイソサイムを分離・染色し、そのバンドの有無及び密度を肉眼によって 6 段階に分ける、異種環境及び組織間で比較した。さらに、各種差異を量的に推定するため、塊茎と葉を用いた場合のそれぞれについて、各バンドの発現程度を形質値として、供試全個体間にユーリッド距離を求めた。

S. pinnatisectum では、合計 14 本のバンド（P1 ～P14）が識別され（Fig. 1 (A)）、これらに対する環境の影響をほとんど認められなかった（Fig. 2）。パターンは供試器官により著しく異なり、特に、葉と塊茎の差異が著しく、葉の場合は、各器官の発現パターンが異なる。したがって、分析材料として最も有用なものは、葉と塊茎のいずれを用いても、種類差異が大きく、結果が指標的なバンドによって検出することができた。

2 種間で得られたユーリッド距離（d）を（Table 1）、分析材料として葉を用いると d=7.4～9.1、葉を用いると d=7.4～10.5、葉を用いた場合について、各種の差異を考慮して、結果を示す。これらはいずれも、同一組織内における個体間や異種環境による差異（d=1.5 ～6.5）に比べ十分大きいので、有効な種間差異と考えられた。

また、パレインショ近縁種のバーオキサーゼアイソサイム分析に従来塊茎が用いられていたが、葉を用いても、同一環境条件下で栽培された同じ生育段階の葉を比較すれば、既に示したように、種特異的なバンドの有無、あるいはユーリッド距離によって、有効な種間差異を検出できることが明らかとなった。

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