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Peroxidases in the Genus *Nicotiana*[1]

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Summary. Leaf peroxidases of 60 *Nicotiana* species, 19 cultivars, autopolyploids, interspecific hybrids, and amphidiploids have been compared by polyacrylamide gel block electrophoresis. At least 19 peroxidase bands, four cathodic and 15 anodic, were detected in the species which varied from two bands in *N. arentsii* to 12 bands in *N. tabacum*. The cultivars of the latter species failed to reveal any intraspecific variation. Specific difference and varietal resemblance in root peroxidase bands were also observed in nine species and 20 varieties analyzed. Zymograms from autopolyploids and amphidiploids appeared to be identical to that of diploid parents, suggesting that peroxidase banding patterns are independent of ploidy levels. An additive manner of parental peroxidase bands without hybrid enzyme formation in interspecific hybrids and the failure of dissociating peroxidases into subunits lead to a hypothesis that peroxidases in *Nicotiana* may be controlled by multiple, dominant genes and/or codominant alleles in chromosomes of different genomes. This is in keeping with the lack of relationship between ploidy level and peroxidase banding pattern. Also, species with different chromosome numbers shared many peroxidases in common that possibly reflects a residual homology of peroxidase loci among *Nicotiana* species. Some species classified in different sections or subgenera but having a common geographic center of origin, showed close similarities in peroxidase zymogram. Results suggest that these species may be closely related in phylogeny, and/or geographic isolation changes the peroxidase genes through mutation and selection. Based on leaf peroxidase zymograms of F_1 hybrids, a putative ancestor of *N. tomentosiformis* was the progenitor of *N. tabacum* at its inception.

Introduction

Peroxidase (EC 1.11.1.7) prevails in plants and has been implicated in disease resistance (Uritani and Stahmann, 1961; Yu and Hampton, 1964), respiration (Chance, 1954; Nicholls, 1965), IAA breakdown (Hinman and Lang, 1965), and many other physiological phenomena. Molecular heterogeneity of this enzyme demonstrable by electrophoresis suggests that peroxidases may differ in physiochemical properties. Shannon et al. (1966) and Kay et al. (1967) have investigated some of these aspects in horseradish root peroxidases. Multiple forms of an enzyme reflect evolutionary adaptions and can therefore be used as a chemical criterion for studying phylogenetic relationship and evolution of plant genera and species. Studies of esterase isozymes from tubers of *Solanum* species, interspecific hybrids, and haploids (Desborough and Peloquin, 1967), and of esterase and leucine amino-peptidase isozymes in the cotyledon of 15 *Phaseolus* species (West and Garber, 1967), are examples that demonstrated the usefulness of plant isozymes in chemotaxonomy.

Leaf peroxidase isozymes in *N. tabacum* cultivars have been compared in healthy and diseased tissues (Novacky and Hampton, 1968) and also in various ontogenetic stages (Sheen, 1969) and during senescence (Sheen and Calvert, 1969). These independent studies agreed with the fact that peroxidase zymograms of young, mature and senescent leaves differ only in banding intensity. Furthermore, Sheen and Calvert (1969) observed similar peroxidase zymograms developed from extracts of burley, flue-cured, and Turkish tobacco leaves. Hart and Bhatia (1967) studied peroxidase isozymes in leaves of six *Nicotiana* species grown under greenhouse condition and failed to detect any activity in *N. tabacum* and *N. glutinosa*; however, variations in peroxidase banding pattern of the other species were apparent. Isozymic comparison between species or varieties by means of Rf values and/or the testing of samples on the same gel column is technically difficult. This can be overcome by polyacrylamide gel block electrophoresis. Samples can be compared side by side on the same gel block and with others on different gel blocks by reference to common internal standards. By this technique, the present paper reports information on leaf peroxidases of 60 *Nicotiana* species, 19 *N. tabacum* cultivars and a number of autopolyploids, interspecific hybrids, and amphidiploids as they relate to each other and to evolution of the genus *Nicotiana*. Data on the root peroxidases of some species and cultivars are also reported herein.

Materials and Methods

Seeds of 62 presently recognized *Nicotiana* species and two formerly known species *N. palmeri* and *N. sanderae* were sown in sterilized sand-loam soil mixture (1:3 by volume) in 4-inch pots and covered with wet filter paper in early spring in a greenhouse. The filter papers were removed as soon as seedlings emerged. Four species, *N. noctiflora*, *N. corymbosa*, *N. attenuata*, and *N. cavicola*, failed to germinate and were not included in this study.

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Burley 21, Ky Iso 1 Ky 16, and Ky Iso 4 Hicks were used as the representatives of *N. tabacum*. Ten seedlings of each entry were transplanted into 2-inch pots and later to 8-inch pots. Greenhouse conditions were maintained at 27 °C during daytime, and 18 °C at night, providing a 16-hour photoperiod by fluorescent and incandescent lights. Axillary buds and inflorescences were removed as they emerged to maintain the plants in a vegetative state. Leaves from a group of five plants of each entry were harvested in the first week of July and bulked as a sample. Four weeks later, the second group of five plants was similarly harvested. A portion of deveined leaf sample was dipped into liquid nitrogen immediately after harvest prior to lyophilization, and the remaining portion was freeze-dried directly. Only the liquid nitrogen-treated samples were used for enzyme preparation. However, it was later found that there was no difference in peroxidase banding pattern between leaf samples with or without liquid nitrogen treatment.

To extend a survey of peroxidases within *N. tabacum*, the air-cured leaves from field grown plants of the following 20 varieties were studied: Ky Iso 1 Ky 16, Ky Iso 2 Ky 151, Ky Iso 3 Burley 37, Ky Iso 4 Hicks, Ky Iso 5 synthetic tobacco (an amphidiploid of *N. sylvestris* × *N. tomentosiformis* Kostoff), Ky Iso 6 Flue-cured 402, Ky Iso 7 Turkish, Burley 21, Ky 10 and Tobacco Introductions 97, 422, 446, 451, 572, 1013, 1310, 1334, 1349, 1352, and 1361 representing a random sample of burley, flue-cured, Turkish, Oriental, and possibly other tobbaco types grown on different continents. Experiments including the same 20 varieties and eight *Nicotiana* species, namely; *N. tomentosiformis, N. otophora, N. sylvestris, N. longiflora, N. rustica, N. pauciflora, N. trigonophylla,* and *N. palmeri,* were conducted in the fall in a greenhouse where temperature and photoperiod were maintained as described above. Seedlings grew in sterilizied sand in 4-inch pots. Forty pots of each entry were divided into four replications and the 28 entries within a replication were randomized. Seedlings were irrigated with one half-strength Hoagland's (Hoagland and Arnon, 1950) solution for three weeks and then changed to full-strength nourishment. One week later, the apical and axillary buds were repeatedly removed over a two-week period. Then, the leaf and root of each entry were harvested by replications and kept at $-90°$ until lyophilized.

Autopolyploids (4 n = 96 and 3 n = 72) of two *N. tabacum* varieties, Burley 21 and Ky 16, together with parents (2 n = 48) were grown in a greenhouse in the manner described above. Their seedling, mature and air-cured leaves were sampled from a randomized block experiment. Interspecific hybrids in the following cross combinations were also used in this investigation: (*N. sylvestris* × *N. tomentosiformis*), (*N. tomentosiformis* × *N. sylvestris*), (*N. sylvestris* × *N. otophora*), (*N. sylvestris* × Burley 21), (Burley 21 × *N. sylvestris*), (*N. tomentosiformis* × *N. otophora*), (Burley 21 × *N. tomentosiformis*), (Burley 21 × *N. otophora*), (*N. palmeri* × *N. tomentosiformis*), (*N. palmeri* × *N. otophora*), (*N. rustica* × *N. solanifolia*), (*N. amplexicaulis* × Burley 21), and (*N. gossei* × *N. glauca*). An amphidiploid of (*N. gossei* × *N. glauca*) (2 n = 60) was produced by colchicine treatment and included for comparison. The identity of autopolyploids, interspecific hybrids, and amphidiploids was based on morphological characters, pollen sterility and/or chromosome number in pollen mother cells. Green leaf samples of the interspecific hybrids were on an individual plant basis. Five or more plant samples were harvested at flowering stage except those involving *N. tomentosiformis* or *N. otophora* as parents which were collected during vegetative growth. All leaf samples were lyophilized immediately after harvest.

The preparation of peroxidase by ammonium sulfate fractionation has been previously described (Sheen, 1969). Polyacrylamide gel block electrophoresis in Buchler's vertical electrophoresis apparatus and the development of peroxidase bands with benzidine-2 HCl, guaiacol or catechol as electron donor in the presence of H_2O_2 (0.015%) are the same as for previous experiments (Sheen, 1969; Sheen and Calvert, 1969). About 100 μg protein of peroxidase fraction was enough to produce satisfactory intensity of bands in most species, whereas double or triple amount of protein was used for those species extremely weak in peroxidase activity. Usually at least three individual plants or replicated samples for each species or variety were tested in triplicate on different gel blocks and two *N. tabacum* varieties, Ky Iso 3 Burley 37 and Ky Iso 4 Hicks, were included in every gel block as references. Species closely resembling each other in zymograms were tested on the same gel block to verify the identity of individual peroxidases. Some experiments were conducted with a modified electrophoresis apparatus which has a sample aperture near the center of the block so that the stacking gel divides an 8 × 12 cm separating gel block on the cathodal end and a 16 × 12 cm block on the anodal end. This facilitates the detection of fast migrating cathodic bands.

Dissociation of peroxidase subunits by repeated freezing and thawing of crude leaf extracts and partially purified enzymes with or without NaCl (1 M) was attempted according to the method of Scandalios (1965). Zymograms obtained were compared with the original gel blocks.

Results

Leaf Peroxidases

Peroxidase zymograms developed from crude extracts of either fresh or lyophilized leaf tissues and from partially purified fractions showed no banding difference. However, the crude leaf extracts produced streaking on the gel after being stored at subzero temperatures, while the results were highly reproducible with partially purified peroxidases. This high reproducibility enabled one to compare a large number of samples during the course of this investigation. A survey of 19 *N. tabacum* varieties by the present technique revealed a similar zymogram which consists of two cathodic and ten anodic bands. This zymogram compared with those of certain *Nicotiana* species on the same gel block is shown in Fig. 1. When seedling, mature, and air-cured leaves of a given variety were compared, banding pattern varied in intensity only. In general, either on a protein content or dry-weight basis, mature leaf extracts showed strong banding intensity, whereas the others were weak. Bands D, F, G, and H were detectable in leaf extracts only when a large amount of enzyme preparation was applied. Regardless of the leaf ontogenetic stages, bands L and N usually were intense. Zymograms of the two burley varieties and their autopolyploids appeared identical, suggesting that ploidy level does not alter peroxidase banding pattern. Desborough and Peloquin (1967) reached a similar conclusion for the esterases from *Solanum* tubers.

Leaf peroxidase zymograms of 60 *Nicotiana* species developed with benzidine-2 HCl and H_2O_2 are illustrated in Fig. 2. The electron-donor benzidine-2 HCl and guaiacol produced identical peroxidase bands, but the benzidine-blue, an oxidative intermediate of benzidine, gave clearer bands than the brown product

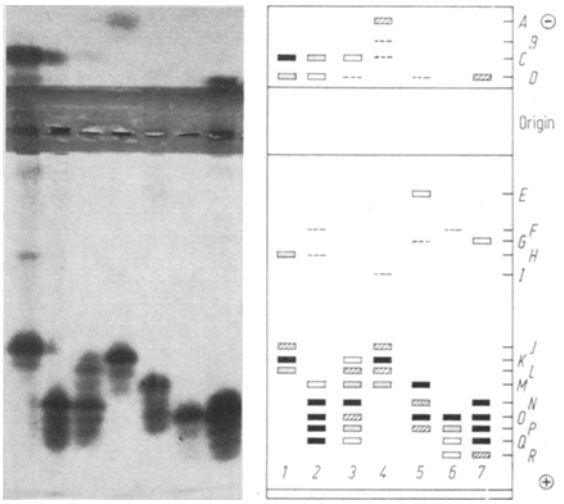

Fig. 1. Leaf peroxidases of some *Nicotiana* species on the polyacrylamide gel block developed with benzidine-2 HCl and H_2O_2. The numerals refer to species:

1. *N. sylvestris*;
2. *N. tomentosiformis*;
3. *N. tabacum* var. Ky Iso 4 Hicks;
4. *N. longiflora*;
5. *N. thyrsiflora*;
6. *N. paniculata*;
7. *N. simulans*

from guaiacol oxidation. When catechol was used, about 70 per cent of the slow moving peroxidases (bands E-I) was not detectable. However, all three electron donors developed bands J to S in common. Comparison between species will be primarily based on this group of peroxidases. With reference to the mobility of anodic pigments as 1.0, these ten anodic bands had relative mobility ranging from 0.55 to 0.94. It should be pointed out that *N. glauca*, *N. benavidesii*, *N. glutinosa*, *N. undulata*, *N. stockonii*, and *N. megalosiphon* possessed peroxidases differing slightly in mobility from bands J to S. In addition, *N. solanifolia*, *N. wigandioides*, *N. palmeri*, *N. acuminata*, *N. angustifolia*, and *N. pauciflora* either completely lacked or were very weak for these bands, although they all possessed at least one intense band moving slowly toward the anode or cathode.

In the section Paniculatae of subgenus *Rustica*, *N. glauca* and *N. solanifolia* differed strikingly in peroxidase banding pattern from the remaining five members. In the same subgenus, *N. thyrsiflora* exhibited four intense bands (M to P) while *N. rustica* had bands N to S. Neither of them resembled any Paniculatae member but most had bands C, F, and an intense O band in common. Of five species in section Tomentosae, four contained identical bands M, N, O, and P. Additional anodic bands in fast mobility were visible in *N. glutinosa* and *N. tomentosiformis*. *Nicotiana otophora* was the only species having bands J, K, and L. Zymogram of *N. tabacum* showed

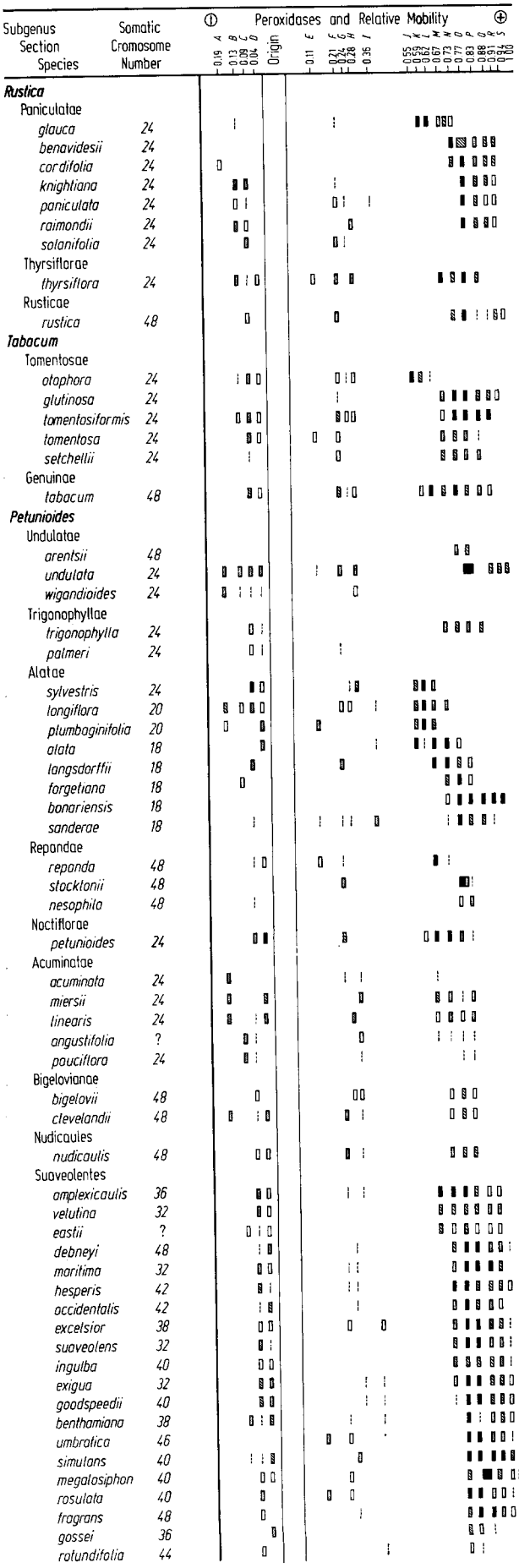

Fig. 2. Diagrammatic representation of leaf peroxidases in 60 *Nicotiana* species developed with benzidine-2 HCl and H_2O_2 on polyacrylamide gel blocks

greater similarity to that of *N. tomentosiformis* than any of the other Tomentosae species.

Peroxidase bands M, N, and O prevailed in most species in subgenus *Petunioides*. Bands P and Q were observed in some Australian species of section Suaveolentes but appeared only in three American species, *N. bonariensis*, *N. trigonophylla*, and *N. undulata*. In the section Undulatae, *N. arentsii* (2 n = 48) showed only two anodic peroxidases, whereas the 2 n = 24 species *N. undulata* and *N. wigandioides* had four identical cathodic bands. The two Trigonophyllae species relegated to synonymy according to Wells (1960) had bands C and D in common. But *N. trigonophylla* had four anodic bands, none of which was detected in *N. palmeri*. Among the eight Alatae species the peroxidases in the 2 n = 18 species migrated faster toward the anonde than those in the 2 n = 20 and 2 n = 24 species. The horticultural strain *N. sanderae*, which originated as a hybrid of *N. forgetiana* and *N. alata* (Smith, 1968), lacked the bands J, K, and L of *N. alata*. Fewer anodic peroxidases were observed among those species from sections Repandae, Bigelovianae, and Nudicaules; all of which have 48 somatic chromosomes. Moreover, *N. bigelovii*, *N. clevelandii*, and *N. nudicaulis* showed a close resemblance in anodic peroxidase pattern. With the exception of *N. acuminata* and *N. pauciflora*, the Noctiflorae and Acuminatae species had at least four fast moving anodic bands. Most of the 20 Australian species in the section Suaveolentes had cathodic bands C and D as well as anodic bands M to R. Three of them also possessed L band, whereas 12 species had M band. Only two and three anodic bands were recorded for *N. rotundifolia* and *N. gossei*, respectively.

Root Peroxidases

Nineteen *N. tabacum* varieties and an amphidiploid of (*N. sylvestris* × *N. tomentosiformis* Kostoff) (2 n = 48) yielded an identical root peroxidase zymogram that consists of two cathodic and three anodic bands. This supports the suspicion that Kostoff's hybrid is heavily introgressed with *N. tabacum* and is not a true amphidiploid. Based on electrophoretic mobility and comparison with tobacco leaf peroxidases on same gel blocks, root peroxidases can be identified as bands C, D, F, G, and H. Different zymograms were obtained in all eight species studied (Fig. 3). Cathodic bands C and D existed in these species but were very weak in *N. pauciflora* and *N. longiflora*. However, the latter two species had a strong A band and several weak bands that moved fast toward the cathode. Additional cathodic bands also appeared in the root extracts of *N. sylvestris*, *N. rustica*, and *N. trigonophylla*. The latter differed from *N. palmeri* by the presence of an intense band A. For anodic peroxidases, all eight species had bands F, G, and H in varying intensity. *N. otophora* and *N. tomentosiformis* had strong F band and relatively weak G and H bands. In contrast, band H was strong while bands F and G were weak in *N. sylvestris*. The major anodic peroxidases in *N. rustica* were the F and G bands. Both *N. palmeri* and *N. trigonophylla* exhibited four identical anodic bands, of which bands F, G, and H were highly intense. Five anodic peroxidases appeared in root extracts of *N. pauciflora* and 11 bands in *N. longiflora*. The four bands in fast anodal migration of the latter species seem to be the same as leaf peroxidase bands J, K, L, and M. Very weak anodic peroxidases of rapid mobility were also obtained from *N. sylvestris*, *N. rustica*, and *N. tabacum* by prolonged incubation of the gel block with substrates.

Leaf Peroxidases Among Interspecific Hybrids

Of the 16 interspecific hybrids, eight belong to hybrid combinations between *N. tabacum* var. Burley 21, *N. otophora*, *N. tomentosiformis*, and *N. sylvestris*. Figures 4 and 5 are illustratives of the zymograms with reference to burleys. The peroxidase banding patterns of these species have been described in previous figures. Zymograms of F_1 hybrids appeared as a combination of parental ones without hybrid band formation. The major bands in parental species were

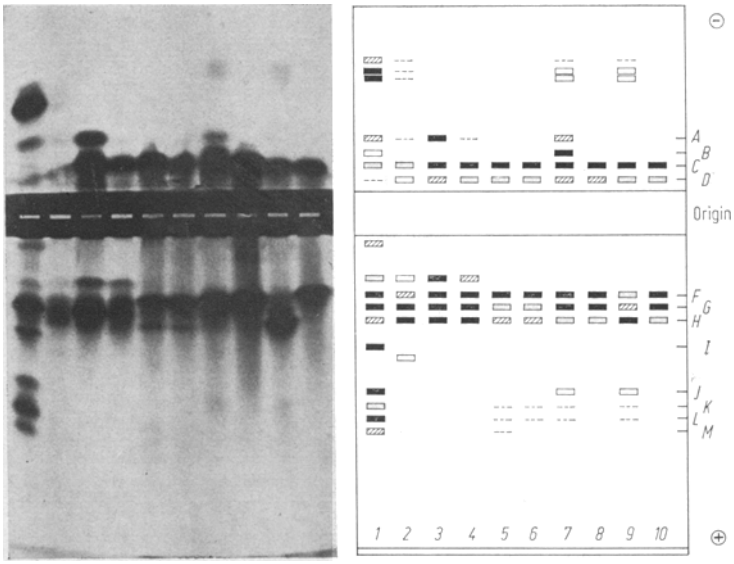

Fig. 3. Root peroxidases of some *Nicotiana* species developed with benzidine-2 HCl and H_2O_2 on the polyacrylamide gel block. The numerals refer to species:

1. *N. longiflora*;
2. *N. pauciflora*;
3. *N. trigonophylla*;
4. *N. palmeri*;
5. Ky Iso 5 synthetic (an amphidiploid of *N. sylvestris* × *N. tomentosiformis* Kostoff);
6. *N. tabacum* var. Burley 21;
7. *N. rustica*;
8. *N. tomentosiformis*;
9. *N. sylvestris*;
10. *N. otophora*

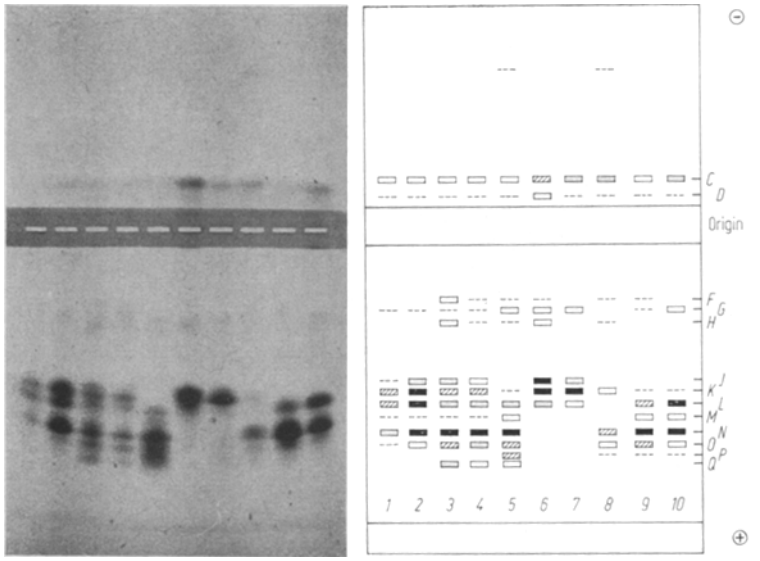

Fig. 4. Leaf peroxidases from the interspecific hybrids of crosses involving the putative progenitors of *N. tabacum* and varieties. The polyacrylamide gel block was developed with guaiacol and H_2O_2 and the numerals refer to the parentage of hybrids and varieties:

1. F_1 (Burley 21 × *N. sylvestris*);
2. F_1 (*N. sylvestris* × Burley 21);
3. F_1 (*N. tomentosiformis* × *N. sylvestris*);
4. F_1 (*N. sylvestris* × *N. tomentosiformis*);
5. *N. tabacum* var. Burley 21;
6. F_1 (*N. sylvestris* × *N. otophora*);
7. F_1 (Burley 21 × *N. otophora*);
8. F_1 (*N. tomentosiformis* × *N. otophora*);
9. F_1 (Burley 21 × *N. tomentosiformis*);
10. Ky Iso 5 synthetic (amphidiploid)

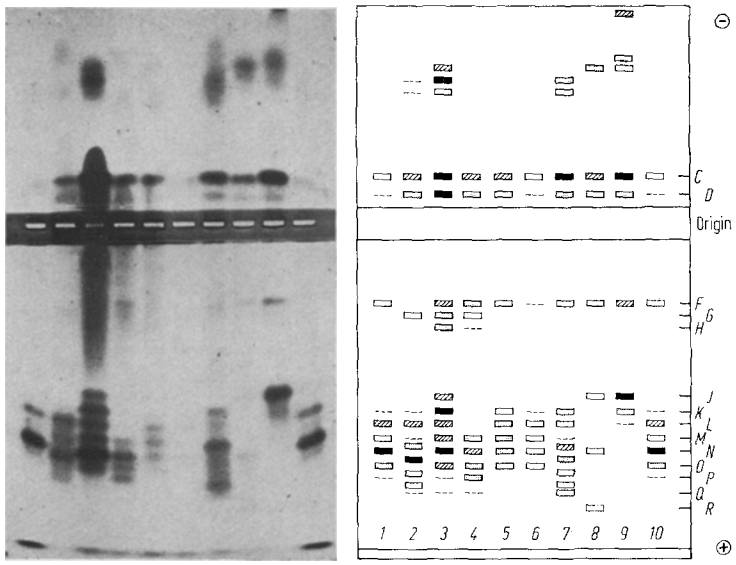

Fig. 5. Leaf peroxidases from interspecific hybrids and an amphidiploid developed with benzidine-2 HCl and H_2O_2. The numerals refer to the parentage of hybrids and varieties:

1. *N. tabacum* var. Ky Iso 3 Burley 37;
2. Amphidiploid of (*N. gossei* × *N. glauca*);
3. F_1 (*N. sylvestris* × Burley 21);
4. F_1 (*N. palmeri* × *N. tomentosiformis*);
5. F_1 (*N. amplexicaulis* × Burley 21);
6. *N. tabacum* var. Ky Iso 4 Hicks;
7. F_1 (*N. gossei* × *N. glauca*);
8. F_1 (*N. rustica* × *N. solanifolia*);
9. F_1 (*N. palmeri* × *N. otophora*);
10. *N. tabacum* var. Ky Iso 3 Burley 37

usually present at high intensities in their F_1 hybrids. Accordingly, the zymogram of (*N. sylvestris* × *N. tomentosiformis*) F_1 hybrids showed a much closer resemblance to that of *N. tabacum* than that of the cross *N. sylvestris* × *N. otophora*. One interesting exception was that in F_1 plants of Burley 21 × *N. otophora* the anodic bands, J, K, and L of *N. otophora* occurred in predominance over the fast migrating peroxidases of Burley 21. Unfortunately, the reciprocal F_1 hybrid was not available for comparison. Anodic bands of different mobility in *N. glauca* were present in the hybrids (2 n = 30) and amphidiploids (2 n = 60) of (*N. gossei* × *N. glauca*). Although there was slight contrast in banding intensity between the sterile F_1 hybrids and the fertile amphidiploids, the number of peroxidases remained the same.

Dissociation Experiments

Noticeable changes in peroxidase intensity have occurred in crude leaf extracts when they were subjected to repeated freeze-thawing treatment with or without the addition of NaCl (1 M). The same treatment did not alter the banding intensity of the partially purified enzymes. Figure 6 gives the results from the crude leaf extracts of *N. tabacum* var. Ky Iso 3 Burley 37, the progenitor species, and an interspecific hybrid. It is clear that by the present dissociation methods, tobacco leaf peroxidases showed no subunits. The production of subunits lacking in enzymic activity seems to be unlikely in view of the fact that the dissociation treatments have neither eliminated single bands, nor reduced banding intensity. An exception is that *N. otophora* did show bands A and B in extremely weak activity in the NaCl treatment. The most striking findings were the three intense bands migrating rapidly toward the cathode in *N. sylvestris*. The same three bands in weak intensity were present in *N. tomentosiformis* and *N. tabacum*. In addition, bands C, D, F, G, and H were very prominent as compared to the zymograms of crude leaf extracts without dissociation treatment or of partially purified peroxidase. Jansen et al. (1960) and Lipetz et al. (1965) have suggested that peroxidases may be either in a soluble form or bound to cell-wall or membrane. The question of whether these peroxidases in crude leaf extracts relate

to those bound to cellular membranes is being investigated.

Discussion

Tobacco peroxidase banding patterns are influenced by temperature (DeJong et al., 1968), hormonal treatment (Galston et al., 1968), and pathogenic infection (Veech, 1969), but are unchanged with respect to leaf ontogenetic stages (Sheen, 1969; Sheen and Calvert, 1969). The reproducibility of leaf peroxidase zymogram by polyacrylamide gel electrophoresis permits making valid comparisons within and between *Nicotiana* species so long as the plants are cultured under a similar environment. Electrophoretic variants in inbred strains of animals and plants have been reported (Shaw, 1965; Hubby and Lewontin, 1966). The present results of similar leaf and root peroxidases in *N. tabacum* varieties did not exclude the possibility of intraspecific variation. Since the prime concern of this investigation is the peroxidases occurring in high frequency in inbreeding populations and they are the basis for intra- and interspecific comparisons, individual plant variation in any variety or species was not studied. One should point out that the source of variation in *Nicotiana* wild species is probably biased as a result of genetic drift and selection, because they are inbreeding progenies of original collections which have been domestically cultivated for years. Nevertheless, the present results indicate that peroxidase variation is greater among species than within species. This stresses the usefulness of electrophoretic enzyme variants in chemotaxonomic studies.

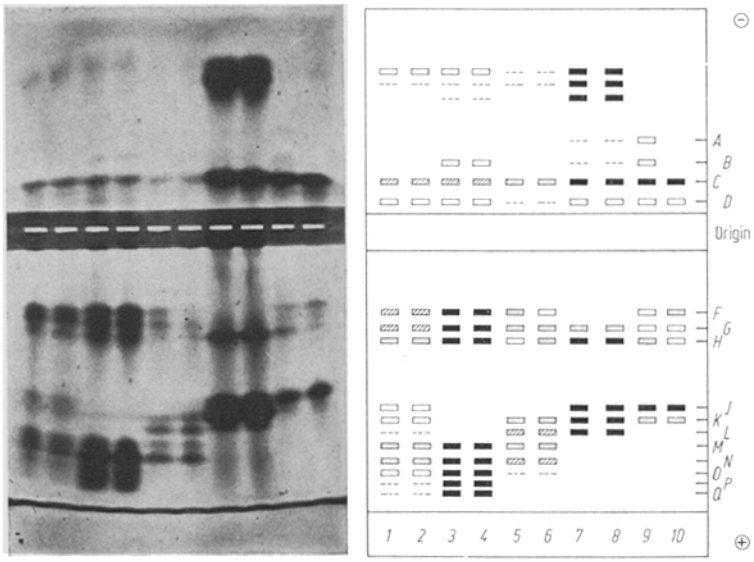

Fig. 6. Results from freeze-thawing and NaCl treatments of leaf peroxidases. The polyacrylamide gel block was developed in benzidine-2 HCl and H_2O_2; and the numerals refer to *Nicotiana* species and treatment:

1. F_1 (*N. sylvestris* × *N. tomentosiformis*), freeze and thawing treatment;
2. F_1 (*N. sylvestris* × *N. tomentosiformis*), freeze-thawing + NaCl treatments;
3. *N. tomentosiformis*, freeze and thawing treatment;
4. *N. tomentosiformis*, freeze-thawing + NaCl treatments;
5. *N. tabacum* var. Ky Iso 4 Hicks, freeze and thawing treatment;
6. *N. tabacum* var. Ky Iso 4 Hicks, freeze-thawing + NaCl treatments;
7. *N. sylvestris*, freeze and thawing treatment;
8. *N. sylvestris*, freeze-thawing + NaCl treatments;
9. *N. otophora*, freeze-thawing + NaCl treatments;
10. *N. otophora*, freeze and thawing treatment

Genetic analysis of peroxidase loci in *N. tabacum* was not pursued in the present study because of lacking intraspecific variation. Searching for allelic variation in inbreeding populations of *N. tabacum* and other species requires time and efforts and subjects to future experiments. Genic relationship between species could only be analyzed with interspecific F_1 hybrids from which segregating generation cannot be produced. Therefore, the interpretation of the present findings has to rest on the following arguments. First, single bands of peroxidase activity on a gel represent the product of two or more loci. *Nicotiana* species are in various ploidy and aneuploidy levels with haploid chromosome numbers 9, 10, 12, and 24. Clausen and Cameron (1944) have, however, postulated that 6 is the basic chromosome number for *Nicotiana*. If this is true, one may expect duplication and multiplication of ancestral peroxidase locus in the present species, especially those in high amphiploidy levels. Gene dosage effect, if present, may not be recognizable if it is only expressed in banding intensity which may fluctuate to a great extent due to environmental effect and physiological state of the plant. Second, molecular heterogeneity resulted from various mechanisms (Shaw, 1968) is likely to increase the degree of divergence rather than similarity among species, so that such biases, if any, will make the analysis of phylogenetic relation more conservative. Third, peroxidase bands of identical mobility in the same or different species reflect similar base sequence in structure genes which contribute polypeptide subunits to a migrating entity. The structure genes likely reside in the same chromosomes of a species but may be in chromosomes of different genomes when different species are concerned. Fourth, if the peroxidase loci of *Nicotiana* species are highly polymorphic, comparison between inbreeding populations of the present species would tend to exaggerate the differences between populations. That is to say, polymorphism tends to bias downward estimates of the genetic identity of peroxidase genes among species. Finally, since a large amount of leaf or root extract and protein fraction was used in checking those species weak in peroxidase activity, missing of certain bands reflects the change or

nearly total inactivation of the related structure genes.

The failure of dissociating peroxidases into subunits and forming hybrid enzymes in the interspecific hybrids indicates that peroxidases may be single molecular entities and are governed by multiple genes in the genus *Nicotiana*. The additive fashion of peroxidase zymograms in interspecific hybrids suggests dominant genes and/or codominant alleles at all loci involved for peroxidase synthesis. Consequently, ploidy level will not alter the peroxidase banding pattern as is the case in autopolyploids and amphidiploids. Moreover, addition and substraction of individual chromosomes may not drastically affect the peroxidase pattern because the occurrence of such events during the course of speciation usually take place in amphiploids. The minor banding contrast in Australian species, which vary greatly in chromosome number, represents a good example. The common mobility of peroxidase bands C, D, M, and N in most *Nicotiana* species may indicate that residual homologies persisted through the process of evolution in this genus. This coincides with Kehr and Smith's (1952) cytogenetic evidence that the genus *Nicotiana* lacks specific genome divergence, not chromosome number.

On the basis of peroxidase zymograms, one may probe the possible origin of *N. tabacum*. It is known that *N. sylvestris* is one of the progenitors and likely contributes cytoplasm to the original hybrid from which *N. tabacum* was derived (Cameron, 1965). There are different thoughts concerning the other progenitor in the Tomentosae group. Goodspeed (1954) favors *N. otophora*, while Gerstel (1960) believes that *N. tomentosiformis* is the closer relative of tobacco. Comparisons of the peroxidase patterns of interspecific hybrids derived from crosses between the possible progenitors and the modern tobacco should be a relevant approach since one can measure the degree of homology between *N. tabacum*, wild species, and hybrids at a molecular level irrespective of the physical arrangement of genes in chromosomes. The present results (Fig. 4) definitely favor *N. tomentosiformis* being the progenitor from the section Tomentosae. Another example of phylogenetic interest is the appearance of anodic band S in only two American species *N. rustica* and *N. undulata*. The latter has been suspected of being a wild ancestor of amphidiploid *N. rustica* (Eghis, 1940).

Several interesting points are worth mentioning in regard to peroxidases and geographic distribution of *Nicotiana* species. First, almost all Australian species have at least five anodic peroxidases in common. Goodspeed (1954) has postulated that *N. suaveolens* ($2n = 32$) and *N. debneyi* ($2n = 48$) represent the modern forms of the original migrants to Australia and that other species have developed as results of hybridization and introgression. Therefore, the close similarity in peroxidases of Australian species could be expected. Second, the endemic species such as *N. stocktonii* and *N. nesophila* which are restricted to the islands of the Revilla Gigedo group off the west coast of Mexico differ from other species in peroxidases. Third, *N. longiflora*, *N. plumbaginifolia*, *N. sylvestris*, *N. otophora*, and *N. glauca* have leaf peroxidase bands J, K, and L in common. Each of them originated in the eastern Andes Mountains of South America. Questions may be raised whether the above five species are closer phylogenetically than their present taxonomic standings as shown in Fig. 2. It could also be pointed out that the leaf peroxidase patterns of *N. otophora* and *N. glauca* completely differ from that of other members in their respective sections. However, on the basis of peroxidases alone, the present results are strongly biased to underestimate the actual amount of genetic homology among these species.

Zusammenfassung

Die Peroxidasen in Blättern von 60 *Nicotiana*-Spezies, 19 Sorten und einer Anzahl Autopolyploiden, interspezifischen Hybriden und Amphidiploiden wurden mittels Polyacrylamid-Gelblockelektrophorese verglichen. In den Species wurden mindestens 19 Peroxidasebanden festgestellt (4 kathodische und 15 anodische) mit einem Variationsbereich von 2 Banden bei *N. arentsii* bis 12 Banden bei *N. tabacum*. Sorten der letztgenannten Spezies ließen keine intraspezifische Variation entdecken. Spezifische Unterschiede und eine Sortenähnlichkeit der Wurzelperoxydase-Banden wurden auch in den untersuchten 9 Species und 20 Sorten beobachtet. Zymogramme von Autopolyploiden und Amphidiploiden schienen identisch zu sein mit denen der diploiden Eltern und lassen darauf schließen, daß die Muster der Peroxidasebanden von der Ploidiestufe unabhängig sind. Eine weitere Art elterlicher Peroxidasebanden ohne Hybrid-Enzymbildung bei interspezifischen Hybriden und die Unmöglichkeit der Trennung der Peroxidasen in Untereinheiten führen zu der Hypothese, daß die Peroxidasen bei *Nicotiana* durch multiple dominante Gene und/oder co-dominante Allele in den Chromosomen verschiedener Genome gesteuert werden. Das paßt gut zu der fehlenden Beziehung zwischen Ploidiestufe und Peroxidasebandenmuster. Auch Spezies mit verschiedenen Chromosomenzahlen hatten viele Peroxidasen gemeinsam, was möglicherweise auf eine Rest-Homologie der Peroxidase-Loci bei den *Nicotiana*-Spezies schließen läßt. Deutliche Ähnlichkeiten im Peroxidase-Zymogramm zeigten einige Spezies, die zwar in verschiedene Sektionen oder Subgenera eingestuft sind, aber ein gemeinsames geographisches Ursprungszentrum haben. Die Ergebnisse lassen vermuten, daß sich diese Spezies phylogenetisch nahestehen und/oder die geographische Isolation die Peroxidasegene durch Mutation und Selektion verändert hat. Nach dem Blatt-Peroxidase-Zymogramm

von F_1-Hybriden war ein vermutlicher Vorfahr von *N. tomentosiformis* der Ahn von *N. tabacum*.

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