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Polyphenol Content, Polyphenoloxidase and Peroxidase Activity in Certain *Nicotiana* Species, Varieties and Interspecific Hybrids*

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Summary. Eight *Nicotiana* species including the putative progenitors of *N. tabacum*, Kostoff's amphidiploid (*N. sylvestris* × *N. tomentosiformis*), and 19 cultivars have been compared for total polyphenols, polyphenoloxidase and peroxidase activity in the leaf and/or root by a small plant technique. Greater variations for these chemical constituents occurred in the species than in the cultivars. *N. tomentosiformis* was highest in polyphenol content. Root extracts contained more polyphenoloxidase than the leaf, but its peroxidase content may not exceed the concentration in the leaf. The Kostoff's amphidiploid tended to resemble more the low oxidase and polyphenol parent. An additional study based on mature green leaves of Burley 21, the progenitor species, and their F_1 hybrids confirmed the quantitative differences of these chemical constituents in the species. The magnitude of the heterosis appeared to be greater in the hybrids of *N. tomentosiformis* or *N. otophora* crossed to *N. sylvestris* than those between the Tomentosae members or involving Burley 21 as the parent. An exception was the hybrid Burley 21 × *N. tomentosiformis* which showed heterosis for oxidase activities.

Introduction

The importance of polyphenols in determining leaf quality of tobacco, *Nicotiana tabacum* L., was originally stressed by Shmuk and Semonova (1927). Later, Koenig and Dorr (1933) related the aroma of tobacco to the levels of chlorogenic acid. This polyphenolic compound together with rutin are oxidized by polyphenoloxidase (PPO) and peroxidase to form brown pigments during curing. Brown coloration has been a major criterion for good leaf quality in air-cured tobaccos. Recently, Schlotzhauer et al. (1967) reported that upon pyrolysis tobacco pigments yield a large amount of phenols which are implicated as the cocarcinogens in cigarette smoke. Pyrolytic products of pigments also contain polynuclear hydrocarbons that may be biologically active as carcinogens. Therefore, polyphenolic constituents in relation to tobacco quality bear a different version as far as the smoke and health problems are concerned.

It has been documented that the concentrations of soluble polyphenols, PPO and peroxidase are drastically reduced in air-cured leaves (Zelitch and Zucker, 1958; Sheen and Calvert, 1969a). The amount of brown pigments in cured leaves is proportional to the polyphenol content in green tissues as reported by Chateau and Albo (1966). Since polyphenols in brown pigments are not quantitatively measurable by current analytical methods, studies of polyphenol content and oxidase activity in green tissue would offer an indirect measurement of polyphenol metabolism and brown pigment accumulation for a given genotype. Quantitative variation in polyphenol content in the immature and mature green leaves of *Nicotiana* cultivars agrees with the results obtained from air-cured samples (Sheen and Calvert, 1969b). Burleys consistently contain less polyphenol than dark tobaccos. In addition to polyphenols, a large amount of PPO and peroxidase was detected in seedling leaves and roots (Sheen et al. 1969). However, little information is available on comparison of *Nicotiana* species and cultivars for polyphenol content and oxidase activity in replicated trials. Genetic diversity in species and varieties may provide desirable germ plasm for altering polyphenol content in commercial tobaccos. Furthermore, genomic contribution on polyphenol metabolism in interspecific hybrids, especially those from crosses between *N. tabacum* (genome designation: SSTT) and the probable progenitor species *N. sylvestris* and *N. tomentosiformis* or *N. otophora* (genome designation: S'S' and T'T', respectively), may give insights on the feasibility of genetic manipulation.

Heterotic response, either negative or positive, for certain agronomic characters and chemical constituents in the interspecific hybrids of the progenitor species crossed to *N. tabacum* has been reported by Mann and Weybrew (1958), Matzinger and Wernsman (1967), and Weybrew and Matzinger (1969), but information on polyphenols and oxidases is lacking. This paper presents data on the variation of these chemical traits in certain species and cultivars as well as on the phenotypic comparison of interspecific hybrids with parental species.

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Materials and Methods

A wide variation of photoperiodic response and morphological features among *Nicotiana* species creates problems for sampling leaves in a competitively physiological state. To minimize it, a small plant technique was employed on one experiment involving eight wild species and 20 cultivars, one of which, Ky Iso 5 synthetic, is an amphidiploid of (*N. sylvestris* × *N. tomentosiformis*, Kostoff). The cultivars representing various tobacco types consist of a sample of Tobacco Introductions (T. I. lines) collected by USDA from different continents and locally adapted varieties. Seeds were sowed in a sand and loam mixture (1 : 3 ratio) in a greenhouse in the fall. Five weeks after germination, 40 seedlings for each entry were transplanted into 3″ pots containing sterile sand and were randomly arranged on greenhouse benches into four replications. Each pot received an equal volume of nutrient solution daily. The greenhouse condition was maintained at 27° during the day and 18° at night, with supplementary incandescent and fluorescent lights three feet above the plants to provide a 16-hour photoperiod. Three weeks after transplanting, the apical buds were repeatedly removed for the following two-week period so as to induce physiological aging of the leaves. Then, the leaf and root of each entry were separately harvested on a replication basis. The fresh tissue was immediately lyophilized, ground into 40 mesh particles and stored at −90° until analysis.

A separate experiment was conducted in a greenhouse in the early spring. The entries in the experiment were Burley 21, three putative progenitor species, and six interspecific hybrids. Six plants of each entry, grown in soil in 6″ pots, were assigned to each of five replications in a randomized block design. Greenhouse conditions were maintained as similar to the small plant experiment. About three months after sowing, when Burley 21, *N. sylvestris*, and their hybrids were in flower-bud or flowering stage, the mature leaves of each entry were harvested on a replication basis. It should be mentioned that at harvest time *N. otophora*, *N. tomentosiformis*, and their hybrids were still in the stage of vegetative growth. The handling of leaf samples was identical to the procedures mentioned above.

Lyophilized leaf samples were analyzed for total polyphenols by Arnow's reagent as previously described (Sheen and Calvert, 1969b). The quantity of polyphenols is expressed as mg chlorogenic acid equivalent/g dry weight. PPO and peroxidase activity in crude tissue homogenates was spectrophotometrically determined by use of 3,4-dihydroxyphenylalanine and a mixture of hydrogen peroxide and *p*-phenylenediamine as substrates, respectively. The procedures of enzyme preparation and assay have been described elsewhere (Sheen and Calvert, 1969a).

Data were subjected to variance analysis, and percent heterosis was calculated as the percent deviation of F_1 hybrid from mid-parent.

Results

Quantitative variations of polyphenol content, PPO and peroxidase activity in seedling leaf and/or root extracts were highly significant ($P < 0.01$) among the species and cultivars analyzed (Table 1). A five-fold difference in polyphenol content existed among the species, whereas a three-fold contrast was observed in the cultivars. In all entries, *N. tomentosiformis* and *N. rustica* represented respectively the high and low extremes, but the amount in the latter species did not differ from that of some cultivars. Among the cultivars, six burleys Ky Iso 1 Ky 16, Ky Iso 3 Burley 37, Burley 21, Ky 10, T.I. 97, and T.I. 1349 contained less polyphenols than most of the dark tobaccos studied. These seedling results are in agreement with the previous findings based on mature and cured leaf samples (Sheen and Calvert, 1969b). Furthermore, the quantity in T.I. 1349 was significantly higher than that of the locally adapted burleys. Such a varietal difference also existed within the dark tobaccos. The amphidiploid Ky Iso 5 synthetic showed a polyphenol level similar to that of the low polyphenol parent *N. sylvestris*. A 36-fold difference in leaf PPO activity appeared in the species but only a one-fold range was observed among the cultivars. Roots contained a higher PPO activity than the leaf extracts in all entries. The high polyphenol burley, T.I. 1349, had the lowest level of root PPO. A close similarity in quantities of polyphenols and PPO in root and leaf extracts of *N. trigonophylla* and *N. palmeri* supports Wells' (1960) proposal that these two species relegate to synonymy. However, they differ significantly in peroxidase concentration. The variation of peroxidase activity in seedling leaves reached 42-fold in species and only seven-fold in cultivars. In comparison of leaf and root peroxidase activity, the pattern of high in leaf and low in root or vice versa is about equally divided among the 28 entries. At a cultivar level, the concentration of both oxidases in either plant parts could not differentiate tobacco types as does the polyphenol content. Some contrast, however, is evident in species by the fact that the leaf extracts of Tomentosae members contained high PPO but low peroxidase activity whereas an inverse relation occurred in *N. sylvestris*. The oxidase concentration in the amphidiploid Ky Iso 5 synthetic tends to resemble the low oxidase parents except that leaf peroxidase concentration approaches a mid-parent value. Correlation was not obtained when all possible combinations of five phenotypes were compared.

Table 2 summarizes the means of five replications for polyphenol content and oxidase activity in mature green leaves of species and interspecific hybrids. Their difference is statistically significant ($P < 0.01$). Among the four species, the comparative quantity of polyphenols agrees with seedling data that *N. tomentosiformis* is highest and Burley 21 lowest. Similarly, low leaf PPO and high leaf peroxidase concentrations remained apparent for *N. sylvestris*, while high PPO but low peroxidase activity was the case for the two Tomentosae species. For F_1 hybrids, the differences in three chemical constituents of the reciprocal crosses involving Burley 21 and *N. sylvestris* were nonsignificant, suggesting that cytoplasmic factors do not influence polyphenol metabolism. However, it should be mentioned that according to Cameron (1965), *N. sylvestris* is likely the contributor of cytoplasm in the original cross from which *N. tabacum* was evolved. Hence, five of the six interspecific hybrids investigated could possess *N. syl-*

Table 1. *Polyphenol content, polyphenoloxidase and peroxidase activity in seedling leaves and/or roots of certain Nicotiana species and varieties* [a]

Species and variety	Polyphenol content in leaf laminae (mg/g tissue)	Polyphenoloxidase activity (ΔO.D./100 mg tissue/min)		Peroxidase activity (ΔO.D./mg tissue/min)	
		leaf extract	root extract	leaf extract	root extract
N. rustica	5.52	.50	5.94	.30	1.52
N. otophora	15.34	5.39	10.88	.35	1.08
N. tomentosiformis	25.40	4.56	23.67	.36	2.51
N. trigonophylla	14.24	.15	7.88	3.91	4.41
N. palmeri	12.35	.14	4.03	.19	2.38
N. sylvestris	18.61	1.20	12.35	7.00	4.22
N. longiflora	11.54	.66	5.48	1.78	.97
N. pauciflora	8.61	1.00	7.58	.20	1.81
N. tabacum var.					
T.I. 97	7.50	2.37	15.60	7.00	.72
T.I. 422	16.62	2.32	28.76	1.15	4.40
T.I. 446	7.71	3.10	24.06	3.22	4.67
T.I. 451	7.67	3.28	27.39	4.44	3.36
T.I. 572	17.34	3.26	21.48	4.46	5.27
T.I. 1013	8.26	2.21	19.03	4.25	1.62
T.I. 1310	6.39	2.24	16.70	1.90	1.66
T.I. 1334	9.53	4.19	19.07	4.56	1.78
T.I. 1349	10.58	4.64	9.54	4.88	1.00
T.I. 1352	13.99	3.37	33.85	7.78	4.29
T.I. 1361	12.50	2.33	20.33	6.68	2.22
Ky Iso 1 Ky 16	6.00	2.68	15.02	3.19	5.75
Ky Iso 2 Ky 151	13.58	2.00	34.00	2.90	3.43
Ky Iso 3 Burley 37	5.48	2.88	15.56	6.89	3.35
Ky Iso 4 Hicks	13.22	2.38	13.84	8.05	1.60
Ky Iso 5 Synthetic	12.37	2.09	14.90	3.58	2.05
Ky Iso 6 F.C. 402	12.60	3.67	32.47	2.39	3.84
Ky Iso 7 Turkish	16.57	4.32	21.62	6.90	1.69
Burley 21	6.00	3.35	21.55	2.68	4.27
Ky 10	7.54	3.01	16.45	3.79	1.59
LSD .05	3.16	1.08	6.08	1.23	.82
.01	4.20	1.44	8.08	1.64	1.09

[a] Moisture-free.

Table 2. *Polyphenol content, polyphenoloxidase and peroxidase activity in mature green leaves of Nicotiana tabacum, its progenitors and their hybrids* [a]

Species and hybrid	Polyphenol content (mg/g tissue)	Polyphenoloxidase activity (ΔO.D./100 mg tissue/min)	Peroxidase activity (ΔO.D./mg tissue/min)
N. tabacum var. Burley 21	7.71	2.89	1.64
N. otophora	29.94	2.94	.67
N. tomentosiformis	43.95	3.93	.70
N. sylvestris	29.06	1.65	5.50
N. sylvestris × *N. tomentosiformis*	12.27	.74	3.39
N. sylvestris × *N. otophora*	11.38	1.96	2.07
N. sylvestris × Burley 21	19.62	2.51	3.71
Burley 21 × *N. sylvestris*	16.13	1.74	3.29
Burley 21 × *N. tomentosiformis*	25.45	2.04	.69
N. tomentosiformis × *N. otophora*	41.35	4.03	.73
LSD .05	4.99	1.08	.62
.01	7.18	1.45	.84

[a] Moisture-free.

vestris cytoplasm in common; consequently, any effect due to plasmogenes for polyphenol metabolism would be comparable. The percent heterosis of the chemical traits in six interspecific hybrids with respect to mid-parent values is given in Table 3. Significant, negative heterosis was obtained for polyphenol content in hybrids of two Tomentosae species crossed to *N. sylvestris*. No heterosis was exhibited in hybrids derived from crosses between the members of the Tomentosae section. PPO activity appeared in negative heterosis in two hybrid combinations involving *N. tomentosiformis* as a common parent. Negative

Table 3. *Percent heterosis of F_1 interspecific hybrids above mid-parent average*

Hybrid	Genomic constitution	Polyphenol content	Polyphenol-oxidase activity	Peroxidase activity
N. sylvestris × *N. tomentosiformis*	S'T'	−82.17**	−73.48**	28.71**
N. sylvestris × *N. otophora*	S'T'	−61.42**	−14.78	−33.77**
N. sylvestris × Burley 21	SS'T	6.69	10.57	3.92
Burley 21 × *N. sylvestris*	SS'T	−12.29	−23.35	− 7.84
Burley 21 × *N. tomentosiformis*	STT'	− 1.47	−40.18**	−41.02*
N. tomentosiformis × *N. otophora*	T'T'	11.91	17.15	5.80

* F_1 deviates from mid-parent at P = .05.
** F_1 deviates from mid-parent at P = .01.

heterosis for peroxidase activity showed in two crosses: (*N. sylvestris* × *N. otophora*) and (Burley 21 × *N. tomentosiformis*). The hybrid of (*N. sylvestris* × *N. tomentosiformis*) was the only one showing positive heterosis for peroxidase activity. In general, the Tomentosae members yielded more heterosis than *N. sylvestris* when crossed to *N. tabacum*, which is in agreement with Matzinger and Wernsman's (1967) observation. Furthermore, the degree of heterosis appeared in decreasing order of *N. tomentosiformis*, *N. otophora*, and *N. tabacum* var. Burley 21 when they were crossed to *N. sylvestris*. This is in keeping with the generalization that an increase of heterosis can be expected with increase of genetic divergence of parents. Therefore, the less heterotic response in (Burley 21 × *N. sylvestris*) hybrid may be due to a high degree of analogy in genomic constitution because *N. sylvestris* is believed to be the progenitor of *N. tabacum*.

Discussion

Based on 28 entries compared for polyphenol content and oxidase activity with the small plant technique, phenotypic variations were greater among species than within cultivars. A same order of rank was obtained for Burley 21 and three putative progenitor species when the chemical characters of immature and mature green leaves were compared. This suggests that the small plant technique is possibly applicable for polyphenol breeding in tobacco. This technique has been successfully employed for breeding burleys for varying alkaloid levels (Dr. R. B. Griffith, personal communication). It may be particularly advantageous when wild *Nicotiana* species, cultivars and interspecific hybrids varying considerably in photoperiodic response, morphological features and susceptibility to disease are compared. Such physiological and morphological differences and environmental variables might be minimized or controlled at an early stage of plant development.

Of interest is the extremely high PPO activity in seedling root but low in seedling leaf extracts of both species and cultivars. Although the presence of endogenous inhibitors in the leaf is possible, a similar trend has been observed in mature burley and flue-cured tobaccos when partially purified enzymes from different plant parts were assayed (Sheen, 1969). It is known that chlorogenic acid is the major polyphenol in tobacco roots and its synthesis takes place in the leaf (Zucker and Ahrena, 1958). Thus, the physiological role of root PPO in relation to chlorogenic acid metabolism in tobacco plants would merit further investigation. Lack of correlation between polyphenol content and oxidase activity in leaf extracts may be explained by spacial separation of enzymes and substrates at one hand and by independent, genetic entities of these chemical constituents at the other. Their quantity in green leaves would, therefore, determine the amount of brown pigments to be formed during the course of curing.

Excluding the hybrid of *N. tomentosiformis* × *N. otophora*, genomic constitution of interspecific hybrids can be symbolized as S'T', SS'T, and STT', although the T' genome of the two Tomentosae members may be different (Table 3). The difference in polyphenol and PPO content in S'T' and STT' may be attributed to ploidy level, gene dosage effect and/or hemizygous gene expression if the T' genome from the high polyphenol species *N. tomentosiformis* is the major contributor for genes responsible for this metabolic pathway. Heterotic response for peroxidase activity toward opposite directions in S'T' and STT' hybrids may reflect the degree of divergence for peroxidase genes in the parental species. With reference to peroxidase zymograms developed by polyacrylamide gel block electrophoresis as illustrated in a separate paper (Sheen, 1970), the great contrast of peroxidase banding patterns in *N. tomentosiformis* and *N. sylvestris* coincides with the positive heterosis of peroxidase activity in their hybrid. On the other hand, the close similarity of peroxidase zymograms between *N. tabacum* and *N. tomentosiformis* and between *N. sylvestris* and *N. otophora* corresponds to the negative heterosis of this oxidase in the respective F_1 plants. Nevertheless, the essence of gene action and genomic interaction in determining the magnitude of heterotic response for polyphenol and oxidase synthesis in *Nicotiana* is far from understanding.

It should be pointed out that the use of Burley 21 as the only representative of *N. tabacum* in the present study of hybrid performance is attributed to the fact that Burley 21 is very low in polyphenol content. Its involvement in the hybridization programs bears an objective that breeding lines lower in polyphenol

content than Burley 21 may hopefully be developed to meet the needs of minimizing smoking hazards due to polyphenols and their derivatives in tobacco leaves. A different result would probably be obtained if a dark tobacco is used.

Zusammenfassung

Acht *Nicotiana*-Spezies einschl. der vermutlichen Eltern von *N. tabacum*, Kostoffs Amphidiploid (*N. sylvestris* × *N. tomentosiformis*) und 19 Sorten wurden auf ihren Gehalt an Polyphenolen und auf die Polyphenoloxidase- und Peroxidaseaktivität in den Blättern und/oder Wurzeln in einem Pflanzen-Kleinversuch verglichen. Bei den Spezies ergaben sich größere Abweichungen für diese chemischen Substanzen als bei den Sorten. *N. tomentosiformis* hatte den höchsten Polyphenolgehalt. Wurzelextrakte enthielten mehr Polyphenoloxidase als Blattextrakte, der Peroxidasegehalt dürfte aber die Konzentration in den Blättern nicht übersteigen. Kostoffs Amphidiploid schien mehr dem Elter mit niedriger Oxidaseaktivität und niedrigem Polyphenolgehalt zu ähneln. Eine weitere Untersuchung anhand von ausgewachsenen grünen Blättern von Burley 21 als Elter-Spezies und ihren F_1-Hybriden bestätigte die quantitativen Unterschiede in diesen chemischen Bestandteilen der Spezies. Das Ausmaß der Heterosis schien stärker in den Hybriden von *N. tomentosiformis* oder *N. otophora* bei Kreuzung mit *N. sylvestris* als innerhalb der Tomentosae oder bei Einbeziehung von Burley 21 als Elter. Eine Ausnahme bildete die Hybride aus Burley 21 × *N. tomentosiformis*, die bezüglich der Oxidaseaktivität Heterosis zeigte.

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