Levels of Chlorogenic Acid in Tobacco Cultivars, Healthy and Infected with Thielaviopsis basicola

S. K. Gayed and Nestor Rosa

Plant Pathologist and Plant Physiologist, Agriculture Canada, Research Station, Delhi, Ontario, Canada N4B 2W9.

Contribution No. 88, Agriculture Canada, Research Station, Delhi, Ontario.

The authors wish to thank D. A. Brown for excellent technical assistance.

Accepted for publication 11 April 1975.

ABSTRACT

In 3- and 4-week-old tobacco seedlings higher levels of chlorogenic acid (CA) were recovered from the immune cultivar Burley 49 than from the susceptible White Mammoth and the tolerant cultivars Hicks Broadleaf and Delhi 34. At the 5-, 6-, and 7-week-old stages, CA was higher in both root and leaf of Burley 49 compared to White Mammoth. At these stages, CA content of the roots of all cultivars was 20 to 25 times higher than that of the leaf. Infection of tobacco by *Thielaviopsis basicola*, either under controlled greenhouse conditions or in the field, resulted in the accumulation of CA not only in the root but also in the leaf. CA accumulation increased with the increase of tolerance to black root rot. The significance of such increase as a possible defensive mechanism is discussed.

Phytopathology 65:1049-1053

Black root rot of tobacco caused by the soil-borne fungus *Thielaviopsis basicola* (Berk. & Br.) Ferr. is one of the common diseases of tobacco in Canada. Black lesions formed on roots of infected plants induce their stunting early in the season, and a delay in their maturity.

Crop plants resistant to certain fungal diseases, as compared with susceptible ones, have been reported to possess higher levels of chlorogenic acid (CA) and other orthodihydric phenols. Examples of these diseases are: potato scab (13), potato verticillium wilt (16, 20), brown root rot of apples (2), coffee (8) and cacao canker (3), and red rot of sugarcane (4).

It has also been established that disease infection increases the polyphenolic content in many host plants. This phenomenon has been discussed and reviewed by several authors during the past decade (7, 14, 15, 21, 22). Similarly, Hampton (11) noticed a rapid increase in CA in carrot tissue inoculated with *T. basicola*. Polyphenols were also discussed as a probable factor in tobacco resistance to black root rot (10).

Phenolic compounds in tobacco have been regarded as potentially important to tobacco smoke flavor (19). Chlorogenic acid forms about 85% of the soluble polyphenols in the leaf (23) and up to 7.7% of leaf dry weight (19). Leaf polyphenols contribute to the phenolic fraction of cigarette smoke which possesses tumor-

promoting activity (27). Therefore, the relationship between disease resistance or infection and the accumulation of polyphenols in tobacco leaves is of vital importance. The aim of the present paper is to trace the change in the CA content of both root and leaf of healthy and infected tobacco cultivars at different stages of growth. These cultivars vary widely in their level of resistance to black root rot.

MATERIALS AND METHODS.—Chlorogenic acid content was determined on healthy seedlings of tobacco *(Nicotiana tabacum* L.) cultivars with different degrees of resistance to black root rot, and on healthy and infected plants raised either in the greenhouse or in the field. Conditions under which plants were raised, as well as methods of sampling at the different stages of growth, are described below in detail:

Healthy seedling stage.—Seeds of tobacco cultivar White Mammoth (susceptible to *T. basicola*), Hicks Broadleaf (moderately tolerant), Delhi 34 (highly tolerant), and Burley 49 (containing the *N. debneyi* immunity factor against black root rot) were sown in a steam-sterilized, fertilized, potted mixture of sand and muck and kept in the greenhouse at a temperature between 22-25 C. Seedlings of all cultivars were dug 3 and 4 weeks after seeding when they were at the 2-leaf and 3-leaf stages, respectively. Adhering soil particles were removed. Shoots of the 3- and 4-week-old seedlings had 2 and 3 leaves, respectively. Entire seedlings were analyzed for CA content since root development was scanty at both stages. Five single seedlings of each cultivar were analyzed for CA, and similar samples were used for moisture determination.

Seedlings were dug after 5, 6, and 7 weeks, thoroughly cleaned, and divided into roots and shoots at the point of transition. Chlorogenic acid was determined separately on roots and leaves.

Healthy and infected mature plants raised under greenhouse conditions.—The fluc-cured tobacco cultivars White Mammoth, Hicks Broadleaf, Delhi 34, as well as the immune species *N. debneyi*, were grown in a steam-sterilized mixture of sand and muck, either infested or noninfested with *T. basicola*. Soil inoculation with *T. basicola* was performed by thorough mixing of the soil with the endoconidia of the fungus at 10,000 endoconidia per gram soil. For each cultivar or species, 20 seedlings were transplanted singly in pots; 10 pots contained noninfested and the other 10 infested soil. Each pot received 20 g of the 2-12-16 fertilizer. Since black root rot is favored by relatively lower temperatures, the temperature in the greenhouse was regulated between 20 and 22 C. Twelve weeks after seeding, at the flowering stage, all plants were removed from the pots, and their roots were washed and longitudinally split in two halves, one for CA analysis and the other for moisture determination. Two leaf disks 12.5 mm in diameter, were excised from each side of the midrib of the fourth leaf. They were cut from comparable positions of the lamina and midway between the tip and base of the leaf. Two disks were used for CA, and the other two for moisture determination.

Healthy and infected plants raised in the field.—Seedlings of tobacco cultivars White Mammoth, Hicks Broadleaf, Delhi 34, and Burley 49 were transplanted in two fields about 8 km apart. One field was a poorly drained sandy loam soil under continuous tobacco-tobacco rotation and heavily infested with *T. basicola*. In the other field the soil was well drained Fox loamy sand; the recommended rate of fumigation and tobacco-rye rotation were being followed. Plants grown in this field were free from black root rot and other disease infestations. Regular cultural practices were followed in both fields. At harvest time, a representative sample of ten plants from each cultivar was carefully dug from each field. Duplicate 400-mg samples of healthy and diseased fibrous root were used for CA and moisture determination, whereas disks cut from the seventh harvestable leaf from the base of the plant were used for the same purposes.

Effect of chlorogenic acid on Thielaviopsis basicola in vitro.—A synthetic medium, suitable for the growth of *T. basicola* contained the following: sucrose 10 g, asparagine 1.0 g, KH_2PO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Ca(NO_3)_2 \cdot 4H_2O$ 0.1 g, thiamine HCl 1.0 mg, water 1 liter, and agar 20 g. Chlorogenic acid (Sigma Chemical Co. #3878) was incorporated in the medium at a rate of 0, 10, 100, or 1,000 μg/ml which was poured in petri dishes, inoculated with standard mycelial disks of the Harrow strain of *T. basicola*, and incubated at 25 C for 1 week. Mycelial spread and cultural characteristics were recorded daily. In another trial, sterilized filter paper disks (6 mm in diameter) soaked in 0, 100, or 1,000 μg/ml aqueous CA solution were placed on the solid synthetic medium at about 1.5 cm from the edge of a *T. basicola* growth. The subsequent growth pattern of the fungus as influenced by the phenolic compound was observed for 1 week.

The effect of CA on the germination of *T. basicola* endoconidia was investigated by suspending the endoconidia in 0, 10, 100, and 1,000 μg/ml CA solutions. After 24 hours, the germinated conidia in 6 microscopic fields, each containing ~25 endoconidia, were counted and the length of germ tubes in these fields were measured using a standardized ocular micrometer.

Influence of chlorogenic acid on the induction of necrotic lesions on tobacco leaf by Thielaviopsis basicola.—Eight leaf disks of the cultivar Hicks Broadleaf were inoculated with *T. basicola* endoconidial suspension (50,000 endoconidia per ml water) as previously described (10). A set of four disks were floated on water in petri dishes and another set was floated on 100 μg/ml CA solution. The two sets were incubated at room temperature for 5 days. Necrotic areas formed on each disk were subsequently rated on a scale 0-10; 0 = no lesions and 10 = disk covered with lesions (10).

Chlorogenic acid determination.—The method described by Zucker and Ahrens (28) for the extraction and determination of CA in tobacco tissue was adopted in the present study. Chlorogenic acid content was calculated both on fresh and dry weight basis. Since results were comparable in both cases, only data based on fresh weight basis are presented in this study.

RESULTS.—*Chlorogenic acid in healthy seedlings of susceptible and immune cultivars.*—At the 3-week and 4-week stages of growth there was no significant difference in the level of CA in the seedlings of the susceptible cultivar White Mammoth as compared with that of the moderately tolerant Hicks Broadleaf and the highly tolerant Delhi 34 (Fig. 1). There was, however, a

significantly lower amount of CA at both stages in those cultivars as compared to the immune cultivar Burley 49. This difference was more pronounced at the 4-week than the 3-week stage.

At the 5-, 6-, and 7-week old stages, levels of CA were 25-50 times higher in the root than in the leaf (Fig. 2). At these stages, leaves of cultivar Burley 49 has a higher level of CA than those of the tolerant Hicks Broadleaf and

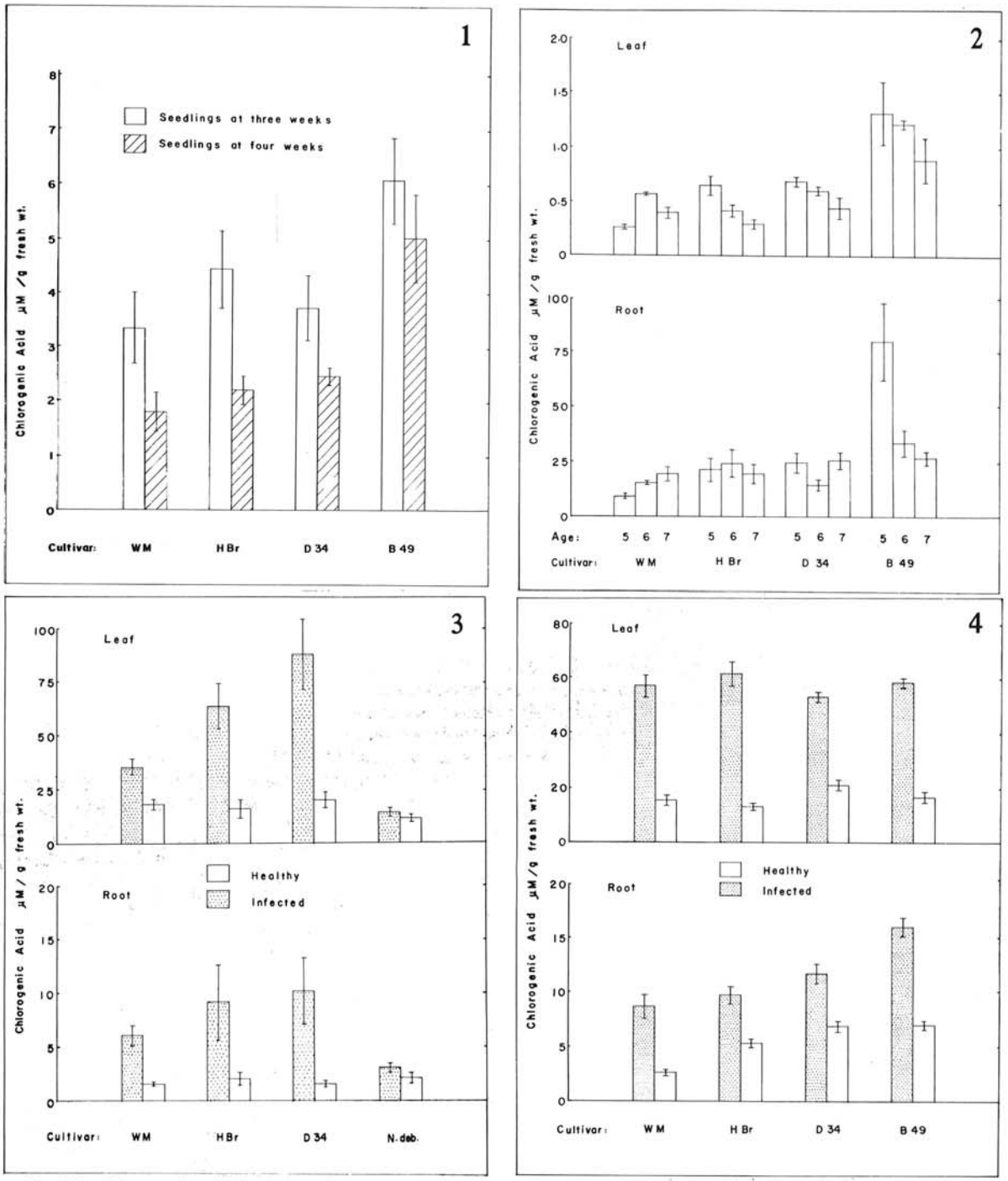

Fig. 1-4. Chlorogenic acid levels (µmoles per gram) in various-aged plants of four tobacco cultivars with different degrees of resistance to *Thielaviopsis basicola* [susceptible, White Mammoth (WM); moderately tolerant, Hicks Broadleaf (HBr); highly tolerant, Delhi 34 (D34); and immune, Burley 49 (B49)]: **1)** entire seedlings 3 and 4 weeks old, **2)** roots and leaves of 5-, 6-, and 7-week-old seedlings, **3)** roots and leaves of 13-week-old plants [the immune *Nicotiana debneyi* (N. deb.) was used instead of B49] grown under greenhouse conditions either in noninfested soil or soil infested with *T. basicola* (10,000 endoconidia per gram of soil), and **4)** roots and leaves of mature (15-week-old) plants grown in field soil noninfested or heavily infested with *T. basicola*.

Delhi 34, or the susceptible White Mammoth. At the 5-week stage, CA content of roots of Burley 49 was significantly higher than that of other cultivars and considerably dropped thereafter, but were still higher at the 6- and 7-week stages than that of the susceptible White Mammoth.

Chlorogenic acid in healthy and infected greenhouse plants.—At the flowering stage of tobacco cultivars tested, CA content of root tissue was considerably lower, and that of the leaf tissue much higher as compared with the seedling stages (Fig. 2, 3). Infection with black root rot induced a significant increase of CA in the roots of White Mammoth, Hicks Broadleaf, and Delhi 34, and increased the CA content of the leaf (Fig. 3). Such increases were most pronounced in the more tolerant cultivar Delhi 34, and decreased with the increase of susceptibility to black root rot. Infected plants were stunted as compared with healthy plants. Stunting increased with the higher susceptibility of the tobacco cultivar. No difference in vigor was noticed between plants of the immune *N. debneyi* raised in noninfested or infested soil and no lesions were formed on the root system. In both cases, levels of CA were comparable due to the lack of infection in *N. debneyi* plants.

Chlorogenic acid plants grown in infested or non-infested field.—A higher level of CA was detected in roots and leaves of tobacco cultivars, including Burley 49, raised in the infested field than in the healthy plants raised in the noninfested field (Fig. 4). Although Burley 49 has the *N. debneyi* immunity factor, under the prevailing experimental conditions several disease lesions of black root rot were noticed on the root system. This indicates the possible infection of this cultivar, which explains the increase in CA content in its tissues.

Increase in CA content due to the black root rot infection was more pronounced in the leaves than in the roots and ranged between 200 to over 350 percent in the different cultivars studied.

Chlorogenic acid and T. basicola in vitro.—Chlorogenic acid incorporated in synthetic medium from 10 to 1,000 μg/ml had no effect on the radial growth of the fungus. Filter paper disks soaked in 100 and 1,000 μg/ml CA solution and placed at 1.5 cm from the edge of *T. basicola* mycelium grown on synthetic medium did not influence the growth pattern of the fungus. The fungus spread under and over the treated disks and both endoconidia and chlamydospores were formed upon and around the disks.

In spore germination tests, 1, 10, and 100 μg/ml CA in water had no adverse effect on the latent period of endoconodial germination which was about 3 hours. The percentage germination after 24 hours was 33, 27, and 36 percent (31 percent for the untreated control). At that stage the length of germ tubes ranged between 795 and 840 μm. At 1,000 μg/ml CA in water there was a reduction since the percentage germination was 11 percent and the length of germ tube was 650 μm.

Chlorogenic acid and formation of necrotic lesions on tobacco leaf by T. basicola.—Leaf disks inoculated with *T. basicola* and floated on distilled water or 100 μg/ml CA solution in water showed no significant differences in the density or size of necrotic lesions; the average rating was 7.0 for both treatments.

DISCUSSION.—Tobacco seedlings are more susceptible to black root rot than mature plants (17), and levels of CA were higher in roots of mature plants than in those of seedlings. Therefore, in healthy tobacco plants CA is not correlated with resistance. This is further confirmed by the fact that Burley 49 seedlings up to 5 weeks of age were found to be susceptible to the disease. Resistance started to build up from that stage on (Gayed, *unpublished*) with a concurrent drop of CA levels in the roots. Higher levels of CA recovered from healthy Burley 49 seedlings might be correlated with the burley type as compared with the flue-cured type of tobacco. Sheen (24) found that amounts of polyphenols in the mature green leaf are not correlated with disease resistance.

Different stresses such as cold, mineral deficiency, or radiation result in increased polyphenols in tobacco plants (26). Similarly, infection of roots with *T. basicola* increased the amount of CA in all tested cultivars, not only in the roots, but also in the leaves. Hampton (11) recorded a marked increase in CA content of carrot root slices following inoculation with *T. basicola*, and showed that CA was toxic to the fungus. The present study indicates that CA is nontoxic to the growth, but suppresses germination of the endoconidia. Such suppression is irrelevant to the protection mechanism since resistance of tobacco to black root rot takes place in the post-penetration phase (6, 12).

High accumulation of phenolic compounds due to formation of necrotic spots at points of infection of tobacco with TMV (9) suggested that those compounds might be involved in the process of lesion formation. Lesion formation was noticed equally on tobacco leaf disks infected with *T. basicola* and floated either on water or CA solution, indicating that excess of the polyphenol is not directly related to lesion formation.

Tobacco tolerance to black root rot is polygenic in nature (5). The higher accumulation of CA in roots of the highly tolerant cultivar Delhi 34, and Hicks Broadleaf, compared to those of the susceptible White Mammoth, might be a manifestation of a defensive mechanism. It is known that tobacco roots respond to *T. basicola* infection by the formation of a periderm, and that such a response is faster in tolerant than in susceptible cultivars (6, 18). Polyphenols, including CA, lead to the formation of lignin by the action of peroxidase (1). Cork cells which are formed as barrier against further pathogen advance contain lignin in addition to suberin.

Early in the season, and under moist and cool conditions, tobacco plants infected with black root rot are stunted, but they reach near normal size at harvesting time although their maturity is somewhat delayed compared to that of healthy plants. In this study, green leaves of infected plants were found to contain triple the amount of CA found in healthy plants. There is evidence that flue-curing does not significantly change the polyphenol content of the leaf (27) and that CA is one of the leaf polyphenols that can contribute to the phenolic fraction of the smoke (25). Since it was shown that the phenolic fraction of cigarette smoke possesses tumour-promoting activity (27), it is essential to consider this fact in marketing tobacco from areas infected with black root rot. Emphasis should also be placed on studies related to the effect of other diseases and stresses on the phenolic content of tobacco leaf.

LITERATURE CITED

1. BROWN, S. A. 1966. Lignins. Annu. Rev. Plant Physiol. 17:223-244.
2. BRYDE, R. J. W., A. H. FIELDING, and A. H. WILLIAM. 1960. The role of oxidized phenols in the varietal resistance of apples to brown root rot. Pages 95-99 *in* J. B. Pridham, ed. Phenolics in plant health and disease. Permagon Press Inc. Oxford, England. 131 p.
3. CAPRILES DE REYES, L., E. SCHULZ SCHOMBURGK, and A. MUÑOZ. 1968. Chlorogenic acid content of different cacao varieties and its relation to Ceratocystis fimbriata. Rev. Appl. Mycol. 47:24 (Abstr.).
4. CHIRANJIVI RAO, K., T. N. KRISHNA MURTHY, E. LALITHA, and V. K. RAJALAKSHMI. 1968. Phenols in relation to resistance of sugarcane varieties to red rot disease. Curr. Sci. (Bangalore) 37:532-534.
5. CLAYTON, R. E. 1953. Control of tobacco disease through resistance. Phytopathology 43:239-244.
6. CONANT, G. H. 1927. Histological studies of resistance in tobacco to Thielavia basicola. Am. J. Bot. 14:457-480.
7. CRUICKSHANK, I. A. M., and D. R. PERRIN. 1964. Pathological function of phenolic compounds in plants. Pages 511-544 *in* J. B. Harbone, ed. Biochemistry of phenolic compounds. Academic Press, New York. 618 p.
8. ECHANDI, E. and C. E. FERNANDEZ. 1962. Relation between chlorogenic acid content and resistance to coffee canker incited by Ceratocystis fimbriata. Phytopathology 52:544-546.
9. FRITIG, B., M. LE GRAND, L. HIRTH, and G. OURISSON. 1972. Biosynthesis of phenolic compounds in healthy and diseased tobacco plants and tissue cultures. Hoppe – Seyler's Z. Physiol. Chem. 353:134-135.
10. GAYED, S. K. 1969. The relation between tobacco leaf and root necrosis induced by Thielaviopsis basicola and its bearing on the nature of tobacco resistance to black root rot. Phytopathology 59:1596-1600.
11. HAMPTON, R. E. 1962. Changes in phenolic compounds in carrot root tissue infected with Thielaviopsis basicola. Phytopathology 52:413-415.
12. JEWETT, F. L. 1938. Relation of soil temperature and nutrition to the resistance of tobacco to Thielavia basicola. Bot. Gaz. 100:276-297.
13. JOHNSON, G., and L. A. SCHAAL. 1957. Chlorogenic acid and other orthdihydric phenols in scab-resistant Russet Burbank and scab-susceptible Triumph potato tubers of different maturities. Phytopathology 47:253-255.
14. KOSUGE, T. 1969. The role of phenolics in host response to infection. Annu. Rev. Phytopathol. 7:195-222.
15. KUĆ, J. 1964. Phenolic compounds and disease resistance in plants. Pages 63-81 *in* V. C. Runeckles, ed. Phenolics in normal and diseased fruits and vegetables. Imperial Tobacco Co., Montreal. 102.
16. MC LEAN, V. G., D. J. LE TOURNEAU, and J. W. GUTHRIE. 1961. Relation of histochemical tests for phenols to verticillium wilt resistance of potatoes. Phytopathology 51:84-89.
17. OHASHI, Y., and T. MURAI. 1961. Studies on black root rot resistance. Rev. Appl. Mycol. 40:561 (Abstr.)
18. OTANI, Y. 1962. Studies on the black root rot disease caused by Thielaviopsis basicola (Berk. et Br.) Ferraris. Okayama Tob. Exp. Stn. Bull. 236. 236 p.
19. PARUPS, E. V. 1967. Plant phenolics and growth of tobacco. The Lighter 37(1):11-16.
20. PATIL, S. S., R. L. ROWELSON, and R. A. YOUNG. 1964. Relation of chlorogenic acid and free phenols in potato roots to infection by Verticillium albo-atrum. Phytopathology 54:531-535.
21. ROHRINGER, R., and D. J. SAMBORSKI. 1967. Aromatic compounds in the host-parasite interaction. Annu. Rev. Phytopathol. 5:77-86.
22. RUBIN, B. A. and E. V. ARTSIKHOVSKAYA. 1964. Biochemistry of pathological darkening of plant tissues. Annu. Rev. Phytopathol. 2:157-178.
23. SHEEN, S. J. 1969. The distribution of polyphenols and peroxidase in different plant parts of tobacco. Nicotiana tabacum, L. Pages 62-64 *in* Proceedings of the University of Kentucky, Tobacco and Health Research Institute, Tobacco and Health Conference, February 5-6. Lexington, Kentucky.
24. SHEEN, S. J. 1970. Comparative quantities of polyphenols and oxidases in Nicotiana species. Tob. Sci. 18:16-19.
25. STECK, W. and S. H. WENDER. 1965. Chlorogenic and neochlorogenic acids in cigarette smoke. Tob. Sci. 9:54-55.
26. WENDER, S. H. 1970. Effects of some environmental stress factors on certain phenolic compounds in tobacco. Rec. Advan. Phytochem. 3:1-29.
27. WYNDER, E. L. and D. H. HOFFMAN. 1967. Tobacco and Tobacco Smoke, Studies in Experimental Carcinogenesis. Academic Press. New York, London. 730 p.
28. ZUCKER, M., and J. F. AHRENS. 1958. Quantitative assay of chlorogenic acid and its pattern of distribution within tobacco leaves. Plant Physiol. 33:246-249.