Relationships between Susceptibility of Field-Grown Burley Tobacco to Blue Mold and Contents of Duvatrienediols

M. N. Rao, M. R. Siegel, R. S. Ferriss, W. C. Nesmith, M. D. Wiglesworth, H. R. Burton, M. Reuveni, S. Tuzun, and J. Kuć

First, second, third, fourth, fifth, seventh, eighth, and ninth authors: Department of Plant Pathology; sixth author: Department of Agronomy, University of Kentucky, Lexington 40546-0091.

Journal paper 88-11-3103 of the Kentucky Agricultural Experiment Station, Lexington 40546.

The research was supported in part by grants from the R. J. Reynolds Corporation, Ciba-Geigy Corporation, and Burley Tobacco Council of Kentucky.

Accepted for publication 8 August 1988 (submitted for electronic processing).

ABSTRACT

Rao, M. N., Siegel, M. R., Ferriss, R. S., Nesmith, W. C., Wiglesworth, M. D., Burton, H. R., Reuveni, M., Tuzun, S., and Kuć, J. 1989. Relationships between susceptibility of field-grown burley tobacco to blue mold and contents of duvatrienediols. Phytopathology 79:267-270.

Greenhouse studies have suggested a role for α- and β-4,8,13 duvatriene-1,3-diols (DVT) in the resistance of burley tobacco to blue mold. To test the validity of this relationship in the field, leaf samples from field-grown plants (cv. Ky 14) were assayed for DVT content and tested for susceptibility to *Peronospora tabacina* under controlled laboratory conditions. The study was conducted in central Kentucky during the 1985 and 1986 growing seasons. In 1985, plants in 11 fields were evaluated six times. Results with leaves from top, middle, and lower stalk positions confirmed the greenhouse findings that susceptibility to blue mold decreases with plant age in both untreated and acetone-dipped leaves, and that dipping leaves in acetone markedly increases susceptibility. There was a significant ($P = 0.022$) negative correlation between disease severity on untreated leaves and DVT content when leaves from all fields and sampling dates were considered. However, when data from individual sampling dates were analyzed, significant ($P = 0.022$, 0.026) negative correlations were detected for only two dates. In 1986, intensive sampling was conducted in a single field. Leaves were sampled from 25 plants at approximately 7-day intervals. Results for two stalk positions and two inoculum concentrations were similar to those in 1985. Although there was a highly significant negative correlation ($P \leq 0.0001$) of disease severity with DVT when all sampling dates were evaluated, no significant ($P < 0.05$) correlations were observed within individual sampling dates. Overall, these results indicate that DVT content probably has only a marginal role in the resistance of tobacco to blue mold. The inability to enhance disease in old plants by dipping in acetone suggests the existence of other age-related defense mechanisms. The relationship between disease severity and DVT content may be affected by interactions with other mechanisms of resistance and with environmental factors such as rainfall, day/night duration, light intensity, and temperature.

Cuticular components of some tobacco genotypes have been reported to have a role in resistance to some insects (9). Although the role of these compounds, notably α- and β-4,8,13 duvatriene-1,3-diols (DVT), in resistance against some insects are established, their role in resistance to fungal pathogens has not been clearly defined. Cruickshank et al (2) reported fungitoxic effects of DVT on spore germination of *Peronospora tabacina* Adam. Reuveni et al (6) observed that dipping leaf strips of tobacco in acetone for 1 sec increased their susceptibility to blue mold and removed 95% or more of the major cuticular diterpenoids, DVT, from the leaf surface. Thus, the change in disease reaction associated with acetone dipping appeared to indicate the contribution of DVT to resistance. Based on greenhouse studies, they suggested that DVT may play a role in resistance of tobacco against blue mold.

To further evaluate the role of DVT in resistance to blue mold, we conducted field experiments during 1985 and 1986 with the blue mold-susceptible tobacco cultivar Ky 14. The relationship between DVT content and susceptibility to blue mold was investigated at various times during the growing season by using a leaf disk assay (5).

MATERIALS AND METHODS

Field experiment conducted in 1985. Burley tobacco, *Nicotiana tabacum* L., (Ky 14) was grown in 11 fields located in different geographic areas of Kentucky. The fields were fertilized according to University of Kentucky Cooperative Extension Service recommendations (10) based on preplant soil tests. Weed control was maintained through cultivation and the application of the preplant-incorporated herbicides pendimethalin (Prowl; American Cyanamid Co., Wayne, NJ) or perbulate (Tillam; Stauffer Chemical Co., Westport, CT). Seedlings were transplanted 45 cm apart in rows spaced 100 cm apart.

Leaves from the top stalk position (first fully expanded leaf, approximately 30 cm in length) were collected at approximately 14-day intervals and brought to the laboratory in polyethylene bags kept in an insulated cooler containing ice. Nine plants per field (three plants from each of three blocks) were assayed at each sampling time, and a plant sampled once was not sampled again. Leaf strips (10–15 × 7 cm) were excised from the middle of each half leaf and traced on paper to measure the area. Strips were dipped in reagent grade acetone for 1 sec and immediately passed through three beakers of distilled water to remove the excess acetone from the surface. The leaf strips were shaken to remove excess water and dried gently with tissue paper. Ten leaf disks, each 18 mm diameter, were cut from untreated and acetone-treated strips and placed in petri plates with inserted filter paper as described previously (5). The upper surfaces of leaf disks were sprayed with a suspension of sporangiospores of *P. tabacina* (15 sporangiospores per cm^2 of disk area) by using an air brush sprayer (5). Sporangiospore germination, determined on glass slides, averaged 65 ± 10% in these experiments.

After inoculation, the plates were incubated at 19 C in the dark for 20 hr and then transferred to a growth chamber (23 C, 60–70 μE $min^{-2}sec^{-1}$, 12 hr of light per day). Four days after inoculation, the leaf disks were transferred to sponge rubber pads as described by Reuveni et al (7). Disease severity was rated 7 days after inoculation on a 0–4 scale: 0 = no symptoms; 1 = ≤ 25% or less of the disk area chlorotic; 2 = 26–50% chlorotic; 3 = 51–75% chlorotic; and 4 = 76–100% chlorotic.

Field experiment conducted in 1986. Burley tobacco (Ky 14) seedlings, approximately 8 wk old, were transplanted into a 2.5-ha field at the Spindletop Research Farm of the University of Kentucky. The field was divided into five blocks, and five plants were sampled at random from each block at each sampling date. A plant sampled once was not sampled again. The first fully expanded leaf from the top, approximately 30 cm in length, was sampled at approximately 7-day intervals throughout the growing season. The first sample was taken at the time of transplanting, and

© 1989 The American Phytopathological Society

the second was taken 18 days after transplanting. From 41 days after transplanting, the first nonsenescent leaf from the bottom was also sampled.

The leaves were brought to the laboratory as described previously. Leaf strips were excised, dipped in acetone, and leaf disks were cut and placed in petri plates. Two inoculum concentrations were sprayed on the upper surfaces of the leaf disks. A low concentration of 15 sporangiospores per centimeter^2 of disk area was sprayed at all samplings, and a high concentration of 750 sporangiospores per centimeter^2 was sprayed on leaf disks starting at the third sampling. The high concentration was chosen because greenhouse experiments had suggested this was the lowest concentration needed to overcome resistance of mature plants. Inoculation of leaf disks and rating of disease severity were done as described earlier.

After disease rating, sporulation was induced by incubating the inoculated leaf disks in the dark at 20 C for 24 hr. The sporangiospores were washed from the leaf disks, stored in a known volume of fixative solution (ethanol, formaldehyde, and acetic acid, 90:5:5 v/v/v), and counted by using a hemocytometer.

Quantification of DVT. Acetone washings of the leaves were filtered, dried, and redissolved in known volumes of methylene chloride (high-pressure liquid chromatography grade). An aliquot of the methylene chloride extract was passed through a PrepSep-Sci extraction column (Fisher Scientific Co., Chicago). The column was washed with 2 ml of methylene chloride and eluted with 1 ml of redistilled acetone into a micro auto sampler vial. Heptadecanol (50 μg) was used as the internal standard. The solution was dried under nitrogen at 40 C, and 300 μl of 1:1 dimethylformamide and N,O-bis (trimethyl) trifluoroacetamide was added to the residue. The sealed vials were heated at 70 C for derivatization. After cooling, 700 μl of methylene chloride was added to each vial. The samples were quantified following the procedure of Severson et al (8) with gas chromatography. In 1985, washings of leaves from the three plants sampled in each block of each field were combined before quantification of DVT. In 1986, washings of leaves from each plant were analyzed separately.

RESULTS

1985 experiment. At all except the first and last samplings, dipping leaf strips in acetone resulted in a significant ($P < 0.05$) increase in the susceptibility of leaf disks to blue mold (Fig. 1). On untreated leaf disks, disease rating declined steadily with time after transplanting to near zero at the fourth and later samplings. On acetone-treated leaf disks, disease rating was high at the first three samplings and then declined to near zero at the sixth sampling. DVT contents increased from the first to third samplings, and then fluctuated at a relatively high level. To detect relationships between disease rating and DVT content, correlations of mean disease with mean DVT were examined for data from each block in each of the 11 fields (Table 1). When data from all samplings were combined, marginally significant negative correlations were detected between disease severity on acetone-treated or untreated leaf disks and DVT content. However, when individual samplings were examined, significant ($P < 0.05$) correlations were detected for only two of six samplings for untreated leaf disks, and for one of the six samplings for acetone-treated leaf disks. Similar results were obtained by using either traditional linear correlation or Spearman's coefficient of rank correlation.

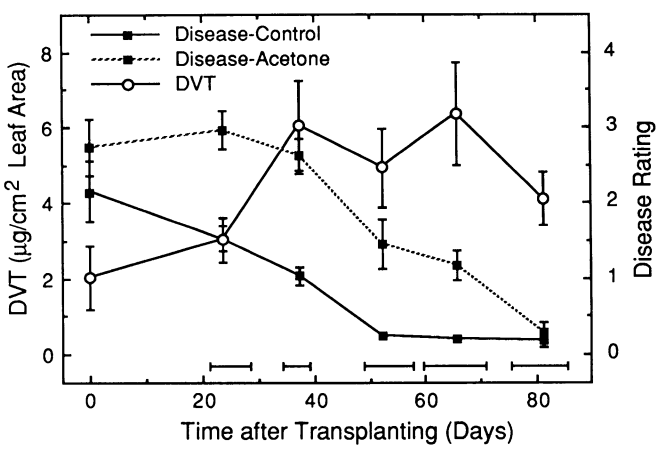

Fig. 1. Disease severity on leaf disks sprayed with 15 sporangiospores of *Peronospora tabacina* per cm^2 of disk area and duvatrienediols content of top leaves from field-grown (1985) Ky 14 tobacco. Leaf disks were prepared from either untreated leaf strips or from strips dipped in acetone. Disease was rated on a 0–4 scale: 0 = no symptoms; 1 = <25% of the disk area chlorotic; 2 = 26–50% chlorotic; 3 = 51–75% chlorotic; and 4 = 76–100% chlorotic. Values are means of one leaf per plant and nine plants per field from nine to 11 fields per sampling date. Duvatrienediols content is expressed as μg/cm^2 of leaf area. Horizontal bars indicate the range of sample collection times for the fields. Vertical bars denote standard error.

1986 experiment. In general, disease ratings of both acetone-treated and untreated leaf disks decreased with time after transplanting (Fig. 2A). Analysis of variance on logistically transformed data indicated that the higher inoculum concentration resulted in significantly ($P < 0.0001$) more disease, and that acetone treatment significantly ($P < 0.0001$) increased disease severity at all except the last three samplings. Disease rating was significantly ($P < 0.0001$) higher for bottom than top leaves where both were sampled. Sporulation on top leaves declined with time after transplanting to near zero at the fifth and later samplings (Fig. 2B). Sporulation on bottom leaves was significantly greater than on top leaves at the fifth sampling. Where greater than a trace amount of sporulation occurred, acetone treatment significantly ($P < 0.0001$) increased sporulation, and significantly ($P < 0.0001$) more sporulation occurred at the higher inoculum concentration on untreated leaf disks. However, on acetone-treated leaf disks, significantly ($P < 0.0001$) less sporulation occurred at the higher than the lower inoculum concentration at the third sampling (the first sampling where the higher inoculum concentration was used), and there was no significant effect of inoculum concentration on sporulation at the fourth and later sampling.

This anomalous effect of inoculum concentration on sporulation was apparently related to the large amount of necrosis that was observed on acetone-treated leaf disks inoculated with the high, but not the low, inoculum concentration. DVT contents in top leaves were near zero at the first, second, and fourth samplings, but were relatively high at the third sampling and generally increased from the fourth sampling on (Fig 2C). A heavy rain occurred one day before the third sampling. In bottom leaves, DVT contents increased steadily with time after transplanting and were significantly lower than in top leaves at three of the four samplings where both top and bottom leaves were assayed.

To detect relationships between disease rating and DVT content, correlations were examined for data from individual plants. When data for all samplings were combined, a highly significant ($P = 0.0024$) negative correlation was found (Table 2). However,

TABLE 1. Linear correlations between disease severity on leaf disks, from acetone-treated and untreated leaves, sprayed with sporangiospores of *Peronospora tabacina* (15/cm^2) and duvatrienediols content of top leaves in 1985 field experiment

Days after transplanting	N[a]	Untreated leaves		Acetone-treated leaves	
		r	Probability	r	Probability
0	9	−0.141	0.717	−0.129	0.741
24	28	−0.421	0.026	−0.448	0.017
38	32	+0.156	0.395	−0.307	0.087
52	28	−0.149	0.450	−0.051	0.796
66	32	−0.403	0.022	−0.189	0.299
80	29	+0.181	0.348	+0.014	0.941
Overall	158	−0.183	0.022	−0.178	0.026

[a] For all except the first sampling, correlations were performed by using mean values for each block in each of nine to 11 fields. The first sampling was performed at the time of transplanting and was not divided by block.

when analyses were performed for individual samplings, no significant ($P \leq 0.05$) correlations were detected. Similar results were obtained for both high and low inoculum concentrations, top and bottom leaves, and acetone-treated and untreated leaves. Similar results were obtained by using either traditional linear correlation or Spearman's coefficient of rank correlation. Analysis of covariance indicated that there was no significant effect of DVT content on disease rating after the effect of sampling had been accounted for.

Wide variation was observed in disease severity and DVT contents in tobacco leaves of any given age. For example, DVT contents of less than 0.5 $\mu g/cm^2$ of leaf area were detected in 83% of bottom leaves sampled 41 days after transplanting, but disease severity for these leaves ranged from 0.1 to 3.0 on the 0–4 scale, with a mean of 1.5 (Fig. 3A). At other samplings wide variation was observed in both DVT content and disease severity. For example, top leaves sampled 49 days after transplanting had a mean DVT level of 3.1 $\mu g/cm^2$ of leaf area, whereas the range was from 0.12 to 7.20 (Fig. 3B). The disease severity for these leaves ranged from 0 to 3.2, with a mean of 1.2.

DISCUSSION

The results that were obtained in this 2-yr field study confirmed previous greenhouse observations that both resistance to *P. tabacina* and DVT content increase with age of tobacco plants (6).

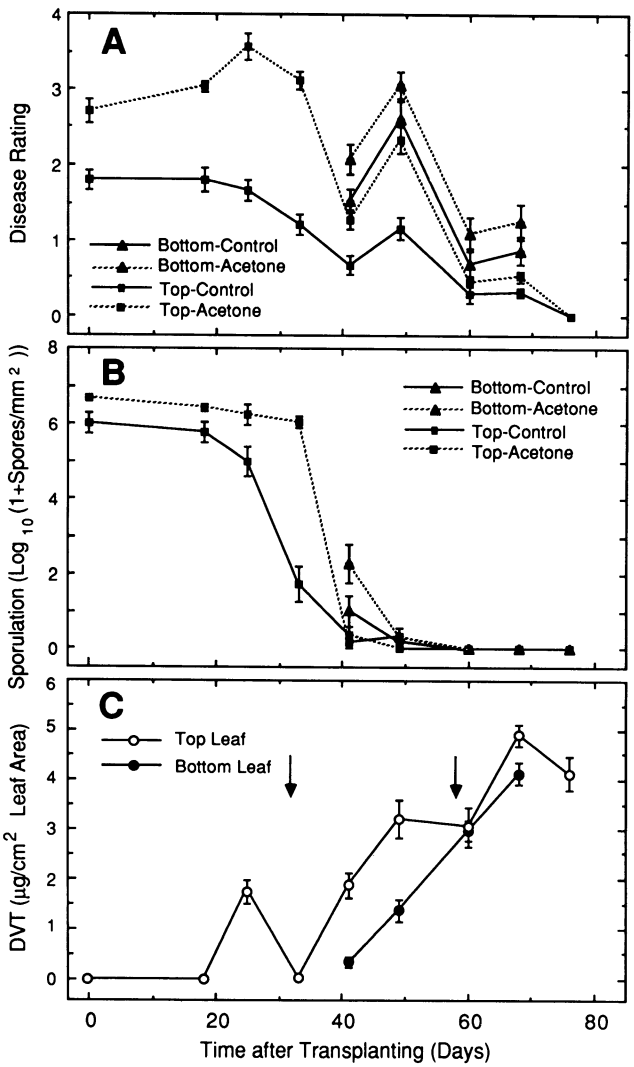

Fig. 2. Effects of time after transplanting in the 1986 field experiment of **A**, disease rating; **B**, sporulation; and **C**, duvatrienediols content. Arrows indicate shower activity. Disease severity and sporulation were determined on leaf disks inoculated with 15 sporangiospores of *P. tabacina* per cm^2 of disk area. Vertical bars denote standard error.

Because duvatrienediols have been shown to be effective inhibitors of germination of sporangiospores of *P. tabacina* (2,6), these results could be considered to indicate that age-related resistance is due to an increase in the content of these compounds. Considering the appearance of metalaxyl-resistant strains of the fungus (1), such a relationship might indicate that DVT could be useful for the control of blue mold. However, our observation that disease resistance also increases with age in leaves from which practically all DVT has been removed indicates that factors other than DVT must play a part in age-related resistance. Furthermore, the low significance of correlations between disease severity and DVT content at individual sampling times indicates that DVT content is

TABLE 2. Linear correlations of disease severity on untreated leaf disks sprayed with sporangiospores of *Peronospora tabacina* (15/cm^2 or 750/cm^2) and duvatrienediols content of top and bottom leaves in 1986 field experiment

Days after transplanting	Sporangiospores (15/cm^2) r	Sporangiospores (15/cm^2) Probability	Sporangiospores (750/cm^2) r	Sporangiospores (750/cm^2) Probability
Top leaves[a]				
18	+0.036	0.863		
25	−0.351	0.085	+0.048	0.820
33	+0.142	0.496	+0.199	0.344
41	−0.290	0.170	−0.149	0.497
49	−0.058	0.788	−0.300	0.148
60	−0.163	0.469	+0.066	0.770
68	−0.395	0.056	+0.198	0.342
Overall	−0.423	<0.0001	−0.320	<0.0001
Bottom leaves[a]				
41	+0.220	0.303	−0.476	0.019
49	−0.116	0.590	−0.375	0.065
60	+0.065	0.803	+0.112	0.679
68	−0.080	0.770	−0.257	0.356
Overall	−0.333	0.0024	−0.563	<0.001

[a]Twenty five plants per sampling period.

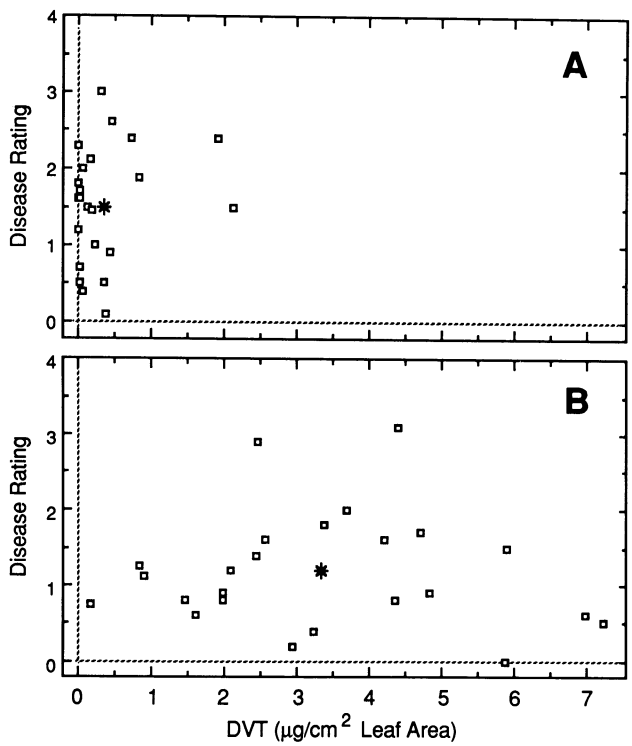

Fig. 3. Examples of variability in duvatrienediols content and disease severity on untreated leaves in the 1986 experiment. **A**, Top leaves, 41 days after transplanting; **B**, Bottom leaves, 49 days after transplanting. Leaf disks were inoculated with 15 sporangiospores of *P. tabacina* per cm^2 of disk area. Each data point represents an individual leaf. Means are denoted by an asterisk.

probably of marginal importance in determining differences in resistance between tobacco fields or individual plants.

Environmental factors such as rainfall, temperature, and duration of light are known to influence DVT content (9). Variation in light and/or temperature may account for the observation that plants grown in the greenhouse from June through September have higher DVT content than those grown from February through May (4). In the 1986 field experiment, DVT content decreased sharply between the 25- and 33-day samplings. This may be accounted for by heavy shower activity that occurred a day before the 33-day sampling. The decrease in DVT content, however, was not accompanied by a noticeable increase in disease severity at the 33-day sampling. This observation is a further indication that DVT does not have a direct effect on disease severity. Environmental effects on non-DVT-related resistance may have been responsible for the significant negative correlation of disease with DVT that was observed for acetone-treated leaves at the second sampling in 1985.

Previous work has shown that DVT is primarily contained in the trichomes of tobacco leaves (3). It is possible that this localization prevents the accumulation of fungitoxic levels of DVT on most of the leaf surface and, therefore, results in poor correlations between disease and the amount of DVT removed from the leaf surface by acetone dipping. It may be that DVT production benefits tobacco plants only in resistance against insects. However, the high fungitoxicity of these compounds makes it attractive to speculate that DVT plays some definite, but as yet unknown, role in resistance to *P. tabacina*. It is possible that removal of DVT from old plants did not make them susceptible because some DVT might be on the leaf surface in an oxidized form, and the oxidized polymers are not extractable in acetone. It thus might be also possible that the mechanism of age-related resistance is independent of DVT content at any particular time, but that acetone-extractable DVT is involved as a precursor for the actual compounds responsible for age-related resistance.

LITERATURE CITED

1. Bruck, R. I., Gooding, G.V., Jr., and Main, C. E. 1982. Evidence for resistance to metalaxyl in isolates of *Peronospora hyoscyami*. Plant Dis. 66:44-45.
2. Cruickshank, I. A. M., Perrin, D. R., and Mandryk, M. 1977. Fungitoxicity of duvatrienediols associated with the cuticular wax of tobacco leaves. Phytopathol. Z. 90:243-249.
3. Keene, C. K., and Wagner, G. J. 1985. Direct demonstration of duvatrienediol biosynthesis in glandular heads of tobacco trichomes. Plant Physiol. 79:1026-1032.
4. Rao, M. N., Siegel, M. R., Nielson, M. T., Wiglesworth, M., Burton. H. R., and Kuć, J. 1989. Evaluation and induction of resistance to blue mold in tobacco genotypes differing in contents of duvatrienediols. Phytopathology 79:271-275.
5. Reuveni, M., Siegel, M. R., and Nesmith, W. C. 1985. Bioassays using detached tobacco leaves to determine the sensitivity of *Peronospora tabacina* to fungicides. Pestic. Sci. 16:244-250.
6. Reuveni, M., Tuzun, S., Cole, J. S., Siegel, M. R., and Kuć, J. 1986. Removal of duvatrienediols from the surfaces of tobacco leaves increases their susceptibility to blue mold. Physiol. Mol. Plant Pathol. 30:44-51.
7. Reuveni, M., Tuzun, S., Cole, J. S., Siegel, M. R., and Kuć, J. 1986. The effects of plant age and leaf position on the susceptibility of tobacco to blue mold caused by *Peronospora tabacina*. Phytopathology 76:455-458.
8. Severson, R. F., Arrendale, R. F., Chortyk, O. T., Johnson, A. W., Jackson, D. M., Gwynn, G. R., Chaplin, J. F., and Stephenson, M. G. 1984. Quantitation of the major cuticular components from green leaf of different tobacco types. J. Agric. Food Chem. 32:566-570.
9. Severson, R. F., Johnson, A. W., and Jackson, D. M. 1985. Cuticular constituents of tobacco: Factors affecting their production and their role in insect and disease resistance and smoke quality. Recent Adv. Tob. Sci. 11:105-174.
10. Smiley, J. H., Nesmith, W. C., Townsend, L. H., Duncan, G. A., and Hourigan, W. W. 1983. Tobacco Handbook. University of Kentucky, College of Agriculture, Cooperative Extension Service, Lexington. 39 pp.