Evaluation and Induction of Resistance to Blue Mold in Tobacco Genotypes Differing in Contents of Duvatrienediols

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ABSTRACT

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The α- and β-4,8,13-duvatriene-1,3-diols (DVT) are fungitoxic leaf-surface components of tobacco. Tobacco Introductions (TI), double haploid breeding lines, and cultivar Ky 14, with different DVT contents, were evaluated for resistance to blue mold caused by *Peronospora tabacina* Adam. DVT contents varied significantly in plants grown at different times of the year and increased with age. TI 1068 and the double haploid breeding lines, DH 944-1, DH 909-2, and DH-960, had higher DVT contents than Ky 14 and were more resistant to blue mold than Ky 14. However, although DH 909-2 was the most resistant genotype in greenhouse tests, it did not have the highest DVT contents. TI 1406, with lower contents of DVT than Ky 14, was somewhat more susceptible in greenhouse tests and considerably more susceptible in field tests. TI 1112, however, with little or no DVT, was highly resistant in greenhouse and field tests. In greenhouse tests, systemic resistance was induced in all types of tobacco by stem injection with sporangiospores of *P. tabacina*, except in TI 1112, which already was highly resistant. DVT contents did not significantly change in stem-injected plants. Removal of DVT by acetone dipping increased susceptibility to blue mold in the early stages of growth, but not in the later stages of field-grown tobacco, which contained DVT. For all genotypes, the oldest plants sampled in the field test (83 days after transplanting) appeared immune. Linear correlations of disease with DVT for individual sampling dates indicated no significant effect of variation in DVT contents among genotypes on disease severity. The disease resistance-DVT relationship is very complex. DVT contents were apparently not responsible for induced resistance or the high resistance of plants sampled late in the season (83 days after transplanting). DVT contents are not the only factors determining resistance to blue mold; however, they may have a contributory role.

Leaf-surface components of some genotypes of tobacco are known to cause resistance to insects and may have a role in resistance to diseases (8). Cruickshank et al (1) reported that α and β isomers of the macrocyclic diterpene 4,8,13-duvatriene-1,3-diol (DVT), present on tobacco leaf surfaces, had an ED_{50} of about 20 μg/ml in inhibiting germination of sporangiospores of *Peronospora hyoscyami* f. sp. *tabacina* in vitro. The α and β isomers of DVT were shown to be synthesized in glandular trichomes present on the leaf surface of tobacco (2). Based principally on greenhouse studies, Reuveni et al (4) suggested a role for DVT in resistance of 14- to 18-wk-old Ky 14, a burley tobacco cultivar, to blue mold.

Systemic resistance to blue mold can be induced in Ky 14 by stem injection with sporangiospore suspensions of *P. tabacina* (10), although the actual mechanism involved in this resistance is not known. The objectives of this investigation were to evaluate various tobacco genotypes for resistance to blue mold and DVT content at different stages of growth, to determine whether resistance could be induced in these genotypes by stem injection with sporangiospores of *P. tabacina*, and to examine the role of DVT in induced and age related resistance.

MATERIALS AND METHODS

Plants. In greenhouse experiments, plants were grown at 20–26 C in fall and winter, and 20–33 C in spring and summer. Sunlight was supplemented with fluorescent and incandescent light to provide a 14-hr photoperiod. Plants were grown in pots containing Pro-Mix BX (Peat Moss Corp. Marketing, New York). They were fertilized twice a week with "Peters Fertilizer" solution (14.8 ml of 20:20:20 [N.P.K] soluble fertilizer per 3.8 L). Genotypes evaluated in the greenhouse experiment for DVT and disease resistance included three Tobacco Introductions (TI): TI 1068 (8), which has high density of secreting glandular trichomes and a high content of DVT; TI 1112 (8), which lacks glandular trichomes; and TI 1406, which has nonsecreting glandular trichomes (8). Also included were the burley tobacco cultivar Ky 14 and three double haploid breeding lines: DH 944-1, DH 909-2, and DH 960, which in preliminary tests were found to have high contents of DVT. DH 909-2 is a double haploid line obtained via anther culture of the F_1 Ky 14 × TI 1068. Similarly, DH 944-1 and DH 960 were derived from the F_1 TI 1406 × TI 1068.

In a field experiment (1986), conducted at Spindletop Research Farm of the University of Kentucky, approximately 8-wk-old seedlings of each of the three Tobacco Introductions and Ky 14 were transplanted on June 13 in two blocks of eight randomized replicated rows, each row having 18 seedlings. Seedlings were transplanted 45 cm apart in rows spaced 100 cm apart. The field was fertilized according to University of Kentucky Cooperative Extension Service recommendations (9). Preplant soil tests were conducted by the University of Kentucky Soil Testing Service, and before planting the soil was brought to approximately pH 6.5 with lime and fertilized (N, P, and K; 280, 0, and 280 kg/ha of extractible nutrient, respectively). Weed control was maintained through cultivation and the application of preplant-incorporated herbicides pendimethalin (Prowl; American Cyanamid Co., Wayne, NJ) or pebulate (Tillam; Stauffer Chemical Co., Westport, CT).

Inducing and challenging inoculations. In greenhouse experiments, for inducing resistance, approximately 9-wk-old tobacco plants, four per variety, were injected with 1 ml of inoculum containing 1×10^6 sporangiospores ml^{-1} of isolate "82" of *P. tabacina* into stem tissue external to the cambium (10). The plants were given a booster injection with 0.5 ml of inoculum 11 days after the first injection. Controls were injected with water.

Plants were challenged 21 days after stem inoculation for induced resistance studies, or at specific stages of growth in a growth chamber (24 C [day]/18 C with a 14-hr photoperiod illuminated at 75 μE $min^{-2}sec^{-1}$). In greenhouse studies, 8-wk-old plants were challenged with 10 ml; 9-wk-old plants with 20 ml;

10-wk-old plants with 30 ml; and 12-wk-old plants with 50 ml per plant of a suspension of sporangiospores containing 5×10^4 spores ml^{-1} of isolate "79" of *P. tabacina*. The upper surfaces of leaves were uniformly sprayed with sporangiospore suspension. The plants, covered with brown plastic bags that had been sprayed inside with water, were placed in the dark at 18 C for 18 hr. Plastic bags were then removed and the plants maintained in growth chambers.

In the field experiment, the first fully expanded leaf from the top (approximately 30 cm in length) was collected from six randomly selected plants for each type of tobacco and brought to the laboratory in polyethylene bags kept in an insulated cooler containing ice. Leaves were collected at 3-wk intervals following transplanting. Leaf strips were excised from the middle of each half leaf, traced on paper to measure the area, dipped in acetone for 1 sec, and immediately passed through 3 beakers of distilled water. Leaf disks (18-mm diameter) were cut and sprayed on the upper surface with a suspension of sporangiospores at the rate of 15 spores per cm^2 of disk area (low inoculum concentration) and 750 spores per cm^2 of disk area (high inoculum concentration) with an air brush sprayer (3). The plates containing disks were incubated as described by Reuveni et al (5).

Disease assessment. For plants in growth chambers, disease was assessed visually on a 0–100% scale for the plants in growth chambers 7 days after challenge. The plants were then sprayed with water, covered with water-sprayed plastic bags, and incubated in the dark for sporulation. The leaves were harvested, their area measured by tracing on paper, spores harvested, and spores counted with the aid of a hemocytometer.

Disease severity was assessed on leaf disks from the field samples on a 0–4 visual scale: 0 = no symptoms; 1 = ≤ 25% of the disk area chlorotic; 2 = 26–50% of the disk area chlorotic; 3 = 51–75% of the disk area chlorotic; and 4 = 76–100% disc area chlorotic.

Determination of DVT. Acetone washings of the first fully expanded leaf from the top were filtered through a folded Whatman No. 1 filter paper into a glass vial, dried, and redissolved in known volumes of high-pressure liquid chromatography grade methylene chloride. The DVT were derivatized and quantified following the procedure of Severson et al (7).

RESULTS

In greenhouse experiments, significant differences ($P < 0.05$) were observed in DVT contents for plants grown at different times of the year (Table 1). Plants grown from June through September and from August through October had higher DVT contents than those grown in February through May and April through July. However, at all sampling dates the relative rankings of the four genotypes for DVT contents were similar. The DVT contents increased with the age of the plants (Table 2). Of the genotypes tested, TI 1068 had the highest DVT content, followed by Ky 14. A significant ($P < 0.05$) increase in DVT contents was observed in TI 1068 and Ky 14 as a function of age. The increase was much less in TI 1112 and TI 1406. In a separate experiment, the DVT contents generally increased with age for the double haploid lines, TI 1068, and Ky 14 (Table 3). Ky 14 consistently had lower contents of DVT than the other tobacco genotypes tested and was most susceptible to blue mold; DH 909-2, however, was consistently the most resistant but did not have the highest contents of DVT. The double haploid line DH 909-2 was highly resistant at 8 and 9 wk of age when DVT contents were low. The DVT contents in TI 1068 and Ky 14 observed in this experiment correspond to the DVT contents in plants grown at the same time the previous year (Table 1).

Resistance was enhanced in all genotypes of tobacco by stem injection with *P. tabacina*, except in TI 1112, which already was highly resistant (Tables 4 and 5). Although stem injection with the fungus protected against blue mold, it did not consistently increase DVT contents.

In the field experiment, DVT contents increased significantly with age in TI 1068 and Ky 14, whereas no detectable DVT was observed in TI 1112 and TI 1406 at any stage of growth (Fig. 1). The plants were topped immediately after the third sampling time,

TABLE 1. DVT content on leaf surfaces of 12-wk-old Tobacco Introductions and Ky 14 grown in the greenhouse at different times of the year

Growth period[a]	DVT content ($\mu g/cm^2$ of leaf area) of various tobacco genotypes (mean ± SE)[b]			
	TI 1068	TI 1112	TI 1406	Ky 14
Feb. 19-May 13	7.5 ± 1.9	0	0	0.2 ± 0
Apr. 28-July 21	2.0 ± 0.3	0.6 ± 0	0.4 ± 0	0.8 ± 0.1
June 26-Sept. 24	33.6 ± 4.3	1.9 ± 0.3	1.9 ± 0.7	12.8 ± 3.8
Aug. 1-Oct. 30	14.1 ± 1.0	2.1 ± 0.3	2.7 ± 0.2	3.4 ± 1.2

[a] Leaves collected for DVT determinations on the last date of the growth period.
[b] Mean ± standard error based on the first fully expanded leaf from four replicates of each tobacco genotype.

TABLE 2. Changes in DVT content with plant age in Tobacco Introductions and Ky 14 grown in the greenhouse

Sampling date[a]	DVT content ($\mu g/cm^2$ of leaf area) of various tobacco genotypes (mean ± SE)[b]			
	TI 1068	TI 1112	TI 1406	Ky 14
Sept. 3	3.7 ± 0.2	0.7 ± 0	0.7 ± 0	0.6 ± 0
Sept. 17	8.5 ± 1.9	1.4 ± 0.1	1.5 ± 0.1	6.6 ± 0.6
Sept. 24	33.6 ± 4.3	1.9 ± 0.3	1.9 ± 0.7	12.8 ± 3.8

[a] Seed planted June 26.
[b] Mean ± standard error based on the first fully expanded leaf from four replicates of each tobacco genotype, three leaves per sample.

TABLE 3. Disease severity and DVT content for different tobacco genotypes at various growth stages in greenhouse

Tobacco genotype and weeks after seeding[a]	Disease severity[b] (percentage of leaf area with symptoms) (mean ± SE)[c]	DVT content ($\mu g/cm^2$ of leaf area[d])
Age ≈ 8 wk		
TI 1068	51 ± 4	0.4 ab
DH 944-1	59 ± 5	0.7 a
DH 909-2	18 ± 4	0.6 a
DH-960	59 ± 4	0.7 a
Ky 14	69 ± 4	0.1 b
Age ≈ 9 wk		
TI 1068	31 ± 6	0.4 ab
DH-944-1	28 ± 6	1.2 a
DH-909-2	10 ± 3	0.7 ab
DH-960	23 ± 5	0.4 ab
Ky 14	58 ± 4	0.2 b
Age ≈ 10 wk		
TI 1068	41 ± 3	0.6 a
DH 944-1	45 ± 4	1.7 b
DH 909-2	33 ± 5	1.4 b
DH-960	38 ± 4	2.6 c
Ky 14	83 ± 3	0.4 a
Age ≈ 12 wk		
TI 1068	31 ± 3	1.7 a
DH 944-1	33 ± 3	1.9 a
DH 909-2	16 ± 3	1.8 a
DH-960	37 ± 5	2.3 a
Ky 14	77 ± 3	0.5 b

[a] Seed planted 4/3/87.
[b] 8-wk-old plants were challenged with 10 ml, 9-wk-old plants with 20 ml, 10-wk-old plants with 30 ml, and 12-wk-old plants with 50 ml per plant of 5×10^4 sporangiospores ml^{-1} of *P. tabacina*. Four plants per time per variety combination.
[c] Mean ± standard error based on the first fully expanded leaf from replicates of each genotype of tobacco.
[d] For each plant age, numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's new multiple-range test.

and no significant ($P < 0.05$) increase was observed in DVT contents of TI 1068 and Ky 14 at the fourth sampling time compared with the third sampling time. Dipping the leaf strips in acetone significantly ($P < 0.05$) increased disease severity on leaf disks from TI 1068 and Ky 14 in the first two samples; no such effect was observed in TI 1112 and TI 1406, which had no DVT (Fig. 2). Acetone dipping did not increase the disease severity by the third sampling time, and all the types of tobacco were highly resistant by the last sampling time, independent of acetone treatment, to both low (Fig. 2) and high (Fig. 3) inoculum concentrations. High inoculum concentration caused more disease compared with low inoculum concentration at the second sampling time in TI 1068, TI 1406, and Ky 14, but little or no increase in disease was observed in TI 1112 (Fig. 4).

Blue mold naturally appeared in the field at approximately the second sampling time, and the disease severity was in the order of TI 1112 < TI 1068 < Ky 14 < TI 1406 (data not shown).

Linear correlations of the relationship between disease severity and DVT contents showed no significant ($P \leq 0.05$) correlation for the genotypes tested in individual samplings.

DISCUSSION

DVT contents are reported to impart resistance to insects (7,8), but their role in resistance against plant fungal pathogens is not clearly established. The availability of the Tobacco Introductions and the double haploid breeding lines, with different DVT contents, and the stem injection technique for inducing systemic resistance to blue mold in tobacco enabled us to investigate the role of DVT not only in resistance against blue mold, but also in induced systemic resistance. Of the genotypes evaluated, TI 1112 contained little or no DVT, but was highly resistant both in greenhouse (Table 4) and field tests (Figs. 2–4). Of the double haploid lines, DH 909-2 was consistently the most resistant in greenhouse tests, although it did not consistently have the highest contents of DVT (Tables 3 and 5). This suggests that mechanisms other than DVT contents influence resistance to blue mold. TI 1068, which had considerably more DVT than Ky 14, was significantly more resistant, whereas TI 1406, with little or no DVT, was highly susceptible compared with Ky 14. At 12 wk after seeding, all the double haploid lines that contained more DVT than Ky 14 were significantly more resistant to blue mold compared with Ky 14 (Table 3). To our knowledge, this is the first report of

TABLE 4. The effect of stem injection with sporangiospores of *P. tabacina* on susceptibility to blue mold and the DVT content on leaf surfaces of greenhouse-grown Tobacco Introductions and Ky 14

Tobacco genotype[a]	Treatment	Percentage of leaf area with symptoms (mean ± SE)[b]	Sporangiospores/cm² of leaf area[c]	DVT content (μg/cm² of leaf area)
TI 1068	Control	20 ± 4	465	14
	Stem injected	5 ± 1	0	16
TI 1112	Control	0	0	2
	Stem injected	0	0	2
TI 1406	Control	72 ± 6	5,655	2
	Stem injected	21 ± 5	92	2
Ky 14	Control	58 ± 5	6,012	3
	Stem injected	24 ± 4	957	4

[a] Seed planted 8/1/86; plant's stem injected 10/9/86; plants challenged 10/30/86.
[b] Mean ± standard error based on four leaves from each of four replicates of each genotype of tobacco.
[c] Indicates number of sporangiospores that were collected from total area of diseased plant.

TABLE 5. The effect of stem injection with sporangiospores of *P. tabacina* on susceptibility to blue mold and the DVT content on leaf surfaces of double haploid breeding lines, TI 1068, and Ky 14 grown in greenhouse

Tobacco genotype[a]	Treatment	Percentage of leaf area with symptoms (mean ± SE)[b]	DVT content (μg/cm² of leaf area)
TI 1068	Control	31 ± 4	1.7
	Stem injected	10 ± 2	1.5
DH 944-1	Control	33 ± 3	1.9
	Stem injected	9 ± 1	2.2
DH 909-2	Control	16 ± 3	1.8
	Stem injected	11 ± 3	2.4
DH-960	Control	37 ± 5	2.3
	Stem injected	23 ± 3	2.3
Ky 14	Control	77 ± 3	0.5
	Stem injected	33 ± 3	0.7

[a] Seed planted 4/3/87; stem injected 6/10/87; plants challenged 7/1/87.
[b] Mean ± standard error based on four leaves from each of four replicates of each genotype of tobacco.

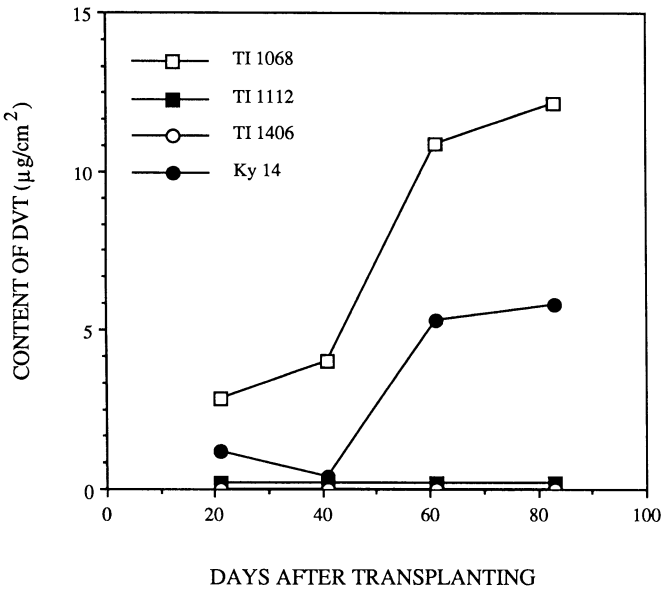

Fig. 1. Changes in DVT contents with age in Tobacco Introductions and Ky 14 in the field.

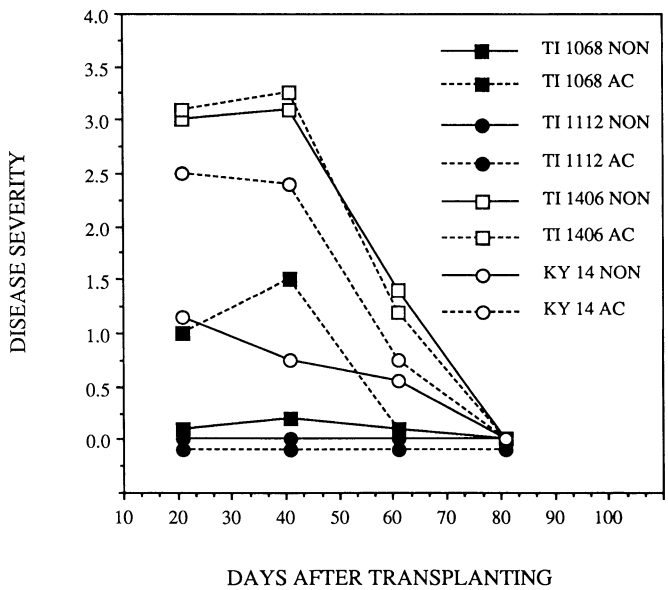

Fig. 2. Effect of removal of DVT from leaves of field-grown tobacco with acetone on susceptibility to blue mold of leaf disks at low inoculum concentration (15 sporangiospores per cm² of leaf disk). NON, leaf strips not dipped in acetone; AC, leaf strips dipped in acetone.

the resistance of these TI types and breeding lines to blue mold.

Systemic resistance against blue mold was induced in all genotypes by using the stem injection technique, except in TI 1112, which was highly resistant (Tables 4 and 5). Although stem injection induced resistance, it did not consistently increase the DVT contents on the leaf surface, thereby excluding the possibility of increased DVT contents as the mechanism of induced resistance. Induced systemic resistance in DH 909-2 was marginal when compared to the control, probably due to the high content of resistance in DH 909-2 control plants (Table 5).

Environmental factors and cultural practices are known to influence the contents of cuticular components of tobacco (8). Our results showed that DVT contents vary significantly at different times of the year in plants grown in the greenhouse under relatively controlled conditions (Table 1). Plants grown in June through September had the highest DVT contents, which coincides with long day period; however, plants grown in April through July had low contents of DVT and a long day period. Although light intensity and quality affect DVT contents, it appears that other factors influenced DVT contents in the experiments reported in this paper. Perhaps temperature during early plant growth had a major influence because plants grown from February through May and April through July had lowest DVT contents. In TI 1068 and Ky 14, which were grown in June through September and sampled at different times in September, the DVT contents increased many fold with age, whereas in TI 1112 and TI 1406 grown under the same conditions the increase was much less (Table 2). This increase in DVT contents with age is in accordance with the results of other investigators (4,5).

The accumulation of low levels of DVT in TI 1112 and TI 1406 during certain periods of growth in the greenhouse (Tables 1 and 2) but not in the field (Fig. 1) was unexpected. All other reports in the literature are that these introductions lack DVT either because of a lack of trichomes (TI 1112) or the presence of nonsecretory trichomes (TI 1406). The data cannot be explained but suggest the possible synthesis and accumulation of DVT in tissues other than the trichomes at low levels under certain growing conditions.

In the field experiment, the leaf-disk bioassay was used to monitor the resistance of the TIs and Ky 14 to blue mold as it reflects the response of intact leaves (3). The TIs and Ky 14 were in the order of severity to blue mold, TI 1112< TI 1068< Ky 14< TI 1406. This same ranking was also found with natural incidence of blue mold observed in the field at approximately the second sampling time (data not shown). Dipping in acetone to remove DVT significantly increased the susceptibility of TI 1068 and Ky 14 to blue mold in the first two samplings for low inoculum (Fig. 2) and in the second sampling for high inoculum concentration (Fig. 3). Dipping leaves in acetone had little or no effect on disease severity of TI 1112 and TI 1406 and they had little or no DVT. All the TI genotypes and Ky 14 were highly resistant to blue mold by the last sampling time independent of acetone treatment and inoculum concentration, suggesting an age-related field resistance mechanism in tobacco to blue mold. Whereas the DVT contents increased significantly with age in TI 1068 and Ky 14, DVT was not detected in TI 1112 and TI 1406 (Fig. 1).

Statistical analysis of the relationship between disease severity and DVT contents for individual samplings did not show significant ($P \leq 0.05$) correlation for any of the genotypes tested. Similar results were obtained in an extensive field experiment in 1986 with burley tobacco cultivar Ky 14 for both high and low inoculum concentrations, top and bottom leaves, and acetone treated and untreated leaves (6). It appears that DVT contents may influence the severity of blue mold as high contents of DVT relative to Ky 14 were consistently associated with resistance, but they do not have a major role as a resistance mechanism in tobacco.

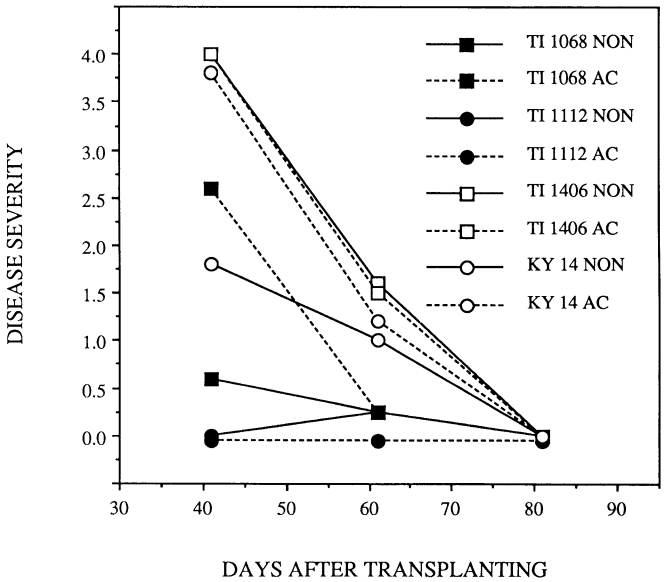

Fig. 3. Effect of removal of DVT from leaves of field-grown tobacco with acetone on susceptibility to blue mold of leaf disks at high inoculum concentration (750 sporangiospores per cm^2 of leaf disk). NON, leaf strips not dipped in acetone; AC, leaf strips dipped in acetone.

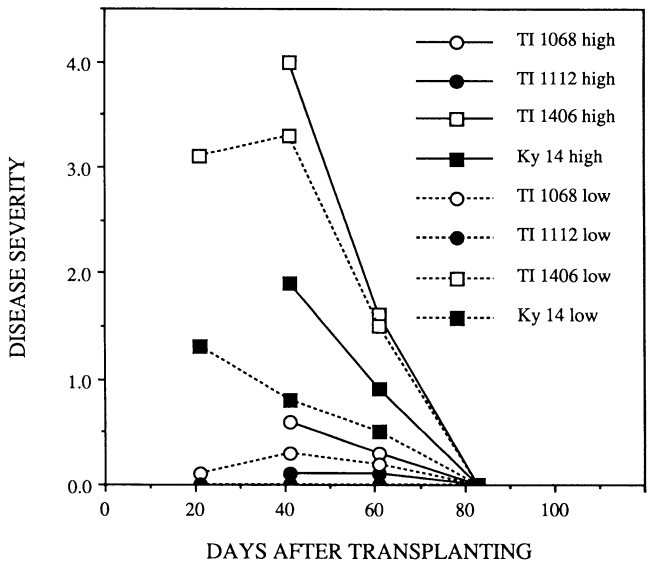

Fig. 4. Disease severity as measured by the leaf-disk assay in field-grown Tobacco Introductions and Ky 14 at low (15 sporangiospores per cm^2 of leaf disk) and high (750 sporangiospores per cm^2 of leaf disk) inoculum concentrations.

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