The Effect of Removing Leaf Surface Components with Acetone from Immunized and Nonimmunized Resistant Tobacco Plants on Their Susceptibility to Blue Mold

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Journal Series Paper 88-11-260 of the Kentucky Agricultural Experiment Station, Lexington, KY 40546.

This work was supported in part by grants from Ciba-Geigy Corporation, R. J. Reynolds Corporation, U. S. Department of Agriculture cooperative agreement 43YK-5-0030, and the Scientific and Technical Research Council of Turkey.

We thank Etta Nuckles, Nancy Doubrava, and Leslee Roberts for technical assistance, Earl Wernsman for providing seeds of the resistant cultivars, and Ray Severson for providing standards of duvatrienediols.

Accepted for publication 4 May 1989 (submitted for electronic processing).

ABSTRACT

Tuzun, S., Reuveni, M., Siegel, M. R., and Kuć, J. 1989. The effect of removing leaf surface components with acetone from immunized and nonimmunized resistant tobacco plants on their susceptibility to blue mold. Phytopathology 79:1024-1027.

The involvement of leaf surface compounds in resistance to blue mold was studied in three tobacco cultivars (Ovens 62, Incekara, and Izmir Ozbaz), bred for resistance to blue mold, and susceptible burley tobacco cultivar Ky 14. In general, plants became more resistant to blue mold with age. A leaf-disk assay supported the results of the whole-plant assays. Dipping leaf strips in acetone significantly increased their susceptibility to blue mold. Stem injection of sporangiospores of *Peronospora tabacina* into Ky 14 plants induced systemic resistance against blue mold (immunized). The susceptibility of immunized and nonimmunized resistant leaf strips was increased after they were dipped in acetone; however, the susceptibility did not reach that of water-injected Ky 14 plants (controls) of the same age that were dipped in acetone. The levels of fungitoxic leaf surface compounds, the duvatrienediols (DVT), on the two resistant varieties Incekara and Izmir Ozbaz were similar to those on Ky 14. The levels of DVT were approximately twice as great on the most resistant variety Ovens 62 and on immunized plants as compared to controls. More than 90% of the DVT was removed by a 1-sec dip in acetone. Duvatrienediols may have a role in resistance of Ovens 62 and immunized plants; however, they are not the sole determinant of resistance or immunization.

Resistance to blue mold of susceptible burley tobacco plants (*Nicotiana tabacum* L. 'Ky 14') increases with plant as well as leaf age (12). Dipping leaves in acetone for 1 sec increased their susceptibility and the susceptibility of disks derived from such leaves to blue mold (13). The increase in susceptibility was accounted for by the removal of α- and β-4,8,13-duvatriene-1,3-diols (DVT) from the leaf surface (13). Other workers reported an increase in susceptibility of tobacco leaves to blue mold by washing for 10 min with water (7). Shepherd and Mandryk (15) reported that germination of sporangiospores of *Peronospora tabacina* Adam on tobacco (*N. tabacum*) leaves was reduced as compared to germination on leaves of *N. debneyi* Domin, which is highly resistant to blue mold. The DVT account for greater than 0.1% of the fresh weight of tobacco leaves, and their ED_{50} for inhibition of sporangiosporal germination of *P. tabacina* in vitro was approximately 20 μg/ml (5,13). Dipping the leaves or leaf strips of greenhouse-grown tobacco in acetone for 1 sec removed approximately 95% of the DVT (13), which were shown to be the only compounds inhibitory at physiological concentrations to the germination of sporangiospores of *P. tabacina* on leaf surfaces.

Stem injections with *P. tabacina* induce systemic resistance to blue mold (2,4,10,17,19) and, in early studies, the phenomenon was associated with stunting (2,4). A modified technique for stem injection overcame stunting and increased growth of tobacco in the greenhouse (10,17,18) and field (19). Whether the mechanisms for resistance and their regulation are identical in immunized plants and nonimmunized resistant plants is not known.

This study investigated the feasibility of using a leaf disk assay for determining nonimmunization-based resistance to blue mold as well as the effect of acetone dipping and DVT on the resistance of nonimmunized resistant cultivars of tobacco (8,11) and a susceptible cultivar systemically immunized by stem injection with sporangiospores of *P. tabacina* (17).

MATERIALS AND METHODS

Plants. A susceptible burley cultivar (*N. tabacum* 'Ky 14'), a resistant Australian cultivar (Ovens 62) (11), and two resistant Turkish cultivars (Incekara and Izmir Ozbaz) (8) were used in the experiments. The genes for resistance were obtained from *N. velutina* Wheeler for the Australian variety and from *N. debneyii* for the Turkish varieties, and the resistance appears to be oligogenic in both cases (14). Plants were grown in the greenhouse (20–26 C in fall and winter, 20–33 C in spring and summer, under daylight supplemented with 14 hr of fluorescent and incandescent light) in 2-L pots containing Pro-Mix Bx (Premier Peat Moss Corp. Marketing, New York, NY). Pots were watered five times a week to saturation with a 0.1% 15:16:17 (N:P:K) fertilizer (Peters Fertilizer, W. R. Grace & Co., Fogelsville, PA) solution.

Fungus and inducing inoculations. Two isolates of *P. tabacina* were used. Isolate 79 was collected from a field near Georgetown, KY, in 1979 and isolate 82 was collected at Spindletop farm, Lexington, KY, in 1982. The fungus was maintained on Ky 14 plants (7–12-wk-old) grown in growth chambers at 20 C. Fresh sporangiospores of isolate 79 were used for challenge inoculations (17). Six or 7 days after plants were inoculated, sporangiospores were brushed from leaves with an artist's paintbrush into distilled water, collected on a 3-μm-diameter Millipore filter, washed with water, and resuspended in distilled water. The concentration of sporangiospores was determined with the aid of a hemacytometer. Frozen viable sporangiospores (1) of isolate 82 were used for stem inoculations (17). Approximately 1 ml of inoculum (5×10^5 sporangiospores/ml of *P. tabacina*) was injected into stem tissue external to xylem of 8–10-wk-old Ky 14 plants as described previously (10,17,18). In preliminary experiments (data not shown), both isolates induced systemic resistance to blue mold and both were satisfactory for challenge inoculations. The choices of inducing and challenging isolates were dictated by inoculum availability.

Acetone treatment and challenge. In all experiments, the third fully expanded leaf from the top was detached from plants and brought to the laboratory. Strips (5 × 7 cm) were excised from the middle of each half leaf with a razor blade. The strips from one side of the leaf were dipped in acetone (reagent grade) once for 1 sec and strips from the other half were not dipped. Immediately after being dipped in acetone, strips were dipped consecutively into three beakers containing 100 ml of distilled water to remove acetone from the leaf surface. The leaf strips then were dried gently with tissue paper. Disks (18-mm diameter) were cut from strips and placed (10 per plate) on Whatman No. 1 filter paper in plastic petri plates. The paper was moistened with an aqueous solution of 1 μg kinetin/ml. The adaxial surfaces of the disks were inoculated by using an airbrush sprayer (Type H 1 w/H-3-oz, Paashe Airbrush Co., Chicago, IL) (12). Leaf disks in petri plates were sprayed with 1.5 ml of a sporangiospore suspension (1.3×10^4 sporangiospores/ml, unless otherwise indicated) over a period of 10 sec. The inner surfaces of petri plate covers were sprayed with distilled water. The petri plates were kept at 16 C for 12 hr in the dark and then placed in a growth chamber (23 C, 60–70 μES/m^2/sec, 12-hr light) for disease development. Plants were challenged with a suspension of *P. tabacina* (5×10^4 sporangiospores/ml) and incubated as described previously (10,17,18).

In another study, leaf disks were cut from the strips and challenged at weekly intervals after stem injection to determine the time course of immunization. Whole plants were challenged 21 days after stem injections as described above.

Symptoms. Lesion development on leaf disks was recorded, unless otherwise indicated, 7 days after challenge. Symptoms on each leaf disk were individually rated using a visual scale of 0 to 5 (0 = no evidence of disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–100% disease, 5 = 76–100% disease with water soaking). Disease also was determined on 10 leaf disks cut from the first leaf below the detached leaf, unless otherwise indicated, 7 days after the leaf was challenged.

To determine the levels of sporulation on disks cut from leaf strips of detached leaves, leaf disks were transferred into glass petri plates 5 days after challenge. The plates contained moistened sponge rubber pads (9-mm thickness × 9-cm diameter), each with 10 14-mm-diameter punched holes. The pads were wetted with an aqueous solution of 1 μg kinetin/ml. Disks were placed adaxial side up over the holes to allow for sporulation on both sides. For sporulation on attached leaves, plants were covered overnight with plastic bags before disks were cut. Sporangiospores from 30 leaf disks were removed as described (12) and placed into a known volume of a fixative solution (ethanol:formaldehyde:acetic acid, 90:5:5, v/v/v) and counted with the aid of a hemacytometer.

Determination of duvatrienediols on leaf surfaces. Strips of approximately 14-wk-old tobacco leaves (10 g), at the same stalk position, were dipped first in a beaker containing 100 ml of acetone for 1 sec and then immediately into a second beaker containing 100 ml of acetone for 2 min. After the first and second dips, acetone on the leaf surface was shaken into the beaker in which it was dipped. Acetone extracts were filtered through Whatman No. 2 filter paper and dried on a rotary evaporator, and the residue was dissolved in 10 ml of chloroform (reagent grade) (13). The DVT were separated and quantified using a gas-liquid chromatography instrument (5880 A level 4, Hewlett-Packard Co., Rockaway, NJ) equipped with a Hewlett-Packard Ultra No. 1 cross-linked methyl silicone capillary column, 25-m length × 0.2-mm internal diameter, 0.33-μm film. Operating conditions were as follows: temperature programming—hold 15 min at 60 C, then to 300 C at 2 C/min and hold; carrier—He; flame ionization detection. n-Eicosane was used as the internal standard. Peak areas were compared to known standards of DVT to determine amounts present in each sample.

RESULTS

Effect of acetone dipping on stem-injected plants. Leaves of plants injected with *P. tabacina* (immunized) were highly protected when challenged with *P. tabacina*, and the degree of protection of leaf disks was similar to that observed on leaf disks cut from plants 7 days after challenge (Table 1, Fig. 1). Sporulation also was reduced on immunized plants and disks obtained from them (Table 1). Acetone treatment increased susceptibility of leaf tissue from plants injected with *P. tabacina* or water (Table 1, Fig. 1). In another study, leaf strips were collected at weekly intervals from plants stem injected with *P. tabacina* or water. Half of the strips were dipped in acetone for 1 sec and half were not dipped. Leaf disks were cut from the strips and challenged. Whole plants were challenged 21 days after stem injections. Leaf strips from both immunized and control plants became more resistant as plants aged, and the resistance of leaf tissue in strips from immunized plants was significantly greater than that from controls 7 or more days after stem injection (Fig. 1). Acetone treatment increased the susceptibility of leaf strips in both control and immunized plants, but strips from immunized plants were significantly more resistant than those from controls at 21 days after plants were injected. As reported earlier (13), dipping leaf strips in water did not increase their susceptibility to blue mold.

Effect of acetone dipping on nonimmunized resistant cultivars. Resistance increased with age in the resistant cultivars Ovens 62, Incekara, and Izmir Ozbaz (Fig. 2). Seedlings, 6- to 7-wk-old, of these cultivars did not show distinct chlorotic symptoms after challenge, but sporulation was very dense. Resistance was

TABLE 1. Blue mold severity and sporulation on disks cut from tobacco leaf strips dipped in acetone and plants from which the strips were obtained[a,b]

	Leaf disks from leaf strips[c]				Leaf disks from attached leaves[d]	
	Plants injected with water		Plants injected with spores			
Disease	Not dipped	Dipped in acetone	Not dipped	Dipped in acetone	Plants injected with water	Plants injected with spores
Rating[e] (0–5)	1.5 b	3.0 a	0.2 c	1.5 b	3.0 a	0.4 c
Sporulation[f] (spores/ml × 10^3)	8.3 b	20.0 a	0.3 c	3.3 b	17.3 a	0.6 c

[a]Leaf strips were from cultivar Ky 14 plants stem injected with water or sporangiospores of *Peronospora tabacina* as described in the Materials and Methods section.

[b]Means are based on 10 disks/plant/treatment and five plants per treatment.

[c]Leaf disks were inoculated with a suspension of 7×10^3 sporangiospores/ml.

[d]Plant leaves were inoculated with a suspension of 5×10^4 sporangiospores/ml. Disease severity and sporulation were rated on leaf disks cut from leaves 7 days after challenge.

[e]Disease ratings were determined 7 days after challenge according to a 0 to 5 scale (0 = no evidence of disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–100% disease, 5 = 76–100% disease with water soaking). Different letters following means indicate a significant difference ($P < 0.05$)according to Duncan's new multiple range test.

[f]For determining sporulation, disks were transferred to sponge rubber pads 5 days after they were challenged. On day 7, sporangiospores were collected and counted with a hemacytometer. Sporulation on whole plants was determined as described in the text. Different letters following means indicate a significant difference ($P < 0.05$) according to Duncan's new multiple range test.

apparent on plants 10 wk old or older, compared with the same age plants of Ky 14, and resistance increased with age. Among these cultivars, Ovens 62 showed the highest resistance. In general, symptoms on the three resistant cultivars resembled symptoms on immunized plants. Resistance was expressed as a restriction of chlorotic and necrotic lesions. The size of necrotic lesions appeared smaller and more confined on Izmir Ozbaz than on other cultivars. The appearance of lesions was delayed, and most disks of resistant cultivars 10 wk old or older were free of spores. A 1-sec dip in acetone increased the susceptibility of leaves from resistant cultivars and Ky 14. The resistant cultivars, however, were generally more resistant after dipping than Ky 14. Acetone dipping of 13- and 16-wk-old plants did not result in full susceptibility as observed in the 10-wk-old plants.

Determination of duvatrienediols on leaf surfaces. Duvatriene-1,3-diols were obtained from leaf strips of all cultivars after a 1-sec dip in acetone. A single dip resulted in recovery of more than 90% of total removable DVT (Table 2). Plants stem injected with *P. tabacina* and the most resistant cultivar, Ovens 62, had approximately twice as much DVT as did Ky 14 injected with water and the other two resistant cultivars (Table 2).

DISCUSSION

Stem injections with *P. tabacina* resulted in development of resistance as well as increased growth as described earlier (10,17–19). Resistance of stem-injected cultivar Ky 14 was comparable to that of nonimmunized resistant cultivars. This study also showed that the disk assay can be used for determining resistance against blue mold. The results obtained by the disk assay paralleled the results obtained with whole plants; however, disease severity was sometimes higher on whole plants compared with disks. This may be due to the higher inoculum density used for inoculating plants or the presence of kinetin in the media used for maintaining disks for the challenge. Greenhouse and large growth chamber facilities are required for inoculating plants, and plants often cannot be inoculated in the field. Thus, the disk assay may be useful to determine the resistance of plants in the greenhouse and field.

The time course study of immunization with the leaf-disk assay (Fig. 1) gave similar results to earlier studies with whole plants (17,18); however, the effect of stem injections on reduction of susceptibility was detected earlier by inoculation of leaf disks. When whole plants were inoculated, the first effects of resistance were observed about 12 days after stem injection (2,17,18); however, with leaf disks, resistance was observed 1 wk after stem injections. The disk assay appears to be more sensitive than the whole-plant assay.

A 1-sec dip in acetone increases the susceptibility of leaf strips from resistant and susceptible cultivars, as well as susceptible cultivar Ky 14 in which resistance was systemically induced. The increase in susceptibility after the acetone dip, however, was less in the resistant cultivars and immunized Ky 14 than in nonimmunized Ky 14. Even three successive 1-sec dips of stem-injected tobacco leaf strips in acetone did not remove all resistance, although a single 1-sec dip removed approximately 90% of total DVT.

Hill (7) reported that washing tobacco leaves with water increased susceptibility of tobacco to blue mold. However, dipping in water did not have an effect on susceptibility of leaf disks in our experiments (data not shown) and those reported by Reuveni et al (13) and Spurr (16). Spurr (16) observed that dipping

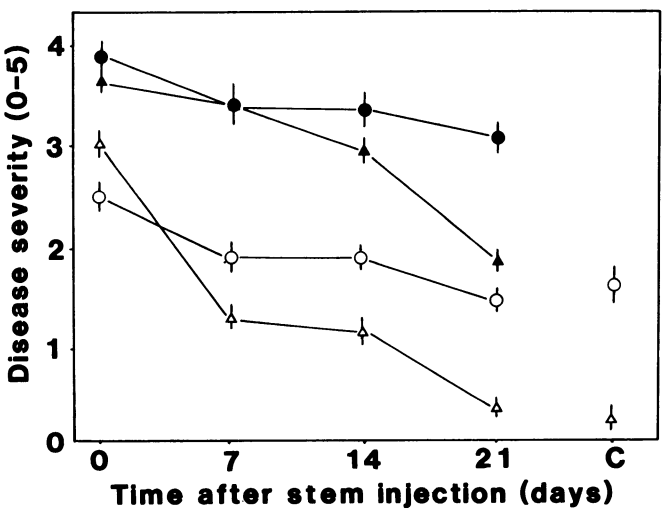

Fig. 1. Effect of dipping tobacco leaf strips in acetone on the susceptibility of disks obtained from the strips to blue mold. Strips from the third fully expanded leaf were collected at weekly intervals after stem injections of approximately 10-wk-old tobacco cultivar Ky 14 plants with *Peronospora tabacina* or water. Disease reactions were determined 7 days after challenge according to a 0 to 5 scale (0 = no evidence of disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–100% disease, 5 = 76–100% disease with water soaking). C = disease severity 21 days after stem injection on plants from which leaf disks were taken. ○—○ indicates disease severity on leaf disks that were taken from plants injected with water; ●—● indicates disease severity on disks from acetone-dipped leaf strips of plants injected with water; △—△ indicates disease severity on leaf disks that were taken from plants injected with *P. tabacina*; ▲—▲ indicates disease severity on disks from acetone-dipped leaf strips of plants injected with *P. tabacina*. Bars indicate standard errors.

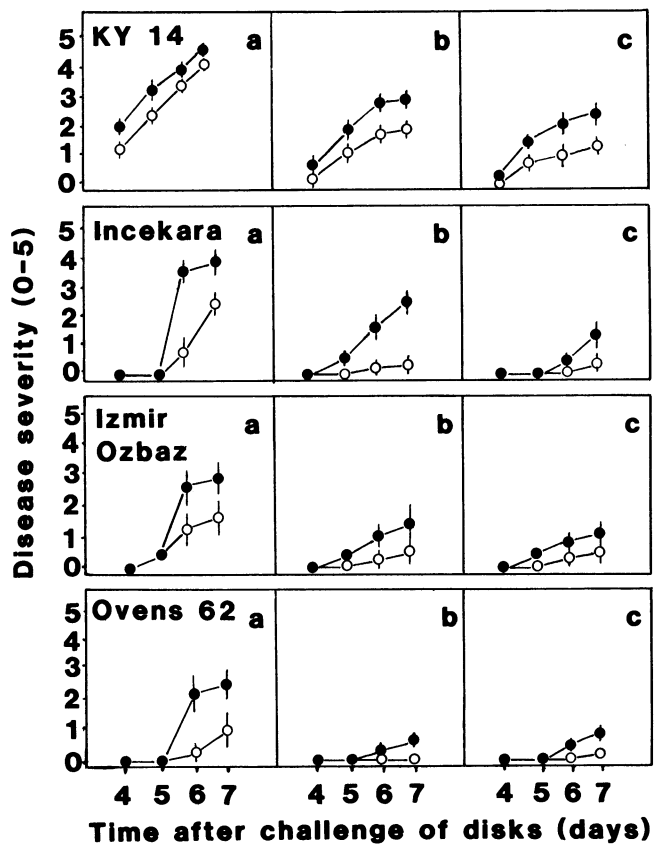

Fig. 2. Effect of age and acetone dipping on the susceptibility to blue mold of four tobacco cultivars. **A,** 10-wk-old plants; **B,** 13-wk-old plants; **C,** 16-wk-old plants. Disease ratings were determined 4–7 days after challenge according to a 0 to 5 scale (0 = no evidence of disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–100% disease, 5 = 76–100% disease with water soaking). Leaf disks were taken from the third fully expanded leaf from the top. Half of the leaves were dipped in acetone for 1 sec (●—●) and the other halves were used as controls (○—○). Leaf strips were rinsed three times consecutively with distilled water and dried with paper towels after acetone dippings. Disks were then cut from the strips, placed on moistened filter papers in petri plates, and inoculated with a spore suspension of *Peronospora tabacina* (1.3×10^4 sporangiospores/ml). Bars indicate standard errors.

TABLE 2. Extraction of α- and β-4,8,13-duvatriene-1,3-diols (DVT) from tobacco leaf strips by dipping in acetone[a]

Plant source (cultivar)	Duvatrienediol (mg/g fresh weight)			
	First dip (1 sec)	Second dip (2 min)	Total	Percent extracted by first dip
Ky 14 control	0.72 b	0.04	0.76 b	95
Ky 14 immunized	1.40 a	0.15	1.55 a	90
Ovens 62	1.50 a	0.09	1.59 a	94
Incekara	0.75 b	0.00	0.75 b	100
Izmir Ozbaz	0.59 b	0.00	0.59 b	100

[a]Leaf strips (10 g) from approximately 14-wk-old tobacco plants were dipped first into a beaker containing 100 ml of acetone for 1 sec and then into a second beaker containing 100 ml of acetone for 2 min. After the first and second dips, acetone on the leaf surface was shaken into the beaker in which it was dipped. Acetone extracts were treated as described in the Materials and Methods section. Quantitative determinations of DVT were done by using a gas-liquid chromatography instrument equipped with a microcapillary column. Peak areas were compared to known standards to determine amounts present in each sample. Different letters following means indicate a significant difference ($P < 0.05$) according to Duncan's new multiple range test.

tobacco leaves into ethanol increased susceptibility of tobacco to *Alternaria alternata* (Fr.) Keissler. He suggested that removal of microflora antagonistic to the pathogen was involved in increased susceptibility. Dipping in acetone for 1 sec also may result in a similar phenomenon; however, removal of a fungitoxic compound, DVT, seems to be a likely explanation for the observed increased susceptibility to blue mold observed in these tests (13).

In earlier work, increased levels of DVT were suggested to be involved in the development of age-related resistance of Ky 14 burley tobacco (13). In this study, DVT levels were higher on one resistant cultivar, Ovens 62, than on susceptible Ky 14; however, levels of DVT on the other two resistant cultivars did not differ from that on Ky 14. Thus, DVT might have a role in the general resistance of Ovens 62 and induced systemic resistance of Ky 14, but other factors affect resistance in both cases. Stresses, such as increased plant population, topping, decreased N_2 fertilization, and soil type have been reported to increase levels of DVT (3,6); however, we are not aware of any such studies that have been related to blue mold resistance. In studies done under low-light intensity during the winter in a greenhouse, protection was observed without a significant increase in DVT in immunized plants, although in every case higher levels of DVT were found in immunized as compared with control plants (authors, *unpublished data*). Cohen and Kuć (2) reported that infiltration of sporangiospores into leaf panels did not overcome induced resistance. Similarly, the highly restricted chlorotic and necrotic lesions formed on immunized leaf disks or leaves after challenge indicate successful penetration. Such lesions do not sporulate or sporulate very sparsely. Removal of DVT from the leaf surface did not make resistant cultivars and immunized Ky 14 "fully" susceptible. These data suggest that resistance does not depend solely on the presence of leaf surface components; however, these components appear to contribute to resistance.

Polarization-interface microscope and electron microscope studies have shown that both resistant and susceptible cultivars are penetrated equally by *P. tabacina* (9). Leaf cells of resistant cultivars (with the exception of epidermal cells) are destroyed quickly after being invaded by *P. tabacina*, whereas susceptible cells are not. Although extensive microscopic studies have not been conducted of the infection process in stem-injected tobacco, the macroscopic reactions of leaf disks from immunized and resistant cultivars are very similar. It is possible that the mechanism of resistance of immunized plants is related to the resistance in nonimmunized resistant cultivars. Stem injection may activate latent resistance genes in susceptible plants and make rapid expression of such genes possible. Further studies on the microscopic and molecular level, comparing resistant and immunized plants, may help to increase our understanding of the mechanism(s) involved in immunization and resistance.

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