# Restricted Colonization by *Peronospora tabacina* and Phytoalexin Accumulation in Immunized Tobacco Leaves

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## ABSTRACT

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*Nicotiana tabacum* 'Kentucky 14' plants were stem injected with either sporangiospores of *Peronospora tabacina* (immunized) or with water (control). For challenge inoculations, filter-paper disks soaked with a sporangiospore suspension of this fungus were placed on the upper surfaces of leaves detached 28 days after stem injection. Nearly all (>99%) germinated spores penetrated both the immunized and the control tissue, as shown after staining with trypan blue. Light microscopy of cleared leaf tissue revealed that colonization of immunized tissue ceased within 5 days postinoculation (dpi) and that mycelium was confined within the chlorotic lesion area ($4.2 \pm 0.2$ mm$^2$ per lesion). Colonization continued in control tissue for about 9 days, and the area per lesion was $314 \pm 3$ mm$^2$ at 7 dpi with hyphae extending about 1 mm beyond the lesion margins. The number of sporangiophores was reduced about 70% in immunized versus control tissue, but the number of sporangiospores per sporangiophore was unchanged. Immunized and control leaves accumulated the sesquiterpenoid stress metabolites phytuberol, phytuberin, rishitin, and capsidiol, but not solavetivone or lubimin, after challenge inoculation. Because the accumulation of these phytoalexins was low compared with reports of accumulations for other solanaceous plants and because they did not accumulate earlier in the immunized leaves than in the controls, it is unlikely that they are the primary cause of restricted colonization by *P. tabacina* in immunized tobacco.

*Additional keywords*: blue mold, resistance.

Immunization of tobacco foliage against the blue mold disease was reported by Cruickshank and Mandryk (4). They observed that injection of sporangiospores of *Peronospora tabacina* Adam into the pith of tobacco stems stunts growth of the plants and systemically protects foliage from disease caused by subsequent challenge with the fungus. Cohen and co-workers (3) studied the development of the fungus from the challenge inoculum on the leaf surface of stem-injected plants. They concluded that, 30 days after stem injection, protection is due to the inhibition of appressorium formation. The method for stem injection reported by Cohen and co-workers (3) results in severely stunted plants (2).

Tuzun and Kuć (13) immunized tobacco plants by injecting *P. tabacina* sporangiospores into stem tissue external to the xylem and overcame the problem of stunting. This technique has been applied successfully in the field (14). Using the technique for stem injection developed by Tuzun and Kuć (13), we now describe the development of *P. tabacina* on and in immunized and control leaf tissues after challenge, and we compare these microscopic observations with measurements of phytoalexin accumulation. Preliminary reports have been published (11,12).

## MATERIALS AND METHODS

**Plants and pathogen.** White Burley tobacco plants (*Nicotiana tabacum* L. 'Kentucky 14') were grown in a peat-vermiculite mixture under a 14-hr photoperiod (sunlight supplemented with fluorescent and incandescent lights) in a greenhouse at 22–30 C. An isolate of *Peronospora tabacina*, obtained in 1979 from tobacco plants in a field near Georgetown, KY, was maintained continuously through weekly transfer on burley tobacco grown in a growth chamber. Inoculum was obtained from freshly sporulating lesions on 7- to 9-wk-old plants 7 days postinoculation (dpi). Sporangiospores were gently brushed into a small quantity of distilled water, collected on a filter (8 $\mu$m pore size), washed twice with distilled water, and resuspended in distilled water to a known concentration of sporangiospores per milliliter of water. Systemic resistance was induced in tobacco plants by injecting 1 ml of a sporangiospore suspension of *P. tabacina* ($10^6$ spores/ml) into the bassal 10 cm of stems with a hypodermic syringe, as described by Tuzun and Kuć (13). Injections were made into tissues external to the xylem of plants 25–30 cm tall about 9 wk after sowing of seed. The largest leaf of each plant was 40–45 cm long. Control plants were injected with 1 ml of distilled water. Longitudinal stem sections at the time of challenge inoculation revealed necrosis of the cambium and external phloem extending 20–25 cm above and 2–5 cm below the point of inoculation. Stems of control plants did not have necrosis.

**Challenge inoculation.** Four weeks after immunization, fully expanded leaves (the second from the top) of the immunized and control plants were broken off at the base of the petiole, and the leaf tips were cut off. Each leaf was placed with its upper surface up on a wet, sponge-rubber pad in a moistened transparent plastic box ($35 \times 27 \times 9$ cm high). Spore suspensions were poured into petri dishes covered with filter-paper disks 6 mm in diameter. Spores

were allowed to settle for 15 min, and the disks were removed from the petri dishes, inverted, and placed about 5 cm apart and about 2 cm from the midrib on the upper surfaces of leaves. This inoculation technique has the advantage of distributing the spores at the inoculation site, as compared with a drop of spore suspension, where spores tend to accumulate around the edges of the drop. The data presented refer to the mean number of spores found on the leaf surface after removing the filter-paper disk. In experiments to determine the effect of leaf detachment on symptom development, disks containing inoculum were placed on leaves of 8- to 10-wk-old plants. Incubation was as described earlier (13).

Detached leaves were maintained initially for 24 hr in the dark at 18 C, then for 6 days at 22 C under fluorescent lights with a 14-hr photoperiod. Six-day-postinoculation sporulation was induced by thoroughly and evenly spraying the upper leaf surfaces and inner surfaces of the boxes with water and keeping the boxes in the dark at 18 C for 15–20 hr. Sporulation occurred on lower leaf surfaces, and sporangiophores were counted using a dissecting microscope. To count sporangiospores, sporulating leaf areas were punched out with a cork borer (1 cm in diameter) and immersed in 1 ml of water. Spores were removed from the leaf tissue with a painter's brush, and the spore concentration was determined with a hemacytometer. For studying further disease development, boxes were kept up to 3 wk at 22 C with a 14-hr photoperiod. Lesions were circular, and lesion margins were more distinct on the lower leaf surfaces than on the upper surfaces. Therefore, lesion diameter was measured on the lower leaf surfaces, and the area was calculated.

**Microscopic observations.** For germination and penetration studies, a drop of trypan blue solution containing 0.1–0.5% dye in 45% acetic acid (1) was placed on inoculated leaf tissue. Within 5 min, fungal cell walls and cytoplasmic contents on the leaf surface were stained dark blue. They were observed in lactic acid under a cover slip using bright-field microscopy. Colonization by the fungus was observed in leaf tissue cleared by boiling in a solution of 70% ethanol, 35% formaldehyde, and glacial acetic acid (90:5:5, v/v/v) for 10 min. To compare lesion area with the leaf area colonized by the fungus, 1-mm leaf strips were cut from chlorotic lesions starting with tissue within the lesion and extending to healthy tissue outside the lesion. Seedlings $<$ 20 cm in height (about 5 wk after sowing seed) served as positive checks on the effectiveness of inoculation, since these young seedlings are highly susceptible. Germination of *P. tabacina* sporangiospores in vitro was determined approximately 20 hr after placing 10-$\mu$l drops of a spore suspension ($5 \times 10^4$ spores/ml) on 2% agar disks.

**Phytoalexins.** Leaves were challenged by spraying the upper surfaces evenly with *P. tabacina* ($5 \times 10^4$ spores/ml, that is, about $10^3$ spores/cm$^2$ of leaf area). Leaves were collected at intervals after challenge; the midribs were discarded; and the remaining tissue was kept frozen until extracted as described previously (6). Thin-layer chromatography of the crude MeOH extracts was performed on 20 × 20 cm precoated silicagel (250 $\mu$m) flexible polyester plates (American Science Products, Columbus, OH) using cyclohexane: ethyl acetate (1:1 v/v) as a solvent system. $R_f$ values of sesquiterpenoid stress metabolites (SSM) were compared to those of known standards. Compounds were detected on thin-layer plates by spraying with a solution containing 2.8 g of vanillin in 100 ml of MeOH containing 0.5 ml of concentrated $H_2SO_4$, or concentrated $H_2SO_4$. Plates were observed before and after heating at 110 C for 2–3 min. The identities of SSM were verified further by gas chromatography (GC) as described previously using methyl arachidate as the internal standard (7). For this purpose, 4-g fresh-weight equivalents of the crude extracts were run on thin-layer plates. Zones of the plate containing SSM were cut off, and SSM were eluted by repeated washing with 5 ml of MeOH. Combined extracts were concentrated under a stream of $N_2$ and dissolved in 100 $\mu$l of MeOH for GC analysis. The GC retention time for methyl arachidate was 16.6 min, and the retention times relative to methyl arachidate for phytuberol, phytuberin, solavetivone, rishitin, capsidiol, and lubimin were 0.34, 0.37, 0.57, 0.83, 1.20, and 1.30, respectively.

## RESULTS

**Germination and penetration.** Nearly all germinated sporangiospores penetrated both the immunized and the control leaves (Table 1), as shown after staining with trypan blue. Penetration of immunized and control leaves occurred within 17 hr of inoculation. In both cases, germ tubes were about 5–40 $\mu$m long. Penetration of the epidermal cells occurred through the periclinal wall near a junction of two epidermal cells. A hypersensitive response of the epidermal cells, that is, necrosis or granulation of cytoplasm within 3 dpi, was not observed. The cytoplasm within the spores decreased (Fig. 1A) during the process of germination and penetration, until the spores and the germ tubes became empty (Fig. 1B). The cytoplasm appeared to migrate from the spore to the germ tube, then into the appressorium and the colonizing hyphae. A blue circle about 5 $\mu$m in diameter, which appeared to be a penetration peg beneath a germinated spore, was indicative of a penetration site. The penetration peg could best be observed after clearing the leaves. Spores germinated on and penetrated equally the leaves from immunized and nonimmunized plants as well as leaves of seedlings at all inoculum densities tested (50, 500, 5,000 spores/disk).

**Colonization.** In this paper, we define colonization as all events between penetration and sporulation: vesicle formation, growth of hyphae from vesicles, and symptom development. Light microscopy of cleared leaves revealed that the fungus invaded the epidermal cells of both the immunized and control leaves. Within 2 dpi, a vesicle developed within the penetrated epidermal cells. Hyphae proliferated intercellularly first through the palisade and then through the spongy parenchyma. Hyphae looked yellowish against the white background of the host tissue. This development of hyphae was observed in immunized and nonimmunized leaves.

Differences in colonization between treatments were detected. In immunized leaves, colonization ceased by 5 dpi, at which time lesions became visible. Lesion diameter did not subsequently increase on immunized leaves (Fig. 2). Mycelium was confined within the lesion area. More than one lesion (usually two to five lesions) were present beneath the majority of the filter-paper disks at sites that developed lesions. Hyphae did not look distorted during the time interval observed (up to 16 dpi) (Fig. 1C).

In control tissue, colonization, and therefore lesion development, continued about 9 days (Fig. 2). Mycelium extended approximately 1 mm beyond the lesion margins. The foremost hyphae were ramifying through the spongy parenchyma close to

TABLE 1. Disease development in immunized and nonimmunized tobacco leaves challenged with *Peronospora tabacina*

| Parameter of disease development | Treatment[a] | |
|---|---|---|
| | Immunized | Nonimmunized |
| Percent germination[b] | 69 ± 11 | 71 ± 12 |
| Percent penetration in relation to germinated spores | >99 | >99 |
| Lesion size (mm$^2$) 7 days after inoculation[c] | 4.2 ± 0.2 | 314 ± 3 |
| Sporangiophores per square centimeter of colonized leaf area[d] | 38 ± 5 | 144 ± 11 |
| Spores per sporangiophore[e] | 40 ± 10 | 40 ± 10 |
| Viability of spores (%) | | |
| Germination on leaves from seedlings[f] | 72 ± 11 | 75 ± 10 |
| Germination on agar[g] | 65 ± 7 | 69 ± 8 |

[a] Four weeks after immunization, leaves were challenged with 6-mm-diameter filter-paper disks containing about 5,000 spores per disk. Data are mean ± standard deviation.
[b] Five leaves per treatment with three leaf disks (1 cm in diameter) per leaf; 100 spores counted per disk. All leaves were taken from different plants. Germination and penetration were determined 24 hr after challenge.
[c] Five leaves per treatment with seven inoculation sites.
[d] Five leaves per treatment with three leaf disks.
[e] The spores collected were counted four times.
[f] Ten filter-paper disks containing spores from immunized or control tissue were placed on leaves from about 5-wk-old seedlings; 100 spores per disk.
[g] Ten agar disks per treatment.

the lower epidermis (Fig. 1D). The lesions from different filter-paper disks often coalesced by 9 days.

With decreasing inoculum concentration, the percentage of inoculation sites (filter-paper disks) with symptoms decreased more rapidly on immunized leaf tissue than on control leaf tissue (Table 2). Lesions developed on leaves from seedlings regardless of spore concentration. The disk inoculation technique was applicable to intact plants and the differences of lesion size between immunized and control plants were similar to those found for detached leaves.

**Sporulation.** The number of sporangiophores was reduced about 70% in immunized versus control tissue, but the number of sporangiospores per sporangiophore was unchanged. Sporulation was not always observed on lesions of immunized plants and was evident in only about 50% of the experiments. More than 90% of the lesions on nonimmunized plants sporulated. Immunization had no effect on in vivo or in vitro germination of the spores harvested (Table 1).

**Phytoalexin accumulation.** Leaves taken from both the immunized and nonimmunized plants accumulated the sesquiterpenoid stress metabolites phytuberol, phytuberin, rishitin, and capsidiol, but not solavetivone or lubimin, after challenge inoculation by spraying with *P. tabacina* spores (Fig. 3). Phytoalexins were first detected on leaves of immunized and nonimmunized plants 5 dpi when chlorosis first was observed. As the disease developed, the amounts of phytoalexins increased. Up to the time of sporulation of the fungus on nonimmunized plants (7 dpi), the total accumulation of phytoalexins was low relative to that reported for other solanaceous plants. At that time, there was about a fivefold greater accumulation in control leaves than in immunized leaves. The chlorotic leaf areas were about 95% for the control tissue and about 20% for the immunized tissue. Phytoalexins were not detected in unchallenged leaves.

## DISCUSSION

*P. tabacina* germinated on and penetrated into leaves of immunized and control tobacco equally. Ungerminated spores had cytoplasm, but spores that had germinated and penetrated were devoid of cytoplasm. This was revealed clearly by trypan blue, and it greatly facilitated counting of germination and penetration. Thus, we prefer trypan blue to calcofluor (3), which stains only the

TABLE 2. Effect of the density of sporangiospores of *Peronospora tabacina* at an inoculation site on the appearance of blue mold on excised leaves of tobacco[a]

| | Tissue and percent infection sites with symptoms | | |
|---|---|---|---|
| Sporangiospores/site | Nonimmunized | Immunized | Seedlings |
| 50 | 37 | 8 | 100 |
| 500 | 63 | 12 | 100 |
| 5,000 | 88 | 61 | 100 |

[a] Filter-paper disks (6 mm in diameter) containing various concentrations of *P. tabacina* sporangiospores were placed on excised tobacco leaves of burley tobacco cv. Kentucky 14 from immunized and nonimmunized plants and nonimmunized seedlings (<20 cm in height about 5 wk after sowing seed).

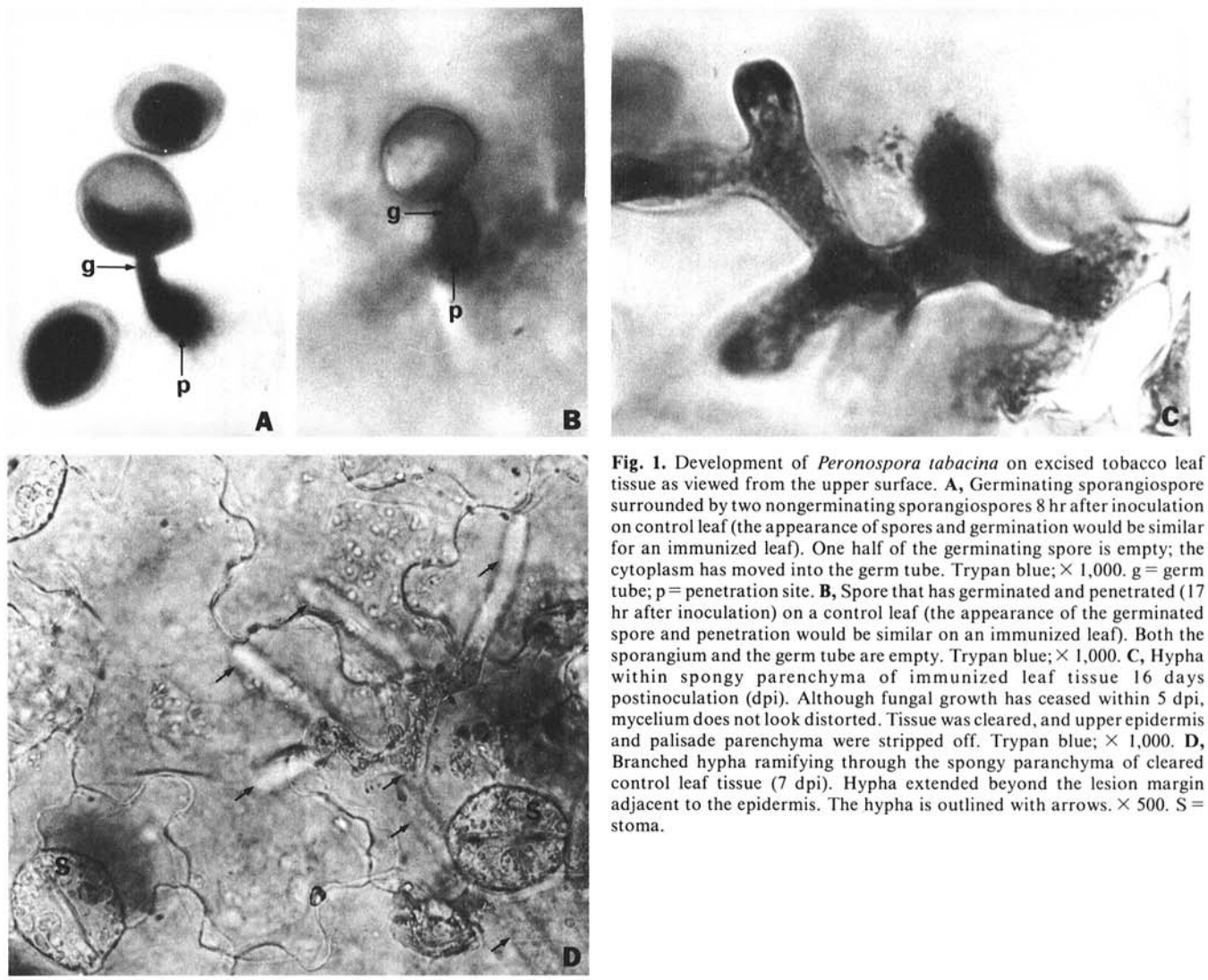


**Fig. 1.** Development of *Peronospora tabacina* on excised tobacco leaf tissue as viewed from the upper surface. **A,** Germinating sporangiospore surrounded by two nongerminating sporangiospores 8 hr after inoculation on control leaf (the appearance of spores and germination would be similar for an immunized leaf). One half of the germinating spore is empty; the cytoplasm has moved into the germ tube. Trypan blue; × 1,000. g = germ tube; p = penetration site. **B,** Spore that has germinated and penetrated (17 hr after inoculation) on a control leaf (the appearance of the germinated spore and penetration would be similar on an immunized leaf). Both the sporangium and the germ tube are empty. Trypan blue; × 1,000. **C,** Hypha within spongy parenchyma of immunized leaf tissue 16 days postinoculation (dpi). Although fungal growth has ceased within 5 dpi, mycelium does not look distorted. Tissue was cleared, and upper epidermis and palisade parenchyma were stripped off. Trypan blue; × 1,000. **D,** Branched hypha ramifying through the spongy paranchyma of cleared control leaf tissue (7 dpi). Hypha extended beyond the lesion margin adjacent to the epidermis. The hypha is outlined with arrows. × 500. S = stoma.

cell wall of fungi, for studies of germination and penetration.

Our data indicate that colonization is the first stage of disease development affected by immunization. Colonization of immunized tissue ceased within 5 dpi and mycelium was confined within the lesion area, whereas colonization continued in control tissue for approximately 9 days and mycelium extended about 1 mm beyond the lesion margins. Because lesions were much smaller on immunized leaves than on control leaves as soon as they were visible (Fig. 2), fungal development was restricted before that time (5 dpi). Thus, the critical events of resistance occur after penetration (1 dpi) but before symptom development (5 dpi).

With decreasing inoculum density, the percentage of inoculation sites, that is, filter-paper disks that resulted in visible lesions, decreased more rapidly on immunized leaves than on control leaves (Table 2). This suggests that the time frame for the critical events of resistance is the period after penetration and the start of hyphal growth from vesicles. A microscopic study of lesion development during this time requires further study. The resistance mechanisms involved also may have been partially active in the control tissue because the number of visible lesions did decline with decrease in inoculum density, whereas lesions were visible under 100% of the disks on seedlings regardless of inoculum density.

Stenzel and co-workers (10) induced resistance against powdery mildew of wheat and barley by spraying the host leaves with culture filtrates of soil microorganisms. The first stage of disease development affected was haustorial formation. Additional effects were reported for the subsequent stages of disease development so that the effect of immunization was greatest on the level of sporulation. The effect of immunization of tobacco against blue mold follows a similar pattern. Fungal development on the leaf surface was not restricted, but colonization ceased within a short period of time. There was an additional adverse effect on sporulation. From these observations, it appears that, once a parasitic relationship is in disorder, the subsequent stages of disease development also are affected by immunization.

It is unlikely that colonization by *P. tabacina* of immunized tobacco is restricted by the sesquiterpenoid phytoalexins phytuberol, phytuberin, solavetivone, rishitin, capsidiol, or lubimin, which have been reported to accumulate in tobacco leaf tissue infiltrated with *Pseudomonas lachrymans* (5). We first detected these compounds in immunized and control plants as cells started to die at symptom development, after the time resistance was expressed by immunized plants. Even at the time of sporulation (7 dpi), the total amount of phytoalexins found (sum of phytuberol, phytuberin, rishitin, and capsidiol) was not greater than about 13 $\mu$g per gram fresh weight in control tissue sprayed with a spore suspension of the fungus. These levels are much lower than those reported for other solanaceous plants (8). Total phytoalexin concentration was about five times higher in control leaves than in immunized leaves; likewise, the fraction of leaf area showing symptoms was about five times higher in control plants than in immunized plants. Therefore, it is unlikely that these phytoalexins are a primary cause of the restricted colonization in immunized tobacco. This conclusion would be strengthened if phytoalexin concentration at the location and time where lesion development ceased could be determined. Because the fungus is a biotrophic parasite, changes in the concentration of host metabolites may be the mechanism by which colonization is restricted. Salt and co-workers (9) reported an increase in total soluble carbohydrates in immunized leaves as compared to control leaves.

An example for reduced infectivity of the spores harvested from immunized plants was reported by von Alten and Schoenbeck (15). Resistance was induced by spraying culture filtrates of soil microorganisms on the lower surfaces of bean leaves. For challenge inoculation, the same surfaces were sprayed with *Uromyces phaseoli* uredospores. The authors claimed that the culture filtrates chelated iron in the leaves causing the production of iron-depleted spores in which the cytochrome system was defective. The direct effect of a resistance-inducing agent solubilized in the inoculum droplet is not likely in our system because the induced resistance is systemic. Indeed, *P. tabacina* spores derived from immunized tobacco plants were as infectious as spores derived from control plants (Stolle et al, *unpublished*). Nevertheless, immunization of tobacco against blue mold has the potential of being very effective from the epidemiological point of view, because it caused greater than a 99% reduction of the amount

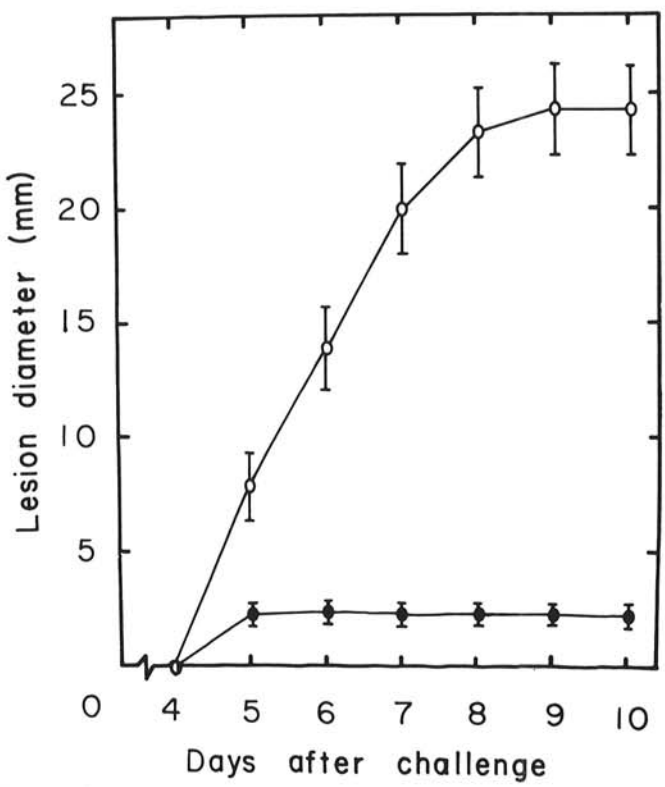


**Fig. 2.** Lesion development on excised immunized tobacco leaves (—●—) and controls (—o—) challenged with *Peronospora tabacina* (about 5,000 sporangiospores per 6-mm-diameter filter-paper disk). Mean ± standard deviation of 10 leaves with 10 filter-paper disks each.

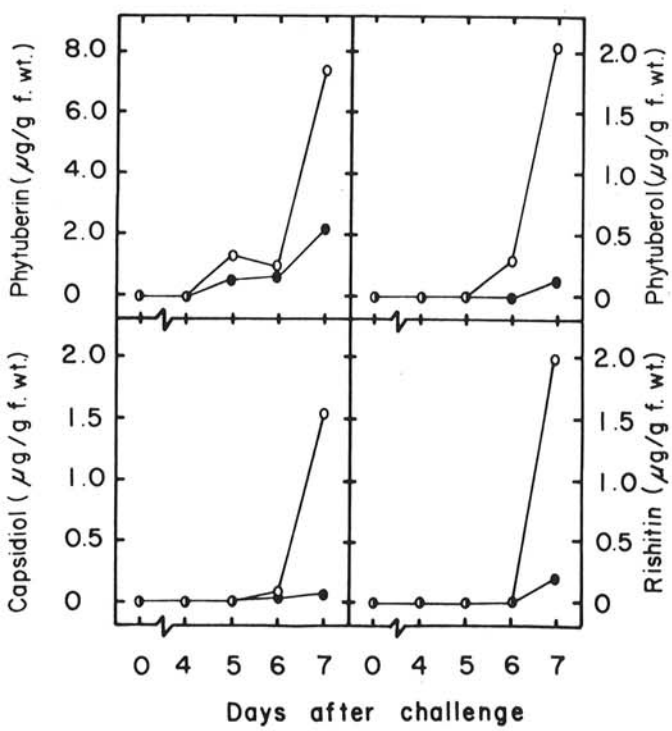


**Fig. 3.** Phytoalexin accumulation in immunized tobacco leaves (—●—) and controls (—o—) at varying time intervals after challenge inoculation by spraying with sporangiospores of *Peronospora tabacina*.

of spores produced per inoculation site.

Injection of *P. tabacina* spores into the pith of tobacco stems, as reported earlier (2), induced resistance to the fungus but also stunted plants. Leaves of such plants were smaller and thicker compared to the leaves of controls. It is reported that such stunted plants escape infection due to an increased percentage of spores with abnormal elongated germ tubes partially incapable of appressorial formation (3). In the present study, spores were injected into stem tissues external to the xylem. Stem necrosis was restricted to the cambium and external phloem. This did not result in stunting except in seedlings (<20 cm in height) where stem necrosis reached the growing tip. Similar to plants immunized by stem injection into the pith, leaves of such immunized but stunted seedlings were smaller and thicker compared to the leaves of controls. When these leaves were challenged with *P. tabacina* spores 4 wk after stem injection, germination was reduced about 80% compared to germination on controls. The reduction of germination on immunized but stunted seedlings is consistent with the reduction of germination on plants stem injected into the pith as reported by Cohen et al (3). The number of lesions on immunized but stunted seedlings inoculated with disks containing 500 spores/disk also was reduced about 50% compared to controls; however, the area of colonization per lesion of such immunized seedlings was reduced only about 20% (Stolle et al, *unpublished*). We conclude that the development of the fungus from the challenge inoculum is restricted on the leaf surface when immunized plants are stunted, but when they are not stunted, immunization primarily affects colonization by the fungus.

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