

The Mode of Action of Phosphite: Evidence for Both Direct and Indirect Modes of Action on Three *Phytophthora* spp. in Plants

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ABSTRACT

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Phosphite, applied as a root drench, provided protection against invasion by *Phytophthora cinnamomi*, *P. nicotianae*, and *P. palmivora* in lupin, tobacco, and paw-paw, respectively. Protection was expressed as a reduction in the rate of lesion extension after wound inoculation. Phosphite concentrations at the site of inoculation were sufficient to reduce mycelial growth in vitro. There was a close relationship between the concentration of phosphite present at the invasion site and the extent to which protection was expressed, although phosphite concentrations

were never fungitoxic. Once accumulated, phosphite remained in the plant for extensive periods. Results suggest that the concentration of phosphate present at the infection site influenced the degree to which phosphite protection was observed in treated plants. In the three fungal-plant combinations examined, phosphite concentrations were sufficient to reduce fungal growth by direct action. However, plant defenses would be important in completely arresting pathogen invasion.

The fungicide fosetyl-Al is an effective control of many plant diseases caused by Oomycetes (2,6,34). Recently, phosphite has been shown to be as effective as fosetyl-Al in the control of several of these diseases (22), and it seems probable that phosphite is formed from fosetyl-Al in the plant and is the active component of the product (4,7,13,28). However, the mode of action of fosetyl-Al, and by implication phosphite, remains unclear.

In low phosphate media, fosetyl-Al or phosphite must be present in millimolar concentrations to reduce the growth of pathogens under some test conditions in vitro (4,7,12,13,31). Diseases caused by these same pathogens are controlled by relatively low-volume (1-5 kg/ha) applications of the fungicides in the field (11,34). To explain this apparent discrepancy, it was proposed that fosetyl-Al and phosphite were able to induce a more rapid response to the invading organisms by the plant's dynamic defense system (3,17,18). Demonstrations of accelerated phytoalexin increases in plants protected by fosetyl-Al or phosphite provided evidence consistent with this view (17,26,27). Inhibition of the enzyme phenylalanine ammonia lyase by the addition of inhibitors such as α -aminoxyacetate reduced the degree of protection provided by fosetyl-Al in plants producing isoflavonoid phytoalexins or compounds derived from cinnamic acid in response to invasion (19). This also could be taken as evidence for a site of phosphite action within the plant or as an essential requirement for host defense systems to function in plants protected by phosphite.

However, the observation that phosphite, in particular, reduced the growth rate of several species of fungi when applied directly during in vitro tests (4,10,13) suggested that a direct action on the pathogen in the host plant was possible if the concentration of phosphite was sufficiently high at the site of fungal invasion.

In this paper, we present evidence to show that in three host/parasite combinations, phosphite applied as a root drench accumulated to concentrations sufficient to reduce fungal growth in vitro. We also show that phosphite-induced protection may be reduced when the phosphate concentration at the infection site rises substantially, e.g., at flowering.

MATERIALS AND METHODS

Fungal strains. *Phytophthora cinnamomi* Rands originated from an infected plant of *Isopogon* sp. in the Brisbane Ranges, Victoria. Its history and the method of culture and maintenance are described in Phillips et al (23). *P. palmivora* (Butl.) Butl. was obtained from the University of California, Riverside, collection. Its history and the method of maintenance are described by Tokunaga and Bartnicki-Garcia (32). Its use in plant infection experiments was carried out under Federal Quarantine permit 6255. *P. nicotianae* B. de Haan var. *nicotianae*, isolate M3049, was isolated from foliar black shank on tobacco by G. Johnson, Department of Primary Industries, Mareeba, Queensland.

Plant cultivars. Lupin (*Lupinus angustifolius* L. 'Unicrop') was grown in gravel-peat moss mixture (7:1, v/v) in a controlled environment chamber (26 and 18 C, day/night temperature; 12-hr photoperiod) as described by Gayler et al (15). Plants were thinned to four per pot before the application of phosphite and were selected for uniformity of size and morphology. Tobacco (*Nicotiana tabacum* L. 'Hicks') was grown for 6 wk in a greenhouse and then transferred to a controlled environment chamber (28 and 20 C, day/night temperature; 16-hr photoperiod) 1 wk before treatment with phosphite (17). Plants were thinned to one per pot at this stage and had six fully expanded leaves. Paw-paw (*Carica papaya* Tourn. ex L.) seedlings were grown in a controlled environment chamber (26 C, 14-hr photoperiod) from seeds obtained from market fruit. Seedlings were thinned to four per pot at the time of phosphite treatment, by which time they were 60 days old and 10-15 cm tall.

Phosphite application. Phosphite was applied to all plants as the potassium salt, prepared from anhydrous phosphorous acid (Albright & Wilson, Australia, Ltd.), shown to be 99% pure by chromatographic analysis (30). Phosphite was applied as a single drench (20 ml per pot). Pots were watered to field capacity 24 hr after the application, and this watering regime continued daily throughout the experiment. Complete fertilizer (Aquadol, Hortico, Melbourne, Australia) was applied every seventh day.

Inoculation. All plants were inoculated through wounds. Apical

meristems were removed and inoculum was placed directly on the wound surface. Lupin (12 to 14 day old) and paw-paw (60 day old) were inoculated with mycelium of *P. cinnamomi* and *P. palmivora*, respectively. Mycelium was grown on V-8 agar in a 1.5-ml microfuge tube, and the tube was placed directly over the cut surface and secured to a supporting stake. The tube prevented desiccation of the wound surface and aided in the establishment of infection. Sham inoculations carried out with tubes containing no mycelium served as controls. Tobacco plants were inoculated with zoospore suspensions of *P. nicotianae* ($5 \mu\text{l}$ containing 5×10^4 spores). Inoculation sites were protected with foil caps to restrict the rate of evaporation. Sham inoculations with $5 \mu\text{l}$ of sterile distilled water served as a control.

Reisolation of pathogens. In one series of experiments (lupin and tobacco), the fungi were reisolated from inoculated plants. Stems were sectioned into 1-cm sections for 5 cm below the most advanced point of the lesion, and the sections were plated on selective agar. Tsao and Ocana's (33) medium was used for *P. cinnamomi* and the medium of Ponchet et al (24) was used for *P. nicotianae*.

Lesion measurement. In lupin and paw-paw, lesions appeared as water-soaked tissue, which then collapsed rapidly. Some necrosis was observed, but the length of the water-soaked section was taken as the measure of lesion extension. In tobacco, external lesions were less distinct, and, therefore, stems were sliced vertically and the internal lesion extension was measured. Lesion extension was marked by browning and necrosis, which spread further in the vascular tissue than in pith or cortex. In paw-paw, complete stem collapse occurred 1 wk after inoculation, limiting the time that lesions could be measured.

Phosphite and phosphate measurement. One-centimeter sections of stem tissue, taken immediately above the site of inoculation, or in some experiments from the lesion front, were extracted with isopropanol-formic acid. Phosphite and phosphate were separated and quantified by ion exchange chromatography (30).

In vitro tests. Potassium phosphite solutions, sterilized by passage through $0.2\text{-}\mu\text{m}$ filters, were added to 50 ml of Ribeiro's (25) defined medium, containing 7 mM phosphate. A single 2-mm^2 mycelial plug, from the edge of a rapidly growing culture on Ribeiro agar was placed in each flask. Mycelia were harvested after 7 days of growth at 26 C by filtration onto preweighed glass-fiber filters, washed twice with distilled water, and dried overnight at 100 C. Mycelial plugs placed on glass-fiber filters at inoculation were used to correct for inoculum mass.

Experimental design and statistics. Measurement of lesion extension after different levels of phosphite application (lupin and tobacco) was carried out two to five times with three to eight replicates for each treatment. The results of each repeated experiment were reproducible except where noted. In vitro tests for phosphite inhibition were carried out eight times for *P. cinnamomi*, three times for *P. nicotianae*, and more than 20 times for *P. palmivora*, each with three replicates for each phosphite treatment. Results from repeated experiments were similar.

In plant experiments where a single concentration of phosphite was applied, a latin square design was used. Replicates (three to eight plants) received phosphite/no phosphite, inoculation/sham inoculation treatments. Plants receiving sham inoculations did not form lesions. Analytical constraints required that samples for phosphite analysis were chosen at random from the total number of plants harvested in each experiment. Data were analyzed by Student's paired *t* tests. Regression coefficients were computed from the Sigmaplot statistics package (Jandel Scientific, Sausalito, CA).

RESULTS

Lupin-*P. cinnamomi*. Phosphite applied to lupin seedlings at the rate of 20 mg per pot reduced the rate of lesion development (Fig. 1). Application of phosphite at this level slowed but did not arrest lesion extension. The site of inoculation was important in determining the outcome of the experiment. Inoculation

between the second pair of leaves and the cotyledons produced lesions, and the rate of extension of these lesions was reduced in phosphite-treated plants (Fig. 1). Differences in lesion lengths were significant ($P \leq 0.05$) from the second day. Inoculation above the second pair of leaves failed to produce a lesion in either phosphite-treated or control plants, whereas inoculation below the cotyledons produced lesions that extended at uniformly rapid rates in both treated and control plants.

Analysis of phosphite-treated plants showed that the highest concentration of the anion was observed in the stem and the lowest in the roots, and there appeared to be a concentration gradient from the upper stem to the roots, although the difference between the levels above and below the cotyledons was not significant (Fig. 2). In a second experiment, phosphite was applied to 14-day-old plants at 20 mg per pot, and phosphite concentrations in the upper stem were measured over an interval of 9 days. Phosphite concentrations reached their maximum ($200 \mu\text{g/g}$) some 4 days after application (Fig. 3). The experiment was repeated with 21-day-old plants. In these older plants, phosphite concentrations exceeded $1,500 \mu\text{g/g}$ in the 1 cm above the first pair of true leaves 7 days after phosphite application, and remained at that level for another 14 days (data not shown). When a range of phosphite levels (0.5–50 mg per pot) corresponding to 0.25–25 kg/ha was supplied to plants, the amount of phosphite present 4 days later at the inoculation site in the stem was proportional to the amount of phosphite applied,

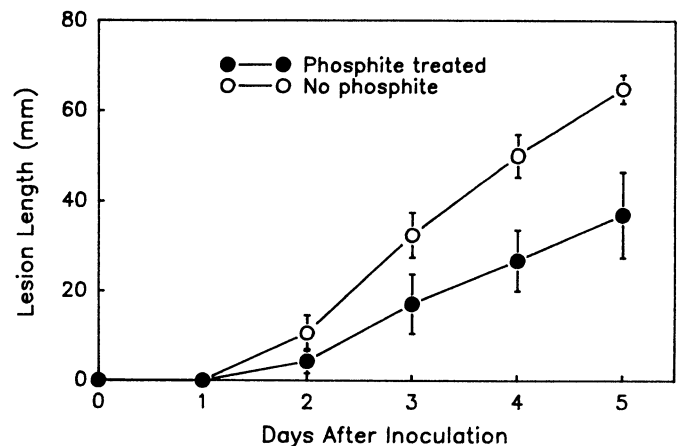


Fig. 1. Extension of lesions after inoculation of lupin stems with *Phytophthora cinnamomi*. Lupin seedlings, 14 days old, were treated with potassium phosphite at 20 mg per pot, and 4 days later they were inoculated with *P. cinnamomi*. Lesion lengths between controls and treated plants were significantly different ($P \leq 0.05$, $n = 5$) from 48 hr onward. Error bars \pm one standard deviation; where no error bars are shown, values were smaller than the symbol size.

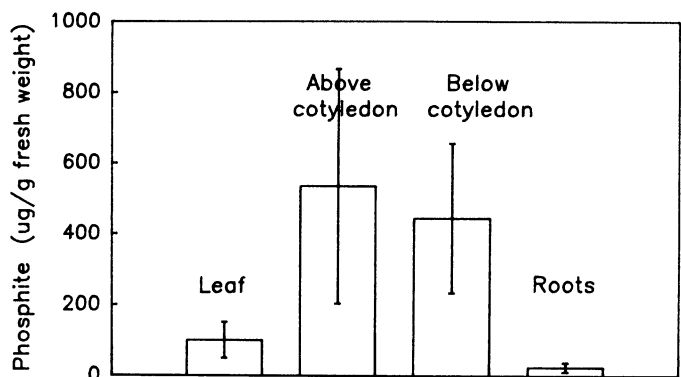


Fig. 2. Phosphite distribution in 14-day-old lupin seedlings, 10 days after a single root drench of 20 mg of phosphite per pot. Data show means \pm standard deviation from eight plants in separate pots.

once a threshold level of 10 mg per pot had been exceeded. The extent of protection, as expressed by decreased lesion extension at 72 hr, increased as the concentration of phosphite present increased above 40 $\mu\text{g/g}$ (Fig. 4). The correlation coefficient between lesion length and log concentration of phosphite was -0.99 . Under these conditions, where all plants were of equal age, there was little variation in the concentration of phosphate present (12.6 mM, 1,200 $\mu\text{g/ml}$), regardless of the amount of phosphite accumulated. Although the concentrations of phosphite present in the lupin stems at the time of inoculation varied from one experiment to the next, the concentrations fell within the range at which phosphite reduced the rate of growth of *P. cinnamomi* when tested in vivo in the presence of 7 mM phosphate (Fig. 5).

Tobacco-*P. nicotianae*. In tobacco, a single application of 20 mg of phosphite per pot 4 days before infection also provided protection against invasion by *P. nicotianae*. In tobacco, as opposed to lupin, lesion extension stopped after 48 hr in phosphite-treated plants, but continued down the entire length of the stem in untreated plants. The protection level afforded by phosphite remained constant as the plants were challenged by inoculation at intervals over a 5-wk period, until it decreased at the onset of flowering (Fig. 6). The concentration of phosphite in the stems at the site of lesion arrest remained constant throughout the whole experimental period (Fig. 7). Phosphate concentrations also remained constant until flowering time when they increased significantly.

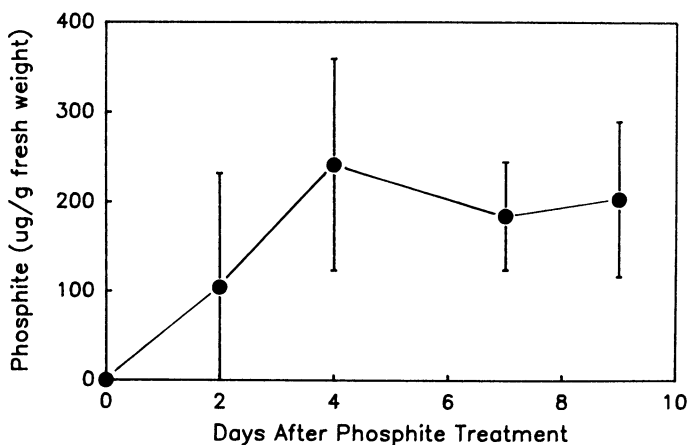


Fig. 3. Levels of phosphite in lupins over a 9-day period after a single application of potassium phosphite at 20 mg per pot; the phosphite concentration in the 1 cm immediately above the cotyledons was measured at intervals over 9 days. The data show means \pm one standard deviation for $n = 3$.

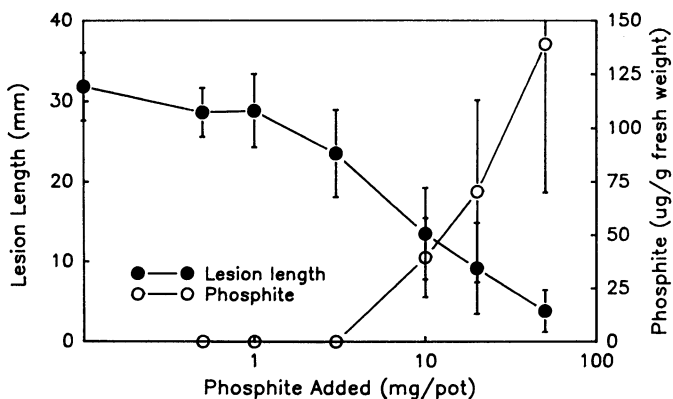


Fig. 4. Phosphite concentrations in lupin stems as a function of phosphite applied to the root system, together with lesion length 72 hr postinoculation with *Phytophthora cinnamomi*. Stem samples for analysis were taken from 1 cm above the inoculation site. Data show means \pm one standard deviation for $n = 5$. Plants for each replicate came from separate pots.

Application of a range of phosphite amounts (0.5–50 mg per pot) showed that in tobacco, as in lupin, the concentration of phosphite present at the inoculation site varied with the amount of phosphite applied (Fig. 8), and that lesion development was

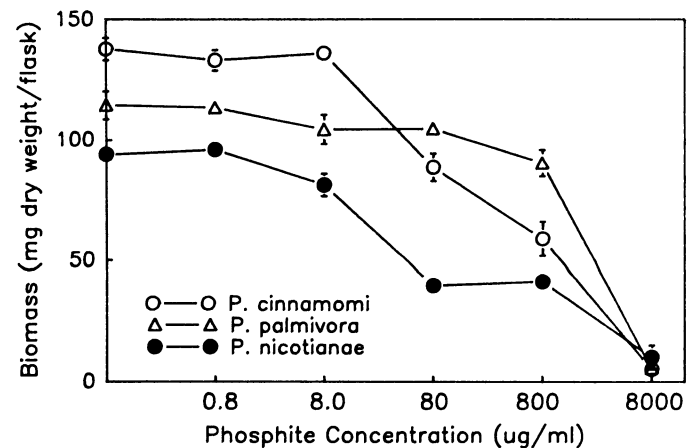


Fig. 5. Biomass of three *Phytophthora* spp. grown in a range of phosphite concentrations. Cultures were grown at 26 C for 7 days on Ribeiro's medium, which contained 7 mM phosphate. Means of eight replicates; bars are \pm the standard deviation.

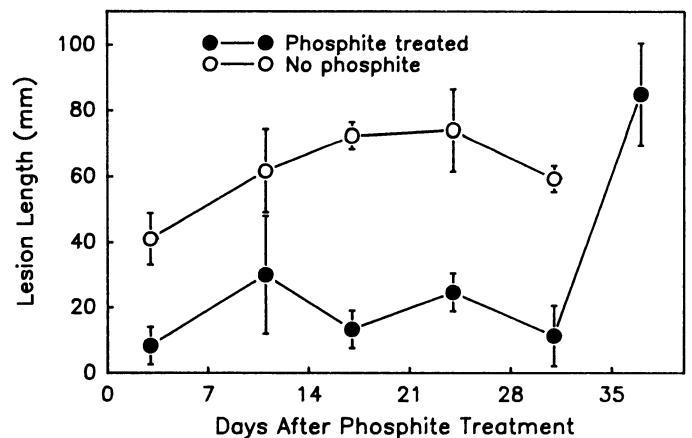


Fig. 6. Lesion lengths in tobacco plants 48 hr after inoculation with *Phytophthora nicotianae* at various times after phosphite treatment, applied at the rate of 20 mg per pot. Means from six plants shown \pm the standard deviations.

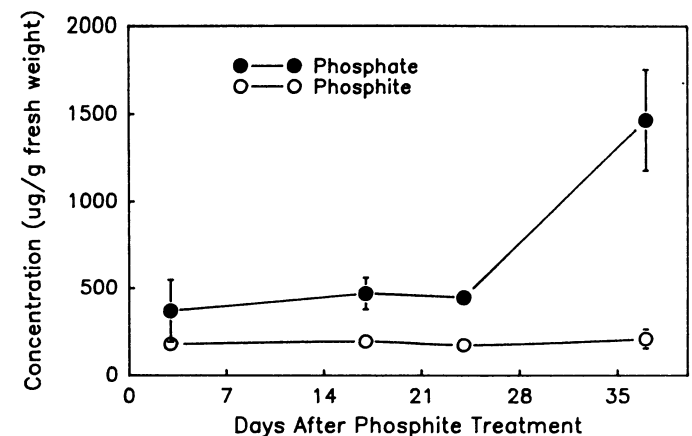


Fig. 7. Phosphite and phosphate concentrations in the stem tissue of phosphite-treated tobacco plants. The plants analyzed were those in which the lesion lengths were presented in Figure 6. Means \pm standard deviation are shown.

inversely proportional to the concentration of phosphite present at the site of inoculation (regression coefficient, -0.95). Where phosphite protection was expressed, concentrations of phosphite at the site of inoculation were in the range required to decrease growth of *P. nicotianae* in vitro (Fig. 5).

Paw-paw—*P. palmivora*. As in the other host/pathogen systems, the application of 20 mg of phosphite per pot resulted in reduced lesion extension in treated plants (Fig. 9). The concentration of phosphite at the site where lesion extension ceased was $125 \mu\text{g/g}$ fresh weight at day 4 postinoculation and $105 \mu\text{g/g}$ fresh weight at day 8 postinoculation. This decrease in phosphite concentration between days 4 and 8 probably reflects variation in phosphite uptake, which is a function of seedling size and vigor rather than any loss of phosphite from plants. The paw-paw seedlings showed much greater variability in size and form than seedlings of either of the two other species tested. Although these phosphite concentrations were also within the range that reduced rates of growth of *P. palmivora* in vitro (Fig. 5), the reduction in growth at $125 \mu\text{g/ml}$ of phosphite was much less than in either of the other two host-parasite combinations, and was just significantly lower than the control ($P \geq 0.05$ with paired Student *t* test, $n = 15$). The concentration of phosphate was $1,196 \pm 164 \mu\text{g/ml}$ at day 4 in phosphite-treated plants and $1,300 \pm 101 \mu\text{g/ml}$ in the control plants, again indicating that the accumulation of substantial amounts of phosphite did not alter the concentrations of phosphate present.

In one set of experiments, the fungi were recovered from the stem sections at least 5 cm ahead of the lesion front, both in untreated plants and those treated with up to 20 mg of phosphite

per pot, and between 3 and 4 cm ahead of the lesion in plants treated with 50 mg per pot.

DISCUSSION

We have shown that phosphite provided protection against invasion by *Phytophthora* spp. in three plant species. Protection was expressed as reduced lesion extension in the stem following wound inoculation. Phosphite accumulated to concentrations exceeding those applied in the root drench ($1,000 \mu\text{g/ml}$) in some treatments with older seedlings, though the concentration was usually lower. Phosphite concentrations at the site where protection was expressed were sufficient to reduce the growth of the pathogens in vitro. Moreover, protection was only observed when phosphite concentrations in the plant reached or exceeded these concentrations. However, phosphite did not arrest lesion extension in lupin, even at the highest concentration used. In tobacco, complete cessation of lesion extension was observed at phosphite concentrations well below the ED_{50} observed ($80 \mu\text{g/ml}$) against *P. nicotiana* in vitro. In paw-paw, the phosphite concentration in the plant was sufficient to slightly reduce the growth of *P. palmivora* in vitro, though much less than was observed in the other two species of *Phytophthora*. The phosphite concentrations observed are comparable to those reported recently by others who have used different plant species and somewhat different methods of administering the phosphite (5,26,29). The presence of phosphite in protected plants at concentrations sufficient to reduce growth in vitro suggests that it is acting directly on these fungi. A similar conclusion was reached by Dercks and Buchenauer (8) from experiments with fosetyl-Al, although these authors used much higher application rates ($4,000$ – $5,000 \mu\text{g/ml}$). In a later study (9), lower rates used in vitro ($250 \mu\text{g/ml}$) showed direct effects, although the concentrations of phosphite in the fungus were not examined.

Our results also show that the effectiveness of phosphite in providing protection may be influenced by the concentration of phosphate present. The coincident increase in phosphate and breakdown of protection in tobacco (Figs. 6 and 7) suggests, though does not prove, that the two events are related. If the increased phosphate concentrations reduced the uptake of phosphite into the fungus, breakdown of protection could be expected. We have shown that this is precisely what happens to *P. palmivora* under in vitro conditions (J. M. Griffith, *personal communication*). Phosphite entry into *P. palmivora* is directly reduced in the presence of phosphate. There appear to be common transport systems for phosphate and phosphite uptake, and mutually competitive inhibition is exhibited between the two types of anion. Phosphite also has been shown to inhibit phosphite uptake in *P. citrophthora* (1), and it was suggested that in this species there might also be two different uptake systems, having different affinities for phosphite. The irregular preinfection control of downy mildew by phosphite (P. A. Magarey, *personal communication*) may be explained by inhibition of phosphite uptake into the fungus in the presence of high phosphate. However, in a recent paper (10), it was reported that a different strain of *P. palmivora*, which was extremely sensitive to phosphite in vitro, responded in the opposite way to that observed in our study, with phosphate enhancing the effectiveness of phosphite. Although the methods used in this latter study do not allow direct comparison with the work described here, the effects of phosphate on phosphite-induced protection are clearly complex. Together with the range of sensitivities to phosphite observed in different isolates of a given species (10), they may explain the variable levels of control exerted by phosphite in other host-parasite combinations (3,7).

Although the results show that in three species of plant treated with phosphite there is sufficient concentration of the ion to reduce the growth of the invading pathogen directly, they also support the concept of a primary role for the natural plant defense systems in bringing the invasion under control. This interpretation differs slightly from that of other workers (8) who argued that increases in phenolic components observed in plants treated with fosetyl-

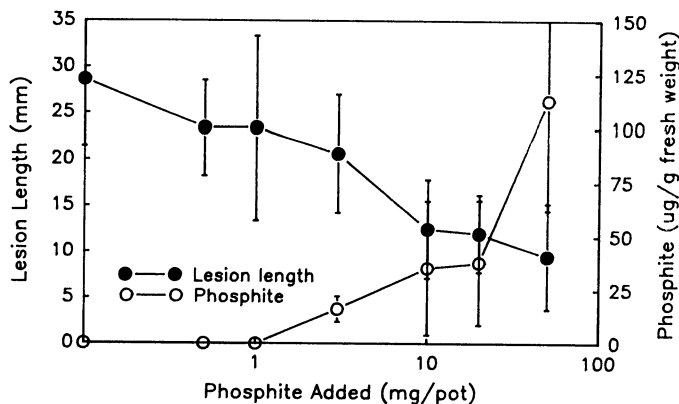


Fig. 8. Phosphite concentrations (means of five) and lesion lengths (means of eight) in tobacco plants treated with various amounts of phosphite and challenged 4 days later with zoospores of *Phytophthora nicotianae*.

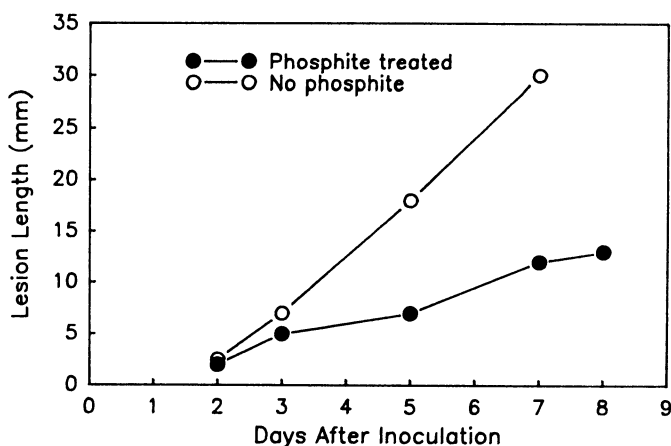


Fig. 9. Lesion extension in paw-paw plants treated with 20 mg of phosphite per pot and inoculated 4 days later with *Phytophthora palmivora*. Seven days after inoculation, control plants had collapsed and further measure of lesion extension was not possible. Data shown are means of two plants.

Al were of secondary importance. However, as noted previously, this may be a consequence of the very high application rates of fosetyl-Al used.

In lupin, even at the highest concentration of phosphite in the plant, there was always some extension of the pathogen, just as at these concentrations of phosphite there was always some growth of the fungus in vitro. It has been suggested that lupin lacks a highly active dynamic defense system, and the plants depend for protection on the constitutive inhibitor luteone (14), which is found in fungistatic concentrations in leaves (20). It has not been clearly established whether other compounds, such as wighteone act as phytoalexins in lupin hypocotyls (21), but in the absence of a true dynamic defense system, the concentration of the constitutive inhibitor would determine whether normal plants were susceptible to wound inoculation with *P. cinnamomi*, and, in turn, whether phosphite protection could be observed in treated plants. In the absence of fungistatic or fungicidal concentrations of constitutive inhibitors, phosphite would provide the only protection against invasion. Since fungal growth was slowed but not stopped under these conditions, it is clear that phosphite did not provide full control. This suggests that a well-developed, dynamic defense system in the plant is essential if phosphite is to halt the fungal attack completely.

The situation in tobacco was different. In this species, lesion extension was arrested in plants treated with phosphite, though the pathogen was not killed and was isolated from apparently healthy tissue ahead of the lesion, both in phosphite-treated and in control plants. Phosphite concentrations in the treated tobacco stems were not markedly higher than those in the lupin plants, and phosphate concentrations were no lower. The strain of *P. nicotianae* was only slightly more sensitive to phosphite in vitro than the strain of *P. cinnamomi*. However, tobacco possesses a complex dynamic defense system, with the capacity to synthesize the sesquiterpenoid phytoalexins capsidiol, rishitin, phytuberin, phytuberol, and lubimin (16). We interpret the complete arrest of pathogen extension in tobacco to be the result of combined action in which phosphite reduced the rate of pathogen extension, allowing time for the host's natural defense systems to arrest, though not kill the invader. A similar conclusion has been reached from the study of cowpea infected by *P. cryptogea* (26).

Too little is known of the dynamic defense systems present in paw-paw to be able to determine their role in the interaction between phosphite, *P. palmivora*, and the host. However, in paw-paw plants treated with phosphite, the rate of growth of the fungus was reduced proportionally more than at the same concentrations of phosphite in vitro. This suggests that in this system, too, plant defense systems act in concert with phosphite to restrict fungal growth.

Our results, therefore, are consistent with a site of action for phosphite in the fungus, not in the plant, but strongly suggest that the plant's natural defense system plays a critical role in arresting pathogen growth. The mode of action of phosphite might, therefore, best be described as mixed, rather than direct or indirect.

To properly understand the mechanism by which phosphite acts to control fungal growth in plants and, specifically, to determine whether there is some specific interaction between phosphite and the host's defense system, it will be essential to determine the site of action of the chemical at the molecular level. We suggest that the place to look for this site is the fungus itself rather than in the host plant. The release of stress metabolites by the fungus following its encounter with phosphite already has been suggested as one means by which the linkage between parasite and host defense system might occur (19). When the site of action has been identified, it will be possible to take the next step in understanding the complex interaction between phosphite, fungus, and plant that leads to control.

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