Evaluation of Single Annual Applications of Sterol-Inhibiting Fungicides for Control of Pine Twisting Rust

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ABSTRACT

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Six sterol biosynthesis inhibiting (SBI) fungicides—cyproconazole, flusilazole, flutriafol, tebuconazole, triadimefon, and triadimenol—were tested against *Melampsora pinitorqua*, the causal agent of pine twisting rust. Triadimefon and cyproconazole provided the highest level of control in seedling and cut-shoot bioassays. At 500 mg a.i./L, 2–4 wk of protective activity and at least 5 days of curative activity were observed. Cyproconazole appeared much more fungitoxic than triadimefon toward seven ectomycorrhizal fungi. The efficacy of a single application of triadimefon was tested under field conditions. Results demonstrate that triadimefon has a good potential for practical control of pine twisting rust. The cut-shoot technique appears to be a valuable tool for screening fungicides. In contrast, basidiospore germination appears to be an unsuitable criterion for assessing SBI efficacy.

Pine twisting rust, caused by *Melampsora pinitorqua* Rostr., is a common disease of young pines, principally Scots pine (*Pinus sylvestris* L.) and maritime pine (*P. pinaster* Aiton) in most European countries (12). Infection causes shoot withering and deformation, mostly on pines between 2 and 5 yr of age, resulting in growth loss and decreased stem quality. In France, the disease is endemic in the Landes forest, which consists of more than 1 million ha of maritime pines. It is feared that the impact of the disease will increase because of intensive silviculture (7). So far, no satisfactory control method has been developed. Genetic breeding for resistance has been investigated in Scandinavia for *P. sylvestris* (13), but breeding for resistance does not look promising for *P. pinaster* (4).

Conditions favorable for infection occur for a period of about 1 mo every year (April–May in France). This is approximately the duration reported for the protective activity of several sterol biosynthesis inhibiting (SBI) fungicides effective against other rust diseases. Triadimefon, for example, is used for control of fusiform rust of southern pines in the United States (17).

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This study was conducted to evaluate and compare the efficacy of several SBIs when applied as a single annual application for the control of pine twisting rust. The protectant activity of the fungicides was the main concern, although the curative activity also was examined. Because of the unreliability of natural infection, most of the work was conducted on artificially inoculated material, both seedlings and excised shoots from older trees being used. In addition, one field experiment was carried out. The nontarget effects of the fungicides on ectomycorrhizal fungi also was examined.

MATERIALS AND METHODS

Six SBI fungicides that are commercially available for rust disease control on various crops in France (1) were tested (Table 1).

Inoculation technique. Inoculum consisted of 6-mm-diameter disks, cut from leaves of aspen, *Populus tremula* L. (the alternate host of *M. pinitorqua*), bearing germinating telia (5). The leaves were collected in spring at teliospore maturity and stored at 4 C to stop further spore development. To assess inoculum density, a basidiospore production test was performed each week from the beginning of the study. For this test, 10 disks from different leaves were laid on moist filter paper in the lid of a petri dish. Basidiospores cast from the disks onto glass slides during a 24-hr period at 15 C were washed off in a known quantity of water and the number of spores was estimated by hemacytometer. A density of 250 ± 80 basidiospores per square millimeter (mean + SD), which corresponds to a high level of inoculum (21), was obtained throughout the season.

Inoculations were performed either on 2-mo-old maritime pine seedlings or on excised shoots taken from the highest whorl on 2- to 3-yr-old trees (8). One inoculum disk was used for each seedling or shoot. On seedlings, the disks were placed in contact with the terminal needles. On shoots, they were placed at the midheight point, and the site was marked to make further observations easier. Seedlings and shoots then were incubated at 15 C and 100% relative humidity (RH) for 24 hr in the dark. Thereafter, seedlings and shoots were placed for 3 wk in a growth chamber at 15 C and 80% RH, with a 16-hr day/8-hr night photoperiod and approximately 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Inoculated seedlings and shoots were examined for sporulation after 3 wk. With the seedlings, assessments were made of the presence of aecia on stems, typical of *M. pinitorqua* infection, but not on leaves (6). For the shoots, the area around the marked inoculation site was examined under a stereomicroscope for both pycnia and aecia. Percent infection refers to the proportion of shoots with pycnia (alone or with aecia). Percentage of aecia sporulation refers to the proportion of shoots or seedlings with aecia.

In vitro activity of fungicides on basidiospore germination. Fungicides were added to water agar (agar = 15 g/L) just before solidification, and the fungicide concentration was adjusted from 1 to 500 mg a.i./L. Tests were performed in petri dishes at 15 C. An inoculum disk was placed for 1 hr on wet filter paper in the lid of each dish, and basidiospores were allowed to fall onto the agar (5). Basidiospore germination was assessed after 24 hr for 100 spores per dish. A basidiospore was considered germinated when the germ tube length was equal to or longer than the spore diameter. Each fungicide × dose combination was replicated three or four times. The experiment was repeated twice.

Protective activity. *Greenhouse evaluation.* An experiment was conducted on maritime pine seedlings grown in plastic trays (35 seedlings per tray) on a peat/sand substrate. Each fungicide was applied once with a hand-held sprayer at concentrations of 250, 500, or 1,000 mg a.i./L (approximately 1 ml per tray, i.e., 5, 11, or 22 mg a.i./m^2). Each treatment and the control (nontreated seedlings) consisted of three trays. Seedlings were inoculated at various intervals up to 4 wk after spraying, as previously described.

Field evaluation. Experimental spraying was conducted on a 2-yr-old maritime pine stand at St Alban, near Bordeaux, in 1989. Shoots in the upper whorl were sprayed to runoff using a pressurized (2–5 bars) backpack sprayer (approximately 35 ml per tree). A single application was made at the beginning of the period of shoot susceptibility (i.e., after bud scale disjunction) (8). The concentrations were the same as those used on the seedlings. Different fungicide × dose combinations were applied to different rows of trees (two rows of approximately 30 trees per combination). Twenty to 30 lateral shoots from the upper whorls were collected at random from trees in each treatment at different times after spraying (one shoot per tree) and inoculated within 2–3 hr using the technique described above. Another experimental spraying, with only three fungicides of one concentration (500 mg a.i./L), was carried out in a 4-yr-old neighboring maritime pine stand in Castillonville. Trees were sprayed during the same week as the St Alban experiment, but the shoots were in a slightly less advanced stage (incomplete disjunction of bud scales). The mean length of pine shoots was approximately 15 cm in both

Table 1. Ergosterol biosynthesis inhibiting fungicides evaluated for control of pine twisting rust

Active ingredient	Commercial fungicide	Formulation[z]
Cyproconazole	Alto (Sandoz)	WP (10%)
Flusilazole	Olymp 10 (Du Pont de Nemours)	EC (100 g/L)
Flutriafol	Impact Sopra (Sopra)	SC (125 g/L)
Tebuconazole	Horizon (Bayer France)	EC (125 g/L)
Triadimefon	Bayleton 100 (Bayer France)	EC (100 g/L)
Triadimenol	Baytan 5 liq. (Bayer France)	EW (5 g/L)
	Bayfidan (Bayer France)	EC (250 g/L)

[z] WP = wettable powder, EC = emulsifiable concentrate, SC = concentrated suspension, and EW = water emulsion.

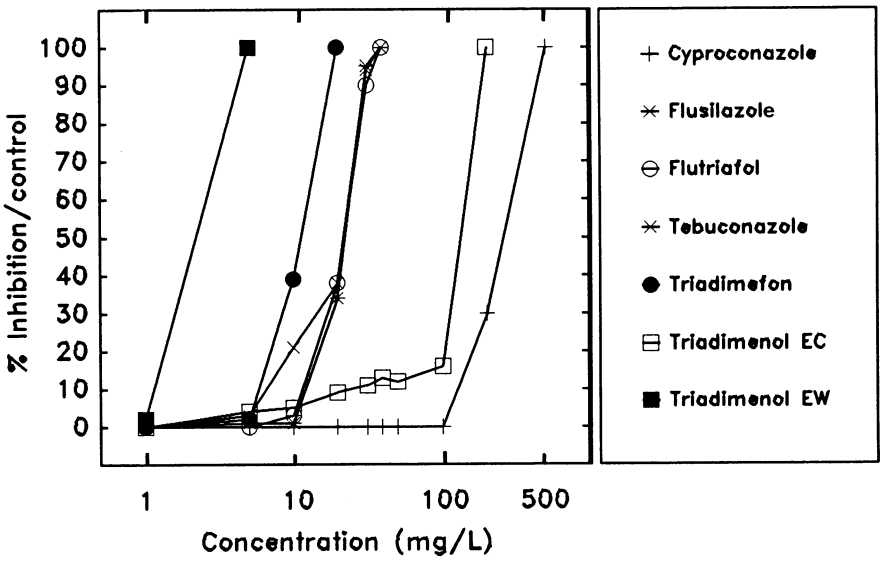

Fig. 1. In vitro inhibition of *Melampsora pinitorqua* basidiospore germination by fungicides in vitro. EC = emulsifiable concentrate, EW = emulsion in water.

Castillonville and St Alban stands at the beginning of the experiments. The mean increase during the following 4 wk was 20 cm in the former and 12 cm in the latter.

The efficacy of each treatment was expressed as the percent reduction of either infection or aecia sporulation on treated shoots (seedlings) compared with nontreated shoots (seedlings). The 2I test (= G^2, likelihood ratio chi-square) was used to compare data in the various treatments (2). Statistical significance was considered at the 5% probability level.

Curative activity. *Seedling experiment.* Fungicides at the rates used in the protectant experiment were applied to seedlings that had been inoculated 5 days previously.

Shoot experiment. This experiment was conducted on pines from the Castillonville stand. Shoots were collected from the upper whorl of pines in nontreated rows and inoculated as previously described. Excised shoots were sprayed with fungicides with a hand-held sprayer either 5 days after inoculation at 500 mg a.i./L or 8 days after inoculation at 1,000 mg a.i./L.

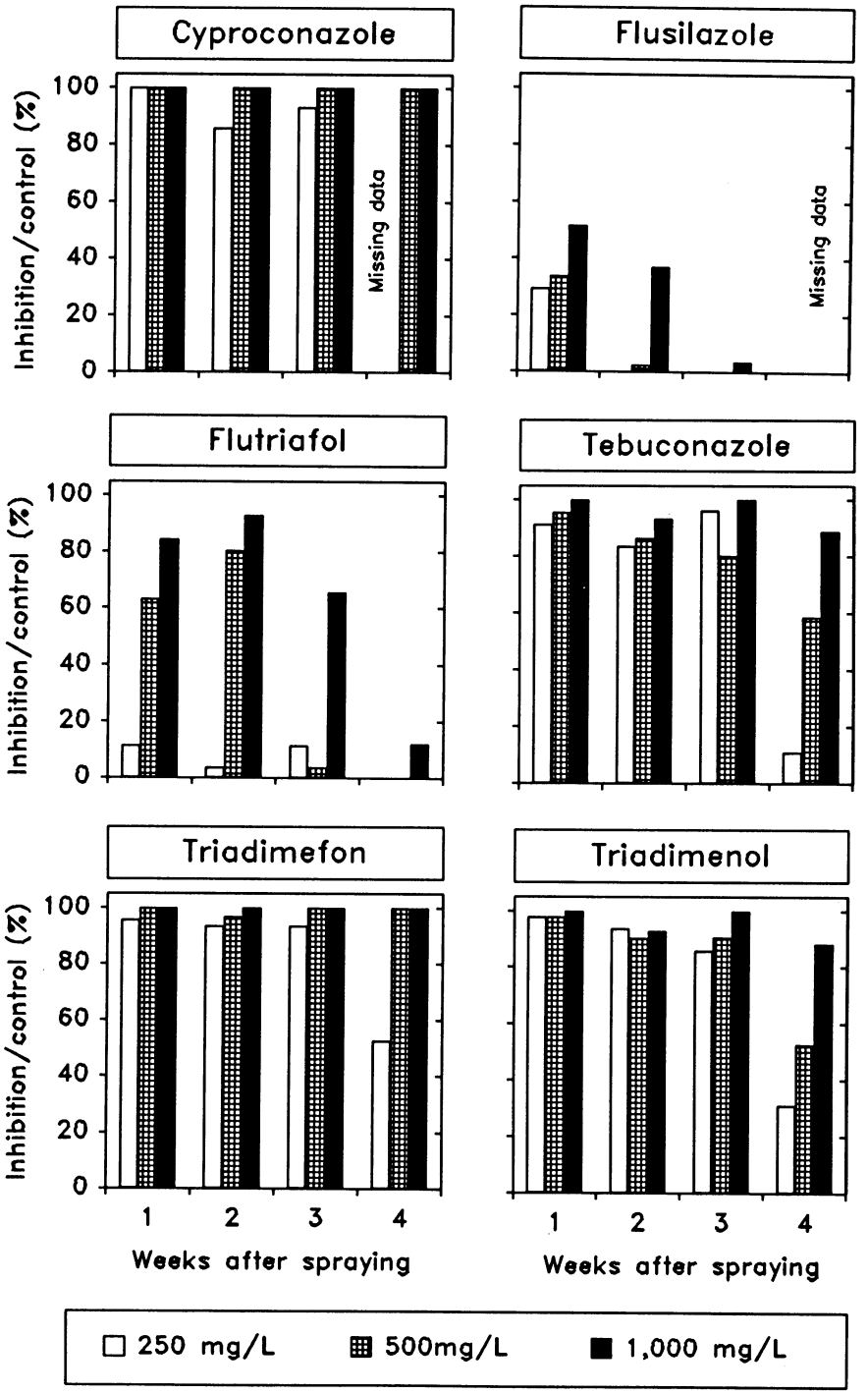

Fig. 2. Inhibition of aecia sporulation on *Pinus pinaster* epicotyls sprayed with fungicides and inoculated with *Melampsora pinitorqua* at various times after spraying.

The efficacy of each treatment was defined as previously described, 3 wk after inoculation. Treatments were compared using the 2I test, as in the case of the protective activity.

Efficacy and influence of time of application under natural conditions. In spring 1988 and 1989, an experiment was conducted in a high rust hazard stand of 3-yr-old maritime pines at Saucats, near Bordeaux. Three blocks were defined according to levels of rust hazard, as estimated by density of aspens and frequency of attacked pines from preceding years. Triadimefon at two concentrations (500 and 1,000 mg a.i./L) was applied at three different times corresponding to different stages of pine development. Within each of the three blocks, six treatments (i.e., five concentration × application time treatments plus one control) were randomly assigned to individual trees to give 10 replications per treatment. This procedure was adopted because of inoculum heterogeneity still existing within the blocks (3). Thus, the experimental design was a randomized complete block design with replications consisting of three blocks with 10 trees per treatment and block.

Fungicide spraying was carried out as previously described for field experiments. Trees were examined at the end of June and the infection of leaders (terminal shoots) and lateral shoots in the upper whorl was recorded.

To estimate probable dates of infections a posteriori, a Burkard spore trap was installed in the stand at the beginning of the experiment. *M. pinitorqua* basidiospores trapped each day were counted at the end of the season.

In vitro toxicity on ectomycorrhizal fungi. Seven isolates from six species of pine ectomycorrhizal fungi were used. The species included *Hebeloma cylindrosporum*, Romagn. *Suillus collinitus* (Fr.) Kuntze, *S. granulatus* (L.:Fr.) Roussel (two isolates), *S. luteus* (L.:Fr.) Roussel, *Laccaria laccata* (Scop.:Fr.) B. K. & Br. (grown on Oddoux medium [15]), and *Lactarius deliciosus* (L.:Fr.) S. F. Gray (on Raper medium [16]). The same fungicides as those evaluated for protective and curative activity against rust were incorporated in the medium at concentrations of 0.01, 0.1, 1, and 10 mg/L. Each fungicide × dose combination was replicated five times for each isolate. Colonies were measured along two diameters on two dates (corresponding to approximately one-third and two-thirds filling of the petri dish by the control) for each fungus. The average linear daily growth of the fungi then was calculated and fungicide toxicity was expressed as percent growth reduction compared with the untreated controls. The experiment was repeated with two species (*H. cylindrosporum* and *L. laccata*). Data were analyzed by means

of an analysis of variance. Treatments were compared to the control by the Dunnett test. Treatment comparisons were done with the Newman-Keuls test. STAT-ITCF (20) and SAS-STAT (18) were used for statistical analysis.

RESULTS

In vitro activity on basidiospore germination. Lethal concentrations varied from approximately 5 to 200–300 mg/L according to the fungicide (Fig. 1, mean of the two replications). The best, and also one of the worst, results was obtained with the same active ingredient, triadimenol, used as two different formulations. Triadimenol used as an emulsion in water was effective, and triadimenol used as an emulsifiable concentrate was not. At concentrations lower than the lethal concentration, deformation and a reduction in germ tube length were observed with all fungicides.

Protective activity. *Greenhouse experiment.* The rate of stem infection with aecia varied from 79 to 97% on control seedlings at the different dates. Four of the six fungicides tested demonstrated a high preventive activity up to 4 wk after application (Fig. 2). Triadimefon and cyproconazole provided significantly better protective activity than all other fungicides, with at least 90% control for up to 4 wk after application at 500 mg/L.

Field experiments. In the St Alban experiment, percent infection on control shoots varied from 81 to 96%, and percent aecia sporulation varied from 52 to 89%, according to the date of inoculation. Results on efficacy followed the same trends when either criterion was considered. Cyproconazole, triadimefon, and triadimenol were significantly the most effective, with a percent control of at least 80% up to 4 wk after spraying, even at the lowest concentration. Flutriafol at 500 and 1,000 mg/L also was highly effective up to 4 wk after spraying. The efficacy of tebuconazole, and particularly that of flusilazole, decreased rapidly with time and dilution (Fig. 3). In the Castillonville experiment, percent infection and percent aecia on control shoots were lower than in the St Alban experiment—53–88% and 17–63% respectively. Efficacy also was significantly lower for triadimenol and tebuconazole than that recorded in the St Alban experiment at the same concentration (500 mg/L) (Table 2). The persistence of maximal efficacy after spraying also was reduced for triadimefon and tebuconazole.

Curative activity. *Greenhouse experiment.* All fungicides demonstrated a good curative activity. Cyproconazole, triadimefon, and triadimenol exhibited the greatest curative activity, which provided almost complete control at the lowest concentration tested (Fig. 4).

Castillonville experiment. Percent infection was 73–100% on control shoots,

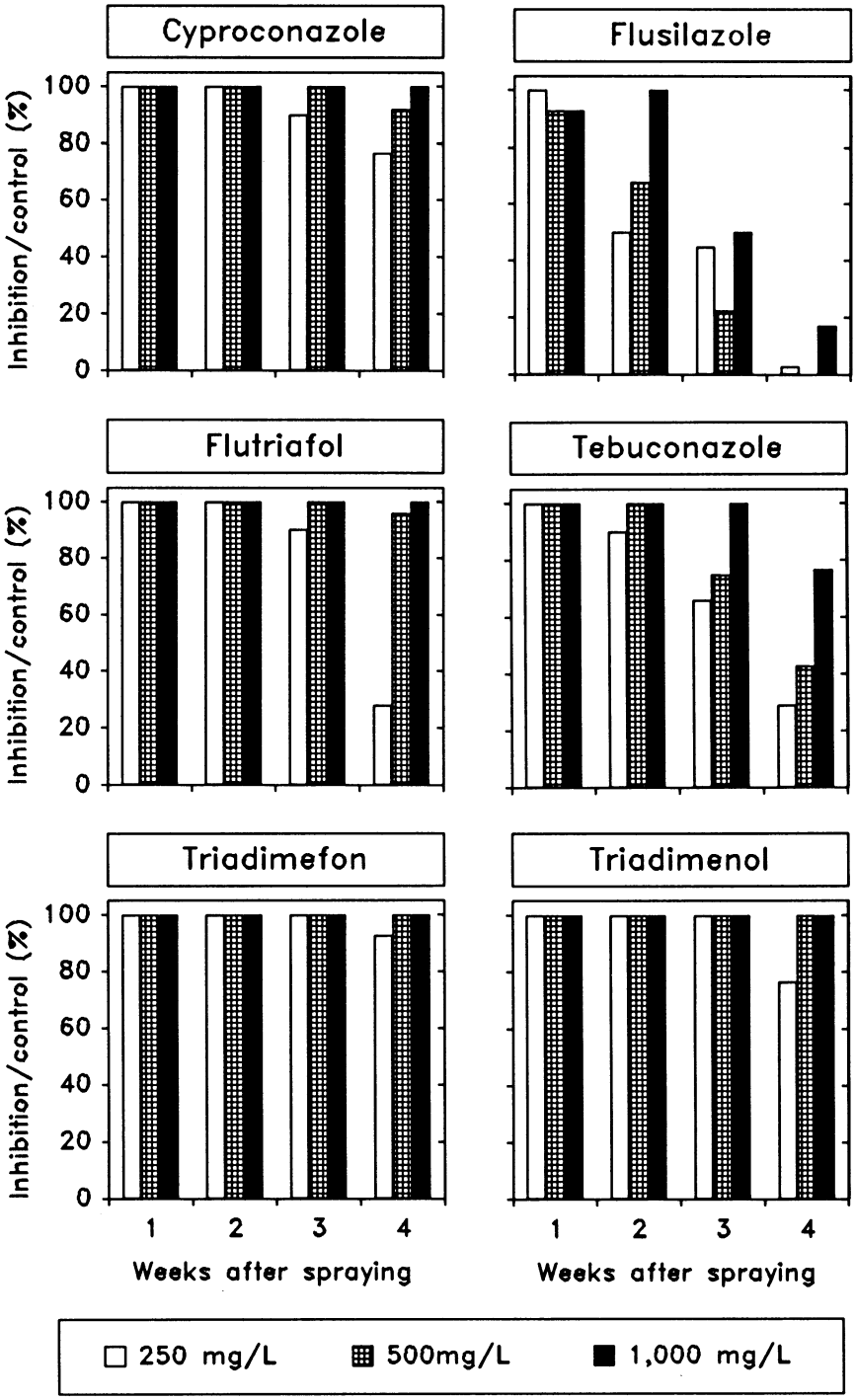

Fig. 3. Inhibition of aecia sporulation on *Pinus pinaster* excised shoots sprayed with fungicides and inoculated with *Melampsora pinitorqua* at various times after spraying (St Alban experiment).

Table 2. Comparison of fungicide preventive efficacies (reduction, as compared to control, of aecia sporulation on shoots excised and inoculated 1–4 wk after spraying) in two field experiments

Fungicide (500 mg/L)	Maximal efficacy (%) (inoculation 1 wk after spraying)		Persistence of maximal efficacy (wk)[y]	
	St Alban	Castillonville	St Alban	Castillonville
Tebuconazole	100 a[z]	76 b	2	1
Triadimefon	100 a	100 a	4	2
Triadimenol	100 a	52 b	4	4

[y] Maximal duration between spraying and inoculation for which efficacy was not significantly different in the 2I test ($P > 0.05$) from maximal efficacy.
[z] Letters denote significant differences in the 2I test ($P < 0.05$).

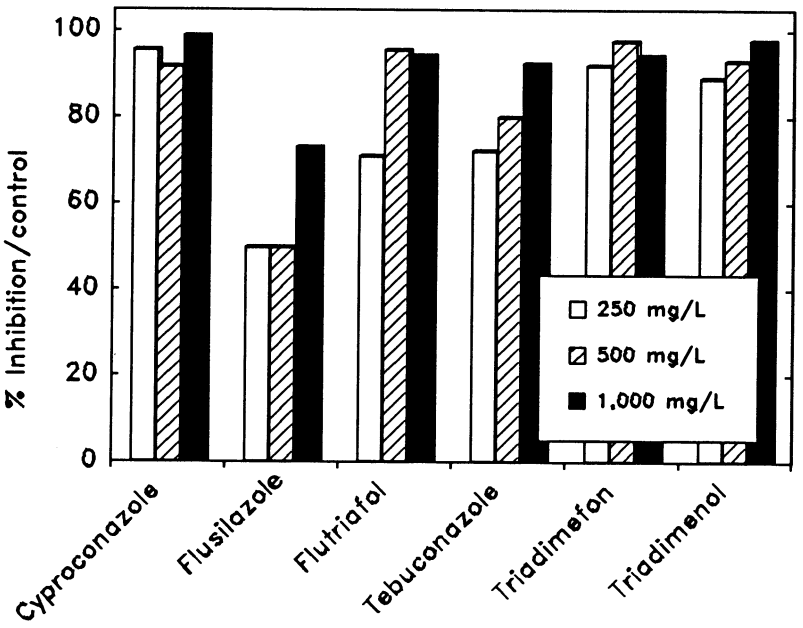

Fig. 4. Inhibition of aecia sporulation on *Pinus pinaster* epicotyls inoculated with *Melampsora pinitorqua* and sprayed with fungicides 5 days after inoculation.

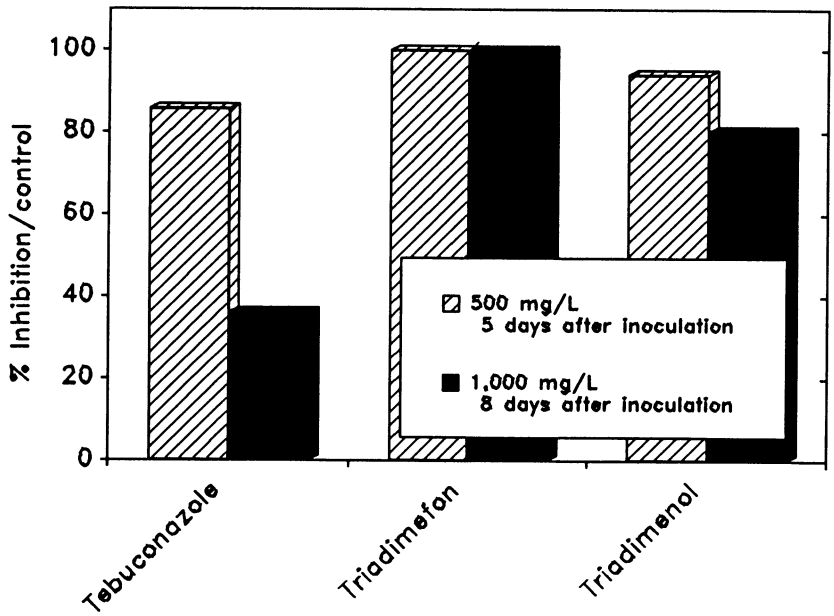

Fig. 5. Inhibition of infection on *Pinus pinaster* excised shoots inoculated with *Melampsora pinitorqua* and sprayed with fungicides at various times after inoculation.

Table 3. Efficacy of different triadimefon spraying schedules for protection of maritime pines against twisting rust under natural infection

Schedule	Concentration (mg/L)	Time of application	Phenological stage of shoots[y]	Percent infected leaders 1988	Percent infected leaders 1989
1 (control)	0	...		52 a[z]	21 a
2	1,000	27 Mar. 1988/17 Mar. 1989	B1	0 b	10 b
3	1,000	6 Apr. 1988/14 Apr. 1989	B2	0 b	3 b
4	500	6 Apr. 1988/14 Apr. 1989	B2	3 b	3 b
5	1,000	21 Apr. 1988/28 Apr. 1989	B3	3 b	0 b
6	500	21 Apr. 1988/28 Apr. 1989	B3	3 b	...

[y] B1 = Beginning of elongation, shoot entirely covered by budscales, shoots nonreceptive to rust; B2 = disjunction of budscales, the shoot surface becoming visible, beginning of shoot susceptibility to rust; and B3 = shoot entirely discovered by budscales, apparition of brachyblasts, near optimal susceptibility of shoots.

[z] Letters denote significant differences in the 2I test ($P < 0.05$) in a column.

and all fungicides proved highly effective in reducing infection, except for tebuconazole applied 8 days after inoculation (Fig. 5). No aecia were observed on the treated shoots with any of the three fungicides applied at 5 or 8 days after inoculation, compared with 29% for the nontreated shoots.

Efficacy of triadimefon under natural conditions, in relation to the time of application. In 1988, there was 52% infection of leaders on the unsprayed plants. Triadimefon applied on all schedules provided a very effective control (Table 3). In 1989, there was little infection on the control plants and infection was reduced with all treatments. According to spore trap slide readings, basidiospore dispersal in 1988 occurred from 4 to 24 April, with peaks on 12 and 16 April, while in 1989 it occurred from 1 to 19 April, with one peak on 2–4 April.

In vitro activity on ectomycorrhizal fungi. An analysis of variance showed that the radial growth of ectomycorrhizal fungi varied significantly with isolates, fungicides, and fungicides × isolates interactions. The lowest concentration of fungicide, which caused a significant reduction of growth on at least four of seven test fungi was 0.01 mg/L for tebuconazole, 0.1 mg/L for cyproconazole and triadimenol, and 1 mg/L for flutriafol, flusilazole, and triadimefon (Dunnett tests). With the combined data for all of the fungi, 50% inhibition of mycelial growth occurred between 0.01 and 0.1 mg/L for tebuconazole and cyproconazole, 0.1 and 1 mg/L for triadimenol, and 1 and 10 mg/L for flutriafol, flusilazole, and triadimefon (Fig. 6). Treatment comparisons for each strain (Newman-Keuls tests) confirmed that cyproconazole and tebuconazole were, in all cases, significantly more toxic than triadimefon. These results were confirmed in the repeated experiment with *H. cylindrosporum* and *L. laccata*.

DISCUSSION

This study shows promising results for chemical control of pine twisting rust, as several fungicides demonstrated high preventive and curative activity. Because only one annual application over a period of 3–5 yr (the period of pine susceptibility) is required, chemical control could be cost effective. This is a rare situation in forest disease management. Moreover, the small number of applications, the restricted spraying site (the highest shoots), and the selective nature of the fungicides should minimize adverse effects on the environment.

Among fungicides tested, triadimefon and cyproconazole proved to be the most effective in all greenhouse and field experiments. The good protective and curative efficacy of triadimefon is in agreement with results on fusiform rust on southern pine seedlings, although

those results were obtained with higher rates or repeated applications (9,17).

Although inhibition of ectomycorrhizae by foliar application of fungicides has not been demonstrated in the field, the toxicity of candidate fungicides toward mycorrhizal fungi could be an additional criterion of choice. In this respect, triadimefon is preferred to cyproconazole because of its significantly lower toxicity toward the ectomycorrhizal fungi tested. Our results on triadimefon and triadimenol are consistent with ED_{50} values reported previously on other ectomycorrhizal fungi (10,14). Our study shows a significantly greater effect produced by more recent SBIs, such as cyproconazole and tebuconazole. Additional studies concerning the effects of these fungicides on mycorrhizae in vivo are required to evaluate the potential problems caused by their use in young pine stands. Even triadimefon, used in three to four applications each at a rate greater than 50 mg/m^2, has been reported to show adverse effects on mycorrhizal development on pine seedlings (11,14).

Differences in protectant activities on the excised shoots from St Alban and Castillonville may be accounted for by differences in pine growth, possibly attributable to age. The earlier flush in the St Alban stand, which resulted in more of the shoot being no longer covered by bud scales at the time of spraying, could have led to improved fungicide penetration. In addition, reduced shoot elongation in this stand may have resulted in a smaller dilution of fungicides during growth. With triadimenol, the main difference between the stands was in terms of the maximal efficacy, which could be explained by a difference in penetration. In contrast, with triadimefon, the difference was only in terms of the duration of the period of maximal efficacy, which could result from a dilution effect. With tebuconazole, there were differences between the stands both in terms of the maximal efficacy and in terms of its duration. These experiments indicate that fungicide doses should be determined in relation to pine growth (vigor, fertilization, etc.). According to these results, a high degree of protection, up to 4 wk, should be obtained with triadimefon at a concentration of 500 mg/L (i.e., a maximum of 17.5 mg a.i. per tree, if 35 ml of spray was retained) in the case of pines with low or medium growth and at 1,000 mg/L (35 mg a.i. per tree) for higher growth.

The high efficacy of triadimefon observed under controlled conditions was confirmed under field conditions, particularly in 1988. During this year, basidiospore dispersal occurred in large amounts in mid-April at a time when shoots were in a susceptible stage (8), the most probable infection date being

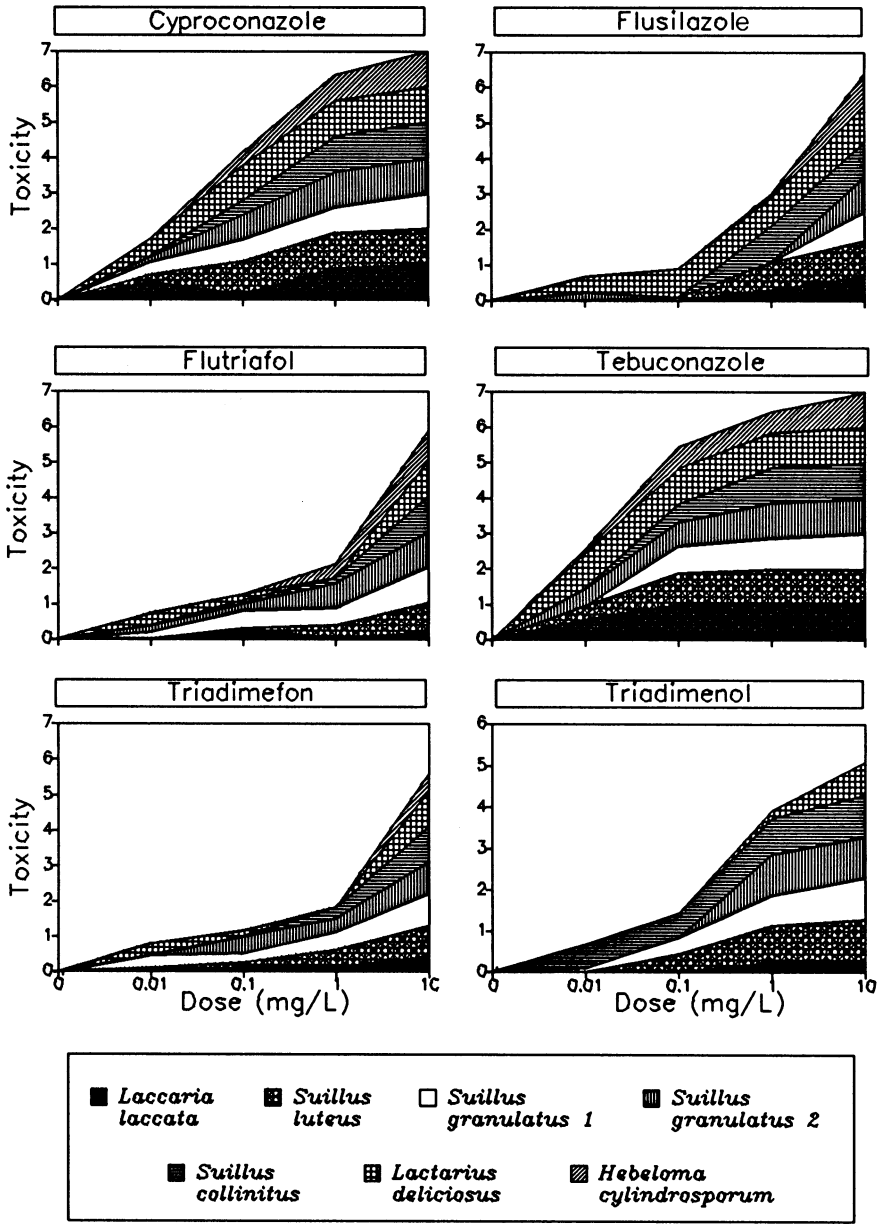

Fig. 6. Inhibition of radial growth of seven isolates of ectomycorrhizal fungi by fungicides. Cumulative toxicity degrees from 0 to 7 were obtained by addition of toxicities against each isolate. 100% inhibition of radial growth = 1 degree (missing data for *Suillus granulatus* isolate 2 and triadimenol).

16 April. The total protection obtained with fungicides applied on 27 March, therefore, indicates a preventive activity of at least 3–4 wk (at 1,000 mg/L). In the same way, it would appear that effective control with a curative application can be obtained using 500 mg/L for up to 5 days after infection.

On the basis of these results, the cut-shoot inoculation technique, with its reliability and simplicity, appears to be a valuable tool in screening fungicides for pine rust control. In contrast, the basidiospore germination test on agar medium proved to be unsuitable for assessing SBI efficacy, because the inhibition of germination in vitro was more affected by formulations than by the nature of the active ingredient. This confirms other studies on SBI sites of action (19).

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