Tricyclazole: A New Systemic Fungicide for Control of Piricularia oryzae on Rice

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ABSTRACT

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Tricyclazole [5-methyl-1,2,4-triazolo(3,4-b)benzothiazole, code number EL-291] is a unique new fungicide for control of *Piricularia oryzae* on rice. In vivo activity was 25 to 35 times greater than in vitro activity. Tricyclazole was systemic in rice according to foliar disease control achieved with root, seed, and soil applications and residue analyses. Field studies indicated that effective, long-term control of *P. oryzae* was obtained from foliar spray, seed coat treatment, soil drench in the transplant flat, and transplant bare-root soak applications. The effectiveness of tricyclazole by different methods of application offers new opportunities for disease control in seeded and transplanted rice.

Additional key words: rice blast.

Rice blast disease, caused by the fungus *Piricularia oryzae* Cav., has been observed in almost every country in which rice is grown and is the most important disease of rice in the world (9, 11). The disease is most serious in temperate climates, such as Japan, resulting in significant yield losses annually (3, 10, 15). In more tropical climates, such as Taiwan and the Philippines, disease outbreaks are more localized, depending on weather favorable for disease development (2). The incidence of rice blast in the United States has been effectively reduced by planting resistant cultivars. However, yield reduction of resistant cultivars from rice blast has been demonstrated in Louisiana (5).

The rice blast pathogen infects rice at all stages of growth, resulting in symptoms which are named according to plant parts infected. For example, leaf blast is a symptom resulting from *P. oryzae* infection of leaves in seedling and tillering stages of rice growth. Neck (culm), panicle branch, and node blast appear later after heading. Infections in the heading stage, hereafter referred to collectively as panicle blast, are the most destructive, reducing the quality of grain and the quantity of yield. In many countries only leaf or panicle blast prevails due to the timing of rice cultivation and prevalence of local weather conditions conducive to infection. In Japan, leaf blast and panicle blast are equally serious (4). Fungicide application is made for control of the former in an attempt to reduce the severity of the latter.

Blast was particularly severe in Japan in 1974 (L. R. Guse, *personal observation*). Fungicides commonly were applied two to three times for leaf blast and panicle blast control, respectively. Current products effectively control blast if applied frequently and at the proper times. All products are foliarly applied, except Kitazin P (O,O-di-isopropyl-S-benzylphosphorothiolate, Ihara Chemical Co., Tokyo, Japan), which is applied two or three times to the paddy water surface (15). All products are of short residual effectiveness, except Rabcide (4,5,6,7-tetra-chlorophthalide, Kureha Chemical Industry Co., Ltd., Tokyo, Japan), necessitating multiple applications (4, 15). Although several products claim systemic activity, the most obvious systemic blast control is obtained from Kitazin P applied to the paddy water surface (4, 15).

Tricyclazole [5-methyl-1,2,4-triazolo(3,4-b)benzothiazole, code number EL-291] a new fungicide for control of *P. oryzae* of rice, is being developed by a research and development team in Asia, South America, and the United States. In addition to representing a chemically unique new class of fungicides, tricyclazole offers several advantages over other rice blast fungicides: (i) it has a long residual effectiveness which eliminates the need for multiple applications; (ii) it is systemic in rice for blast control; and (iii) it provides effective rice blast control using methods of application other than foliar dust or spray.

The objective of this study was to characterize the unusual activity of this new experimental fungicide in laboratory, greenhouse, and field studies.

MATERIALS AND METHODS

Piricularia oryzae U.S. race 1 [international race IB-5 (1)] was used in all artificial inoculations and in vitro studies. Rice cultivars Nato or Early Colusa II were used in all studies. Rates of tricyclazole are expressed as active ingredient even though a 75% wettable powder (WP) formulation was used in some studies. All treatments were replicated three or four times unless otherwise indicated.

In vitro studies.—To study the in vitro response of *P.*

oryzae to tricyclazole, modified medium A of Tanaka (14) was used. The medium contained 15 g sucrose, 0.5 g NH_4Cl, 0.5 g KNO_3, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 6H_2O$, 7.5 mg $ZnCl_2$, 0.75 mg $FeSO_4 \cdot 7H_2O$, 0.6 mg $CuSO_4 \cdot 5H_2O$, 0.22 mg $MnSO_4 \cdot 5H_2O$, 0.06 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.09 mg H_3BO_4, 2.5 mg thiamine, and 5 μg biotin per liter of deionized water. The pH was 6 after autoclaving. Since tricyclazole is stable at high temperatures, technical grade compound (99+% pure) in ethanol was added to the medium before autoclaving. Mycelial growth was studied in 250-ml flasks containing 100 ml medium on a shaker at 60 oscillations per minute with an 8-cm length of travel. Mycelium was harvested after 13 days, dried at 45 C, and weighed. Percent growth inhibition was based on mycelial weight in the control. There were six replicated flasks per treatment, and the study was repeated with similar results. Spore germination studies were conducted in petri plates on Tanaka modified medium A (14) plus 2% agar. Percent spore germination and germ tube length were determined by observing 100 spores per six replicated plates per treatment, then compared with the control to determine percent inhibition. Spores were counted as germinated if germ tube length equalled spore length. To determine whether tricyclazole was fungicidal in vitro, mycelia and spores were exposed to 75, 150, and 300 μg/ml technical tricyclazole in water for 2 hours, then harvested, washed with water, and placed in a tricyclazole-free medium. Fungal growth response was compared with the growth of mycelium and spores not exposed to tricyclazole. All in vitro studies were conducted at 25-27 C.

In vivo studies.—Tricyclazole was evaluated for control of a *P. oryzae* of rice by various methods of application in the greenhouse and in two field locations. In the greenhouse, Nato rice was grown in autoclaved soil in 10-cm diameter round pots without drainage holes. In field studies at Greenfield, Indiana, Early Colusa II rice was either seeded or hand-transplanted to 38-cm diameter round plastic containers containing nonsterile soil and flooded with 3-6 cm water. In the field at Crowley, Louisiana, Early Colusa II rice was seeded at 155 kg/ha in 20 × 2.5-meter plots which were kept flooded with 5-10 cm water after emergence.

Foliar application of tricyclazole 75 WP was made once (booting stage of growth) or twice (booting and early heading) in 480 liters H_2O/hectare (ha) with a 2.3-meter hand-carried spray boom fitted with nine nozzles with Tee Jet TX-12 tips (Spraying Systems Co., Bellwood, Illinois) and pressurized to 2.46 kg cm^{-2} with CO_2. Hinosan (O-ethyl-S,S-diphenyldithiophosphate, Nihon Noyaku Seizo K.K., Tokyo, Japan), a popular rice blast fungicide, was applied as a reference fungicide.

Rice seed was treated by suspending tricyclazole 75 WP in water (2-3% of seed weight) and spraying the suspension on seed rotating in a tumbler. Application efficacy was approximately 90% based on chemical assay.

Soil drench application was made by suspending tricyclazole 75 WP in 0.5 liter of water and uniformly pouring the suspension over the soil surface of 21-day-old rice plants growing in a 28 × 58 × 3 cm transplant flat. Although rice grown in flats in Japan normally would be transplanted by machine, treated plants were removed from the flats by cutting 1 cm^2 blocks of soil and hand-transplanting roots plus soil into flooded plastic containers in the field. Treatment variables were tricyclazole rates and hours between treatment and transplanting.

Root-soak applications were made by suspending tricyclazole 75 WP in 1 liter of water, placing the soil-free roots of 28-day-old plants in the treatment for 10 minutes, and hand-transplanting them to flooded field plots.

Plants were artificially inoculated in the greenhouse and in the field at Greenfield with spores of *P. oryzae* harvested from 10- to 14-day-old cultures grown on rice polish agar (30 g rice polish consisting of pericarp and aleurone layers plus germ of the seed, 20 g agar/liter H_2O). Greenhouse plants were inoculated 14 days after planting by atomizing a suspension of spores on the foliage (spores from 25 culture plates in 525 ml H_2O/100 pots). Inoculated plants were incubated for 48 hours at 21 C in a moist chamber. Leaf blast severity was recorded 7 days after inoculation using a 1-5 scale: 1 = severe, 2 = moderate, 3 = slight, 4 = very slight, and 5 = no disease. Field plants in plastic containers were brought into a barn on a wooden sled, a plastic sheet was draped over the sled, and two humidifiers were placed under the plastic. When the air was saturated with water, the plants were inoculated using spores from 1.5 plates in 50 ml H_2O per container. After 4 days, the plastic was removed, and 2 days later the sled was returned to the field. Plants were inoculated at various stages of growth after planting. Leaf blast severity was recorded 5-10 days after inoculation using the Barratt-Horsfall 0-11 scale and converting those data to percent disease incidence (13). Panicle blast incidence was determined by counting all diseased and healthy plants per plot and converting to percent disease incidence. Plants in field studies at Crowley were subjected to natural infection by unknown races of *P. oryzae*. Panicle blast incidence was determined as stated for Greenfield, but in 1.4 m^2 per plot.

Assays for tricyclazole in leaves.—Tricyclazole was identified in rice leaves from the greenhouse seed treatment study by subjecting solvent extracts of leaves to two chromatographic procedures. One procedure involved column extraction and detection by gas-liquid chromatography (GLC) using a flame photometric detector. Assay sensitivity was 0.01-0.02 μg/g.

The second procedure was the bioautographic assay technique using *Cladosporium cucumerinum* Ellis and Arth. as the bioassay organism (12). Leaf samples (3- to 4-grams) were homogenized in 100 ml acetone/ethanol (1:1, v/v) for 3 minutes in a blender, filtered, and the filtrate was evaporated to dryness. The residue was resuspended in 2 ml of chloroform and spotted (10 μliters) on silica gel thin-layer chromatographic (TLC) plates. References included technical tricyclazole in ethanol and control leaf extracts to which were added (spiked) known amounts of tricyclazole. Tricyclazole was separated from plant constituents by developing the TLC plates with methylethylketone/benzene (4:6, v/v). After drying, the plates were inoculated with *C. cucumerinum* in potato-dextrose broth and incubated 48 hours in a moist chamber. Tricyclazole was identified as a red spot at R_f 0.13. The fungicide was colorless in solution, but the red color appeared when tricyclazole was in contact with an actively growing culture of *C. cucumerinum*. Tricyclazole did not inhibit fungus growth within the red spot, although the compound was present according to GLC verification. No degradation products of tricyclazole are known which produce a color in contact with *C. cucumerinum* at R_f 0.13. The concentration of tricyclazole in rice leaves was determined by measuring TLC spot diameters and referring to a standard curve derived from tricyclazole-spiked plant samples.

RESULTS

The concentrations of tricyclazole required to produce 0, 50, and 95% reduction in mycelial growth in vitro were <15, 65, and 200 μg/ml, respectively. Concentrations associated with similar percent reductions in spore germination were <10, 100, and 200 μg/ml, respectively. For 0, 50, and 95% reduction in germ tube length of those spores that did germinate, it required <1, 60, and 300 μg/ml of tricyclazole, respectively.

Tricyclazole was weakly fungicidal to spores with a 2-hour exposure at 300 μg/ml (24% reduction in growth was not reduced after a 2-hour exposure to 75-300 μg/ml tricyclazole.

One foliar application of 0.56 kg/ha tricyclazole at the booting stage of growth or a split application of 0.28 + 0.28 kg/ha at booting plus early heading provided approximately 90% control of panicle blast and 10-15% yield increase compared with the untreated control (Table 1). No crop injury was observed from any rate.

In the greenhouse study, a tricyclazole seed treatment rate of 0.2 g/kg seed (0.179 g/kg actually on the seed) effectively controlled leaf blast 21 days after planting and resulted in 8.2 μg/g tricyclazole in the leaves at that time according to GLC assay (Table 2). Although lower concentrations were associated with reduced disease incidence, 8.2 μg/g is assumed to be the concentration of tricyclazole necessary for approximately 100% leaf blast control until later information may prove otherwise.

Bioassay results were similar to GLC assay results for tricyclazole concentrations in rice leaves (Table 2). Bioassay sensitivity was 0.05 μg in spiked leaf extracts and less than 0.01 μg in ethanol. Percent recovery decreased with decreasing tricyclazole concentrations, and consid-

TABLE 1. Control of *Piricularia oryzae* on rice from one and two foliar applications of tricyclazole 75 WP at the Crowley, Louisiana, field site[x]

Time of application and treatment	Rate (kg/ha)	Panicle blast (%)	Yield (% of control)
Booting stage			
Tricyclazole	0.28	11 de[z]	111 a
Tricyclazole	0.56	5 e	110 a
Tricyclazole	1.12	3 e	119 a
Hinosan[y]	0.35	19 cd	111 a
Control, WP blank	0.0	30 b	96 b
Booting and early heading			
Tricyclazole	0.28	3 e	115 a
Tricyclazole	0.56	3 e	116 a
Tricyclazole	1.12	3 e	119 a
Hinosan	0.38	5 e	112 a
Control, WP blank	0.0	26 bc	95 b
Control, untreated	0.0	38 a	100 b

[x]Treatments applied in 480 liters H_2O/hectare with a hand-carried spray boom.
[y]Hinosan, applied at recommended rate in Japan, is not registered for use on rice in the U.S.
[z]Values not followed by the same letter are significantly different ($P = 0.05$) by Duncan's multiple range test.

TABLE 2. Control of *Piricularia oryzae* on rice and tricyclazole residues in leaves from plants grown from tricyclazole-treated seeds in the greenhouse[x]

Tricyclazole rate (g/kg seed)		Leaf blast severity rating[z]	Tricyclazole leaf residues (μg/g ± SE)	
Theory	Actual[y]		GLC assay	Bioassay
0.0	0.0	2−	NDR	NDR
0.025	0.022	1	0.41 ± 0.02	NDR
0.05	0.051	3+	1.40 ± 0.2	NDR
0.1	0.094	3+	2.40 ± 0.2	2.9 ± 1.5
0.2	0.179	4+	8.20 ± 0.6	11.9 ± 2.0
0.4	0.331	5	23.90 ± 0.7	26.3 ± 4.8

[x]Plants were inoculated with *P. oryzae* and leaves were harvested for residue assays 14 days after planting. Gas-liquid chromatographic assay sensitivity for tricyclazole was 0.01-0.02 μg/g and residue values were corrected for 62% recovery in tricyclazole-spiked plant samples. More recent techniques have improved the percent recovery of tricyclazole from plant tissues. Bioassay sensitivity was approximately 0.05 μg/g. NDR = no detectable residue.
[y]According to GLC assay of seed.
[z]Rating was estimated on a 1-5 disease severity scale: 1 = severe, 2 = moderate, 3 = slight, 4 = very slight, and 5 = no disease.

erable within-test and test-to-test variability has been encountered with this technique when attempting to quantitate tricyclazole residues. Although the bioassay data (Table 2) are similar to GLC assay data, they are exceptional. The bioautographic assay technique for tricyclazole in plant tissues should be used as a qualitative assay only.

In the field studies, tricyclazole seed treatment rates of 1 g/kg seed and above provided good leaf blast control for 5 weeks after planting but not after 9 weeks when panicles were emerging (Table 3). Panicle blast incidence was reduced by seed treatment, but control was not competitive with the reference foliar spray. No crop injury was observed from 2 g tricyclazole/kg seed, but 3 g/kg resulted in stand and yield reductions.

Tricyclazole applied as a soil drench treatment to rice seedlings in flats 2 hours before transplanting provided excellent leaf blast control 6 and 10 weeks later from application rates of 2.5 and 5.0 g/flat (Table 4). When transplanting was delayed 24 hours after treatment, disease control did not improve significantly. No panicle blast developed in this test. Initial crop injury (leaf chlorosis and necrosis) was not evident in new leaves developing after transplanting.

Ten-minute soaks of rice roots in 3 and 5 g/liter suspensions of tricyclazole provided good leaf blast

TABLE 3. Control of *Piricularia oryzae* on rice and crop response from tricyclazole 75 WP seed coat treatments at field sites in Greenfield (planted 7 June) and Crowley (planted 11 June)

Treatment	Rate (g/kg seed)	Greenfield site[x] Leaf blast (%) 23 July	21 August	Crowley site Panicle blast (%)	Stand count (25 June) (% of control)	No. of panicles (% of control)	Yield (% of control)
Tricyclazole	1.0	9 b[z]	30 a	27 b	104 a	101 a	100 a
Tricyclazole	2.0	15 ab	15 a	13 c	101 a	102 a	100 a
Tricyclazole	3.0	9 b	15 a	12 c	75 b	89 b	89 b
Hinosan	0.35[y]			6 c	114 a	102 a	102 a
Control, WP blank	0.0	37 ab	30 a	37 a	107 a	94 a	106 a
Control, untreated	0.0	44 a	30 a	37 a	100 a	100 a	100 a

[x]Plants were artificially inoculated with *P. oryzae* on 13 July and 13 August.
[y]Hinosan applied as a foliar spray at 0.35 kg/480 liters H_2O at booting and early heading stages of growth.
[z]Values not followed by the same letter are significantly different ($P = 0.05$) by Duncan's multiple range test.

TABLE 4. Control of *Piricularia oryzae* on rice and crop response from tricyclazole 75 WP soil drench application to the transplant flat at the Greenfield site[x]

Tricyclazole rate (g/flat)	Time from treatment to transplanting (hr.)	Leaf blast (%) 30 July	Leaf blast (%) 30 August	Crop injury 29 June[y]
1.0	2	62 a[z]	24 ab	0.0 c
2.5	2	9 b	4 cd	0.7 c
5.0	2	2 c	3 d	3.7 a
1.0	24	44 a	10 bc	0.6 bc
2.5	24	4 ab	3 d	2.7 ab
5.0	24	1 c	2 d	4.0 a
Control		73 a	44 a	0.0 c

[x]Tricyclazole was suspended in 500 ml of H_2O and drenched on the soil surface of a 28×58×3 cm flat. Transplanting took place on 19 June and artificial inoculation on 25 July and 24 August.
[y]Crop injury was estimated on a 0-10 rating scale: 0 = none, 1-3 = slight, 4-6 = moderate, 7-10 = severe. Injury was chlorosis and necrosis of leaves.
[z]Values not followed by the same letter are significantly different ($P = 0.05$) by Duncan's multiple range test.

TABLE 5. Control of *Piricularia oryzae* on rice and crop response from 10-minute root-soak exposures to tricyclazole 75 WP at the Greenfield field site[x]

Tricyclazole rate (g/liter)	Leaf blast 30 August (%)	Panicle blast 17 October (%)	Crop injury 29 June[y]
1	23 ab[z]	21 a	0.0 b
3	5 b	14 a	0.6 ab
5	9 b	16 a	1.3 a
Control, WP blank	37 a	17 a	1.3 a
Control, untreated	37 a	21 a	0.0 b

[x]The roots of soil-free transplants were soaked in 1 liter suspensions of tricyclazole. Transplanting took place on 20 June and artificial inoculations on 20 August and 29 September.
[y]Crop injury was estimated on a 0-10 rating scale, where 0 = none, 1-3 = slight, 4-6 = moderate, 7-10 = severe.
[z]Values not followed by the same letter are significantly different ($P = 0.05$) by Duncan's multiple range test.

control 10 weeks after transplanting and slight panicle blast control (Table 5). Slight crop injury (leaf chlorosis and necrosis) appeared soon after transplanting, but was not apparent 4 weeks later because of new growth and natural senescence of old leaves.

DISCUSSION

The systemic distribution of tricyclazole in rice plants was demonstrated indirectly by control of foliar disease symptoms by seed, root, and soil applications, and directly by identification of tricyclazole in leaves of plants grown from treated seed. The concentration of tricyclazole in leaves associated with 100% disease control was 25 to 35 times less than the concentration associated with in vitro inhibition of *P. oryzae*. Enhanced in vivo activity has been reported for other fungicides, such as kasugamycin (8), Rabcide (6), and Dowco 269 (7). Tricyclazole may control *P. oryzae* by methods other than direct fungicidal action.

The possibility exists that tricyclazole induces a resistance mechanism in rice that prevents establishment of *P. oryzae*. If this were a generalized resistance mechanism, other rice pathogens should be inhibited also. Greenhouse and field data (J. D. Froyd, *unpublished*) demonstrated that foliar applications of tricyclazole to rice at 1 kg/ha or 1,000 μg/ml did not reduce the incidence of *Cochliobolus miyabeanus* Dickson (*Helminthosporium oryzae* Breda de Haan), *Corticium sasakii* (Shirai) T. Matsu, or *Xanthomonas oryzae* (Uzeda and Ishiyama) Dowson. The in vitro response of these pathogens to tricyclazole was similar to the response of *P. oryzae*. These findings suggest that either a host resistance specific to *P. oryzae* is induced by tricyclazole or the fungicide has direct inhibitory activity against *P. oryzae* in the host which is not expressed in vitro.

The systemicity and residual fungitoxicity of tricyclazole provides control of *P. oryzae* by various methods of application to rice. Seed treatment, soil drench in the transplant flat, or transplant root-soak applications provided good to excellent leaf blast control until panicle emergence when leaf infection normally diminishes. These types of application have the potential for replacing the one to three foliar applications of fungicides currently used for leaf blast control.

Seed and transplant root-soak applications of tricyclazole provided partial control of late season panicle blast. Soil drench to the transplant flat also has given partial control of panicle blast (L. R. Guse, *unpublished*). One foliar application of 0.56 kg/ha at booting stage of growth provided excellent panicle blast control and was competitive with two applications of the recommended rate of a reference fungicide which is commercially used in Japan. A combination treatment of either the seed or a transplant application plus one foliar application of tricyclazole has the potential for providing season-long protection of rice from *P. oryzae*. Future publications on tricyclazole will present data on the efficacy of these applications in comparison with several commonly used rice blast fungicides.

Soil drench application of tricyclazole in the transplant flat offers a unique method of blast control in Japan. Machine transplanting is rapidly replacing hand transplanting of rice as a time- and labor-saving practice. The application of pesticides, such as tricyclazole for *P. oryzae* control, to the soil in the flat prior to transplanting has obvious advantages over multiple foliar applications for pest control.

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