Translocation of Fungicides and Their Efficacy in Controlling *Phellinus noxius*, the Cause of Brown Root Rot Disease

Ting-Zhi Liao,[1] Yu-Hsuan Chen,[2,3] Jyh-Nong Tsai,[4] Chieh Chao,[2] Tzu-Pi Huang,[2,3] Cheng-Fang Hong,[2,3] Zong-Chi Wu,[5] Isheng Jason Tsai,[6] Hsin-Han Lee,[6] Ned B. Klopfenstein,[7] Mee-Sook Kim,[8] Jane E. Stewart,[9] Ndeme Atibalentja,[10] Fred E. Brooks,[11] Philip G. Cannon,[12] A. Mohd Farid,[13] Tsutomu Hattori,[14] Hoi-Shan Kwan,[15] Regent Yau Ching Lam,[15,16] Yuko Ota,[17] Norio Sahashi,[14] Robert L. Schlub,[18] Louise S. Shuey,[19] Alvin M. C. Tang,[16,20] and Chia-Lin Chung[1,5,†]

[1] Master Program for Plant Medicine, National Taiwan University, Taipei 10617, Taiwan
[2] Pesticide Residue Analysis Center, National Chung Hsing University, Taichung 40227, Taiwan
[3] Department of Plant Pathology, National Chung Hsing University, Taichung 40227, Taiwan
[4] Taiwan Agricultural Research Institute, Council of Agriculture, Taichung 413008, Taiwan
[5] Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 10617, Taiwan
[6] Biodiversity Research Center, Academia Sinica, Taipei 11529, Taiwan
[7] United States Department of Agriculture (USDA) Forest Service, Moscow, ID 83843, U.S.A.
[8] USDA Forest Service, Corvallis, OR 97331, U.S.A.
[9] Department of Agricultural Biology, Colorado State University, Fort Collins, CO 80523, U.S.A.
[10] American Samoa Community College, Western Dist. 96799, American Samoa
[11] 1961 Westwood Pl., Pomona, CA 91768, U.S.A.
[12] USDA Forest Service, State and Private Forestry, Forest Health Protection, Vallejo, CA 94592, U.S.A.
[13] Forest Health and Conservation Programme, Forest Research Institute Malaysia, Selangor 52109, Kepong, Malaysia
[14] Forestry and Forest Products Research Institute, Tsukuba 305-8687, Japan
[15] School of Life Sciences, The Chinese University of Hong Kong, Central Ave, Hong Kong
[16] Muni Arborist Limited, Lam Tsuen, Hong Kong
[17] College of Bioresource Sciences, Nihon University, Kanagawa 252-0880, Japan
[18] Cooperative Extension and Outreach, University of Guam, Mangilao 96923, Guam
[19] Department of Agriculture and Fisheries, Queensland Government, Brisbane 4001, Australia
[20] Division of Applied Science, College of International Education, School of Continuing Education, Hong Kong Baptist University, Hong Kong, China

Abstract

Brown root rot disease (BRRD), caused by *Phellinus noxius*, is an important tree disease in tropical and subtropical areas. To improve chemical control of BRRD and deter emergence of fungicide resistance in *P. noxius*, this study investigated control efficacies and systemic activities of fungicides with different modes of action. Fourteen fungicides with 11 different modes of action were tested for inhibitory effects in vitro on 39 *P. noxius* isolates from Taiwan, Hong Kong, Malaysia, Australia, and Pacific Islands. Cyproconazole, epoxiconazole, and tebuconazole (Fungicide Resistance Action Committee [FRAC] 3, target-site G1) inhibited colony growth of *P. noxius* by 99.9 to 100% at 10 ppm and 97.7 to 99.8% at 1 ppm. The other effective fungicide was cyprodinil + fludioxonil (FRAC 9 + 12, target-site D1 + E2), which showed growth inhibition of 96.9% at 10 ppm and 88.6% at 1 ppm. Acropetal translocation of six selected fungicides was evaluated in bishop wood (*Bischofia javanica*) seedlings by immersion of the root tips in each fungicide at 100 ppm, followed by liquid or gas chromatography tandem mass spectrometry analyses of consecutive segments of root, stem, and leaf tissues at 7 and 21 days posttreatment. Bidirectional translocation of the fungicides was also evaluated by stem injection of fungicide stock solutions. Cyproconazole and tebuconazole were the most readily absorbed by roots and efficiently transported acropetally. Greenhouse experiments suggested that cyproconazole, tebuconazole, and epoxiconazole have a slightly higher potential for controlling BRRD than mepronil, prochloraz, and cyprodinil + fludioxonil. Because all tested fungicides lacked basipetal translocation, soil drenching should be considered instead of trunk injection for their use in BRRD control.

Keywords: *Bischofia javanica*, bishop wood, gas chromatography tandem mass spectrometry (GC-MS/MS), liquid chromatography tandem mass spectrometry (LC-MS/MS), *Phellinus noxius*, systemic fungicide

[†]Corresponding author: C.-L. Chung; clchung@ntu.edu.tw

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Brown root rot disease (BRRD) causes serious damage to many woody plant species in plantations, agroforests, and landscapes in tropical and subtropical areas (Ann et al. 2002; CABI 2022). Over the last 10 years in Taiwan, BRRD comprised approximately 40% of all tree diseases reported to the Forest Disease Information Center (https://health.tfri.gov.tw/fhsnc/), which reflects the prevalence of the disease and the high public awareness. The pathogen, *Phellinus noxius* (Corner) G. H. Cunningham, is a white-rot fungus with a broad host range. Over 200 species of forest, fruit, and ornamental trees have been reported as hosts of *P. noxius* (Ann et al. 1999, 2002). The wide host range is likely associated with a comprehensive array of carbohydrate-active enzymes encoded in the genome of *P. noxius* (Chung et al. 2017; Ibarra Caballero et al. 2020). Field observations and molecular evidence suggested that *P. noxius* disseminates between adjacent trees by mycelia via root-to-root contact, and its long-distance dispersal is via basidiospores and

the transport of infected plants or debris by human activities (Akiba et al. 2015; Chung et al. 2015, 2017; Kozhar et al. 2022). *P. noxius* usually infects the roots and basal stem of trees, degrading cellulose and lignin, leaving dry, whitish remnants. Diseased trees with decayed root systems can easily topple over, causing economic losses and endangering public safety. The aboveground symptoms of BRRD vary, depending on the tree species. Banyan (*Ficus microcarpa*) trees with BRRD typically show small and chlorotic leaves, defoliation, and gradual withering. Many other trees with BRRD such as camphor (*Camphora officinarum*), loquat (*Eriobotrya japonica*), and flame trees (*Delonix regia*) typically show quick decline symptoms. Their leaves frequently turn reddish brown and wilt within a few months (Ann et al. 1999, 2002; Chung et al. 2015, 2017). The optimal temperature for the mycelial growth of *P. noxius* on potato dextrose agar (PDA) is 30°C (Ann and Ko 1992), and global warming could increase the incidence and severity of BRRD. Together, effective control and management of BRRD has become an important and urgent issue for tree protection and public safety.

Various measures have been developed and tested for BRRD management, including soil fumigation using dazomet for 1 month (Fu et al. 2012) or flooding for 1 to 2 months (Chang 1996). Both approaches have efficacy after the removal of a *P. noxius*-infected tree's stump and roots. A recent study by Wu et al. (2020) suggested that, because *P. noxius* cannot survive in the soil without host tissues, the soil-disinfestation step can be omitted as long as diseased roots and wood debris are removed carefully and thoroughly. Microbial antagonists of *P. noxius* can be used for biological control. *Trichoderma asperellum* isolate TA forms mycoparasitic coils around the hyphae of *P. noxius*. Preventive drenching with *T. asperellum* TA protected the highly susceptible loquat cuttings from wilting caused by BRRD (Chou et al. 2019). Soaking fresh cuttings of Ceylon myrtle (*Phyllanthus myrtifolius*) seedlings with *Bacillus* sp. and *Streptomyces padanus* suspensions significantly promoted the shoot and root growth and reduced the BRRD severity (Leung et al. 2020). Chemical control or surgery (removal of infected tissues) can be conducted to reduce impacts of BRRD in diseased trees: fungicides have been typically applied by soil drenching and trunk injection (Sun et al. 2020; Tsai et al. 2008) or sprayed on the wounds after surgery treatment.

Several studies have been conducted in vitro and in planta on chemical control of BRRD. Tsai et al. (2005) tested the sensitivity of four isolates of *P. noxius* (from Tainan and Taitung, Taiwan) to 46 fungicides with 11 modes of action (MoAs) (Fungicide Resistance Action Committee [FRAC] 1, 2, 3, 4, 5, 7, 14, 20, M01, and NC). They observed that 4-4 Bordeaux mixture, propiconazole, triadimefon, and prochloraz at 10 ppm caused 98.6 to 100% inhibition of mycelial growth. These fungicides were also effective in potted plant assays at reducing the incidence of decline in loquat seedlings artificially inoculated with *P. noxius* (Tsai et al. 2005). Li (2016) tested the sensitivity of one *P. noxius* isolate from Taipei, Taiwan, to six fungicides (FRAC 3, 11, and 40), and propiconazole, difenoconazole, and difenoconazole + propiconazole showed 98.9 to 100% inhibition of mycelial growth. Lim et al. (1990) tested the sensitivity of a *P. noxius* isolate from Malaysia to four fungicides, and penconazole and triadimenol, tridemorph, and quintozene (FRAC 3, 5, and 14) at 10 ppm resulted in 97.2 to 100% inhibition of mycelial growth. In a vineyard naturally infested with *P. noxius*, grapevines treated with soil drenching of triadimefon + urea + $CaCO_3$ (l0 g each in 1 liter of water) every 3 months for 2.5 years did not develop BRRD, whereas 14.3% of untreated plants were killed by BRRD (Tsai et al. 2008).

Commonly, fungicides are applied by foliar spraying, trunk injection, soil drenching, and soil mixing. The bioavailability of fungicides at the target sites can be significantly affected by their uptake and translocation in plants. Nonsystemic fungicides remain on the surface of the plant after application; thus, preventive applications are needed for disease control. Systemic fungicides can be absorbed and show long-distance translocation ability. Local systemic or translaminar fungicides can move only a short distance by diffusion. Different analytical techniques have been used to assess the systemic movement of fungicides, mostly in young seedlings of herbaceous plants. For instance, the movements of fungicides were observed using an isotope-labeling imaging technique at the two- to three-leaf-stage of tomato, Chinese flowering cabbage, and water spinach seedlings grown in nutrient solution containing ^{14}C-labeled fungicides (Chen et al. 2020; Kubicki et al. 2019). After root or leaf treatments of the 3- to 42-day-old seedlings of wheat, leafy vegetables, rice, and castor bean, the translocations of pesticides were determined by high-performance liquid chromatography or gas chromatography–mass spectrometry (GC/MS) (Ge et al. 2017; Li et al. 2018; Yang et al. 2011; Yu et al. 2022). In addition to direct quantification, the translocation or translaminar activity of fungicides could also be inferred by evaluating the efficacy of disease control (Augusto and Brenneman 2012; Klittich and Ray 2013). Studies on the translocation of fungicides in woody plants have been relatively limited. Thomidis (2003) developed a quick in vitro method to evaluate the translocation of metalaxyl, fosetyl-Al, captan, dimethomorph, cymoxanil, and copper hydroxide in excised peach shoots. The woody shoots inoculated with *Phytophthora cactorum* or *P. citrophthora* were inserted into vermiculite containing the tested fungicides, and the systemic movement of fungicides was determined by measuring the length of pathogen-induced necrosis. The acropetal and basipetal translocations of propiconazole have been verified in peach trees using trunk infusion and GC/MS, and higher levels of basipetal movement were detected following the infusion in the spring or fall than in the summer (Amiri et al. 2008). Trunk infusion of peach with propiconazole significantly inhibited the occurrence of Armillaria root disease in a commercial orchard (Amiri and Schnabel 2012).

In Taiwan, prochloraz has been the only fungicide officially recommended for emergency control of BRRD (https://pesticide.baphiq.gov.tw/information/). Prochloraz is a demethylation inhibitor (DMI) that targets the 14α-demethylase enzyme (CYP51) (FRAC 3, target-site G1) in the ergosterol biosynthetic pathway of fungi (Sierotzki and Scalliet 2013). In addition to prochloraz, other DMIs such as triadimefon, propiconazole, and difenoconazole have also been used in soil drenches or root or basal stem injections of BRRD-diseased trees and the neighboring trees (Sun et al. 2020; Tsai et al. 2008). The long-term and extensive use of fungicides with the same MoA can increase the risk of emergence of fungicide resistance in fungal pathogens. Although the sensitivity of *P. noxius* to fungicides with different MoAs has been evaluated, the assays were conducted on culture media and used only a few isolates of *P. noxius* (Li 2016; Lim et al. 1990; Tsai et al. 2005). In addition, tree roots are widely distributed and sometimes extend beneath pavement or construction barriers, making it difficult to apply fungicides to the whole root system via drenching or injection. Fungicides with strong systemic activity may enhance their distribution across the root systems to help prevent or ameliorate infection by *P. noxius*. The translocation efficiency of fungicides is dependent on the physical and chemical properties of the compound in combination with the biological characteristics of the plant (Bromilow et al. 1990; Kleier 1988; Satchivi et al. 2001; Tyree et al. 1979). However, most current knowledge on the systemic activity of fungicides is derived from studies with herbaceous plants (Ge et al. 2017; Liu et al. 2014; Namiki et al. 2018; Wang et al. 2021).

The objectives of this study were to (i) evaluate alternative fungicides for chemical control of BRRD, (ii) assess acropetal and basipetal translocation patterns of *P. noxius*-effective fungicides, and (iii) determine the control efficacy of the fungicides for BRRD in planta. We first tested the inhibitory effects of fungicides with different MoAs on the mycelial growth of 39 *P. noxius* isolates collected across 12 global regions. Convenient root-tip immersion and stem-injection methods were developed to evaluate systemic activities of fungicides in bishop wood (*Bischofia javanica*) seedlings. The intraplant distribution and control efficacies of selected fungicides were evaluated by soil drenching of bishop wood seedlings artificially inoculated with *P. noxius* on the stem. Information on the spatiotemporal distribution of the fungicides and their inhibitory effects on *P. noxius* provides insights on potential fungicides and application methods for controlling BRRD and other tree diseases.

Materials and Methods

P. noxius isolates and plant materials

In total, 39 *P. noxius* isolates collected from 12 geographic regions (i.e., Taiwan, Hong Kong, Malaysia, Australia, Yap, Kosrae, Rota,

Pohnpei, Palau, American Samoa, Saipan, and Guam) were used in this study. Details of the isolate collection are available in Table 1. *P. noxius* was preserved as mycelial discs in sterile, distilled water (dH_2O) at room temperature (Richter et al. 2010). Before conducting experiments, each isolate was revived by culturing on PDA (HIMEDIA) for 5 to 7 days at 30°C in the dark.

Seedlings of bishop wood (*Bischofia javanica*), 6 to 12 months old, were used in this study because of their abundant and longer roots (suitable for the root-tip immersion treatment), tender and brittle tissues (relatively easy to homogenize for extracting fungicides in the translocation assay), and high susceptibility to *P. noxius* (suitable for the control assay) (Pang 2020). In the translocation assays, the bishop wood seedlings (Mu-Sheng Horticulture Company) were 40 to 60 cm in height; in the control efficacy assays, the seedlings (Tian-Wei Rose Garden) were 50 to 60 cm in height. Seedlings were first transplanted into pots (18 cm high by 12 cm wide) with a mixture of 3:1 peat to perlite. Each seedling was drenched with 200 to 300 ml of 1,000-fold diluted Hyponex No. 2 fertilizer (Hyponex Japan Corp., Ltd.), and grown for at least 2 weeks before the experiments.

Table 1. *Phellinus noxius* isolates used in this study[a]

Collection site, isolate	Host	Collection year	Collector or provider
Taiwan			
2248*†	*Ficus microcarpa*	2013	Schung-Ching Chiu
2252*†	*F. microcarpa*	2013	Schung-Ching Chiu
NTU5-7*†	*F. benjamina*	2011	Yu-Ching Huang
A42*†	Basidiocarp	2011	Yu-Ching Huang
Hong Kong			
CUPN053*†	–	2018	–
CUPN098	–	2018	–
JCHS	*Celtis sinensis*	2014	Alvin Tang
SBR*†	*F. microcarpa*	2014	Regent Lam
Malaysia			
FRIM 003*	*Acacia mangium*	1991	Mohd Farid
FRIM 055*	*A. mangium*	1994	Mohd Farid
FRIM 557†	*A. mangium*	2004	Mohd Farid
FRIM 616	*Tectona grandis*	2003	Mohd Farid
FRIM 620†	*T. grandis*	2003	Mohd Farid
FRIM 727	*Elateriospermum tapos*	2010	Mohd Farid
Australia			
AusPn-1*†	*Araucaria cunninghamii*	2014	Louise Shuey
AusPn-6	*Cajanus cajan*	2014	Louise Shuey
AusPn-12	*Mangifera indica*	2014	Louise Shuey
AusPn-16	*Persea americana*	2014	Louise Shuey
AusPn-21	*F. benjamina*	2014	Louise Shuey
Yap			
Yap 5A	*M. indica*	2013	Phil Cannon
Yap 5D	*M. indica*	2013	Phil Cannon
Kosrae			
PN KOSRAE #5-1-1104	–	–	Phil Cannon
Rota			
ROTA 8B	–	–	Phil Cannon
Pohnpei			
P919-02W.5*†	*Ficus tinctoria*	2013	Yuko Ota
P919-02W.7	*F. tinctoria*	2013	Yuko Ota
P919-08W.4	*Artocarpus altilis*	2013	Yuko Ota
P919-08W.7	*F. tinctoria*	2013	Yuko Ota
Palau			
Palau 20P*†	–	2013	Phil Cannon
Palau 28P	–	2013	Phil Cannon
Palau 33P	–	2013	Phil Cannon
American Samoa			
AS-1.2-PN†	*Pometia pinnata*	–	Fred Brooks et al.
AS-2B.2-PN*†	*Swietenia macrophylla*	–	Fred Brooks et al.
AS-4.2-PN	*Macaranga* sp.	2014	Fred Brooks et al.
AS-5A.2-PN†	*Cananga odorata*	2014	Fred Brooks et al.
AS-7.1A-PN†	*Ceiba pentandra*	2014	Fred Brooks et al.
Saipan			
S916-07C	*Delonix regia*	2013	Yuko Ota
S916-03W.1	*D. regia*	2013	Yuko Ota
S916-04W.1	*D. regia*	2013	Yuko Ota
Guam			
UOG3	*Ochrosia mariannensis*	2013	Ashley Lehman
UOG17	*O. mariannensis*	2013	Ashley Lehman
GUAM-SCHLUB 17-28A	–	–	Robert L. Schlub

[a] All isolates except for FRIM 557 and FRIM 620 were tested for sensitivity to 14 fungicides at 10 ppm. For the fungicides showing high inhibitory effects at 10 ppm, a set of 12 selected isolates (labeled with bold and *) was used for sensitivity assays at low concentrations (1 and 0.1 ppm). A set of 15 isolates (labeled with bold and †) was used for SDHI sensitivity assay. Symbol – indicates unknown. Import Permit Number 107-F-505 and 108-F-501.

Fungicide sensitivity assays

The "poisoned food technique" (Nene and Thapilyal 2002) was used to determine the sensitivity of *P. noxius* to different fungicides. Mycelial agar discs (6 mm in diameter) were cut from the edge of 5- to 7-day-old *P. noxius* colonies, then transferred to the center of a 90-mm Petri dish containing 20 ml of PDA and the fungicide to be tested. PDA without a fungicide was used as the control. After a 3-day incubation at 30°C in the dark, the diameters of the resulting *P. noxius* colonies were measured. The mycelial growth rate (millimeters per day) was calculated as (colony diameter − diameter of the mycelium-agar disc)/3. The percentage of inhibition of mycelial growth (%) was calculated as [(the average colony diameter of the control − the average colony diameter of the treatment)/(the average colony diameter of the control − the diameter of the mycelium-agar disc)] × 100%.

The sensitivity of 39 *P. noxius* isolates was tested with active ingredient (a.i.) at 10 ppm for 14 fungicides with 11 MoAs: mepronil (suspension concentrate [SC]) (FRAC 7, target-site C2); trifloxystrobin (FRAC 11, target-site C3); quinoxyfen (FRAC 13, target-site E1); procymidone (FRAC 2, target-site E3); isoprothiolane (FRAC 6, target-site F2); cyproconazole, epoxiconazole, tebuconazole, triadimenol, triadimefon, and prochloraz (FRAC 3, target-site G1); and tricyclazole (FRAC 16.1, target-site I1), and two coformulants: ametoctradin (FRAC 45, target-site C8) + dimethomorph (FRAC 40, target-site H5) and cyprodinil (FRAC 9, target-site D1) + fludioxonil (FRAC 12, target-site E2) (Table 2). After preliminary screening, the effective fungicides (>90% inhibition rate) were selected for subsequent assays at lower concentrations. In a diseased tree, the amount of fungicide that can be translocated to infected tissues can be limited; therefore, screening out the fungicides that are highly effective at low concentrations is essential. Six fungicides (mepronil [SC], cyproconazole, epoxiconazole, tebuconazole, triadimenol, and cyprodinil + fludioxonil) were tested at 1 ppm a.i., and four fungicides (cyproconazole, epoxiconazole, tebuconazole, and cyprodinil + fludioxonil) were tested at 0.1 ppm a.i. against 12 representative *P. noxius* isolates. The 12 representative isolates were selected based on phylogenetic analysis of the 39 isolates using whole-genome sequencing data (two isolates selected from each phylogenetic cluster) (I. J. Tsai, unpublished), which also show broad representation across the phylogenetic groups determined in other studies (Kozhar et al. 2022; Stewart et al. 2020). Potential cross-tolerance to different succinate dehydrogenase inhibitors (SDHIs) was evaluated by testing the sensitivity of 15 isolates (12 representative isolates plus additional American Samoa isolates) with mepronil (SC and wettable powder [WP]), flutolanil, and boscalid at 10 ppm (Table 2).

The experiments were conducted in two independent trials, each containing four plates per treatment. Data from 10-, 1-, and 0.1-ppm fungicide assays were analyzed separately. Analysis of variance (ANOVA) was performed to determine the blocking effect (trial) and the effect of fungicide on the percent inhibition of mycelial growth. For each fungicide (10-ppm data), ANOVA was conducted to determine the blocking effect (trial) and the effect of geographical region (*P. noxius* source) on the inhibition of mycelial growth. For the SDHI assay, ANOVA was conducted to determine the blocking effect (trial) and the effect of fungicide, geographical region, and their interactions (fungicide × region) on the percent inhibition of mycelial growth. Differences among treatments were analyzed by Tukey's honestly significant difference test at $P < 0.05$ using SAS Enterprise Guide 7.1 (SAS Institute Inc.).

Translocation assays

Acropetal and bidirectional translocation assays were conducted for fungicides with strong inhibitory effects on *P. noxius*, including mepronil, cyproconazole, epoxiconazole, tebuconazole, cyprodinil + fludioxonil, and prochloraz (nonsystemic). Imidacloprid and spirotetramat were used as positive controls for acropetal and bidirectional translocation assays, respectively. Their translocation activities have been confirmed in herbaceous and woody plants (Brück et al. 2009; Buchholz and Nauen 2002; Tattar et al. 1998; Turcotte et al. 2017; Vermeer and Baur 2008). The experiments were conducted at 35 and 30°C (12-h day and night temperatures, respectively) and 55 to 95% relative humidity under natural light in the phytotron at National Taiwan University. Bishop wood seedlings (40 to 60 cm in height) were used as the plant materials. A week before the experiment, the leaves were trimmed to three to five trifoliate leaves per plant (depending on leaf sizes).

Table 2. Pesticides used in this study

Pesticide	Formulation	Manufacturer	MoA (FRAC target-site code)	FRAC code	Systemic activity[a]	Rate of acute toxicity (LD_{50} [mg/kg])[b]
Mepronil	40% SC	Kumiai Chemical Industry Co., Ltd.	C2	7	S	10,000
	75% WP	Kumiai Chemical Industry Co., Ltd.	C2	7	S	10,000
Flutolanil	50% WP	Taiwan Nihon Nohyaku Co., Ltd.	C2	7	S	10,000
Boscalid	50% WG	Wonderful Agriculture Co., Ltd.	C2	7	LS	>5,000
Trifloxystrobin	50% WG	Great Victory Chemical Industry Co. Ltd.	C3	11	N	5,000
Quinoxyfen	22.58% SC	Dow AgroSciences Taiwan Ltd.	E1	13	N	5,000
Procymidone	50% WP	Lih-Nung Chemical Co., Ltd.	E3	2	S	7,000
Isoprothiolane	40% WP	Taiwan Nihon Nohyaku Co., Ltd.	F2	6	S	1,190
Cyproconazole	8.8% SL	Rich Country Chemical Corp.	G1	3	S	1,020
Epoxiconazole	7.5% EC	BASF SE	G1	3	S	3,160
Tebuconazole	25.9% EW	Great Victory Chemical Industry Co. Ltd.	G1	3	S	3,352
Prochloraz	25% EW	Great Victory Chemical Industry Co. Ltd.	G1	3	N	1,600
Triadimenol	23% DC	Bayer AG	G1	3	S	3,800
Triadimefon	25% WP	Chia Yi Chemical Industries Co., Ltd.	G1	3	S	363
Tricyclazole	75% WP	Corteva Agriscience	I1	16.1	N	250
Ametoctradin + dimethomorph[c]	52.5% SC	BASF SE	C8, H5	45, 40	LS, N	2,000, 3,900
Cyprodinil + fludioxonil[c]	62.5% WG	Syngenta Group Co., Ltd.	D1, E2	9, 12	S, N	2,000, 5,000
Imidacloprid[d]	9.6% SL	Shui Fong Crop Protection Corp.	4A	–	S	410
Spirotetramat[d]	10% SC	Bayer AG	23	–	S	2,000

[a] S = systemic, N = nonsystemic, and LS = local systemic.

[b] Sources of information: PubChem and CompTox Chemicals Dashboard (United States Environmental Protection Agency) databases. LD_{50} = 50% lethal dose.

[c] Mode of action (MoA) (Fungicide Resistance Action Committee [FRAC] target-site code), FRAC code, systemic activity, and toxicity of different active ingredients in the coformulant are listed in order.

[d] Imidacloprid and spirotetramat were used as positive controls for acropetal and bidirectional translocation assays, respectively. Imidacloprid has been confirmed to have a superior acropetal translocation efficiency in herbaceous and woody plants (Buchholz and Nauen 2002; Tattar et al. 1998; Turcotte et al. 2017). For spirotetramat, its basipetal translocation has been confirmed in cabbage (Brück et al. 2009), and it could be detected in the xylem and phloem of the leaves of *Allium* spp., rape, wheat, and citrus after dropping treatment (Vermeer and Baur 2008). "4A" and "23" are Insecticide Resistance Action Committee (IRAC) target-site codes.

Acropetal translocation assays were conducted by a root-tip immersion method (Fig. 1A). Five absorbing roots were separated from the soil at the bottom of the pot. Root tips were immersed in each of the test fungicide solutions (50 ml of 100 ppm a.i.), which was placed in a container underneath the original pot. After incubation for 7 and 21 days, the whole plant was sampled in consecutive 5-cm segments. Root samples treated with the test fungicide (R+) were washed continuously with tap water for 3 min to remove the fungicide residues from the root surfaces. Samples were placed in plastic bags, flattened with a hammer, and stored at −80°C before use (flattened and frozen tissues are amenable to homogenization). The experiment was performed in two independent trials; each trial contained six plants per treatment (three plants processed at each time point). The first and second trials were conducted in July and December 2020, respectively. For the first trial, all root, stem, and leaf segments were analyzed; for the second trial, only R+, R1 (the first 5-cm segment of the root), and S2 (the second 5-cm segment of the stem) were analyzed. Root concentration factor (RCF) was used to evaluate the efficiency of the test fungicide being absorbed by the roots of bishop wood seedling, where RCF = the concentration of the fungicide (ppm) measured in R+ (the root segment treated with the test fungicide)/the application concentration of the fungicide (100 ppm) (Li et al. 2018, 2022).

Bidirectional translocation assays were conducted by a stem-injection method (Fig. 1B). The stem was surface disinfested with 75% alcohol, and a shallow hole (1 mm in diameter, 3 to 4 mm deep) was made with a pushpin. For each test fungicide, 3 μl of the fungicide stock solution was injected into the stem hole with a pipette, and the opening was sealed with Parafilm. The concentrations were 100,000 ppm of spirotetramat, 400,000 ppm of mepronil, 88,000 ppm of cyproconazole, 75,000 ppm of epoxiconazole, 259,000 ppm of tebuconazole, 250,000 ppm of prochloraz, and 625,000 ppm of cyprodinil + fludioxonil. At 7 and 21 days posttreatment, the 5-cm segment containing the injection site (S + 0), the 5-cm segment of 7.5 to 12.5 cm above the injection site (S + 2), and the 5-cm segment of 7.5 to 12.5 cm below the injection point (S − 2) were collected. Samples were placed in plastic bags, flattened with a hammer, and stored at −80°C before use. The experiment was performed in two independent trials; each trial contained six plants per treatment (three plants processed at each time point). The first and second trials were conducted in October 2020 and January 2021, respectively.

The fungicide concentrations in the samples were determined by liquid chromatography (LC)- and GC-tandem mass spectrometry (MS/MS) (described below). Because "0" (not detected [ND]) was frequently contained in the datasets, the translocation assays did not conform to a normal distribution. For this reason, 0.01 was added to all data points and the data were logarithm transformed before statistical analysis. Transformed data from R1, S2, S + 2, and S − 2 were analyzed separately. ANOVA was performed to determine the blocking effect (trial) and the effect of fungicide on the fungicide concentration detected in the segment. ANOVA was also performed to determine the blocking effect (trial) and the effect of fungicide on RCF. Differences among treatments were analyzed by Tukey's honestly significant difference test at $P < 0.05$ using SAS Enterprise Guide 7.1 (SAS Institute Inc.).

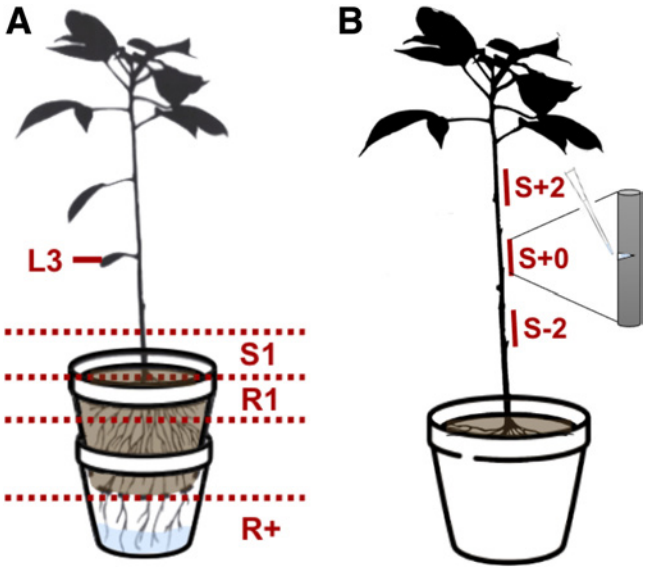

Fig. 1. Schematic diagram of the translocation assays. **A,** Acropetal translocation assay by the root-tip immersion method. Five absorbing roots were immersed in 50 ml of test fungicide solution at 100 ppm. **B,** Bidirectional translocation assay by the stem-injection method. A shallow hole was made by a pushpin, and 3 μl of the stock solution of the test fungicide was injected into the hole by a pipette. Shaded area indicates the test fungicide solution. R+: root segment treated with the test fungicide, R1: first 5-cm segment of the root, S1: first 5-cm segment of the stem, L3: leaf attached to the third 5-cm segment of the stem (S3), S + 0: 5-cm stem segment containing the fungicide injection site, S + 2: stem segment 7.5 to 12.5 cm above the injection site, and S − 2: stem segment 7.5- to 12.5 cm below the injection site.

LC- and GC-MS/MS

Each frozen sample was placed into a 50-ml, polycarbonate cryovial (SPEX), then homogenized with a stainless-steel grinding ball (12.7 mm in diameter; SPEX) at 1,500 rpm for 3 to 5 min using Geno/Grinder 2010 (SPEX). The FaPEx multipesticide residue extraction method was conducted following Chuang et al. (2019), with modifications. Each pulverized sample (1 g) was suspended in 5 ml of acetonitrile with 1% acetic acid and vigorously shaken on the shaker GETPP3000 (GETECH, Taiwan) at room temperature for 1 min. The suspension was filtered through FaPEx-chl (fast pesticide extraction kit, for chlorophyll samples; GETECH) and a 0.2-μm polytetrafluoroethylene (PTFE) filter membrane (GETECH) at a flow rate of 1 drop/s by using a constant-speed extractor (GETPP3200; GETECH). The filtered extract was directly used for LC- and GC-MS/MS in the first trial of the acropetal translocation experiment. For the purpose of instrument maintenance, an additional purification step was performed for the rest of the translocation assays. Our preliminary test showed that the purification step greatly reduced the color in the leaf and stem extracts (Supplementary Fig. S1) but would not significantly affect the results. The abovementioned filtered extract was added to a purification tube (225 mg of primary and secondary amine, 450 mg of anhydrous $MgSO_4$, 150 mg of C18 end-capped, and 25 mg of graphitized carbon black; GETECH), and vigorously shaken on the shaker GETPP3000 for 1 min. After

Table 3. Schedule of different treatments in the control assay

	dbi[b]			dpi[b]					
Treatments[a]	**21**	**14**	**7**	**0**	**7**	**14**	**21**	**28**	**Trial[c]**
Preventive treatment									
F-Pn	▲[b]	▲	▲	◎	△	△	△	☒	1, 2
Curative treatment									
Pn-F	△	△	△	◎	▲	▲	▲	☒	1, 2
Control									
Pn	△	△	△	◎	△	△	△	☒	1, 2
Gr	△	△	△	○	△	△	△	☒	1, 2
Bk	△	△	△	–	△	△	△	☒	1, 2
Fungicide analysis									
F-FA	▲	▲	▲	–	☒	–	–	–	1, 2
F-Gr-FA	▲	▲	▲	○	☒	–	–	–	2
F-Pn-FA	▲	▲	▲	◎	☒	–	–	–	2

[a] F = fungicide, Pn = *Phellinus noxius*, Gr = autoclaved grain, Bk = blank, and FA = fungicide analysis.

[b] Abbreviations and symbols: dbi = days before inoculation and dpi = days postinoculation; ▲ = drench application of 300 ml of the fungicide (10 ppm of cyproconazole and 50 ppm of the other test fungicides), △ = drench application of water, ◎ = inoculation with *P. noxius*-colonized grains, ○ = inoculation with autoclaved grains, and ☒ = sample collection.

[c] Treatments were applied in the first or second independent trials.

centrifuging at 3,000 × *g* for 2 min at 15°C, the supernatant was filtered through a 0.2-μm PTFE membrane (GETECH). The extracts were stored at −20°C until use.

All extracted samples were analyzed using LC-MS/MS or GC-MS/MS at the Pesticide Residue Analysis Center of National Chung Hsing University. For each analyte, the standard curve was established by mixing a serial dilution of each analytical standard at 200, 100, 50, 20, 10, and 4 ppb with a blank matrix extract. In the first trial of the acropetal translocation experiment, extracts from bishop wood stem tissues without any pesticide treatment were used as the blank matrix extract. For the remaining translocation experiments, this blank matrix extract was used at twofold dilution. When the concentration of the analyte was outside the range of the standard curve, the sample was diluted and reanalyzed.

The extracts from samples treated with mepronil, epoxiconazole, tebuconazole, cyprodinil + fludioxonil, prochloraz, imidacloprid, and spirotetramat-enol were analyzed by LC-MS/MS on the Agilent 1290 Infinity LC system coupled to an AB Sciex QTRAP 5500 system (AB Sciex Pte. Ltd., Taipei, Taiwan). For each sample, 2 μl was applied to the LC system on an analytical C18 reverse-phase column (Agilent Poroshell 120 EC-C18, 3 × 150 mm, 2.7-μm particle size; Agilent Technologies Inc., Germany) with a guard column (Agilent EC-C18, 3 × 5 mm, 2.7-μm particle size; Agilent Technologies). Separation was performed at 40°C using an oven with the mobile phase A consisting of 5 mM ammonium acetate (aqueous) and 0.1% (vol/vol) formic acid, and mobile phase B consisting of 5 mM ammonium acetate in methanol using the gradient listed in Supplementary Table S1. The analytes were ionized and detected by electrospray ionization in positive mode using multiple reaction monitoring with the following conditions: curtain gas flow at 15 psi (1.05 kg/cm^2), high nitrogen gas collision, ion spray with a voltage of 5,500 V, temperature at 350°C, and ion source gas at 55 psi (3.87 kg/cm^2). The data were acquired with the Analyst V software (AB Sciex). For ion source optimization for each analyte, two product ions were selected for quantification and qualification for identification purposes (Supplementary Table S2). Data were acquired in multiple reaction monitoring mode by the software Analyst V (AB Sciex).

Sample extracts treated with cyproconazole were analyzed by GC-MS/MS on the Agilent 7890A GC system with a programmed vaporization temperature injector, coupled to an Agilent 7000A triple quadrupole mass spectrometer (Agilent Technologies). The extracts (from FaPEx and purification tube) were blow dried with nitrogen gas, redissolved with 1 ml of acetone/hexane (1:1, vol/vol), and filtered through a 0.2-μm PTFE filter membrane (GETECH). For each sample, 1 μl was injected into the GC-MS/MS system in a splitless mode for analysis. Two Agilent DB-5 ms Ultra Inert GC columns (15 m by 0.25 mm by 0.25 μm) were coupled and preceded by a guard column (0.5 m by 0.25 mm by 0.25 μm). The flow for the first column was helium gas (99.99% purity) at 1.2 ml/min and the second column was 1.4 ml/min. The oven temperature for the GC column was programmed as follows:

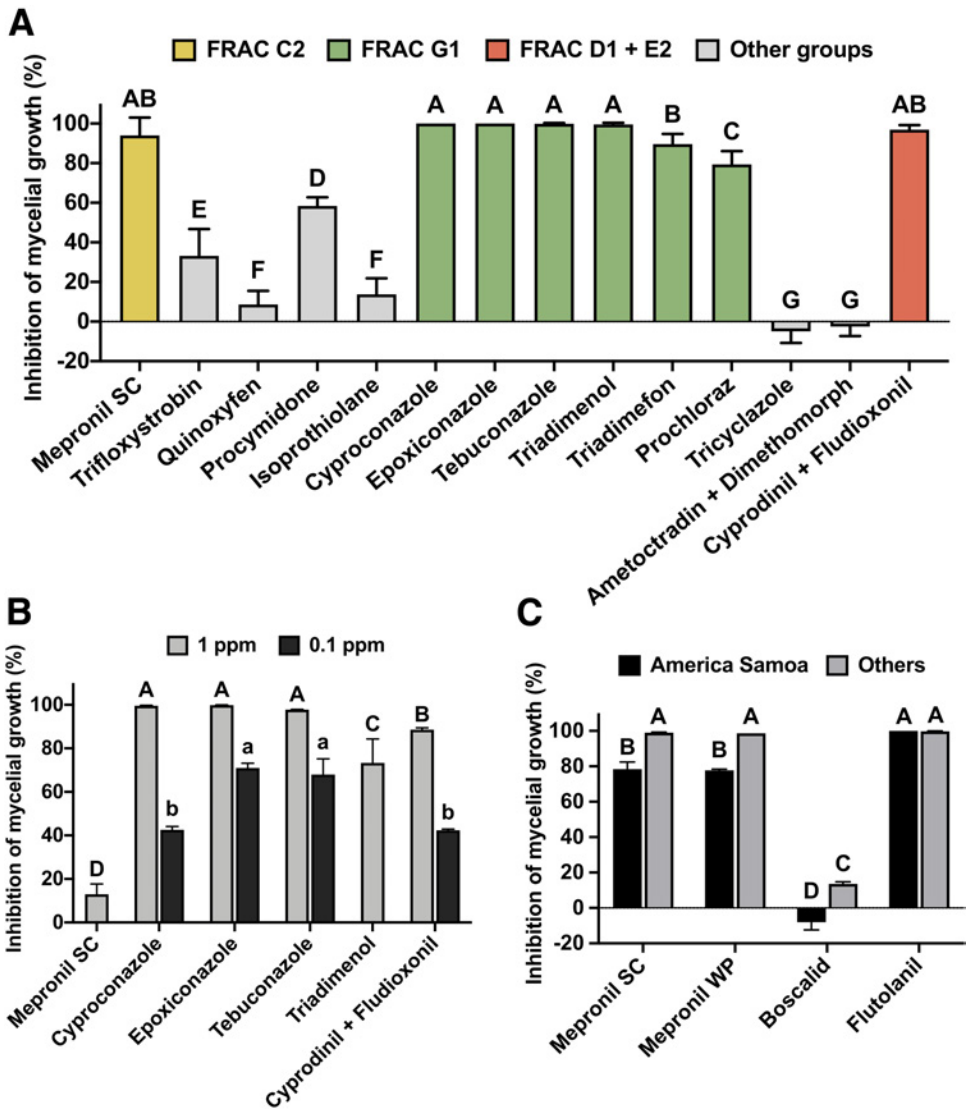

Fig. 2. Inhibitory effects of fungicides on the mycelial growth of *Phellinus noxius*. **A,** Inhibitory effects of 10-ppm fungicides on 39 *P. noxius* isolates. FRAC = Fungicide Resistance Action Committee. **B,** Inhibitory effects of 1- and 0.1-ppm fungicides on 12 representative *P. noxius* isolates. Data from 1 to 0.1 ppm were analyzed separately. **C,** Inhibitory effects of 10-ppm succinate dehydrogenase inhibitor fungicides on 15 *P. noxius* isolates. Two formulations of mepronil (SC = suspension concentrate and WP = wettable powder) were tested. Percent inhibition of mycelial growth (%) was calculated as [(the average colony diameter of the control − the average colony diameter of the treatment)/(the average colony diameter of the control − the diameter of the mycelium-agar disc)] × 100%. Data (mean ± standard error) with different letters are significantly different according to Tukey's honestly significant difference test at $P < 0.05$.

initial 60°C (for 1 min), increased to 170°C at 40°C min^{-1}, then ramped to 310°C at 10°C min^{-1}, and held for 2.25 min. Nitrogen (1.5 ml/min) was used as collision gas and ion source temperature was 300°C. The data were acquired using multiple reaction monitoring by the MassHunter GC/MS Acquisition software (Agilent Technologies). The mass transitions of cyproconazole are listed in Supplementary Table S2.

Control assays in the greenhouse

The control efficacies of mepronil, cyproconazole, epoxiconazole, tebuconazole, prochloraz, and cyprodinil + fludioxonil were tested using bishop wood seedlings in the greenhouse of Taiwan Agricultural Research Institute (natural light). The experiment was conducted in two independent trials, each containing six plants per treatment. The two trials were conducted in October to November 2020 and February to March 2021, respectively. The fungicides were applied by soil drenching once a week for 3 weeks before or after the inoculation of *P. noxius* (F-Pn = fungicide application followed by *P. noxius* inoculation and Pn-F = *P. noxius* inoculation followed by fungicide application). For use as the inoculum, *P. noxius* 2248 was cultured on wheat-oat grains (50 g of wheat, 50 g of oat, 50 ml of dH_2O, and 5 mg of chloramphenicol) at 30°C for 3 weeks (Chou et al. 2019). Plants inoculated with *P. noxius* (Pn), inoculated with sterile wheat-oat grains (Gr), and treated with water (Bk) were used as control (Table 3). According to our preliminary tests (soil drenching of 300 ml of fungicides at 10, 50, and 100 ppm, once a week for 3 weeks), 50 and 100 ppm of cyproconazole caused little and moderate levels of fascicled, distorted, and curled leaves, respectively; 100 ppm of epoxiconazole and tebuconazole caused slightly fascicled leaves. For these reasons, 300 ml of cyproconazole at 10 ppm and the other test fungicides at 50 ppm were used for each application; water was applied for the control treatments.

Inoculation was conducted following the methods of Ann et al. (1999), with modifications. The inoculum was wrapped around the S2 segment, which was wounded by gently scraping the epidermis around half of the circumference using the back side of a razor blade (Supplementary Fig. S2A). Our preliminary tests on plants with fixed-size wounds (5 by 0.5 cm^2) showed that the incidence of wilting was positively correlated with the stem diameter; therefore, the wounding treatments on different plants were normalized by considering the circumferences of their stems. To the wounded S2 segment, 8 g of *P. noxius*-colonized grains or autoclaved grains was applied with a plastic sheet, wrapped with Parafilm to retain moisture, then wrapped with aluminum foil to exclude light (Supplementary Fig. S2B to D).

Seedlings for each treatment (F-Pn, Pn-F, Pn, Gr, and Bk) (Table 3) were examined and photographed at 0, 7, 14, 21, and 28 days post-inoculation (dpi), and the results from the two trials were combined to determine the incidence of wilting [(the number of wilted plants/total number of plants) × 100%]. At 28 dpi, the inoculated 5-cm segment and its adjacent 5-cm segments above and below the inoculated segment were cut longitudinally and photographed. Image J (Abràmoff et al. 2004) was used to assess the total longitudinal-sectional area and the area of discoloration for each plant. The percentage of discoloration area of the stem was calculated as (the area of discoloration/total longitudinal-sectional area) × 100%. For each stem segment, one of

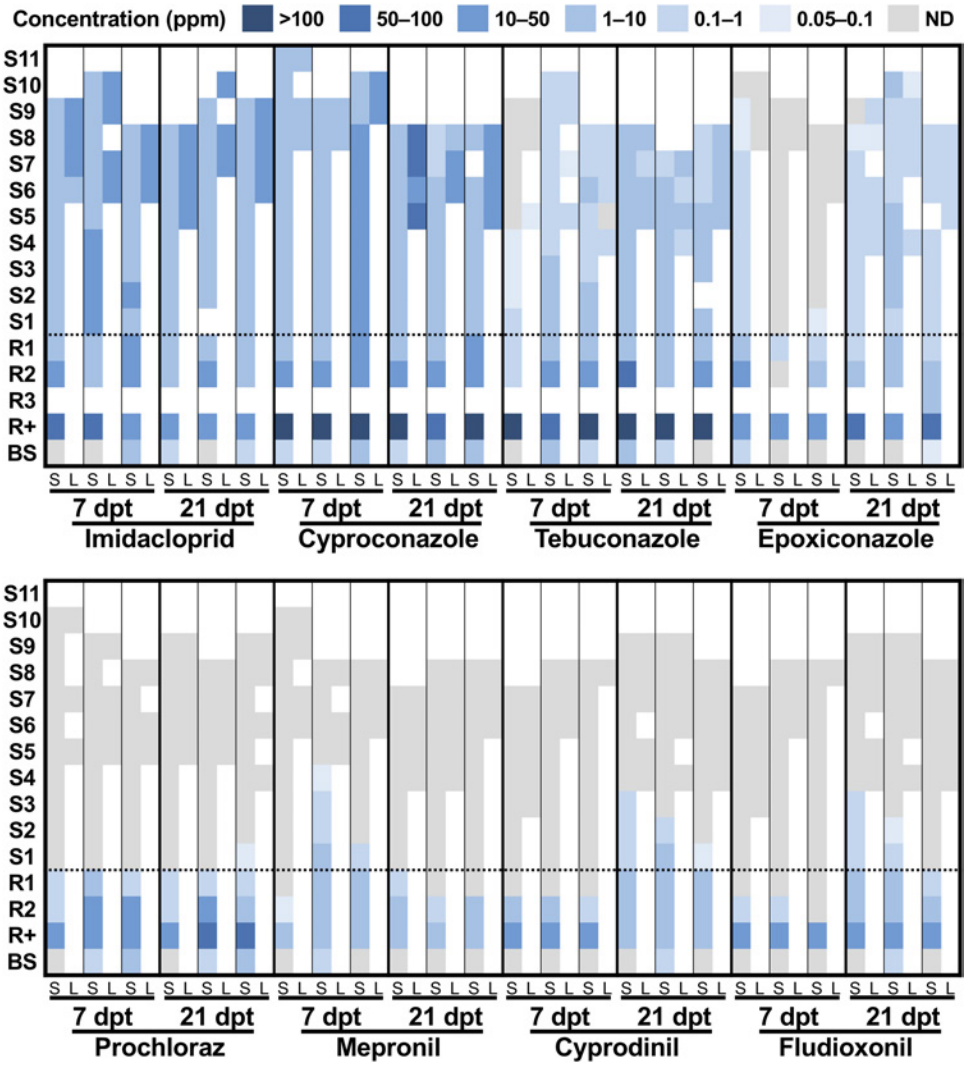

Fig. 3. Acropetal translocation and whole-plant distribution of fungicides detected by the root-tip immersion method. Each bishop wood seedling was treated with 50 ml of 100-ppm fungicide. Imidacloprid was used as the control. Concentrations of fungicides in consecutive 5-cm segments were detected. R, S, L, and BS represent the roots, stems, leaves, and bulk soil, respectively. R+: root segment treated with the test fungicide, R#: segment number of the root, S#: segment number of the stem, dpt = days posttreatment, and ND = not detected.

the longitudinal halved parts was used for reisolation of *P. noxius*. For the reisolation of *P. noxius*, each halved part was surface disinfested by immersing one time in 75% EtOH for 30 s, 1% sodium hypochlorite for 30 s, and three times in sterile dH2O for 30 s. The surface-disinfested segment was longitudinally halved again, cut into 1-cm segments, then transferred to a modified *P. noxius*-selective medium, MA + 4 (Chang 1995; Wu et al. 2020). After incubation in the dark at 30°C for 4 days, the isolation frequencies of *P. noxius* were determined as (the number of segments from which *P. noxius* colonies were isolated/total 30 segments per plant) × 100%. ANOVA was conducted to determine the blocking effect (trial) and the effect of treatment on the percentage of discoloration area and the isolation frequencies of *P. noxius*. Differences between each treatment and the Pn group were analyzed by a two-tailed Student's *t* test at $P < 0.05$ using SAS Enterprise Guide 7.1 (SAS Institute Inc.).

To examine whether the fungicides could be translocated to the inoculation position in the control assay, an additional set of bishop seedlings was used for fungicide analysis (Table 3). The experiment was conducted in two independent trials, together with the two trials in the control assay. Each trial contained four to six plants per treatment. In both trials, all of the test fungicides were applied by soil drenching once a week for 3 weeks, and the S2 segments were subjected to fungicide analysis (F-FA). In second trial, plants treated with cyproconazole and tebuconazole before mock inoculation with autoclaved grains (F-Gr-FA) or inoculation with *P. noxius*-colonized grains (F-Pn-FA) were also analyzed. The extraction was conducted by FaPEx and a purification tube, and the extracts of four plants per treatment were pooled and analyzed by LC- and GC-MS/MS, as described above.

Results

Fungicide sensitivity assays

The inhibitory effects of 14 fungicides (10 ppm) with different MoAs on the mycelial growth of 39 *P. noxius* isolates are shown in Figure 2A and Supplementary Fig. S3. *P. noxius* showed the highest sensitivity to DMIs, especially cyproconazole, epoxiconazole, tebuconazole, and triadimenol, with average inhibition rates of 100, 100, 99.9, and 99.5%, respectively. Slightly lower inhibition rates were observed from cyprodinil + fludioxonil (96.9%) and mepronil (94.0%). Two DMIs, triadimefon and prochloraz, showed significantly lower inhibition rates of 89.6 and 79.4%, respectively. Less than 60% of inhibition rates were observed from the other remaining fungicides, which included procymidone (58.4%), trifloxystrobin (33.2%), isoprothiolane (13.7%), quinoxyfen (8.6%), ametoctradin + dimethomorph (–2.5%), and tricyclazole (–4.9%).

The fungicides with higher inhibitory effects (>90% inhibition rate) were tested at 1 and 0.1 ppm on 12 representative *P. noxius*

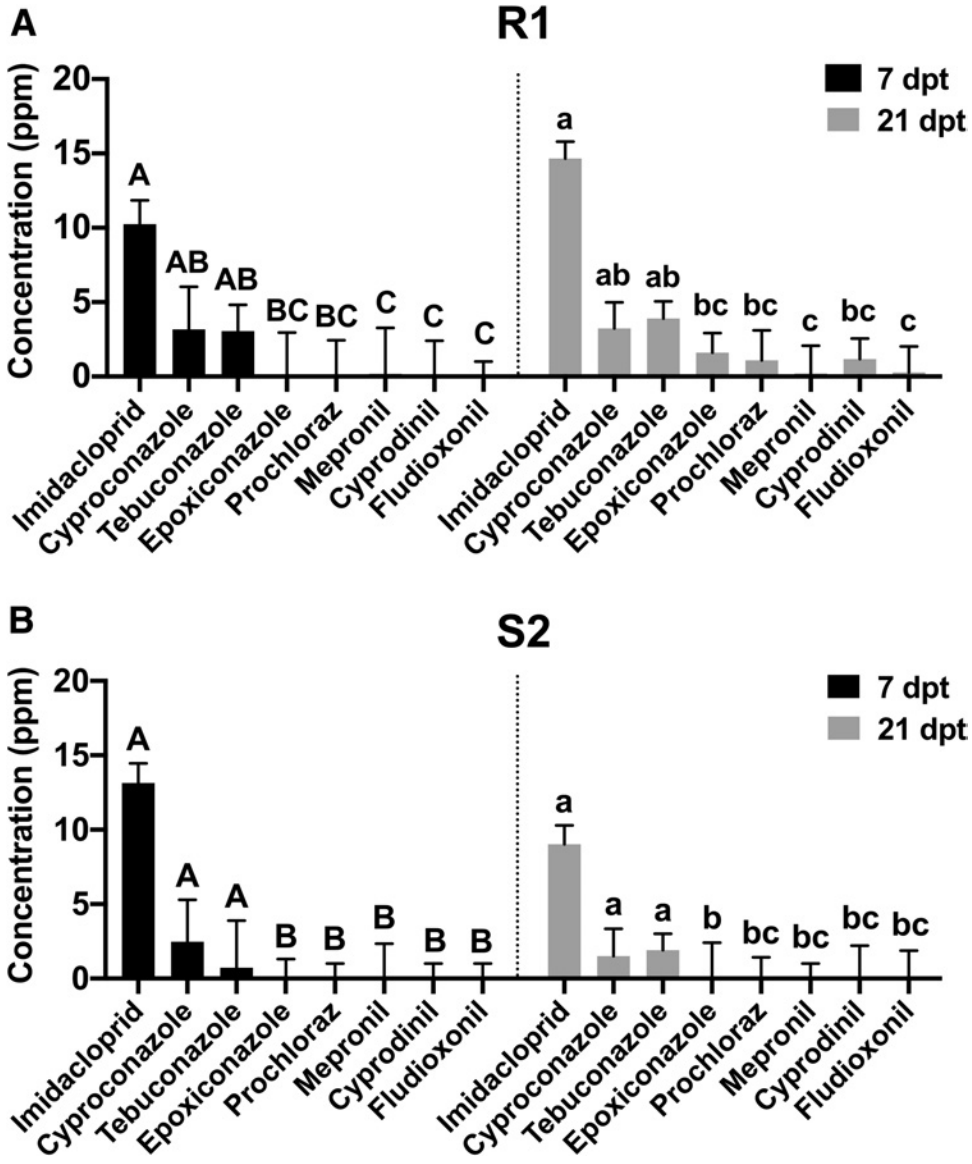

Fig. 4. Concentrations of fungicides in the root and stem segments detected by the root-tip immersion method. **A,** Fungicides in the first 5-cm segment of the root (R1) and **B,** fungicides in the second 5-cm segment of the stem (S2). Each bishop wood seedling was treated with 50 ml of 100-ppm fungicide. Imidacloprid was used as the control; dpt = days posttreatment. Data (mean ± standard error [SE]) with different letters are significantly different according to Tukey's honestly significant difference test at $P < 0.05$. Data were logarithmic transformed [log (concentration + 0.01)] for statistical analysis, and the corresponding means and SE were back transformed to original scale before plotting. Intervals of SE are different from significance levels due to asymmetry resulting from back transformation.

isolates (Fig. 2B). At 1 ppm, the ranking of inhibition rates of six tested fungicides was as follows: epoxiconazole (99.8%), cyproconazole (99.6%), and tebuconazole (97.7%) > cyprodinil + fludioxonil (88.6%) > triadimenol (73.3%) > mepronil (13.0%). At 0.1 ppm, the ranking of inhibition rates of four tested fungicides was as follows: epoxiconazole (71.0%) and tebuconazole (68.0%) > cyproconazole (42.6%), and cyprodinil + fludioxonil (42.3%). None of the tested fungicides showed a strong inhibitory effect at 0.1 ppm.

For individual fungicides, except for epoxiconazole and cyproconazole (100% inhibition at 10 ppm for all 39 isolates), the inhibition of mycelial growth was significantly associated with geographical region. In particular, the inhibitory effect of mepronil at 10 ppm on *P. noxius* isolates from American Samoa (67.4%) was significantly lower than the isolates from other regions (91.1 to 100%) (Supplementary Table S3). For the other remaining fungicides, significant differences of inhibition rates were observed among different regions but these differences were relatively minor (<15% difference of inhibition) or the fungicides were not effective (<50% of inhibition) (Supplementary Table S3). The sensitivity of *P. noxius* to different SDHIs is shown in Figure 2C. Compared with the isolates from other regions, American Samoa isolates showed significantly lower sensitivity to two different formulations of mepronil (SC: 78.4% and WP: 77.7%) at 10 ppm. Isolates of *P. noxius* from American Samoa and other regions showed similarly high sensitivity to flutolanil (99.8 to 100.0% inhibition) and low sensitivity to boscalid (−8.0 to 13.6% inhibition).

Translocation assays

In the acropetal translocation assay, the distributions of tested fungicides across the whole seedling are shown in Figure 3, and the results of fungicide translocation to the S2, R1, and R+ segments are contained in Supplementary Fig. S4. In the whole-seedling analysis (Fig. 3), the uptake of imidacloprid (positive control) and cyproconazole was rapid, with distributions across the whole plant as early as 7 days posttreatment (dpt). Cyproconazole was found to accumulate in leaves at 10 to 100 ppm by 21 dpt. Tebuconazole and epoxiconazole were found to be distributed across the whole plant at 21 dpt, with the accumulative concentrations of epoxiconazole lower than tebuconazole. Limited acropetal translocation was observed with prochloraz, mepronil, cyprodinil, and fludioxonil, which were either not detected in the aboveground part or only detected at trace amounts in the basal stem either at 7 or 21 dpt. According to the quantities of fungicides detected in R1 and S2 segments in both trials (Fig. 4), cyproconazole and tebuconazole showed significantly higher acropetal translocation efficiencies than the other tested fungicides. In addition, tebuconazole and prochloraz were most efficiently absorbed by the root systems of bishop wood seedlings, with RCF values of 6.6 and 5.4 at 7 dpt, respectively (Fig. 5). Epoxiconazole, mepronil, cyprodinil, and fludioxonil showed poor absorption efficiencies by roots, with RCF values of 1.6, 1.0, 0.8, and 0.4, respectively.

In the bidirectional translocation assays, fungicides detected in S + 0, S + 2, and S − 2 are shown in Figures 6 and 7. Similar translocation patterns were observed from two independent trials. Whereas spirotetramat-enol (the major metabolite of spirotetramat in plants; positive control) showed both basipetal and acropetal translocation patterns, consistent basipetal translocation was not evident for any of the tested fungicides. In S + 2 at 7 dpt, tebuconazole, cyproconazole, and epoxiconazole could be detected with average concentrations of 2.85, 1.04, and 1.19 ppm, respectively; cyprodinil was detected only in the first trial (average 3.28 ppm); prochloraz was stably detected only in the second trial (average 0.75 ppm); a trace amount of fludioxonil was detected only in the first trial (average 0.14 ppm); and mepronil was not detected in either trial. In S + 2 at 21 dpt, increased concentrations of epoxiconazole and cyprodinil were detected, and the concentrations of tebuconazole, epoxiconazole, and cyprodinil were significantly higher than other fungicides (Fig. 7A). The lowest acropetal translocation efficiency was observed with mepronil and fludioxonil, which were not stably detected even at 21 dpt.

Control assay

To evaluate whether the fungicides can translocate to nontreated tissues and inhibit *P. noxius*, the control efficacies of soil-drenching treatments of different fungicides were tested on bishop wood seedlings inoculated with *P. noxius* on the wounded S2 segment (Fig. 8). Plants without treatment (Bk) and plants inoculated with autoclaved grains (Gr) remained healthy throughout the experiment (0% wilting, 0% discoloration area of the stem, and 0% isolation of *P. noxius*). Plants inoculated with *P. noxius* started to decline at 14 to 21 dpi. Leaves first turned droopy and brownish, then totally wilted. At 28 dpi, plants in the inoculated Pn group were all wilted, with average 75.8% of discoloration area of the stem and 77.3% of the isolation frequency of *P. noxius*. Compared with the Pn group (no fungicide treatment), lower incidences of wilting were observed on

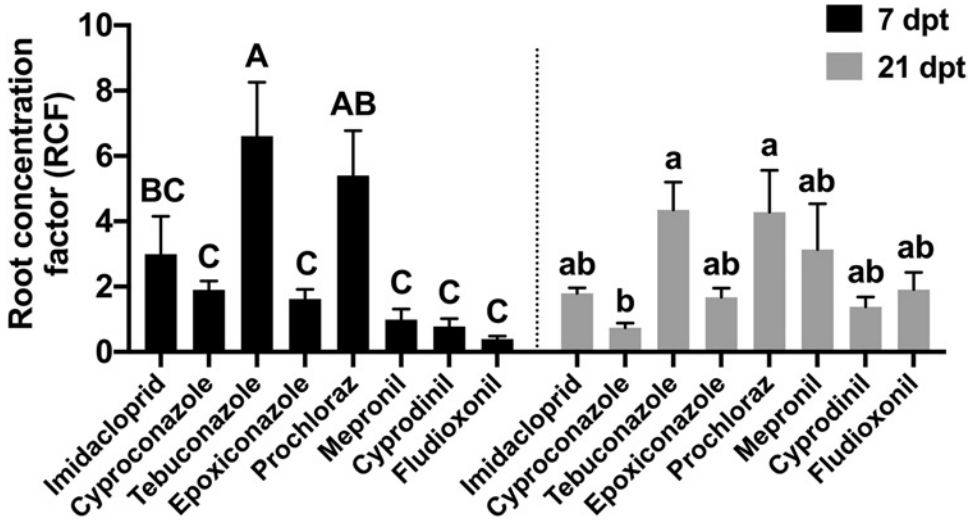

Fig. 5. Root concentration factor (RCF) determined by the root-tip immersion method. Each bishop wood seedling was treated with 50 ml of 100-ppm fungicide. Imidacloprid was used as the control. RCF was calculated as the concentration of the fungicide (ppm) measured in R+ (the root segment treated with the test fungicide)/the application concentration of the fungicide (100 ppm). Data (mean ± standard error) with different letters are significantly different according to Tukey's honestly significant difference test at $P < 0.05$; dpt = days posttreatment.

preinoculation and postinoculation treatments with cyproconazole (F-Pn: 83.4% and Pn-F: 91.7%) and epoxiconazole (F-Pn: 90.0% and Pn-F: 87.5%) (Fig. 8A). Preventive treatment of cyproconazole resulted in significantly lower percentage of discoloration area (average 59.2%) (Fig. 8B). Preventive treatments of tebuconazole and epoxiconazole showed significantly lower isolation frequencies of *P. noxius* (both averaged 61.4%) (Fig. 8C). For mepronil, prochloraz, and cyprodinil + fludioxonil, averages of 71.3 to 78.6% discoloration area (Fig. 8B) and averages of 71.7 to 82.2% isolation frequency of *P. noxius* (Fig. 8C) were observed.

The amounts of the fungicides translocated into S2 in the control assay are shown in Supplementary Table S4. In the F-FA (fungicide soil drench) group, the ranking of fungicides based on their average concentrations in S2 was tebuconazole (0.73 ppm) > epoxiconazole (0.43 ppm) > cyproconazole (0.09 ppm) > fludioxonil (ND in the first trial and 0.34 ppm in the second trial) > cyprodinil (ND in the first trial and 0.33 ppm in the second trial) > mepronil (ND) = prochloraz (ND). In the F-Gr-FA (mock inoculated and fungicide soil drench) and F-Pn-FA (inoculated and fungicide soil drench) groups, the concentrations of tebuconazole were 2.59 and 1.41 ppm, respectively, which were higher than the respective concentrations, 0.14 and 0.06 ppm, of cyproconazole in the same treatments.

Discussion

BRRD is a serious threat to most broadleaved and coniferous trees in many tropical and subtropical areas (CABI 2022). A few DMIs have been used to treat BRRD-affected trees by soil drenching, soil mixing, or root or basal stem injection (Sun et al. 2020; Tsai et al. 2005, 2008). However, information on the effectiveness of fungicides with different MoAs and their translocation efficacy in woody plants has been limited. In this study, fungicides were first screened for effectiveness against *P. noxius* using conventional in vitro assays on fungicide-containing PDA. The spatiotemporal distributions of

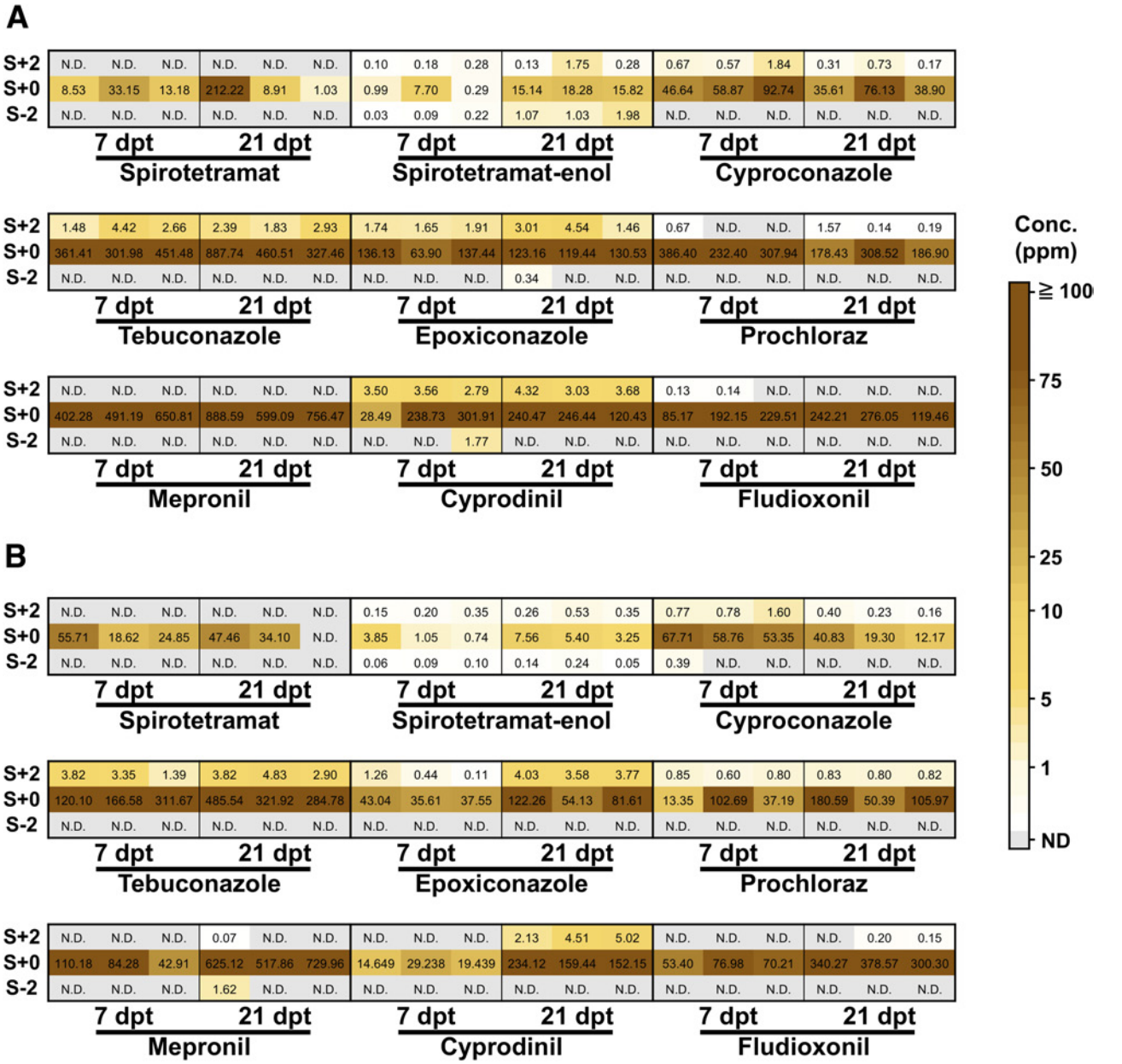

Fig. 6. Bidirectional translocation of fungicides detected by the stem-injection method. **A,** Results from first trial and **B,** results from second trial. Each bishop wood seedling was injected with 3 μl of the fungicide stock solution on the stem. Spirotetramat was used as the control (spirotetramat-enol is the major metabolite of spirotetramat in plants). S + 0: 5-cm stem segment containing the fungicide injection site, S + 2: stem segment 7.5 to 12.5 cm above the injection site, S – 2: 5-cm stem segment 7.5 to 12.5 cm below the injection site, dpt = days posttreatment, and ND = not detected.

fungicides in a host plant were investigated using newly developed root-tip immersion and stem-injection systems in bishop wood seedlings, and subsequently sampling different segments of the whole plant at different time points. Compared with soil drenching (treatment applied to the whole root system), our method of treating specific roots from the root tips allowed further differentiation of fungicide movement to the upper part of roots. As a simplified system to simulate trunk injection, trace amounts of fungicide stock solution were injected into the stems of bishop wood seedlings, and acropetal and basipetal translocation fungicides were evaluated by the highly sensitive LC- and GC-MS/MS methods.

For our screening to identify fungicides effective against *P. noxius*, 39 isolates from diverse geographic regions were tested for their sensitivity to 14 fungicides (11 MoAs) at 10 ppm. For fungicides showing high inhibitory effects, six fungicides were further evaluated for effectiveness at 1 ppm, and four fungicides were evaluated at 0.1 ppm, using 12 representative *P. noxius* isolates from seven geographic regions. Similar to previous observations with five isolates from Taiwan and one isolate from Malaysia (Li 2016; Lim et al. 1990; Tsai et al. 2005), DMIs were highly effective against *P. noxius* isolates from different geographic regions. It is worth noting that, because *P. noxius* uses hyphae to extend within a diseased tree and spread to the neighboring trees, only the inhibitory effects on mycelial growth were tested in our and other related studies. DMIs function by inhibiting the biosynthesis of ergosterol, which is required for the formation of cell membranes during hyphal growth (Mueller et al. 2017). SDHIs and quinone outside inhibitors (QoIs), which inhibit mitochondrial respiration by acting on different target sites, usually have better inhibitory effects on spore germination than on mycelial growth (Balba 2007; Mueller et al. 2017). This could be the reason that SDHIs and QoIs didn't perform as well as DMIs.

Among the six DMIs tested at 1 ppm, cyproconazole, epoxiconazole, and tebuconazole showed the highest inhibitory effects (97.7 to 99.8%). Prochloraz, which is the only officially recommended fungicide for BRRD control (since 2011) in Taiwan, was the least effective (79.4%) among the tested DMIs at 10 ppm. Notably, cyprodinil + fludioxonil and mepronil were more effective (94.0 to 96.9% at 10 ppm) than the two DMIs triadimefon and prochloraz

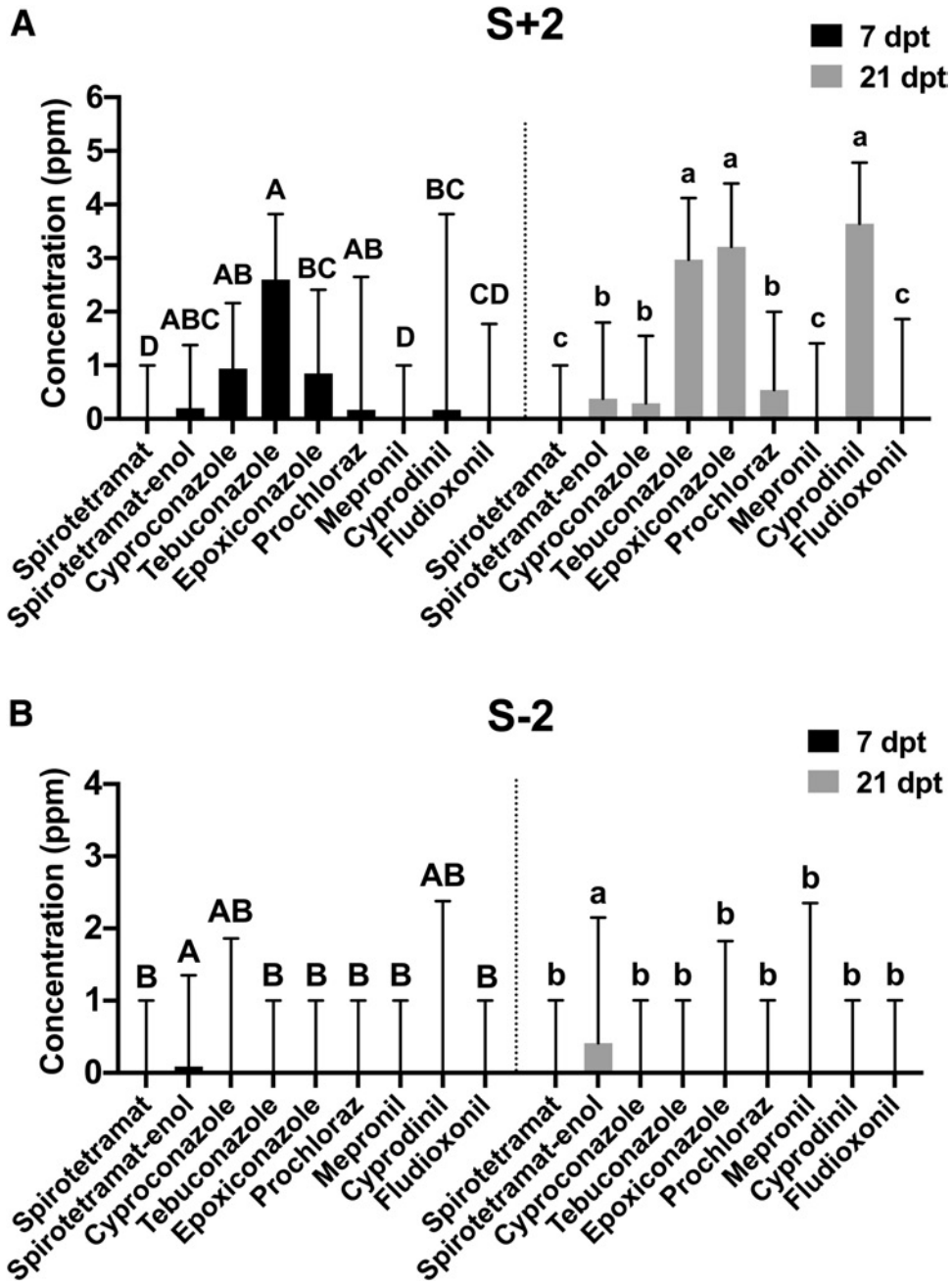

Fig. 7. Fungicides in the stem segments detected by the stem-injection method. **A,** The 5-cm stem segment 7.5 to 12.5 cm above the injection site (S + 2) and **B,** the 5-cm stem segment 7.5 to 12.5 cm below the injection site (S – 2); dpt = days posttreatment. Each bishop wood seedling was injected with 3 µl of the fungicide stock solution on the stem. Spirotetramat was used as the control (spirotetramat-enol is the major metabolite of spirotetramat in plants). Data (mean ± standard error [SE]) with different letters are significantly different according to Tukey's honestly significant difference test at $P < 0.05$. Data were logarithmic transformed [log (concentration + 0.01)] for statistical analysis, and the corresponding means and SE were back transformed to original scale before plotting. Intervals of SE are different from significance levels due to asymmetry resulting from back transformation.

(79.4 to 89.6% at 10 ppm). However, while cyprodinil + fludioxonil remained effective (83.9 to 94.4%) at 1 ppm, the inhibitory rate of mepronil was greatly reduced to 0 to 28.3% at 1 ppm. Although mepronil did not show the greatest inhibitory effect on *P. noxius*, it could likely be used in combination with *T. asperellum* TA, a mycoparasite of *P. noxius*, because 100 ppm of mepronil was previously shown to produce no inhibition of *T. asperellum* TA mycelial growth (Chou 2018).

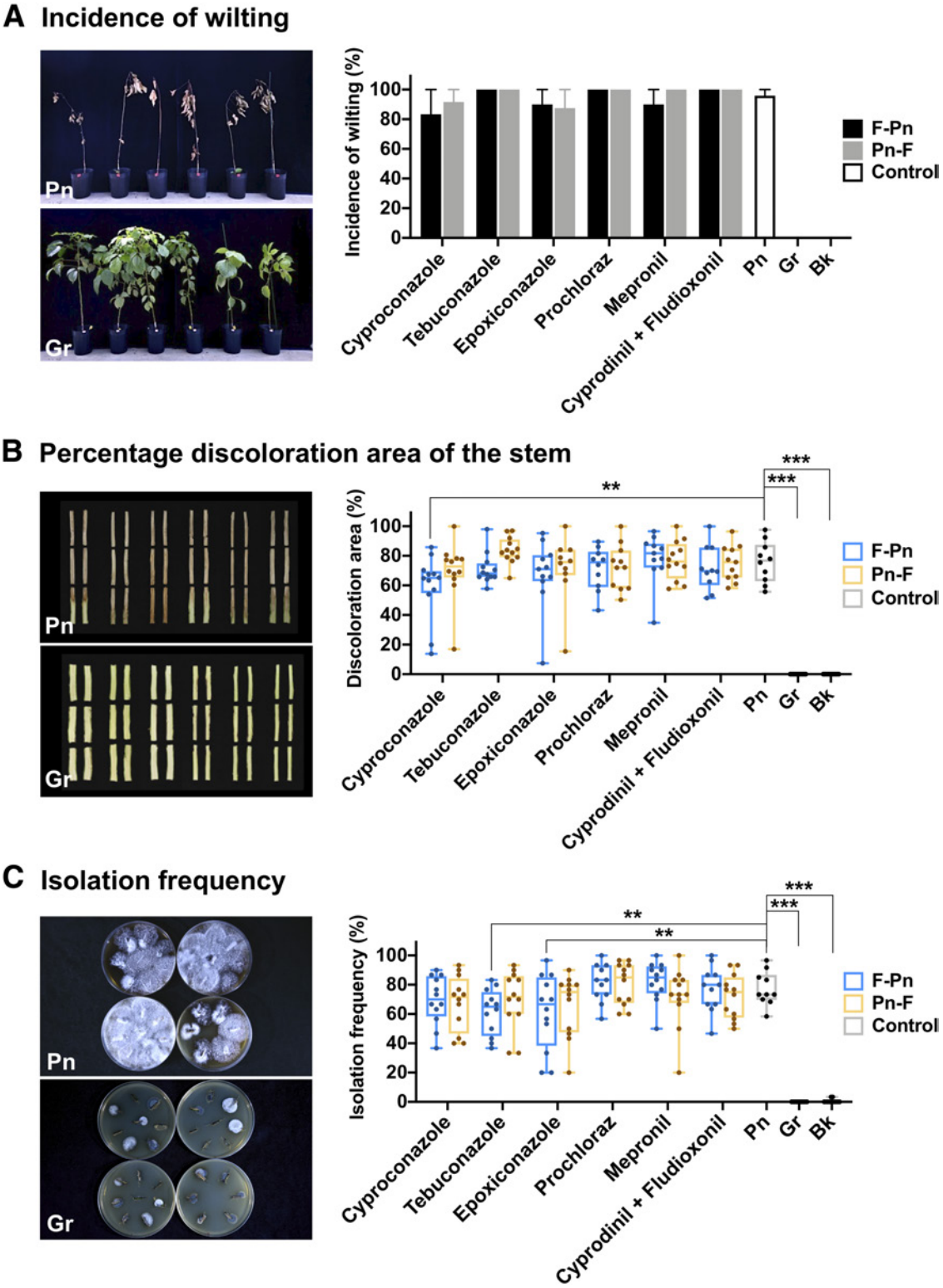

Fig. 8. Results of the control assay. Acropetal translocation activities and control efficacies of the fungicides were evaluated by soil drenching of bishop wood seedlings artificially inoculated with *Phellinus noxius* on the stem. **A,** Incidence of wilting at 28 days postinoculation (28 dpi), which was calculated as (the number of wilted plants/total number of plants) × 100%; **B,** percentage discoloration area of the stem at 28 dpi, which was calculated as (the area of discoloration/total longitudinal-sectional area) × 100%; **C,** isolation frequency of *P. noxius*, which was calculated as (the number of segments from which *P. noxius* colonies were isolated/total 30 segments per plant) × 100%. Photos on the left are the results from positive (*P. noxius*-inoculated [Pn]) and negative (mock-inoculated [Gr]) controls. Gr: inoculation with autoclaved grains; Pn: inoculation with *P. noxius*-colonized grains; F-Pn: plants were treated with the fungicide once per week for 3 weeks, then inoculated with *P. noxius*-colonized grains; Pn-F: plants were inoculated with *P. noxius*-colonized grains, then treated with the fungicide once per week for 3 weeks; and Bk: treated with water (blank). Differences between each treatment and Pn were analyzed by two-tailed Student's *t* test. Asterisks *, **, and *** indicate $P < 0.01$, 0.05, and 0.001, respectively.

Isolates of *P. noxius* from American Samoa showed reduced sensitivity to mepronil. Interestingly, this phenomenon was not observed with the other two SDHIs tested (flutolanil and boscalid). SDHIs can compete with ubiquinone (Q10) for the ubiquinone-binding site (Qp site) in succinate dehydrogenase in fungal mitochondria. Succinate dehydrogenase is composed of four subunits (A, B, C, and D), and point mutations in the subunits B, C, and D can result in fungicide resistance. In different fungal species, the mutations associated with fungicide resistance were quite variable (Sierotzki and Scalliet 2013). To understand the potential cause of mepronil resistance in *P. noxius*, a preliminary analysis was conducted using our unpublished whole-genome sequencing data of the 34 *P. noxius* isolates. Alignment of the sequences encoding succinate dehydrogenase subunits B, C, and D showed that American Samoa isolates possess three nonsynonymous point mutations in the subunit C sequences (A32V, S85A, and V173I) (Supplementary Fig. S5). Mepronil and flutolanil, both phenylbenzamides, were found to bind with 17β-hydroxysteroid dehydrogenase by forming hydrogen bonds at different amino acid positions (Mol et al. 2019). In *P. noxius*, whether the changes of three amino acids in subunit C altered succinite dehydrogenase binding with mepronil (but not flutolanil), or mutations in other metabolic pathways caused the reduction in sensitivity to mepronil, warrants further exploration.

The root absorption and acropetal or basipetal translocation efficiencies of mepronil, cyproconazole, tebuconazole, epoxiconazole, prochloraz, and cyprodinil + fludioxonil in bishop wood seedlings can be compared in Table 4. According to the Pesticide Manual (https://www.bcpc.org), mepronil, cyproconazole, tebuconazole, and epoxiconazole are considered systemic fungicides, and prochloraz and fludioxonil are considered nonsystemic fungicides. Our investigations revealed that cyproconazole and tebuconazole were efficiently absorbed by roots, and effectively transported upward to the stem and leaves. In particular, cyproconazole accumulation was detected at 21 dpt in the leaves (Fig. 3). Epoxiconazole and cyprodinil showed weak root absorption but moderate acropetal translocation efficiency. Prochloraz could be effectively taken up by roots but its acropetal movement was very limited. The lowest levels of acropetal translocation were observed with mepronil and fludioxonil, which could only be detected from the treated segment in the stem-injection system. Importantly, basipetal transport was not detected in any of the tested fungicides.

With the exception of mepronil, our results were generally consistent with previous observations on tebuconazole, epoxiconazole, prochloraz, and fludioxonil from rice, wheat, or lettuce (Li et al. 2022; Šudoma et al. 2019; Yumita et al. 1981); however, related studies on cyproconazole and cyprodinil are apparently lacking in the literature. Higher acropetal translocation efficacy was previously observed in tebuconazole compared with epoxiconazole, when used as a treatment on roots of hydroponically grown rice seedlings (Li et al. 2022) and premixed in soils used to cultivate lettuce seedlings (Šudoma et al. 2019). Prochloraz mixed in the soil could not be detected in the leaves of lettuce, indicating that the acropetal translocation of prochloraz was rather limited (Šudoma et al. 2019). No acropetal translocation was found for fludioxonil in wheat leaves treated with drops of fungicides (Klittich et al. 2008). With radiolabeled mepronil, acropetal but not basipetal translocation was observed from three- to four-leaf-stage rice seedlings treated by root immersion, stem injection, and leaf painting. The radiolabel was detected throughout the plant at 5 days after root treatment (Yumita et al. 1981). Although mepronil has long been considered a systemic fungicide with protective and curative properties (Pesticide Manual; https://www.bcpc.org), it exhibited very limited acropetal translocation efficacy (similar to the nonsystemic fungicides prochloraz and fludioxonil) in bishop wood seedlings. The translocation of mepronil in different plant species merits further investigation.

Cyproconazole, tebuconazole, and epoxiconazole, the fungicides with higher translocation efficiencies, showed a slightly higher potential than mepronil, prochloraz, and cyprodinil + fludioxonil in controlling *P. noxius* in our greenhouse assay. Preventive treatments of cyproconazole, tebuconazole, and epoxiconazole slightly reduced the incidence of wilting, the percentage of discoloration area in the stem, or the isolation frequency of *P. noxius*. However, these three fungicides were ineffective as a curative treatment and their efficacies were quite limited as a preventive treatment. Compared with the S2 data in the translocation assays (Fig. 4B), lower concentrations of cyproconazole, tebuconazole, and epoxiconazole were detected in the control assay (Supplementary Table S4). This finding could be due to higher evapotranspiration rates caused by more leaves on the seedlings and higher temperatures in the greenhouse of the Agricultural Research Institute during the experimental time. Although the overall concentration was low, the accumulation of tebuconazole in S2 was still detected at 0.72 to 2.59 ppm, which could be sufficient to effectively inhibit *P. noxius* (tebuconazole at 1 ppm showed 100% inhibitory effect on the mycelial growth of *P. noxius*). Inoculation by wrapping *P. noxius*-colonized grains on the stem wounded by half-circumference may provide an inoculation potential that is too high for the seedlings. If this is the case, fungicides may fail to effectively prevent the infection and colonization of *P. noxius* even if the fungicides did translocate to the inoculation site. The results also suggest that fungicide treatment may be ineffective for managing severe BRRD cases.

Although more tests will be needed to further determine the efficacies of preventive and therapeutic treatments of fungicides on BRRD-affected trees under different levels of inoculum potential and natural infection, this research provides an important basis for the application of fungicides for tree diseases. For BRRD control, our studies showed that tebuconazole produced the highest inhibitory effect on mycelial growth, could easily be absorbed by roots, remained at high concentration in roots, and was efficiently translocated to nontreated tissues. Epoxiconazole, which showed the same high inhibitory effect but relatively lower RCF and systemic activity, is another fungicide that is a strong candidate to consider for BRRD control. To reduce the risk of resistance development for DMIs, cyprodinil + fludioxonil, which showed lower root absorption and translocation efficiencies but high inhibitory effect, can be considered for use in combinations or rotations. Considering the lack of basipetal translocation property for all of the fungicides tested in this study, soil drenching or soil injection should be more effective than trunk injection for the application of such fungicides. Soil-drenching applications are typically performed in a manner that covers the root zone (1 to 1.5 times the distance of the dripline from the stem [tree canopy]) to achieve better control efficacy, and the health of trees should be monitored to avoid phytotoxicity. Because cyproconazole showed the best acropetal systemic activity, rapid absorption by roots, and high accumulation concentration in the canopy, it seems to also have a high potential for controlling foliar diseases. The control effects of cyproconazole on foliar diseases of trees such as anthracnose, rust, and needle cast merit further examination.

Table 4. The uptake and translocation of tested pesticides in bishop wood seedlings in this study[a]

	Root-tip immersion		Stem-injection	
Pesticide[b]	**RCF**	**Acropetal**	**Acropetal**	**Basipetal**
Imidacloprid	+++	++++	NT	NT
Spirotetramat-enol	NT	NT	++	+
Mepronil	++	+	+	–
Cyproconazole	++	++++	++	–
Tebuconazole	++++	+++	++++	–
Epoxiconazole	+	++	+++	–
Prochloraz	++++	+	++	–
Cyprodinil	+	+	+++	–
Fludioxonil	+	+	+	–

[a] RCF = root concentration factor, – = no mobility, + = little mobility, ++ = slight mobility, +++ = moderate mobility, ++++ = high mobility, and NT = not tested.

[b] Imidacloprid and spirotetramat-enol were used as the positive control in the root-tip immersion and stem-injection assays, respectively.

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