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ABSTRACT

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Single and mixed infections by *Pyrenophora teres* and two isolates of barley yellow dwarf virus (BYDV) were evaluated for their effects on the resistance to freezing stress of crowns of the winter barley cultivar Pennrad. Plants received one of several treatments: *P. teres*; either the RMV-NY or PAV-NY isolate of BYDV; RMV+ *P. teres*; PAV+ *P. teres*; or infestation with either nonviruliferous *Rhopalosiphum maidis* or *R. padi*. After the treatments, foliage and roots were harvested from 4-wk-old plants to evaluate the effects of infection(s) on top and root growth before freezing. The crowns were subjected to a controlled freezing regime, and resistance to freezing stress was evaluated by assessing retardation of shoot and root regrowth after freezing (crown injury). The treatment combinations resulted in decreased plant growth before freezing and in increased crown injury, relative to control plants. Infection by *P. teres* did not reduce top and root growth, or lead to crown injury, to the extent of the other treatments. Feeding by viruliferous aphid species, in comparison to feeding by nonviruliferous aphid species, led to decreased top and root growth and to further crown injury. Infections by PAV+ *P. teres* or RMV+ *P. teres* did not reduce top and root growth, but led to increased crown injury relative to plants exposed only to viruliferous aphids.

Additional key words: *Drechslera teres*, freezing stress, *Helminthosporium teres, Hordeum vulgare,* net blotch.

Winter hardiness is an important consideration in the production of fall-sown barley in much of Pennsylvania. One aspect of winter hardiness is the capacity of plants to survive freezing stress (5,13). In the case of fall-sown cereals, crown tissue must survive if the plant is to survive (8).

Infections of barley by barley yellow dwarf virus (BYDV) decrease resistance to freezing injury (2,3,9). Winter barley in Pennsylvania may be infected in the fall by two isolates of BYDV, characterized as RMV-NY and PAV-NY (F. E. Gildow, *personal communication*).

Barley seedlings also may be infected in early fall by *Pyrenophora teres* Drechs. (anamorph = *Drechslera teres* (Sacc.) Shoem. syn. *Helminthosporium teres* Sacc.), the causal agent of net blotch. Seed-transmitted mycelium, and ascospores and conidia produced on infested debris are sources of inoculum (4,7). The effect of *P. teres* on the resistance of winter barley to freezing injury has not been established.

Because both BYDV and *P. teres* commonly infect barley during the fall, the interaction between these pathogens and the subsequent effect on winter survival should be considered. The objectives of this study were to evaluate the effect of infections and potential interactions between *P. teres* and BYDV on the resistance to freezing injury in a winter barley cultivar.

MATERIALS AND METHODS

Winter barley (*Hordeum vulgare* L. emend. Bowden) cultivar Pennrad, although susceptible to infection by *P. teres* and BYDV, has good winter hardiness and is recommended for planting throughout Pennsylvania (1). Untreated seed were germinated for approximately 72 hr in darkness on moistened blotter paper in glass petri dishes. Germinated seed were planted at a depth of 3 cm in black plastic cylinders (2.5 cm diameter, 11.5 cm depth), in a mixture of equal parts (v/v) of sphagnum peat moss, perlite, and sand, at the rate of one seed per cylinder (6). Thirty-six cylinders were placed in a plastic pan containing a measured amount of a balanced nutrient solution (6). The solution was changed at weekly intervals. Plants were grown for 3 wk in a greenhouse supplemented with lighting from metal halide lamps (12-hr daylength). At the end of the 3-wk period, the plants were subjected to the hardening-freezing procedure developed by Marshall and Kolb (6). All plants were placed in a controlled environment chamber for 1 wk of hardening, with a day/night temperature of 13/1 C and illumination from fluorescent and incandescent lights (11-hr daylength, 350 μE m^{-2} sec^{-1}). Three hundred sixty plants (10 pans with 36 cylinders each) were grown for each of three replications.

During the growth period in the greenhouse, 30 plants in each pan were selected for one of several treatments, with one pan per treatment. The treatments were: 1) plants infested with *Rhopalosiphum maidis* Fitch, carrying the RMV-NY isolate of BYDV; 2) plants infested with *R. padi* L., carrying the PAV-NY isolate; 3) plants infested with nonviruliferous *R. maidis*; 4) plants infested with nonviruliferous *R. padi*; 5) plants inoculated with the fungus *P. teres;* 6) plants infested with viruliferous *R. maidis* and later inoculated with *P. teres*; 7) plants infested with viruliferous *R. padi* and later inoculated with *P. teres*; 8) healthy plants that were caged and then frozen; 9) healthy plants not caged and then frozen; and 10) healthy plants, not caged and not frozen.

All plants receiving aphid treatments were infested when one leaf was fully expanded. (All aphids used in this study were obtained

from Dr. W. F. Rochow, ARS-USDA, Cornell University.) Five to 10 aphids (mixture of adults and nymphs) were applied to each plant, and plants were caged, four each in a large nylon-mesh aphid cage. After 2 days, the aphids were killed by a 6-hr fumigation with 2,2-dichlorovinyl 0,0-dimethyl phosphate. Aphid-free plants in one pan were caged and fumigated to serve as a control.

Inoculations with *P. teres* were made when plants had three to four leaves by spraying with a conidiospore suspension of *P. teres* (10^4 spores per milliliter). Inoculum was prepared from an isolate of *P. teres* collected in 1984 from barley leaves near State College, PA, and maintained on potato-dextrose agar.

After the hardening period, each plant was removed from its plastic cylinder. Root tissue was removed approximately 0.5 cm below the base of the crown for dry weight determination. Foliage was cut approximately 2 cm above the crown base for fresh weight determination. After weighing, samples of leaf tissue were frozen and tested later by the enzyme-linked immunosorbent assay (EIA) for the presence of BYDV. Each crown was placed in a plastic vial, covered with a gas-permeable cap, and placed in a specially-designed freezing unit (6). After 8 hr at 2 C, the temperature was lowered 1.5 C/hr to −3.8 C and maintained for 8 hr. This temperature was warmer than that used by Marshall and Kolb (6). Preliminary freezing tests indicated that colder temperatures damaged a large proportion of healthy crowns and would have made separation of effects due to aphids or *P. teres* difficult or impossible. The temperature was raised 1.5 C/hr to 2 C for an 8-hr thaw. The crowns then were warmed gradually to 20 C over a 24-hr period. Crowns that were not frozen were refrigerated at 4 C for the length of the freezing and warming period.

The crowns were transferred from the freezing chamber and refrigerator to the greenhouse and placed under supplemental lighting from metal halide lamps. Two days later, 0.5 ml of distilled water was added to each vial. Nine days after freezing, the crowns were rated simultaneously for top and root regrowth. Regrowth was used as an indicator of crown injury. The degree of crown injury was assessed on a scale of 0 to 6, where 0 indicated no regrowth (dead) and 6 indicated no visible injury when compared with crowns that were not frozen (6).

The three replications were grown on three different dates. Data from 30 plants were averaged by treatment for each replication. Crown injury, fresh weight of foliage, and dry weight of roots data were subjected to an analysis of variance that treated the experimental design as a randomized complete block. Orthogonal contrasts were used for separation of treatment effects. Because the experiment did not contain the full complement of factorial treatments, the usual contrasts could not be used. The 10 treatments, their orthogonal contrast coefficients, and treatment means are listed in Table 1.

TABLE 1. Experimental treatments, orthogonal contrast coefficients, and treatment means for evaluation of the effects of *Pyrenophora teres* and barley yellow dwarf virus on the winter barley cultivar Pennrad

	Orthogonal contrasts and coefficients									Treatment means		
Treatment[w]	C1	C2	C3	C4	C5	C6	C7	C8	C9	Crown injury[x]	Top growth[y]	Root growth[z]
Healthy, not frozen	−7	−2	0	0	0	0	0	0	0	5.93	2.18	0.103
Healthy, caged, frozen	−7	1	1	0	0	0	0	0	0	3.43	2.28	0.106
Healthy, uncaged, frozen	−7	1	−1	0	0	0	0	0	0	3.43	2.53	0.108
V *R. maidis* + *P. teres*	3	0	0	1	1	1	1	1	0	2.50	1.17	0.058
V *R. padi* + *P. teres*	3	0	0	1	1	1	−1	−1	0	2.57	1.51	0.068
V *R. maidis*	3	0	0	1	1	−1	1	−1	0	2.70	0.76	0.045
V *R. padi*	3	0	0	1	1	−1	−1	1	0	2.87	0.91	0.047
NV *R. maidis*	3	0	0	1	−2	0	0	0	1	3.10	1.81	0.086
NV *R. padi*	3	0	0	1	−2	0	0	0	−1	3.07	1.81	0.089
P. teres	3	0	0	−6	0	0	0	0	0	3.13	1.55	0.101

[w] Plants infested with viruliferous (V) *Rhopalosiphum maidis* (RMV) or *R. padi* (PAV), with nonviruliferous (NV) *R. maidis* or *R. padi*, caged for 2 days and fumigated with 2,2-dichlorovinyl 0,0-dimethyl phosphate, at one-leaf stage. Plants inoculated with *Pyrenophora teres* at three- to four-leaf stage.
[x] Crowns evaluated for injury 9 days after freezing at −3.8 C. Rating scale: 0 (no regrowth) to 6 (regrowth similar to healthy, not frozen crowns).
[y] Fresh weight (g) of tops from 4-wk-old plants, before freezing (30 plants/treatment, three replications).
[z] Dry weight (g) of roots from 4-wk-old plants, before freezing (30 plants/treatment, three replcations).

TABLE 2. The effects of infection by *Pyrenophora teres* and two isolates of barley yellow dwarf virus on the crown injury, top growth, and root growth of the winter barley cultivar Pennrad, based on analysis of variance and orthogonal contrasts

		Crown injury[x]	Top growth[y]	Root growth[z]
Source of variation	df	SS	SS	SS×10^2
Blocks	2	0.033	0.027	0.001
Treatments[w]	9	2.936***	1.034***	0.181***
Contrasts				
C1—Healthy controls vs. all others	1	12.610***	5.927***	0.776***
C2—Healthy (later frozen) vs. healthy (not frozen)	1	12.500***	0.101***	0.003
C3—Caged plants vs. uncaged plants	1	0.000	0.094***	0.001
C4—*P. teres* vs. NV and V aphid species	1	0.277***	0.126***	0.324***
C5—V aphids vs. NV aphids	1	0.722***	2.088***	0.436***
C6—BYDV isolate vs. isolate + *P. teres*	1	0.187***	0.765***	0.087***
C7—V *R. maidis* vs. V *R. padi*	1	0.043	0.180***	0.011
C8—Interaction between BYDV isolates	1	0.007	0.027	0.005
C9—NV *R. maidis* vs. NV *R. padi*	1	0.001	0.000	0.001
Error	18	0.220	0.154	0.040

[w] Plants infested with viruliferous (V) *Rhopalosiphum maidis* (RMV) or *R. padi* (PAV), with nonviruliferous (NV) *R. maidis* or *R. padi*, caged for 2 days and fumigated with 2,2-dichlorovinyl 0,0-dimethyl phosphate, at one-leaf stage. Plants inoculated with *Pyrenophora teres* at three- to four-leaf stage.
[x] Crowns evaluated for injury 9 days after freezing at −3.8 C. Rating scale: 0 (no regrowth) to 6 (regrowth similar to healthy, not frozen crowns).
[y] Fresh weights (g) of tops from 4-wk-old plants, before freezing (30 plants/treatment, three replications).
[z] Dry weight (g) of roots from 4-wk-old plants, before freezing (30 plants/treatment, three replications). *** Indicates significance ($P < 0.001$) based on analysis of variance and orthogonal contrasts.

To confirm the presence or absence of RMV and PAV in all treatments, top growth of 10 plants per treatment per replication (total of 30 plants per treatment) were selected at random for analysis by EIA. This number of plants was a manageable subsample of the population for use in the EIA. Approximately 1 g of tissue per plant was chopped and extracted with a PT-20 probe of a Brinkman Polytron Homogenizer, and the samples were clarified as described previously (11). Each of the 30 samples was assayed by the direct (double-sandwich) EIA procedure. Immunoglobulins prepared from antisera specific for RMV and PAV, provided by Dr. W. F. Rochow, were used in the EIA (11). The EIA was conducted as described previously (10,11). After 60 min at room temperature, alkaline phosphatase reactions were measured at 405 nm with a Dynatech microELISA reader Model MR-580. The criterion for a positive reaction was an absorbance of at least 0.1 (12).

RESULTS AND DISCUSSION

The contrast of healthy plants versus those exposed to aphids, virus, *P. teres*, or a combination of the three had the largest sums of squares value of all of the contrasts for each of the characters measured (Table 2, C1). Plants not exposed to aphids, virus, or *P. teres* had less mean crown injury (4.3 vs. 2.8), greater top growth (2.33 vs. 1.36 g per plant), and greater root growth (0.106 vs. 0.071 g per plant). Thus the treatment combinations had a general negative effect on survival and plant growth.

As expected, the freezing treatment had a significant effect on the crown injury score (Table 2, C2). Plants subjected to the freezing treatment without aphids, virus, or *P. teres* had an average crown injury score of 3.4 compared with 5.9 for those that were not frozen. Although top and root growth measurements were made before the freezing treatment was applied, the contrast between treatments of frozen plants versus those not frozen was highly significant for top growth (2.41 g per plant for frozen vs. 2.18 g per plant for those not frozen). The cage effect (Table 2, C3) was also significant for top growth. The unexpected significant effects for top growth were the result of an abnormally large mean for the healthy, uncaged frozen treatments (2.53 g per plant), and we have no suitable explanation for the observed result.

The contrast of *P. teres* versus treatments receiving combinations of aphids, virus, and/or *P. teres* was highly significant for each of the characters measured (Table 2, C4). Plants receiving only *P. teres* had less crown injury (3.13 vs. 2.80), greater top growth (1.55 g per plant vs. 1.33 g per plant), and greater root growth (0.101 g per plant vs. 0.066 g per plant) than those receiving combinations of aphids, virus, and/or *P. teres*.

The effect of viruliferous versus nonviruliferous aphids was significant for each of the characters measured in the experiment (Table 2, C5). Plants exposed only to nonviruliferous aphids had less crown injury (3.08 vs. 2.66), greater top growth (1.81 vs. 1.09 g per plant), and greater root growth (0.088 vs. 0.055 g per plant). The results indicate that the virus reduced survival and plant growth more than exposure to aphids alone.

The average of treatments receiving viruliferous aphids was significantly different from those receiving viruliferous aphids plus *P. teres* (Table 2, C6). Plants exposed to viruliferous aphids plus *P. teres* had more crown injury (2.54 vs. 2.78), greater top growth (1.340 vs. 0.835 g per plant), and greater root growth (0.063 vs. 0.046 g per plant). The addition of *P. teres* to virus-infected plants increased crown injury.

The effect of virus isolates among those treatments receiving only viruliferous aphids or viruliferous aphids plus *P. teres* was significant only for top growth (Table 2, C7). Plants receiving viruliferous *R. padi* and those with viruliferous *R. padi* + *P. teres* had greater top growth than those receiving viruliferous *R. maidis* or viruliferous *R. maidis* + *P. teres* (1.210 vs. 0.965 g per plant). The results of our experiment indicate that the RMV isolate caused a greater reduction in top growth than did the PAV isolate.

The treatment combination permitted one orthogonal contrast for the interaction of RMV versus PAV and of each virus isolate plus *P. teres* treatments. This contrast was not significant for any character measured in our experiment (Table 2, C8). An additional contrast possible with the treatment combinations was nonviruliferous *R. maidis* aphids versus nonviruliferous *R. padi* aphids, which also was not significant for any character measured in our experiment (Table 2, C9).

EIA. Plants infested with viruliferous aphids developed characteristic symptoms of BYDV infection. Plants presumably infected with RMV were stunted slightly, in comparison with healthy control plants. Plants presumably infected with PAV were stunted markedly and had chlorotic leaves, when compared with healthy plants. Plants infested with nonviruliferous aphids, those infected by *P. teres* alone, and presumably healthy controls were negative for the presence of either RMV or PAV antigens. All of the tested barley plants that had been infested with viruliferous *R. padi* gave positive reactions for PAV. However, in cases of plants infected by either PAV + *P. teres* or RMV + *P. teres*, the reactions were negative for PAV and RMV. In the case of plants presumably infected with RMV, only one sample tested positively for RMV.

Despite the variable results of the EIA, the significant effect on crown injury, top and root growth suggested that BYDV was present in those plants treated with either RMV, RMV + *P. teres*, or PAV + *P. teres*. These suggestions are further supported by the fact that aphids used in the PAV + *P. teres* treatment (EIA-negative reaction with the plant samples) came from the same population as those used in the PAV treatment (EIA-positive reaction with the plant samples). We suggest a possibility that the presence of *P. teres* has interfered with the EIA.

This study has provided additional evidence that infections by *P. teres*, BYDV, and feeding by nonviruliferous aphids all increase freezing injury to barley crowns. In addition, this work has provided specific information regarding the effect of two distinct BYDV isolates on the growth and resistance to freezing stress of a barley cultivar.

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