Effects of Freeze Damage on Soybean Seed Mycoflora and Germination

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ABSTRACT

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Pods of soybean (*Glycine max*) plants grown in three environments (pots under greenhouse conditions, pots exposed to weather, or field plantings) were inoculated at growth stage R5 with isolates of *Fusarium graminearum*, *Alternaria alternata*, or *Phomopsis longicolla*. The applied fungus became the dominant colonist of the pods in all environments. Plants grown in pots were exposed to freezing at −4.5 or −2.5 C at growth stage R6 under controlled-environment conditions, and the latest of three field plantings experienced a natural frost of −2.0 C at the same growth stage. On inoculated plants, freezing was associated with increased seed infection by *F. graminearum* and *A. alternata* and with reduced seed infection by *P. longicolla*. Pod inoculations by *F. graminearum* and *A. alternata* caused greater reductions in seed germination than that which occurred in noninoculated plants when plants were subjected to freezing at −4.5 C. These fungi, however, had little effect on seed germination in plants not subjected to freezing. Inoculum of *P. longicolla* reduced seed germination for both frozen and nonfrozen plants in all environments; however, there was no evidence that damage from this fungus was exacerbated by freezing. Survey data indicated that *Fusarium*, *Alternaria*, and *Phomopsis* spp. are common residents of soybean pods in Iowa.

Freezing injury adversely affects germination of soybean (*Glycine max* (L.) Merr.) seed (8) and may predispose plant tissues to invasion by microorganisms (2). Extensive infection by *Fusarium* spp., *Alternaria* spp., and other microorganisms has been reported in frost-damaged soybean seeds (10,14). Seedborne fungi also are an important cause of soybean seed quality problems. *Phomopsis longicolla* T. W. Hobbs, the cause of Phomopsis seed decay, can greatly reduce seed germination (9). Infection of seed by *Fusarium* spp. has been associated with reduced germination in laboratory tests (11,13,15) and with pre- and postemergence blights (16). *Alternaria* spp. normally are not pathogenic to soybean seeds (11) unless seeds have previously been damaged by insects (17). *Phomopsis*, *Fusarium*, and *Alternaria* spp. commonly colonize soybean pods (12), but the effects of these fungi on germination of frost damaged seeds are not known.

The objective of this study was to elucidate interactions between soybean pod mycoflora and freeze damage with respect to soybean seed quality.

MATERIALS AND METHODS

Seed tests. Fungal infection of harvested seeds was determined by surface-disinfesting seeds in 0.5% sodium hypochlorite for 1 min, rinsing in sterile water, and placing them on potato-dextrose agar (PDA). After incubation for 7 days in the dark at 25 C, the number of seeds from which colonies of *Phomopsis*, *Fusarium*, and *Alternaria* species grew was counted. Seed germination was determined in a standard warm germination test (1) and in a cold germination test in which seeds were placed on a moist paper substrate, covered with a mixture of unsterilized soil and acid-washed sand, and grown out for 7 days at 10 C in the dark followed by 7 days at 25 C under alternating cycles of 12 hr of fluorescent light and dark. Seed germination also was estimated by imbibing seeds for 16 hr on saturated paper towels then placing them in a 0.075% solution of 2,3,5-triphenyltetrazolium chloride (TZ) for 4 hr at 37.5 C. The extent of tissue damage, determined by tetrazolium staining patterns, indicated potential seed germination (6). Each of the above fungal infection and germination tests was carried out on a sample of 100 seeds from each replicate of each treatment.

Controlled freezing experiments. Plants of the soybean cultivar Corsoy 79 were grown in pots (23 cm in diameter with five plants per pot) in growth chambers at 25 C, on a 14-hr light, 10-hr dark regime. At growth stage R5 (5), plants in sets of 18 pots were sprayed to runoff with suspensions of *P. longicolla*, *Fusarium graminearum* Schwabe, or *Alternaria alternata* (Fr.:Fr.) Keissl., or with distilled water. The cultures of each fungus originally were isolated from soybean seeds produced in Iowa. *P. longicolla* was applied as a suspension of 1.5 × 10^8 conidia per milliliter. The other fungi were applied in mycelial suspensions standardized to a 10% transmittance at 400 nm in a spectrophotometer. Plants were placed in a mist chamber at 24 ± 3 C for 72 hr after inoculation, transferred to the greenhouse, and kept at 80-90% relative humidity and 24 ± 3 C under plastic covers. After the mist chamber treatment, pod infection was determined on 20 pods detached from plants in each inoculation treatment. The pods were incubated in on moist blotters at room temperature for 5 days, and the number of pods supporting growth of each fungus was recorded. At growth stage R6, two replicates of three pots from each inoculation treatment were subjected to freezing at −2.5 or −4.5 C or were not frozen. Freezing treatments were applied in a growth chamber programmed to cool from 12 C at a rate of 2 C/hr to the appropriate freezing temperature, to maintain this for 4 hr, and then to raise the temperature at a rate of 5 C/hr to the original 12 C. The two replicates were treated in sequence

at a 24-hr interval in the same chamber. Plants were returned to the greenhouse and arranged in two randomized blocks. At harvest maturity (seed moisture content approximately 14%), seeds from all treatments were assayed for fungal infection and for germination in a warm test.

In a second experiment, the same inoculation and freezing treatments were applied to each of three replicates of six pots and, except for freezing periods, plants were exposed to weather for the duration of the experiment from 25 May to 26 September 1986. Treatments were arranged in three randomized blocks. As in the first experiment, freezing treatments were applied one replication at a time. At harvest maturity, seeds from all treatments were tested for fungal infection and for germination in warm, cold, and tetrazolium tests.

Field experiment. Seed of the soybean cultivar Corsoy 79 (maturity group II) was planted into four blocks of 24 rows (6 m long and 75 cm wide) on 23 May, 13 June, and 10 July 1986 near Ames, IA. For each planting date, separate four-row plots in each block were treated with inoculum of *P. longicolla, F. graminearum,* or *A. alternata,* or with distilled water at growth stage R5 by spraying to runoff all plants in 0.6-m sections in each of the two middle rows. Inoculum concentrations were the same as those used in the controlled-environment experiments. Three days after treatment, 40 pods were sampled from each plot and tested for pod infection as described previously. At harvest maturity, all seeds were removed from plants in treated sections of each plot and tested for fungal infection and seed germination by warm, cold, and tetrazolium tests. A split-plot experimental design was used, with planting dates as the main plots and inoculations as the subplots. There were four replications.

Precipitation, relative humidity, and temperature at the height of the canopy were measured continually with a rainfall gauge and hygrothermograph located at the experimental site. After seeds formed in pods, seed moisture was measured twice a week by sampling all the pods on two plants in each plot. Seeds then were removed, weighed, dried at 105 C for 4 hr, and reweighed. Seed moisture was expressed on a wet weight basis. A freeze of −2.0 C occurred on the night of 13 October, when the seed moisture contents averaged 23.3, 33.4, and 66.0% for the May, June, and July plantings, respectively. Plants in the first two plantings were at growth stage R8, and the July planting was at R6.

In all experiments, separate analyses of variance were made for seed infection by each pathogen or for germination values in each test in each experiment.

Survey of pod infection by *Phomopsis, Fusarium,* and *Alternaria* spp. in Iowa. Samples of 100 pods were randomly collected from plants at the R6 growth stage in 27 soybean fields widely distributed throughout Iowa during the 1986 growing season. Numbers of pods infected by *Phomopsis, Fusarium,* and *Alternaria* spp. were determined in a pod test (9,12). Isolates of typical colonies of *Fusarium* spp. and *Alternaria* spp. on the pods were identified to species (4,7).

RESULTS

Statistical analyses. Main effects were significant at the 0.01 or 0.001 levels of probability in all of the experiments (Fig. 1–3), with the exception of the measurement of the TZ test made in the field experiment (Fig. 3). In Figures 2 and 3, significant (0.05 probability level) interactions are indicated by an LSD value below the X-axis that relates to all values directly above it in each graph.

Establishment of fungal inoculum in pod tissues. The fungus applied as inoculum became the dominant colonist of pod tissues 72 hr after inoculation at growth stage R5 in all three experiments (Fig. 1A–L). In field inoculations, pod infection by both *A. alternata* and *P. longicolla* declined as the planting time was delayed (Fig. 1C and I), but infection by *F. graminearum* was similar for all plantings (Fig. 1F). *Alternaria, Fusarium,* and *Phomopsis* spp. were detected at low levels of pod infection in noninoculated treatments in each experiment (Fig. 1J–L), except for the extensive pod infection by *A. alternata* in the June and July field plantings (Fig. 1L).

Seed infection in plants subjected to different freezing treatments. Freezing at growth stage R6 significantly increased seed infection by *F. graminearum* in the two pot experiments for both inoculated (Fig. 2D and E) and noninoculated plants (Fig. 2J and K). In the field, seed infection by *F. graminearum* was greater in inoculated plants in the July planting, which had sustained a natural freeze of −2.0 C at growth R6, than it was in the June and May plantings, which had been frozen at growth stage R8 (Fig. 2F). Pod inoculation by *F. graminearum* had no effect on seed infection in nonfrozen plants in the two pot experiments (Fig.
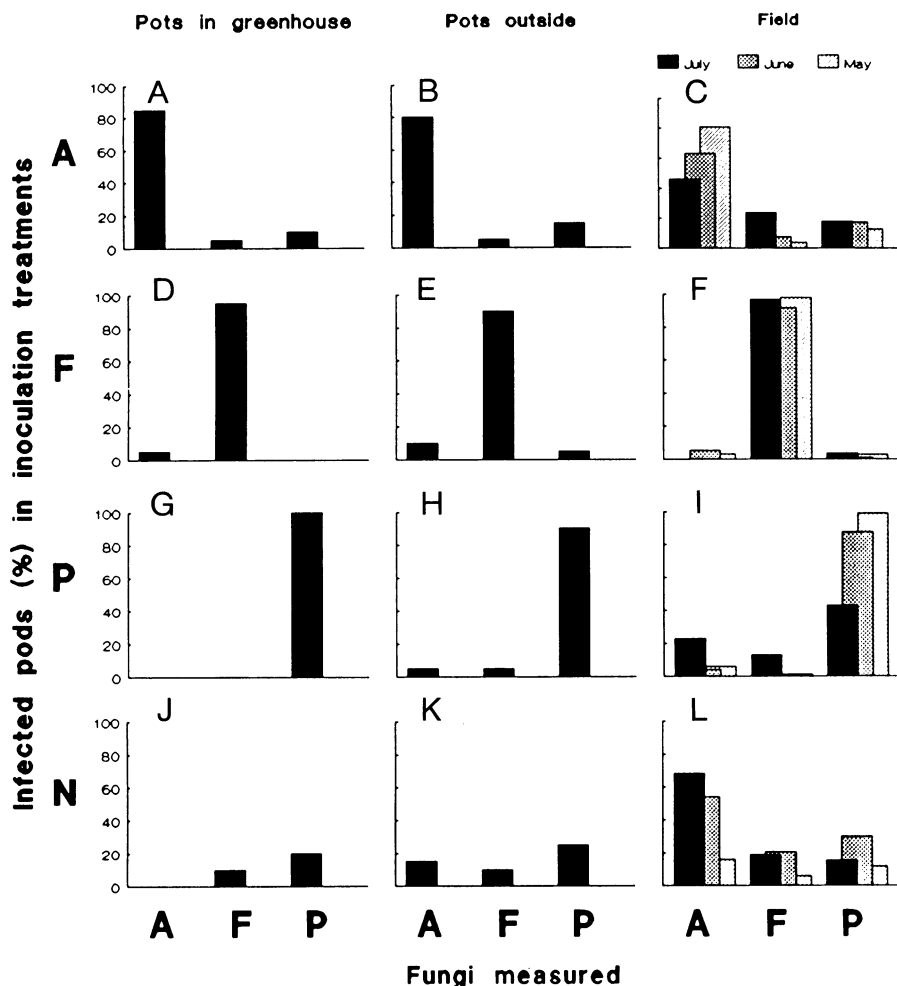

Fig. 1. Pod infection by *Phomopsis, Fusarium,* and *Alternaria* spp. measured 72 hr after inoculation at growth stage R5 by isolates of *Phomopsis longicolla* (P), *Fusarium graminearum* (F), or *Alternaria alternata* (A), or no inoculum (N) in soybean plants grown in pots under greenhouse conditions, in pots exposed to weather, or in three field plantings. Fungal infection was determined by incubating detached pods on moist blotters at room temperature for 5 days. The number of pods supporting fungal growth of each fungus was then recorded.

2D, E, and J).

Seed infection by *A. alternata* was significantly increased by freezing at −4.5 C on plants inoculated with this fungus in the two pot experiments, but the effect was not detected in plants frozen at −2.5 C or in the field plantings (Fig. 2A–C). Pod inoculation by this fungus had no effect on seed infection of nonfrozen plants in any experiment (Fig. 2J–L).

Pod inoculation by *P. longicolla* significantly increased seed infection by this fungus in all three experiments, in both freezing and nonfreezing treatments (Fig. 2G–L). Seed infection by this fungus was consistently reduced, however, by freezing treatments within experiments (Fig. 2G–I).

Seed germination in plants subjected to different freezing treatments. All three test methods showed a decline in germination in seeds harvested from noninoculated plants subjected to freezing at growth stage R6, either at −4.5 C in the two pot experiments or by natural freezing in the July field planting (Fig. 3J–L). The tetrazolium test provided direct evidence of physiological damage to seed tissues.

Warm germination test values were significantly lower in nonfrozen plants inoculated with *A. alternata* than that in nonfrozen, noninoculated plants in the greenhouse experiment (Fig. 3A and J). There was no evidence that *F. graminearum* significantly reduced germination in nonfrozen plants in any of the experiments (Fig. 3D–F and J–I). Warm and cold germination test values for seeds in plants inoculated with *A. alternata* and subjected to −4.5 C in the two pot experiments were significantly lower than those in corresponding noninoculated plants (Fig. 3A, B, J, and K). This effect was seen for *F. graminearum* only in the warm test values in the greenhouse experiment (Fig. 3D and J). Neither fungus elicited this effect in the field plantings (Fig. 3C, F, and L).

Pod inoculation by *P. longicolla* significantly reduced germination in seeds from plants not subjected to freezing in all three experiments (Fig. 3G–L). There was no evidence in any of the experiments for this fungus causing a significant decline in germination in addition to that caused by freezing alone (Fig. 3G–L).

Fungal species commonly colonizing soybean pods in Iowa. The percentage of pods infected by *Alternaria, Fusarium,* and *Phomopsis* spp. averaged 56.5, 54.6, and 45.5%, respectively for 27 fields sampled across Iowa in 1986. *F. graminearum, F. tricinctum* (Corda) Sacc., and *F. semitectum* Berk. & Ravenel were the most prevalent and widely distributed species of *Fusarium.* Other species found included *F. equiseti* (Corda) Sacc., *F. solani* (Mart) Sacc., *F. poae* (Peck) Wollenweb., and *F. moniliforme* J. Sheld.

f. sp. *subglutinans* Wollenweb. & Reinking. *P. longicolla* and *A. alternata* were the only species found in these genera.

DISCUSSION

Freezing soybean plants at growth stage R6 clearly influenced both soybean seed mycoflora and seed germination. Survey data indicated that *F. graminearum* and *A. alternata* are common colonists of soybean pods in Iowa. The induction of high populations of these fungi on soybean pods by inoculation generally did not lead to increased seed infection in nonfrozen plants, and, apart from a slight reduction in germination in the greenhouse experiment by inoculation with *A. alternata,* had no effect on seed germination. These data are in agreement with previous findings (11,13, 15,16) that these fungi have a minimal effects on seed germination. Freezing of plants at R6, however, resulted in sig-

nificant increases in seed infection by both of these fungi; the extent of seed infection increased with the severity of the freeze. The two pot experiments provided evidence that *A. alternata* and, to a lesser extent, *F. graminearum* caused a decline in germination in addition to that caused by freezing injury. Both fungi are facultative parasites, and it is likely that the disruption of defense mechanisms and the release of nutrients by freezing injury led to their increased growth and colonization of seeds. Damage to soybean seeds by *Alternaria* spp. can be aggravated by insect injury (17). This would explain the extensive infection of dead seeds by these fungi in frost-damaged seeds observed in germination tests in 1984 (10).

P. longicolla differs from the other common colonists of soybean pods in that it is a major pathogen of soybean seeds. As expected, extensive seed infec-
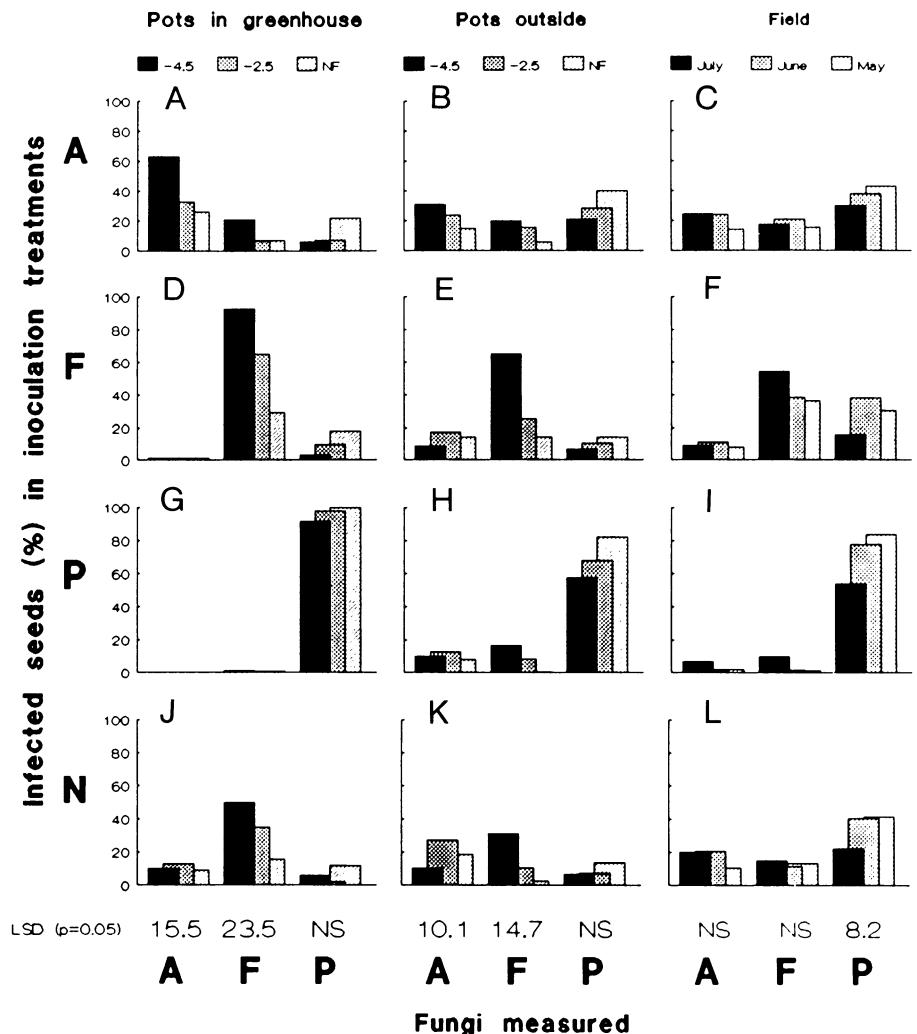

Fig. 2. Seed infection by *Phomopsis, Fusarium,* and *Alternaria* spp. measured at harvest maturity in soybean plants inoculated at growth stage R5 by isolates of *Phomopsis longicolla* (P), *Fusarium graminearum* (F), or *Alternaria alternata* (A), or no inoculum (N), subjected to different freezing conditions at growth stage R6, and grown in pots under greenhouse conditions, in pots exposed to weather, or in three field plantings. Analyses of variance for seed infection by each pathogen in each experiment indicated that all main effects (inoculation and freezing treatment or planting date) were significant at the 0.01 or 0.001 levels of probability. Significant (0.05 probability level) interactions are indicate by an LSD value below the axis that relates to all values directly above it in each graph.
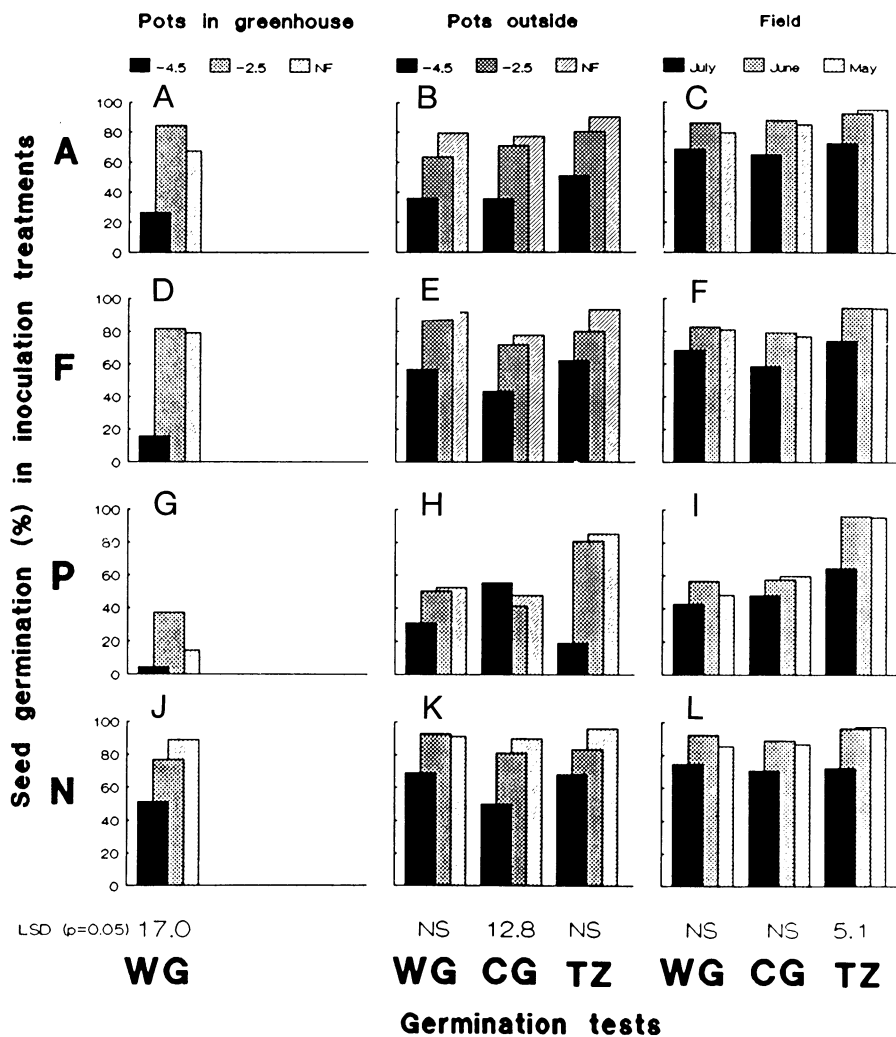
Fig. 3. Seed germination at harvest maturity in soybean plants inoculated at growth stage R5 by isolates of *Phomopsis longicolla* (P), *Fusarium graminearum* (F), or *Alternaria alternata* (A), or no inoculum (N), subjected to different freezing conditions at growth stage R6, and grown in pots under greenhouse conditions, in pots exposed to weather or in three field plantings. Seed germination was determined in standard warm germination test (WG) and a cold germination test (CG) in which seeds were placed on a moist paper substrate, covered with a mixture of soil and sand, and grown out for 7 days at 10 C in the dark, followed by 7 days at 25 C under alternating cycles of 12 hr of fluorescent light and dark. Seed germination was also estimated by imbibing seeds for 16 hr on saturated paper towels, placing them in a 0.075% solution of 2,3,5-triphenyltetrazolium chloride (TZ) for 4 hr at 37.5 C. The extent of tissue damage, determined by tetrazolium staining patterns, indicated potential seed germination. Analyses of variance for each germination test in each experiment indicated that all main effects (inoculation and freezing treatment or planting date) were significant at the 0.01 or 0.001 levels of probability. Significant (0.05 probability level) interactions are indicate by an LSD value below the axis that relates to all values directly above it in each graph.

tion and significant reductions in seed germination occurred in inoculated treatments with high levels of pod infection by *P. longicolla* (3,9). The decline in seed infection observed in freezing treatments may be attributable to competition from other common seedborne fungi (11).

Reduced germination due to inoculation of pods with *F. graminearum* and *A. alternata* was detected only in plants

frozen at −4.5 C under controlled environment conditions. The −2.5 C freezing treatment or the natural freeze of −2 C in the field presumably were not severe enough to cause substantial physiological damage. Whether additional damage to freeze-damaged seeds by these fungi is of any practical concern is questionable. The reduced quality resulting from freezing alone probably would

make the germination of the seed lot unacceptable for commercial use. If such seeds were to be grown, however, seed treatment fungicides might improve emergence by reducing fungal inoculum and by protecting seeds, in which vigor had been reduced by freezing, from soilborne pathogens.

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