Pathological Factors Affecting Survival of Winter Barley Following Controlled Freeze Tests

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ABSTRACT

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Recovery from freeze injury is one component of winter hardiness in barley. The intent of this study was to determine to what extent pathogens and nutritional factors introduced during the recovery phase would modify survival of barley following a freeze test. Hardiness, as measured by development of new roots from crown meristems after controlled freeze tests, was depressed by inoculation with *Fusarium roseum* f. sp. *cerealis* 'Avenaceum'. Supplementation of the fungus with glucose and amide nitrogen, compounds known to accumulate in hardened plants, further reduced survival.

Additional key words: predisposition, plastic strain, stress.

Winter hardiness of barley (*Hordeum vulgare* L.) depends on traits that protect the plant from freeze injury either by modifying stresses that develop as water freezes or by increasing resistance to injury (8). Hardiness also depends on genetic traits that promote recovery and disease resistance. Essentially, winter hardiness involves all aspects of survival. Since 1935, the U.S. Department of Agriculture has coordinated uniform hardiness nurseries for rating barley cultivars at more than 50 sites throughout the United States and Canada (17). Each season, as many as 50 cultivars are planted at each location. Such tests are characterized by great variability caused by the interaction of plant growth, crown moisture, snow cover, freeze-thaw sequences, and disease occurence. Rating is based on percent survival in years of differential kill. This procedure requires many replications to obtain statistically significant data.

Controlled freeze tests would reduce the magnitude of such genotype-environment interaction. Harvey (4), in 1917, introduced the concept of controlled freeze tests in which genetic differences could be characterized. The literature on hardiness evaluation up to 1956 was summarized by Dexter (1). During this period, vitality tests such as Luyet's neutral red test or assays based on solute leakage from injured protoplasts improved the precision of rating hardiness. Recognition of lateral crown meristems as tissues critical for plant survival resulted in more accurate tests (5, 7, 8). In freeze tests, the critical tissue is the first essential tissue to be killed.

Recently, the accuracy of freeze testing has been improved by characterization of different freezing stresses (3, 11). Controlled freeze tests can be separated conveniently into four phases: growth, hardening, freeze stress, and recovery. Several distinct forms of freeze stress can be imposed in the third phase. These stresses differ in the form of energy involved (10), the manner in which crystallization energy is dissipated (9, 13), the temperature range in which the stress causes injury, and the pattern of injury that develops in the plant (11). As a result, freeze hardiness can be defined in terms of specific cryoprotectant systems required for survival. Procedures have been developed for testing plant response to each form of stress, and test statistics for discrimination between genotype have been published (3, 11). Differences also may exist in the ability of cultivars to recover from freeze injury.

That facultative plant pathogens can initiate disease by entry through even minute lesions is well known (15). The large lesions caused by ice damage in the lower crown during a freeze test are ideal infection sites. Once established in the area of freeze injury, a fungus easily can invade the adjacent meristem. We have attempted to determine the importance and interrelations of a facultative parasite, glucose-amide nitrogen nutrition, and toxicity accompanying tissue deterioration on the recovery of moderately freeze-injured plants.

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MATERIALS AND METHODS

Barley roots from the East Lansing nursery were plated-out eight times during the spring of 1974. The first collection was made during March from plants in frozen soil. Collections continued twice a month until stem elongation in June. Fifteen species from 10 genera were isolated and identified; *Fusarium* was the most frequently occurring genus. All isolates of *Fusarium* were screened for low-temperature tolerance on acidified potato-dextrose-agar (PDA) at 1 C. A strain, isolated from the frozen barley roots of the earliest collection, and identified as, *Fusarium roseum* Lk. emend. Snyd. and Hans. f. sp. *cerealis* (Cke) Snyd. and Hans. 'Avenaceum' was the most low-temperature tolerant. On this basis it was selected for the study, and further testing showed that it grew well up to 20 C.

The Avenaceum inoculum in which the plants were grown during the recovery phase was prepared as follows: the fungus was cultured in petri plates on PDA at 14 C under continuous light. After 3 wk, the mycelium was scraped from the agar surface of each plate with a microscope slide and blended with the healthy, washed, chopped, roots from eight, 8-wk-old barley plants. The mycelium-root mixture was blended into washed sterilized sand at 1:5 and incubated, with daily aeration, for 3 wk at 8 C in heavy, black, plastic bags. At this time, 12 to 18 random samples of the mixture, subsequently referred to as the inoculum, were removed and plated on PDA; the Avenaceum developed from all samples.

After the 3-wk incubation, the inoculum was tested to determine whether it had developed any toxicity. The material was bioassayed using *Bacillus subtilis* (Cohn) em. Prazm. with a test procedure developed at the University of Minnesota. Clear potato-dextrose medium prepared with 2% agar was cooled to 55 C, seeded with an aqueous suspension of *B. subtilis* spores, and a thin layer was poured into petri plates. Twenty small samples of inoculum were placed on the surface of the plates and incubated for 18 to 36 hr. Toxic products secreted from the colonized roots would produce a zone of bacterial inhibition around the sample. A second method used to detect toxicity was to germinate Hudson barley seeds in the inoculum; toxicity would reduce germination.

Products which accumulate in or are released into the intercellular liquid of hardened plant tissues could provide substrate for invading microorganisms. This possibility was tested by collecting guttation water. The hardened plants were grown in chambers at 0 to 1 C and nonhardened plants at 15 C. Each chamber was programmed to provide 14 hr of light (24,000 lux) followed by 10 hr of dark each day. Prior to the dark phase, during which the collections were made, the plants were heavily watered. With the lights out, the humidity in the chamber rapidly climbed to saturation. The guttation water was collected in test tubes as it dripped from the leaf tips. By using a standard Warburg respirometer procedure described by Umbreit (16), O_2 uptake of *Saccharomyces cerevisiae* (Mesen) Hansen was measured using a basal medium supplemented with the guttation water from hardened or nonhardened barley.

Hudson barley was selected as the test plant for these studies because it is a commercial, winterhardy, cultivar to which all others are routinely compared in our hardiness assay. The stability and uniformity of this cultivar are basic to the success of the method. The plants were grown in washed, steamed sand in 10-cm-diameter pots, eight plants per pot. They were watered with modified Hoagland's solution for 5 wk at 15 C followed by 3 wk at 2 C. At this hardening temperature (2 C) plants metabolize slowly, gradually acquire resistance to propagation of ice crystals through the plasmalemma, and attain maximum hardiness after 3 wk.

In preparation for the freeze test, the leaves and roots of hardened plants were trimmed to a few centimeters; these plants then were frozen in a water-saturated medium. Increasing the water content before freezing increased the latent heat flow and the crystallization energy of the test (8, 10, 11). Supercooling never was permitted to exceed 2 C and the rate of cooling never exceeded 1 C/hr, in the standard freeze test. The plants were held at a minimum temperature of -10 C for 1 hr, after which they were warmed at less than 1 C/hr until thawed. Minus 10 C was selected for the freeze test to induce a 20% reduction in recovery of control plants. The crystallization energy generated in this process was especially destructive to thick-walled rigidly structured tissues. Therefore, the test caused severe damage in the lower crown and roots of the plants, but little damage in the leaves and upper crown (8). The completely thawed plants then were ready for the recovery phase. At this stage, part of the plants were inoculated with Avenaceum.

Inoculum was introduced at a level high enough to establish infection quickly during the 2-wk recovery phase. In each test the 720 freeze-injured plants were divided into three sets. Set A was repotted in washed sand. Sets B and C were repotted in the inoculum described earlier. In each set, the roots of half of the plants were excised at the crown. Sets A and B were watered every other day with modified Hoagland's solution. Set C was watered with modified Hoagland's to which was added 2% glucose and 10^{-4}M asparagine. All of the plants were kept in a growth chamber at 14 C for the 2-wk recovery period. This temperature is near optimum for barley growth and within the range of early spring temperature, following thaw, in the field.

After the 2-wk recovery, plants were removed from the pots and survival was determined on the basis of new root development from the crown meristems. Bright new roots were clearly visible and easily counted on surviving plants. Survival was evaluated on a scale of 1 to 5 based on the new root generation. Plants rated 1 had only one new root; plants rated 2 had two or three short new roots; plants rated 5 had many vigorous new roots.

RESULTS

Factors contributing to recovery.—Introducing a pathogen into a plant already in the recovery phase of a freeze test required a series of special control experiments. These were designed to determine the effects of pathogenic, nutritional, and toxic factors that might make results misleading.

Earlier studies had shown that excising the freeze-damaged roots of frozen Hudson plants immediately after thawing, before toxins could develop, increased plant survival (12). To insure maximum recovery in the

present studies, frozen roots were excised from half of the plants in each set. In the presence of the fungus, excision was no longer beneficial and actually may have promoted infection by facilitating entry. As an additional control, the inoculum was tested for toxicity. If present, toxins could interfere with root initiation from the lateral meristems. Of the 20 samples assayed for inhibition of *Bacillus subtilis* growth or Hudson seed germination, none showed any evidence of toxicity.

Intercellular liquid of Hudson barley, collected as guttation water, stimulated respiration of *S. cerevisiae* (Table 1). In a preliminary experiment, buffered basal medium containing no carbon energy source, other than the essential vitamin thiamine, and no ammonium or amide nitrogen, gave a minimum rate of respiration for the yeast. Supplementing this basal medium with increasing concentrations of glucose stimulated respiration which reached a plateau at 0.05 M glucose. This level was elevated with additions of glutamine, and the maximum rate achieved at 10^{-4}M glutamine. Table 1 presents data from an experiment in which duplicate samples of guttation water were collected from 24 barley plants. Respiration rates were uniform over a 30-min period of measurement. Guttation water from hardened plants was more stimulatory than that from tender plants. Guttation water from the hardened barley approached the effectiveness of combined carbon-nitrogen supplementation.

The effect of glucose-asparagine supplementation on noninoculated freeze-stressed plants was evaluated using a routine freeze test without introduction of fungal inoculum during the recovery phase. Survival of 240

TABLE 1. Effects of glutamine, glucose, and guttation water (collected from hardened and nonhardened Hudson barley) on respiratory activity of *Saccharomyces cerevisiae*

	Respiratory rate[a]	
	No glucose	+ Glucose
Basal medium	7	22
Basal medium + guttation water from nonhardened plants	26	33
Basal medium + guttation water from hardened plants	31	37
Basal medium + glutamine	18	36

[a]Oxygen uptake at 37 C (μliters/liter/mg wet weight of yeast). Rates were uniform over the 30-min period of measurement. Data are expressed in significant numbers.

TABLE 2. Effect of excision, pathogen (*Fusarium roseum* 'Avenaceum'), and nutrition on survival of hardened Hudson barley plants after freezing at high crown moisture. Survival is measured as a function of new root formation from lateral crown meristems. The chi square values were obtained by comparison of the mean values with that of the Set A-excised

	Vigor of recovery[a]	Set A[b]		Set B[c]		Set C[d]	
		Excised	Non-excised	Excised	Non-excised	Excised	Non-excised
Test I:							
Survival (%)[e]		88	64	62	42	35	43
Recovery rating partition[f]	1	5	8	8	7	12	12
	2	23	14	25	11	10	15
	3	18	17	18	17	13	15
	4	31	22	9	5		1
	5	11	3	2	2		
Test II:							
Survival (%)[e]		85	75	62	62	41	42
Recovery rating partition[f]	1	15	13	9	7	16	24
	2	13	9	11	8	16	7
	3	42	25	28	31	9	11
	4	14	24	13	14		
	5	1	4	1	2		
Mean survival (%)		86	70	57		40	
χ^2			17.5***	60***		135***	

[a]Rating system for vigor of recovery from freeze injury, based on root regeneration from crown meristems on a scale of 1-5 (1=weak, and 5=vigorous).

[b]Noninoculated and watered with Hoagland's solution. "Excised" and "nonexcised" refer to whether freeze-damaged roots were removed immediately after the plants were thawed.

[c]Avenaceum-inoculated and watered with Hoagland's solution.

[d]Avenaceum-inoculated and watered with glucose (2%) + asparagine (10^{-4}M)-supplemented Hoagland's solution.

[e]Percent survival figures each represent results from 120 tested plants.

[f]Recovery rating partition presents the distribution of numbers of plants that were classified into the rating classes described in footnote *a*.

plants watered during the recovery phase with glucose (2%) plus asparagine (10^{-4}M)-amended Hoagland's solution, was the same as the set watered with nonsupplemented Hoagland's solution. Two percent glucose caused some leaf chlorosis, but did not alter the pattern of root regeneration or survival.

In a final experiment, the pathogenicity of Avenaceum to nonfrozen plants was determined. The plants were grown, hardened, trimmed, and inoculated as for a routine test. Avenaceum was only weakly pathogenic as tested on the trimmed crowns of nonfrozen plants. There was no significant difference in the generation of new roots between inoculated and noninoculated sets (180 plants/set). Control plants had no disease lesions; inoculated plants developed a few root leasions from which the fungus was isolated. When inoculated plants were supplemented with glucose and asparagine, although all survived, the roots were distinctly discolored and more lesions developed.

Recovery following a freeze test.—Table 2 presents the results of two experiments in which freeze-stressed barley was inoculated with Avenaceum during the recovery phase. The reference set, to which all treatments were compared, is labeled Set A-excised. In this set, where survival was the highest, there was no nutritional supplementation or fungal inoculum and toxicity was minimized by excision.

Removal of injured roots following freeze stress increased survival of the plants which were not inoculated with Avenaceum by 16%, but it had little effect on inoculated plants. Disease had a more pronounced effect and survival was reduced by 29%. Glucose and asparagine increased disease and suppressed survival, compared to that of the control set, by 46%.

DISCUSSION

The freeze stress imposed in these experiments is an example of predisposition defined by Yarwood (18) as the tendency of a nongenetic factor, acting prior to infection, to affect the susceptibility of plants to disease. Most low-temperature studies of plants have dealt with the hardiness of the plant. The effect of freezing stress on disease expression has been little noted in work with winter cereals. Several groups have studied this problem in woody plants (14). These studies used both natural and controlled freezing procedures and showed that cold stress did predispose to disease. In most cases, defense reactions limited disease development and the strain that resulted from freezing was elastic in relation to disease susceptibility (14). Levitt (6) characterized two types of cold injury; he termed irreversible physical or chemical change induced by stress, such as our freezing procedure, a plastic strain, in contrast to the reversible changes of an elastic strain.

The relative potential of facultative parasites to colonize host tissue Schoeneweiss (14) has defined as aggressiveness. Broadly speaking, the Avenaceum used in our study is an aggressive pathogen; i.e., one capable of causing disease in nonstressed plants. But in a limited sense, as tested in our experiments, it was nonaggressive because nonstressed plants did not develop disease. Only freeze-stressed plants subjected to plastic strain were killed by the pathogen.

The damage sustained by the barley plants in these freeze tests would be described as plastic injury. The more rigidly structured tissues of the roots and lower crown were much more susceptible to injury from this form of stress than were the leaves and upper crown tissue. New roots, originating from lateral meristems at the base of the barley crown, developed after the freeze test and initiated the plant's recovery. The meristems, although not injured by the freeze test, were rendered vulnerable by their proximity to dead tissues which underwent toxic deterioration during recovery. Furthermore, these dead tissues were ideal sites for entry of pathogens. The facultative parasite became established in the dead tissue from which it then invaded the adjacent healthy meristems.

Survival of these freeze-stressed plants was influenced by disease, nutrition, and toxicity which had to be evaluated separately and with nonfrozen plants before their combined effect on frozen plants could be determined. Independent evaluation of these factors, before freeze-testing, established parameters for evaluation of the freeze-test recovery.

Nutritional studies in this work were limited to glucose and amide nitrogen because these products are known to be associated with cold-hardened plants. As used, they did not affect recovery of freeze-stressed barley. Predictably, these products stimulated pathogenicity of the Avenaceum. Toussoun et al. (15) reported that organic nitrogen, especially amide nitrogen, stimulated early penetration and pathogenesis of *Fusarium solani* f. *phaseoli*, and that glucose favored germination of conidia and saprophytic development of mycelium on the host.

It was necessary to determine whether the inoculum or the plant generated any toxicity which could be confused with disease. Tests of the Avenaceum inoculum showed it to be nontoxic and the toxicity of decaying roots was eliminated in our controls by root excision after freezing. Lastly, the Avenaceum, as tested on the nonfrozen barley, was only weakly pathogenic. This was essential in order to measure the predisposing effect of freezing on disease expression of the pathogen.

Allowing for the interaction of contributing factors, Avenaceum infection significantly reduced recovery of freeze-stressed barley plants. With high levels of inoculum, the disease was quickly established and reduced survival during the 2-wk postfreeze recovery period. Supplementation of the system with amide nitrogen and glucose further reduced recovery. Winter hardiness of barley is a complex trait that involves many genes (2). Complex traits tested in poorly controlled experiments have low heritability, which limits progress in plant breeding. Results from this research emphasize the importance of disease control when breeding for cryoprotective systems, for freeze-testing procedures produce a predisposing plastic strain in the test plants. Even normally nonaggressive faculative pathogens can cause disease in such plants, and disease resistance of predisposed plants may be a component of winter hardiness.

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