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# Twenty-Four-Hour Induction of Freezing and Drought Tolerance in Plumules of Winter Rye Seedlings by Desiccation Stress at Room Temperature in the Dark[1]

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## ABSTRACT

**Exposure of seedlings of winter rye (*Secale cereale* L., cv. Puma) for 2 weeks or 24 hours to desiccation stress (40% relative humidity) at room temperature (21°C) in the dark induced degrees of freezing and drought tolerance in the plumules comparable to those produced by cold conditioning for 2 weeks at 3°C. The induction was associated with repression of growth and could not be produced in plumules excised from the seedlings indicating a requirement for translocation of nutrients from the endosperm. Rapid increase in osmotic pressure, soluble proteins, and phospholipids in plumules in association with the development of freezing and drought tolerance and the requirement of endosperm suggested diversion of nutrient from use in extension growth, to use in augmentation of protoplasm in plumule cells. Since cold acclimation slowed or arrested growth and is associated with augmentation of protoplasm, it is suggested that the common element in the induction of freezing tolerance by cold and drought is the necessity for producing a condition of augmented protoplasm and membranes in cells thus reinforcing a similar conclusion reached from seasonal studies on woody plants.**

A number of studies have established that freezing tolerance can be induced in plants by mere desiccation stress without exposure to low temperature (2, 4, 8, 10, 23, 24). During the course of studies of cold hardening of Puma winter rye seedlings to determine the extent of correlation of their dehydration tolerance with freezing tolerance we observed that the resistance of the cells of the plumules to plasmolysis injury had increased during the desiccation stress. In accordance with correlations which have been established between freezing tolerance and plasmolysis injury resistance (14), it was expected that the freezing tolerance of these cells would also increase and this was confirmed in preliminary studies on rye and wheat (6, 21).

The humidities used in these first desiccation experiments were in the range of 85% to 90%. The hardening response can be elicited over a much larger range of humidities extending even down to 40% RH, equivalent to a dehydrating stress of approximately 1000 atm osmotic pressure, without affecting survival of the seedlings. More significantly, freezing tolerance and extreme desiccation tolerance can be induced in only 24 h. In the present paper, we report the details of our findings both on the 2-week and the 24-h induction of freezing tolerance in plumules of winter rye seedlings by desiccation stress in 40% RH (21°C) in the dark and on processes and events related to this induction. We will attempt to show the significance of these results for the study of adaptation of plants to freezing.

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## MATERIALS AND METHODS

**Seedlings.** Winter rye (*Secale cereale* L., cv Puma) seedlings were used. Seed was washed with 2% NaOCl for 3 min and germinated by immersion of the seeds for 6 h in water and then spreading on moistened blotting paper in trays placed at 24°C. Sprouting was allowed to proceed for 2 days until the plumules had reached 5 to 7 mm.

**Desiccation Stress.** For the desiccation stress, the seedlings were transferred to open Petri dishes placed in a desiccator on a perforated steel plate just above a solution of $H_2SO_4$, the concentration of which has been adjusted to produce an atmosphere of 40% RH. Desiccations were allowed to proceed for 2 weeks or 24 h in the dark at 21°C.

**Test of Viability.** At the termination of the desiccation the seedlings were removed and reimbibed. Viability was assessed by regrowth of whole seedlings after planting in vermiculite or by regrowth of excised plumules incubated for 2 weeks at 3°C with shaking in White's salt solution (22) containing 2% sucrose (referred to as WS + S). In one experiment (Fig. 9), simultaneous greening of the epicotyl occurring during an extended incubation period under light was also used as an indication of survival. Viability was determined in some instances by vital staining of tissue slices with a 0.1% solution of neutral red and plasmolysis in 0.6 M balanced salt solution.

**Desiccation Tolerance Test.** Desiccation tolerance tests were performed only on excised plumules. These plumules had been excised from whole fresh seedlings (control) or from imbibed seedlings which had been conditioned previously either by the desiccation stress for 2 weeks or 24 h or by low temperature at 3°C for 2 weeks. Excised plumules were placed on Petri dishes and subjected to desiccation in 40% RH at 21°C. At the end of 10 days, viability of excised plumules was assessed by imbibition and regrowth of the plumules after immersion and incubation for 2 weeks in WS + S and by vital staining and plasmolysis.

**Plasmolysis Injury Resistance.** Resistance or tolerance to plasmolysis injury was determined by vital staining of cells after being subjected to a series of balanced salt solutions of final molarities ranging from 1 M to 5 M.

**Freezing Tolerance.** Seedlings and excised plumules were frozen in a controlled temperature freezing cabinet by gradual lowering of the temperatures at the rate of 1°C/h to −12°C at which temperature the samples were held for 12 h before being slowly thawed in a cold room at 3°C. Samples were ice seeded at −3°C

to prevent supercooling.

**Cold Conditioning.** Conditioning in the cold consisted of exposure to seedlings or plumules placed on moist blotting paper in covered trays to a temperature of 3°C for 2 weeks.

**Analyses.** Analyses on plumule for soluble sugars, soluble protein, and phospholipids were performed according to procedures described in previous publications (15, 17, 20). Values for these constituents obtained under the various conditions were expressed and compared on the basis of 10 plumules of equal dimensions (5 to 7 mm length). Osmotic pressures were determined by the incipient plasmolysis method in the balanced salt solutions described above.

## RESULTS

**Two-Week Desiccation Induction (21°C).** The progressive loss of water from plumules of Puma rye seedlings during the course of 2 weeks exposure at 21°C to an atmosphere of 40% RH is shown in Figure 1. Water content of plumules was reduced from approximately 85% initially to about 5% during this period. Appearance of the original seedlings and of the shrunken state of the desiccated seedlings at termination of the 2-week desiccation stress is portrayed in Figure 2. As indicated in Figure 2, exposure to 40% RH caused total repression of growth. Immersion and imbibition of these desiccated seedlings in tap water at 3°C for 2 days restored the seedlings to original water content and turgidity. After planting in vermiculite under lighting at room temperature, imbibed seedlings grew and leafed normally (Fig. 3). Directly after completion of imbibition of the desiccated seedlings in water at 3°C, seedlings could be redesiccated again at 40% RH for 2 weeks without any loss of viability. Viability in plumules of the desiccated seedlings could be demonstrated separately by excision from the seedlings and incubation in shaking flasks containing WS + S where, after 10 days, considerable regrowth occurred (Fig. 4). Regrowth of the rehydrated plumules was not different from that of plumules excised from control seedlings incubated under the same conditions. Thus, plumule regrowth was used as a test of recovery in subsequent experiments. Plumules of seedlings subjected to 40% RH for 2 weeks also exhibited freezing tolerance. While the whole seedling did not develop normally after thawing and planting, plumules excised from the thawed seedlings showed the same turgidity and regrowth potential of fresh normal plumules or of desiccation-stressed seedlings when incubated for 10 days at 3°C in WS + S (Fig. 5E). The viability of these plumules was confirmed also by vital staining and plasmolysis. Plumules excised from control seedlings which had no previous desiccation stress and after the seedlings had been frozen to −12°C and

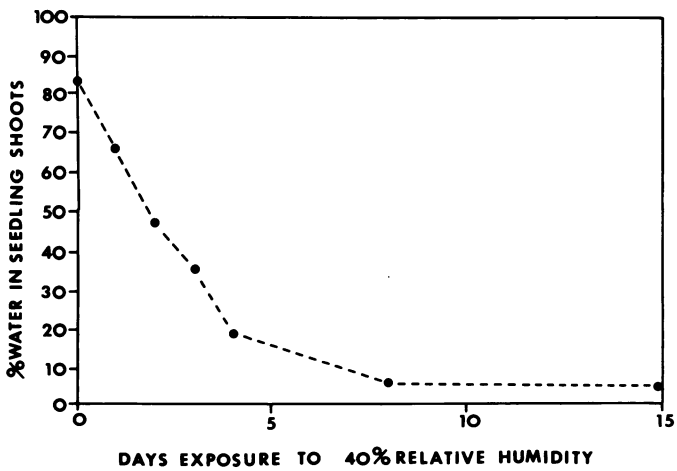


FIG. 1. Progressive loss of water (%) in seedlings of Puma winter rye during 2 weeks exposure to 40% RH at room temperature (21°C).

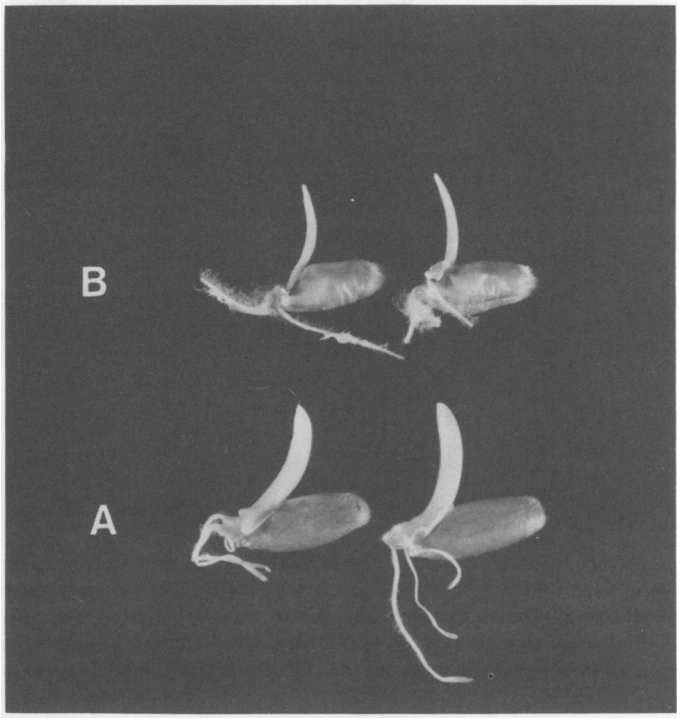


FIG. 2. Appearance of seedlings before (A) and after (B) 2 weeks desiccation in 40% RH.

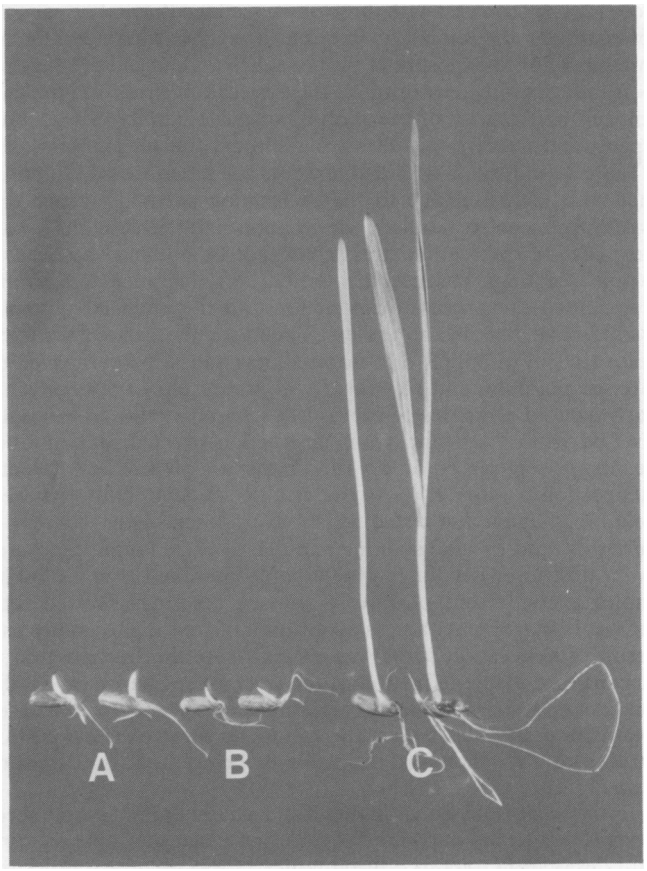


FIG. 3. Appearance of seedlings. A, Before desiccation; B, after desiccation; C, growth in 8 days of imbibed desiccated seedlings after planting in vermiculite.

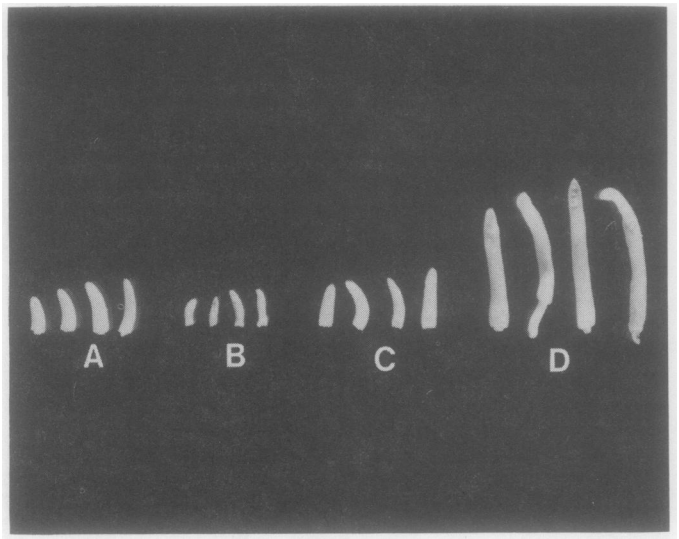


FIG. 4. Regrowth by incubation in WS + S of excised seedlings after desiccation stress for 2 weeks at 40% RH. A, Plumules excised from fresh seedlings; B, plumules excised from desiccated whole seedlings; C, plumules excised from desiccated imbibed seedlings; D, regrowth of plumules excised from imbibed desiccated seedlings after incubation for 2 weeks at 3°C in WS + S.

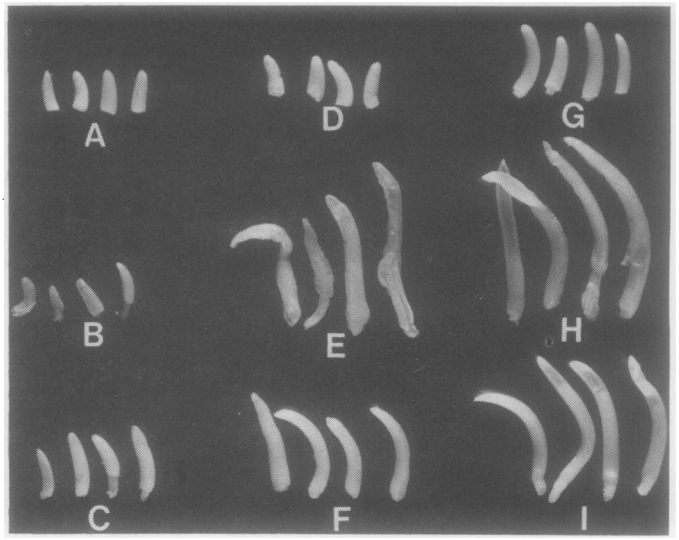


FIG. 5. Survival as determined by extent of regrowth in WS + S of plumules excised from fresh, from imbibed 2-week desiccation-conditioned (40% RH) and from 2-week cold-conditioned (3°C) seedlings after (a) freezing of these seedlings to −12°C and (b) after exposure of the plumules excised from these seedlings, to a second desiccation for 10 days at 40% RH. A, Plumules of fresh seedlings; B, lack of regrowth in WS + S of plumules excised from fresh seedlings after freezing of these seedlings to −12°C. C, lack of regrowth in WS + S of plumules excised from fresh seedlings and then desiccated in the excised state for 10 days at 40% RH; D, plumules excised from imbibed 2-week desiccation-conditioned (40% RH) seedlings; E, growth in WS + S of plumules excised from imbibed 2-week desiccation-conditioned (40% RH) seedlings after freezing of the seedlings to −12°C; F, growth in WS + S of plumules excised from imbibed desiccation (40% RH) seedlings and desiccated a second time in the excised state at 40% RH, for 10 days; G, plumules excised from 2-week cold-conditioned (3°C) seedlings; H, growth in WS + S of plumules excised from 2-week cold-conditioned (3°C) seedlings after freezing of these seedlings to −12°C; I, growth in WS + S of plumules excised from 2-week cold-conditioned (3°C) seedlings and desiccated a second time in the excised state at 40% RH, for 10 days.

thawed showed complete killing as indicated by failure to grow under the same culture conditions (Fig. 5B) and to stain vitally with neutral red or undergo plasmolysis.

Plumules of unstressed fresh seedlings failed to survive freezing to −12°C by contrast with those of stressed seedlings. The capacity of plumules for tolerating freezing to −12°C was therefore not present prior to exposure of the seedlings to desiccation stress but was induced only as a result of the desiccation stress and dependent on the integrity of the seedling and availability of the endosperm during the stress. Plasmolysis injury test on plumules cells (Table I) indicated that dehydration tolerance in salt solutions had also increased due to the desiccation stress. Evidence that tolerance to dehydration by desiccation had increased as a consequence of desiccation stress could not be obtained by tests on whole seedlings because the desiccation tolerance test simply constituted the process of induction and the attached plumules would have survived regardless. Therefore, the desiccation tolerance tests of plumules of fresh seedlings had to be performed on excised plumules and compared with the results of tests on similarly excised plumules of previously stressed seedlings. Plumules excised from fresh and from desiccation-stressed and imbibed seedlings were placed on dry filter paper and exposed to a second desiccation at 40% RH. At the end of 10 days, both sets were reimbibed again and tested for survival by regrowth, recovery of turgidity, and by vital staining and plasmolysis. Plumules exicsed from unstressed seedlings were completely killed (Fig. 5C). Plumules excised from previously stressed seedlings showed total survival (Fig. 5F). Although acquisition of freezing tolerance as a result of desiccation stress was evident from the freezing tests, a dependence of the induction of the freezing resistance on availability of endosperm could not be demonstrated because the excised plumules of unstressed seedlings would not survive the desiccation stress and were therefore incapable of being used for a freezing test.

**Two-Week Cold Conditioning.** Cold acclimation of whole seedlings (3°C) in the dark for 2 weeks, produced the same freezing and desiccation tolerance in plumules as was obtained under the desiccation stress conditions (Fig. 5, H and I). Whole seedlings and plumules survived and regrew after the freezing test. Plumules excised from cold-conditioned seedlings survived completely to −12°C as shown by regrowth tests (Fig. 5H). Plumules excised from cold-conditioned seedlings and subjected in the excised state to the desiccation tolerance test also survived (Fig. 5I). Plumules

Table I. *Survival (%) of Plumular Cells*

Survival was determined by vital staining of cells of plumules of fresh, imbibed 2-week and 24-h desiccation-conditioned (40% RH), 2 week conditioned (3°C) seedlings, and cells of excised plumules cold-conditioned at 3°C for 2 weeks after plasmolysis in balanced salt solutions ($NaCl/CaCl_2$, 9:1) of increasing molarity and deplasmolysis in 0.1 M salt solution.

| Cells of Plumules From | Molarity of Salt Solutions | | | | |
|---|---|---|---|---|---|
| | 1 M | 2 M | 3 M | 4 M | 5 M |
| Fresh seedlings | 90–100 | 10–20 | 0 | 0 | 0 |
| Imbibed 2-week desiccation-conditioned | 100 | 100 | 60–80 | 20–40 | 10–20 |
| Imbibed 24-h desiccation-conditioned seedlings | 100 | 100 | 60–80 | 20–40 | 10–20 |
| Two-week cold-conditioned seedlings | 100 | 100 | 40–50 | 40–50 | 10–20 |
| Cells from plumules cold-conditioned (3°C) for 2 weeks after excision from fresh seedlings | 50–70 | 0 | 0 | 0 | 0 |

excised from thawed, nonacclimated seedlings frozen to −12°C showed no viability in the regrowth test (Fig. 5B). Plumules excised from nonacclimated seedlings subjected to desiccation tolerance test also did not survive (Fig. 5C). Traces of growth exhibited in plumules of these seedlings (Fig. 5C) after 10 days of desiccation are spurious and probably due to differential swelling and emergence of the dead epicotyl during imbibition in the regrowth test because vital staining and plasmolysis tests directly after imbibition of the dried plumules showed no trace of living cells.

**Twenty-Four-Hour Desiccation Induction (21°C).** The detection of rapid increases in osmotic pressure and plasmolysis injury tolerance in cells of plumules of young seedlings within hours after exposure to 40% RH led to a study of the effects of a 24-h desiccation stress on induction of freezing and drought tolerance. Water loss from plumules over a period of 3 days to 40% RH and 20% RH are illustrated in Figure 6. Water losses in the first 24 h from plumules of whole seedlings and excised plumules (shoots) are not greatly different at 40% RH and 20% RH, but subsequent water losses from excised plumules exceed those from plumules of whole seedlings and the losses and the differences become more pronounced at 20° RH.

Seedlings reimbibed after 24-h desiccation at 40% RH recovered completely. Fresh and desiccation-stressed seedlings and plumules were subjected to freeze or desiccation tests. Whether excised from thawed seedlings after freezing to −12°C or rehydrated from the desiccated state, plumules of 24-h stressed seedlings recovered completely as shown in Figure 7, C and D. Plumules taken from frozen and thawed fresh seedlings or desiccated after being excised from fresh seedlings were killed (Fig. 7, A and B). Therefore, similar degrees of tolerance, as far as they were measured, to freezing and desiccation, were induced by 24-h and by 2-week desiccation stresses. Plumules of seedlings that were stressed at 20% RH for 24 h showed the same freezing and desiccation tolerance as those stressed at 40% RH. Osmotic pressure and amounts of soluble sugars, soluble proteins, and phospholipids increased in the plumule tissues during the first 24 h of desiccation even though the plumules had already undergone partial dehydration and ceased to grow (Table II).

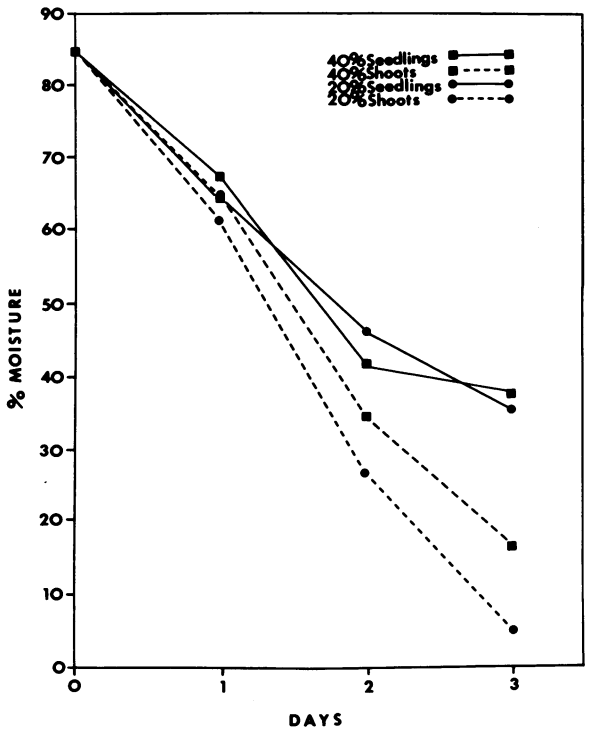


FIG. 6. Progressive loss of water (%) from plumules of whole seedlings and from excised plumules (shoots) during 3 days desiccation at 40% RH and at 20% RH.

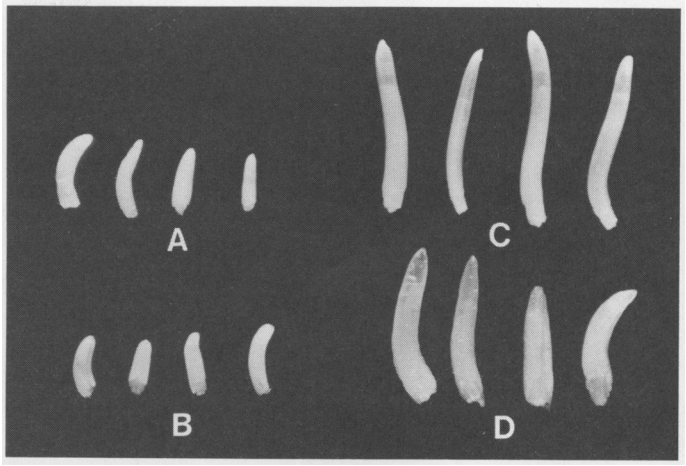


FIG. 7. Survival as determined by extent of regrowth in WS + S of plumules excised from fresh seedlings and from 24-h desiccation-conditioned seedlings (40% RH (a) after freezing of the seedlings to −12°C, and (b) after desiccation of the plumules excised from these seedlings for a second time for 10 days at 40% RH A, Lack of regrowth in WS + S of plumules excised from fresh seedlings and desiccated in the excised state for 10 days at 40% RH; B, lack of regrowth in WS + S of plumules excised from fresh seedlings subsequent to freezing of these seedlings to −12°C; C, growth in WS + S of plumules excised from imbibed 24-h desiccation-conditioned (40% RH) seedlings and desiccated a second time in the excised state for 10 days at 40% RH. Original dimensions of these plumules were equal to those shown in A. D, Growth in WS + S of plumules excised from imbibed 24-h desiccation-conditioned (40% RH) seedlings after freezing of the seedlings to −12°C. Original dimensions of these plumule were equal to those shown in B.

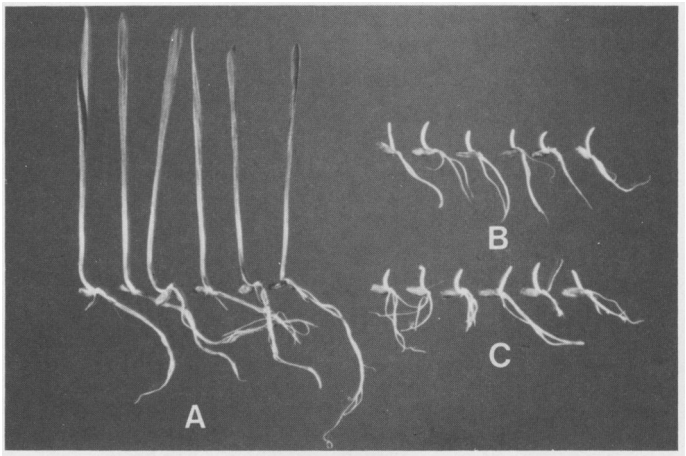


FIG. 8. Repression of growth of seedlings by desiccation stress and by cold-conditioning. A, Growth of normal seedlings at room temperature (21°C) in the light after 7 days planting; B, imbibed seedlings after 7-day desiccation at 40% RH at room temperature (21°C) in the dark; C, seedlings after 7-day cold-conditioning at 3°C in the dark.

**Two-Week Cold Conditioning: Whole Seedlings *versus* Excised Plumule.** Exposure of seedlings to 3°C produced nearly the same repression of growth as 40% RH, but over longer periods of time, growth ceases entirely at 40% due to extreme desiccation (Fig. 8). Fresh seedlings and excised plumules from fresh seedlings were cold-conditioned at 3°C in the dark for 2 weeks. Cells of these excised plumules showed the same viability as the plumules of intact seedlings as determined by vital staining and plasmolysis.

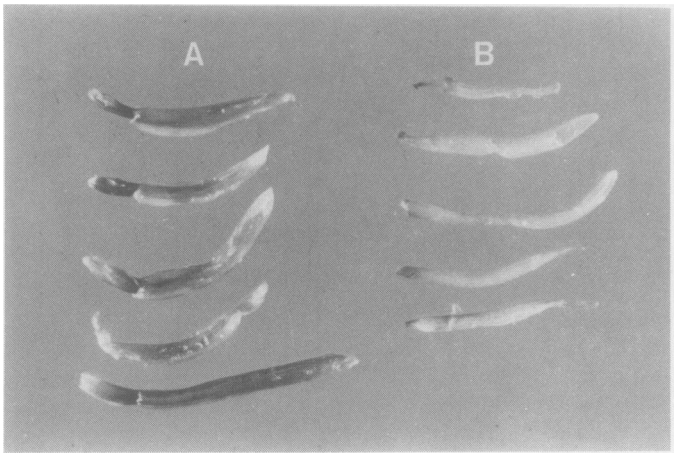


FIG. 9. Survival as determined by extent of regrowth and greening (dark aspect) of epicotyl in extended incubation (3 weeks) under light in WS + S of excised plumules frozen to −12°C after conditioning at 3°C for 2 weeks while (A) integral with seedling and (B) while in the excised state. A, Regrowth and greening in WS + S of plumules frozen to −12°C after excision from whole seedlings which had been subjected to normal conditioning at 3°C for 2 weeks. Original dimensions of plumules prior to freezing and incubation in WS + S were similar to plumules of cold-conditioned plumules shown in Fig. 5G. B, shrunken and bleached condition of WS + S incubated plumules killed by freezing to −12°C after being conditioned for 2 weeks at 3°C in the excised state only. Greater length of these plumules after incubation compared to other plumules killed by freezing in earlier experiments (Fig. 7B) not a consequence of incubation but due to persistence of some extension growth of plumules on moist paper while being conditioned in the excised state at 3°C previous to killing by freezing.

Table II. *Increases in Osmotic Pressure, Amounts of Total Soluble Sugars, Soluble Proteins, and Phospholipids (lipid-P) in Plumules of Puma Winter Rye Seedlings after 24 h Desiccation-conditioning at 40% RH at 21°C and in Plumules Attached to Seedlings but not when Excised from them after Cold-conditioning at 3°C for 2 weeks*

Values in table are duplicate analyses from tests and on, (a) plumules of fresh seedlings (control), (b) on plumules of seedlings imbibed after 24 h desiccation-conditioning in 40% RH, at room temperature, (c) on plumules of whole seedlings after these seedlings had been cold-conditioned at 3°C for 2 weeks, and (d) on plumules which had been just excised from fresh seedlings and then subjected to cold-conditioning at 3°C for 2 weeks in the excised state.

| Plumules from | Osmotic Pressure | Soluble Sugars | Soluble Proteins | Lipid-P |
|---|---|---|---|---|
| | *atm* | *mg/10 plumules* | | *μg/10 plumules* |
| Fresh seedlings (control) | 9.6–12.0 | 3.18 | 1.63 | 11.7 |
| | | 4.08 | 1.75 | 11.0 |
| 24-h Desiccated Seedlings | 14.1 ± 16.2 | 6.86 | 2.50 | 23.1 |
| | | 6.56 | 2.45 | 16.6 |
| Cold-conditioned at 3°C for 2 weeks while attached to seedlings | 16.8–19.2 | 13.19 | 2.25 | 26.3 |
| | | 14.72 | 2.30 | 22.0 |
| Cold-conditioned at 3°C for 2 weeks after excision from seedlings | 4.8–7.2 | 1.71 | 0.40 | 10.0 |
| | | 1.93 | 0.55 | 11.0 |

Plumules excised from the cold-conditioned seedlings and frozen to −12°C showed normal regrowth, greening of the epicotyl (dark color, Fig. 9), and normal staining and plasmolysis ability after thawing. Plumules exposed to cold conditioning in the excised state showed no regrowth or greening (Fig. 9) or capacity to take up neutral red. Plasmolysis tolerance, osmotic pressure, and amounts of soluble sugars, soluble proteins, and phospholipids increased in plumules of the intact seedlings during the cold-conditioning indicating augmentation, whereas in the excised plumules these tolerances and levels of the cell constituents had decreased (Tables I and II). Therefore low temperature conditioning of itself, without impairing viability, cannot induce freezing tolerance in cells of plumules of winter rye seedlings as long as these plumules are deprived of endosperm.

## DISCUSSION

It has been demonstrated that freezing tolerance in plumules and extreme tolerance to desiccation in both plumules and intact seedlings of winter rye could be induced by only 24 h of desiccation stress at room temperature in the dark. Since the seedlings experienced only a limited loss of water (Fig. 6) during this short period of exposure to desiccation, it is evident that even a mild dehydration stress is sufficient to evoke, within the limits of the freezing and desiccation tests, the same capacity in the plumule cells for withstanding freezing and desiccation as plumules of seedlings which had been exposed for 2 weeks to the same desiccation stress. The induction was dependent on availability of endosperm and failure of excised plumes was not due to more rapid water loss because the amount of water lost from plumules intergral with the seedlings and plumules that were excised was not greatly different in the first 24 h of desiccation (Fig. 6). Also the plumules of seedlings which were exposed to 20% RH and had undergone losses of water almost identical with those of excised shoots exposed to 40% in 24 h (Fig. 6) developed the same freezing and desiccation tolerance as plumules of seedlings exposed to 40% RH.

This dependence of the process of induction of freezing and desiccation tolerance in plumules on maintenance of the integrity of the seedlings and the achievement of the same induction in 24 h as in 2 weeks indicated that, whatever the requirement was for induction in the form of some factor or nutrient translocated from the endosperm, this requirement must have been satisfied even in the first 24 h. The translocation of sugars and of precursors from endopserm for protein and phospholipid synthesis in the plumule (Table II) is part of the normal processes of seedling development providing the necessary energy and substrates for extension growth of the shoot prior to the initiation of active photosynthesis. Under the circumstances of arrest of growth by dehydration but with some translocation unimpeded for 24 h at least, the carbohydrate energy sources and precursors for protein and phospholipid synthesis were not dissipated in extension growth in the plumule but were diverted instead into accumulation and synthetic processes in existing cells.

Data obtained on increases in soluble proteins and phospholipids, although limited, indicate that protoplasmic augmentation took place in cells of plumules of cold and desiccation stressed winter rye seedlings, by analogy with similar events in bark cells of woody plants (12, 13, 19, 25). Beyond the first few days of exposure to 40% RH, translocatory and synthetic processes would also be expected to cease so that any effects which these augmentative events would have on the properties of plumule cells would have to be completed in the first few days of dehydration. Augmentation in plumule cells, as a result of repression of growth, seems to be an underlying factor in the development of freezing and desiccation tolerance in these plumules as well as a common element contributing to hardening of these plumules by drought

stress of cold temperatures. Many indications of similar involvements in the acclimation of herbaceous plants to freezing have been obtained (3, 5, 7, 9) although the evidence of augmentation in herbaceous plants is still conflicting (11). Increases in osmotic pressure and soluble sugars occurring in rye plumules during desiccative stresses or cold conditioning cannot be excluded from consideration as part of the augmentative processes even in terms of an added supply of energy. Some protective effects can be ascribed to increases in osmotic pressure and soluble sugars but the effects which these augmentations might have on freezing tolerance are perceived here, on the basis of evidence obtained in studies of woody plants (13, 16, 18, 19) to derive principally from the effects of increases in the more structural components of protoplasm and membranes. The induction of freezing tolerance and also of desiccation tolerance in rye seedlings is subject to conditions similar to those that determine the development of freezing tolerance in mature plants with the important exception that, due to the availability of the endosperm, periods of illumination for photosynthesis are not required. The inductions can proceed fully in the dark whether produced by desiccation stress for 24 h or as shown here and elsewhere (1, 5) by cold conditioning for 2 weeks. The longer period required for cold conditioning of the seedling may reflect lower rates of translocation from the endosperm and lower rates of synthetic processes at the lower temperature. Whether the same degree of desiccation tolerance can be induced in mature rye plants as in the plumule of seedlings by cold conditioning is not known.

Intact seedlings cannot be fully acclimated by a 24-h desiccation stress to tolerate freezing and will not regrow after freezing to −12°C while similar seedlings conditioned at low temperature will withstand such freezing with impunity. There is a possibility that the meristematic cells of the whole seedlings did not undergo the desiccation stress induction of cold hardiness. Regardless, the inductions with desiccation stress at room temperatures do proceed fully in the plumules and the significance attached to protoplasmic augmentation observed in the plumules cells caused by repression of extension growth should be weighed only in the context of the effects which those augmentations might have on tolerance of plumule cells and not on the whole seedling. The viability of the cells of plumules of seedlings conditioned by desiccation stress after freezing to −12°C is just as great as the cells of plumules normally conditioned by low temperatures and similarly frozen to −12°C.

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