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Physiology of Oil Seeds. VIII. Germination of Peanut Seeds Exposed to Subfreezing Temperatures while Drying in the Windrow.[1] [2]

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ABSTRACT

During November 1976, freshly dug, high-moisture (30-40%) peanuts drying in the windrow in North Texas were exposed to subfreezing overnight temperatures for 6 days. The effects of that exposure on germination of the seeds were studied. Samples of the subsequently cured and hand-shelled peanut seeds were tested for germination, seedling emergence, ethylene and carbon dioxide production, and certain enzyme activities. Laboratory germination was 42%, greenhouse seedling emergence 32%, and most of the freeze-damaged seeds that germinated grew at a slow rate. Germination and greenhouse seedling emergence of controls were 96 and 100%, respectively. At their maximum rates, ethylene and carbon dioxide production by freeze-damaged seeds were reduced 83 and 36%, respectively. Mean enzyme activities measured from protein extracts of the freeze-damaged seeds were reduced, but they were not always significantly different from the control. However, isocitric lyase activity, which depends on *de novo* protein synthesis, was significantly less for freeze-damaged than for control seeds, particularly during initial stages of germination. Thus, low-temperature exposure of high-moisture peanut seeds interfered with the initial biochemical and developmental processes, such as synthesis of new proteins, that determine seedling growth.

Key Words: Seedling growth, ethylene, carbon dioxide, enzyme activities.

Plants of subtropical origin such as peanut (*Arachis hypogaea* L.) and cotton (*Gossypium hirsutum* L.) are injured by cool temperatures of about 0 to +10°C (1, 3, 10, 12). Christiansen (3) listed some effects of chilling on metabolic processes of cotton and other plant species. Although chilling and freezing injury are not the same, the structural changes in membranes that occur during chilling of sensitive plants probably also are involved in freezing injury (1, 10). Similar disruption of cellular functions could be expected for both types of injury.

Intracellular ice formation in living protoplasm destroys cellular integrity causing death. Plants that tolerate freezing acclimate by gradual cellular dehydration with extracellular ice formation in the vicinity of the cell walls (1, 13). Freezing injury results in membrane destruction (plasmalemma and organelle), denaturation of proteins, which play a major role in cell structure and function (1, 12, 13), and probably combinations of these with other factors that contribute to cell and tissue destruction. The manner in which plants are subjected to cooling temperatures as well as the subsequent warming rate determines the extent of survival (13). Too rapid cooling followed by slow warming is one means of freezing injury to plants (13). In November 1976, high-moisture (30-40%) peanuts drying in the windrow were exposed to such temperature changes during passage of a weather system from the North. Subfreezing, overnight temperatures for 6 days were followed by a gradual warming trend. This research was done to assess the effect of this exposure on germination, growth, and metabolic activity of the peanut seeds.

Materials and Methods

Materials.

Nicotinamide adenine dinucleotides (NADH and NAD), nicotinamide adenine dinucleotide phosphate (NADP) monosodium salt, D-glucose-6-phosphate monosodium salt, D-fructose-1, 6-diphosphate tetracyclohexylammonium salt, cis-oxalacetic acid (OAA), D,L-isocitric acid trisodium salt, α-N-benzoyl-D,L-arginine-p-nitroanilide • HCL (BAPA), phenylhydrazine • HCL cysteine • HCL, glutathione (GSH), glyceraldehyde-3-phosphate dehydrogenase, Trizma base, and Na_2HAsO_4 were obtained from Sigma Chemical Co.

Seed Material.

The peanut (*Arachis hypogaea* L. 'Tamnut 74') seeds were from freshly dug, high-moisture peanuts grown in North Texas in 1976 that were subjected, while drying in the windrow, to subfreezing overnight temperatures for 6 days. These seeds were supplied by Dr. C. E. Simpson, Associate Professor, Texas Agricultural Experiment Station, Texas A&M University, Stephenville, Texas 76401. Seeds grown in 1976, but not exposed to low temperature (designated Control in Table and Figures) were used for comparison. Samples of cured seeds were hand-shelled and tested for germination, seedling emergence, ethylene and carbon dioxide production, and certain enzyme activities as indicators of the metabolic capability of the seeds.

Germination and Ethylene and Carbon Dioxide Production Procedures.

Methods of germination and sampling for ethylene and carbon dioxide production have been described (5, 6, 7, 8). In these tests, 50 seeds per replication were germinated at 30 ± 1°C in 2-liter flasks fitted with two sheets of 15-cm diameter Whatman No. 5 filter paper. Seeds were imbibed in 50 ml of distilled water for 16 hours. Excess water was decated and 10 ml of distilled water readded to maintain moist conditions. Germination and categorization of the seeds for vigor (estimated by growth of the hypocotyl-radicle) were determined at 69 hours.

At about 21, 45, and 69 hours of germination, 10 and 1 ml samples of the gas phase above the seeds were taken for deter-

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[2]Mention of a trademark name or proprietary product does not constitute endorsement by the United States Department of Agriculture or Texas A&M University and does not imply its approval to the exclusion of other products that also may be suitable.

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mining ethylene and carbon dioxide production, respectively. A Beckman Model GC-4 gas chromatograph was used to determine ethylene by flame ionization and carbon dioxide by thermal conductivity (5, 6, 8).

Ten seeds per replicate were planted 5 cm deep in a mixture of vermiculite/agricultural vermiculite/sand 1:1:1 (v/v/v) in 21-cm diameter plastic pots to determine seedling emergence in the greenhouse.

Preparation of Protein Extract.

Cotyledons were randomly selected from several seedlings that were uniform in development for the time period assayed (16, 40, 64, 88 or 112 hours of germination). The seedcoats were removed, cotyledons trimmed to remove embryo tissue, washed briefly three times in distilled water, blotted to remove excess moisture, and cut into 1 to 2 mm slices. The slices from all cotyledons were pooled and about 2.0 g of material was weighed for protein extraction. Proteins were extracted in 20 ml of cold 0.1 M KH_2PO_4 buffer, pH 7.6, containing 10^{-4}M GSH by homogenizing for 1 minute, at a speed setting of 10, using a Sorvall Omni Mixer. The brei was squeezed through two layers of cheesecloth and centrifuged for 30 minutes at 27,000 x g in a Sorvall RC-2B refrigerated centrifuge at 0°C. Ten ml of the supernatant fraction was removed, made 95 to 100% saturated with $(NH_4)_2SO_4$ and placed at 0 to 3°C for 1 to 2 hours to precipitate the proteins. The $(NH_4)_2SO_1$ solution was centrifuged at 27,000 x g for 10 min at O°C, the supernatant discarded, and the pellet dissolved in 10.0 ml of cold 0.1 M KH_2PO_4, pH 7.6, was used as the source of enzymes. The enzyme source was kept on ice for the duration of the enzyme assays. Protein content was determined by Biuret.

Enzyme Assays.

Malate Dehydrogenase was assayed by the procedure of Ochoa (14). The reaction mixture contained in μmoles: 100, tris buffer, pH 7.5, containing 10^{-4}M GSH; 0.25, NADH; 1.0, OAA; 0.1 ml of 1:10 dilution of protein extract; and water to 3.0 ml.

Protease activity was determined by the procedure of Mainguy, *et al.* (11). The reaction mixture contained 2.5 ml of 0.5 mM BAPA in 0.2 M KH_2PO_4, pH 7.0, and 0.5 ml of protein extract.

Isocitric lyase activity was assayed by the procedure of Dixon and Kornberg (4, 15). The reaction mixture contained in μmoles: 200, KH_2PO_4 buffer, pH 7.6; 5, cysteine • HCL; 5, $MgSO_4$; 10, phenylhydrazine • HCL; 20, D, L-isocitric acid trisodium salt; 0.2 ml of protein extract; and water to 3.0 ml.

Fructose-1, 6-diphosphate aldolase was assayed by the procedure described by Benedict and Ketring (2). The reaction mixture contained in μmoles: 100, tris buffer, pH 7.5, containing 10^{-4}M GSH: 0.25 NAD; 50, Na_2HAsO_4; 10, D-fructose-1, 6-diP tetracyclohexylammonium salt; 1.0 mg of crystalline glyceraldehyde-3-phosphate dehydrogenase in a 2.7 M $(NH_4)_2SO_4$ suspension; 0.2 ml of protein extract; and water to 3.0 ml.

Glucose-6-phosphate dehydrogenase was assayed after the procedure of Kornberg and Horecker (9). The reaction mixture contained in μmoles: 20, tris buffer, pH 7.5; 40, $MgCl_2$; 0.6, NADP; 10, D-glucose-6-phosphate monosodium salt; 0.2 ml of protein extract; and water to 3.0 ml.

Statistical Analyses.

"Students" t-test was used to determine whether differences between mean values for the control and freeze-damaged seeds were significant. Data for greenhouse germination and seedling emergence are the means of five replicates of 10 seeds each for control and freeze-damaged seeds. Categorization of the seedlings for vigor (estimated by the length of the hypocotyl-radicle at 69 hours), and ethylene and carbon dioxide production are the means of two experiments with four replicates of 50 seeds each per experiment. Growth rate and enzyme analyses are the means of two experiments with 6 to 10 seeds used for each data point (hour of germination) per experiment. Each enzyme analysis was repeated twice per experiment.

Results and Discussion

Color of seedcoats was visibly lighter for intact, freeze-damaged than for control seeds. Cotyledonary surfaces were pitted for many freeze-damaged seeds, but were smooth for most control seeds. The exterior texture of freeze-damaged seeds differed from that of wrinkled immature seeds, but resembled that of overly mature seeds. Inspection of the cotyledonary surfaces at low magnification (10 - 20X) with the seedcoats removed confirmed the above observations. By examination of the radicles of both control and freeze-damaged seeds at 10 to 20X, the seeds were separated into two common categories: a "normal" appearing radicle which was rounded or somewhat pointed with a uniform, smooth surface; and an "abnormal" appearing radicle that was flattened with concave indentations. In a third category, found only among the freeze-damaged seeds, the radicle was "abnormal" in appearance and discolored from light to dark brown. For these observations, the seedcoats were removed and one cotyledon was detached from the embryo. These halfseeds (embryo with one cotyledon) were then tested for germination. Ten halfseeds from each category were placed on two layers of Whatman No. 5 filter paper in 9-cm petri dishes, 7 ml of distilled water was added and they were incubated at 30°C. The test was run twice. At 24 hours from the start of imbibition, less than 30% of the seeds in the "normal" appearing category and none of the seeds in the other two categories of freeze-damaged seeds showed signs of germination (radicle elongation). Also, 90% of the radicles of freeze-damaged seeds in all three categories were discolored from light to dark brown. For control seeds, germination was 100% in both categories and less than 25% of the radicles were discolored. Thus, the morphological appearance of the radicles of cured seeds from these two lots before imbibition was not a valid criteria of their germinability. However, discolored radicles or darkening of the radicles of cured seeds following imbibition was a distinct characteristic of these freeze-damaged seeds.

Further studies more completely defined the effects of exposure of high-moisture peanut seeds to subfreezing temperatures while they were drying in the windrow. The control seeds germinated 96% with 100% seedling emergence in the greenhouse and 72% of the seedlings grew rapidly (seedlings with a hypocotyl-radicle length greater than 20 mm in length) (Table 1). In contrast, the freeze-damaged seeds only germinated 42% with 32% seedling emergence and only 6% of the seedlings were capable of rapid growth (Table 1).

Previous reports showed that ethylene production by peanut seeds is a sensitive indicator of

Table 1. Effect of exposure to subfreezing temperatures on vigor, germination and emergence of 'Tamnut 74' peanut seeds.

Treatment	Extent of growth in mm of hypocotyl and radicle at 69 hr of germination (estimate of vigor), < 5	5-10	10-20	> 20mm	Germination	Emergence
	%					
Control	8a[1/]	4a	12a	72a	96a[1/]	100a[2/]
Freeze Damaged	14a	12a	10a	6b	42b	32b

1/ Data are means of 2 experiments with 4 replicates per experiment and 50 seeds per replicate. Germination was determined at 69 hr.

2/ Data are the means of 5 replicates of 10 seeds each planted in the greenhouse. Emergence was determined at 14 days. The same letter adjacent to each datum indicates no significant difference at the 0.01 level of confidence.

damage or deterioration of these seeds (6, 7, 8). Similarly, for freeze-damaged seeds with reduced germinability and vigor (Table 1), maximum ethylene production was reduced 83% and delayed 24 hours in comparison with control seeds (Fig. 1, left). This pattern of ethylene production was typical of Spanish-type peanut seed samples with a low proportion of rapidly growing seedlings (6, 7, 8). In agreement with these data, seedlings from freeze-damaged seeds had a significantly slower growth rate than the control seedlings (Fig. 1, right).

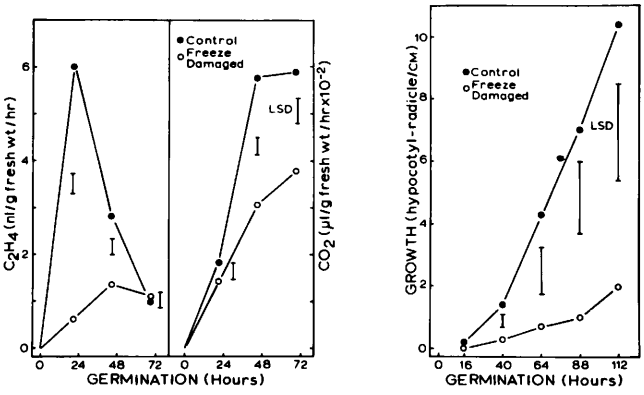

Fig. 1. Effect of exposure to subfreezing temperatures on ethylene production (left), carbon dioxide production (center), and seedling growth (right). LSD: Least Significant Difference.

At 69 hours of germination, respiration of the freeze-damaged seeds was only about two-thirds that of the control (Fig. 1, center). As a means to further assess the effect of freezing on metabolism of the seeds, a series of enzyme activities were assayed. All enzymes measured (except isocitric lyase) are present in protein extracts from dry seeds, but activities do not increase until seeds are imbibed. These proteins are synthesized during seed maturation and are possible sites of damage from freezing of high-moisture peanut seeds.

Malate dehydrogenase activity in protein extracts from freeze-damaged seeds declined until 88 hours but was not significantly less than activity for the control until 112 hours of germination (Fig. 2).

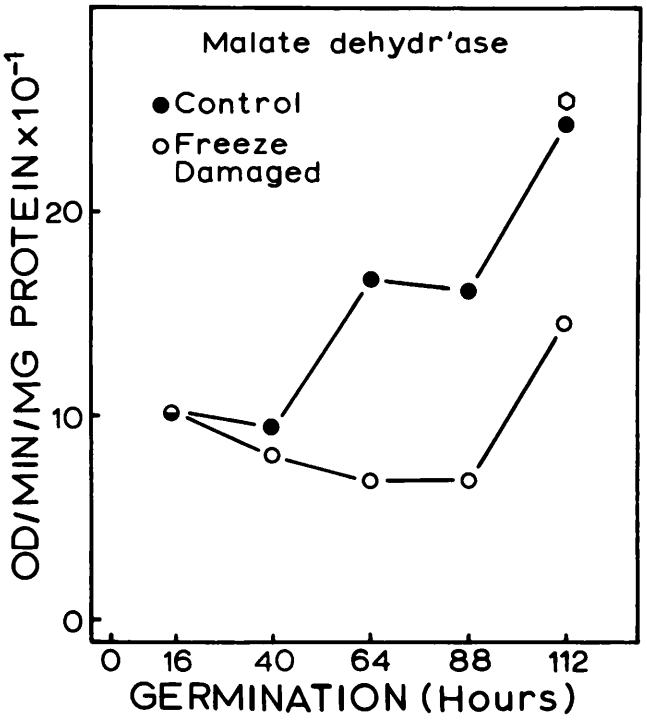

Fig. 2. Effect of exposure to subfreezing temperatures on the development of malate dehydrogenase activity during germination. The hexagon symbol (⬡) indicates a significant difference between data points at the 0.05 level of confidence.

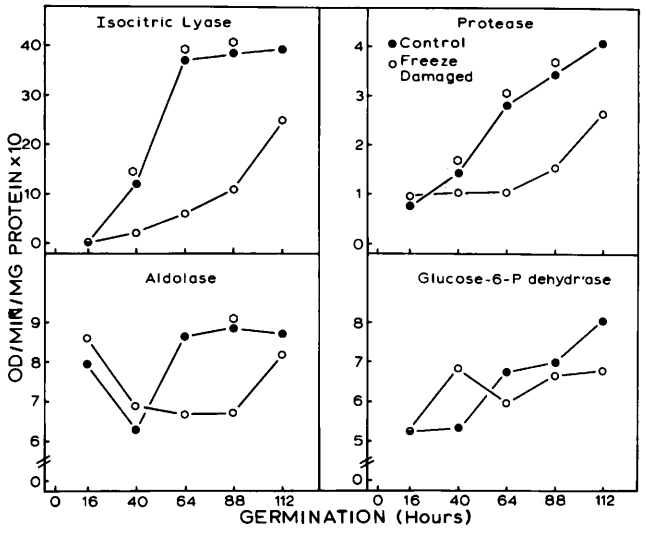

Fig. 3. Effect of exposure to subfreezing temperatures on the development of isocitric lyase (upper left), fructose-1,6-diphosphate aldolase (lower left), protease (upper right), and glucose-6-phosphate dehydrogenase (lower right) activities during germination. The hexagon symbol (⬡) indicates a significant difference between data points at the 0.05 level of confidence.

Fructose – 1,6 diphosphate aldolase had a similar pattern of activity (Fig. 3, bottom left). Glucose-6 phosphate dehydrogenase activity (Figure 3, bottom right) did not differ significantly between freeze-damaged and control seeds. In contrast, isocitric lyase and protease activities of freeze-damaged seeds were lower than those of control seeds during early hours of germination when the

seeds were initiating metabolic processes to provide energy and substrate for growth (Fig. 3, top left and right).

Enzyme activities from protein extracts of control and freeze-damaged seeds were equal at 16 hours of germination (Fig. 2 and 3). This suggests that the proteins present at the time of low-temperature exposure either were not affected by or were protected in some way from damage by freezing. However, except for glucose-6-phosphate dehydrogenase, new protein synthesis by freeze-damaged seeds apparently lagged, since enzyme activities declined or increased slowly until 88 to 112 hours of germination. This occurrence was particularly evident for isocitric lyase which is synthesized *de novo* in peanut and other oil seeds and had no detectable activity at 16 hours of germination (Fig. 3, top left). Also, at 88 to 112 hours of germination when enzyme activities of protein extracts from freeze-damaged seeds substantially increased (Fig. 2 and 3), there was a noticeable increase in growth rate of the seedling (Fig. 1, right).

The data suggest that exposure of high-moisture peanut seeds to freezing injured the protein-synthesizing system. This system is partially a membrane-bound sequence of biochemical reactions and membranes have been found to be damaged by freezing (1, 12, 13). Carbon dioxide production from mitochondrial activity also could have been reduced by damage to these membranes. The results were reduced ethylene production, germination, and seedling growth or complete loss of most of these functions by a majority of the seeds.

Because these seeds were exposed to subfreezing temperatures during a natural occurrence, the exact parameters (time of exposure, limits of seed moisture and temperature, and rate of rewarming) that would either prevent or cause freeze-damage to peanut seeds were not defined. But the data clearly indicate the types and extent of damage that were associated with freezing injury of peanut seeds.

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