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Induction of Freezing Tolerance in Spinach during Cold Acclimation[1]

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ABSTRACT

Spinach (*Spinacia oleracea* L.) seedlings, grown in soil or on an agar medium *in vitro*, became cold acclimated when exposed to a constant 5°C. Plants subjected to cold acclimation, beginning 1 week postgermination, attained freezing tolerance levels similar to that achieved by seedlings that were cold acclimated beginning 3 weeks after sowing. Seedlings at 1 week of age had only cotyledonary leaves, while 3-week-old seedlings had developed true leaves. Plants grown *in vitro* were able to increase in freezing tolerance, but were slightly less hardy than soil-grown plants. These results suggest that spinach, a cool-season crop that begins growth in early spring when subzero temperatures are likely, can undergo cold acclimation at the earliest stages of development following germination. Axenic seedlings, grown *in vitro*, were used to develop a noninjurious radiolabeling technique. Leaf proteins were radiolabeled to specific activities of 10^5 counts per minute per microgram at 25°C or 5×10^4 counts per minute per microgram at 5°C over a 24 hour period. The ability to radiolabel leaf proteins of *in vitro* grown plants to high specific activities at low temperature, without injury or microbial contamination, will facilitate studies of cold acclimation.

Spinach, a cool-season herbaceous species, becomes more tolerant to freezing upon exposure to low, but nonfreezing temperatures (5, 7, 8). Examination of the kinetics of freezing-tolerance induction during cold acclimation of spinach has revealed increased hardiness after only 1 d exposure to low temperature (5). Similarly, when cold-acclimated spinach was subjected to deacclimation, freezing tolerance began to decline during the first day's exposure to warm temperature. This rapid induction and loss of hardiness in spinach suggests it could be useful as a model system to study the metabolic changes that occur during cold acclimation and deacclimation.

Several model systems have been developed to facilitate the study of cold acclimation (2–4, 6, 11, 12). Most of these systems consist of callus cultures, cell suspensions, or protoplasts. These cellular based systems have been most useful in the study of membranes (6) and the role of ABA in freezing-tolerance induction (3, 4, 11). However, a major disadvantage with these cellular systems is that they may not show the same responses to environmental change or have the physiological capabilities possessed by an intact plant. To overcome this disadvantage, we have developed a model system using intact spinach seedlings, cultured *in vitro*, that can be useful in studies of metabolism associated with cold acclimation.

MATERIALS AND METHODS

Plant Materials. Spinach seeds (*Spinacia oleracea* L. cv Bloomsdale, Stokes Seed, Inc.) were disinfested and sown in a moist soil-mix (Metromix, W. R. Grace) or on an agar medium. Seeds were disinfested by immersing in 95% ethanol for 30 s, followed by vigorous stirring in 5.25% NaOCl for 5 min. The NaOCl solution was diluted with four volumes of sterile distilled water and stirred for 30 min. The NaOCl solution was removed and the seed was immersed in 0.01% NaOCl in sterile distilled water and stirred for 2 h. The disinfested seed was then germinated at 25°C. Emergent seedlings were grown on a light bench at 25±2°C or in a controlled environment chamber (Puffer-Hubbard, model CEC8VHL) at 25±1°C with a 12h photoperiod. Illumination was provided by cool-white fluorescent lamps on the light bench and by cool-white fluorescent lamps providing 60% input wattage and incandescent lighting providing 40% input wattage in the growth chambers. The average irradiance (400–700 nm) over the growing area at plant height was 186 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the light bench and 466 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the growth chamber. Seedlings grown in the soil mix medium were watered as needed with a Hoagland nutrient solution (9).

***In Vitro* Culture.** Surface-sterilized seed were germinated in foam-stoppered 2 × 9 cm vials containing 8 ml sterile inorganic medium solidified with 0.7% (w/v) agar. The agar medium was based on the nutrient formulation of Hoagland and Arnon (9). The nutrient medium consisted of the following ingredients in mg/L: $Ca(NO_3)_2 \cdot 4\ H_2O$, 500; KNO_3, 125; $MgSO_4 \cdot 7H_2O$, 125; KH_2PO_4, 125; Na_4EDTA, 37; $FeCl_3$, 25; $MnSO_4 \cdot H_2O$, 2.3; $ZnSO_4 \cdot 7H_2O$, 0.5; H_3BO_3, 0.5; $CuSO_4 \cdot 5H_2O$, 0.02; $NaMoO_4 \cdot 2H_2O$, 0.02; $CoCl_2$, 0.01. The nutrient medium pH was adjusted to 6.0. For long duration experiments, *in vitro* grown plants were irrigated with 0.5 ml sterile one-tenth Hoagland nutrient solution as needed to prevent dehydration and depletion of nutrients.

Cold Acclimation and Hardiness Assessment. Seedlings were cold acclimated in a growth chamber at a constant 5°C with a 12 h photoperiod. The average irradiance at plant height was 470 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with an abrupt transition from light to dark. The plants were cold acclimated for periods up to 14 d. Nonacclimated plants were maintained at 25°C for the duration of the experiment.

Freezing tolerance was determined by freezing intact plants in a controlled temperature bath (Forma Scientific model 2425). Seedlings, to be frozen, were removed from the soil or agar medium with as little damage to the root system as possible and

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immediately wrapped in tissue paper. Three to five plants were wrapped together and placed in a 2.2 × 22 cm test tube. Water was added to saturate the tissue paper and prevent the tissue from desiccating. Stoppered tubes were placed in the controlled temperature bath and equilibrated for 30 min at 0°C. Following temperature equilibration, a chip of ice was placed in contact with the tissue paper and the temperature lowered at a rate of 2°C h^{-1}. Tubes were removed at predetermined temperatures and kept at 4°C overnight. Leaf injury was determined by visual observation of the degree of tissue water soaking and the loss of turgidity and by measurement of electrolyte leakage. Leaf tissue was immersed in 10 ml distilled water and shaken at room temperature for 4 h. After the conductivity of the solution was measured, the samples were frozen at −80°C overnight, thawed, and the conductivity measured again. Freezing tolerance was calculated as the temperature (LT_{50})[2] that resulted in 50% tissue injury. Visual ratings and electrolyte leakage tests almost always gave the same LT_{50} values. For this reason, all LT_{50} values reported are those from the electrolyte leakage tests. All cold acclimation experiments were replicated at least four times.

Microbiology. Leaf tissue from soil or *in vitro* grown plants was aseptically weighed and homogenized with a sterile glass homogenizer in 2 ml sterile distilled water. Serial dilutions of the homogenate were plated out on nutrient agar plates (Difco Manual). The plates were incubated at room temperature for up to 7 d. Microbial populations were expressed on the basis of colony forming units per g fresh weight.

Radiolabeling Kinetics. Leaf proteins of *in vitro* cultured seedlings were radiolabeled at 5 and 25°C with [^{35}S]methionine and the kinetics of uptake and incorporation into protein determined. Sterile L-[^{35}S]methionine 1205 Ci/mmol (Amersham) was diluted with sterile distilled water containing 0.01% v/v Triton X-100 to a concentration of 1 μCi/μl. The day prior to addition of label, the plant was trimmed to a single leaf. To a single-leaf plant, 5 μl of the radiolabel solution was applied to the underside (abaxial) of the leaf and the plant was incubated at 5 or 25°C. At 1, 2, 3, 4, 6, 8, 12, and 24 h after application of the radiolabel, the leaf (average fresh weight 8.7 mg) was removed and homogenized in 200 μl buffer: 120 mM Tris-HCl (pH 6.8), 1 mM DTT, 1% v/v glycerol, 0.2% w/v SDS. The sample was boiled for 5 min, then centrifuged for 10 min at 15,000*g* at 4°C to pellet cell debris and the supernatant was analyzed for protein, total and TCA insoluble radioactivity. Duplicate aliquots of the sample (50 μl) were spotted onto Whatman GF/C filter disks. One filter disk was dried and the radioactivity determined by liquid scintillation counting. The other filter disk was washed with 10% cold TCA, 5% cold TCA, then twice with 95% ethanol, dried, and the radioactivity determined. Protein was determined by the Bradford method (1).

Gel Electrophoresis. Leaf proteins were extracted into buffer (120 mM Tris-HCl [pH 6.8], 20 mM DTT, 20% v/v glycerol, and 2% SDS). Samples containing 25 μg protein were applied to one-dimensional 10% polyacrylamide gels containing 1% SDS and subjected to electrophoresis with a constant current of 25 mA. The gels and buffer solutions were prepared as described by Laemmli (10).

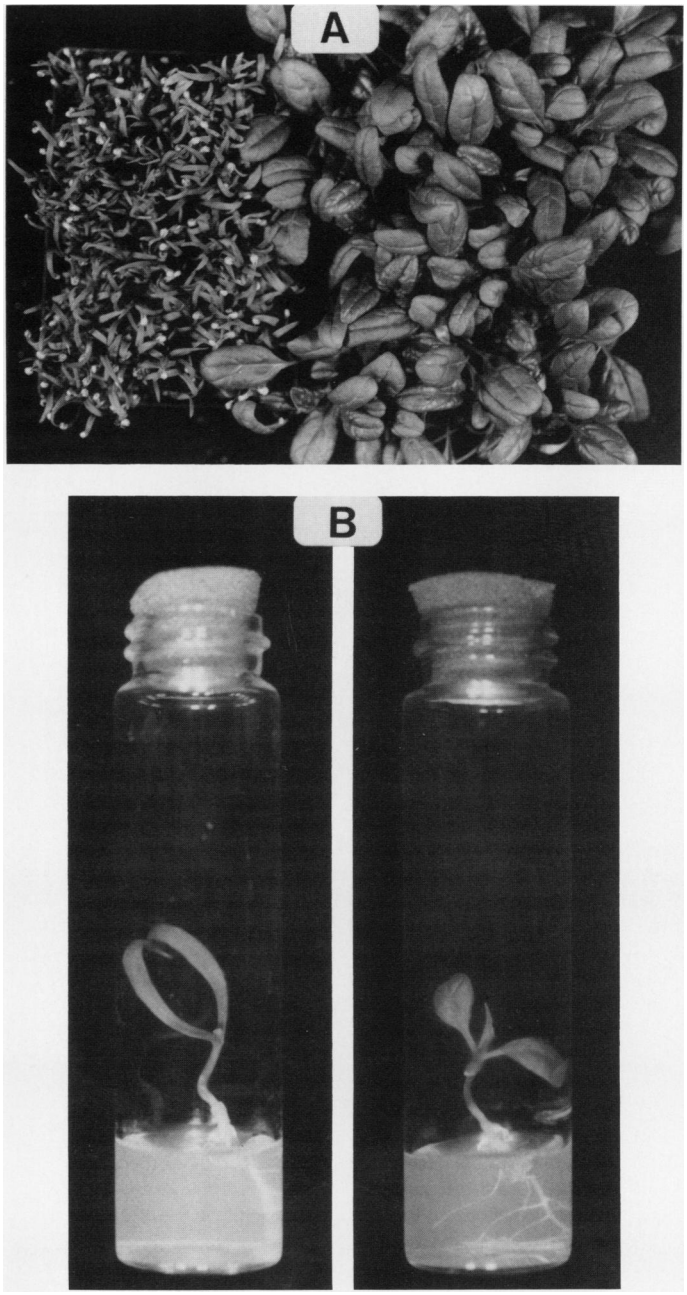

FIG. 1. Spinach developmental stages and growth conditions. A, Soil grown plants; B, *in vitro* grown axenic plants. Plants were grown for 1 week (cotyledonary stage) or for 3 weeks (true leaf stage). Plants grown *in vitro* had the cotyledons removed after the true leaves emerged.

RESULTS AND DISCUSSION

Examination of spinach seeds obtained from commercial sources revealed they were heavily infested with fungi and bacteria. In order to successfully culture seedlings *in vitro*, the microbial contamination had to be minimized or eliminated. A methodology was developed whereby spinach seed could be partially disinfested and germinated on an inorganic agar me-

Table I. *Microbiology of Soil Grown and in Vitro Cultured Spinach Seedlings*

Values are the mean and SE.

Growth Medium	CFU/g fresh wt	Replicates
Soil	$3.2 \times 10^4 \pm 1.5 \times 10^4$	7
Agar	$2.0 \times 10^1 \pm 2.0 \times 10^1$[a]	6

[a] Five of the six replicates did not contain detectable microbial populations.

[2] Abbreviations: LT_{50}, lethal temperature for 50% of tissue; CFU, colony forming unit.

Table II. *Freezing Tolerance of Cotyledons and True Leaves*

Plants were germinated and grown for 1 week (cotyledonary stage) or for 3 weeks (true leaf stage) at 25°C before exposure to 5°C for 7 d. LT_{50} values are expressed as mean ± SE.

Treatment	LT_{50} Values of Growth Stage and Growth Medium			
	Cotyledons		True leaves	
	Soil	Agar	Soil	Agar
	°C			
Nonacclimated	−2 ± 1	−3 ± 1	−5 ± 1	−4 ± 1
Cold acclimated	−10 ± 1	−7 ± 1	−9 ± 2	−9 ± 2

Table III. *Kinetics of Freezing Tolerance Induction during Cold Acclimation*

Seedlings cultured *in vitro* for 1 week or for 3 weeks were frozen and the hardiness of the cotyledons and true leaves was determined as described in the text. LT_{50} values are expressed as mean ± SE.

Treatment	Days	Cotyledons		True Leaves	
		LT_{50}	Replicates	LT_{50}	Replicates
		°C		°C	
Nonacclimated		−3 ± 1	5	−4 ± 0	4
Cold acclimated	2	−5 ± 1	4	−7 ± 1	4
	7	−8 ± 1	6	−8 ± 0	4
	14	−8 ± 2	3	−9 ± 2	4

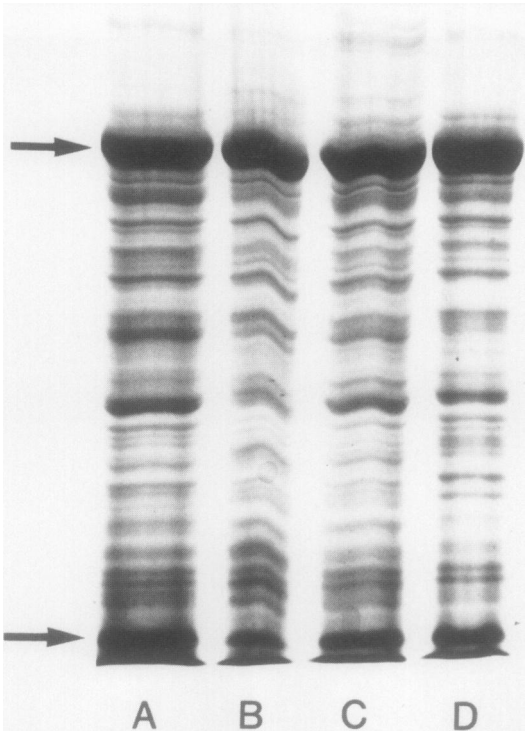

FIG. 2. Protein profiles of cotyledons and true leaves. Proteins were extracted from nonacclimated and cold-acclimated tissue. Plants were cold acclimated for 7 d at 5°C. Nonacclimated plants were kept at 25°C as described in the text. Proteins from true leaves: A, cold acclimated; B, nonacclimated. Proteins from cotyledons: C, cold acclimated; D, nonacclimated. Arrows point to the large and small subunits of ribulose bisphosphate carboxylase oxygenase.

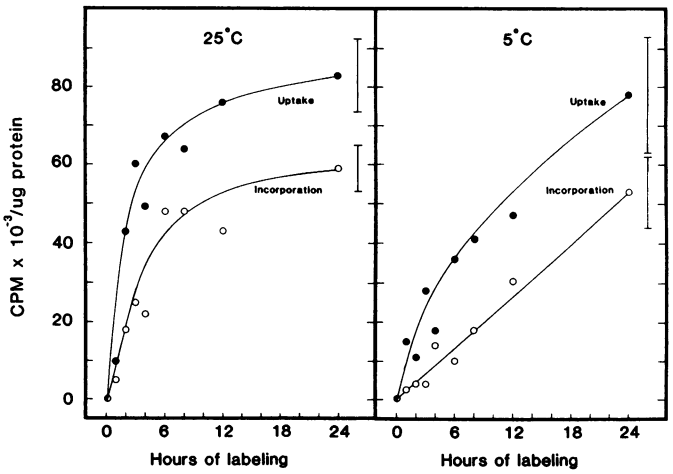

FIG. 3. Kinetics of [^{35}S]methionine uptake and incorporation. Axenic plants were trimmed to a single leaf and aseptically radiolabeled at 25 or 5°C by placing 5 μl of sterile [^{35}S]methionine (1 μCi/μl) in 0.01% Triton X-100 on the abaxial side of the leaf. The vertical bars represent the maximum SE of the mean for each treatment.

dium (Fig. 1). However, the microbial contamination was present on both the outside and inside the seed coat and resisted the disinfestation procedure. In a typical experiment where 500 seeds were germinated on agar, about 80% of the seedlings would be visibly contaminated. These plants were discarded. Approximately 15% of those seedlings not visibly contaminated had bacterial populations (ranging from 10^1 to 10^8 CFU/g fresh weight), while 85% of the seedlings had no detectable populations. Seedlings grown on agar that were not visibly contaminated, on the average, supported microbial populations roughly a thousand-fold lower than seedlings grown on soil mix in the laboratory (Table I). With proper sample replication the confounding effects of the low level microbial contamination were eliminated without necessitating determination of the population levels for each experimental plant. We did not determine whether the microorganisms were on or in the leaf tissue, but only their presence. The fact that a small percentage of seedlings could support a high bacterial population, appear normal, and still acclimate to cold, points to the need to determine population levels associated with soil and *in vitro* grown plants.

Soil-grown spinach seedlings, 1 week of age, were able to cold acclimate when subjected to a constant 5°C (Table II). Plants that had developed true leaves, 3 weeks postgermination, acclimated to the same levels of freezing tolerance as the 1-week-old plants having only cotyledonary leaves. The protein profiles of the cotyledons and true leaves are basically similar with both having high amounts of presumptive large and small subunits of ribulose bisphosphate carboxylase (Fig. 2). This suggests that the spinach cotyledons function as photosynthetic organs until true leaves can develop and become functional. As a photosynthetic organ, the cotyledons would be vitally important in the establishment of the young seedling in the early spring. Thus, it is not surprising that cotyledons and true leaves would have similar levels of freezing tolerance in spite of their substantial structural and biochemical differences. Furthermore, *in vitro* cultured seedlings with true leaves were able to cold acclimate to hardiness levels equivalent to that of soil grown plants. The cotyledons of *in vitro* grown plants, however, were not as hardy following cold acclimation as the cotyledons from soil grown plants. Nevertheless, true leaves and cotyledons from *in vitro* cultured seedlings did show freezing tolerance induction kinetics (Table III) similar to that of older soil grown plants (5, 8). In this study, the hardiness levels observed for cotyledons and true leaves from both soil-

and agar-grown seedlings are comparable to those observed by others using leaf tissue from much older spinach (5, 7). This suggests that spinach is capable of cold acclimation at the earliest stages of growth. Spinach is a cool-season crop that begins growth early in spring when temperatures are cool and frosts are likely. Therefore, it would be valuable for very young seedlings to be able to cold acclimate to avoid the likelihood of injury or death caused by freezing. Additionally, this ability is expressed when the plants are grown on an agar medium *in vitro*. The fact that small, young plants can be grown on a defined medium and still retain the capacity to cold acclimate makes it possible to begin radiotracer studies of the biochemistry of cold acclimation. These studies can be accomplished without relying entirely on protoplasts or cell suspension cultures and will more closely approximate processes occurring in whole plants as they undergo cold acclimation.

When a solution of [^{35}S]methionine was applied to the abaxial side of a leaf of *in vitro* cultured plants, the methionine was readily absorbed into the tissue and incorporated into protein. Uptake and incorporation of radiolabel, as expected, was much more rapid at 25°C than at 5°C (Fig. 3). This difference could be simply explained by a number of mechanisms: lower metabolic and protein synthesis rates, altered methionine pool size, or reduced membrane transport. Another explanation could be that greater photosynthetic activity at the higher temperature depleted intracellular CO_2 and caused the stomata to open earlier or for longer periods than at the lower temperature. Alternatively, exposure to low temperatures has been shown to lead to increased ABA content (4) which may cause the stomata to remain closed for longer periods. The plateau observed in the uptake and incorporation of label at 25°C was a function of the labeling technique and not a physiological phenomena. The methionine solution applied to the leaf at 25°C evaporated to dryness within 8 h after which no additional uptake was possible, while the same volume of solution added to a leaf at 5°C did not evaporate over a 24 h period due to the higher humidity inside the vial at low temperature. As a result, the leaf labeled at low temperature continued to absorb radiotracer up to 24 h and required about three times as long to take up and incorporate about the same amount of methionine as a leaf at 25°C.

In summary, we present evidence that spinach seedlings can cold acclimate at very early stages of growth and that plants cultured *in vitro* on an inorganic nutrient medium retain the capacity to cold acclimate. Furthermore, leaf proteins of *in vitro* cultured axenic plants can be radiolabeled to high specific activities at low temperature without injury or complications caused by epiphytic microorganisms. In the next communication we use these techniques to study leaf protein metabolism during cold acclimation.

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