Electrophoretic and Immunological Comparisons of Soluble Root Proteins of Medicago sativa L. Genotypes in the Cold Hardened and Non-Hardened Condition[1]

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Summary. Electrophoretic and immunological properties of the soluble root protein complement of 6 *Medicago sativa* L. genotypes in the cold hardened and non-hardened physiological condition were compared. These 6 genotypes were chosen to represent a range of abilities to survive exposure to subfreezing temperatures when in the cold hardened condition.

A zone of highly charged and/or low molecular weight protein components were found to be more prevalent in the protein complements of the cold-hardened material than the non-hardened material.

Immunodiffusion plate tests were not so definitive as the electrophoretic patterns for identifying the genotypes or physiological conditions, but did corroborate the electrophoretic interpretations.

The intimate association of proteins, either as components of enzyme systems or the structural organization of cellular organelles, with cellular metabolism implicates the important role which soluble proteins may have in the physiological adaptation of plants to environmental stresses. Mertz and Matsumoto (3) observed changes in electrophoretic patterns of soluble proteins of leaf cytoplasm of alfalfa plants when subjected to the physiological stress of nutrient deficiencies. Perry (6) reported distinctly different banding patterns of acetone powder extracts from active and dormant tissue of *Pinus thunbergii* L. Briggs and Siminovitch (1) related variations in the electrophoretic patterns of the water soluble proteins of black locust bark to the development of cold-hardiness in this tissue.

It is generally accepted (2) that the ability of plants to undergo the cold-hardening process is genetically controlled. This coincides with current concepts of gene-protein relationships. The increased sensitivity of gel electrophoresis and immunochemical tests in separating cytoplasmic protein components of plants should provide more definitive information than moving-boundary electrophoresis concerning changes in the protein complement that may be associated with the physiological adaptation of a plant during cold hardening.

This paper reports comparative gel electrophoretic patterns and immunochemical relationships of the soluble root protein complement from alfalfa plants which have specific genetic capacities to undergo cold-hardening.

Materials and Methods

Six *Medicago sativa* L. genotypes, representing various levels of ability to develop cold resistance, served as the experimental material. Vegetative cuttings were made from the 6 plants. After rooting and establishment in pots, 50 plants of each genotype were transplanted to the field in June 1963, and 50 plants were retained in the greenhouse. Plants retained in the greenhouse, harvested as needed, provided the non-hardened plant material. The plants grown in the field were removed on December 9, 1963 and immediately stored at −30°. These plants provided the cold hardened plant material. Comparative tests of cold hardened material that had not been frozen at −30° with frozen material showed no alteration of the electrophoretic or immunochemical tests due to freezing.

The top 10 cm portion of the root was washed in a water-ice mixture, rinsed in distilled water, cut into discs, and dropped immediately into cold 0.1 M Tris-maleic acid buffer; to this was added 2 mM cysteine-hydrochloride and adjusted to pH 7.0

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with KOH. The root discs were homogenized in a Waring blender, filtered through parachute silk, and centrifuged at 2000 × *g* for 20 minutes to remove cell debris. This supernatant fraction was centrifuged again at 60,000 × *g* for 20 minutes to remove cell organelles. The supernatant material was then dialyzed through 5 changes of buffer, the final medium was 0.01 M Tris-maleic buffer adjusted to pH 7.0. The dialyzed protein suspensions were concentrated with polyethylene glycol (Carbowax 4000) to a final concentration of approximately 2.5 mg of protein N per ml, as determined by the micro-Kjeldahl method.

Electrophoresis was carried out in polyacrylamide gel, according to the procedure outlined by Ornstein (4). The sample gel consisted of a 0.1 ml mixture of protein suspension and concentrated sample gel. Electrophoretic runs were made at 1.67 ma per tube for 1 and one-half hours, or until the tracking dye approached the lower end of the tube. Proteins in the gel column were stained with Amido Schwartz (1 g/200 ml of 7.5 % acetic acid).

Antisera to the protein suspensions of M 5-44 and C-630 genotypes, representing the extremes in sensitivity to subfreezing temperatures, were prepared in rabbits. The animals were injected intramuscularly with 0.5 ml of the appropriate protein suspension; which contained 7.5 mg of protein N, emulsified in 0.5 ml of Complete Freund adjuvants. The animals were given booster injections of the same protein-adjuvant mixture 10 and 50 days after the initial injection. The animals were bled 10 days after the last injection to provide immune sera. Double diffusion plate tests in 2 dimensions were carried out as described by Ouchterlony (5).

Results

Electrophoretic Comparisons. A schematic diagram of the general banding pattern of the gel columns is shown in figure 1. All the stained bands represent anodic moving proteins at a pH maximum of 9.5. An anomaly invariably present at the leading edge of the column was arbitrarily referred to as group A. Two or 3 bands immediately behind this anomaly are referred to as group B. Four bands midway in the column were usually the most dense and are referred to as group C. Five bands were generally found in the upper portion of the column and are referred to as group D. Three thin, distinct bands were present in the upper most portion of the column, and are referred to as group E.

Groups referred to above were common to all 6 genotypes but the number and location of the bands varied among genotypes and the 2 physiological conditions. From 17 to 20 protein bands, excluding the anomaly, were observed in the columns. The patterns were precisely reproducible with aliquots from the same extraction as well as replicated extraction of the genotypes in either the cold hardened or non-hardened condition.

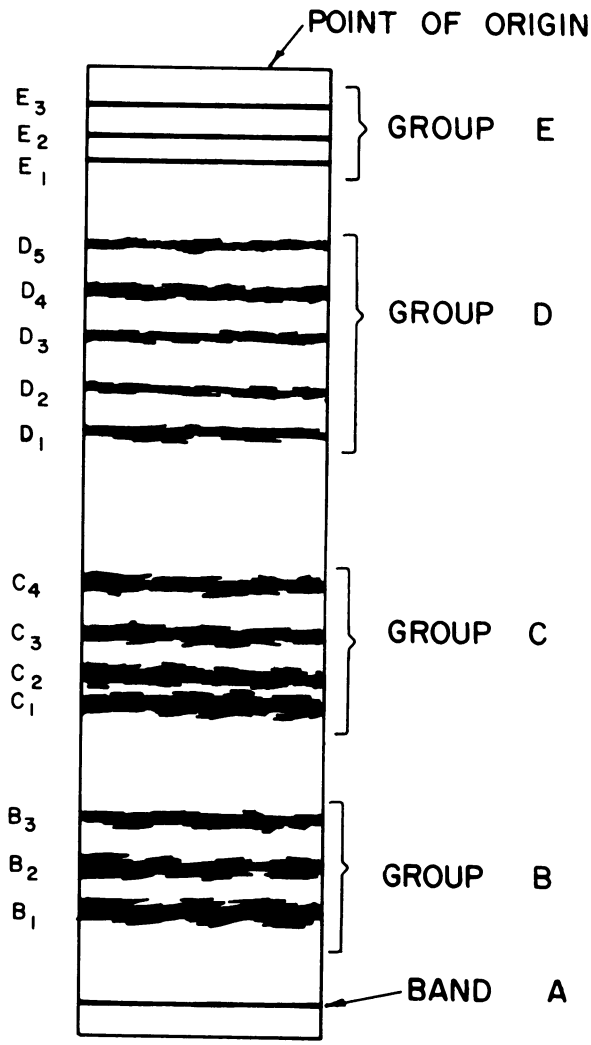

FIG. 1. Schematic diagram of general banding pattern of electrophoresis gels of alfalfa root proteins.

The gel columns shown in figure 2A represent the electrophoretic pattern of the soluble proteins from the roots of genotype M 5-44 in the cold-hardened (II) and non-hardened (I) condition. This genotype is severely injured or killed by exposure to overwintering conditions which freeze the soil. The gel columns represent the electrophoretic pattern of the soluble proteins from the roots of genotype C-630 in the cold-hardened (II) and non-hardened (I) condition (fig 2F). This genotype is capable of withstanding severe overwintering temperatures without any visible damage to the plants the following spring.

The gel columns shown in figure 2B, C, D, and E represent the electrophoretic pattern of the protein complement of genotypes capable of developing in-

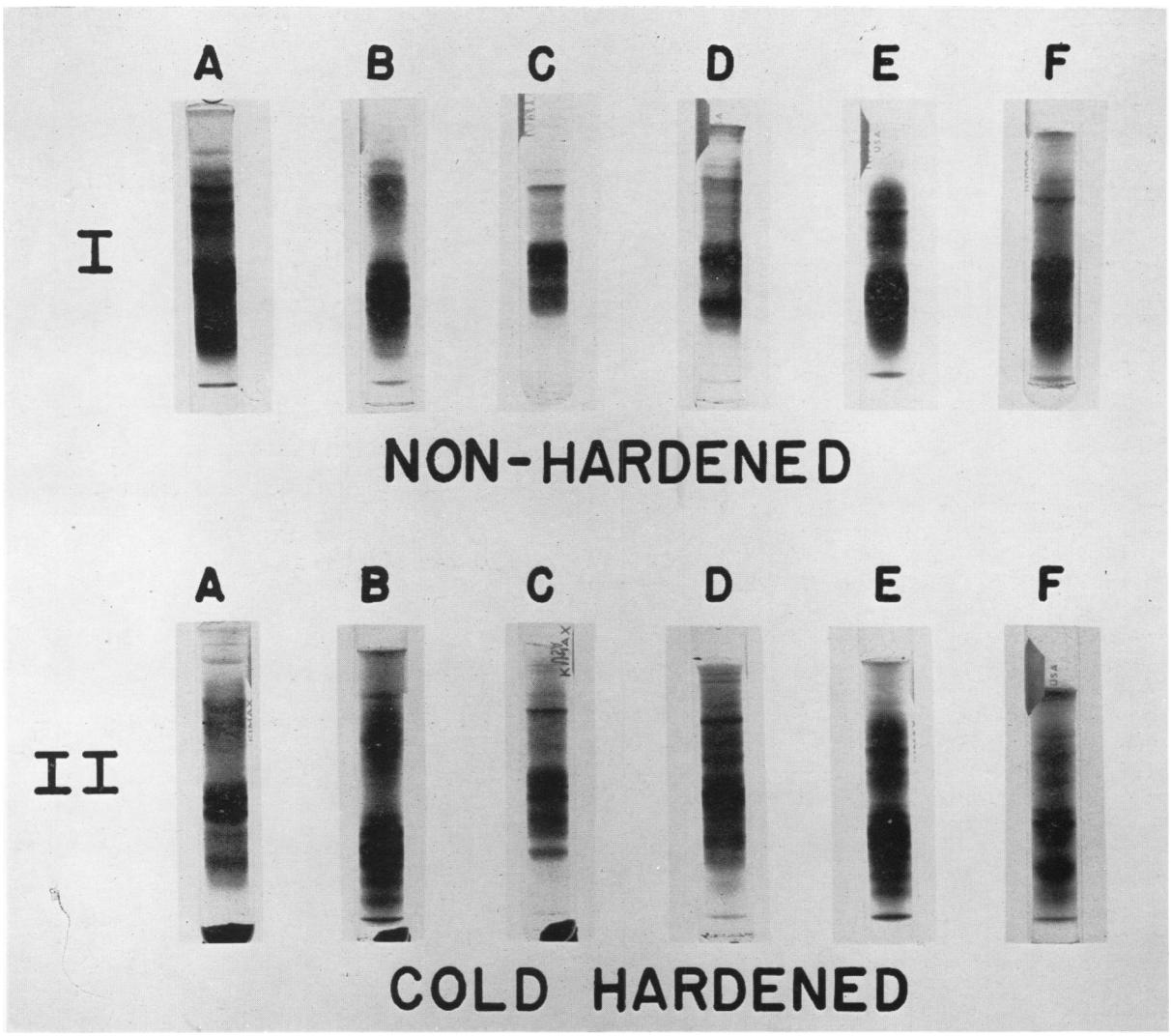

FIG. 2. Gel electrophoretic patterns of protein complements of 6 alfalfa genotypes arranged in increasing abilities to develop cold hardiness from left to right, A to F, genotypes M 5-44, 54-30, 61-238, M-589, 48-55, and C-630, respectively; I, non-hardened condition, II, cold hardened condition.

Table I. *Number of Similar (S) and Different (D) Antigen-Antibody Precipitin Lines Apparent in the Immuno-Diffusion Plate Tests Comparing Genotypes M5-44 and C-630 in the Cold Hardened and Non-Hardened Condition*

	Antigen from:							
	M5-44				C-630			
	Hardened		Non-hardened		Hardened		Non-hardened	
	S	D	S	D	S	D	S	D
Antiserum produced from:								
M5-44, hardened	6	—	4	2	3	2+		
M5-44, non-hardened	5	1	6	—			5	1
C-630, hardened	3+	1+			5	—	3+	2
C-630, non-hardened			5	1+	3	1+	6	—

termediate levels of hardening to the extremes, genotypes M 5-44 and C-630.

The most striking difference in the electrophoretic patterns of the protein complements of the roots in the cold-hardened and non-hardened physiological condition was the more intense B_1 protein band evident in the protein complement of the cold-hardened material. This band appears to be present in the protein complement of the non-hardened material but in lower quantity. A second conspicuous characteristic of the electrophoretic patterns of the cold-hardened material was the presence of distinct protein bands in the E groups.

Immunological Comparisons. The immunodiffusion plate tests comparing the antigens and corresponding antisera produced with the root protein complement of the 2 clones are summarized in table I. Five precipitin lines of the double diffusion plate tests appeared to be common to the protein complements of both genotypes in the non-hardened condition. Comparative double diffusion plate tests of the protein complements of these 2 genotypes in the cold-hardened condition showed 3 precipitin lines common to both genotypes. At least 2 precipitin lines evident in the antigen of C-630 in the cold-hardened condition were not evident in the antiserum of M 5-44 in the cold-hardened condition.

Discussion

The electrophoretic patterns of the soluble root protein complement of non-hardened alfalfa showed a zone of highly charged and/or low molecular weight protein components in trace amounts. This zone was more highly concentrated and definitive when the roots were in the hardened condition. It seems plausible that the protein components present in this region of the electrophoretic pattern reflect the degree to which a plant has been cold-hardened.

Another striking characteristic of the electrophoretic patterns of the protein complement from the cold-hardened material was the more definitive banding of the protein complements. Considerable tailing of the protein bands was observed in the electrophoretic patterns of the protein complement from the non-hardened material.

The immunodiffusion plate tests did not identify the soluble protein complements of the genotypes or physiological conditions as distinctly as the electrophoretic patterns. However, these tests did confirm protein complement similarities and differences as in the electrophoretic interpretations. It is possible that if selected components of the total protein complement were used to produce the antiserum, the immunological tests would have been more informative. The double diffusion plate tests confirmed the presence of antigens, in the cold-hardened condition which were not present in the non-hardened condition. If these antigens were present in the protein complement of the non-hardened plant, they were present in insufficient quantities to elicit adequate antibody reactions.

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