Plant Physiol. (1967) 42, 895–899

PLANT PHYSIOLOGY

Vol. 42 No. 7

July 1967

# Soluble Proteins in Alfalfa Roots as Related to Cold Hardiness[1, 2]

Eldean D. Gerloff,[3, 4] Mark A. Stahmann, and Dale Smith

Department of Biochemistry and Department of Agronomy, University of Wisconsin, Madison, Wisconsin 58706

Received February 13, 1967.

*Summary.* Soluble proteins extracted from alfalfa roots of hardy and nonhardy varieties were studied in relation to cold hardiness with polyacrylamide gel electrophoresis and quantitative enzyme analysis. Soluble protein content of alfalfa roots increased during hardening in all varieties. Two new isoenzymes with peroxidase activities were found in the fully hardened samples but no large shifts in the electrophoretic pattern were detected with polyacrylamide gel electrophoresis. Peroxidase and catalase activities increased during hardening in all varieties, but only small differences among hardy and nonhardy varieties were detectable. The studies indicated that protein metabolism was altered during the hardening process.

Soluble proteins have been studied and appear to be closely associated with cold hardening in plants (1, 2, 3, 12, 13). Siminovitch and Briggs (12) reported increases in soluble protein content during hardening in the bark of the black locust tree. Electrophoretic analysis indicated that an increase in 2 out of 5 soluble protein components occurred prior to hardening (1). Wilding et al. (13) demonstrated an increase in free amino acids and nonamino acid nitrogen as hardy alfalfa plants gained cold resistance. The nonhardy alfalfa plants showed little change in both nitrogen fractions as hardiness developed. Wilding et al. (13) suggested that specific increases in some of the free amino acids might have resulted from changes in protein synthesis as cold hardiness developed. An increase, as well as a shift, in proteins may have been responsible in part for cold hardiness and the protection of plant cells against the damage from low temperatures.

The present study was conducted to more clearly characterize differences that occur in soluble proteins during hardening. The enzymes studied are some that have been implicated in other plant-stress interactions. Gerloff (7) previously was unable to detect differences in the soluble proteins of hardened and nonhardened alfalfa root tissue with diethylaminoethyl cellulose chromatography or with urea-starch gel electrophoresis.

## Materials and Methods

*Materials.* Three bacterial wilt-resistant alfalfa varieties that differed in hardiness, nonhardy Caliverde (C), intermediately hardy Buffalo (B), and very hardy Vernal (V), were seeded in rows 18 inches apart during mid-May at Madison, Wisconsin, in 1961, 1962, 1963, and 1964. The plants were weeded and sprayed for as needed throughout the growing season. The top growth was never cut but allowed to develop normally into the autumn. Samples of approximately 500 to 1000 roots of each variety were dug from the soil each year in mid-August (A), mid-October (O), and late November, hereafter referred to as December (D). The roots harvested in August and October were washed free of soil and the 4-inch section just below the cotyledonary node was frozen in liquid nitrogen, placed in a plastic bag and stored at −20° until analysis. The samples taken in late November were sampled in a different manner (8). The plants were harvested, washed free of soil, and the top growth removed, leaving the crown and root intact. The plants were wrapped in cheesecloth, moistened with tap water, covered with waxed paper, and placed

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] This work was supported in part by grants from the Herman Frasch Foundation for Agricultural Research.

[3] The data are taken from a thesis by E. D. Gerloff submitted to the Graduate Faculty of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. Degree.

[4] Present address: Crops Research Division, Agricultural Research Service, United States Department of Agriculture, Northern Grain Insects Research Laboratory, Brookings, South Dakota.

in a freezer at −2.5° for approximately 1 month to develop a higher degree of hardiness. Every 2 weeks, the plants were removed from the freezer, the cheesecloth peeled away, and air blown across the roots to remove gaseous products of respiration. At the end of the artificial hardening period, the plants were removed and the 10 cm root section just below the crown was frozen in liquid nitrogen and stored at −20° until analysis. To assure no death of plants had occurred during the artificial hardening period, 10 roots of each variety were planted in warm soil in a greenhouse and observed for regrowth. There was 100 % recovery in all cases.

*Methods. Extraction of Soluble Protein from Lyophilized Alfalfa Roots.* Frozen root samples, used only for the quantitative peroxidase and catalase analysis, were lyophilized and ground through a 40-mesh screen in a Wiley mill. Soluble proteins were extracted from 2 g dry weight of each sample into 25 ml of 0.1 M tris-HCl (pH 8.0) containing 12 % sucrose, 1 % ascorbic acid, and 0.1 % cysteine. The extraction was performed in a cold room at 4° with a mortar and pestle. The extraction was carried out by grinding the lyophilized tissue in the buffer using ten 360°-strokes. After a 10 minute interval, 5 g of acid washed sea sand were added and the extraction continued in alternating 10 stroke, 10 minute intervals until a minimum of 50 strokes had pulverized the tissue. The extract was filtered through 2 thicknesses of cheesecloth and centrifuged for 30 minutes at 10,000 × *g*. After centrifugation, the extract was decanted into chilled test tubes and stored at −20° until analysis.

*Extraction of Soluble Proteins from Frozen Alfalfa Roots.* Soluble proteins were also extracted from nonlyophilized alfalfa root samples which had been stored in plastic bags at −20° after being frozen at the time of sampling. Five grams wet weight (approximately 2 g dry wt) were thinly sliced with a razor blade and the soluble proteins were extracted in the same manner as employed in the extraction of the lyophilized root samples.

*Determination of Soluble Protein Content.* Soluble protein was analyzed according to the method of Lowry (9), using a standard protein, rabbit serum albumin, which was stored over $P_2O_5$ until constant weight.

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis as described by Ornstein (11) and Davis (6) was used to study the soluble proteins of alfalfa roots. All electrophoretic separations were conducted on standard 7.5 % acrylamide gels. The electrophoresis was carried out in a cold room at 4° using 2.5 milliampere per gel tube until the bromphenolblue band had migrated near the lower end of the gel column. The gels were removed from the tubes and immediately analyzed.

*Amido Schwartz Assay.* After the proteins were separated by polyacrylamide gel electrophoresis, the gels were immersed in 7 % acetic acid containing 1 % amido schwartz and allowed to react for 1 hour at room temperature. The unbound dye was removed from the gel by the destaining method described by Davis (6). The results were photographed for permanent records.

*Peroxidase Assay.* Peroxidase activity was measured directly on the gel surface. The gels were immersed in 0.02 M guaiacol for 30 minutes before being transferred to a solution containing 0.3 % hydrogen peroxide. Peroxidase activity produced brown bands which were photographed during their formation.

An alternate method used to measure peroxidase was to immerse the gels in 0.1 M catechol for 30 minutes before transferring the gels to the 0.3 % hydrogen peroxide. Brown bands were produced as in the guaiacol determination.

*Quantitative Analysis of Peroxidase.* Quantitative assays of peroxidase were conducted according to the method of Chance and Maehly (4) on the 1963 and 1964 samples. For this analysis, proteins were extracted using a 0.1 M tris-HCl (pH 8.0) containing 12.5 % sucrose. One ml of 0.3 % hydrogen peroxide was rapidly pipetted by blowing into a 1.7 cm i.d. test tube containing 1 ml 0.1 M phosphate buffer pH 7.0, 1 ml 0.02 M guaiacol, 3 ml redistilled water, and 100 μg soluble protein in 1 ml water. The reaction was monitored, at 10 second intervals for 2 minutes, by recording the optical density at 470 mμ. Results were expressed as enzyme units on an equal protein and equal dry weight basis. One enzyme unit was defined as the amount of enzyme necessary to produce a change of 0.1 OD at 470 mμ per minute, using a total volume of 7 ml with a light path of 1.7 cm at initial velocity conditions.

*Quantitative Analysis of Catalase.* Quantitative catalase assays of the 1963 and 1964 samples were conducted according to the method of Maehly (10) using a 0.1 M tris-HCl pH 8.0 buffer containing 12.5 % sucrose. Catalase was measured by the manometric method using a Recording Gilson Differential Respirometer. The main chamber of a Warburg flask contained 3 ml 0.01 M phosphate buffer pH 7.0, and 0.2 ml of 0.2 M hydrogen peroxide. The side arm contained 500 μg protein in 0.1 ml. The determination was conducted at 4° with a shaking rate of 160 cycles per minute. The results were expressed as μl $O_2$ produced on an equal protein and dry weight basis.

## Results and Discussion

*Soluble Protein Content of Alfalfa Roots.* The amount of soluble protein extracted from the roots of all varieties studied increased during the autumn as the plants developed hardiness. On a dry weight basis the soluble protein content for nonhardy Caliverde, medium hardy Buffalo and very hardy Ver-

Table I. *Soluble Protein Content of Alfalfa Roots as Related to Cold Hardiness*

Soluble protein content was measured according to the method of Lowry et al. (9). Values are the average of duplicate analysis.

| Sample* | Grams soluble protein extracted per 100 g root tissue (dry wt) | | | |
|---|---|---|---|---|
| | 1961 | 1962 | 1963 | 1964 |
| CA | 9.0 | 6.6 | 10.3 | 10.4 |
| CO | 13.6 | 8.7 | 12.2 | 9.6 |
| CD | 10.8 | 11.0 | 13.9 | 11.5 |
| BA | 8.5 | 5 9 | 9.8 | 9.3 |
| BO | 5 7 | 8.9 | 11.9 | 9.4 |
| BD | 9.8 | 10.0 | 14.5 | 11.1 |
| VA | 7.9 | 7.5 | 9.2 | 8.4 |
| VO | 7.0 | 9.2 | 10 0 | 9.3 |
| VD | 10.6 | 10.8 | 14.0 | 11.1 |

* Alfalfa varieties Caliverde (C), Buffalo (B), and Vernal (V) were sampled in August (A), October (O), and December (D). Proteins were extracted with pH 8.0, tris-HCl buffer.

nal varieties increased each of the 4 years studied (table I).

Other workers who have used distilled water as an extraction medium have suggested that differences in soluble protein content was due to the hardening capacity of the varieties. The use of distilled water may allow endogenous differences in salt and organic acid concentrations to influence the amount and type of protein extracted. In the present study, differences among the varieties due to hardiness were not apparent. The use of 0.1 M tris-HCl buffer pH 8.0 controlled the pH of the extraction medium and helped provide a high ionic medium so changes due to solubilization of ionic substances from the plant tissue would tend to be negligible.

## Polyacrylamide Gel Electrophoresis

*Amido Schwartz Analysis.* The polyacrylamide gel electrophoretic patterns of the soluble proteins of alfalfa roots are shown in figure 1. Only the data for the nonhardy Caliverde and the hardy Vernal varieties sampled in the unhardened condition of August and in the hardened conditions of December are presented. Without exception, the same trends were observed in each variety tested during the hardening period. Five hundred μg of each soluble protein sample were analyzed. After separation, the proteins were stained with amido schwartz. There were approximately 20 bands visible under ideal optical conditions. Two bands showed changes that appeared to be associated with hardening. Band 1 was a fast moving band that increased during the hardening period from August to December in all varieties. Band 2 was a slower-moving band, and it also increased during hardening in all varieties. These results appear to collaborate the electrophoretic findings of other workers (5). Coleman et al. (5) concluded that differences in soluble protein existed between non hardened and cold hardened conditions. Their findings were based on electrophoretic and immunological evidence. It is the contention of the authors of the present paper that the amido schwartz analysis showed no shifts large enough to account for the large inherent differences in the hardening capacity that exist among the varieties studied.

*Peroxidase Analysis.* The peroxidase activities of soluble proteins of alfalfa roots in relation to cold hardiness are shown in figure 2.

Catechol (fig 2, top) and guaiacol (fig 2, bottom) were used as substrates to measure peroxidase activity on the gel surfaces. The results showed that peroxidase activity, using catechol as a substrate, increased during the hardening period from August to December. The increase was observed in all varieties without exception during all 4 years. The increase in peroxidase activity was located primarily in 3 bands. Band 1, a darkly stained band, had the same $R_F$ as band 2 seen with the amido schwartz stain in figure 1. This study also showed the development of 2 new bands of

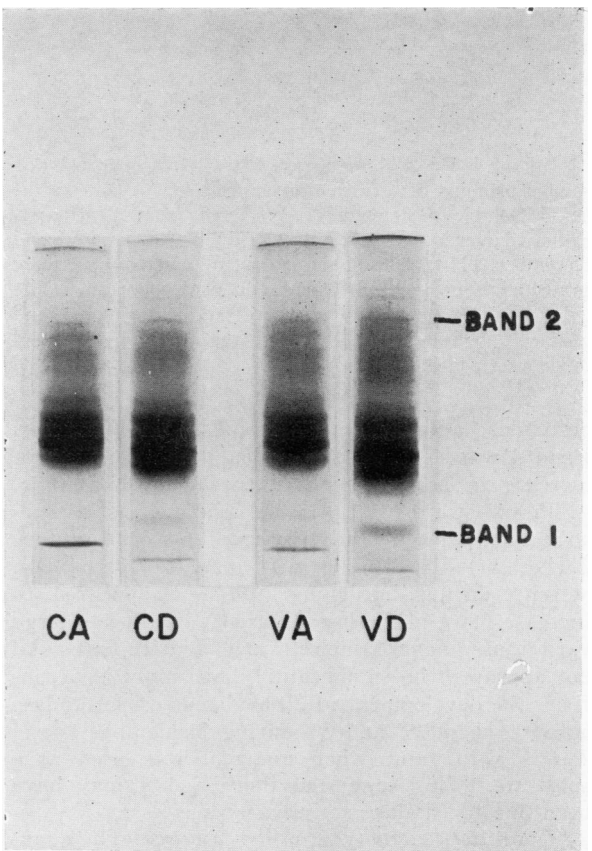


FIG. 1. Polyacrylamide gel electrophoresis of soluble protein of alfalfa roots in relation to cold hardiness. Five hundred μg of the soluble proteins from Caliverde (C) and Vernal (V) varieties sampled in August (A) and December (D) were separated with polyacrylamide gel electrophoresis and stained with Amido Schwartz.

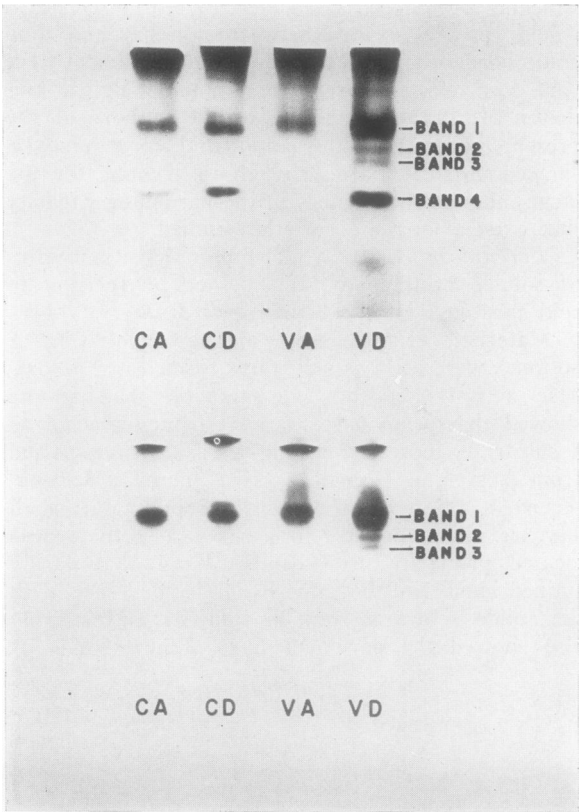


FIG. 2. Peroxidase assay after electrophoresis of soluble proteins of alfalfa roots in relation to cold hardiness. Soluble proteins were extracted from alfalfa varieties Caliverde (C) and Vernal (V) in August (A) and December. 1500 $\mu$g soluble protein of each sample were separated with polyacrylamide gel electrophoresis. The gels were immersed in catechol substrate (top) and guaiacol (bottom) for 30 minutes before hydrogen peroxide was added.

peroxidase activity (bands 2 and 3) in December. Band 4 was a faster-moving band that showed an increase in peroxidase activity during hardening.

Peroxidase activity, using guaiacol as a substrate, showed a slightly different pattern. Figure 2 (bottom) also demonstrates increased peroxidase activity during hardening. Band 1 appeared to increase more in the hardy variety, but due to large variations among samples, varietal differences did not appear to be significant. This study also confirms the development of 2 new bands of peroxidase activity (bands 2 and 3) during hardening. Band 4 was very faint when guaiacol was used as a substrate. This suggested that band 4 may have been due to catalase activity.

*Quantitative Analysis of Peroxidase.* The quantitative analysis of peroxidase activity in alfalfa roots as related to cold hardiness are presented in figure 3. The results are expressed on an equal protein and equal dry weight basis. One unit of enzyme was defined as the amount of enzyme which produced a change of 0.1 OD at 470 m$\mu$ in a total volume of 7 ml with a light path of 1.7 cm under initial velocity conditions. Boiled protein extracts with substrate and hydrogen donor, extracts with no substrate, extracts with no hydrogen donor, and buffer extraction blanks with substrate and hydrogen donor all failed to show activity. These data confirm the observations made in the gel electrophoretic study. In all varieties, marked increases in peroxidase activity occurred during the hardening period. The trend on an equal dry matter basis appeared the same as on an equal protein

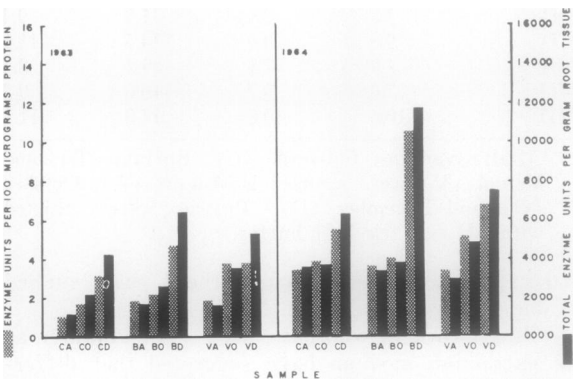


FIG. 3. Peroxidase enzyme activity of alfalfa roots as related to cold hardiness. Alfalfa varieties Caliverde (C), Buffalo (B), and Vernal (V) were sampled in August (A), October (O), and December (D). Proteins were extracted with pH 8.0 tris-HCl buffer and activities measured using guaiacol as a substrate. Solid bars indicate total enzyme units per g dry weight root tissue. Cross-hatched bars indicate enzyme units per 100 $\mu$g protein.

basis, but with higher increases. The data indicated that the hardy variety was capable of reaching a higher maximum of peroxidase activity in October compared with the other varieties. The medium hardy variety showed the highest peroxidase activity in December, but relatively little increase in October.

These results may indicate that one of the important aspects of the hardening process is the speed at which it occurs. The only varietal difference that seemed to be related to hardiness was the capability of the hardy variety to induce change in peroxidase sooner than the other varieties. The medium hardy variety showed a higher enzyme activity in December than the nonhardy or hardy variety. This increase in peroxidase activity on an equal protein and dry weight basis indicated that the increase in soluble protein content (table I) was reflected by an increase in peroxidase activity.

*Quantitative Analysis of Catalase.* The catalase activities of alfalfa roots as related to cold hardiness are shown (fig 4). The results are expressed as $\mu$l of oxygen produced per minute on an equal protein and equal dry matter basis. Boiled extracts plus hydrogen peroxide, extraction buffer plus hydrogen peroxide, and extracts without hydrogen

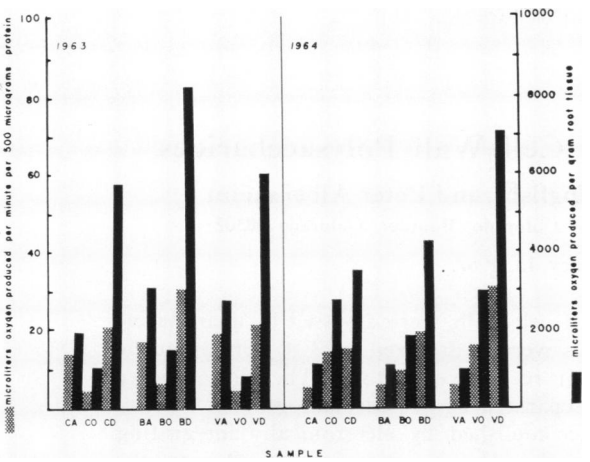


FIG. 4. Catalase enzyme activity of alfalfa roots as related to cold hardiness. Alfalfa varieties Caliverde (C), Buffalo (B), and Vernal (V) were sampled in August (A), October (O), and December (D). Proteins were extracted with pH 8.0 tris-HCl buffer and activities measured using hydrogen peroxide as a substrate. Solid bars indicate $\mu$l $O_2$ produced per minute per g dry weight root tissue. Cross-hatched bars indicate $\mu$l $O_2$ produced per 500 $\mu$g protein.

peroxide, all failed to produce oxygen. On an equal protein basis the catalase activity of alfalfa roots showed marked increases during hardening in all alfalfa varieties. When these data were expressed on an equal dry matter basis, the trends remained unchanged. In 1964, the increase in catalase activity was progressive in all varieties with the hardy variety showing the most pronounced increase and the nonhardy showing the least.

## Conclusions

The overall picture observed in the soluble proteins of the alfalfa roots reconfirmed that soluble proteins increased in content during hardening. There were no large shifts in the polyacrylamide gel electrophoretic patterns of the soluble proteins in relation to cold hardiness. Increases in soluble oxidative enzymes and the formation of 2 new isoenzymes were demonstrated. Quantitative increases during hardening in peroxidase and catalase were demonstrated on an equal protein and a dry weight basis. This indicated that increases in the total soluble protein content were associated with a preferential increase of specific enzyme activities. These enzymes, although increasing in activity, did not clearly differentiate among the inherent hardening capacities of the varieties. The only indication of a varietal difference occurred in peroxidase activity where in the hardy variety an increase occurred sooner than in the other varieties. The data demonstrated, however, that there was a profound alteration in plant metabolism due to the hardening process. The effect that this alteration may have on the overwintering plant cell may determine whether or not the cell survives or succumbs to the stresses and strains encountered. It appears that the surviving plant cell has to adapt itself fast enough to survive the cold and alternating temperatures in order to avoid death, especially when the temperature change is rapid. The hardiest variety may be the one which is more sensitive, and therefore more responsive, to the conditions which promote the hardening process.

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