Plant Leaf and Stem Proteins. II. Isozymes and Environmental Change¹,²

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Abstract. The activity of 10 enzymes separated by acrylamide disc gel electrophoresis of leaf and stem extracts from Dianthus grown under summer and winter conditions was studied. While banding was constant and highly reproducible under each environment, differences between the 3 cultivars and between the tissues were evident. No significant differences in the isozyme patterns of glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, and catalase were observed between the 2 environments. Loss of activity was observed under winter conditions with amylase and lactate dehydrogenase and loss of certain isozyme components was evident with acid phosphatase and esterase. Prominent changes were observed in peroxidase isozymes, the hardy cultivars developing additional isozymic components under winter conditions. Only minor changes in the total protein banding were seen. The enzymes showed considerable stability in those tissues killed by the freezing conditions.

Protein metabolism has been associated with the adaptation of plants to environmental change and stress in many studies. Briggs and Siminovitch (1), studying the soluble proteins of the bark of Robinia pseudoacacia, found that 2 of 5 fractions changed with winter hardening. Coleman et al. (2) separated the soluble protein fractions of 6 genotypes of Medicago sativa roots and found the proteins of high Rf values were more prevalent in hardened tissues. Even though total soluble proteins have often been associated with hardening, some workers have not found a significant correlation (13).

Some studies have attempted to correlate specific enzymes with hardening. Gerloff et al. (4) found changes in peroxidase and catalase with hardening of alfalfa roots; however, they were not able to distinguish varietal differences in hardness. Roberts (14) also associated peroxidase isozymes in wheat with hardening. Unfortunately, many of the studies attempting to correlate specific enzymatic action with hardening have been severely limited by extraction techniques (13), which restricted them to the non-green portions of the plant.

With the development of a very reliable protein extraction technique for green tissues (11), the individual isozyme components can now be definitively studied during stress conditions. This technique has yielded active enzymes from a number of unrelated genera (5). Using this method, we have studied the enzyme components of both the leaf and stem of 5 Dianthus cultivars during summer and winter conditions.

Materials and Methods

Three cultivars of Dianthus were studied: D. caryophyllus L. 'Scania', a greenhouse cultivar that was not potentially winter hardy; D. sp. PI 303284, a hardy selection referred to as "Wyoming"; and D. × plumarius L., a selection from northern Wisconsin which is also winter hardy. Dianthus is an evergreen herbaceous perennial, thus affording opportunity to study both the leaf and stem during hardening. No dormancy has been observed in Dianthus and thus changes observed were not involved with this phenomenon.

Plants were grown both in greenhouses with long photoperiods and a minimum temperature of 10°, and also in the field. No winter protection was afforded the plants in the field. The plants were sampled 3 times in the summer (June-July) and 4 times in the winter (November, December, and twice in January); the minimum temperature of 30° occurred in January. Winter samples were taken when the plants were thawed. All tissues were randomly selected, extracted immediately, and electrophoresed. The methods of separation and electrophoresis have been detailed (11). Extracts could be stored at 20° for at least 2 weeks without degradation as long as the dithiothreitol concentration was increased and maintained at 1 to 5 mM.
Fig. 1 and 2. Gels stained for total protein with aniline blue-black. (Fig 1 top) Gels of leaf tissues from 3 cultivars of Dianthus (D. sp. "Wyoming" = W; D. × plumarius = P; D. caryophyllus 'Scania' = S). Few differences were evident between the summer or unhardened condition (U) and the winter or hardened condition (H) except in 'Scania' where the late January sample (J) lost banding because of severe bleaching of the leaves. N = November sample; D = December sample. (Fig 2 bottom) Gels of stem tissue proteins. Bands of very high R_f became prominent on hardening. Again the January 'Scania' sample lost banding.
Peroxidase, acid phosphatase, esterase, catalase, amylase, and 5 dehydrogenases were assayed. The dehydrogenases were identified by the procedures of Honold et al. (7), however hepes (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid) buffer (pH 7.4) replaced tris in all dehydrogenase reactions. Catalase and amylase determinations essentially followed the procedures of Rudolph and Stahmann (15). Where starch was required in the gel, only a 0.05% concentration was used, otherwise the banding was readily obscured. Esterase was assayed by the method of Desborough and Peloquin (3) using tris buffer at pH 7.4. Incorporation of Tween-20 into the esterase reaction solution stabilized the α-naphthylacetate and thus reduced the alcohol to very small amounts for initially dissolving the substrate. Peroxidase was analyzed using benzydine or pyrogallol, 0.5% H2O2 and 1 m potassium acetate buffer at pH 5.0. The peroxidase isozymes all reacted within 2 min at this pH, while requiring up to 30 min to give good intensities at a neutral pH. Benzydine gave stable and reproducible banding; patterns obtained using pyrogallol were similar, but not as intense or stable. For all reactions except amylase the reaction solution without substrate served as the control. All reactions were conducted at room temperature (22°C) and were allowed to proceed until definite banding was evident. This time was never greater than 60 min and usually less than 30 min. All the gels were rinsed twice in the reaction buffer before being added to the reaction mixture.

Much better resolution of the isozyme pattern could be obtained when the gels were photographed with color film rather than black and white film. Color slides and prints were thus used to record the data, black and white pictures being produced from these.

Results and Discussion

The soluble protein and enzyme banding for each cultivar was similar from all 3 tissue samples of the field grown plants during the summer. These banding patterns were similar to those from tissues of comparable plants grown in the greenhouse. In contrast, differences were found both in activity and in isozyme pattern among cultivars and tissues. Lactate dehydrogenase was present only in the leaves (fig 6). Amylase was present intensely in the leaves, but only weakly in the stems (fig 7). A strong band of Rp 10 to 12 was present in all the leaf samples, but not in the stems. This band could be seen without staining the gel. It stained intensely with aniline blue-black (fig 1) and also non-specifically absorbed the colored products of many of the enzyme reactions, especially the dehydrogenases (figs 5 and 6). A very active phosphatase isozyme of Rf 23 to 25 appeared in the leaves, but only weakly in the stems (fig 4). Differences between cultivars were observed with many of the enzymes, 6-1-gluc

conate dehydrogenase (fig 6) and acid phosphatase (fig 4) being good examples.

The number of isozymes obtained varied markedly with the specific enzyme. Glutamate dehydrogenase had only 1 isozyme component (fig 5) whereas esterase (fig 3) had up to 10 components. Most of the isozymes were located in the upper one-half of the gel (Rf 0-50) but the esterase bands occurred also in the lower portion. This isozyme distribution corresponded with the aniline blue-black stain for total protein where most of the coloration occurred in the upper one-half of the gel (fig 1). However, correlation of a specific isozyme band with a specific total protein band was very difficult since many of the isozymes of different enzymes had similar Rp values. In no case were isozyme patterns for different enzymes identical, nor was there banding without substrate, which differs from the results of Scandalios (16) with maize. The sensitivity of the enzyme tests accounted for more total bands being seen with the enzyme reactions than with the total protein stain. This also accounted for changes being resolved with the enzymes but not with the total protein stain.

Winter samples of the field tissue of each hardy cultivar showed very similar isozyme patterns for all sampling dates except for lactate dehydrogenase where the banding decreased in activity until none was observed in the January samples (fig 6). Winter samples of the unhardy 'Scania' were similar until the late January sampling when much of the enzyme activity was absent. Regrowth tests of cuttings showed that the 'Scania' was dead at the time of the early January sampling, but the enzymes remained unaffected. Only during the prolonged thaws of late January when the dead tissues became bleached, did much of the enzyme activity disappear. This demonstrated that death of these plants was not due directly to the inactivation of the enzymes surveyed.

Since lactate dehydrogenase was not present in any of the winter tissues after the December sampling, a study was conducted to determine if the activity would regenerate upon regrowth of the plant. Cuttings from the hardy field plants were rooted in the greenhouse and after 2 weeks, the over-wintering leaves were extracted. Lactate dehydrogenase activity was found to be partially restored, although the activity never reached that of the summer tissues. The new growth from these shoots showed full enzyme activity.

Generally, only the data from the early January sampling of the winter tissues was used in the figures since 'Scania' isozymes were still present and no changes occurred in the 2 hardy cultivars after this sampling.

Besides the loss of lactate dehydrogenase activity, amylase activity was markedly reduced in the winter field tissues (fig 7). All the other enzyme components either showed no loss of activity or the dis-
FIG. 3. Esterase isozyme patterns from leaf (L) and stem (S) tissue of 3 cultivars of *Dianthus* (*D. sp. "Wyoming" = W; *D. x plumarius* = P; *D. caryophyllus 'Scania' = S) from summer or unhardened (U) and winter or hardened (H) plants. Loss of and shifts in the bands of high $R_p$ were evident in the winter tissues.

FIG. 4. Acid phosphatase isozyme patterns. These patterns show loss of the higher $R_p$ bands in the winter tissue. Note the differences in cultivar patterns, especially in the unhardened condition. Labeling as for figure 3.
Fig. 5. Isozyme patterns of 4 enzymes (MDH = malate dehydrogenase; G-6-PDH = glucose-6-phosphate dehydrogenase; GDH = glutamate dehydrogenase; Cat = catalase) where no major differences in banding were observed between the 2 environments. The band of Rf 10 to 12 in leaf G-6-PDH and GDH is an artifact resulting from the non-specific absorption of dye onto a band associated only with leaf tissue. Labeling is the same as in figure 3.

Fig. 6. Isozyme patterns of 6-phosphogluconate dehydrogenase (6-P-GDH) and lactate dehydrogenase (LDH). Note the marked differences between the 6-P-GDH patterns for D. x plumarius and “Wyoming” as contrasted to that of ‘Scania’. No changes in banding for 6-P-GDH from different environments were detected. LDH lost activity in the winter. The band of Rf 10 to 12 in LDH resulted from non-specific absorption noted for figure 5. Labeling is the same as in figure 3.
appearance of only certain isozyme components during winter. This was evident with the acid phosphatase (fig 4) and esterase (fig 3) patterns where the higher \( R_F \) bands generally lost activity in winter.

Loss of activity as described above occurred in all 3 cultivars and thus was not associated with the development of full hardiness. Lactate dehydrogenase and many of the higher \( R_F \) esterase isozymes have been shown in this laboratory to be easily cryo-denatured (12). Thus the loss of these bands during freezing was to be expected. However, glucose-6-P dehydrogenase is also cryosensitive, but its activity was fully protected in the winter tissues (except in the late January 'Scania' sample).

Some change in banding in the stem extracts was seen in the total protein staining (fig 2) where bands of very high \( R_F \) became more prominent under hardening conditions. This was observed in all 3 cultivars.

Figure 8 shows the peroxidase isozyme components of the various samples. While the general isozyme pattern remained constant, 2 prominent isozymes of \( R_F \) 36 to 39 appeared in the winter samples extracted from stems of the 2 hardy cultivars. These isozymes were present in the first winter sample but not present or only very faintly present in the summer and greenhouse tissues. Therefore, they must have appeared after July and before November, which includes the hardening period.

It is of particular significance that the peroxidase isozymes of \( R_F \) 36 to 39 were not produced under these environments in 'Scania', a cultivar incapable of developing sufficient hardiness for winter survival. With no other enzyme studied was new isozyme production initiated during the change in environment. Since there was a correlative difference in only 2 isozymes of 1 enzyme of all the 10 enzymes
studied, differences in total soluble protein would not disclose such changes with hardening.

The new peroxidase isozyme appeared clearly as 1 or 2 bands early in the peroxidase reaction (1–3 min), whether benzidine or pyrogallol was used. When the reaction was allowed to proceed longer (up to 10 min), the whole region from RP 0 to RP 39 was intensely darkened (Fig 8). This indicated that a complex involving a number of isozyme bands, many of which were too low in concentration to visualize, may be initiated during fall and winter conditions. No such darkening with longer reaction times was observed in the separations from summer tissues.

No leaf tissues ever developed the peroxidase complex although most of the leaves of the hardy cultivars survived the coldest part of the winter. It is interesting that the stem tissues survived the winter without injury whereas the leaves showed considerable bleaching in spring.

That the other enzymes studied did not show new isozyme components does not necessarily exclude them from environmental modification during hardening. Gerloff et al. (4) found quantitative changes in catalase as well as peroxidase. Such quantitative changes may be difficult to resolve in acrylamide gels. 'Scania' was able to harden enough to survive $-15^\circ$. The changes in total protein banding observed in all 3 cultivars and the quantitative changes that have been reported for other plants may possibly be important for this degree of hardening. This cultivar may also be unable to harden rapidly enough to survive the winter conditions as has been suggested by Weiser (18) in Cornus selections.

The regulation of several aspects of peroxide metabolism in plants during fall and winter is essential. The effects of peroxides on membranes (17) would indicate the requirement for a peroxidase control system, if only to prevent membrane damage. Since changes in membrane permeability have been correlated with the development of hardiness (9), it is fascinating to speculate if specific peroxidases could be associated in the process. Peroxidative action has been correlated with ethylene synthesis (10) and IAA metabolism (6,8). Whether or not these latter functions are involved in cold hardiness is unknown. These aspects along with possible red: far-red interactions with the system are now being investigated in this laboratory.

**Literature Cited**

1. Briggs, D. R. and D. Siminovich. 1949. The chemistry of living bark of the black locust tree in relation to frost hardiness. II. Seasonal varia-