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Plant Leaf and Stem Proteins. I. Extraction and Electrophoretic Separation of the Basic, Water-Soluble Fraction[1, 2, 3]

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Abstract. **Simplified but highly reproducible extraction and electrophoretic procedures have been developed for plant stem and leaf proteins using recent chemical and technical advances. The method is applied to the separation of the basic, water-soluble proteins found in stem and leaf tissues of 3 *Dianthus* clones. While most of the highly reproducible protein bands appear in all 3 selections, and many are also common to both leaf and stem tissue, others are characteristic for the variety or tissue.**

During studies on stress physiology the protein fraction of mature green tissues became of particular interest. While studies into plant protein metabolism have recently intensified, the extraction and separation of the protein fractions frequently presents seemingly formidable problems, this being especially the case with mature green tissues. It was found that, for our material, none of the available techniques gave reliable extraction and identification procedures. The relatively recent introduction of certain protective reagents has permitted the development of successful extraction methods, which we have found to yield stable and reproducible protein fractions, while avoiding such extreme dehydration procedures as lyophilization.

The instability of proteins from green tissue is of general knowledge. The problem has been reviewed and in some cases, solutions suggested (11, 12). Using electrophoresis and various extraction techniques, proteins have been isolated from green tissues of beans (13), wheat (8, 15), and peas (14).

Loomis and Battaile (6) thoroughly reviewed the use of insoluble PVP (polyvinylpyrrolidone) for the removal of plant phenols and quinones from extracts. Using this compound as an additive, they successfully extracted peppermint, thistle, and apple leaf proteins. Cleland (2) described the use of dithiothreitol as a protective agent for the sulfhydryl groups of enzymes and this compound has since been used successfully in the solubilization, isolation, and activation of a number of protein components (1, 5, 9). With the use of such protective agents as these, it seemed possible to produce plant protein preparations of high quality and stability.

Materials and Methods

After a careful screening of many plant genera and species, 3 selections of *Dianthus* were chosen as the research material: *Dianthus caryophyllus* L. 'Scania', *D. caryophyllus* PI 303284 (thereafter referred to as Wyoming clone), and *D. plumarius* L. These selections were easily grown under growth chamber conditions, field cultivation, and greenhouse culture. The semi-woody stems allowed for easy maceration and the evergreen character of the leaves increased the scope of use over deciduous material. Although the stems as well as the leaves are green, the latter have more chlorophyll.

The plants for this study were grown under long photoperiods in air-conditioned greenhouses. The minimum temperature was 10°. Identical cultural methods were used for all plants.

Stem tissue was selected from the nonwoody, terminal 2 to 8 cm of the shoots. The leaves sampled were from the same terminal shoots, but only fully expanded leaves were used. The tissue was prepared for electrophoresis by the following procedure: I) Extraction: (all steps conducted at 4°). A) Extraction solution: 1) 10 g leaf tissue or 7 g stem tissue (fr wt). 2) 0.1 M Hepes buffer at pH 7.3 to 7.4. 3) A minimum concentration of 0.1 mM dithiothreitol. 4) 2 to 3 g insoluble PVP (Polyclar-AT powder, General Aniline Film Corporation) per g of tissue, *i.e.* enough to make a heavy slurry. B) Grind by hand in a mortar and pestle. C) Strain through fine acetate cloth; re-extract twice with buffer; add approximately 1 g

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[3] Reported in part at the meetings of the American Society of Plant Physiologists, 1967.

PVP. D) Centrifuge at 20,000 × *g* for 30 minutes. E) Decant into dialysis tubing (previously soaked in 10 mM EDTA and rinsed in water).

II) Dialysis and concentration at 4°. A) Dialyze against 0.05 M tris-glycine buffer at pH 8.9 for 6 hours. Change solution at least twice. B) Concentrate against Aquacide I or II. C) Replenish dithiothreitol to maintain a minimum 0.1 mM concentration.

The total procedure including electrophoresis and destaining required 2 days. The extracts remained a pale green throughout all the steps. If PVP and dithiothreitol were not included in the procedure, the extracts became amber to brown in color within a few hours. This was especially true of the leaf extracts and changes in banding were evident. In all cases the extract was electrophoresed within 24 hours after extraction.

Hepes buffer at pH 7.0 to 7.5 was used initially because of its superior qualities at this pH (4). The pH must be kept below 7.5 for the PVP to be effective in absorbing the phenols (6). During dialysis, tris-glycine buffer was used because the buffering capacity is high at pH 8.5 to 9.0.

The final volume of extract was 2 ml, which contained approximately 15 to 25 mg of stem protein or 20 to 30 mg of leaf protein. From 200 to 1000 μg of protein was applied to a gel. The protein was estimated by the Folin reaction (7).

The electrophoresis was adapted from Ornstein and Davis (10). All gels were cooled to 4° before the protein extract was applied and all subsequent steps were conducted at this temperature. The gels were larger than the standard gels, the length of the running gel being 8.5 cm. This was prepared and run in plexiglas tubes 10 cm long and slightly less than 7 mm ID. The plexiglas tubes allowed for easier removal of the gels and were more convenient to handle than the standard glass tubes. No sample gel was used, the protein extract being placed directly on the spacer gel and layered with reservoir buffer. The concentrated protein extract would often deform the surface of the spacer gel; this was completely prevented by preparing the spacer gel with 20% sucrose solution. Electrophoresis was conducted at 4 ma/tube for approximately 3 hours or until the marker protein was less than 1 cm from the bottom of the gel. Using our equipment, 48 gels could be conveniently run simultaneously.

Gels were stained immediately for 1 hour in 0.5% aniline blue black. The gels were destained electrophoretically by applying 12 volts and 10 amps to 24 gels simultaneously in a specially designed apparatus that circulated 7% acetic acid around the gels and through a charcoal filter.

The leading protein band (R_F 100) was present in all preparations and was visible without staining. Since the location of this band was highly dependable as checked by bromophenol blue marker dye and the band invariably appeared as a major band after staining, it was used to calculate R_F values. R_F values were estimated visually using a caliper. This method proved to be reproducible within 0.5 R_F value. The bands also were grouped into major and minor bands according to their relative intensity in a gel (3).

For gels that had weak banding, a technique was devised to intensify the bands by dehydrating (shrinking) the gel with glycerol. Gels were placed in 100% glycerol in 10 dram capped vials and shaken overnight. If further dehydration was desired, the gels were placed in fresh glycerol and further dehydrated.

The gels were photographed using Ansco Versipan film, an adjustable fluorescent-lighted background and no filters. It was not possible to photograph an entire gel and retain the resolution of all the bands, for the minor bands were usually

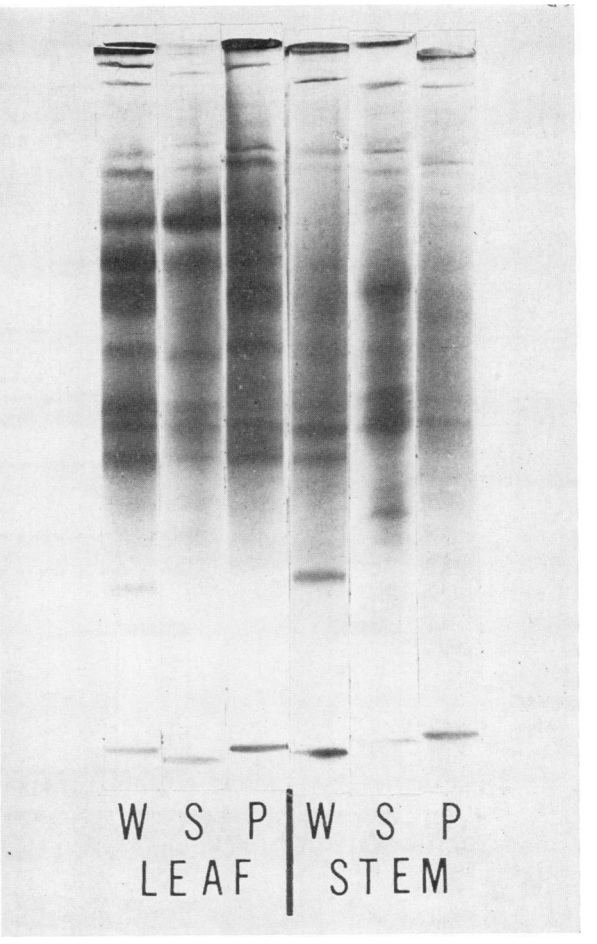

FIG. 1. Gels showing protein bands from stem and leaf tissue of 3 *Dianthus* clones (W-*D. carophyllus,* a selection referred to as Wyoming; S-*D. caryophyllus* 'Scania'; P-*D. plumarius*). Note the marked similarities in banding, especially the presence of the leading protein band which was used to calculate R_F values. Many of the minor bands are not visible in these photographs.

not visible unless the restricted area containing the bands of interest was specifically photographed.

Results and Discussion

Figures 1 and 2 show gels with bands obtained from the leaf and stem proteins of all 3 clones of *Dianthus*. As many as 28 bands were distinguishable within a gel, and generally a minimum of 20 bands could be clearly resolved. At least 9 bands (R_F 100.0, 57–58, 54–55, 51–53, 44–46, 16–18, 14–16, 4–6, 1–3) occurred in all the gels regardless of the tissue or the clone. While the relative intensity of these bands was constant for a tissue or a clone, differences between clones and tissues did occur.

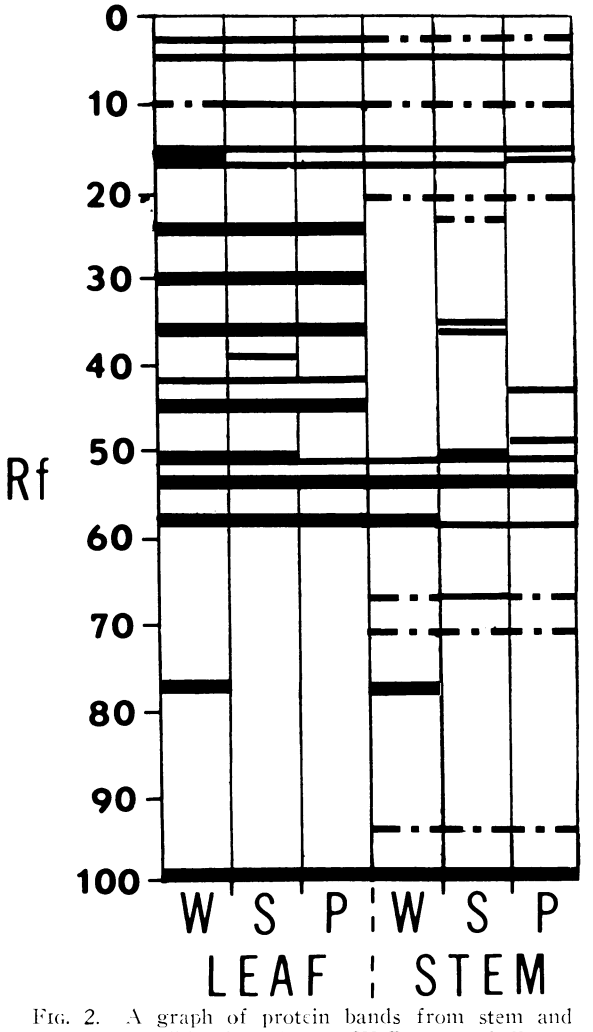

FIG. 2. A graph of protein bands from stem and leaf tissue of 3 *Dianthus* clones (W-*D. caryophyllus*, a selection referred to as Wyoming; S-*D. caryophyllus* 'Scania'; P-*D. plumarius*). Only the major bands (solid lines) and some of the important minor bands (broken lines) are shown here to simplify the figure. Note the bands that are specific for a tissue or a clone.

At least 4 additional bands (R_F 94–95, 71–72, 66–69, 21–23) were seen in all the gels of the stem tissues, although these were also present as very minor bands in some of the gels of leaf tissue. Major bands (R_F 30–31, 24–25) were seen in all the gels of leaf tissue, but these appeared only as minor bands when present in stem tissue. These similarities indicate that many soluble proteins of the leaf and/or stem tissue of these 3 clones are similar, if not identical.

Clonal differences in banding also were observed. These differences were primarily with the minor bands, however some major band differences were evident (figs 1 and 2). A band of relatively high mobility (R_F 77–79) was particularly apparent, for this protein occurred in all the Wyoming tissues but was either not present or only very weakly present in the tissues of the other clones.

A large green band (R_F 44–46), which stained dark blue with the protein stain, was seen in the gels of *D. caryophyllus* 'Scania' leaf extracts. A band with the same R_F was present in all the stained gels but of much lower intensity in the gels of the stem tissue. Bands of low mobility (especially R_F 14–16) which have been suggested to be fraction I protein (8, 15) were also present in all gels.

The differences in banding could not be attributed to differences in the amount of protein applied to a gel because increasing the amount of protein above a minimum affected mainly the intensity of the bands, not their presence. This was true of the major bands and most of the minor bands, however some minor bands did vary with the amount of protein added to a gel. Increases in the amount of protein would cause a considerable increase in the background coloration, obscuring the minor bands. Those minor bands close to major bands were easily obscured. For these reasons a concentration series of an extract was routinely used (usually 300–1000 μg) to accurately determine the total banding present.

The R_F of a band was not affected by the amount of protein applied to a gel. This was only true when the leading edge of the band was used to calculate the R_F. If the center of the band was used for R_F determination, considerable variance in the R_F resulted with changes in the amount of protein applied. This effect was the result of changes in band width.

Little resolution was lost when using the longer gels. Bands of very low mobility, which in the shorter gel remained very close to the spacer gel, were separated. Bands having small differences in R_F values were separated by greater distances, making for more precise R_F determination.

In those gels where the banding was very weak, dehydration (shrinking) of the gels proved valuable (fig 3). This did not alter the R_F values, and in fact made them easier to determine because the bands were sharper and thinner. In some instances,

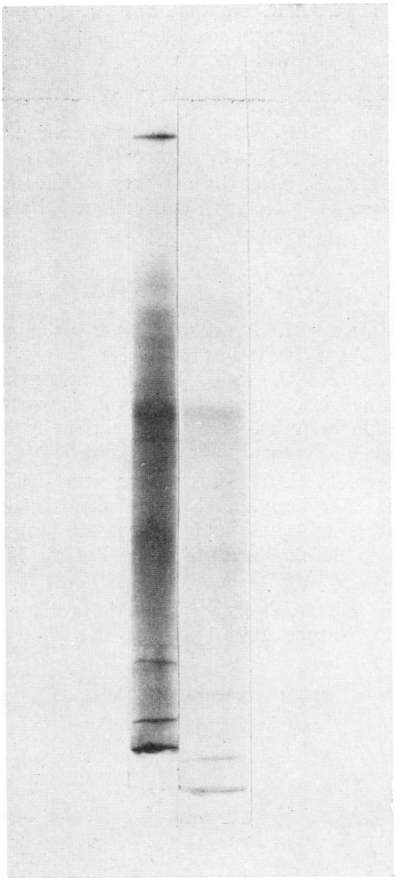

FIG. 3. Gels showing the intensification of banding by dehydration (shrinking) of the gel in glycerol. The gel on the right was dehydrated for 4 hours in 100 % glycerol. No change in R_F values was apparent, but the banding was markedly intensified. Note the increase in background coloration with shrinking. Both gels initially had the same amount of protein applied.

dehydration permitted identification of bands that could not be seen in the undehydrated gels. Possibly these bands widened considerably during a run and thus the shrinking of the gels intensified them enough to be visible. This was applicable only to those gels that were low initially in background coloration for the background was strongly intensified in the process. Bands of very close R_F values often became difficult to resolve when the gel was dehydrated: It has become a common practice in this laboratory to prepare both dehydrated and non-dehydrated gels since the benefits of both can then be realized.

The total banding was highly reproducible (fig 4). The variability in R_F values of the bands of a clone or tissue within a run was less than $\pm$ 0.5 R_F value. The variability between runs was less than $\pm$ 1 R_F value. This high reproducibility was also shown in the ability to constantly detect those bands common to all the gels within 2 R_F units. Such dependability in the R_F values is the result of stable protein preparations and the established constancy of the gel electrophoresis.

With the successful extraction and separation of plant leaf and stem proteins, many further extensions of the technique are indicated. Studies on the specific enzyme components and the influence of environment on their turnover and synthesis are highly feasible. Investigations of other components such as the acid-soluble fraction are needed. These adaptations of this technique will be the basis for further papers in the series.

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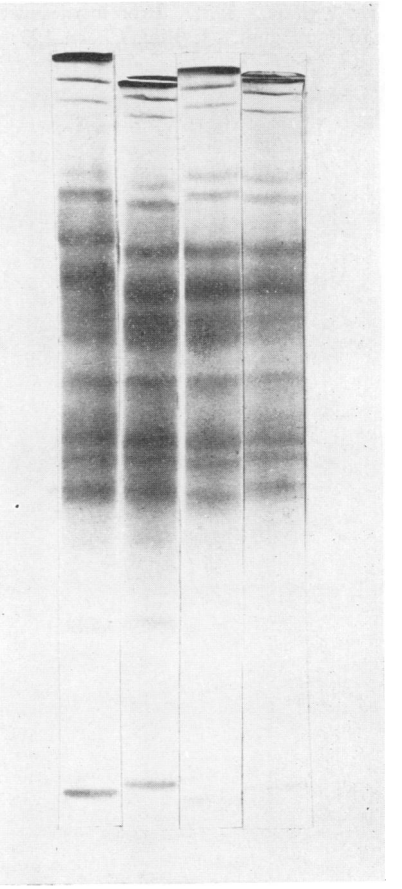

FIG. 4. Gels of leaf tissue extracts of the Wyoming selection of *Dianthus caryophyllus* showing the reproducibility of the method. The first 3 gels are from the same extraction and electrophoretic run, the fourth gel (right) is from another extraction and run, performed 7 days after the first. Note the close agreement in band location and intensity.

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