EXTRACTION OF PROTEIN FROM PINUS TISSUE FOR ANALYSIS BY ELECTROPHORETIC AND SEROLOGICAL TECHNIQUES

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ABSTRACT

The extracts obtained by this method of extraction of protein from mature tissues of some **Pinus** species are suitable for analysis of protein by electrophoresis and isoelectric focusing, isozyme analysis, and serological techniques. The relative freedom from interfering substances makes the method suitable for taxonomic studies.

Keywords: Conifer; protein; electrophoresis, serology; graft incompatibility.

INTRODUCTION

Graft incompatibility is a serious impediment to traditional methods of tree improvement but is often detected only after several years of apparent grafting success. Severe incompatibility losses have occurred in established seed orchards in New Zealand, where increasingly more graft incompatibility symptoms were found as graft age increased 10%, 24%, 35%, 52–57%, and 70–75% of 1-, 3-, 5-, 8-, and 13- to 18-year-old grafts respectively (Copes 1980).

Graft incompatibility may be due to cells of a particular genotype recognising foreign cells and reacting against them. In incompatible *Pseudotsuga menziesii* (Mirb.) Franco grafts, this hypersensitive reaction resulted in unusually high activity of oxidative enzyme systems (Copes 1978). The early detection of this reaction using enzyme analysis techniques may allow graft incompatible clones in *Pinus radiata* D. Don to be recognised and rogued out early, saving time and resources. Further, determination of incompatible genotypes by protein identification will allow the selection of compatible clones before grafting commences.

Protein extraction from mature conifer tissue is more difficult than from most plant tissues. Techniques suitable for other plant groups produced extracts from *Pseudotsuga menziesii* foliage which could not be resolved on acrylamide gels (McMullan & Ebell 1970).

Starch gels have often been used to separate the protein components of conifer extracts for isozyme analysis where relatively crude extracts can be separated. Successful

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separation on acrylamide gels, where finer resolution can be obtained, was achieved using 8 molar urea in the extraction, and low pH gels (McMullan & Ebell 1970). Some success was also reported by Hamaker & Snyder (1973) using *Pinus taeda* L. and *P. palustris* Mill. extracted with 15% urea, although not all the enzyme systems analysed showed activity.

The electrophoretic techniques used in this study require protein extracts to be free of substances which interfere with the electrophoretic process or damage the proteins, but this is made difficult by the presence of cellular structures, inhibitory chemicals, and the lower metabolic rate of plants (Feret & Bergmann 1976). The inhibitory chemicals include various organic acids, phenolic compounds, and tannins (Walker 1980). When these are released during tissue homogenate preparation, cross-linking reactions are particularly problematical. The bond formed between phenols and N-substituted amides of proteins is one of the strongest types of hydrogen bonds (Loomis & Battaile 1966). The amount of bound phenolic material may be up to one-third the dry weight of protein concerned. The serological techniques used particularly require extracts to be free of toxic substances, further restricting the number of suitable extraction procedures. Bud and shoot tip material was selected for the relative ease of extraction due to the higher protein : "interfering substance" ratio (McMullan & Ebell 1970).

The aim of this study was to develop an extraction procedure which produced protein extracts from mature *Pinus* tissue which could be used for graft incompatibility and clone identification studies.

MATERIALS AND METHODS

Separate shoot tip samples were taken from individual trees or clones of mature (>20 years) *Pinus radiata, P. elliottii* Engelm., *P. taeda,* and *P. monticola* Dougl. Protein was extracted in a mixture containing polyvinylpolypyrrolidone (7%), ascorbic acid (0.5%), sodium diethyldithiocarbamate (0.3%), and 2-mercaptoethanol (1%) in distilled water. This extraction mixture was stored at 6°C and used within 5 days of preparation. Approximately 200 g of frozen material was chopped into 3-mm pieces and placed immediately into 500 ml of the chilled extraction mixture with continuous stirring. The mixture was homogenised with an Ultra-Turrax homogeniser (type 45, Janke and Kunkle K.G. Staufen I. Breisgau) at full speed for 1 minute and centrifuged at 15 000 g for 30 minutes at 4°C. The supernatant was filtered through Whatman No. 1 filter paper and sucrose was added to make a 20% solution. The filtrate was either passed through a Sephadex column as follows or stored at –17°C for use later. A 100-mm-diameter glass chromotography column filled with hydrated Sephadex G25 (column height of 300 mm) enabled volumes up to 250 ml to be fractionated at one time. The column was washed between runs with at least 40 *l* of distilled water. All runs were carried out at 6°C. The void volume (600 ml) containing the high molecular weight material was lyophilised and the dried product taken up in as little distilled water as was necessary to dissolve the material (usually 3–5 ml). This solution was stored at –17°C until required. The solution was assayed for protein and carbohydrate using the methods of Bradford (1976) and Hodge & Hofreiter (1962), respectively.

Electrophoresis was performed according to the method of Ornstein (1964) and

Davis (1964), on polyacrylamide gels comprising a stacking gel (pH 8.9) and a separating gel (pH 9.5) using an Acrylophor apparatus (Pleuger, Belgium) and Pleuger (CVC-D) power supply. A current of 2 mA per tube was used until the tracking dye was within 5 mm of the bottom of the gel. The position of the tracking dye was marked by inserting a small syringe dipped in black drawing ink into the centre of the tracking dye. Protein was stained by immersing the gels in a solution containing 0.5 g coomassie brilliant blue G250, 40 ml ethanol (95%), 7 ml glacial acetic acid, and distilled water to make 100 ml. The gels were destained in a solution containing 250 ml ethanol (95%), 70 ml glacial acetic acid, and distilled water to make 100 ml. The perchloric acid protein stain method of Reisner *et al.* (1975) was used when results were required immediately.

Isoelectric focusing (IEF) was performed on LKB 2117 'Multiphor' apparatus (LKB Sweden) using an LKB 2103 power supply. 'Ampholine PAG' plates with a pH range of 3.5–9.5 were used when available. Laboratory gels were cast using the method of Görg *et al.* (1978) and covering the same pH range. A constant current of 10 mA was used for a complete 'PAG' plate and proportionately less if only parts of a plate were used.

Laboratory-made gels were run using a constant current of 2 mA. Tap water was used for cooling. Samples (15–30 μl) were applied at the cathode end of the gel using LKB sample applicator wicks. The gels were fixed and stained for protein according to the method of Winter *et al.* (1977). Peroxidase isozymes were visualised using a method modified from Brewbaker *et al.* (1968) (D. L. Copes, pers. comm.). A solution containing 200 ml 1 M sodium hydroxide adjusted to pH 4.0 with 1 M acetic acid (approximately 900 ml) was prepared, and 70 ml of this added to 30 ml of a solution containing 100 mg of 3,3'-dimethoxybenzidene (o-dianisidine) dissolved in 30 ml ethanol (95%). Hydrogen peroxide (2 ml of a 3% solution) was added immediately prior to adding the gels.

IEF gels were stained for acid phosphatase isozymes using the overlay method of Ross (1976).

Antisera were produced by injecting samples of the protein extracts into New Zealand white rabbits using Freunds complete and incomplete adjuvants at a 14-day interval. Serum collected from blood samples were used in double diffusion and immunoelectrophoresis analysis.

Ouchterlony double diffusion and Graber and Williams immunoelectrophoresis were performed as described by Wallenborg & Andersson (1978), except that electrophoresis was performed at 5 V/cm and cooled by tap water until the tracking dye (in a separate well) had reached the end of the trough.

RESULTS AND DISCUSSION

The freeze-dried product was analysed for protein and carbohydrate content to determine sample purity and sample loadings for each technique. The product represented approximately 0.1% of the initial fresh weight (dry weight of the sample material = 34.8% fresh weight) and consisted of approximately 25% protein and 70% carbohydrate.

A typical electrophoresis gel of separated native protein of an extract of *P. radiata* is shown in Fig. 1. The bands are clear and sharp, with little evidence of distortion allowing an accurate determination of intensity and relative mobility. The number of bands detected was similar to that reported for conifers by McMullan & Ebell (1970) and the number and definition of the bands were greater than that reported by Prager *et al.* (1976). As expected, when the same extracts were separated using IEF more bands were detected than with electrophoresis (Fig. 2). IEF, while allowing greater resolution, is particularly susceptible to interference by non-protein extract components such as salts. The level banding and clarity of the gel illustrate the lack of interfering substances in these extracts. Sharp clear banding was also obtained using peroxidase enzyme

FIG. 1—Polyacrylamide gel with proteins extracted from **Pinus radiata** separated by electrophoresis.

FIG. 2—Proteins extracted from **Pinus radiata** separated by isoelectric focusing.

stain after electrophoresis (Fig. 3). The bands were less diffuse and the position more precise than has apparently been found for enzyme staining in starch gels (Rudin & Rasmuson 1973; Rudin 1975; Copes 1978; O'Malley *et al.* 1979). Isozymes of acid phosphatase were detected in one sample of *Pinus radiata* after separation by isoelectric focusing (Fig. 4). The positions of the main bands were clearly visible although some streaking of the protein had occurred.

FIG. 3—Polyacrylamide gel with proteins extracted from **Pinus radiata** separated by electrophoresis and stained for peroxidase activity.

FIG. 4—Proteins extracted from **Pinus radiata** separated by isoelectric focusing and stained for acid phosphatase.

In the Ouchterlong double-diffusion analysis (Fig. 5) the serum containing the antibodies was placed in the central well and aliquots of the protein extracts in the outer wells. The proteins and antibodies were allowed to diffuse into the agar gel. When proteins and antibodies specific to those proteins meet in the correct proportion they form a stable complex which precipitates and can be stained (the bands in Fig. 5).

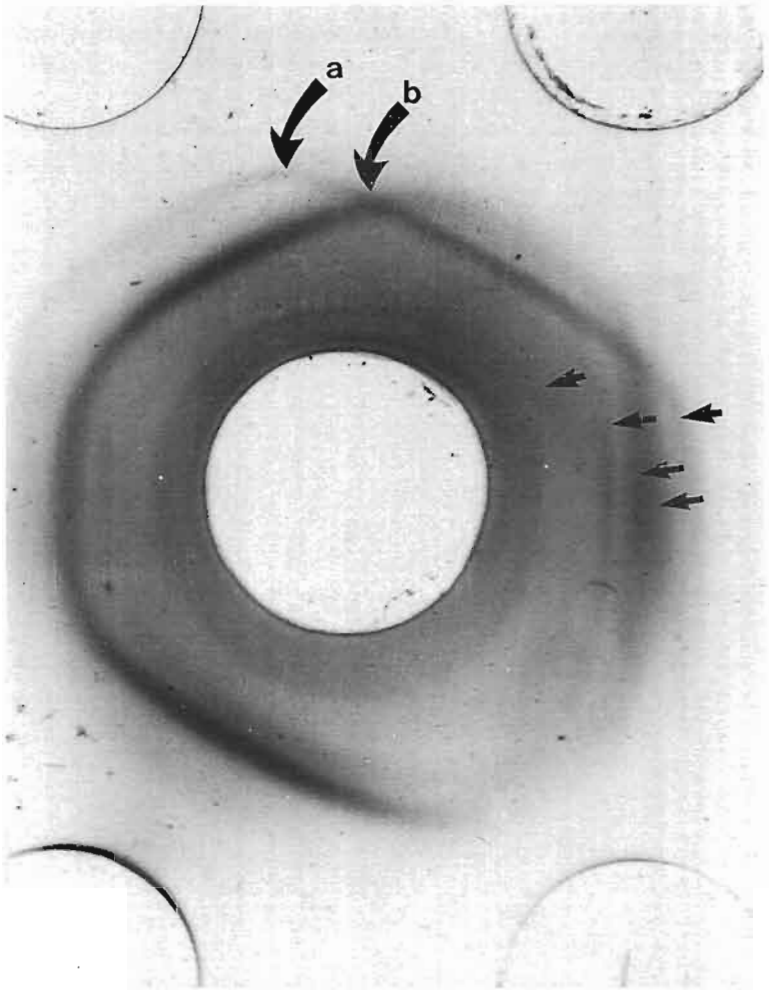

FIG. 5—Proteins extracted from four species of **Pinus** challenged with antiserum to **Pinus radiata.**

The sharp bands show complete recognition of a protein by an antibody produced against that protein, whereas the diffuse bands show incomplete recognition, i.e., a precipitate formed by a protein and an antibody produced against a similar but not identical protein. Bands which meet between the wells (arrow "a") indicate the same protein/antibody complex produced the precipitate, hence the same protein was present in both samples. Where bands cross (arrow "b") the precipitates are produced by different protein/antibody complexes.

The purified protein exhibited good antibody activity showing that the extracts were free of toxic substances and produced high titre antisera indicated by the small

volumes of protein and antisera required to produce good bands. The number of bands (arrows) and the clarity were similar to those detected on certain angiosperm genera (El-Lakany *et al.* 1977; El-Tinay *et al.* 1979).

A greater number of bands was detected using immunoelectrophoresis (Fig. 6) and the bands were clear and sharp compared to published data for other genera (El-Tinay *et al.* 1979). In this technique, an aliquot of the protein sample was placed in the well and electrophoresis performed in the direction indicated. After electrophoresis, troughs were cut on both sides of the gel along the same axis as the electrophoresis, and these were filled with antiserum. The antibodies and proteins were allowed to diffuse and precipitate as described for the double diffusion analysis. Each band in Fig. 6 represents a distinct protein recognised by an antibody specific to that protein. The number, position, and degree of staining of these bands can be used to characterise the protein complement of an extract and to compare species or clones.

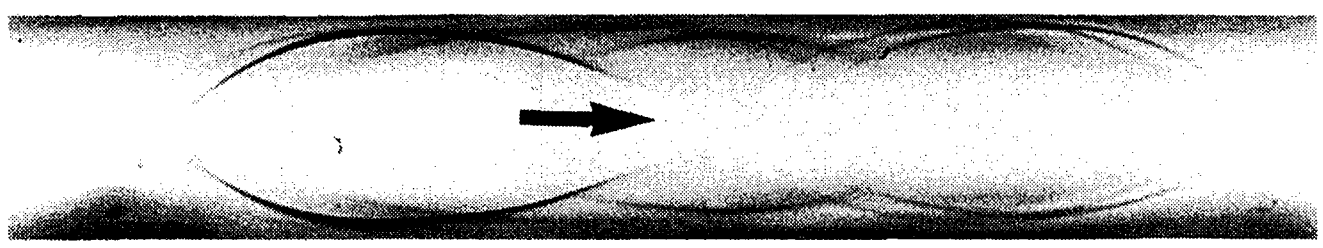

FIG. 6—Protein extracted from **Pinus radiata** separated by electrophoresis and challenged with anti-serum to **P. radiata**.

The techniques used in this study enabled similarities and differences to be detected amongst the four *Pinus* species used, and between the different clones within a species. These findings are the subject of a separate communication.

This paper reports a simple and rapid method for protein extraction from *Pinus* tissue which will enable taxonomic investigations for systematic and breeding purposes to be carried out. The major advantages of the method are the apparent control of interfering substances and their rapid removal by a relatively large-scale gel filtration step to yield a high molecular weight fraction which is amenable to analysis by electrophoretic and serological techniques.

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