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Electrophoretic Comparisons of Soluble Optical Shoot Proteins in *Pinus Thunbergii* Trees having Different Resin Exudation Amounts

(Preliminary report)

By

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Summary: Electrophoretic properties of the soluble protein complements extracted from the optical shoots of *Pinus Thunbergii* trees having different resin exudation amounts were compared.

The 4 planting individuals with non-resected root system consist of 2 of the 1st class (+++) and 2 of the 3rd class (+) in resin exudation, and 2 of the 1st (+++) or the 2nd class (++) in resin exudation 2 days after the artificial resection to root system were chosen as test materials to identify the physiological condition.

A zone of highly charged and low molecular weight protein components was found to be more oppressive in the protein complements of one material of the 3rd class in resin exudation and one resected root system among all those in the test.

The electrophoretic patterns of soluble optical shoot protein complements may be useful for identifying the physiological condition of the standing trees.

Introduction

In the plant growing in condition, green leaves synthesize not only proteins for their own growth, but in addition break them down catalytically into peptides and amino acids[2]. The breakdown continues to ammonia or other N-rich compounds. The end products have low molecular weight and are therefore easily transported into the site of active growth or the downwardly directed stream.

While green leaves are subjected continuously to the hydrolysis and synthesis of constitutive protein, they are preserved to be very much of a constant concentration in protein extending over a long time, because there is a balance between hydrolysis and synthesis of protein in the living cell. The balance between anabolic and catabolic processes of protein in green leaves is closely connected with not only available N·P·K macro- and other micro-nutrient supplies but also the respiratory and photosynthetic metabolism of the plant. It is influenced by such environmental factors as light, photoperiodism, temperature, ground water level, permeability of soil and so on surrounding the plant. MERZ et al.[3] observed changes in electrophoretic patterns of soluble proteins of alfalfa plants when subjected to nutrient

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deficiencies. In the studies on the electrophoretic and immunological comparisons of soluble root proteins of *Medicago sativa* L. Genotypes in cold hardened and non-hardened condition, COLEMAN et al.[1] found a zone of highly charged and/or low molecular weight protein components that proved to be more prevalent in the protein complements of the cold hardened material than the non-hardened.

From these facts, it is presumed that the information on the qualitative and quantitative analysis in the soluble proteins from plants reflect significantly the current physiological conditions of the plants. It is accordingly possible that electrophoretic properties in soluble proteins from the tissues of *Pinus Thunbergii* trees serve for identifying the vigor class of the trees.

From among the standing trees of *Pinus Thunbergii* afflicted with insect damage caused by pine bark beetles, the percentage of dead trees among them in the current year seems to be quite correctly estimated with the classifications of resin exudation amounts produced by circular perforation reaching to the sapwood of the butt in August[4]. This paper reports the comparative gel electrophoretic properties of soluble protein complements from the optical shoots of the standing trees of *Pinus Thunbergii* having different amounts of resin exudation in August 1969 and mechanically resected root system except tap root, unrelated directly with the insect damage.

Materials and methods

A. Experimental plot and estimation of resin exudation amount

Experimental plot was established in the middle of June 1969 on 14-year-old planted forest located in the north-western part of Uranouchi Bay, Suzaki City, Kochi Pref. in Japan. Soils under the plot had fair to good internal drainage but were low in natural fertility.

The estimation on resin exudation amounts of standing trees within the plot were made according to the procedure outlined by NITTO et al.[4] at late in August 1969.

One circular hole of 2 cm in diameter reaching the bast zone of butt was perforated on every tree. The aspects of resin exudation were observed about 3 hrs after the working. The resin exudation amounts in every tree were classified into 6 classes, namely, ##, #, +, ±, − and 0 in plentiful order. The individuals having such better flow as correspond to the former 2 class were judged as normal condition, but the others were regarded as abnormal. The perforation workings were carried out newly as the investigation progressed.

B. Artificial interference to root system

To investigate the electrophoretic properties of soluble protein after artificial interference to root system, the following workings were practised about the 9 individuals within the same plot ranked to the 1st class in resin exudation in the middle of June 1969.

Soils around the trees were dug down to reach the underlying rock or about 50 cm deep, and then the root system except tap root was cut down vertically at the place of 15 cm long from the butt of trees. The dug soils were put back again. Some trees showed a decreasing amount of resin exudation just after the resection of root system, but all trees except 2 windfalls were restored to the 1st class in resin exudation by the middle of July 1969.

C. **Collection of optical shoots**

The sampling of material optical shoots was made in late August 1969 individually from 4 trees with non-resected root system consisting of 2 of the 1st class, and 2 of the 3rd class in resin exudation, and 2 with resected root system still judged as the 1st class in resin exudation 2 days after the resection of root system. The sampling trees were chosen so as to be in those having leaves judged as normal by externals.

D. **Preparation of extracts**

Some optical shoots having scaly leaves, removed were washed with ethyl-ether and frozen by dry ice. The frozen materials were dropped into some cold acetone kept at below zero, and then immediately broken to pieces for 3 min. by homogenizer cooled with cold acetone and dry ice. The broken matelials in cold acetone kept at below zero were immediately filtered by a suction filter. The filtrate was deserted. The residue was washed in cold ethyl-ether using a suction filter. When the ethyl-ether was removed from the residue by volatilization in the inside of a freezer, the residue was dehydrated on P_2O_5 at 0°C for 2 days. The dehydrated residues were screened out using 20 mesh sieve, and then acetone powders were obtained.

The powders corresponding to 2 g were weighed and added to 50 m*l* cold tris-hydrochloric acid buffer (1 M; pH 7.5) including 0.04 M cystine, 0.002 M EDTA, 12.5% glucose, 1% ascorbic acid, 0.39 g thioglycol and 0.5 m*l* Tween 80, and then homogenized speedily for 5 min. with homogenizer. The resulting homogenates were kept for 1 hr at 0°C and then centrifuged at 3,000 g for 15 min. to remove cell debris in cold room. The supernatant fraction was centrifuged again at 10,000 g for 20 min. to remove cell organelles at 0°C. The supernatant fraction was salted out with ammonium sulfate at a point of 90% saturation and left for 20 min. at 0°C, and centrifuged at 10,000 g for 20 min. The supernatant fraction was deserted. The residue was dissolved again to 20 m*l* cold tris-hydrochloric acid buffer (0.1 M; pH 7.5). And then both the procedures of the salt out and the centrifugation were repeated again. The supernatant fraction was deserted. The residue was dissolved to 10 m*l* tris-hydrochloric acid buffer (0.03 M; pH 7.5) and then dialyzed 48 hrs at 0°C against cold tris-hydrochloric acid buffer (0.03 M; pH 7.5) through 3 exchanges of buffer. The dialyzed protein suspension were preserved in some ampoules and lyophilized, and stored in a freezer kept at 0°C.

Previous to the electrophoretic separations of protein extracts, the lyophilized protein samples were prepared with deionized water to give a final concentration of approximately 6.0 mg of protein per m*l*, following the protein content per ampoule estimated by the micro-Kjeldahl method.

E. **Preparation of gel and electrophoresis**

Electrophoretic separations were carried out by disc-electrophoresis using polyacrylamide gel as a supporting medium, according to a few modified froms of the procedure outlined by STEWARD et al.[5] The following solution instead of the stock solution *d* was prepared.

1 N-Hydrochloric acid	24.0 m*l*
Tris	2.99 g
TEMED	0.46 m*l*
Deionized water to	100 m*l*

And the electrode buffer was as follows:

Tris	6.0 g
Glycine	28.8 g
Deionized water to	10 *l*

The electrophoretic runs just after the light polymerization of gel were made at 1.67 mA per column until the tracking dye (front band) by bromophenol blue approached at a distance 10 mm to the lower end of tube.

F. **Detection of separated bands**

Protein in the gel column were fixed and stained with amido Schwarz 10 B (10 g/mixed solution consists of 400 m*l* of deionized water, 500 m*l* of methanol and 100 m*l* of acetic acid) for 60 min. after the removal of gels from each tube, and then washed with several exchanges of deionized water.

The gels were washed repeatedly with 7% acetic acid solution. When the part of gel which contained no protein was completely decolorized of dye, the gels having stained bands were analyzed densitometrically by use of a model DMU-2 Type Densitorol (439 mμ filter) designed and manufactured by Toyo Kagakusangyo Co.

The densitometric traces and diagramatic interpretations of each gel column are shown in Fig. 1～3. As it was very difficult to arrange the runs to a same length from the origin to the front during the electrophoresis, the diagrammatic interpretations with relative movement (comparable to the Rf values) to front of individual bands (movement of the front; 100) in the diagrammatic interpretations (Fig. 1～3) obtained from all the gels were represented again in Fig. 4 so that the electrophoretic patterns from every sample were compared directly. The signs marked with arrows mean peaks on the traces. The diagrammatic banding patterns consisting of broad densed (A), narrow densed (B), dotted (C) and linear (D) bands were used to indicate various standards of the staining portions on gels. The A, B and C bands mean visible bands on gels, although C band is a less-densed band. The last D band means not visible, very fine and faint band.

Results

A. **Electrophoretic separation of trees with normal resin flow**

The electrophoretic separation carried out on 2 individuals wtih non-resected root system of the 1st class in resin exudation are shown in Fig. 1-1 and 1-2.

In Fig. 1-1, one A band, 7 B bands as well as a similar one which constituted the front and 6 D bands excluding the front were observed. Other sample in Fig. 1-2 had 1 A band, 8 B bands, 1 C bands and 4 D bands excluding the front.

The variation in the position of each band was found between individuals within the 1st class in resin exudation, but there was not much variation between individuals in the number of visible bands on gel.

Both the individuals hold in common relatively many visible bands.

A zone of lowly charged or high molecular weight protein complements was found to be more prevalent in both the densitometric traces.

Both the 2 individuals continued to support the normal resin flow until the middle of May 1970.

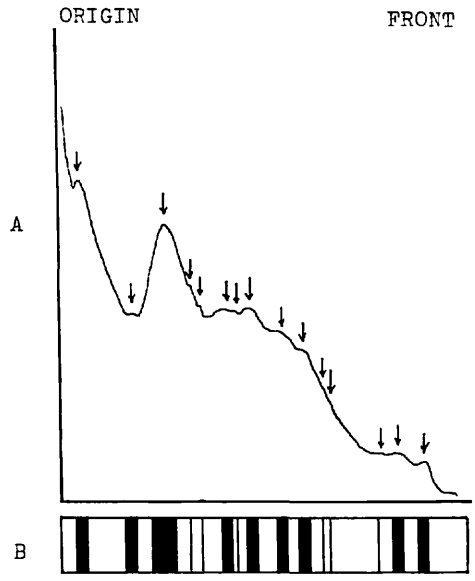

Fig. 1-1 The electrophoretic separations of the soluble protein of individuals of the 1st class in resin exudation. A is a densitometric trace of the gel; B is a diagrammatic interpretation of the gel.

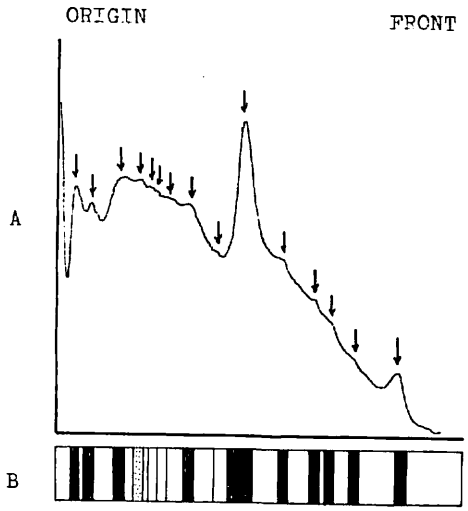

Fig. 1-2 (Continued)

B. **Electrophoretic separation of trees with resected root system**

The electrophoretic separation practised on 2 individuals with resected root system kept up the normal resin flow 2 days after the resection of root system and are shown in Fig. 2-1 and 2-2.

It could be seen that one individual (Fig. 2-1) had 1 A band, 4 B bands, 1 C band and 3 D bands, and the other individual (Fig. 2-2) had 1 A band, 5 B bands, 1 C band and 5 D bands, excluding the front.

The individuals with resected root system having a number of lower A, B and C visible bands than ones with non-resected root system, yielded the best flow in resin exudation.

The individual investigated in Fig. 2-1 continued to support normal resin flow until the middle of May 1970, but the other one in Fig. 2-2 died during the same season in 1970.

The densitometric trace in Fig. 2-1 represented a similar pattern to Fig. 1-1 or 1-2, while the other trace (Fig. 2-2) of investigated individual that died by the middle of May in the following year differed from them in having a more lowly concentrated trace amount of the zone of lowly charged or high molecular weight protein components.

C. **Electrophoretic separation of tree with abnormal resin flow**

The electrophoretic separation of individuals of the 3rd class in resin exudation are shown in Fig. 3-1 and 3-2.

From Fig. 3-1, 1 A band, 1 B band, 3 C bands and 8 D bands excluding the front were obtained on gel. In Fig. 3-2, 1 A band, 9 B bands and 2 D bands excluding the front were

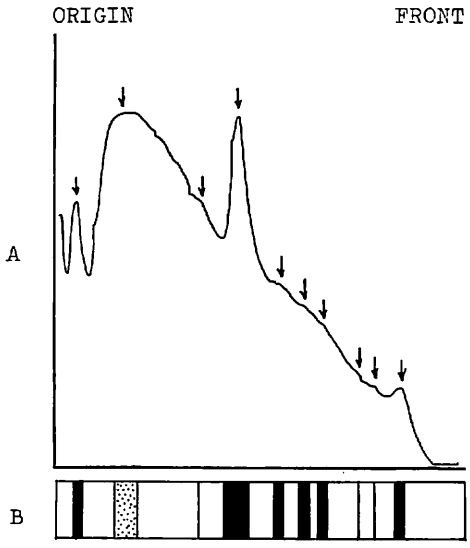

Fig. 2-1 The electrophoretic separations of the soluble protein of individuals with resected root system.

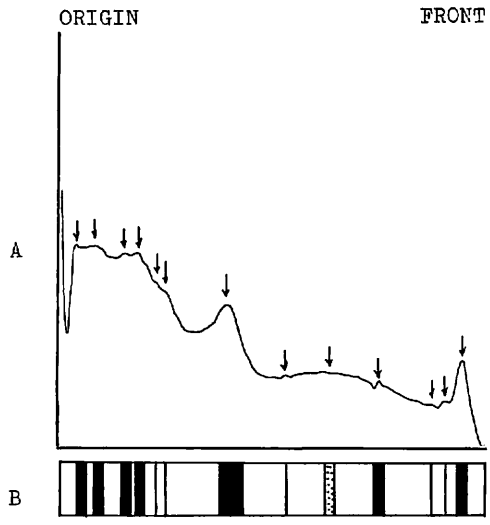

Fig. 2-2 (Continued)

represented.

The individual used in Fig. 3-1 was unable to be observed for the relation between little resin flow and protein metabolic block, because the standing tree had been felled just after the sampling of optical shoots. The individual used in Fig. 3-2 had been restored to the best resin flow by the middle of May the next year.

From these separations, it was observed that the number of A, B and C visible bands varied between individuals. The pattern in Fig. 3-1 had the fewest among test individuals in the number of A, B and C visible bands, while the other pattern had as many visible bands similar to the number of bands in individuals having the best resin flow.

The densitometric trace in Fig. 3-1 showed a similar pattern to Fig. 2-2 from between individuals with resected root system, but the trace in Fig. 3-2 showed a similar pattern to ones observed in individuals having the best resin flow.

Discussion

It has been shown that disc-electrophoresis technique using polyacrylamide gel as a supporting medium is sensitive and useful when applied to separate the soluble optical shoot protein of *Pinus Thunbergii*.

The densitometric traces showed such general pattern as maintain more quantitatively predominant in a zone of slowly migrated and high molecular weight protein components, and oppressive in a zone of rapidly migrated and low molecular weight protein components in relation to trace amounts.

It seems that a zone of slowly migrated and high molecular weight protein components was more highly concentrated and definitive when the material optical shoots were in normal

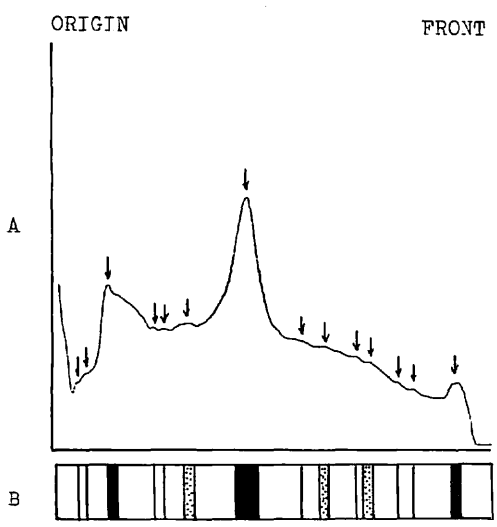

Fig. 3-1 The electrophoretic separations of the soluble protein of individuals of the 3rd class in resin exudation.

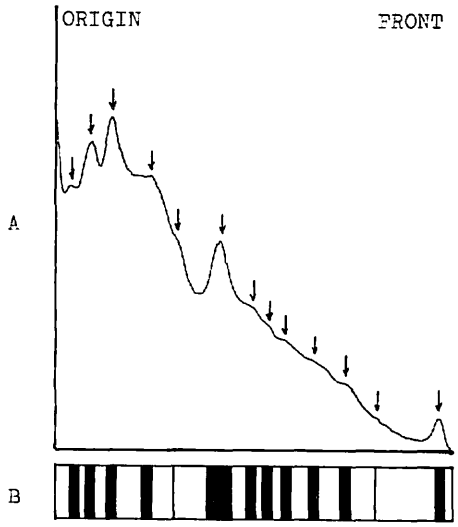

Fig. 3-2 (Continuous)

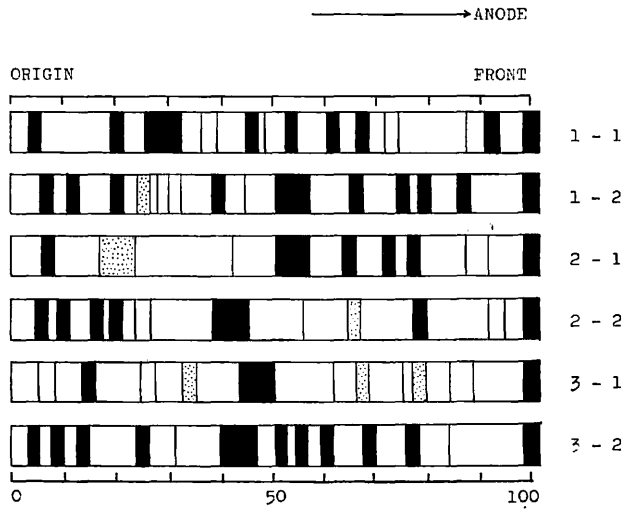

Fig. 4 Diagrammatic interpretations by relative movement to front (movement of the front = 100) of each bands in electrophoretic separations of soluble protein from individuals of the 1st class in resin exudation (1-1, 1-2), ones with resected root system (2-1, 2-2) and ones of the 3rd class in resin exudation (3-1, 3-2).

physiological condition. On the contrary, both the densitometric traces in individuals with resected root system led to death by the middle of May the next year (Fig. 2-2) and one of the 3rd class in resin exudation (Fig. 3-1) were more poorly concentrated and indistinct in the same zone.

It seems that the constitutive proteins of green leaves are preserved to be very much of a constant concentration extending over a long time, because there is a balance between the anabolic and catabolic processes of proteins in the living cell[2], but when once the photosynthetic activity of green leaves becomes more oppressive owing to the influence of a certain environmental element and so on, the balance is broken, and then the falling of protein content occurs.

From these points of view, it appears that both the densitometric traces on an individual of the 3rd class in resin exudation used in Fig. 3-1 and one with resected root system used in Fig. 2-2, mean such condition as the hydrolysis of protein is superior in metabolic speed to synthesis.

The positive correlation was not obtained between the number of A, B and C visible bands and the amounts of resin exudation, but it brings forward an interesting problem in relation to the vigor class of standing tree in that some individuals among those of the 3rd class in resin exudation and those with resected root system showed a peculiar densitometric trace considered to be relatively abnormal in protein metabolism.

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(研　究　資　料)

樹脂流出量のことなるクロマツ立木における頂芽の可溶性蛋白質の電気泳動パターン

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摘　　要

樹脂流出量のことなるクロマツ立木における，頂芽の可溶性蛋白質の電気泳動による泳動パターンのちがいをしらべてみた。

高知県須崎市浦の内の14年生クロマツ人工林（民有林）において，1969年8月下旬に日塔ら[4]の方法によって，樹脂の流出量から健全木とされた2個体，異常木とされた2個体，さらに1969年6月下旬に樹脂の流出がおう盛な健全木に，根系の切断処理をおこなった個体から2個体，合計6個体から頂芽をあつめた。

頂芽をアセトンパウダーとしたあと，シスチン，EDTA，グルコース，アスコルビン酸，チオグリコールならびにツィーン80含有トリス塩酸緩衝液（1 M; pH 7.5）でホモゲナイズし，遠心分離，硫安塩析，透析などをおこなって可溶性蛋白質を抽出した。

電気泳動は，Steward ら[5]によって報告されたポリアクリルアマイドゲルを支持媒質とする，ディスク電気泳動法に順じておこなった。

泳動後のゲルを染色ならびに脱色したあとの分離帯の検出は，東洋科学産業製DMU-2型デンシトロール（439 mμ フィルター）でおこなった。

Fig. 1～3は，各ゲルカラムのデンシトメトリーによる泳動パターンと模式図である。また泳動に際しては，原線からフロントまでの泳動距離を同一の長さに統一できなかったので，Fig. 1～3の模式図で原線からフロントまでの距離を100とした場合の，各バンドのフロントまでの相対的移動度（Rf値に相当する）による模式図を，サンプルごとのパターンが直接比較できるように，一括してふたたびFig. 4に示した。

デンシトメトリーによる泳動パターンは，泳動速度の小さい高分子の蛋白質成分が量的に優占し，泳動速度の大きい低分子の蛋白質成分が，量的に少ないという一般的な傾向を示したが，そのなかでとくに供試翌年に枯死につながった根系処理木（Fig. 2-2）ならびに異常木（Fig. 3-1）のパターンではこの傾向があきらかでなく，泳動速度の小さい高分子蛋白質成分の量的優占の程度が弱かった。したがって，Fig. 2-2 と Fig. 3-1 の例は，クロマツ立木の健全度との関連で興味ある問題を提起する。

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