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THE USE OF POLYACRYLAMIDE GEL ELECTROPHORESIS FOR RAPID DIFFERENTIATION OF Gremmeniella abietina ISOLATES

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ABSTRACT

Soluble proteins from crude mycelial extracts of Gremmeniella abietina isolates from Europe, North America and Japan were compared using non gradient and gradient polyacrylamide gel-slab electrophoresis. With Coomassie blue, a total of 18 bands were recognized when results from all isolates were grouped (standard type). With reference to this standard type, isolates could be classified into six groups: 1) Isolates from Europe, New York State (US7-US11), and some from red pine from Quebec, are characterized by the presence of band 12 and the absence of bands 4 and 10. 2) Isolates from Ontario (C1) and others from pine from Quebec, show the presence of band 4 and the absence of bands 10 and 12. 3) Isolates from Japan lack band 12. 4) Isolates from New Brunswick and some from Quebec (MER) show the greatest number of bands and combining both European and American characteristics. 5) Isolates from spruce from Quebec are relatively similar to the isolates from the second group but distinguishable by at least three additional bands. 6) Isolates from balsam fir from Quebec are characterized by the lack of several bands and especially band 13. The importance and the interest of these two tests as complementary or even as a possible substitute to serology is discussed.

INTRODUCTION

For several years, the criteria used for differentiating races or strains of Gremmeniella abietina (Lagerb) Morelet were based on symptomatology of infected hosts, conidium morphology and geographic and site differences. (Ettlinger 1945; Gremmen 1968; Dorworth 1971). In 1975, by means of immunological tests, Dorworth and Krywienczyk described the occurrence of three serotypes of G. abietina, heterologous among, but homologous within, the geographic

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range of this fungus in Europe, North America, and Asia. Later, by comparing symptomatology, growth and spore dispersal patterns, Skilling (1977) suggested that most of the isolates found associated with mortality of large trees in New York State, were similar to the European isolates. Dorworth et al. (1977) confirmed Skillings' report, and their serological study provided strong evidence that the New York isolates were identical to the European ones and different from the standard North American type. Serological methods were, therefore, considered to be of great interest for differentiating among these various isolates. Considering the threat in the New York state plantations, Canada and the United States applied quarantine regulations to stop import of trees from areas where the European race occurred. Because of the strong governmental measures and the complexity of the serological techniques (Dorworth et al. 1982), it became necessary to develop other techniques for differentiating G. abietina isolates. This need was also stressed by the variability of the results obtained by serology with G. abietina isolates from spruce from Quebec (D. Lachance, personnal communication), and the difficulties encountered assigning some Canadian isolates, e.g. those from New Brunswick, with a particular race. For these reasons and others, electrophoretic techniques were considered as a possible substitute to serology. For the last fifteen years, gel electrophoresis of soluble fungal proteins has been used as an aid to resolve taxonomy problems (Glynn and Reid 1969), or to evaluate relationships between species (Dorworth 1974) or among several strains of one species (Webb et al. 1972; Torp and Andersen 1982). The objective of the present study was to evaluate the use of polyacrylamide gel-slab electrophoresis, for a rapid and reliable differentiation of races of G. abietina. We also tried to gain more information about some Canadian isolates which could not be identified through serology.

MATERIAL AND METHODS

Origin of Isolates and Cultural Methods

Isolates of G. abietina from different geographical areas, except those from Quebec, were obtained from the culture collection of Dr. C.E. Dorworth, Great Lakes Forest Research Centre, Sault Ste. Marie, Ontario (Dorworth 1982) (Table 1). Isolates were grown at 4C on agar medium made of 25 ml of Campbell's V8 juice; 15 g Bactoagar; 7.5 g malt extract and 475 ml of distilled water, dispensed at 25 ml per Petri dish. Pieces $1cm^2$, cut from actively growing mycelium on agar were transferred in 125 ml Erlenmeyer flasks containing 45 ml of an autoclaved liquid growth medium [containing for one liter]: 100 ml of each of the following: 0.5% KH_2PO_4; 0.5% NaH_2PO_4; 0.5% $MgSO_4$ 0.5% KCl; 2% asparagine; 1% glucose; 2% $FeCl_2$; 4.5% HCl plus distilled water. This 45 ml liquid medium was sterilized and complemented with 5 ml of a filter sterilized vitamin solution (100 ml of 12.10^{-5}% biotine; 4 ml of 25.10^{-4}% thiamine; 4 ml of 25.10^{-4}% pyridoxine). The mycelium was allowed to grow at 14C for 4 to 5 weeks and then ground in a sterilized homogenizer. Freshly prepared flasks of autoclaved liquid medium (same composition as above), were inoculated with 1 ml of the ground mycelial suspension and maintained at 14C for at least 4 but not more than 8 weeks.

Preparation and Gel Electrophoresis of Crude Mycelial Extracts

The mycelium was harvested by filtration through a Buchner funnel, washed with distilled water and ground to a powder with autoclaved acid-washed silica gel (Silicon dioxide S-150) using a prechilled mortar and pestle (Bucher 1974).

Table 1. Origin of isolates of Gremmeniella abietina tested by polyacrylamide gel-slab electrophoresis

Isolate No.	Place of Origin	Host	Serological Identification
US 2	Michigan - USA	Pinus banksiana	Am
US 6	New York - USA	Pinus strobus	Am
US 7	New York - USA	Pinus resinosa	E
US 8	New York - USA	Pinus resinosa	E
US 9	New York - USA	Pinus resinosa	E
US 10	New York - USA	Pinus resinosa	E
US 11	New York - USA	Pinus sylvestris	E
F 2	Virolahti-Finland	Pinus sylvestris	E
F 3	Ylikiiminki-Finland	Pinus sylvestris	E
F 4	Virolahti-Finland	Larix sibirica	E
N 2	Ostfold - Co- Norway	Picea abies	E
N 3	Reykjavik - Norway	Pinus aristata	E
G 1	Germany	Pinus strobus	E
J 1	Hokkaido - Japan	Abies sachalinensis	As
J 2	Hokkaido - Japan	Abies sachalinensis	As
C 1	Ontario - Canada	Pinus resinosa	Am
79 - 253	Armtown-Quebec-Canada	Pinus resinosa	E
79 - 353	St.Lambert-Quebec-Canada	Pinus resinosa	-
79 - 347	East Pimade-Quebec-Canada	Pinus resinosa	E
79 - 384	Sutton-Quebec-Canada	Pinus resinosa	E
79 - 3456	MTF-Quebec-Canada	Pinus resinosa	Am
79 - 257	Power court-Quebec-Canada	Pinus resinosa	E
79 - 376	Sutton-Quebec-Canada	Pinus resinosa	E
79 - 1039	Mont-Oxford-Quebec-Canada	Pinus resinosa	E
79 - 333	Power court-Quebec-Canada	Pinus resinosa	E
79 - 622	Pont rouge-Quebec-Canada	Pinus resinosa	Am
79 - 623	Chute Panet-Quebec-Canada	Pinus sylvestris	-
79 - 419	Lac Gauthier-Quebec-Canada	Pinus resinosa	-
79 - 1041	Memphremagoz-Quebec-Canada	Pinus resinosa	E
MER 3677	MTF-Quebec-Canada	Pinus resinosa	Am
MER 961	MTF-Quebec-Canada	Pinus resinosa	-
MER 1973	Hereford-Quebec-Canada	Pinus resinosa	E
MER 1887	Hereford-Quebec-Canada	Pinus resinosa	E
NB 138	New Brunswick-Canada	Pinus resinosa	Problem
NB 192	New Brunswick-Canada	Pinus resinosa	Problem
NB 202	New Brunswick-Canada	Pinus resinosa	Problem
NB 047	New Brunswick-Canada	Pinus resinosa	Problem
81 - 867	Montmorency-Quebec-Canada	Picea mariana	Problem
79 - 606	Launiere Parc-Quebec-Canada	Picea mariana	Problem
79 - 211	Lac Gregory-Quebec-Canada	Abies balsamea	Problem
79 - 218	Lac Jumeau-Quebec-Canada	Abies balsamea	Problem
79 - 502	Zacharie-Quebec-Canada	Pinus banksiana	E
79 - 077	St.Magloire-Quebec-Canada	Pinus sylvestris	Intermediate
79 - 063	Launiere Parc-Quebec-Canada	Picea mariana	E
C 36	Valcartier-Quebec-Canada	Pinus contorta	-
C 94	Newfoundland-Canada	Pinus sylvestris	-
C 77	New Brunswick-Canada	Pinus resinosa	-

E: European race - Am: North American race - As: Asian race

ıe soluble proteins were extracted by adding to the mortar 0.3 ml cold 0.1 M ris-glycine buffer pH = 7.5, containing 10% sucrose and 1 mM of a protease hibitor (Phenyl methylsulfonylfluoride: PMSF;). The material was centrifuged 10,000 x G for 15 min at OC and the supernatant was collected with a Pasteur pette avoiding the presence of a lipid layer floating on the surface. Soluble roteins were separated by polyacrylamide gel-slab electrophoresis. Best results were obtained using a 14% acrylamide separating gel: 9.4 ml of acrylamide 30%) - bisacrylamide (0.8%) solution; 5 ml of Tris-HCl buffer, pH 8 ; 5.5 ml f distilled water; 0.2 ml of ammonium persulfate 10%; 8 ul of Temed. The tacking acrylamide was made with: 1.7 ml of an acrylamide (30%) - bisacrylaide (1.037%) solution; 1.25 ml of Tris-H_3PO_4 buffer: pH = 6.7; 7 ml of disilled water; 0.3 ml of ammonium persulfate; 40 ul of Temed. For each experient, 120 ul of crude extracts of G. abietina isolates was placed directly into ıe preformed slots.

A voltage of 50v was applied for 30 min, then increased to 150v for about hours. After the electrophoretic run, the polyacrylamide gel was carefully emoved and stained, for 30 min, with 2% Coomassie brilliant blue R-250 in 50% ethanol, 40% distilled water and 10% acetic acid. Excess coloration was eliinated by incubation of the gel in a 50% methanol, 40% distilled water, 10% cetic acid solution for 2 hours. The gel was then stored in a 7% acetic acid nd dried later under vacuum. Silver staining was used in some cases according o Morrissey (1981).

Gradient Gel Electrophoresis

Gradient polyacrylamide gel-slab consisted of a 7.5-15% separation polyaylamide gel. The 15% gel solution (15.5 ml of an acrylamide (30%) bisacrylaide (0.8%) solution; 3.85 ml of Tris HCl buffer pH 8.9; 8.95 ml of sucrose 0%; 2.7 ml of distilled water; 110 ul of ammonium persulfate; 8 ul of Temed) as progressively diluted with the 7.5% solution (7.9 ml of acrylamide (30%) - isacrylamide (0.8%); 3.85 ml of Tris - HCl buffer pH 8.9; 2.6 ml of sucrose 0%; 16.5 ml of distilled water; 110 ul of ammonium persulfate; 8 ul of Temed) nd poured at one drop per second with a peristaltic pump.

After polymerization of the saccharose gradient gel, a 5% concentration el consisting of 3.34 ml of acrylamide (30%) bisacrylamide (1.037%); 2.5 ml of ris H_3PO_4 buffer pH 6.7; 13.95 ml of distilled water, 0.8 ml of ammonium perulfate and 80 ul of Temed, was added. A constant current of 3 mA was applied or about 16 hours. After the electrophoretic run, the gel was removed and reated with Coomassie brilliant blue R-250 as described above.

ESULTS

Electrophoretic tests carried out with many different G. abietina isolas showed that the patterns of protein bands obtained from mycelial extracts uld vary with culture age. The best patterns were obtained with 4 to 8 weekd (5 week-old cultures were mostly used) cultures and 14% polyacrylmaide ls.

From the analysis of the results obtained with Coomassie blue staining, a andard type pattern (ST), grouping all identifiable bands contained in all e isolates tested (representatives of which are shown in Figure 1), was esblished and constituted our point of reference. Band 7 was one of the most onounced bands found which was constantly associated with all these isolates;

therefore whatever sight variations in band positioning might have occurred between runs this band constituted a reliable marker for identifying other bands. Frequently, a variability in band thickness and color intensity was observed, indicating a variation in the amounts of extracted proteins. These differences were not investigated and our study was based on the comparative

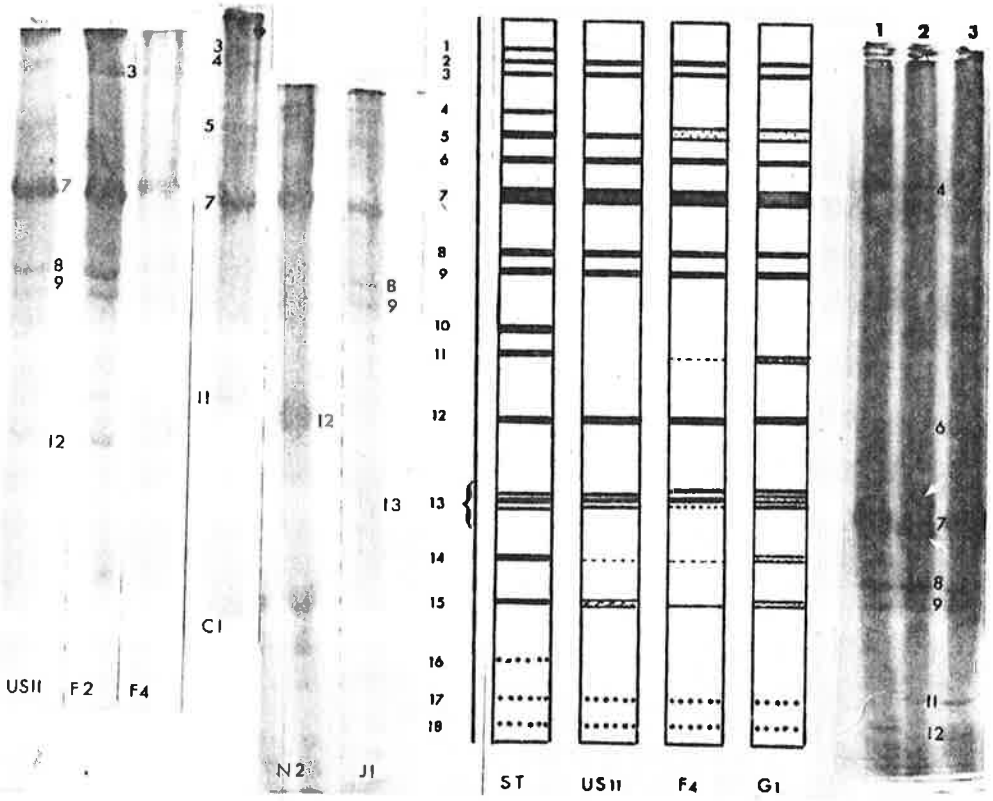

Figure 1. Illustrations of typical protein patterns otained with Commassie blue and 14% acrylamide gel (left part) and gradient gel (right part). Left part: the numbers from top to bottom, in the direction of the separation front, correspond to the main bands identified and considered to be of significance in the present study. Note: band 12 missing in C1 and J1; band 11 missing in F4, N2, and J1; presence of band 4 in C1 and and not in others. Although band 13 is visible only in J1, it was also present in the others (except N2) but did not reproduce well here. Right part: numbers 1, 2 and 3 correspond respectively to isolates US11, 81-867, and C77. The numbered bands are those believed to correspond to the same bands shown in the left part. Note: absence of band 12 in 81-867; and absence of band 11 in US11. The band identified as 4 is present in 81-867 but not in others: the faint line visible at this level in US11 is probably a background effect as it really could not be detected in the gel itself. Middle part: a histogram representation of some of the isolates constituting Group I.

evaluation of the number and the positioning of identifiable electrophoretic bands. Bands 16-18 were considered as non specific and their presence or absence was considered non reliable.

Analysis of representative electrophoretic patterns for soluble proteins indicated that the G. abietina isolates tested could be divided into six well defined groups (Table 2). Pine isolates (79-253, -353, -347, -384, -3456, -267, -374, -376, -1039, 1071 and 81-133 from Quebec exhibiting affinities with one another, with the other pine isolates from New York (US7, 8, 9 ,10, 11) or Europe (F2, F3, N3, G1) and with a larch isolate from Europe (F4), were placed in the first group. Qualitative comparisons with the standard type, showed that bands 4 and 10 were constantly missing. Band 11 was prominent in some isolates (G1, 791039), but was, most often, very weak, and sometimes not recognizable. Bands 12 and 13 were always present.

Other pine isolates from Quebec (79-622, -623, -419, -1064) gave patterns similar to that of isolate C1 (a pine isolate from Ontario, identified as American by serology) and formed the group II. This group was characterized by the lack of bands 10 and 12 while band 13 was always present.

In group III, fir isolates from Japan (J1, 2) gave similar patterns showing the total absence of band 12, the weakness of bands 6 and 11 and the constant occurrence of bands 10 and 13.

Four pine isolates from Quebec (MER3677, 961, 1973, 1887) and four from New Brunswick (NB 138, 192, 202, 047) formed the group IV. They were demarcated from the other groups by patterns exhibiting the greatest number of bands. Only band 14 was missing.

Table 2. Classification of isolates of Gremmeniella abietina according to patterns obtained after polyacrylamide gel electrophoresis

	Bands														
	1	2	3	(4)	5	6	7	8	9	(10)	(11)	(12)	(13)	14	15
Group I - Isolates from Europe, New York, Quebec	-	+	+	(-)	+	(+)	+	+	+	(-)	(±)	(+)	(+)	±	+
Group II - Isolates from Ontario, Quebec	-	+	+	(+)	+	(+)	+	+	+	(-)	(+)	(-)	(+)	+	+
Group III - Isolates from Japan	-	+	+	(+)	+	(-)	+	+	+	(+)	(±)	(-)	(+)	+	+
Group IV - Isolates from Quebec (MER), New Br.	±	+	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	(+)	-	+
Group V - Isolates spruce from Quebec	-	+	+	(+)	+	(+)	+	+	+	(-)	(+)	(-)	(+)	+	+
Group VI - Isolates balsam fir and one from pine from Quebec	-	±	+	(-)	+	(+)	+	+	+	(-)	(+)	(-)	(-)	+	+

Data in parenthesis are considered as the most reliable results in grouping isolates.

Examination of electrophoretic patterns from spruce isolates from Quebec (81-867; 79-606) revealed differences with groups I and III but had a close relationship with group II. They were, however, placed in a fifth group because bands 10 and 12 were missing. Two light bands, not observed in other isolates, appeared between bands 12 and 13.

Group VI is composed of G. abietina isolates from balsam fir (79-211,-218) and pine (79-502) from Quebec. The electrophoretic patterns of these three isolates were easily distinguishable by the lack of bands 4, 10, 12 and especially band 13, which was present in the other groups.

From all the European isolates tested, the spruce isolate N2 from Norway, revealed an atypical pattern. Bands 7 and 12 were always recognizable, but not bands 8, 9, 11 and 13.

Patterns of soluble proteins obtained from US2 and US6 were totally dissimilar from those resulting from all the other US isolates . By the number and relative positions of the bands, they could be more or less considered as related with group II. The pine isolate 79-077 from Quebec lacked bands 1-4, 8-10 and 14 and had no affinity with any of the other isolates.

Silver staining was found to be efficient and yielded the same patterns as with Coomassie blue. However results were not always consistent. With improvements, this technique could be very useful since its sensitivity is much greater than the Coomassie blue staining method.

Isolates: 81-867 from spruce from Quebec, US11, C36, C94, C77 from pine respectively from New York, Quebec, Newfoundland and New Brunswick produced various patterns with saccharose gradient polyacrylamide gel-slab electrophoresis. Electrophoretic patterns confirm the constant presence of one large band, corresponding to band 7 as obtained with polyacrylamide gel-slab electrophoresis. However the Rf of this band was displaced towards the anode, bands beyond 13 becoming somewhat crowded at the run end. The main differences described above for the same isolates were well confirmed: the band corresponding to band 12 was present in US11, C94, and C77, but not in 81-867 (from spruce) and C36; three extra bands, one as band 4 and two as satellites to band 7 in 81-867, separated it from C36 (these two satellite bands were also absent in the others); C94 differed from the others by lacking a band corresponding to band 6 (which however was weak in US11 and 81-867) and by having a band corresponding to band 4 (in 81-667) and another one corresponding to band 3 (in US11).

DISCUSSION AND CONCLUSION

We have shown that non gradient and gradient polyacrylamide gel-slab electrophoresis can be used to discriminate among isolates of G. abietina. Electrophoretic comparisons of the different isolates (Table 1) tested generally confirmed the inital results obtained using serology (Dorworth 1974; Dorworth and Krywienczyk 1975; Dorworth 1982) concerning the existence of three distinct races of this fungus and the affiliation of the New York isolates to the European race (Skilling 1977; Dorworth et al. 1977). The protein patterns obtained from European isolates, except for isolate N2, were dissimilar to those from North American strains represented by C1. Thus, among other possible differences, band 4 only present in the American and band 12 only present in the European isolates, were considered as good criteria of differentiation between these two groups. The absence of band 12 (and generally band 6) and the pre-

sence of band 10 in Japanese isolates made them easily distinguishable from both European and American types. They were also different from all other isolates (Table 1) and therefore could be identified without any problem. In the light of these results, three groups of isolates with distinctive protein patterns are proposed and correspond to previous groupings obtained by serology. The New York isolates, except US2, US6, and a large number of isolates from red pine from southern Quebec, exhibited very close affinities with the European type. A few isolates from this same part of Quebec gave patterns similar to the standard American type C1. These results do not indicate, however, that the European strain is more abundant in Quebec, because only a limited number of isolates from the northern part of this Province were tested.

The evidence for the establishment of three more groups came from the examination of particular patterns obtained with some red pine isolates from New Brunswick and spruce and balsam fir isolates from Quebec which did not show homology with any of the groups already described. Patterns of some isolates from New Brunswick and Quebec (MER) revealed the interesting feature to combine both bands 12 and 4 considered to be specific for European and American isolates respectively. This may explain why, using serology, the identification of these isolates was problematic and not consistant (D. Lachance, personal communication). Since characteristics of both European and American isolates are present with these isolates, it is not surprising that they might be antigenically indistinguishable. By non gradient and gradient electrophoretic tests, isolates from spruce from Quebec were found to be dissimilar to European ones. Although they showed a relative homology with the American race, they had to belong to a separate group because of the presence of a few additional bands in their patterns. It was not possible to explain why these isolates were sometimes identified as European by serology, since, band 12, characteristic for all the European isolates was never present. Isolates from balsam fir gave patterns different from all others, even with fir isolates from Japan. The lack of a great number of bands and especially band 13 which was found associated to other isolates, constituted a good criterium of identification and classification of these isolates in a particular group.

The present data indicates that a good correlation could be established between results obtained with serology and electrophoresis tests with G. abietina isolates. There are, however, a number of discrepancies that deserve further consideration. Thus, isolates C94 from Newfoundland, C77 from New Brunswick, 79-077 from Quebec which was serologically classified as intermediate, failed to fit in any one of the well defined groups. More studies are necessary to identify these isolates. To determine the extent of Scleroderris canker in Quebec, G. abietina isolates from different sites should be electrophoretically tested.

Present results have shown precise and specific differences among the various isolates of G. abietina. However, before being able to regard this method as an ideal substitute to current methods of identification, the proposed criteria of differentiation should be confirmed by other more refined tests such as immunoelectrophoretic techniques.

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