THE USE OF ANTIGENIC PROTEIN POLYMORPHISM AS A TOOL FOR EVALUATING SELECTED BLACK LOCUST (ROBINIA PSEUDO-ACACIA) CLONES

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Abstract .--Antigenic proteins of several clones representing the so-called dominant-stem form of black locust (Robinia pseudo-acacia var. rectissina (L.) Raber) were compared to clones representing the typical form (R. pseudo-acacia L.).

The probability analysis of proteins from dominant-stem forms vs. typical forms showed that there were no significant differences in double diffusion precipitin bands attributable to bole form. Analysis of immunoelectrophoretic results likewise indicated that there were no significant differences in antigenic proteins among the trees studied. It is proposed that, based on antigenic root proteins the dominant stem form is an ecological variant.

Additional analyses were performed on clonal selections grown on strip mine spoils on two diverse sites. The samples collected at Clarksburg, W.Va. consistently generated more precipitin bands than those from Maidsville, W.Va. indicating that environmental differences are expressed in antigenic proteins.

It is suggested that the additional precipitin bands are a reflection of an epidemic attack of locust borer on one of the strip mine locations.

Additional Key Words: Double diffusion, immunoelectrophoresis, environmental effects.

Recent developments in the field of antigen protein analysis have indicated that it is a useful tool for taxonomic, pathological, genetic and physiological investigations (Clarkson and Fairbrothers , 1970; Vaughn, et al. , 1967; Vaughn, et al ., 1969; Ziegenfus and Clarkson , 1971; Pickering, et al ., 1965; Hillebrand , et al. , 1970; Esposito , et al ., 1965; 1966; Bozzini , et al ., 1970; Lester, et al ., 1965; Cristofolini, et al ., 1970; Saito , 1968; Klotz, et al. , 1963; Cristofolini , 1971;).

The basic purpose of this study was to determine the suitability of serological techniques for detecting antigenic protein differences due to form of black locust, first, in connection with subspecific separation of Robinia pseudo-acacia L. and R. p. var rectissima (L.) Raber, and second in connection. with possible antigenic protein variation between well formed and poorly formed trees found on the same site.

METHODS

Roots were collected from one ramet of each of eight clones from a

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strip mine planting near Maidsville, W.Va. Each of these clones was originally selected by the Soil Conservation Service because of its well-formed dominant stem. Four of these clones were originally selected in the vicinity. of Harman, W.Va., the area recognized for outstanding dominant-stem individuals, while the other four clones were selected near Byrantsburg, Indiana, an area of essentially diffuse branched individuals. Roots were also collected from all but two of the same clones planted on a strip mine near Clarksburg, W.Va. To determine within-clone variation, collections were made from more than one ramet of several clones on the Clarksburg strip. Collections were also made from a clonal planting in the Clements Nursery near Point Pleasant, W.Va. These clones (originally selected by West Virginia University Personnel), were paired superior and inferior phenotypes from the same location.

Methods of Collecting Roots

Roots were dug and frozen immediately in dry ice and methonal. They were then placed in polyethylene bags and transported in an ice chest with dry-ice. Within a few hours after collection, they were transferred to a freezer where they were stored at -20° C until ready for use.

Sample Preparation

Roots were removed from the freezer, and scrubbed with tap water in a cold room at 4° C to. remove any attached soil. This was followed by several washings with tap water. Finally, they were rinsed in cold distilled water.

Clean roots were chopped into the smallest possible pieces (approximately 2 - 4 mm.) A Prolabo crusher pre-cooled with dry-ice, was then used to pulverize the roots. The pulverized roots were then stored in glass jars at -20° C until used.

Protein extractions were accomplished by soaking 5 g of root meal in 10 ml of 2.5 percent saline solution at 4° C for 18 hr. with constant agitation using a magnetic stirrer. After one hour of extraction, the pH value of the extracting solution was adjusted to the original pH of 7.0 using 1N NaOH.

Following the 18 hr extraction period, the mixture was filtered through a millipore filter (diameter 1.2µ) and centrifuged for 20 min at 9500 x G at 0° C.

The precipitate was discarded and the supernant analyzed for its protein concentration according to the method devised by Lowry, et al.,(1951). Without further purification, the extract was used for immunization.

Immunization of Rabbits

New Zealand white rabbits were used for immunization. Normal rabbit sera, i.e. serum obtained before injections were made, were tested against each antigen to determine whether any non-specific precipitating systems were present.

For subcutaneous injections, one ml. of protein extract was added to one

ml Freunds incomplete adjuvant (8.5 ml paraffin oil plus 1.5 ml Arlacel A) and mixed using a Vortex-Geni mixer. This mixture was injected in the neck region of the rabbit. For intravenous injections one ml of protein extract was injected in the marginal vein of the rabbit's ear.

Two injection series were made, each consisting of subcutaneous injections on four succesive days, and intravenous injections on the last two of the four days. A rest period of ten days was allowed between the first and second series of injections.

Five days after the completion of the second series of injections, 20 ml of blood were drawn from each rabbit. The blood was put into a refrigerator at 4° C overnight. The following day, the blood samples were centrifuged (5,000 x G for 20 minutes at 0° C) and the serum was decanted into plastic vials and stored at -20° C.

Double Diffusion

Five ml of one percent Ionagar in a 2.5 percent saline buffer solution at pH 7.0 were poured into a plastic petri dish (55 mm diameter) and allowed to solidify at room temperature. Using a Grabar auto-gel agar cutter, six perimeter wells (6 mm diameter) and one center well (10 mm diameter) were cut simultaneously. The distance between any one perimeter well to the center well (edge to edge) was 6mm. Two-tenths ml of the antiserum was placed in the center well and 0.1 ml of the corresponding antigen was placed in alternate peripheral wells. Finally, one ml of each of three foriegn antigens was placed in each of the three remaining wells.

The petri dishes were then incubated for a period of three days at 4° C, and the double diffusion patterns read at the end of this period. The precipitin bands were diagrammed and the patterns photographed for future references. Numbers of bands of identity, partial identity, and non-identity were recorded and analyzed.

Immunoelectrophoresis

Immunoelectrophoresis was performed using a Gelman electrophoresis apparatus according to the Grabar method (1959), modified as follows: The 0.7 percent Ionagar solution was made by adding 0.7g of Ionagar to 100 ml of veronal-citrate buffer at pH 8.2 (51.95g sodium barbital; 2.0g citric acid; 2.25g oxalic acid; 14.7g sodium chloride and distilled water were mixed to make five liters of solution). Twenty ml of this solution were poured into a frame containing six 3 in x 1 in microscope slides. The frame was placed in a humidity tank which was then put into a cold room at 4° C for two hrs while the gel solidified. When the gel had solidified, the frame with the slides was removed from the cold room. A Gelman gel punch was used to make a 2 x 65 mm trough down the center of the slide and two 3 mm wells a distance of 5 mm from the trough.

A Pasteur pipette joined to a vacuum pump was used to remove the excess agar from the wells. Antigens were placed in the wells and electrophoresis was performed at 45 mA for 3.5 hr. Upon completion of electrophoresis, the agar in the trough was removed and antisera was placed in the troughs. The slides were then stored at 4° C for two days while the precipitin bands developed.

Following the incubation period, the slides were stained in amido black. After staining the slides were dried, precipitin bands were diagrammed and the slides stored at room temperature.

Numbers of precipitin bands were counted and analyzed.

RESULTS AND DISCUSSION

Although there were too few pairs of the well vs. poorly-formed trees in the Clements samples for a valid statistical treatment, a probability analysis of the double diffusion data showed that results were non-significant (Table 1), and from this it is inferred that there are no differences in double diffusion bands due to bole form. A careful examination of the data is sufficient to establish the fact that this method is relatively inefficient. Paired samples have a great deal of variation, the range equalling that from among samples. Additionally the maximum number of bands for all trees was four except for tree 203X where the maximum was five. Apparently there was a very significant effect due to test animals. This also may have obscured tree-to-tree effect.

The same criticism can be made for the Maidsville-Clarksburg samples as made for those from Clements except that there was a sufficient number available for a valid probability analysis (Table 2). The results are non-significant, indicating that there is no difference in double diffusion band number due to site. However, because of the great variation within samples this interpetation should not be accepted as conclusive.

The analyses of the immunoelectrophoresis results are presented in Tables 3 and 4. A probability analysis was used to compare the three pairs of well vs. poorly-formed trees on the Clements Nursery. As with the previous portion of the study, the results have indicated that there is no real difference in immunoelectrophoretic patterns between well and poorly-formed trees.

Anti-	Antigen					
	220s	220x	203s	203x	pa-s	Pa-x-1
220s	3	4	3	3	4	3
220x	3	4	4	5	4	4
220x	3	3	3	4	3	4
203s	3	1	3	4	2	3
203x	3	2	4	4	3	3
Pa-s	2	2	2	3	2	2
Pa-s	4	4	4	4	3	2
Pa-x-1	2	2	2	4	2	2
Pa-x-1	1	3	3	4	2	3

s=well-formed x=poorly-formed

Using the binomial frequency distribution; number of bands "x" greater than "s" is 12, less than "s" is 4, and equal to "s" is 11. Since Prob.$(x,n,p) = (n/x)(p)^{x}\bullet(1-p)^{n-x}$, so Prob. $= 2x\{p(x=12)+p(x=13)+p(x=14)+p(x=16)\}=2x\{(16/12)+(16/13)+(16/14)+(16/15)+(16/16)+\}x(1/2)^{16} = (1820+560+120+16+1)(1/2)^{15} = 2517/32768=$ .0768127441 larger than critical value .05 , a null hypothesis of no difference was accepted, i.e., there was no difference in the number of double diffusion bands between the well-formed and poorly-formed trees.

Table 2.--Total number of double diffusion bands; Clarksburg vs. Maidsville.

Anti-sera	c 4191	m 4191	c 4194	m 4194	c 8449	m 8449	c 8450	m 8450	c 8452	m 8452	c 8470	**m 8470**
c4191	4	4	5	4	5	3	4	4	4	4	3	**3**
	3	2	3	2	2	2	2	2	2	2	3	**3**
m4191	4	3	3	3	3	3	3	3	3	3	3	**3**
	5	3	3	3	3	3	3	5	5	5	4	**5**
c4194	6	5	4	5	6	6	6	5	4	3	5	**5**
m4194	5	5	5	5	4	5	4	5	5	5	5	**4**
	5	5	5	4	5	5	5	5	5	5	5	**5**
c8449	5	3	4	4	3	3	3	5	5	5	5	**5**
	5	5	5	5	4	4	4	5	5	5	5	**4**
m8449	4	4	5	4	4	4	5	4	4	4	4	**14**
	6	5	4	4	6	6	6	6	6	6	6	**6**
c8450	6	5	5	5	6	6	6	6	4	6	6	**6**
	5	5	5	6	5	5	5	5	5	5	3	**5**
m3450	5	4	4	3	5	4	3	5	5	5	4	**5**
	4	4	5	4	4	4	4	4	4		4	**4**
c8452	5	5	5	4	4	4	4	4	4	4	5	**4**
	5	5	5	5	5	5	5	6	6	5	4	**4**
m8452	6	6	6	6	6	6	6	4	6	5	6	**5**
	4	4	4	4	4	3	3	4	4	4	4	**3**
c8470	6	5	5	5	6	6	6	5	5	5	6	**6**
m8470	5	4	4	4	5		4	4	5	5	5	**5**
Total	103	91	94	88	96	91	92	96	96	95	94	**94**

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Probability = 2x{p(x=5) + p(x=6)}= 2x{6(69)+(64)}=.218. Therefore, probability .218 is not less than =.05, so we accept the null hypothesis, no difference between Clarksburg samples and Maidsville samples. This may be due to the fact that double diffusion is not as sensitive as immunoelectrophoresis.

s= Well-formed C=Clarksburg
x= Poorly-formed M=Maidsville

Table 3.--Total number of bands by immunoelectrophoresis. Clements Nursery samples.

Anti-	Antigen					
sera	220s	220x	203s	203x	Pa-s	Pa-x-1
220s	3	4	3	4	4	4
220x	4	6	5	4	4	3
220x	3	3	3	4	4	3
203s	4	5	1	4	3	5
203x	4	3	3	5	4	3
Pa-s	3	6	3	4	3	3
Pa—s	3	5	2	6	5	5
Pa—x-1	1	1	1	2	1	1
Pa—x-2	3	3	4	3	3	4

s= well-formed x= poorly-formed

Using the bionomial frequency distribution: the number of bands "x" is greater than "s" is 14, smaller than "s" is 6, and equal to "s" is 7.

Thus: when b(x,20,p) x=6 or less is .06, x=14 or larger is .06.

Since this was a two-tail test the probability of getting 6 or less and 14 or larger than "s" or "x" is .12. Since .12 was not less than ^{cc} = .05; a null hypothesis was accepted. This means that there was no difference in the number of immunelectrophoresis bands between the well-formed and poorly-formed trees. This result was complementary to the double diffusion, peroxidase, and the total protein studies.

The analysis comparing the clones planted at Clarksburg and Maidsville indicated that there were real differences in the immunelectrophoresis patterns due to site.

The samples from Clarksburg generated a greater number of bands than those from Maidsville, indicating that there are environmental differences expressed biochemically. If these are due to soil-site differences, it would be difficult to quantify possible associated phenotypic differences at this time.1/ If however, as has been suggested, these differences are a reaction to the locust borer attack at Clarksburg, it would seem possible to document phenotypic evidence. No attempt was made to determine the causes for the environmental differences.

It is apparent from this study that the immunelectrophoresis technique is more sensitive than Ouchterlony's double diffusion method.

CONCLUSION

From an analysis of black locust root extracts, we could not differentiate between well and poorly-formed trees or between dominant stem and diffuse branching types using the Ouchterlony double diffusion or the immunelectrophoresis techniques. The between-tree variation within treatment was often greater than that due to tree

1/ Top kill was universal on the Clarksburg planting as a result of the severe borer attack. Sprouts are too small for analysis.

Table 4.-- Total number of bands by immunelectrophoresis. Clarksburg vs. Maidsville.

Anti-sera	Antigen											
	c 4191	m 4191	c 4194	m 4194	c 8449	m 8449	c 8450	m 8450	c 8452	m 8452	c 8470	m 8470
c4191	6	4	7	6	5	5	5	5	6	5	7	6
	6	4	5	5	5	5	7	6	5	5	6	5
m4191	5	5	7	4	7	4	6	4	6	6	5	4
	5	5	7	4	7	5	5	5	6	4	5	4
c419	3	4	7	3	5	4	6	6	5	5	5	5
m419	6	6	6	5	6	6	6	5	6	5	5	6
	6	5	6	7	7	5	8	6	7	6	6	4
c8449	4	4	6	5	8	5	5	5	5	4	5	3
	6	5	6	4	6	6	5	4	5	4	5	5
m8449	8	5	8	5	9	7	5	5	6	6	6	6
	5	4	8	7	8	4	8	7	6	6	6	5
c8450	6	5	6	4	6	5	9	9	6	6	7	5
	6	5	6	4	6	5	9	8	5	5	9	6
m8450	6	4	8	7	7	6	8	9	6	4	5	5
	6	4	7	4	6	7	7	10	8	4	6	5
c8452	5	4	6	4	7	5	6	6	6	3	6	5
	6	4	8	4	6	4	6	7	7	4	6	6
m8452	6	5	6	4	7	5	6	6	6	6	6	5
	5	5	7	5	5	3	6	6	5	5	6	4
c8470	5	5	7	6	6	5	7	5	5	5	6	6
m8470	5	5	7	5	6	5	7	7	5	5	5	6
Total	116	97	140	102	135	106	137	131	122	103	123	106

c= Clarksburg m=Maidsvillen

Since probability (x,n,p)=(n/x)(p)x(1-p)n-x= (6/6)(1/2)6(1/2)0= 1/64. Therefore, the probability ('c' larger than "m") = 2x(1/64) = .0312. Since .0312 is less than infinity = .05, reject the null hypothesis, accept Ha, that we have a greater number of bands at Clarksburg than Maidsville. This result is also complementary with the general protein pattern study. Although there was a difference in terms of the immunoelectrophoretic bands between the Clarksburg and Maidsville plantings of the same clonal material, they still had very similar immunoelectrophoretic patterns. Double diffusion is less sensitive than imunoelectrophoresis. Therefore, in this study we detected a difference between the Clarksburg and Maidsville plants, but we did not obtain a difference in the previous study by Ouchterlony's double diffusion method.

form. Although the Ouchterlony double diffusion and the immunelectrophoresis patterns were consistent from one run to the other, there were differences associated with test animals. Conversely, banding differences due to site were evident from the immunelectrophoresis results. It is postulated that these differences are due to an epidemic locust borer attack on one site. From this evidence, it is proposed that the so-called dominant stem variety is merely an ecological variety.

While the double diffusion technique was least sensitive in this study, it is a more simple method than the immunelectrophoresis technique and probably should be tested to determine its value before utilizing the immunelectrophoresis method. If it is found to be inadequate (i.e. too many overlapping bands, excessive variation within samples, etc.) then the immunelectrophoresis technique should be used. In spite of the disadvantages inherent to antigenic type tests, the authors feel that they are a useful tool for taxonomic and genetic investigations.

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