Comparison of Anastomosis Groups of *Rhizoctonia solani* by Polyacrylamide Gel Electrophoresis of Soluble Proteins

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ABSTRACT

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Soluble proteins were extracted from isolates of the five anastomosis groups (AG) of *Rhizoctonia solani* and compared by electrophoresis in polyacrylamide gels. The protein profiles for the five AG were distinctive and could be distinguished from one another. Particular emphasis was placed on determining the variability of protein patterns of isolates belonging to AG3 and AG4 from different hosts and geographic locations. The protein patterns from isolates belonging to AG3 were uniformly distinct, regardless of isolate source. Protein patterns of isolates belonging to AG4 varied in minor bands, but the general pattern was recognizable as AG4. It is suggested that polyacrylamide gel electrophoresis of soluble proteins can be used to help distinguish anastomosis groups of *R. solani*.

Additional key words: Gossypium hirsutum, Solanum tuberosum, Thanatephorus cucumeris.

Rhizoctonia solani Kuehn (*Thanatephorus cucumeris* (Frank) Donk) is a worldwide pathogen on a large number of crops. It has been widely studied and has been shown to occur in four main anastomosis groups (AG) (11). Recently, a fifth group has been described in Japan (10). The anastomosis groups are genetically distinct, and attempts to bridge these groups have been unsuccessful (2). Although the anastomosis groups are different morphologically, there is enough overlap to prevent AG determination based on colony characteristics alone (11,13). Adams and Butler (1) have shown that the anastomosis groups are serologically distinct.

There is some correlation of AG with host or host range, but overlap is considerable using this criterion (9). Anastomosis grouping does, however, correlate with ecological behavior in the soil. In California, members of AG4 are readily isolated from soil and occur as seedling pathogens of many crops including bean and cotton (17). Members of AG3 are less frequently isolated from soil and are not seedling pathogens, but cause stem and stolon canker and black scurf on potato. In culture, however, there is enough morphologic similarity to make it difficult to assign some isolates to either AG. In addition, when isolating *R. solani* from plant material and soil, fungi, which are morphologically similar to the pathogen, are often obtained. A rapid and reliable method of determining whether these belong to known AG would be very useful in studies on the ecology of *R. solani*.

Gel electrophoresis of fungal proteins has been used as an adjunct to morphologic criteria in taxonomy. The method has been applied in delineation of genera, species, and subspecies of various fungi. The use of electrophoresis in fungal taxonomy has been reviewed by Schechter (12) for medically important fungi, by Hall (6) for fungi in general, and by Snider (14) for phytopathogenic fungi.

Although the usefulness of the electrophoretic protein profile technique is well documented for species delimitation of plant pathogens, there have been few reports of its applicability at the subspecies level. Workers have found that patterns at the subspecies level are not sufficiently distinctive to be useful (4,5,8).

To date, there are two reports of gel electrophoresis of the soluble proteins of *R. solani*. The results of Clare et al (3) indicated that there was considerable variation in the banding patterns from isolate to isolate and this was interpreted as evidence that *R. solani* is a collective species. Welch (18) surveyed the proteins from representative isolates of the five AG and concluded that, with

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further work, electrophoresis could prove useful in identifying the various groups.

The purpose of this study was to compare soluble protein patterns among the five AG, with the objective of developing a reliable procedure for AG determination. Particular emphasis was placed on the members of AG3 and AG4, which commonly occur in California.

MATERIALS AND METHODS

AG tests. Representative isolates used for polyacrylamide gel electrophoresis were tested for anastomosis to a tester isolate of AG3 and one of AG4. Mycelium from the tester isolates and from an isolate of unknown anastomosis grouping were placed 1 cm apart on glass microscope slides coated with a thin film of potato-dextrose agar (PDA). The slides were supported on glass rods in petri dishes and incubated at 30 C until the hyphae had grown together and intermixed (E. E. Butler, *personal communication*). After incubation, the slides were stained with a mixture of lactic acid and fuchsin and examined with a light microscope to determine whether anastomosis between isolates had occurred.

Pathogenicity tests on potato and cotton. Dormant tubers of *Solanum tuberosum* L., soaked in 2% a.i. formaldehyde for 5 min and air-dried at room temperature for 3 days, were cut into seed pieces, each piece having at least two eyes. The seed pieces were planted in 25-cm-diameter pots, two seed pieces per pot, in the greenhouse. Plugs (1-cm-diameter) were cut from 5-day-old cultures of *R. solani*, and a plug was placed next to each eye at the time of planting. Disease was evaluated after 30 days. The pathogenicity of isolates of *R. solani* on potato was rated by the Rhizoctonia Index (RI) developed by Weinhold and Bowman (16), which is an expression of the stem surface area covered by lesions. Isolates were tested for pathogenicity on cotton (*Gossypium hirsutum* L.) using 5-day-old seedlings and the sand-glass plate method of Weinhold et al (15). For each isolate, 10 seedlings were used for each of two tests.

Culture methods for electrophoresis. Isolates of *R. solani* were grown on PDA at 30 C for 4–6 days. Two plugs approximately 1.0 cm in diameter were transferred to 100 × 25-mm petri plates containing 40 ml of a defined liquid medium (15) (essential salts plus 20 g of glucose and 2 g of asparagine per liter). After 5 days incubation at 25 C, mycelial mats were placed on Whatman qualitative filter paper in a 9-cm-diameter Büchner funnel, and a vacuum was applied to remove excess liquid.

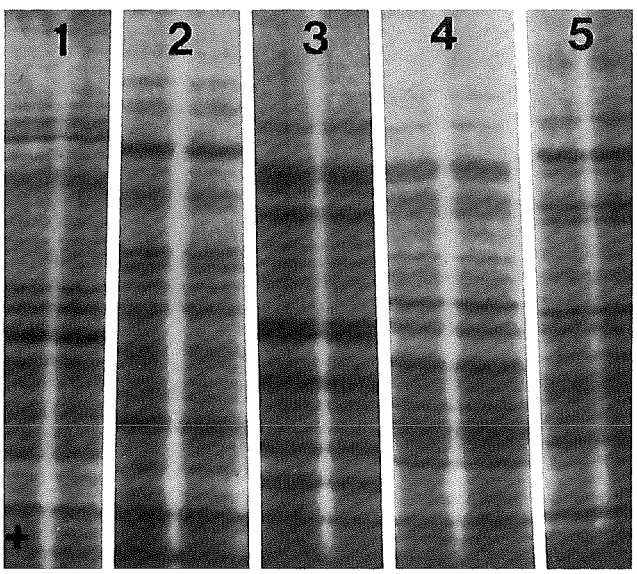

Fig. 1. Comparison of the polyacrylamide gel pattern obtained by electrophoretic separation of soluble proteins from *Rhizoctonia solani* anastomosis groups (AG) 1, 2, 3, 4, and 5. Paired lanes are from two separate isolates.

Protein extraction and sample preparation. Soluble proteins were extracted by the "aqueous" method (7). Mycelium (2.5 g fr. wt.) was placed in 15-ml centrifuge tubes and homogenized in 5 ml of distilled water with a Brinkman polytron at maximum speed for 1–2 min. The extracts were then clarified by two successive centrifugations at 4 C for 30 min at 12,100 *g* and 2 hr at 98,000 *g*. Protein was precipitated from the clarified supernatant by addition of an equal volume of 7.5% trichloroacetic acid, and the pellets, collected by centrifugation, were washed with acetone and air-dried. Prior to electrophoresis, the pellets were resuspended in 400 μl of a solution containing 0.01 M tris-HCl, pH 6.7, 10% gylcerol, 1% sodium dodecyl sulphate, 2% 2-mercaptoethanol, and 0.002% bromophenol blue, and heated to 100 C for 2 min. About 20 μg of protein was applied to the gel per sample.

Electrophoresis. Electrophoresis was performed with a discontinuous system (4,12) in 1.5-mm-wide polyacrylamide slabs. A 10-cm-long, 10% resolving gel at pH 8.9, and a 1-cm, 3% spacer gel at pH 6.7 were used, and electrophoresis was carried out at 150 V (constant) for 3.5–4 hr at 10 C. The electrode buffer was 0.1 M tris-HCl and 0.76 M glycine at approximately pH 8.2. After electrophoresis, the gels were stained overnight in a general protein stain containing 0.2 g of Coomassie brilliant blue, 50 ml of methanol, 7.0 ml of glacial acetic acid, and 43 ml of water. They were destained in a solution of 50% methanol and 7% acetic acid in water and photographed.

RESULTS

Anastomosis and pathogenicity tests. Thirteen isolates were tested to determine their AG. Four were found to be AG4 and nine were AG3. Three of the AG4 isolates (two were obtained from cotton plants and one from soil) were highly pathogenic to cotton. The fourth isolate, obtained from carrot, was weakly pathogenic to cotton. None of these isolates caused stem or stolon canker on potato. They did, however, cause necrotic flecks that occasionally coalesced to form superficial lesions. Seven of the nine AG3 isolates were pathogenic on potato, and two were nonpathogenic. These isolates did not infect cotton.

Fifteen additional isolates were tested for pathogenicity on cotton and potatoes, and their cultural characteristics were compared with authentic AG3 and AG4 isolates. Based on growth rate, colony morphology and pathogenicity, five were tentatively designated as AG3, and 10 were tentatively designated as AG4. Those designated as AG3 were nonpathogenic to cotton. Three were pathogenic and two were nonpathogenic to potato. Of the 10 isolates designated as AG4, seven were highly pathogenic to cotton, whereas three only caused slight superficial necrosis on cotton. Two of the weakly pathogenic isolates were from soil from Tulelake, CA, and the other was isolated from cucumber in Japan. None of the isolates tentatively identified as AG4 caused stem and stolon canker on potato, but several caused necrotic flecking.

Protein pattern comparison of isolates belong to the five AG. Sixteen isolates from different hosts and geographic locations representing the five AG were compared electrophoretically. The number of isolates tested for each group was: AG1, 3; AG2, 2; AG3, 4; AG4, 4; and AG5, 3. The protein patterns produced by isolates from the five AG differed markedly (Fig. 1). Each group appeared to have a distinct pattern, but not enough isolates were tested to determine the variability within a particular group, except with AG3 and AG4 (see following section).

Upon electrophoresis of proteins from three isolates belonging to AG1, considerable variation of banding pattern was noted for isolates from different geographic locations. Two isolates from California were identical, whereas the third isolate from Japan showed little homology with the banding pattern of the California isolates. The protein patterns of the two AG2 isolates tested were identical, although they came from different subgroups of AG2 (10). The patterns produced by the isolates of AG5, all from Japan, were identical.

To determine whether the variability of banding patterns observed was the result of intrinsic variability in the gel system, growth and extraction of selected isolates was repeated several

times and the soluble proteins were electrophoresed. It was found that for each isolate, the banding patterns were exactly the same or differed at most by one or two minor bands.

Protein banding patterns of isolates belonging to AG3 and AG4. The electrophoretic patterns of soluble proteins were obtained for nine isolates determined to be AG3 by the slide anastomosis group test. Seven prominent, dark-staining, recurring bands were chosen from the protein profiles of the AG3 isolates. Rather than measuring the migration distances relative to the bromophenol blue tracking dye front, the bands were measured relative to an internal reference band, which occurred in all of the protein profiles, including those of isolates from AG4. Distances in location from this band, designated "f," were taken for the six other prominently recurring bands, and labeled "a" through "g" (Fig. 2).

Distance (in millimeters) of the lettered bands from the reference band "f" was taken from the middle of the reference to the middle of each lettered band. Measurements from nine isolates in three gels were taken and averaged. This average was used to describe the position of a particular lettered band relative to the reference band "f." The distance of the lettered bands from band "f" were as follows: a, 26.3 ± 0.02 mm; b, 24.3 ± 0.02; c, 17.9 ± 0.02; d, 13.7 ± 0.01; e, 10.1 ± 0.01; and g, −3.1 ± 0.01. All bands with positive values occurred above the standard band in the gels, and the one band with a negative value occurred below the standard band.

The overall banding pattern of the AG3 isolates tested was consistent and distinctive. The characteristic darkly staining bands made the profiles of the AG3 isolates readily distinguishable from the AG4 isolates (Fig. 2). The effect of geographic location on the banding patterns of the AG3 isolates was minimal. An isolate from Japan was identical to isolates from various locations in the United States. No differences in banding pattern were noted between isolates that were pathogenic on potato and those that did not attack potato.

The electrophoretic pattern of soluble proteins was determined for five isolates of *R. solani*, which belonged to AG4 on the basis of the slide anastomosis test. The protein patterns of 10 isolates suspected to be AG4 based on source and culture morphology were compared with known isolates of AG4. On the basis of protein patterns, the unknown isolates were designated as AG4.

The same system of identifying an internal reference band and comparing other prominent bands to it was used to identify isolates from AG4. Since a band migrating the same distance as the internal standard band "f" for the AG3 isolates was found to occur universally in the AG4 isolates, it was used as the internal standard band and labeled "m" (Fig. 2). Band "m" had the same mobility in the gels as band "f" in the AG3 profiles and had an approximate molecular weight of 22,400. The distance of the six bands from the reference band "m" were as follows: h, 27.8 ± 0.2 mm; i, 24.6 ± 0.02; j, 21.4 ± 0.003; k, 16.5 ± 0.01; l, 10.8 ± 0.01; and n, −1.1 ± 0.01.

The bands used for characterization of AG4 were present in extracts from all isolates tested, but there was variation in staining intensity. There was no darkly staining distinctive band common to the AG4 isolates as there were with the AG3 isolates. No consistent differences in banding pattern were noted among isolates obtained from different hosts or geographic locations, nor were there any bands to distinguish the patterns of isolates highly virulent on cotton from those that had little or no effect on cotton.

DISCUSSION

Reproducible and distinctive protein patterns were obtained from polyacrylamide gel electrophoresis of the soluble proteins from the five AG of *R. solani*. Particular emphasis was placed on comparison of banding patterns of isolates belonging to AG3 and AG4. It was found that the patterns of isolates within AG3 varied very little, and although the patterns from the AG4 isolates tested were slightly more variable, the patterns of the two groups were readily distinguishable from each other and from those produced by the other AG.

The protein patterns obtained from the AG3 isolates were very homogeneous, which possibly can be attributed to the fact that all of the AG3 isolates came from one kind of host plant, the potato. The patterns of the AG4 isolates were not as homogeneous, and there was variability in the pattern of minor bands as well as in the intensity of the bands used for characterization from isolate to isolate. The AG4 isolates came from a number of host plants and from soil, and this may, in part, explain some of the variability observed in the protein banding patterns within this group.

Measurements of electrophoretic differences between these groups relative to an internal reference band were less variable than comparing their mobilities relative to the tracking dye front. Because only seven bands in each group were used for identification of a particular isolate, the time involved in assigning an isolate to either group was greatly reduced.

Choosing a few distinctive bands to identify AG3 and AG4 makes the method of polyacrylamide gel electrophoresis a practical laboratory technique as an adjunct to agar-coated slide AG determination. It is recommended that when an isolate of unknown AG is electrophoresed, an isolate from AG3 and one from AG4 be electrophoresed with it in the same gel. Standard bands "a-g" for the AG3 isolate and "h-n" for the AG4 isolate can then be calculated, and the unknown isolate compared for homology to these isolates.

Preliminary evidence is presented that the other AG (AG1, AG2, AG5) differ in protein patterns from each other and from the isolates of AG3 and AG4 tested. The variability of protein banding patterns within these groups is not known, but evidence from the three isolates of AG1 indicate that, in some cases, the variation may be quite large.

Several workers (4,5,8) have been unable to distinguish among subspecies and formae speciales of fungi by using polyacrylamide gel electrophoresis. That the patterns of the five AG of *R. solani* differ is evidence that perhaps this is a "collective" species, or at least would support the suggestion that the groups are genetically distinct (10). Differences in electrophoretic protein patterns are an indication, in part, of genomic differences between organisms, but environmental circumstances affect the proteins that are synthesized. Therefore, it is stressed that growth, extraction, and electrophoretic procedures must be carried out under standardized conditions when making comparisons.

The results of this study are consistent with those of the serological relationships among AG reported by Adams and Butler (1). The groups are distinctive both on the basis of serology and electrophoresis of soluble proteins, suggesting they are biological

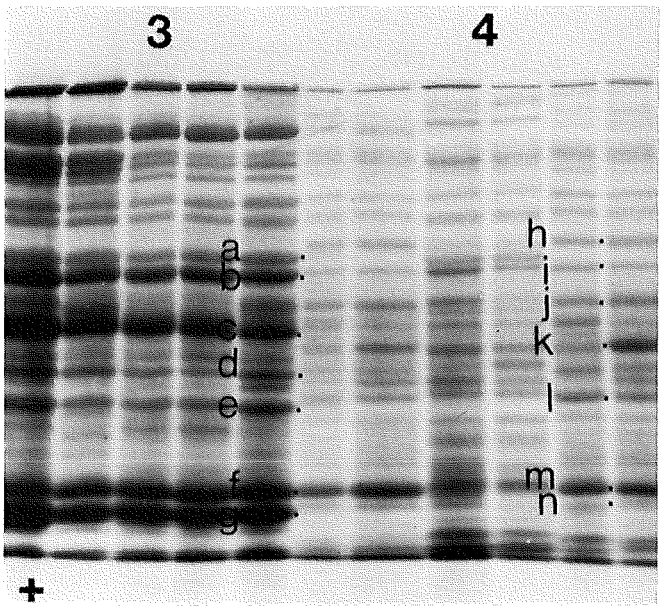

Fig. 2. Polyacrylamide gel pattern obtained by electrophoretic separation of soluble proteins from five isolates of AG3 and six isolates of AG4 of *Rhizoctonia solani*. Letters identify the bands used to characterize the groups.

species. It is of interest that the subgroups within AG2 could not be distinguished by either serology or electrophoresis.

Results of this study indicate that AG groups can be distinguished by comparing soluble protein patterns in polyacrylamide gels. However, none of the protein pattern differences observed among the isolates tested could be related to their virulence. Further work to resolve protein differences at this level could be useful, since cultural characteristics of a particular isolate cannot generally be used as an indication of virulence.

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