Techniques

Clean Slide Technique for the Observation of Anastomosis and Nuclear Condition of *Rhizoctonia solani*

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ABSTRACT

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A simple, rapid technique for simultaneous observation of anastomosis and nuclear condition of *Rhizoctonia solani* employing clean glass slides and a moist chamber is described. The use of cellophane overlays and agar-coated slides is eliminated. Anastomosis and nuclear condition can be determined in less than 24 hr.

Additional keywords: *Medicago sativa*, soilborne disease.

The anastomosis group (AG) concept of *Rhizoctonia solani* Kühn has become an important principle in the study of this species. Anastomosis (hyphal fusion) occurs only between isolates of the same AG. The significance of this concept is that each AG can be considered to be a separate, non-interbreeding population (1). In addition, the isolates of each AG differ somewhat in host range and pathogenic habit from other AG (11). For example, rice sheath blight is caused by isolates belonging to AG-1, whereas isolates of AG-3 are mainly found on potato (1). Determination of the anastomosis group of *R. solani* has become an important step in studying the specific strain of the fungus involved in a particular disease. Since the anastomosis group concept was first suggested in 1937 by Schultz (13), nine anastomosis groups have been proposed, along with two subgroups within AG-1 and AG-2 (1,3,7).

A second characteristic that is consistent with all strains of *R. solani* and which distinguishes them from most other *Rhizoctonia* species is the nuclear condition of the vegetative cells. All isolates of *R. solani* are multinucleate and are considered to be the imperfect stage of *Thanatephorus cucumeris* (Frank) Donk. *R. zeae* Voorhees is also multinucleate but can be distinguished from *R. solani* by differences in sclerotial morphology (10). Other *Rhizoctonia*-like fungi are binucleate and many of these belong to the genus *Ceratobasidium* Roberts (12).

There are several methods reported for observation of anastomosis or nuclear condition of isolates of *R. solani*. Parmeter et al (11) grew isolates on 2% water agar or on cellophane resting on 2% water agar. He cut out sections of the agar or cellophane where the hyphae of the paired isolates overlapped and mounted them on microscope slides and stained them. Herr and Roberts (6) made pairings on sterile glass slides coated with 2% water agar, and placed the slides on water agar plates to maintain moisture. Castro et al (4) grew isolates on cellophane dipped in soft potato-dextrose agar before overlaying onto water agar to promote growth. Martin (9) and others (5,15) use stains such as DAPI (4', 6'-diamidino-2-phenylindole) and acridine orange, which fluoresce upon excitation by UV light. Although these methods work satisfactorily, they can be time-consuming and somewhat cumbersome to use or require the use of specialized equipment.

The objective of this study was to develop a simpler method of determining the anastomosis groups and nuclear condition of isolates of *R. solani* collected from alfalfa roots with black root canker. Black root canker is a disease of alfalfa roots that occurs during the hot summer months in the irrigated desert areas of Arizona and California. The disease is characterized by stunting,

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lack of secondary roots, and stand decline. The fungus causes round to oval cankers, which vary in color from yellow to brown, on the surface of the taproot. The lesions frequently occur where the lateral roots emerge from the taproot and may also occur on the lateral roots. Although it has been known since 1943 that *R. solani* is the causal organism of black root canker (14), nothing is known about the anastomosis group or groups that are involved. A preliminary report has been published (8).

MATERIALS AND METHODS

Alfalfa roots (cultivars Lew and Maxidor) with cankers were collected from infested fields and washed under running tap water in the laboratory for 20 min. Pieces from the advancing margin of the lesion were placed on water agar and incubated at 27 C for 1 or 2 days. Hyphal tips of *Rhizoctonia*-like hyphae were transferred to potato-dextrose agar and incubated at 27 C. After 2 or 3 days, 7-mm-diameter disks were cut from the perimeter of the colony and placed on a clean glass slide (cleaned by dipping in 95% ETOH and wiping dry). A 7-mm-diameter disk of a known AG tester isolate was placed 2–3 cm away (Fig. 1A). Tester isolates were obtained from Earl Ruppel, Colorado State University, Ft. Collins, CO. The slides were placed in a moist chamber and incubated at 27 C for 24–28 hr (Fig. 2). When the hyphae from the two disks overlapped, slides were removed from the moist chamber, the excess moisture was wiped from the bottom of the slide, the mycelium immediately adjacent to the disk was sliced, and the disk lifted from the slide. The overlapping hyphae were then stained with safranin O and 3% KOH according to the method reported by Bandoni (2) and covered with a 22-mm coverslip (Fig. 1B). The stained area was then examined microscopically to determine the number of nuclei per cell and anastomosis with the tester isolate. Anastomosis was determined at 200× and fusion confirmed at 800×. All pairings were repeated at least once and all anastomosing hyphae were traced back to their source to avoid any false positives (self anastomosis).

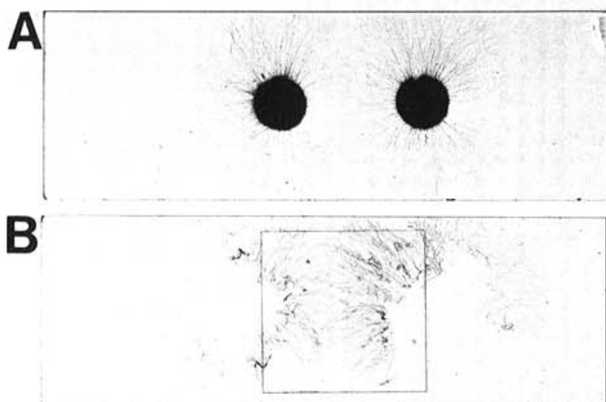

Fig. 1 Microscope slides with paired isolates of *Rhizoctonia solani*. **A,** After 24 hr growth. **B,** With coverslip after removing agar plugs and staining with safranin O.

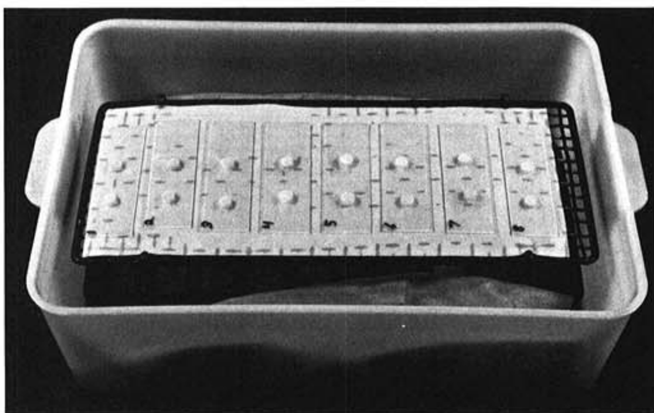

Fig. 2. Microscope slides with paired isolates in moist chamber (cover not shown).

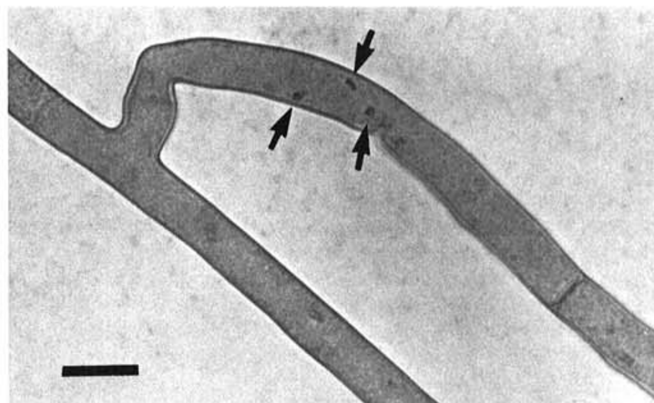

Fig. 3. Anastomosing hyphae of *Rhizoctonia solani* showing multiple nuclei stained with safranin O. Bar = 10 μm.

RESULTS AND DISCUSSION

Fourteen isolates of *R. solani* were collected from taproot lesions and feeder roots of alfalfa plants with black root canker. All but one isolate were multinucleate and anastomosed with an AG-4 tester isolate. In addition, random pairings between the multinucleate isolates from alfalfa all resulted in anastomosis. Anastomosis with tester isolates from AG-1, -2, -3, and -5 were all unsuccessful. These results indicate that the black root canker isolate of *R. solani* belongs to AG-4.

This report describes a simultaneous method for the observation of both the nuclear condition and anastomosis of *R. solani*. Other methods involved the use of two separate preparations to determine both the anastomosis group and nuclear condition (6,12). We choose to use safranin O as a nuclear stain because of simplicity and speed. With this stain, nucleoli were stained within 2 min; other staining methods (5,9,15) are much more time consuming or require the use of fluorescence microscopy. Because safranin O stains the nucleoli rather than the entire nucleus (2), the red-stained bodies are smaller than nuclei stained by other methods, but this does not pose any problems (Fig. 3).

The advantages of this technique over other methods are its simplicity and rapidity. There is no need to coat slides with agar, autoclave cellophane, or cut out and transfer sections of overlapping mycelium to a microscope slide. The preparations can be stained and observed as early as 18–24 hr after the disks are placed on the slide. Observations can be made on an ordinary light microscope. Because growth of the mycelium of each isolate in the overlapping region is primarily unidirectional, it is easy to trace the source of the anastomosing hyphae. In addition, the mycelium is mainly on one plane on the surface of the slide, so there is a minimum of focusing required to find an anastomosis and trace its origin.

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