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9. ABSTRACT

This paper presents a rather comprehensive review of the research literature concerning the media and methods used for isolating and culturing R. solani. More than 70 past studies are described. The author's conclusions: R. solani appears to be a good organism for exploring chemical tolerance in fungi. Tolerance to fungicides has been established in this fungus, but better techniques and further studies are needed. Tolerance to a larger number of chemicals needs to be determined. A chemical to which strains of R. solani are tolerant, and that would overcome some of the disadvantages of PCNB and TCNB, needs to be found. Additional in vitro and in vivo studies need to be made to determine the extent, potential, and nature of this resistance. Effects of prolonged exposure of R. solani to these fungicides should be studied. The mechanism of variation in R. solani has been reported by Flentje, et al, in Rhizoctonia Solani, Biology and Pathology, 1970, edited by J. R. Parmeter. If fungicide resistance is due to mutation, as suggested by Flentje and Stretton (1964) and Ricaud (1965), then definite proof could be obtained by genetical analysis.

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Rhizoctonia Solani: Special Methods of Study

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ISOLATION AND CULTURE OF RHIZOCTONIA SOLANI. *Media used for isolation.—Rhizoctonia solani* is comparatively easy to isolate from infected plant tissues, soil, and rhizospheres. The fungus grows on a wide range of solid and liquid media ranging from water agar to highly specialized media (see Table 1). Recipes for most of the common media were presented by: Riker and Riker (1936), *Difco Manual* (Anon., 1953), Johnson [L. F.] et al. (1959). Many workers prefer to use simple or modified potato-dextrose agar (PDA) or other decoction agars. Maier and Staffeldt (1963), for example, found *R. solani* isolates from cotton varied in their ability to grow on carrot-decoction agar, PDA, and nitrate-dextrose agar. All isolates, however, grew well on PDA. Ashour and El-Kadi (1959) compared the growth of *R. solani* on five different media. They found that the fungus grew most rapidly on PDA and Richard's media.

For routine isolation of *R. solani*, these agars are often modified with acids, bacteriostatic or fungistatic compounds. Acidified PDA helps control bacterial contamination and may favor growth of *R. solani*, since the fungus prefers an acid medium (Samuel and Garrett, 1932; Sanford and Marritt, 1933; Allington, 1936; Kotila, 1945*a*; Houston and Kendrick, 1949; Warcup, 1950).

Three acids are commonly used for acidification of agar: acetic, lactic, and phosphoric. A few drops of a 10% solution of one of these may be added to a culture plate prior to pouring of agar or directly into the medium before dispensing. Acidification usually gives a pH range of 3.5-5.0. The response of *R. solani* to pH is highly variable. In general, the optimum for growth of most isolates of *R. solani* is between 5.5-7.0 (Ragheb and Fabian, 1955; Elarosi, 1957*b*; Bateman, 1962). There is probably some reduction in growth on media with a pH lower than that for optimum growth. Latham and Linn (1961) found media at pH 4.0 using lactic acid suppressed growth of the fungus. Slower growth isolates may be lost in the presence of more rapidly growing ones.

Some workers (LeClerg, 1939; Papavizas and Davey, 1959*a*; Latham and Linn, 1961; Schmitthenner, 1964) incorporated antibiotics into media to decrease bacterial contamination and to increase frequency of isolating *R. solani*. Papavizas and Davey (1959*a*) used a mixture of fresh solution containing 50 ppm each of aureomycin hydrochloride, neomycin sulfate, and streptomycin sulfate in water agar to increase the frequency of isolating *Rhizoctonia* spp. from soil. Latham and Linn (1961) found that PDA containing either streptomycin sulfate at 100 μg/ml or vancomycin at 50 μg/ml was superior in all instances to media containing rose bengal. They reported also that novobiocin did not suppress the growth of *R. solani*. Schmitthenner (1964) reported 50 mg/ml of streptomycin sulfate added to media helped reduce bacterial contaminants. Rose bengal (Schmitthenner, 1964) and methylene blue (LeClerg, 1939*a*) also were used to reduce contamination.

Isolates of *R. solani* should be routinely grown on a liquid medium, such as potato-dextrose or potato-sucrose broth, to detect contamination by either bacteria or yeasts that may not be apparent on agar.

Isolation of R. solani from infected plant tissue.—Standard procedures for isolation of plant pathogenic fungi are generally used to obtain *R. solani* from infected plant tissues. Small sections of infected roots stems, leaves, or reproductive organs are aseptically removed from the plant after either surface sterilization or washing in tap or distilled water. Rao (personal communication) found that *R. solani* was unable to tolerate highly competitive conditions on agar plates and classed the fungus as having limited "competitive saprophytic ability." Diseased tissue therefore should be either thoroughly washed or surface sterilized before plating.

Parmeter (personal communication) found that plating isolation pieces on water agar minimizes competition, allows *R. solani* to grow away from most competitors, and facilitates hyphal-tip isolation.

Solutions of 1.0% sodium hypochlorite (Kernkamp, et al., 1949; Schmitthenner, 1964) or 0.1% mercuric chloride (Mohamed, 1962) have been used to disinfest plant tissues. Luttrell (1962), however, stated that since *R. solani* is frequently killed by usual procedures of surface sterilization, isolations from infected leaves, stems, and roots should be made by washing bits of tissue in sterile water before plating out fragments on agar media. Bourn and Jenkins (1928*a*) washed infected portions of various aquatic plants before plating out. Hansen and Curl (1964) prepared clover stolons for isolation by cutting them

TABLE 1. Media used for isolating and culturing *R. solani* and literature cited.

Medium	Citation
Decoction and extract agars	
Bran	Ray (1943)
Bean hypocotyl	Bateman (1963*a*, 1964*b*)
Carrot	Maier and Staffeldt (1963)
Coen's	Person (1944*a*)
Cornmeal	Ullstrup (1936); Saksena (1960); Schmitthenner (1964)
Dextrose-yeast extract + V-8 juice	LeClerg (1939*a*)
Leomin	Person (1944*a*)
Lima bean	Ullstrup (1936), Schmitthenner (1964)
Malt	Ullstrup (1936); Gibson, et al. (1961)
Nutrient	Chesters (1948); Warcup (1950)
Nitrate-dextrose	Maier and Staffeldt (1963)
Potato	Shephard and Wood (1963)
Potato-cerelase	Kilpatrick, et al. (1954)
Potato-carrot-dextrose	Maier (1962)
Potato-dextrose	Samuel and Garrett (1932); Allington (1936); Ullstrup (1936); Weber (1939); Neal (1944); Kotila (1945*a*); Chesters (1948); Houston and Kendrick (1949); Allison (1952); Papavizas and Davey (1959*a*, 1962*b*), Saksena (1960); Martinson and Baker (1962); Bateman (1963*a*); Shephard and Wood (1963)
Potato-dextrose + germinating radish-seed exudate	Martinson and Baker (1962)
Potato-marmite	Whitney (1964*a*)
Potato-sucrose	Shatla and Sinclair (1963), Elsaid and Sinclair (1964)
Richard's	Ullstrup (1936), Ashour and El-Kadi (1959)
Soil-decoction	Carpenter (1949)
Tromer's malt	Neal (1944)
Water	Boosalis and Scharen (1959); Shephard and Wood (1963); Whitney (1964)
Water + germinating radish-seed-exudate	Martinson and Baker (1962)
Water + cold-sterilized aster straw	Flentje and Stretton (1964)
Synthetic	
Czapek's	Allington (1936); Maier (1961*b*); Ruppel, et al. (1965)
Czapek-Dox	Kaufman and Williams (1965)
Czapek-Dox + yeast-extract	Warcup (1950), Gibson, et al. (1961)
Edward's synthetic	Edwards and Newton (1937)
Liquid	
Fries' nutrient solution	Millikan and Field (1964)
Potato-broth	LeClerg (1939*a*), Shephard and Wood (1963)
Potato-dextrose-broth	Bateman (1963*a*), Ruppel, et al. (1965)
Potato-sucrose-broth	Shatla and Sinclair (1963); Elsaid and Sinclair (1964)
Other	
Barley-grain	Creager (1945)
Cornmeal-vermiculite	Kernkamp, et al. (1949)
Cornmeal-sand	Gibson, et al. (1961)
Cornmeal-water	Dickinson (1930)
Corn, sand, or soil	Tervet (1937), LeClerg (1941*b*); Ray (1943); Demaree (1945); Kotila (1945*b*); Boosalis (1956); Flentje and Stretton (1964)
Oat	Ullstrup (1936); Person (1944*a*)
Oatmeal-Sand	Shephard and Wood (1963); Ruppel, et al. (1965)
Oat-wheat and water	Person (1944*a*)
Sorghum	Brodie and Cooper (1964)
Sterile moist black soil	Bruehl (1951)
Vermiculite + nutrients	Varney (1961)

into segments 3-6 inches long and washing in tap water for 20 minutes; then through two changes of sterile, demineralized water on a mechanical shaker for 10 minutes each and finally by hand for 2 minutes in 50 ml of sterile water. The excess water was blotted aseptically and the stolons were then cut into three segments. These segments were plated on PDA at pH 4.2 and incubated at 26°C for 6 days.

Schmitthenner (1964) washed diseased alfalfa seedlings for 10 minutes under running tap water in a stainless steel 5-inch sieve inserted in a beaker. Approximately 1.0 ml Tween 20 (Hercules Powder Company) was added at the beginning and halfway through the washing cycle. Excess liquid was blotted with sterile filter paper. The seedlings were placed on agar media in petri plates and the agar disk was inverted over the tissues.

The fungus could be either transferred to new agar plates after it grew out from the tissue and hyphal-tip isolates recovered, or, if the fungus appeared to be in pure culture, hyphal-tip isolates could be made directly from the original plate.

Isolation of R. solani from soil.—There were four books and six references published recently on the biology and ecology of soil-borne microorganisms and plant pathogens, portions of which are pertinent to this subject (Garrett, 1956; Burgess, 1958; Parkinson and Waid, 1960; Park, 1963; Maciejowska, 1964; Baker and Snyder, 1965). There were several review articles (Montegu, 1960; Warcup, 1960; Durbin, 1961; Menzies, 1963*a*,*b*) and a handbook (Johnson, [L. F.] et al., 1959) published on techniques for isolating and assaying microorganisms from soil. Those techniques that have had some degree of success for the isolation of *R. solani* shall be discussed.

Dilution plate (Johnson [L. F.], et al., 1959).—A water suspension using a 25 gram sample of dry soil was shaken for 30 minutes. A 10 ml sample was added to 90 ml sterile-water blank and followed through a successive dilution series until the final test dilution was reached. After shaking, 1.0 ml of suspension was aseptically transferred to agar medium just above solidifying temperature. After incubation, the average number of colonies per dish was multiplied by the dilution factor to obtain the number of microorganisms per gram in the original soil.

Maier (1961*b*) reported fairly reliable recovery of *Rhizoctonia* spp. from soil by making initial dilutions of 1:10 in pasteurized sand, with subsequent dilution in distilled water to $1:10^3$ or $1:10^4$, pipetted into sterile plates and swirled into potato-carrot-dextrose agar.

Microscopic detection on plant debris (Bourn and Jenkins, 1928*a*; Boosalis and Scharen, 1959).—This method was based on the distinct morphological characters of *R. solani*, which make it easy to identify. Plant debris fragments screened from field soil were plated directly onto modified water agar, and the fungus was then detected by direct microscopic observation. The principal disadvantage of the technique was that it dislodged all of the sclerotia from some debris tissues and thereby introduced error in estimating the number of infested substrate sites. Another disadvantage was the difficulty in distinguishing the mycelium of *R. solani* from certain other fungi. These authors found that the standard soil-dilution plate method was unsatisfactory, since it did not yield *R. solani* from the same soil samples.

In the technique of Bourn and Jenkins (1928*a*), soil to be tested was suspended in tap water and then passed through a 600-mesh screen. The settled soil was resuspended and repeatedly passed through the screen until the supernatant was clear. Pieces of wood, root, straws, lumps of soil, and other foreign objects were removed with forceps, then a strong jet of tap water was run through the screen to remove any soil adhering to the organic debris particles. Washed debris particles were blotted dry between sterile paper towels. From the towels, 100 particles were picked at random and transferred to 25 plates of water agar at pH 4.8. Plates were marked at four points on the bottom and one drop of 2.0% solution of streptomycin sulfate was placed above each mark. A debris particle was placed on top of the streptomycin sulfate drop and incubated at 24°C for 48 hours. *Rhizoctonia solani* grew from the particles, and hyphal-tip isolates were transferred to PDA.

Hyphal isolation method (Warcup, 1955).—Soil was suspended in beakers of water and allowed to settle. The supernatant was poured off and the soil resuspended and allowed to settle until the supernatant was clear. The soil was spread in a small amount of water in a culture dish. The individual hyphae or groups of hyphae were detected under a binocular dissecting microscope and 20-50 were removed with a fine forceps to sterile culture plates with a small amount of sterile water. Cooled, but still liquid, agar was poured into the plate and the hyphae were dispersed by rotating the agar before it solidified. Hyphae showing growth were transferred to fresh media.

Improved immersion plate.—This technique was described by Wood [F. A.] and Wilcoxson (1960) as a variation of Chesters (1940) technique. After 25 ml of water agar had solidified in sterile culture plates, a sterile disk was placed on the agar surface and the cover was closed (Fig. 1). To isolate fungi from soil, the covers were removed and the plates were pressed against the soil profile so that soil and disk were in contact. The plates were covered with aluminum foil and held in place with soil. After 2 days or more, the plates were taken to the laboratory, the disks were removed immediately, and the fungi isolated were transferred to fresh agar plates. This technique favored the isolation of nonsporulating fungi, such as *Rhizoctonia* spp.

Plate-profile.—*Rhizoctonia* spp. were isolated 15-18% of the time by the plate-profile method and 0-1% by the dilution plate method in studies by Anderson and Huber (1962). The plate-profile method used a 20 × 20 × 1.5 cm autoclavable plastic plate with 77 holes 1.0 cm deep and 0.5 cm in diameter spaced at

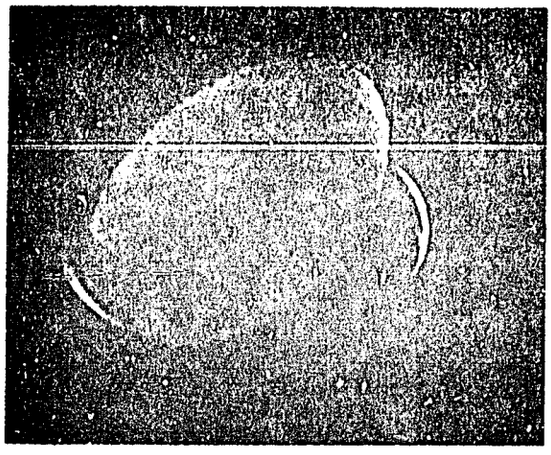

Fig. 1. Improved immersion plate used for isolating fungi from soil showing placement of sterile, perforated disk in agar plate (Wood and Wilcoxon, 1960).

2.5 cm intervals (Fig. 2). There were 11 horizontal rows of seven holes each. The plate was autoclaved and, after cooling, each hole was filled aseptically with sterile agar. The holes were covered with autoclaved plastic electricians' tape. The plate was exposed for about 5 days to a soil profile after small holes were punched in the tape directly above the agar-filled holes. The tape was removed in the laboratory and the agar plugs were transferred to agar plates. The frequency of isolation of various soil fungi and their location at specific levels in the soil profile were determined with this method. The method lends itself to a study of microorganisms active in the vicinity of plant roots.

Glass-cloth and nylon-mesh traps.—Maier (personal communication), employing 1 × 1 cm square of multifilament glass cloth embedded in soil, reported that *R. solani* readily colonized the cloth and formed abundant sclerotia (Fig. 3). He found that percentage colonization was a more reliable method for estimating populations than the immersion-tube method described below. Monofilament nylon mesh with 400 squares per square centimeter was used by Old and Nicolson (1962) to study the spread of *R. solani* in soil. These authors suggested that the method might be used to isolate the fungus from the soil. It might also be used to make population estimations.

Immersion tubes.—This method was devised by Chesters (1940), who used a large glass tube, closed at one end with holes blown out of the lower half of the tube. The tube was filled with nutrient agar and placed in soil. Pieces of agar from the immersion tube were plated on PDA after 7 days. *Rhizoctonia* spp. were among the fungi collected. Mueller and Durrell (1957) used agar-filled plastic centrifuge tubes with holes arranged in a spiral. The holes were covered with electrician's tape, which was pierced with a sterile needle just before immersion into the soil. Martinson and Baker (1962) increased the frequency of isolation of *R. solani* by adding exudate of radish seed to agar placed in the tubes.

Thornton (1958) designed another modification of the immersion-tube technique, using perforated rods and tubes. Sterile water agar was dispensed aseptically with a hypodermic syringe into holes in a sterile rod. The rod was inserted into a perforated tube with the agar filled holes turned away from the tube apertures. A hole was dug in the soil, the tube inserted horizontally into the soil profile, and the rod was then turned so that the agar-filled holes were opposite the tube apertures. Just before removal, after 4-7 days, the rod was rotated to turn the agar-filled holes away from the apertures. The agar plugs were punched out of the rod in the laboratory and plated on nutrient agar.

A modification of Chesters' immersion tube was designed by Gochenaur (1964). This method reduced selectivity for fungi that grow rapidly and tolerate low oxygen tension. Pyrex immersion tubes were invaginated with 2-4 capillaries and then filled with 1.0 g of dry soil in place of nutrient agar. The tube was moistened, plugged with cotton, capped with aluminum foil, and autoclaved. Tubes were placed in soil for 7 days, then withdrawn. After the exterior was cleaned with 70% ethanol, the contents were removed and dispensed on three agar plates. The technique provided a well aerated medium in which moisture content rapidly came into equilibrium with surrounding soil. This technique has not been tested specifically for the isolation of *R. solani* from soil, but it should be tried since it offered some advantages over other immersion-tube methods.

Varney (1961) showed that *R. solani* grew satisfactorily in vermiculite saturated with nutrients. Substituting nutrient vermiculite for agar or soil in immersion tubes might improve recovery of *R. solani* and should be explored. The advantages would be a standardized medium that would be loose and well aerated and in which moisture levels could be controlled. The mixture could easily be broken apart and uniformly distributed on agar plates for assay.

Trapping and baiting.—Plant segments of bean, cotton, wheat, barley, oat, carrot, and potato have been used successfully as plant baits for *R. solani* (Sneh, et al., 1966). They found that autoclaved green segments buried in nonautoclaved soil both increased and decreased the acceleration in percentage of colonized plant fragments, whereas ripe cereal segments were less influenced by autoclaving.

Sterile lima bean segments (Chu, 1966) and split-stem segments of rush (Downes and Longhnane, 1965) were successfully used to isolate *Rhizoctonia* spp. from various soils.

The use of buckwheat stems for trapping *R. solani* was worked out by Papavizas and Davey, 1959*b*, 1961, 1962*b*). Segments of mature buckwheat stems about 5.0 mm long were mixed with soil and incu-

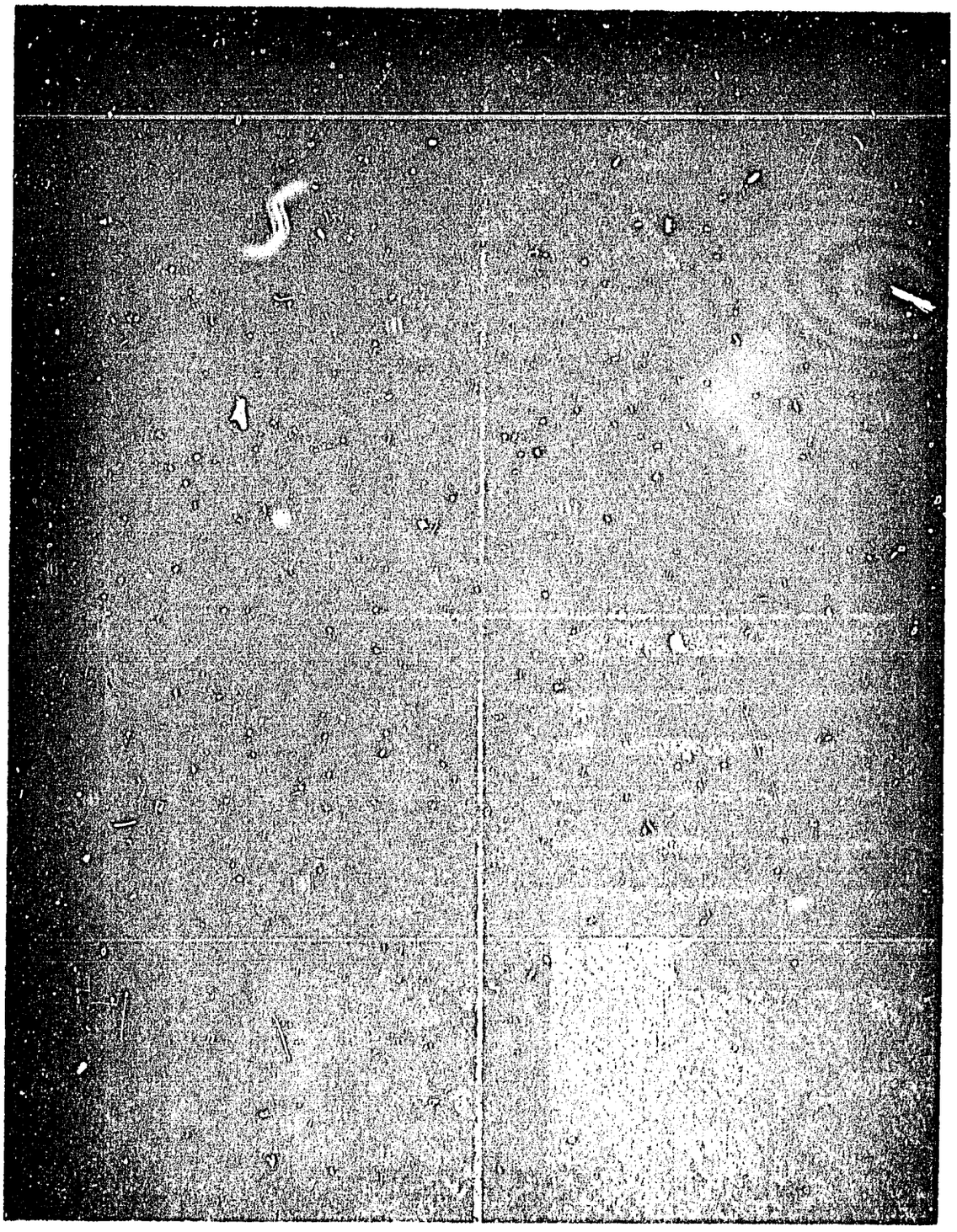

Fig. 2. Plate-profile technique for isolating soil microorganisms. (*A*) Preparation of profile. (*B*) Pin holes punched through tape over agar-filled holes. (*C*) Plate placed against soil profile in vicinity of roots. (*D*) Electricians' tape stripped from plate for transferring invaded agar samples to petri plate (Anderson and Huber, 1965).

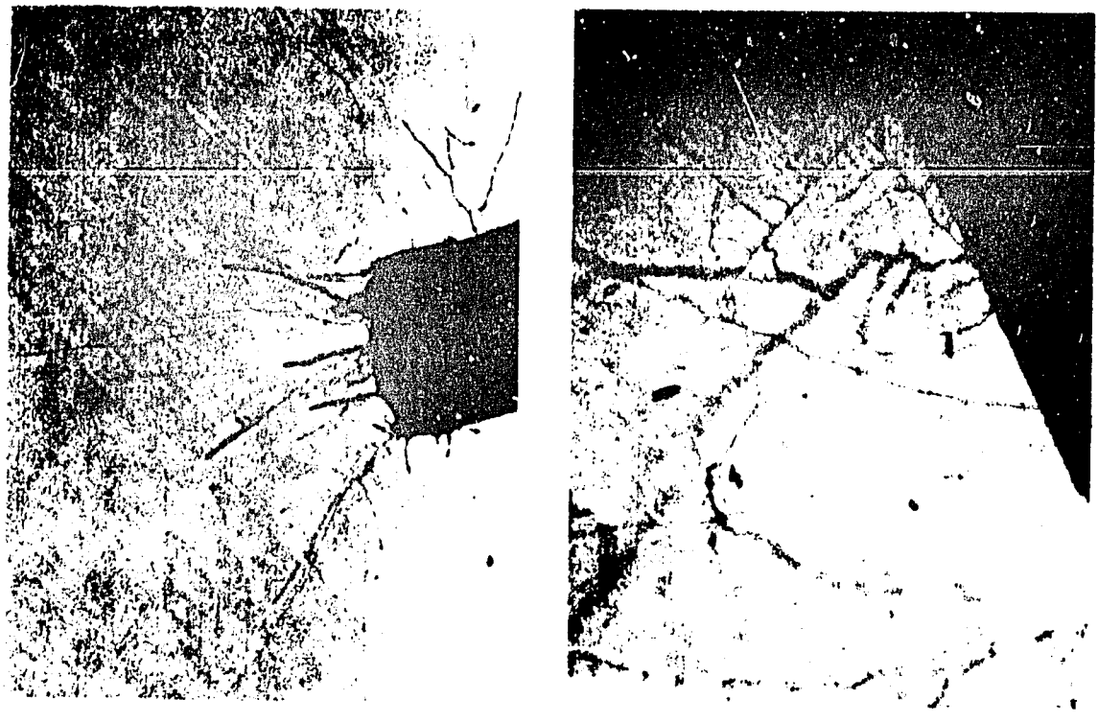

Fig. 3. Low-power photomicrograph of fungus hyphae growing from colonized glass-cloth fiber 24 hours after planting. Several different fungi were recovered in this isolation (× 40), Maier (1967).

bated for 4 days at 25°C. The segments were recovered from soil, placed on 10-mesh screens, and washed thoroughly under tap water. Excess water was removed by blotting between sterile paper towels. Segments were either soaked in an antibiotic solution before plating to reduce contaminants or plated on 2% water agar containing 50 ppm of streptomycin sulfate, aureomycin hydrochloride, and neomycin sulfate. Hyphal-tip isolations were made after 20-24 hours incubation.

Sneh, et al. (1966) reported stem segments of cotton and bean gave higher percentage of colonization than buckwheat.

El-Zarka (1963) developed a modification of the buckwheat method using *Cochorus olitorius* L. as a substrate. Stainless steel insect pins 4 cm long were inserted perpendicularly through pieces of the substrate. The stem pieces were placed in the soil and recovered after 24 hours. These were washed thoroughly in tap water and surface sterilized in 1.0% sodium hypochlorite solution for 1-2 minutes, rinsed in several changes of sterile water, and soaked for 2 hours in a fresh solution of 100 ppm terramycin hydrochloride at room temperature. The pieces were removed, dried in paper towels, and plated on agar.

Messiaen (1957) compared five different methods for the isolation and culturing of fungi from soil in France. He found that *R. solani* was detected best by incubating corn grains in soil and then isolating on prune agar. A similar technique was found satisfactory by Frick and Johnson (1958) when they recovered *R. solani* from corn seed buried in soil and later plated on water agar.

Bourn and Jenkins (1928*a*) planted disinfected seed of the host plant in *R. solani*-infested soil. The soil and seed were incubated in total darkness for 7-10 days. Seedlings were then lifted from the soil and examined for lesions. Segments with lesions were cut from the stems and roots and plated on agar. A variation of this technique was used by Wilhelm (1956) to recover *R. solani* from soil in strawberry fields in California. Nonblemished roots were immersed in 0.1% mercuric chloride for 1.5-2.0 minutes, rinsed, and incubated in coarse, sterile, moist sand in petri dishes covered with lids containing a layer of 2.0% water agar. Smith [J. F.] and Ashworth (1965) used the number of lesions on seedlings of small white bean to measure inoculum potential of *R. solani* in greenhouse experiments.

Evaluation of soil isolation techniques.—Thornton (1956) compared the screened immersion-plate technique and Warcup's plating method for isolating *R. solani* from grassland soils. He reported that typical mycelia of *R. solani* were predominant in all sites and levels tested by the immersion-plate technique. Warcup's technique failed to yield comparable results. Since results with the Rossi-Cholodny slide, agar-soil film, and direct examination methods demonstrated the presence of the fungus, he concluded that War-

cup's technique did not give a true picture of the fungus flora in soil.

Davey and Papavizas (1962*b*), using two naturally infested soils, compared four methods: debris-particle, immersion-tube, buckwheat-colonization, and infected-host. The infected-host method was found best for studying the range of fungus isolates pathogenic to certain hosts. The immersion-tube method recovered only a limited number of fungus isolates. The debris-particle method in conjunction with the buckwheat-colonization method gave the most complete information. The debris-particle method measured the quiescent isolates in soil and the buckwheat-colonization method measured the active. Isolates of the fungus obtained by the four methods varied from highly virulent to avirulent.

Further studies by Papavizas and Davey (1962*b*) used saprophytic-colonization methods to determine frequency of isolation of *Rhizoctonia* spp. from 15 naturally infested soils. They found, in general, that stem segments of buckwheat, cotton, and lima bean were satisfactory, while those of corn, oats, and soybean were not. Livingston, et al. (1962) reported the plant-debris method and the buckwheat-stem method were comparable in the frequency of isolation of *R. solani*.

Ui and Ogoshi (1964) compared several methods for frequency of isolation of *R. solani* from both artificially infested and naturally infested soils. They reported a higher frequency was consistently obtained with the flax-trap technique when compared to the soil-dilution plate, Warcup's soil-plate, LaTouche's slide-trap, Mueller and Durrell's immersion-tube, and Blair's contact-slide techniques. Mueller and Durrell (1957) stated that the soil-dilution plate method was not suitable for the isolation of *R. solani*.

Agnihothrudu (1962) compared the following methods for the isolation of various fungi, including *R. solani*, from tea soils in India: direct-isolation, Rossi-Cholodny slide, washed-root, Warcup's soil-plate, and dilution-plate. He was able to recover the fungus with the first three methods, but not with the last two.

A high degree of correlation was found between infestation level and colonization potential of *Rhizoctonia* spp. in soil with the plant-colonization, plant debris particle, or immersion-tube methods (Sneh, et al., 1966).

One should not therefore rely on any single method for studying *R. solani* populations in soils. Warcup (1951) pointed out that "different methods of sampling soil fungi are complementary rather than exclusive."

Media used for culturing.—*Rhizoctonia solani*, as shown above, can be recovered on a wide range of substrates. Shaw (1912) cultured the fungus on raw plant material, such as carrots and potatoes, and on bread, meal, and filter paper. Balls (1906) grew the fungus on filter paper containing 2% of either asparagin or sucrose. Varney (1961) successfully grew the fungus in vermiculite saturated with nutrients. *Rhizoctonia solani* is commonly cultured on agar or liquid media ranging from water agar to highly specialized media (Table 1). The basic nutrient requirements of the fungus were discussed by Sherwood (This vol.).

Many workers prefer to use a nonmodified PDA or slightly modified PDA for culturing the fungus. Successful culturing of certain isolates may require the use of an infusion broth or agar using host tissue similar to that from which the original isolates were obtained. Other specialized media may be required for specific studies concerned with nutrient requirements or physiologic studies and are reviewed in other sections of this book.

Czapek's agar or modifications of it and Edward's medium were the chief synthetic media used for culturing *R. solani*.

Rhizoctonia solani can be cultured successfully on various liquid media. It usually forms a tough, crusty mycelial mat on the surface of potato-sucrose broth in nonshake cultures. The mat is easily removed and washed. Most staling products can be separated from the fungus mat by decanting off liquid medium before the mycelial mat is broken up. *Rhizoctonia solani* also grows well in liquid shake cultures.

Steamed-sterilized soil was used successfully by a number of workers to increase inoculum of *R. solani* (Sanford, 1952). This substrate is available and easy to prepare. The constituents of soils are variable, however, and may not be repeatable from one area to another. Cornmeal sand medium (Gibson, et al., 1961) could be used as a standard for this method.

Recent descriptions of the use of foam rubber (Domsch and Schicke, 1960) and synthetic sponge (Sulba Rao, 1964) for the culturing of fungi should be noted. These synthetic materials were moistened with liquid-nutrient media, autoclaved, and inoculated with various fungi including *R. solani*. The advantages of using these techniques were that the fungus penetrated readily and the substrate afforded abundant aeration and increased surface area for mycelial growth. The use of these synthetic media for culturing *R. solani* should be further explored.

Rhizoctonia solani is therefore a relatively easy organism to isolate and culture on a wide variety of media. Some of these are easy to prepare and the growth obtained is suitable for most studies. It would be of material advantage to future studies of *R. solani* not requiring specialized media if either one or two agar media could be universally used as standards for in vitro studies. Variation in *R. solani* attributed to the variety of media used to study this fungus might be better understood if routine culture methods could be standardized.

Incubation and storage.—Incubation conditions for isolating and culturing *R. solani* are not as critical as for some other pathogenic fungi. Most isolates will grow well in vitro over a relatively wide range of temperatures, relative humidity, pH, CO_2 concentrations, light, and other environmental factors. The lit-

erature concerning the effects of environment was reviewed by Sherwood (this vol.).

Much variability in optimum temperatures for in vitro growth of *R. solani* is reported. Incubation temperature might play an important role in recovering and maintaining the fungus only if high- or low-temperature isolates were studied. Cochrane (1958) stated that temperature optima and ranges reported were valid under the specific conditions of the experiment, with regard to time, medium, other environmental factors, and the method of measurement. He concluded that there was no single temperature optimum for either growth or any enzymatic type reaction in *R. solani*.

Most in vitro experiments in closed culture dishes would have close to 100% relative humidity, the optimum for the growth of the fungus. The fungus is known to survive in air-dried soil and may be considered tolerant to low relative humidities in soil. The author observed the fungus surviving on desiccated agar in culture tubes for many weeks, which would indicate the fungus could survive low relative humidities in vitro (unpub.).

Most isolates of *R. solani* prefer an acid medium, with optimum pH for growth being less variable than that reported for temperature. The optimum pH for in vitro growth of most isolates of *R. solani* is between 5.5-7.0 (LeClerg, 1934; Jackson, 1940; Blair, 1943; Khan, 1950; Ragheb and Fabian, 1955; Elarosi, 1957*b*; Bateman, 1962). Matsumoto (1963) found that the effect of pH varies according to the nature of the media used and thought it was almost impossible to designate a definite optimum pH value for growth of the fungus.

Blair (1943) reported that aeration of soil was necessary for optimum growth of *R. solani* as CO_2 accumulation was very toxic. The summary statement of Garrett (1956): "the toxic effect of carbon dioxide upon a fungus is greatest when the energy growth is least" is appropriate.

Light had an important effect on in vitro growth (Durbin, 1959*b*; Weinhold and Hendrix, 1962), sclerotia production (Valdez, 1955; Durbin, 1959*b*, and basidial production (Whitney, 1964*a*) of *R. solani*

Storage and maintenance of stock-culture collections of *R. solani* and other phytopathogenic fungi involves certain problems. Stock cultures of *R. solani* stored in a refrigerator or at room temperature require frequent subculturing mainly because of desiccation. This may involve monthly transfers depending on environmental conditions. Viability and pathogenicity may then be altered. Shaw (1912) reported that *R. solani* remains viable for 5-6 months on agar media in culture plates and tubes. This was the experience of the author (unpub.), who recovered the fungus from 4–6-month-old agar slants by plating on fresh agar. It was not determined, however, if the fungus survived as mycelium or as sclerotia. Parmeter (personal communication) believed that most isolates will remain viable on PDA for at least 1 year. Sims (1960) found certain isolates of the fungus lost virulence following culture on amended media.

Loss of pathogenicity after prolonged storage in vitro may be a problem. Schroeder and Provvidenti (1961) found that tomato-fruit-rot isolates of *R. solani* lost their infectivity after 2 months in culture. They reported that 4-day-old cultures gave five times the infectivity on tomato that 2-month-old cultures did. Sherwood (personal communication) stated that certain cultures of the fungus stored in a refrigerator could not be recovered after 3 months. He maintained his stock culture collection of the fungus on PDA fortified with 0.5 gram each of malt extract, yeast extract, and casein hydrolysate. These cultures stored in a cabinet at room temperature and transferred at least once every 3 months have maintained their morphological and pathogenic characters for 6-12 years. Parmeter (personal communication) reports having similar results.

There are a number of techniques for the storage of phytopathogenic microorganisms. A review of these techniques was made by Iyenger, et al. (1959). One of the best methods for the preservation of agar cultures of *Rhizoctonia* spp. is with sterile mineral oil. They found that the fungus remains viable under oil after 5 but not after 7 years. Schneider [C. L.] (1957) reported that 70 of 76 cultures of *Rhizoctonia* spp. survived 6-7.5 years on PDA amended with vitamins B_1 and C, covered with sterile mineral oil, and sealed with cotton plugs and Parafilm. In a similar experiment, he reported that 31 of 40 cultures survived 5.5-9.0 years. Pathogenicity studies on the surviving cultures were not made. Pumpyanskaya (1964) showed that a mineral-oil depth of 0.2-0.5 cm did not prevent oxygen penetration, while 1.0 cm gave relatively anaerobic conditions, and 6.0 cm gave complete anaerobiosis.

The fungus could also be preserved either on grain or in soil. Kreitlow (1950) showed that *R. solani* could be stored for at least 16 months by growing isolates on a steamed mixture of two parts wheat and one part oats in a 250 ml flask for 3 weeks at 15°C and allowing them to dry at room temperature. The material was ground to no less than 40 mesh and stored at 5°C.

Soil has been used as a storage medium by Tolmsoff (personal communication). He reported that sugar beet isolates were stored for several months in soil by infesting autoclaved soil with cultures grown on sterile sugar beet seed and incubated until a high population built up. The soil was then air-dried and stored at cool temperatures. If the soil was maintained at room temperature in a moist condition, cultures lost infectivity to sugar beet within 2-3 months. This is opposite to results found using agar. Vaartaja (1964*b*) isolated *R. solani* from a sand-soil mixture stored for 2 years with only 0.2% by weight of water.

Sulba Rao (1964) recommended the use of a washed, synthetic sponge cut into the shape of agar slopes, inserted into test tubes, moistened with 3-5 ml of Richard's medium, and autoclaved, as an alternative to agar culture of fungi. It afforded abundant

aeration and increased surface areas of mycelial ramification.

The best methods of incubation of *R. solani* in vitro are to grow the fungus: (1) only on fresh standardized, buffered medium (pH 6.0); (2) at temperatures optimum for growth of the particular isolates being studied; and (3) in a closed cabinet to prevent due exposure to sunlight or artificial light.

The best conditions for long-term storage of the fungus are fresh, standardized, buffered medium, immersion of the culture under sterile mineral oil, and storage at temperatures below 0°C. Containers should be sealed before storage. Comprehensive studies are needed, however, to determine the effects of prolonged storage under oil on pathogenicity of *R. solani* isolates.

The use of foam rubber (Domsch and Schicke, 1960) and synthetic sponge (Sulba Rao, 1964) moistened with liquid nutrients for storage of *R. solani* should be explored.

METHODS USED IN DETERMINING INOCULUM POTENTIAL AND POPULATIONS OF *R. solani* IN SOIL.—The concept of inoculum potential was reviewed by several authors (Garrett, 1960; Horsfall and Dimond, 1963; Dimond and Horsfall, 1960, Baker [R.], 1965) and is discussed in other sections of this volume. The term inoculum potential was first used by Horsfall (1932) to define "variations in the content of pathogenic fungi or inoculum in the soil." The meaning of the term has changed and become broader, but it still carries the concept that is a measure of the ability of a soil-borne organism to cause disease in a susceptible host under certain conditions, i.e. to act as inoculum. It connotes measurement of this ability in a carrier, such as soil, sand, or an artificial medium infested with the organism. Inoculum potential should not replace "pathogenicity" or "virulence," terms used to describe characteristics of organisms.

Rhizoctonia solani exists in soil both as a potential parasite and a saprophyte and is probably distributed throughout the soil (Garrett, 1956; Livingston, et al., 1964). Some *Rhizoctonia* spp. are active saprophytes in soil and can survive in the absence of hosts by competitive colonization of dead organic matter (Garrett, 1956; Boosalis and Scharen, 1959; Papavizas and Davey, 1961). Papavizas and Davey (1962*b*) emphasized that pathogenic and nonpathogenic strains in soil were morphologically indistinguishable from one another. *Rhizoctonia solani* has been classed as a primitive parasite (Garrett, 1956) and as a weakly competitive saprophyte (Rao, personal communication). Papavizas (1964) pointed out that the fungus may lose its pathogenicity after passage through soil or following changes in nutritional levels.

Susceptibility of hosts may be influenced by environmental conditions. These facts create a problem for developing an accurate method of measuring the inoculum potential of *R. solani* in soil. This is discussed below under testing for pathogenicity.

Since *R. solani* exists as mycelium in the soil, most immersion methods are useful in estimating populations of this pathogen. Martinson (1963) found that the frequency for isolation of *R. solani* with soil microbiological sampling tubes correlated with preemergence damping-off of radish. The correlation held when inoculum density, temperature, and concentration of pentachloronitrobenzene (PCNB) were varied. These experiments were conducted under controlled conditions; an isolate of the fungus known to be pathogenic to radish and watermelon was used. Under these conditions, particularly where soil was infested with a pathogenic isolate of the test fungus, the inoculum potential was measured adequately. Under field conditions, the sampling tube method would measure only population density and thus not give a true picture of the inoculum potential of the soil.

Some studies and techniques measuring the inoculum potential of *R. solani* in field soil have in reality estimated only the population of the fungus in soil, including saprophytic and pathogenic isolates. In order to measure the true inoculum potential of *R. solani* in a given soil sample, it must be shown that all isolates of the fungus in the sample are pathogenic. If an isolate is not pathogenic, it cannot be considered part of the inoculum. The problem of selective pathogenicity among isolates of *R. solani* may also become involved.

Some of the methods for isolation of *R. solani* from soil were used to compare the amount of disease with estimated populations of the fungus. Papavizas and Davey (1959*b*), using buckwheat stem pieces and naturally or artificially infested soil, found some correlation between infection index and frequency of isolation of *R. solani*. Their infection index, however, took into account only invisible symptoms and not preemergence damping-off. They found that 60% of 50 *R. solani* cultures isolated by the buckwheat-stem method were pathogenic. They concluded, however, that the infection index was not always reliable for detecting differences in soil infestation by the pathogen.

Livingston, et al. (1964) found general agreement between the plant-debris fragment and the buckwheat-stem-segment methods for indexing *Rhizoctonia* spp. inoculum levels. The buckwheat technique was particularly sensitive. Comparisons made by them in 1960 and 1961 showed that disease severity was greatly influenced by seasonal differences, soil types, and general field conditions.

The most successful method for predicting the severity of either Rhizoctonia root or crown rot is by estimating the inoculum potential of soil from the number of infested plant debris particles or mycelial fragments on the roots of host plants. Rich and Miller (1963) showed that the severity of Rhizoctonia root rot of strawberry was related to the number of *Rhizoctonia* spp. fragments adhering to a 5.0 cm length of strawberry root.

The debris-particle method was used by Boosalis and Scharen (1959) to compare the incidence of sugar beet crown rot in two soil samples from the

same field. The first sample came from an area of the field that had a high incidence of crown rot the previous season, the second from an area with low incidence. The soil with the highest infection index also had the highest percentage of disease plant debris particles supporting strains of *R. solani* pathogenic to sugar beet. Both soil samples were infested with the same pathogenic strain of the fungus. The second sample, however, had a mixture of at least two strains, the second being pathogenic only to potato stems. This technique gave both a population estimate and an estimate of the inoculum potential of the soil, i.e. indicating the ratio of pathogenic to nonpathogenic strains.

Smith [L. F.] and Ashworth (1965) estimated inoculum potential under field conditions by counting the number of lesions that developed within a 96-hour period on small, green tomato fruit buried in soil. Tomato fruit were found to be immune to *Pythium* spp. which obscured *R. solani* damage to tomato and bean seedlings. Sanford (1952) found the disease rating of host plants to be a satisfactory measure of the persistence of *R. solani* in soils, whereas data from buried glass slides and root colonization of indicator plants were unreliable.

The application of the theory of inoculum potential to *R. solani* may best be studied under controlled conditions using artificially infested soil. Sanford (1952), Papavizas and Davey, 1959*b*), and Martinson (1963) infested soil with pathogenic strains of *R. solani*. Measured inoculum mixed with a known quantity of soil was varied and correlated with a disease index. The relationship among the pathogen, host, and environment then could be expressed in mathematical terms (Dimond and Horsfall, 1965). Study of inoculum potential and variables affecting it under controlled conditions might lead to methods for predicting disease severity.

STUDYING PATHOGENICITY.—Pathogenicity tests are necessary in fulfilling Koch's postulates. It is particularly important that pathogenicity be established for isolates of *R. solani* being studied.

Tolba and Moubasher (1964) stated that *Rhizoctonia* isolates are nonspecific in their pathogenicity. Most standard techniques used to establish pathogenicity of other phytopathogens must therefore be modified for *R. solani*. The fungus does not produce conidia. Basidiospore production is either rare, difficult to obtain in culture, or lacking. Basidiospore infection in nature appears to be rare, but this may be due to ignorance rather than fact. Strong (1961) attributed stem canker of greenhouse tomatoes in Michigan to basidiospore infection. Pinckard and Luke (1967) thought basidiospore infection of cotton bolls played an important role in boll rot initiation. Basidiospore infection appears to be common in the tropics (Echandi, personal communication). More studies are needed to determine the role of basidiospores in spread of disease.

Mycelia and/or sclerotia are widely used in place of spores. Some isolates produce few, if any, sclerotia in culture and mycelia must be used. If mycelia are used for testing of pathogenicity, hyphal-tip cultures should be obtained. These can be obtained by plating mass transfers of the fungus on water agar and, after several days' incubation, examining under a dissecting microscope. Single hyphal-tip cells can be cut from hyphae with a sharp dissecting knife or razor-blade chip. Cut cells become almost transparent, while intact cells remain translucent and are easily selected (Whitney and Parmeter, 1963).

Use of basidiospores.—The perfect state of *R. solani* forms more or less freely in nature on a wide range of hosts (Kotila, 1929, 1947; Exner, 1953; Pinckard, 1964; Whitney, 1964*a*; Pinckard and Luke, 1967). Successfully induced fruiting of isolates in vitro would aid materially in taxonomic and cytological, as well as pathogenicity studies. A number of methods were derived to induce basidiospore formation.

The common method is to transfer the fungus from a rich medium to a poor one (Fig. 4). The transfer of the fungus directly from host tissue or enriched agar cultures to 2% water agar was reported to be successful by a number of workers (Kotila 1929, 1947; Ullstrup, 1939; Exner and Chilton, 1944; Carpenter, 1949, Exner, 1953; Sims, 1956). Sims (1956) recorded basidiospore formation after transfer of the fungus from amended potato broth to alligator weed (*Alternanthera philoxeroides*) stems and roots. Kilpatrick (1966) induced sporulation of *R. solani* by transferring agar plugs of the fungus to a small "moist chamber" consisting of filter paper, moistened with distilled water, placed on the top and bottom of a culture plate.

A second method is to place fungus hymenium and/or mycelium on the inside of a culture dish cover and place it over either sterile water, water agar, or amended agar (Exner, 1953; Hawn and Vanterpool, 1953; Sims, 1956; Whitney, 1963).

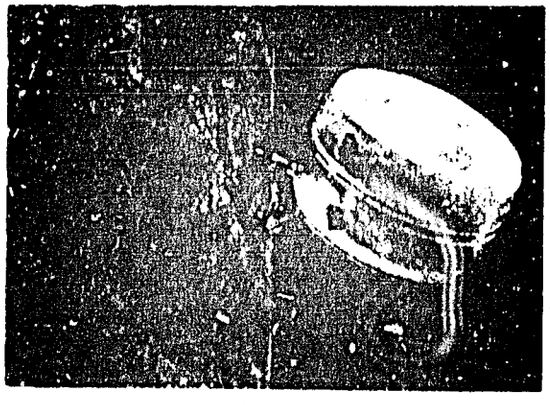

Fig. 4. Cluster of *Rhizoctonia* basidia produced on water agar (*left*) (Whitney and Parmeter, 1963) and experimental set-up for collecting basidiospores (*right*). Upper plate (*A*) contains perfect state of the fungus and lower plate (*B*) contains water agar (Whitney, 1963).

Basidiospore formation was successful when the fungus was grown directly on either Coons' medium (Kotila, 1929) or alligator weed decoction agar (Sims, 1956). Stretton, et al. (1964) and Whitney and Parmeter (1964) found that either covering or mixing an agar culture of the fungus with soil or sand would induce sporulation (Fig. 5.).

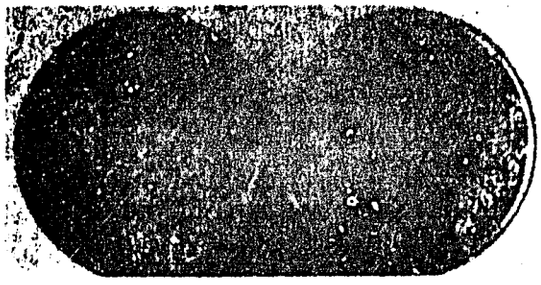

Fig. 5. R. solani fruiting in culture plates after being covered with soil to induce sporulation (Whitney and Parmeter, 1964).

Warcup and Talbot (1962) successfully used modifications of three methods to induce fruiting of basidiomycetous fungi isolated from soil. These were: Warcup's chaff method (Warcup, 1959); Tamblyn and Da Costa's wood-block method (Tamblyn and Da Costa, 1958); and Flentje's soil method (Flentje, 1956).

The perfect state of *R. solani* usually occurs in nature at the base of plants and on the underside of soil clods during periods of high rainfall and humidity (Exner and Chilton, 1943; Warcup and Talbot, 1962, Pinckard, 1964, 1967). This suggested that humidity and light were important factors in inducing fruiting. The requirement of high RH for spore production was shown by Kotila (1929), Carpenter (1949), Exner (1953), Hawn and Vanterpool (1953), and Sims (1956). Heaviest sporulation and discharge by the fungus in nature was shown to occur during the first 6 hours of darkness at night with little or none occurring during the day (Carpenter, 1949). Several investigators suggested that light inhibited the initiation of the perfect state (Kotila, 1929; Carpenter, 1949; Hawn and Vanterpool, 1953; Flentje, Kerr, Dodman, McKenzie, and Stretton, 1963; Whitney, 1963, 1964*a*). Whitney (1964*a*) suggested that alternating light and dark periods would produce a correspondingly rhythmatic cycle of sporulation.

Temperature, pH, CO_2, and oxygen requirements for sporulation are not critical (Kotila, 1929; Carpenter, 1949; Hawn and Vanterpool, 1953; Whitney, 1963).

A technique to induce sporulation at will may have to be developed for particular isolates. Pathogenicity studies using cultures from single spores could then be made. Once an isolate is stimulated to fruit, single basidiospores can be harvested by several methods. Kotila (1929) described a method of collecting single basidiospores using a micromanipulator. Even with modern equipment, this is tedious and is recommended only where the basidiospores from a single basidium are required. Olsen et al. (1967) removed single basidiospores from a tetrad by touching them with a small water agar block on the tip of a fine glass needle. The block with spore was then placed in the center of a culture plate containing agar.

Allowing the discharge of basidiospores from the lid of a culture dish to an agar medium below was shown to be a successful means of obtaining single basidiospores (Exner, 1953; Hawn and Vanterpool, 1953). Isolated germinating spores were located, marked, and removed by using a "biscuit cutter" technique as described by Keitt, et al. (1915). Each spore was then transferred to suitable media in a culture tube.

Other methods used for single-spore isolations of fungi may be applicable to *Rhizoctonia*. These are the dry-needle technique (Hildebrand, 1938); or dog hair attached to a metal probe or needle (Guthrie, 1963). These workers picked single spore from either agar or glass slides. Khair, et al. (1966) found it difficult to recover spores from agar, because some types of spores became embedded in the water film on the agar surface, and from glass slides because of their smoothness. They found that single spores, deposited on dialysis tubing cellophane taped over a rectangle of 4% water agar, were more easily harvested.

Use of mycelium for inoculating aerial plant parts.—Young plant tissue is generally considered more susceptible than mature tissue to *R. solani*. Testing for pathogenicity on aerial plant parts necessitates, in most cases, the use of either seedlings or newly formed leaves and stems. Luttrell (1962), using fescue and soybeans, found that spraying plants with a suspension of macerated agar plate cultures of *R. solani* was effective on tender seedlings and ineffective on mature leaves. Alfalfa seedlings in the unifoliate-leaf stage were found by Schmitthenner (1964) to be less susceptible to *R. solani* than were seedlings in the cotyledonary leaf stage. Parmeter (personal communication) found that *R. solani* readily attacked older leaves, but that *Ceratobasidium* sp. attacked only young leaves.

Schroeder and Provvidenti (1961) and Ruppel, et al. (1965) worked with *Tephrosia vogelii* and were successful in establishing infection on 6-week-old seedlings by atomizing a mycelial suspension from 1-week old PDA cultures onto plants held for 24 hours in a constant mist. They both recorded variation in virulence among isolates of the fungus. Atkins and Lewis (1954), however, obtained infection of all aerial plant parts of soybeans, regardless of age, with *R. solani* in a moist chamber at about 30°C, 24 hours after spray inoculation. Tandon (1961) reported systemic infection on guava seedlings in a greenhouse after inoculation with *R. solani*. After inoculation, small, irregular spots occurred on upper leaves. These spots coalesced and spread downward until the entire plant succumbed.

Luttrell (1962) compared methods of foliar inoculation with *R. solani* on fescue grass. Good infection

was obtained by spraying plants with a suspension of macerated agar-plate cultures. Better infection resulted, however, when alfalfa meal was added to the suspension. The best and most convenient method was dusting moistened plants with dried oat-grain cultures (Kreitlow, 1950). Allison and Hanson (1951) sprayed plants with a blended suspension of sclerotial fragments and mycelium and placed them in a moist chamber.

In order to successfully establish infection on tomato fruit, Schroeder and Provvidenti (1961) found that a PDA biscuit with mycelium from a 4-day-old culture had to be placed over an epidermal puncture made with a fine-pointed sterile needle. Their inoculation studies showed that the type of symptoms produced depended on fruit maturity at the time of infection.

Two additional techniques described by Lutrell (1962) were the use of single oat grains from whole oat-grain cultures and single sclerotia on leaves. The use of sclerotia permitted quantitative comparisons in terms of size of lesion produced from a single point of inoculation during a specific period in an incubation chamber.

Schuster, et al (1958) described a toothpick method for determining the pathogenicity of *R. solani* isolates on sugar beet seedlings in the field. They found, however, that the reaction of the seedling was not correlated with mature plant reaction to crown rot.

Regardless of the method used, infection seems to be influenced by available nutrients in either sclerotia or in nonliving substrates (Lutrell, 1962), and this should be taken into consideration in making inoculations, especially on aerial plant parts.

Inoculating plant parts at or below the soil line - There are a number of standard techniques used to test pathogenicity of *R. solani* isolates on plant parts either at or below the soil line. Most involve using infested soil or other media sown with seed or seedlings of host plants either before or after soil infestation. Methods have been designed for laboratory, greenhouse, and field experiments. Media can be infested with fungus hyphae, either in suspension or growing on a suitable medium, or sclerotia.

Infection of stem and hypocotyls of jute and avocado was accomplished by techniques worked out by Shaw (1912) and Mircetich and Zentmyer (1964) respectively. Shaw tested for pathogenicity by placing small pieces of an agar culture on stems of susceptible jute plants, while Mircetich and Zentmyer placed 2.0 mm disks of agar with fungus in cuts in avocado stems and covered with adhesive tape. Portions of the host were surface sterilized and placed on glass slides with the test organism in a moist chamber, using a technique described by Flentje (1965).

Root inoculation with *R. solani* has received consideration, particularly on fleshy-root crops such as potato and sugar beet. Schwinn (1961), to establish the causality of *R. solani* for dry core of potato, inoculated both mechanically wounded and natural lenticel excrescences. Cormack and Moffatt (1961) studied the pathogenicity of *R. solani* on sugar beet roots by removing a core of tissue, placing the inoculum in the hole and replacing the core. The amount of decay at different incubation temperatures was recorded.

Mircetich and Zentmyer (1964) studied the pathogenicity of *R. solani* on avocado roots by using seedlings grown in aerated, complete nutrient solution in 5-gallon ceramic crocks at pH 6.5. One-half petri dish cultures of the test fungus were blended with 100 ml of deionized water for 1.0 minute and poured into the crocks with the seedlings. The other half of the culture was placed in cheesecloth and suspended in the nutrient solution. At 35 days after inoculation, 66.95% of the roots were infected (Fig. 6).

Infesting soil and other media - There are a variety of methods using infested soil for testing pathogenicity of *R. solani*. Some techniques have been developed in connection with the evaluation of soil fungicides for control of Rhizoctonia seedling diseases. A few of these were selected for review.

Infested oat and wheat grain were used by LeClerg (1941) to infect the underground stems of potato and sugar beet. The grain inoculum was placed in direct contact with the stems. He also mixed the grain inoculum with steamed soil and allowed it to stand for 2 weeks. The resulting infested soil was added to tops of greenhouse pots containing potato seed pieces. Infested oat, wheat, and/or barley grains were used to infest soil or to infest underground stems of crop plants (Newton and Mayers, 1935; Le Clerg, 1941*a*, Ashworth, et al, 1964).

Staten and Cole (1948) prepared infested soil by mixing diseased plant tissue with the soil. To study *R. solani* on cotton seedlings, Lehman (1940) infested soil by stirring a culture of *R. solani* in half of the soil contained in greenhouse flats.

Sinclair (1958, 1960) used 2 week old macerated broth cultures of *R. solani* to infest greenhouse flats for the study of cotton seedling damping-off by the fungus. After 2 weeks incubation in the greenhouse, the soil was sown with cotton seed.

Brueil (1951) prepared inoculum by incubating the fungus in flasks containing 150 grams of black soil at 20-23 C for 8 days. A portion of this inoculum was mixed in the top half of sterile soil in greenhouse pots and used to test for pathogenicity on oat and wheat seedlings.

Root canker of alfalfa was studied by Smith [O. F.] (1944) by placing transplants directly in infested soil or by allowing plants to grow about a month in non-infested soil and then placing inoculum in holes in the soil.

The pathogenicity of *R. solani* on celery was tested by pouring a suspension of macerated agar cultures around the crown and between the petioles before soil was pushed up around the base of the plants (Houston and Kendrick, 1949).

The pathogenicity of various fungi to turfgrass seedlings was tested by Endo (1961) by growing seedlings in steam-sterilized soil in clay pots. The soil

was infested after germination with small plugs of washed mycelium placed in the soil. Various isolates of *R. solani* were tested for their potential to incite damping-off of *Tephrosia vogelii* (Ruppel, et al., 1965), by growing fungus isolates on sand-oatmeal medium for 3 weeks, then mixing with 300 grams of sterile sand in partially filled greenhouse pots, and covering them with a thin layer of inoculum.

Schmitthenner (1964) used three different methods to study the virulence of *R. solani* isolates on alfalfa seedlings: (1) The vermiculite-culture technique employing a 1 liter flask plus 500 ml of vermiculite moistened with autoclaved yeast-extract–glucose solution; (2) the injection-infestation technique using shake cultures of the fungus filtered, resuspended in distilled water, blended, and injected into the soil around roots of 10-day-old seedlings; and (3) the inoculum-layer technique, using fungus cultures placed in a layer below, but separated from, the seed at sowing. Using the inoculum-layer and injection-infestation methods, differences in virulence were detected.

Owen and Gay (1964) used greenhouse ground beds artificially infested with a mixture of seven isolates of *R. solani* from cotton seedlings to study control of the fungus with soil fungicides (Fig. 7). Temperature and moisture were controlled to assure reasonable kill of plants in nontreated soil. They obtained good results in field trials by mixing *R. solani*-infested cottonseed and seedlings into the soil three weeks before planting and by using overhead irrigation.

Whether inoculations should be made with sterilized soil or with soil containing a normal soil microflora is open to question.

The advantages of using pure culture inoculations are that they provide: (1) effective means of controlling inoculum levels; (2) precise measurement for fungus effects; (3) severest disease conditions possible; (4) reproducible results; and (5) elimination of stimulatory and antagonistic effects of other ecosystems. The disadvantages are that, because of the alteration of soil ecosystems, conditions are not comparable to field situations because: (1) disease will likely be more severe than might be expected under field conditions; and (2) plant vigor may be increased or decreased.

The advantage of using field soil with or without infestation of the test organism is that it approximates the disease situation encountered in the field. The disadvantages may be: (1) difficulty in obtaining reproducible results because of variability in the ecosystems from experiment to experiment; (2) data may be appropriate only for the area from which the soil

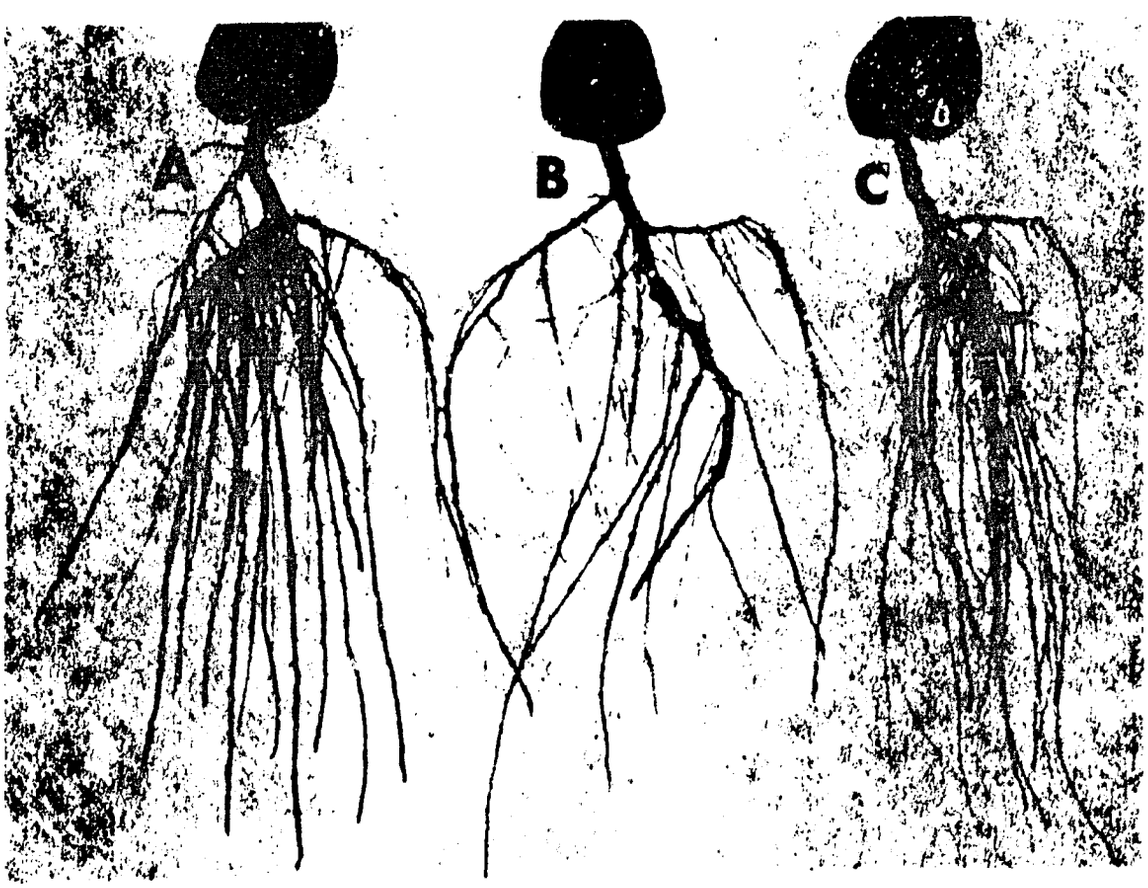

Fig. 6. A, B. Typical root lesions produced by *R. solani* on avocado seedlings in inoculated nutrient solution. (C) Noninoculated control. (After Mircetich and Zentmyer, 1964.)

sample was indigenous; and (3) the test may not represent the severest disease situation possible.

Use of infested sand and agar media.—Endo (1961) was able to detect nonpathogenic and pathogenic isolates of *R. solani* from turfgrass by sowing seed on sterile quartz sand in culture plates watered with sterile water. Newton and Mayers (1935) tested the susceptibility of plants to *R. solani* by growing surface-sterilized seed on nutrient agar previously inoculated with the fungus. A similar technique was used by Bruehl (1951), who placed germinated wheat and oat seed in culture plates of the fungus. Hansen and Curl (1964) found that differences in pathogenicity among isolates of *R. solani* could be detected on white clover seedlings established on low-nutrient substrates. Another method, used by Vaartaja et al. (1961), detected differences in pathogenicity by placing aseptically germinated tree seed in tubes containing half-strength cornmeal agar and the fungus.

Sims (1960) showed that various amended agar media significantly reduced virulence and dry weight below that of nonamended media among three isolates of *R. solani*.

Flentje (1965), in summarizing the various methods for testing pathogenicity of *Rhizoctonia* isolates, outlined three complementary approaches: (1) The direct addition of the test organism to natural or

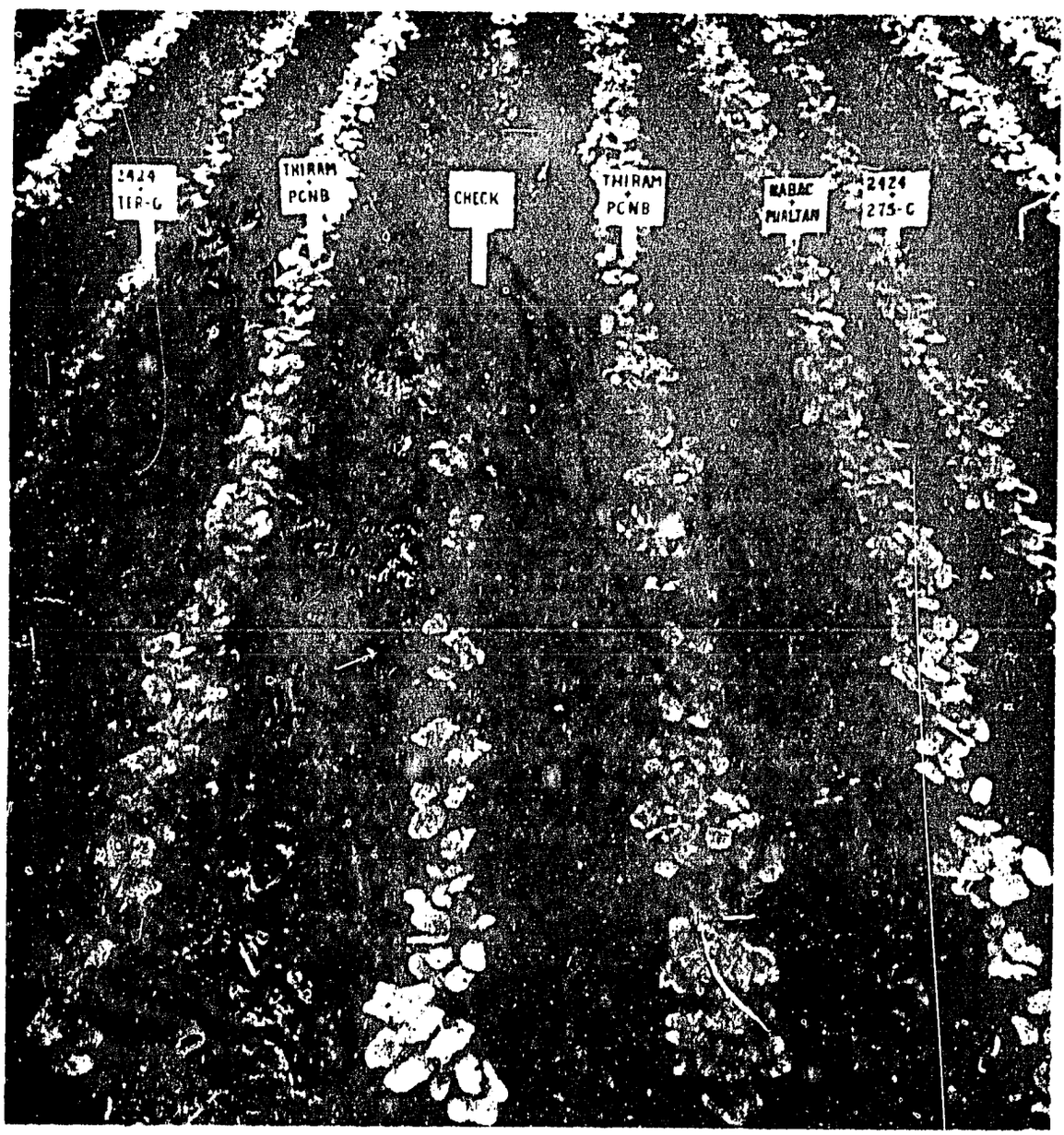

Fig. 7. Soil in floor of greenhouse beds was artificially infested with 7 isolates of *R. solani* to evaluate soil fungicides. (Owen and Gay, 1964.)

amended soil in which the host plants are grown and the natural soil microflora is present. This method is often used to study the ability of the fungus to grow through soil. It does not indicate how the organism enters the host or how pathogenesis is effected (2). The direct opposition of the test organism to the host in sterile sand or soil or on surfaces such as glass slides in a moist chamber, allowing for direct study on host penetration, type of tissue damage, and rate of damage in the absence of other organisms (3). The indirect opposition of the test organism to the host by testing culture filtrates or extracts of the organism on host tissues, providing for the study of possible mechanisms involved in pathogenesis.

Effects of environment on pathogenicity studies.—Classically, the study of any plant disease and the pathogenicity of the pathogen involved must take into account the interaction of host and pathogen and the effect of the environment on this interaction. Much of this information on factors affecting pathogenicity is summarized elsewhere in this volume.

There seems to be no limit to the host range of the fungus. The whole concept of host range for *R. solani* may be confused because the host range can be altered by time, kind of plants used, varietal resistance and susceptibility, and by a number of soil microecological factors (Papavizas and Ayers, 1965).

Rhizoctonia solani can survive, grow, and remain pathogenic in soil under a wide range of environmental conditions because of its variability. Shephard and Wood (1963) thought pathogenicity of *R. solani* depended mainly on intrinsic properties of the host and isolate, and environmental factors were rarely of more than secondary importance in determining whether an isolate would attack a particular host plant. But the environment does play more than a secondary role in the infection of *R. solani*. The effect may not be directly on the fungus, but an indirect effect on the host plant, as with soil temperature and soil reaction.

Khan (1950) stated that the generalization that *R. solani* is either a high- or low-temperature pathogen or is destructive in an acid or alkaline soil, is not justified.

Results from studies concerning the effect of soil moisture on *R. solani* and disease development are not in agreement. This may be due to lack of standardization in technique. In a study by Bateman (1964) in which he made a factorial analysis of soil temperature, moisture, and pH, using *R. solani* and other soil-borne organisms, he found no combination of the three factors prevented infection of poinsettia by the fungus. Shephard and Wood (1963) showed that high humidity appreciably increased pathogenicity of *R. solani*.

The effect of soil moisture on the susceptibility of host plants needs study. Standardization experiments using field capacity (field-moisture capacity) as defined by the Soil Science Society (Anon., 1962) should be used in future studies. Couch, et al. (1967) reviewed the application of soil-moisture principles to the study of plant disease.

The effect of nutrient balance on saprophytic activity of *R. solani* was considered by Papavizas and Davey (1961), Papavizas, et al. (1962), and Papavizas (1964). Gibson (1956) demonstrated that the activity of *R. solani* as a damping-off pathogen is largely dependent on the nutrients available in the soil.

Predisposition to infection.—Testing for pathogenicity of *R. solani* is not always successful, as was pointed out by Huber and Finley (1959) and Brigham (1961). Such variable results may be due, in part, to the fact that the fungus was pathogenic on weakened seedlings under field conditions. Several agents are reported to predispose plants to infection by *R. solani*.

Certain plant viruses predisposed hosts to infection by *R. solani*, such as cucumber mosaic virus (Bateman 1961), barley yellow-dwarf virus (Smith, 1962); and beet curly top virus (Erwin and Flack, 1963).

Neal and Newsom (1951) showed a relationship between top (*Leptosphaeria fusca*) injury and cotton wilt caused by *R. solani*.

Smith (A. M.) and Prentice (1929) observed that an association existed between *R. solani* and *Heterodera schachtii*. Grainger and Clark (1963) reported a synergistic effect between the fungus and *H. rostochiensis*. Mountain and Benedict (1956) in greenhouse and field experiments found that the combination of *R. solani* and *Pratylenchus minyus* reduced growth of wheat plants by half. In greenhouse tests, Fielding and Mathie (1959) showed a combination of either *Meloidogyne hapla* plus *R. solani* or *M. javanica* plus *R. solani* reduced emergence of soybean to 2% and 17% of the check, respectively. Sayed (1961) showed a significant increase in damping-off of pea caused by *R. solani* in association with *M. hapla*. Reynolds and Hanson (1957) and White (1962) showed an increase in Rhizoctonia disease of cotton seedlings associated with an increase in the incidence of both *M. incognita* and *M. incognita acrita*. Similar results were obtained by Brodie and Cooper (1964), who showed that cotton seedlings grown in soil infested with either of three *Meloidogyne* spp., *Rotylenchus reniformis*, or *Hoplolaimus tylenchiformis* were susceptible longer to *R. solani* than were seedlings in nematode-free soil.

Fulton and Hansen (1950) clipped foliage of red clover plants at frequent intervals and increased the susceptibility of the host to *R. solani*. Reynolds and Hanson (1957) and Brodie and Cooper (1964) showed that excising either all or portions of cotyledons of cotton seedlings increased their susceptibility to *R. solani*. Webster and Roth (1965) increased susceptibility of lima bean seedlings to *R. solani* by damaging the seed coat before planting.

Plant damage due to agricultural chemicals was shown to increase the incidence of *R. solani* on cotton seedlings (Erwin and Reynolds, 1958; Miller and Ahrens, 1964; Ramey, 1964; Pinckard and Standifer, 1966).

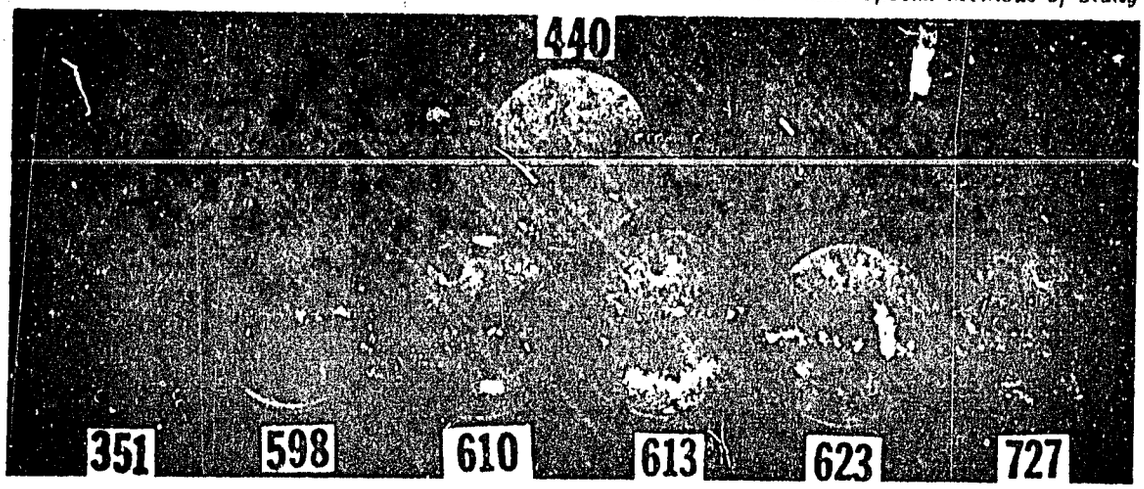

Fig. 8. Example of paired homokaryons forming tufts at their common line of convergence. Upper petri dish shows reaction of homokaryon 440 when paired with itself: colonies completely merge but no tufts are produced. Lower 6 petri dishes show results of pairing homokaryon 440 with 6 other homokaryons in which tuft formation is induced. Tufts can be seen as erumpent cottony wisps of mycelium at line of convergence between paired colonies. Sclerotium initials can also be seen in some plates; these later harden and darken. Colonies photographed after growth on Fries's agar for 10 days (Parmeter, et al., University of California, Berkeley).

The physiologic responses of host plants to these predisposing factors should be studied to determine the exact nature of increased susceptibility to *R. solani*.

Heterokaryosis and pathogenicity.—The effect(s) of heterokaryosis on pathogenicity and other characters of *R. solani* isolates also needs study.

The common technique for developing heterokaryotic lines is to make mycelial transfers of two homokaryotic lines on opposite sides of culture plates containing agar. When marked tufts of aerial mycelium form at the lines of contact, bits of mycelia are transferred to agar tubes or plates (Fig. 8). The characters from hyphal tip isolates are then compared with that of the two original strains (Fig. 9).

Whitney and Parmeter (1963) used a semiliquid medium in which equal amounts of two strains of *R. solani* were blended. Droplets of the mixture were spotted on water agar and hyphal tips from subsequent growth were transferred to agar slants and studied.

Cellophane was used by Whitney and Parmeter (1963) as a substrate for anastomosis studies and the development of heterokaryons in *R. solani*. Sterile pieces of cellophane cut to fit culture plates were placed on the surface of water agar. Single spore strains that had given tuft reactions were plated at opposite sides of the cellophane. When anastomosis had occurred, a small piece of cellophane bearing the anastomosis figure was cut from the cellophane and transferred to agar slants.

METHODS OF STUDYING TOLERANCE TO CHEMICALS.—*Rhizoctonia solani* is highly variable. This fact has been emphasized throughout all sections of this book. This variability allows for rapid adaptation to changes in the environment including tolerance to chemicals. The development of tolerance in microorganisms to drugs is well documented in human medicine beginning with the work of Kossiakoff (1887). Only within the past 25 years have plant pathologists examined

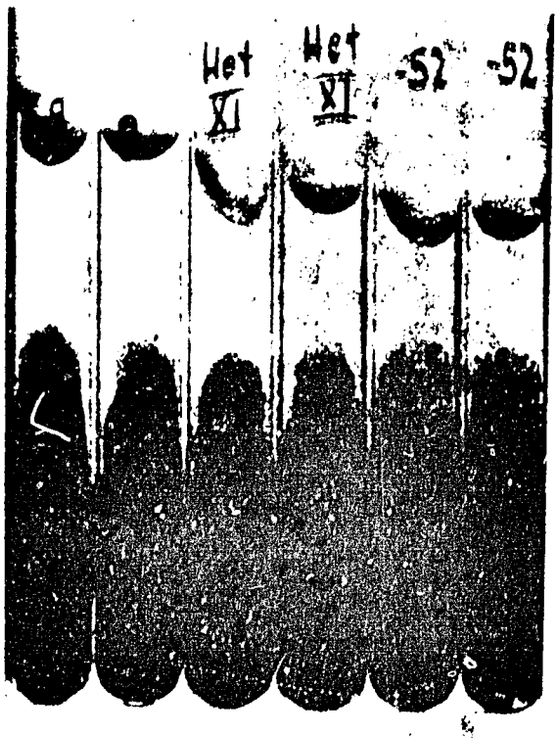

Fig. 9. Comparison of characteristics of heterokaryon (XI) with 2 original strains (8 and 52). (Whitney and Parmeter, 1963.)

the development of tolerance to fungicides used for the control of plant-disease-causing organisms. The literature of fungicide adaptation was reviewed recently in theses written by Shatla (1959), Elsaid (1962), Raffray (1965), and Ricaud (1965). Kernkamp, et al. (1952) noted three papers dealing with tolerance to toxic substances in their review.

Rhizoctonia solani, like other members of the Mycelia Sterilia, offers some limitations in methods that can be used to study tolerance to fungicides and other chemicals, because of the lack of sufficient spore production. This fungus does not produce asexual spores. Basidiospore production is either lacking in most isolates or is difficult to obtain in sufficient amounts for use in techniques requiring spore suspensions. This limitation may be overcome in the future with better methods of inducing basidiospore production. Fungicide tolerance was studied using both in vitro and in vivo methods.

It is a prerequisite for any *R. solani* isolate used for chemical-tolerance studies that it be in pure culture, stable in cultural characteristics, and able to withstand continued subculturing. The author has found that all isolates of *R. solani* are not capable of repeated subculturing, particularly where weekly serial transfers may be involved. Much of the work has been done using cultures from hyphal-tip isolations. Studies on chemical tolerance in *R. solani* were conducted by growing the fungus on a suitable agar medium containing various concentrations of the test chemical.

Test fungicides can be incorporated into nutrient media by several means. Most workers add chemicals in known concentration to the agar medium. Ricaud (1965) recrystallized PCNB and TCNB (tetrachloronitrobenzene) from acetone from commercial preparations and used them in either the vapor phase or dispersed them as a fine suspension in the medium. These fungicides were added to the medium before autoclaving. Concentration was based on ppm of actual ingredient. For studies in our laboratory, commercial fungicides were added to media after autoclaving just before the agar became solid (Sinclair, 1960; Shatla and Sinclair, 1963; Elsaid and Sinclair, 1964; Raffray and Sinclair, 1965). Concentration also was based on ppm of active ingredient. These different approaches may have an effect on results.

The use of radial growth as a measure of adaptation requires that either a standardized "seeding" or "plant inoculation" method is used. A sterile cork-borer should be used to cut plugs, biscuits, or cores of agar with mycelium from agar plates. For *R. solani*, cores should be cut as uniformly as possible from the outer growth of the colony in an old culture (more than 7-10 days old at optimum incubation). Various studies have shown that the older mycelium loses its vigor and therefore might not be suitable for culture plate studies. Domsch (1958) stated, however, that age of mycelium does not substantially influence its sensitivity. If very young cultures (2-5 days old at optimum temperature) are used, most of the colony is suitable for inoculation. Use of a standardized mycelial suspension for seeding plates is not satisfactory because the inoculum does not spread uniformly over the agar. Agar plugs should be placed in the center of each culture plate. Radial growth is measured either at the radius or diameter. If growth is irregular, the widest part of the colony should be measured.

Isolates should be incubated at a constant, controlled temperature optimum for growth. Incubation should be in a closed cabinet, since light has been shown to have an adverse effect on growth of *R. solani* in vitro. The length of incubation will be determined, in part, by the rate of growth of fungus isolates in nontreated plates. This may range from 3-6 days.

An obvious limitation of this technique is that radial-growth measurements may not give a true picture of differences in mycelial growth between treatments (Brown, 1923). Some chemicals may limit horizontal growth, but not vertical growth; others may prevent the fungus from penetrating into the agar. Check plates may have fungus mycelium growing in quite a different manner and may thus influence final results. Brown (1923) compared dry-weight measurements and radial growth for studying growth of fungi and found the use of radial growth: (1) made it possible to carry out experiments on a large scale; (2) allowed for abundant repetition, and (3) provided for effective controls. In radial-growth measurement, a culture can be followed throughout all its stages while dry weight methods involve destruction of a culture for each measurement taken. Brancato and Golding (1963) concluded that the use of diameter growth of fungi was sufficiently reliable for determining growth rates and for comparing the effect of environmental factors.

The use of liquid shake cultures was explored by Ricaud (1965). He inoculated 100 ml of medium with five agar plugs taken 2 mm from the margin of a 2-day-old culture of *R. solani*. PCNB was added to the medium at either 2.5, 5.0, or 10.0 ppm before autoclaving. Only a single isolate was studied because another isolate proved unsuitable for growth in shake culture. He found that the percentage of inhibition was almost constant for the three concentrations. Only a slight increase in inhibition with increased fungicide concentration was noted. This confirmed his results on agar. From these results, it appeared that all isolates of *R. solani* would not be suitable for shake-culture studies. The possibility of using broth media for this type of study and comparing results on agar should be explored further. Nonshake-culture growth should be compared with shake-culture and agar cultures grown simultaneously under identical conditions.

It has been the experience of this author that most isolates of *R. solani* will form a thick mat on the surface of nonshaken potato-dextrose broth. This mat is easily removed for either the wet and or dry weight.

Factors affecting studies on tolerance.—Shatla (1959), Elsaid (1962), Maier (1962), Shatla and Sinclair (1963), and Elsaid and Sinclair (1964)

found as concentrations of PCNB in agar were increased, there was a corresponding decrease in growth among isolates of *R. solani*.

Shatla and Sinclair (1963) compared the growth of 6 of 36 isolates that had differed in their growth rates on agar with 1,370 ppm PCNB. The amount of PCNB required to inhibit completely the growth of these isolates ranged from 6,000-10,000 ppm. Ricaud (1965) found different concentrations of PCNB and TCNB incorporated in agar produced only a slight response with increased concentration beyond the saturation point (10 ppm). The solubility of chlorinated nitrobenzenes is less than 2.5 ppm (Reavill, 1950). The actual amount needed to obtain saturation of assay medium may exceed this value through gradual loss by diffusion (Ricaud, 1965). There seems to be no question that *R. solani* isolates vary in their ability to grow on agar containing different concentrations of PCNB and TCNB. It appears that this effect may involve more than a response to fungicide concentration.

Many studies of chemical tolerance assume that either reduction or absence of growth indicates toxicity and that increased growth in agar with the toxicant indicates tolerance to it. In cases where the toxicant has limited solubility, increased concentrations may have little effect on increase in the quantity of toxic material. Increased concentration of a fungicide in powder form, for example, may increase the osmotic value, change the pH, or affect available nutrients of the agar medium (Ricaud, 1965).

Ricaud (1965) found when PCNB and TCNB were suspended in agar at different concentrations, there was only a very slight response with increasing concentration beyond the saturation point. The fungicides also were only slightly more effective when impregnated in the medium than when applied in the vapor phase. These results suggested that the effect on mycelial growth of contact toxicity associated with undissolved solids in an assay medium was slight.

Many of the techniques described for fungicide evaluation under greenhouse conditions could be modified to study fungicide tolerance among isolates of *R. solani*. Greenhouse techniques for evaluating fungicides to control *R. solani* on cotton have been described by Ranney and Bird (1956), Sinclair (1958, 1960, 1965*a*), Elsaid and Sinclair (1962), Maier and Staffeldt (1963), Owen and Gay (1964), Gayed and Temple (1965), and Raffray and Sinclair (1965). A clay-pot technique was used to screen soil fungicides for control of root-rotting organisms isolated from infected lily, poinsettia, and other ornamentals by Schaffer and Haney (1956) and also to screen soil fungicides for control of bean-root-rot organisms by Davison and Vaughn (1957).

A simple and rapid technique for the evaluation of chemicals for control of *R. solani* was reported by Gayed and Temple (1965). Dried flax stems were placed in infested soil treated with a chemical. After 24 hours, the "traps" were removed and laid on 2% water agar containing bacterial-growth inhibitors. The extent of fungal growth after 24 hours was used to measure the efficacy of the chemical.

In general, soil in either greenhouse pots or flats is mixed with known quantities of either fungicide, fungus, or both and used to study the tolerance in the fungus to the fungicides. Greenhouse environmental conditions are not usually as controlled as in laboratory experiments. Experiments of this nature, conducted in controlled environmental chambers will give valuable information about fungicide tolerance in *R. solani* and other phytopathogens. For instance, Raffray and Sinclair (1965) described a modification of a technique of Elsaid and Sinclair (1962) using greenhouse flats to determine if three isolates of *R. solani* became more tolerant to 1,370 ppm PCNB captan, and maneb after three serial transfers through treated soil.

Flats of steam-sterilized soil were either nontreated or treated with each of the three fungicides. A strip of soil was removed from one side of the flat and replaced with soil infested with one of the isolates of *R. solani*. The preparation of inoculum to infest steam-sterilized soil was essentially the same as described by Sinclair (1960). Fifty seeds of the susceptible cotton variety Deltapine 15 were sown in each of five rows in a greenhouse flat. Rows were approximately equidistant from one another. The progress of the fungus through the soil was measured by the progressive kill of the cotton seedlings. The fungus was reisolated from infected seedlings on the opposite side of the flat and plated on potato-sucrose agar, without and with the respective fungicides. A strip of infested soil from the first flat then was taken from the side opposite the original strip of infested soil, placed in a second flat, and the procedure repeated.

In a technique described by Domsch (1958), a soil-compost mixture was placed in shallow trays, the fungicide worked in or applied by drenching, and *R. solani* introduced by watering with a mycelial suspension on the same day. Alderman peas were sown and covered, and trays kept for 6 days at 8°C, and then for 6 days in the dark at 20°C. Captan, PCNB, and an organic mercurial gave results reproducible to an exceptional degree.

Shatla (1959) for 2 years collected cotton seedlings showing symptoms of infection by *R. solani* from various locations in cotton-growing areas of Louisiana. Seedlings came from either nontreated soil or soil treated with PCNB alone or in combination with other soil fungicides. The isolates were plated on agar with 1,370 ppm PCNB. The mean diameter of the isolates from PCNB-treated soil was significantly greater than of isolates from nontreated soil after 7-day incubation at room temperature.

The advantages and disadvantages of using either pure culture inoculation or inoculation in the presence of normal soil microflora were discussed earlier in this volume.

CONCLUSIONS.—*Rhizoctonia solani* appears to be a good organism for exploring chemical tolerance in

fungi. Tolerance to fungicides has been established in this fungus, but better techniques and further studies are needed. Tolerance to a larger number of chemicals needs to be determined. A chemical to which strains of *R. solani* are tolerant, and that would overcome some of the disadvantages of PCNB and TCNB, needs to be found.

Additional in vitro and in vivo studies need to be made to determine the extent, potential, and nature of this resistance. Effects of prolonged exposure of *R. solani* to these fungicides should be studied. The mechanism of variation in *R. solani* has been reported earlier in this volume (Flentje, et al., this vol). If fungicide resistance is due to mutation, as suggested by Flentje and Stretton (1964) and Ricaud (1965), then definite proof could be obtained by genetical analysis.