Rhizoctonia Decline: A Degenerative Disease of Rhizoctonia solani

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ABSTRACT


Several isolates of Rhizoctonia solani (= Thanatephorus cucumeris) were shown to be affected by Rhizoctonia decline, a degenerative disease. Studies were made with isolate 189 (= ATCC 13248), anastomosis group 1, and 189a, a diseased isolate obtained from 189 during routine transfers. Cultural characteristics of 189a are a white-tan color, floccose texture, irregular appearance, production of few or no sclerotia, and a slow growth rate. Healthy 189 cultures are brown with mycelium appressed to the agar surface, uniform in appearance, produce numerous dark sclerotia, and grow rapidly. Healthy cultures were recovered at a low frequency (1-5%) from 189a by hyphal-tip isolations; these cultures were repeatedly hyphal-tip transferred and to date no symptoms of disease have been observed. Attempts to cure 189a using elevated incubation temperatures, hot water exposures, antibiotics, and acridine dyes failed. None of these treatments was any more efficient in the recovery of healthy cultures than hyphal tipping. The disease was not due to bacterial contamination or toxins. Basidiospore analysis of 189a showed inheritance of the disease agent to be cytoplasmic. Transmission of the agent occurs by hyphal anastomosis. Fusion between 189a and healthy 189 results in the conversion of healthy 189 cultures into the disease-type. The movement of the disease agent throughout the hyphae is complete since all transfers from infected 189 are diseased. Attempts to transmit the disease agent from 189a to other anastomosis group 1 isolates failed.

Additional key words: cytoplasmic inheritance, mycovirus, double-stranded RNA.

Several reports have been published on degenerative diseases of fungi (4, 5, 6, 10, 11, 12, 13, 14, 17, 18, 19, 20, 23, 24, 26), but among the plant-pathogenic fungi only five species have been reported to exhibit degenerative-type symptoms. Lindberg (14) described a disease of Helminthosporium victoriae characterized by hyphal lysis, slow growth, reduced sporulation, and reduced virulence (15). He showed that the disease agent was transmitted by hyphal fusion and mechanically using an inoculum phenol-extracts of diseased mycelium (16). Later he reported similar diseases in H. maydis (18) and H. oryzae (17). Grente (10) described diseased cultures of Endothia parasitica that were hypovirulent, produced fewer pycnidia and conidia, and less pigmentation than healthy cultures. Van Allen et al. (26) showed the disease agent to be transmitted by hyphal anastomosis. Lapierre et al. (13) reported that isolates of Gaumannomyces graminis var. tritici containing viruslike particles (VLPs) rarely formed perithecia, tended to sector, were difficult to maintain on agar, and were weakly pathogenic. However, Rawlinson et al. (23) were unable to confirm the relationship between culture degeneration and the presence of VLPs.

In the present paper we describe Rhizoctonia decline, a degenerative disease of Rhizoctonia solani Kuehn (= Thanatephorus cucumeris (Frank) Donk). In addition, evidence is given for the recovery of healthy cultures from diseased cultures and transmission of the disease agent by hyphal anastomosis. A preliminary report describing this disease has been published (2).

MATERIALS

Isolates.—The work reported here is based on isolate 189 (= ATCC 13248), anastomosis group 1, of R. solani obtained from J. R. Parmeter, Jr. (22). Isolate 189a, a severely diseased culture, was obtained from 189 during routine transfers. Isolates 189HT3 and 189HT5 were obtained by hyphal-tip isolation from 189a. Each was apparently healthy after the first hyphal tip isolation, but successive hyphal tipping was continued, three times for 189HT3 and five times for 189HT5. Both of these isolates have remained healthy for 4 yr.

Media.—All cultures were maintained and stored on potato-dextrose agar (PDA) at 24-28 C. Various other media were used: potato-dextrose broth (PDB), potato marmite agar (PMA) (25), soil extract agar (25), malt extract agar-Kaufman (25), Weinhold's Medium A (27), Blue Ribbon malt extract agar (BRMEA) [3% Blue Ribbon malt extract (Premier Malt Products, Inc., Milwaukee, WI 53201) in distilled water, pH 4.5-5.0], Blue Ribbon malt extract broth (BRMEB), yeast extract

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agar (2% Difco yeast extract in distilled water), acidified-PDA (PDA adjusted to pH 4.2-5.0 with 25% lactic acid), and water agar.

**Chemicals.**—To determine whether diseased isolates could be cured by chemical treatments, PDA was supplemented with various antibiotics and dyes. Antibiotics used were streptomycin sulfate, chloramphenicol, tetracycline (all from Sigma Chem. Co., St. Louis, MO 63178), and gentamicin sulfate (Schering Diagnostics, Port Reading, NJ 07064). These antibiotics were added to make a final concentration of 50 and 100 μg/ml depending on the toxic level of the dye. All dye-supplemented PDA was kept in the dark.

**METHODS AND RESULTS**

**Disease description.**—Cultural characteristics of the severely diseased 189a are a white-tan color, floccose, irregular appearance, production of few or no sclerotia, and an extremely reduced growth rate. In comparison, healthy 189 cultures are brown with mycelium pressed to the agar surface, uniform in appearance, produce numerous dark sclerotia, and grow rapidly (Fig. 1). The level of disease expression for 189a can be variable. Severely affected cultures (Fig. 1) upon transfer usually give rise to similar-appearing diseased cultures; however, at times subcultures may fail to grow. Upon transfer, mildly affected cultures may range in appearance from healthy to severely diseased. In some cases a healthy appearing culture may yield only diseased subcultures. Because of the variability in disease expression for mildly affected 189a cultures, we used only severely diseased 189a cultures in our work; usually they were viable upon mass transfer and consistently yielded only severely diseased subcultures. No changes were noticed in the stability of disease expression for 189a when tested on the various liquid and solid media at temperatures from 23-30 C.

Although disease expression in isolates of *R. solani* varies, each appears to fall into one of three categories: (i) those in which disease is mild, exhibiting symptoms such as a reduced growth rate, patches of cottony or mealy mycelium, and reduced sclerotium production; (ii) isolates in which disease is lethal; such isolates may appear healthy but upon transfer fail to grow, resulting in the loss of the isolate; and (iii) those isolates which, though severely debilitated by disease, remain stable upon transfer (189a exemplifies this type of isolate).

**Growth-rate comparisons were made of 189HT3 and 189a based on radial expansion of colonies on PDA at 25 C. Average values of 40 + 1 mm/day and 4 + 1 mm/day were obtained for 189HT3 and 189a, respectively. These values represent averages of 10 replications and show that the radial growth rate on 189a is one-tenth that of 189HT3.**

Based on the degenerative nature of this disease we propose the name Rhizoctonia decline.

**Recovery of healthy cultures from 189a by hyphal-tip isolations.**—Hyphal tips were excised from cultures of 189a grown on PDA for 5 days at 25 C. Of 150 hyphal tips, only six grew to form cultures which appeared healthy. All other hyphal tips failed to grow. This low level of recovery (3%) indicates that the disease agent is unequally distributed within the hyphal tips of 189a and that the agent is lethal. In comparison, 95-100% of the hyphal tips from 189HT3 grow to form healthy cultures. Thus, to insure isolate survival, 189a as well as all severely diseased isolates were mass transferred.

**Basidiospore analysis.**—Single basidiospores of isolate 189a and 189HT5 were produced and isolated as described by Whitney and Parmeter (28). Germinated basidiospores were placed on PDA slants and incubated at 23-27 C. Each culture was mass-transferred every 2 mo onto PDA slants. At that time the number of healthy and diseased cultures was recorded. Basidiospores of 189a were isolated on two occasions; 40 the first time and 326 the second. In the cultures that developed directly from basidiospores, only three showed signs of disease and 363 appeared healthy; nevertheless subsequent mass transfer resulted in progressive decline. By the fourth generation, in the case of the 40 basidiospores, five were healthy, 25 diseased, and 10 dead. Similarly, by the sixth transfer of cultures derived from the 326 basidiospores, 207 were healthy, 110 diseased, and nine were dead. Although cultures in the second trial did not decline as rapidly as

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Fig. 1-(A to C). *Rhizoctonia solani*. Cultural characteristics of diseased isolate 189a, left column of plates, and healthy isolate 189HT3, right column. Age of cultures: A) 2 days, B) 2 wk, and C) 2 mo.
the 40 cultures in trial 1, the pattern was identical; it seems likely that most cultures would become diseased.

Ninety-eight single basidiospore cultures of 189HT5 were established; all appeared healthy in the first 2 mo of growth. However, by the sixth transfer-generation 12 had become diseased and 86 remained healthy. Thus, as with 189a, the basidiospore isolates of 189HT5 progressively declined, indicating that the phenotypically healthy 189HT5 may contain the disease agent.

**Bacterial contamination.**—The possibility that the disease may be due to bacterial contamination was explored. Microscopic observations of 189a mycelium at 1,280 using bright-field and phase-contrast optics failed to reveal the presence of bacteria. Growth of 189a in broth media (PDB and BRMEB) at 28 C showed no signs of bacterial growth, even after an incubation of 1 mo. Of 300 hyphal tips isolated from 189a grown on antibiotic PDA (streptomycin, tetracycline, chloramphenicol), and gentamicin added individually and in combination to warm PDA (only eight grew to form healthy appearing cultures after 5 days at 27 C. From acidified-PDA (pH 4.2-5.0), 100 hyphal tips were made of which only two were healthy. From these results we conclude that the disease is not due to bacterial contamination and that the four antibiotics have no curative effects on 189a when added to growth media.

**Exposure of 189a to elevated temperatures.**—The potential curative value of elevated temperatures was tested by growing 189a at a temperature near the maximum for survival or by exposing young and mature cultures to hot water. In the first trial, 189a was transferred to PDA and incubated at 30 C. The maximum incubation temperature that 189a can survive is about 33 C. After 1 wk, hyphal-tip isolations were made and transferred to PDA at 25 C. Of 63 hyphal tips, only two developed into healthy appearing cultures—a 3% recovery.

Tests were made to determine the potential of hot water to cure disease cultures. Isolate 189a was transferred to PDA in tubes and incubated 4 days at 25 C. Then the slants were covered with sterile-distilled water, preheated to 52 C, and placed in a 52 C water bath to maintain the elevated water temperature. Each minute after flooding, the tubes were removed from the hot water bath, the water poured out, and a mass transfer made to PDA. The maximum exposure to the hot water was 5 min. In the controls the water temperature was 24 C. All subcultures, regardless of exposure time, the hot water, developed into typical 189a cultures as did the controls.

In another attempt to cure 189a, mycelium essentially free from agar was treated with hot water using the following method. Sterile coverslips were placed on the surface of PDA in petri plates and 189a was inoculated near the edge of each coverslip. After the mycelium grew over the glass, usually after 4 days, the coverslips with the adhering mycelium were removed with forceps and immersed in water at 52 C from 1 to 5 min at 1-min intervals. Following treatment, the coverslips were placed on the surface of PDA with the mycelium in contact with the agar. In the controls the mycelium was immersed in water at 24 C for the same time intervals for treatment. Each treatment was replicated six times.

At the 5-min exposure there was no survival; at 4 min, three of the six replicates survived, and at exposure times less than 4 min, the mycelium of all immersed coverslips produced viable cultures. Of the surviving cultures, all were diseased showing that hot water treatment had no curative effect on 189a.

**Exposure of 189a to acidrine dyes.**—Plasmids of bacteria and fungi have been shown curable by exposure to acidrine dyes (7, 9). Hyphal tips, obtained from 189a cultures grown 5 days on PDA at 25 C, were placed on dye-supplemented PDA (concentrations of 1, 10, and 100 µg/ml) and incubated in the dark at 25 C. After the hyphal tips developed into micro-colonies (3 days) transfers were made to PDA. Of 250 dye-exposed hyphal tips, only 10 developed into healthy appearing cultures, a 2.5% recovery. Hyphal tips not exposed to the dyes showed a 4% recovery rate. Thus, there appears to be no curative effect from treatment with acidrine dyes.

**Toxin production by 189a.**—To test if 189a cultures produce toxins which in turn cause disease, 250-ml Erlenmeyer flasks each containing 50 ml of Blue Ribbon malt extract broth (pH 6.5) were inoculated either with 189HT3 or 189a and incubated 5 or 10 days, respectively, at 27 C in the dark. Eight replications of each were made. The contents of the flasks for each isolate were pooled, blended for 10 sec in a Waring Blender, filtered through Whatman No. 1 filter paper and then through No. 41 filter paper. The filtrate was centrifuged at 8,500 g for 25 min, and the supernatant passed through a millipore filter (0.45 µm pore diameter). This sterile filtrate was then tested for toxic properties by growing 189HT3 in flasks containing the filtrate from 189a and 189HT3 only, and the filtrate of each mixed 1:1 with fresh malt broth. Controls were fresh malt broth inoculated with 189HT3. Four replicates for each of the five test solutions were made.

The flasks were inoculated with a 4-mm-diameter plug taken from a young culture of 189HT3 and incubated at 27 C for 7 days. The mycelium in each flask was harvested. A small piece of this mycelium was placed on PDA at 27 C; the remainder was dried overnight at 77-80 C and weighed.

There were no significant differences (according to Duncan's multiple range test, P = 0.01) in the dry weights of the harvested mycelium grown on 189a filtrate, 189a filtrate plus malt broth, and 189HT3 filtrate plus broth. The growth on fresh malt broth, however, was significantly greater. This seems logical since this medium was undpleted and probably contained more nutrients than any of the other test solutions. On the other hand, the least amount of growth occurred on 189HT3 filtrate probably because the nutrients were depleted the most due to the growth of 189HT3. All transfers from the 189HT3 mycelium were healthy.

The results show that the disease is not caused by a toxin and that the agent, if present in 189a growth media, was not transmitted to healthy 189 cultures.

**Transmission by hyphal anastomosis.**—Anastomosis between 189a and 189HT3 was demonstrated by placing mycelial plugs of each taken from the advancing margin of young cultures, 2 cm apart on a microscope slide coated with a thin layer of PDA. The slides were incubated at 25 C until the hyphae of the isolates intermingled. The occurrence of anastomosis between 189HT3 and 189a was expected since they are probably genetically identical; 189HT3 being merely a hyphal-tip isolate of 189a. Thus, fusion between these isolates could
be considered a selfing response and not an anastomosis between two entirely different isolates within the same anastomosis group. In fact, we have observed that fusion between 189a and 189HT3 occurs much more often than between 189 and other isolates of anastomosis group one.

Initial transmission experiments involved the coinoculation of 189a and 189HT3 on opposite sides of PDA plates. Isolate 189a was inoculated prior to 189HT3 and incubated for 7 days. Fifteen 4-mm-diameter plugs were taken from the side of the plate inoculated with 189HT3 and five from the side with 189a for each of three replicates and transferred to PDA.

All samples produced diseased cultures, regardless of the part of the plate from which they had come. This indicated that the 189HT3 had become diseased as a result of hyphal contact with 189a. The fact that all samples were diseased illustrates that the disease agent moved throughout the mycelium of the 189HT3 resulting in the inability to recover the fast-growing healthy-type culture. We observed that the mycelium of 189a by growth alone could not have grown more than half way across the plates. Yet the question could be asked: Did 189a grow completely across the plate masking the presence of the healthy-type in subsampling? Another experiment using compartmented petri plates (Y-plates, Falcon Co., Oxnard, CA 93030) was devised to answer this question.

In all of the Y-plates, compartment one (Fig. 2) was half-filled with PDA, compartment two was filled with PDA, and the third compartment was left empty. Then compartment one was inoculated with 189a in the corner farthest from compartment two. Following a 2-day incubation at 25 C, compartment two was inoculated in the center with 189HT3. Each day following the inoculation of 189HT3, representative plates were selected and the media in compartment one containing 189a was removed aseptically and discarded. Contact between 189a and 189HT3 occurred only in compartment one. Thus, in order for transmission to occur the disease agent must pass into the 189HT3 mycelium and via this route into the mycelium occupying compartment two. Following incubation for 7 days, five 4-mm-diameter mycelial plugs of the 189HT3, taken at random, were removed from each plate and placed on PDA slants. Transmission was determined by disease symptoms (i.e., slow growth, cottony appearance, and lack of sclerotia). Controls for this experiment were plates inoculated with 189a or 189HT3 only, in the appropriate compartments. Growth of 189a across compartment one was monitored in the 189a control plates and samples from 189HT3 control plates were made to check for spontaneous disease development. All control plates were treated the same as test plates.

Representative plates for each day following the inoculation of 189HT3 in compartment two are shown in Fig. 2. Two separate trials were made with a total of 10

Fig. 2-(A to F). Transmission of the decline agent of Rhizoctonia by hyphal fusion. Compartmented petri plates show the mycelial advancement of diseased 189a (compartment one on the left) and healthy 189HT3 (compartment two on the right). A) 2 B) 3 C) 4 D) 5, and E) 6 days after the inoculation of 189HT3 in compartment two. Plate (F) shows advancement of 189a after 7 days. Samples of 189HT3 from compartment two became diseased only after 189HT3 contacted 189a (C-E).
test plates that were sampled each day for 7 days.

No transmission occurred during the first 2 days. This was not surprising, since hyphal contact had not occurred in any of the test plates (Fig. 2-A). On the 3rd day, 189HT3 had grown across the barrier and into compartment one, but only one of the 10 plates showed transmission. However, in most cases the two cultures had not made contact (Fig. 2-B). In the one plate that showed transmission, hyphal fusion was observed, explaining the results. On the 4th day there was hyphal contact between 189a and 189HT3 (Fig. 2-C) and transmission had occurred in three of the 10 plates tested. By the 5th day, the number and duration of hyphal contacts had increased (Fig. 2-D) and all transmission tests for day five were positive. Likewise, test plates for days 6 and 7 showed 90 and 100% transmission, respectively.

Several points should be made concerning these results: (i) the 189HT3 in control plates showed no signs of disease and all transfers of 189HT3 from plates not containing 189a were healthy; (ii) the control 189a plates which were not inoculated with 189HT3 showed that the 189a mycelium never advanced beyond half-way across the compartment into which it was inoculated even after the 7th day (Fig. 2-F). Therefore, 189a never entered compartment two where 189HT3 had been inoculated; (iii) transmission appears to be a plus or minus reaction based on our observations that samples from a single test plate were either all diseased or all healthy. In no case were samples of both healthy and diseased-type cultures obtained from a single test plate; (iv) in plates which were positive for transmission (i.e., in which all samples were diseased) the converted 189HT3 showed no apparent symptoms of disease. Thus, transmission of the disease agent from the 189a to the 189HT3 can occur followed by the movement of the agent throughout the mycelium of the 189HT3 without producing any signs of disease. However, all transfers from such cultures were diseased. This situation illustrates how a healthy-appearing culture in fact can be completely infected.

Attempts to transmit disease from 189a to other anastomosis group 1 isolates of R. solani were made using tricompartmented petri plates by the methods described previously; the only difference being the replacement of 189HT3 with isolates 43, 65, 239, and 65 (1). In no case was there any indication of transmission to any of the isolates.

**DISCUSSION**

*Rhizoctonia solani* usually is very stable; sectors are rarely observed and isolates kept in culture for as long as 30 years on PDA retain a high level of virulence. In fact, highly virulent 189 was isolated in 1962. Nevertheless, pathologists who have worked with *R. solani* have observed cultures in a state of decline. Records kept by J. R. Parmeter, Jr., and now in our possession, indicate that he observed sick cultures as early as 1965. Because decline cultures could not be cured by antibiotics or other means they were discarded or failed to grow upon transfer.

Our results show hyphal-tipping as a means of curing diseased cultures and the value of hyphal-tip isolation before and during research with *R. solani*, especially with regard to maintenance of virulence (2). Our studies began in 1973 and since that time we have accumulated over 30 diseased isolates from all anastomosis groups including group 5 proposed by Ogoshi (21).

We have shown that transmission of the disease agent by hyphal anastomosis from diseased to healthy cultures of the same isolate (i.e., 189a to 189HT3) is extremely efficient resulting in the total conversion of the healthy culture into the diseased-type. Although to date we have no evidence for mechanical, insect, or mite transmission (Castanho, unpublished), it seems likely that such means of transmission for Rhizoctonia decline is unnecessary. Adequate spread of the disease agent could readily be attained because of the frequency of anastomosis between diseased and healthy mycelium of the same isolate. Thus, any hyphal fusion, between isolates of *R. solani*, may result in transmission of the agent causing Rhizoctonia decline. The negative results for transmission from 189a to other anastomosis group 1 isolates indicates that the disease agent of Rhizoctonia decline is isolate-specific.

The nature of the agent responsible for Rhizoctonia decline is unresolved. However, three lines of evidence show that a nuclear factor is not involved: (i) cells of *R. solani* are multinucleate and these nuclei divide conjugatively; the result being that all daughter nuclei move forward into the hyphal tips (8). Thus, if disease were due to a nuclear factor, one would not expect to escape disease by hyphal-tip isolations; (ii) basidiospore analysis shows that disease is not inherited in a Mendelian fashion. If a dominant, suppressive nucleus was the cause of disease, it would be in relatively high frequency and expressed immediately in basidiospore cultures, but that was not the case; (iii) there is a strong correlation between disease of 189a and three segments of double-stranded ribonucleic acid (ds-RNA) (3). Based on this information, we feel Rhizoctonia decline is cytoplasmic and perhaps mycoviral in nature (3).

**LITERATURE CITED**


