Plant-Based Culture Media for Improved Growth and Sporulation of *Cercospora janseana*

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Abstract

Cercospora janseana is the causal agent of narrow brown leaf spot, one of the major diseases of rice in the United States. In vitro studies were conducted to evaluate various plant-based agar media in comparison with potato dextrose agar (PDA) for their effects on radial growth and sporulation of *C. janseana*. The agar media were evaluated individually with the following plant-extract ingredients: fresh rice leaves, dried rice leaves, dried rice stems, dried rice roots, dried rice hulls, dried rice straw, rice bran, dried sorghum leaves, and dried barnyard grass leaves. Plant-based extracts obtained from 25, 50, 100, or 200 g/liter (plant material/water) were tested either alone or in combination with 10 and 20% clarified V8 juice. PDA served as the control medium. Media with 10 and 20% V8 juice were also included for comparison. Media were inoculated

with a disc of 1-week-old actively growing *C. janseana* at the center of Petri plates and incubated at 28°C with a 12/12-h (light/dark) cycle for 2 weeks. Radial growth was measured at 7 and 14 days after inoculation (DAI), whereas sporulation was measured at 14 DAI. Radial growth and sporulation differed among various media and their amendment concentrations. Media with extracts of fresh rice leaves, dried rice leaves, dried rice stems, dried barnyard grass leaves, and clarified V8 juice enhanced radial growth compared with the PDA control. Of all the agar media evaluated, fresh rice leaf extract amendment at 25 g/liter with no V8 supplementation consistently supported the greatest radial growth, and 10% V8-only medium resulted in greater sporulation of *C. janseana* than other media evaluated.

Cercospora janseana (Racib.) O. Const. (syn. *Cercospora oryzae* Miyake; teleomorph *Sphaerulina oryzina* K. Hara) is the fungal pathogen that causes narrow brown leaf spot (NBLS), an economically significant disease in rice. The association of NBLS pathogen with rice plants was independently discovered by Raciborski from Java in 1900 (Raciborski 1900) and Metcalf from North America in 1906 (Metcalf 1906). The pathogen was originally named *Napicladium janseanum* by Raciborski (Ou 1985). However, Miyake was the first to describe this disease and named the fungus *C. oryzae* Miyake (Miyake 1910). In 1982, the fungus was renamed as *C. janseana* (Racib.) O. Const. by Constantinescu (Constantinescu 1982). NBLS was first reported in the United States in 1937 (Ou 1985). At present, NBLS occurs in almost all major rice-growing regions of the world, except Europe (Ganguly 1946; Groth and Lee 2003; Overwater 1960; Ryker and Jordon 1940; Tuli et al. 2017).

NBLS historically was considered a minor disease owing to its sporadic occurrence in the United States. However, in recent years, the occurrence and severity of NBLS have increased in Louisiana, Texas, and Arkansas (Groth 2007, 2008; Yingling et al. 2007; Zhou and Jo 2014). This disease became more severe especially when warm springs and wet summers were coupled with excessive overwintered inoculum and large acreage of susceptible cultivars (Groth 2007, 2008; Yingling et al. 2007; Zhou and Jo 2014). NBLS symptoms manifest on the leaves, sheaths, internodes, and panicles of the rice plant. On the leaves and sheaths, the symptoms include narrow, brown, elongated, linear, reddish-brown lesions ranging from 2 to 12 mm in length and 1 to 2 mm in width running parallel to the leaf veins (Groth and Lee 2003; Zhou and Jo 2014). As the disease progresses, net blotch or brown blotch symptoms occur on leaf sheaths owing to the browning of veins on tan to yellow discolored leaf sheaths. Light brown to tan discoloration of the internodal areas at the base of the panicle is another symptom of NBLS. Under

conductive conditions, NBLS causes grain discoloration, premature ripening, chalkiness, reduced milling recovery, and lodging of plants, especially on extremely susceptible cultivars (Ryker and Jordon 1940; Zhou and Jo 2014). The control of NBLS has been primarily through race-resistant cultivars. However, *C. janseana* is highly variable in pathogenicity, and cultivar resistance often breaks down owing to the genetic adaptation of the fungus and the development of new physiological races (Chilton and Tullis 1946; Estrada et al. 1981; Groth and Lee 2003; Ryker and Jordon 1940; Sah and Rush 1988). Therefore, fungicides remain as an important option for control of this disease (Mani et al. 2016; Uppala and Zhou 2018).

Culturing and conidial production of *C. janseana* inoculum for laboratory and field studies is crucial to better understand the biology and epidemiology of NBLS, screen rice germplasm for resistance, and develop effective disease management strategies. *C. janseana* grows slowly and does not sporulate well on potato dextrose agar (PDA) (Kaur and Hollier 2013; Mew and Gonzales 2002). The development of plant-based culture media to improve growth and sporulation of various species of *Cercospora* has been carried out. These include the extracts of peanut leaves (Abdou and Cooper 1974) and hulls (Starkey 1980) for sporulation of *C. arachidicola*; soybean and crape myrtle leaf extracts for sporulation of *C. kikuchii* (El-Gholl et al. 1982); green corn leaves, senescent corn leaves, and V8 juice for sporulation, spore germination, and germ tube growth of *C. zeae-maydis* (Beckman and Payne 1983; Brunelli et al. 2006); and V8 juice for radial growth and spore germination of *C. beticola* (Secor and Rivera 2012). Plant-based media can also be cost-effective compared with commercially available dehydrated culture media (Basu et al. 2005; Jha and Dikshit 2017; Osman et al. 2013). In the current study, we evaluated various plant-based agar media in comparison with PDA for their effects on radial growth and sporulation of *C. janseana*.

Materials and Methods

Fungal strain. Isolate BMT-41 of *C. janseana* was used in this study. This isolate was recovered from a rice leaf showing symptoms of NBLS in Beaumont, Texas, in 2011. The culture was maintained on Difco PDA (Beckton, Dickinson and Company, Sparks, MD).

Effects of plant-based media and amendment concentrations on radial growth of *C. janseana* (experiment 1). In this study, plant extracts obtained from the following plant materials were evaluated

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individually in an agar medium: fresh rice leaves, dried rice leaves, dried rice stems, dried rice roots, dried rice hulls, dried rice straw, dried sorghum leaves, and dried barnyard grass (*Echinochloa crus-galli*) leaves. Four concentrations (25, 50, 100, and 200 g/liter) of these plant materials were evaluated in the form of plant extracts. Rice (cultivar Presidio), barnyard grass, and sorghum (hybrid DKS 3707) at reproductive growth stages were collected from fields located at Texas A&M AgriLife Research Center, Beaumont, Texas. Plant materials were thoroughly washed under running tap water and were partitioned into roots, stems, and leaves. For media with dried plant extracts, plant materials were dried at 70°C for 48 h and ground into powders. Individual plant extracts were obtained by processing the dried ground plant tissue powders or fresh rice leaves in a kitchen blender (Hamilton Beach Brands, Glen Allen, VA). For this, plant material (25, 50, 100, or 200 g) was added to 750 ml of deionized water, processed in a kitchen blender for 2 min, and filtered through two layers of cheesecloth into a measuring cylinder. Plant material in the cheesecloth was moved to the blender, and 250 ml of water was added; it was ground for 30 s and then filtered through two layers of cheesecloth into the same measuring cylinder that contained filtrate. At both these stages of grinding and filtering, the volume of the filtrates was less than the initial amount of water added, owing to the absorption by plant material. After the second grinding and filtering, water was poured through the plant material in the cheesecloth until the final volume of the filtrate was 1,000 ml. These filtrates were used as plant extracts or liquid source for agar media. Respective media were prepared by combining plant extracts with agar at 15 g/liter.

V8 medium from commercially available V8 juice (Campbell Soup Company, Camden, NJ), containing blends of tomatoes, carrots, celery, beets, parsley, lettuce, watercress, spinach, salt, ascorbic acid, and citric acid as major ingredients, was also evaluated in this experiment. V8 medium was prepared using the method described by Ferguson and Jeffers (1999). Calcium carbonate at the rate of 1% was added to commercially available V8 juice and centrifuged at 6,000 rpm for 10 min. The supernatant was collected as clarified V8 juice, and the pellet was discarded. Twenty percent V8 medium was prepared by adding 200 ml of clarified V8 juice to 800 ml of deionized water and 15 g of agar. PDA medium was included as a control. The pH of each medium was measured by a Mettler Toledo LE-438 pH meter (Mettler Toledo, Columbus, OH), and all media were autoclaved for 20 min at 121°C. Autoclaved media were cooled to 50°C in a water bath, and filter sterilized 1 ppm of thiamine (VWR International, Radnor, PA) and 2 ppm of streptomycin sulfate (Thermo Fisher Scientific, Waltham, MA) were added to the media and dispensed into sterile 60 × 15-mm Petri plates at 15 ml per plate.

Each medium was inoculated in the center with a 0.6-cm agar disc, topside down, from a 1-week-old PDA culture of *C. janseana*. Cultures were incubated at 28°C under diurnal fluorescent light (12/12-h light/darkness cycle) for 2 weeks. Each medium treatment was replicated three times. The treatments were arranged according to a split-plot design, with plant extracts evaluated as the main-plot factor and amendment concentration as the subplot factor. Radial growth was measured at 7 and 14 days after inoculation (DAI). For radial growth measurements, two perpendicular straight lines (diameters) were drawn on the bottom of each Petri dish containing fungal growth. The crossing point coincided with the center of the initial 0.6-cm fungal disc. Colony diameters were measured on the perpendicular lines, and mean colony diameter was obtained by averaging both transects. Net radial growth was obtained by subtracting 0.6 cm from the mean colony diameter. This experiment was conducted three times.

Effects of plant-based media in combination with V8 juice on radial growth and sporulation of *C. janseana* (experiment 2). This study was conducted to evaluate the effects of plant extracts in combination with clarified V8 juice on the growth and sporulation of *C. janseana*. Individual agar media were prepared using plant extracts (each at 25 g/liter) of fresh rice leaves, rice bran (stabilized rice bran from Bob's Red Mill, Milwaukie, OR), dried rice leaves, dried rice stems, and dried barnyard grass leaves, either alone or in combination

with 10 or 20% clarified V8 supplementation. PDA and 10 and 20% V8 media without any plant extracts were included as the controls. All the media were prepared and inoculated with 0.6-cm discs of actively growing *C. janseana* as described in experiment 1. Inoculated plates were incubated at 28°C under diurnal fluorescent light (12/12-h light/darkness cycle) for 2 weeks. Each medium treatment was replicated three times. The treatments were arranged according to a split-plot design, with plant extracts evaluated as the main-plot factor and V8 supplementation level as the subplot factor. Radial growth measurements were taken at 7 and 14 DAI as described above. Conidial counts were obtained at 2 weeks after inoculation using a whole mycelium harvest technique. For each treatment, the whole culture of *C. janseana* (total growth of the fungus along with the medium) from each Petri plate was cut into small pieces using a heat-sterilized stainless-steel scalpel and placed into a sterile 15-ml test tube (Cole-Palmer, Vernon Hills, IL) containing 10 ml of water. The tubes were vortexed for 60 s and filtered through double layers of cheesecloth for retention of media and mycelial fragments. The number of conidia per milliliter of the filtrate was enumerated by using a hemocytometer under a compound microscope. The data were extrapolated to obtain the number of conidia per Petri plate. Two conidial counts were obtained from each sample. This experiment was conducted twice.

Data analysis. Data on the net radial growth and sporulation (conidia per Petri plate) of *C. janseana* were analyzed by generalized mixed linear model analysis (Proc Mixed; SAS 9.4, SAS Institute, Cary, NC) for differences among the media, amendment concentrations of plant extracts, V8 juice supplementation levels, and assessment intervals. Mean separations were performed using Fisher's protected least significant difference test at $P = 0.05$. Transformations were performed when data did not fit a normal distribution. After statistical analysis, means were back-transformed to original units.

Results

Effects of plant-based media and amendment concentrations on radial growth of *C. janseana* (experiment 1). Net radial growth data were logarithmically transformed to meet the criterion for normal distribution. At 7 and 14 DAI, significant differences in radial growth were observed among the media ($P < 0.0001$) but not among the plant extract amendment concentrations ($P = 0.61$) or medium by plant extract amendment concentration interactions ($P = 0.81$). Therefore, the data at each interval were pooled over the amendment concentrations of plant extracts (Fig. 1). At both the interval assessments, the media amended with the extracts of fresh rice leaves, dried rice leaves, dried rice stems, dried barnyard grass leaves, and 20% V8 supported significantly greater radial growth of *C. janseana* (average of 8.2 and 15.1 mm at 7 and 14 DAI, respectively) than the PDA control (average of 5.6 and 8.8 mm at 7 and 14 DAI, respectively).

Fig. 1. Radial growth of *Cercospora janseana* at 7 and 14 days after inoculation on eight plant-based agar media amended with plant extracts (experiment 1). Data were pooled over all amendment concentrations (25, 50, 100, and 200 g/liter) of the plant extracts because there were no significant differences in the radial growth among the four concentrations ($P = 0.61$) tested, or their interaction with plant-based media ($P = 0.81$) ($n = 9$). At each of the same assessment dates, bars with different letters were significantly different ($P < 0.05$).

Effects of plant-based media in combination with V8 juice on radial growth and sporulation of *C. janseana* (experiment 2). Net radial growth data were square-root transformed to normalize the data distribution. At 7 DAI, radial growth differed among the media ($P = 0.0025$), but not owing to V8 supplementation levels ($P = 0.77$) or medium by V8 supplementation interactions ($P = 0.45$). Thus, data from all V8 supplementation levels within a medium treatment were combined (Fig. 2A). All the media with plant extracts and the 10 and

20% V8-only media resulted in greater net radial growth (average of 9.1 mm) than the PDA control (5.8 mm). At 14 DAI, radial growth differed among the media ($P < 0.0001$) and medium by V8 supplementation interactions ($P = 0.0383$), but not owing to V8 supplementation alone ($P = 0.06$). All the plant-based media at all V8 supplementation levels and V8-only media had a greater radial growth (ranged from 13.6 to 21.8 mm) compared with the PDA control (9.8 mm); the greatest radial growth occurred in the medium with fresh rice leaf extract with no V8. Fresh rice leaf extract supplemented with V8 had less radial growth. In other media, V8 supplementation effects were not consistently negative or positive (Fig. 2B).

Sporulation data were natural log transformed to stabilize the variance. Sporulation differed among the media ($P = 0.03$), but not owing to V8 supplementation levels ($P = 0.48$) or medium by V8 supplementation interactions ($P = 0.97$). Thus, data from all the V8 supplementation levels within a medium treatment were combined. The 10% V8 medium resulted in greater sporulation than all other media with 1×10^8 spores per plate (479% more conidia than the PDA control). The 20% V8 medium had 8.3×10^7 spores per plate (371% more conidia than the PDA control) (Fig. 3). Other plant-based media resulted in conidial yields in the range of 2.4×10^7 to 4.3×10^7 spores per plate, whereas the PDA control had a conidial yield of 1.8×10^7 spores per plate. The growth of *C. janseana* in various plant-based media with and without V8 supplementation is presented in Figure 4.

Fig. 2. Effects of five plant-based agar media amended with plant extracts (at 25 g/liter) in combination with 0, 10, and 20% of clarified V8 juice on the radial growth of *Cercospora janseana* at 7 (A) and 14 (B) days after inoculation (DAI) (experiment 2). At 7 DAI, data within a medium treatment were pooled over all the three V8 supplementation levels (0, 10, and 20% of V8), because there were no significant differences in the radial growth among these three levels ($P = 0.77$) and the interactions of plant-based medium by V8 supplementation ($P = 0.45$) ($n = 6$). At each of the same assessment dates, bars with different letters were significantly different ($P < 0.05$).

Fig. 3. Effects of five plant-based agar media amended with plant extracts at 25 g/liter on sporulation (conidia per plate) of *Cercospora janseana* at 14 days after inoculation (experiment 2). Data within a medium treatment were pooled over all the three V8 supplementation levels (0, 10, and 20%), because there were no significant differences in the sporulation among these three levels ($P = 0.48$) and the interactions of plant-based medium by V8 supplementation ($P = 0.97$) ($n = 6$). Bars with different letters were significantly different ($P < 0.05$).

Fig. 4. Colony morphology of *Cercospora janseana* on agar media amended with five plant extracts at 25 g/liter in combination with 0, 10, or 20% of clarified V8 juice supplementation at 14 days after inoculation (experiment 2).

Discussion

The fungus *C. janseana* grows slowly and produces sparse conidia on PDA, commonly used in laboratories. An improved cultural medium that can stimulate growth and sporulation of the fungus could facilitate research on the biology of *C. janseana*, screening rice germplasm for disease resistance, and evaluating various disease management strategies. Several investigators reported improved growth, sporulation, and cultivability of various fungal pathogens and of beneficial endophytic and rhizospheric microorganisms in plant-based culture media (Beckman and Payne 1983; Brunelli et al. 2006; Murphy et al. 2015; Nour et al. 2012; Yadav et al. 2014; Youssef et al. 2016). The current study was initiated with an objective to develop an effective plant-based culture medium for *C. janseana*. For this, we evaluated the extracts obtained from different tissues of the host plant (rice), leaves of nonhost plants such as barnyard grass and sorghum, and commercially available V8 juice at various concentrations in agar medium for their effects on the radial growth and sporulation of *C. janseana*.

Plant-based media with extracts of fresh rice leaves, dried rice leaves, dried rice stems, dried barnyard grass leaves, and V8 juice consistently resulted in greater radial growth compared with PDA throughout the current study. At 7 DAI, media with these four plant extracts and V8 juice resulted in statistically similar radial growth in both experiment 1 and 2. However, at 14 DAI, in both the experiments, the medium with the extract of fresh rice leaves alone was consistent in supporting greater radial growth than all other media evaluated. Differences in the radial growth between the medium with the extract of fresh rice leaves and media with dried plant products (including dried rice leaves) could be explained by the possible alterations in nutritional profiles owing to drying (Alakali et al. 2015; Clement et al. 2017; Sharma et al. 2004). The reasons for the plant-based media with the extracts of dried rice hulls, dried rice roots, dried rice straw, and dried sorghum leaves being less favorable for the radial growth are unknown, but differences in nutritional composition profiles of these media may be a factor contributing to the different responses (Gao et al. 2007; Griffin 1994; Hawker 1957; Walker and White 2011). Carbon, nitrogen, and phosphorus accumulation is reported to be different among various plant parts, with leaves accumulating more than stems or roots (Tang et al. 2017; Ye et al. 2014). Concentrations of carbon, nitrogen, carbon/nitrogen ratio, vitamins, and minerals in culture media have been known to affect the growth and sporulation of other fungi (Gao et al. 2007; Griffin 1994; Hawker 1957; Walker and White 2011). In experiment 1, when the extracts from 25 to 200 g/liter were evaluated, no significant increase in radial growth was observed with an increase in the concentration of plant material beyond 25 g/liter. Thus, 25 g/liter can be concluded as high enough concentration for enhancing radial growth to the levels reported in the current study.

At 2 weeks after inoculation, net radial growth in media with plant extracts was in the range of 10 to 22 mm. In PDA and V8 media, average net radial growth was 10 and 15 mm, respectively. At 17 DAI, when incubated at 28°C under a 12/12-h (fluorescent light/dark) cycle, Mew and Gonzales (2002) reported *C. janseana* diameters of 14 and 23 mm on PDA and V8 agar media, respectively. However, direct comparisons cannot be made with our study because they did not provide details on the size of the initial inoculum discs or concentrations of V8 juice in the culture medium.

Greater sporulation was observed in 10% V8 medium (with average conidial yields of 1×10^8 per Petri plate) than in all other media. The current study is the first to report conidial yields of *C. janseana* in culture media. Beckman and Payne (1983) reported 2.4×10^5 conidia per plate on 30% V8 medium at 14 DAI of *C. zeae-maydis*. Brunelli et al. (2006) reported 2.2×10^6 conidia per plate (or 22.4×10^4 conidia/ml of harvested spore suspension) of *C. zeae-maydis* on 20% V8 medium at 9 DAI. The current study differs from Beckman and Payne (1983) and Brunelli et al. (2006) in that they used higher concentrations of V8 compared with our study; they harvested spores by adding 10 ml of sterile water and scraping the surface of the agar medium with a sterile glass slide, whereas we employed the whole

mycelia harvest technique. Unlike the current study, for which prior clarification of V8 juice was performed with 1% CaCO_3 before preparation of media, Brunelli et al. (2006) and Beckman and Payne (1983) incorporated 0.32 and 0.4% CaCO_3 directly to 20 and 30% V8 media, respectively. While we evaluated 10 and 20% V8 juice individually and in combination with plant extracts, Beckman and Payne (1983) and Brunelli et al. (2006) tested a single concentration of V8 juice in agar medium.

When the plant extracts were evaluated in combination with 10 and 20% V8, none of the plant extract and V8 amendment combinations had any additional stimulating effect on the radial growth of *C. janseana* than the media without V8 amendment. In media with the extracts of fresh rice leaves, V8 juice supplementation resulted in significant reduction in radial growth. In other plant-based media, the effects of V8 juice supplementation were not consistently negative or positive. With reference to sporulation, agar media with individual plant extracts either alone or in combination with V8 did not result in enhanced sporulation compared with the media with V8 juice alone or the PDA control. This indicates that the addition of plant extracts to V8 had a negative effect on sporulation. The current study is the first to evaluate V8 juice in combination with plant extracts for radial growth and sporulation of fungi.

In the current study, *C. janseana* was incubated under 12/12-h (light/dark) cycle, because this was reported to induce more sporulation of other *Cercospora* species compared with constant light (Beckman and Payne 1983; Brunelli et al. 2006; Gómez and Reis 2013; Latterell and Rossi 1983). Incubation temperature and medium pH affect the growth and sporulation of *Cercospora* species (Beckman and Payne 1983; Brunelli et al. 2006; Cooperman and Jenkins 1986; Djebali et al. 2010; Gómez and Reis 2013; Silva et al. 2016; Tasugi and Ikeno 1956; Verma and Agnihotri 1972). The optimum temperature and pH for the growth of *C. janseana* were reported to be 25 to 28°C and 5.7 to 7.1, respectively (Tasugi and Ikeno 1956). In the current study, *C. janseana* was incubated at 28°C, and the pH values of various agar media (data not presented) ranged from 5.7 to 6.8, which is within the optimum range. Further studies on the effects of culture medium pH, light regimes, the time required for sporulation initiation in vitro, and nutrient composition of effective plant-based media might provide a better understanding of the optimum cultural conditions for *C. janseana*. One of the limitations of the current study is that only one isolate of *C. janseana* was evaluated for radial growth and sporulation. The inclusion of multiple isolates could have addressed variation among the isolates, if any, in any specific culture medium.

The results of this research demonstrate that medium with fresh rice leaf extract amendment at 25 g/liter is the best medium to improve the radial growth of *C. janseana*, and 10% V8 medium is the best medium to improve the sporulation of the fungus.

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