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Conidial germination and germ tube elongation of *Phomopsis amaranthicola* and *Microsphaeropsis amaranthi* on leaf surfaces of seven *Amaranthus* species: Implications for biological control

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Abstract

Microsphaeropsis amaranthi and *Phomopsis amaranthicola* are potential biological control agents for several *Amaranthus* species. In an effort to understand the initial infection processes with these pathogens, a study was conducted of the conidial germination and germ tube length (μm) on the weed leaf surfaces at 21 °C and 28 °C. Weeds included *Amaranthus rudis*, *A. palmeri*, *A. powellii*, *A. retroflexus*, *A. spinosus*, *A. hybridus*, and *A. albus*. For *P. amaranthicola*, conidial germination and germ tube length varied among the seven weed species at both temperatures, while for *M. amaranthi* the differences in germ tube lengths were significant among weed species only at 21 °C. While the conidia of *M. amaranthi* and *P. amaranthicola* germinated on the leaf surfaces of all seven weed species, temperature appeared to impact the number and length of germ tubes on the leaf surfaces. The percentage of germinated conidia and the length of germ tubes at both temperatures were often greater for *M. amaranthi* than for *P. amaranthicola.* In order for the fungal pathogen to successfully infect and kill a weedy host, conidia must germinate and form a germ tube, two processes that vary with host species and temperature for *M. amaranthi* and *P. amaranthicola.* The extent to which successive infection processes, e.g., penetration, invasion and colonization, contribute to host specificity warrants study.

Keywords: *Microsphaeropsis amaranthi*; *Phomopsis amaranthicola*; *Amaranthus rudis*; *A. palmeri*; *A. powellii*; *A. retroflexus*; *A. spinosus*; *A. hybridus*; *A. albus*; Bioherbicide; Mycoherbicide; Biological control; Spore germination

1. Introduction

Several species in the genus *Amaranthus* are weeds in cropping systems throughout the United States and the world (Holm et al., 1997; Wax, 1995) and some biotypes have developed resistance to a number of herbicide families (Heap, 2005). Recently, a population of *A. palmeri* in Georgia has developed resistance to glyphosate (Haire and Culpepper, 2005), and a potentially resistant biotype of *A. rudis* has been found in Missouri (Dailey and Bradley, 2005). Research on alternative weed management tactics for *Amaranthus* species (Mintz et al., 1992; Ortiz-Ribbing and Williams, 2006; Rosskopf et al., 2000b; Smith, 2003) has shown that two indigenous fungal pathogens, *Phomopsis amaranthicola* Rosskopf, Charudattan, Shabana & Benny (Rosskopf, 1997; Rosskopf et al., 2000a,b) and *Microsphaeropsis amaranthi* (Ell. & Barth.) Heiny & Mintz (Heiny et al., 1992), as well as a mixture of these two organisms (Ortiz-Ribbing and Williams, 2006) could provide effective control of several *Amaranthus* species in the field and under controlled environmental conditions.

While these pathogens have potential for use as biological control agents for managing weedy *Amaranthus* species, efficacy and disease-response trials have indicated that differences in susceptibility exists among *Amaranthus* species (Rosskopf et al., 2000b; Mintz et al., 1992; Ortiz-Ribbing and Williams, 2006). The varying levels of symptom expression and plant mortality remain unex-

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plained. Rosskopf et al. (2000b) found that mortality of four *Amaranthus* species in the field resulting from applications of *P. amaranthicola* varied from species to species and from year to year, with between-year differences influenced by environmental conditions, and within-year differences between species more likely due to host specificity of the pathogen. Ortiz-Ribbing and Williams (2006) also found a range of host responses in disease expression, seedling mortality, plant biomass, and height reduction among eight *Amaranthus* species under controlled and field conditions from applications of conidial suspensions of *P. amaranthicola* and *M. amaranthi*.

Disease expression in response to temperature during post-inoculation dew period has been examined for *P. amaranthicola* and *M. amaranthi* individually on several *Amaranthus* species. Smith (2003) reported the highest levels of disease severity on *A. rudis* seedlings occurred after an application of *M. amaranthi* followed by a 18-h dew period at 18–23 °C. Dew period temperatures ranging from 20 to 28 °C were conducive for 100% mortality of *A. albus* seedlings caused by *M amaranthi*, but at 32 °C mortality dropped to zero (Mintz et al., 1992). Temperature during the dew period significantly impacted the mortality of *A. hybridus* resulting from applications of *P. amaranthicola* (Rosskopf, 1997). A temperature range of 25–35 °C was conducive, but a dew period temperature of 20 °C resulted in significantly lower levels of *A. hybridus* mortality. A 24-h dew period with temperatures of 20 °C or 30 °C also favored disease severity and mortality of field bindweed (*Convolvulus arvensis* L.) by the bioherbicide agent *Phomopsis convolvulus* Ormeno, (Ormeno-Nuñez et al., 1988).

Germination of *P. amaranthicola* conidia and infection of *A. viridus* and *A. spinosus* leaf surfaces appeared highly host-specific and required between 15 and 24 h to complete (Wyss and Charudattan, 2000). Conidia of *P. amaranthicola* attached, germinated, and invaded the leaf tissue of *A. viridus*, but not leaf tissue of *A. spinosus* or the two nonliving surfaces tested. Chandramohan and Charudattan (2003) illustrated that *P. amaranthicola* spores germinated on *A. hybridus* but not on sicklepod (*Senna obtusifolia* [L.] Irwin & Barneby) or showy crotalaria (*Crotalaria spectabilis* Roth.). Smith (2003) measured *M. amaranthi* conidial germination in potato dextrose broth and colony diameter on V8 agar in response to temperature, and found the greatest conidia germination at 20 °C, with reduced germination occurring at temperatures below 15 °C or above 25 °C. He concluded that the optimum temperature for *M. amaranthi* colony growth (near 30 °C) differs from that for abundant germination (near 25 °C). However, germination of *M. amaranthi* conidia was not evaluated on *Amaranthus* leaf surfaces. The infection processes of *M. amaranthi* and *P. amaranthicola* on leaf surfaces may account for differential response among *Amaranthus* species.

A greater understanding of infection processes of *M. amaranthi* and *P. amaranthicola* would help define the role of these organisms as bioherbicides for weedy *Amaranthus* species. The objective of this research was to assess initial infection processes by estimating conidia germination and subsequent germ tube elongation on the leaf surfaces of seven different *Amaranthus* species under two temperatures. Successful infection requires several steps from infection to death of the host plant. Because some steps must be completed for subsequent processes to proceed, our research has focused on conidial germination of *M. amaranthi* and *P. amaranthicola*.

2. Materials and methods

2.1. Experiment background

Cultures of *M. amaranthi* and *P. amaranthicola* were obtained from Raghavan Charudattan, University of Florida, Gainesville, Florida. *P. amaranthicola* was originally isolated from stems and leaves of a blighted *Amaranthus* species collected in Florida (Rosskopf et al., 2000a); *M. amaranthi* was isolated from an *Amaranthus* species in Fayetteville, Arkansas (Heiny et al., 1992). Cultures were received and maintained for long-term storage in sterilized soil in sterile glass tubes in the dark at 4 °C. The soil was SB300 Universal Mix 60% by weight mixed with 40% fine silica sand.

Seven *Amaranthus* species were evaluated in this study. These included: *A. rudis* Sauer., (common waterhemp); *A. palmeri* S. Wats., (Palmer amaranth); *A. powellii* S. Wats., (Powell amaranth); *A. retroflexus* L., (redroot pigweed); *A. spinosus* L., (spiny amaranth); *A. hybridus* L., (smooth pigweed); and *A. albus* L., (tumble pigweed). The *Amaranthus* seed used in this study was previously collected in Champaign and adjacent counties of Illinois. The seed was stored in glass jars under low humidity at 4 °C.

2.2. Inoculum preparation

Phomopsis amaranthicola and *M. amaranthi* were grown on V8 juice agar using the methods and starting concentrations of Ortiz-Ribbing and Williams (2006). Both organisms were incubated at 25 ± 2 °C with a 12-h light period for 14 to 21 days. Conidia were rinsed from individual plates using a modified method of Mintz et al. (1992) by using 15 ml sterile, distilled water and straining through 2 layers of cheese cloth. Conidial suspensions were prepared in 30 ml sterile, distilled water (SDW) and contained 3.0×10^6 conidia per ml for *P. amaranthicola* and 1.0×10^6 conidia per ml for *M. amaranthi*. Fungal spore suspensions were amended with 0.5% psyllium mucilloid (Metamucil®, Procter and Gamble, Cincinnati, OH). A control treatment with the mucilloid was prepared using 30 ml sterile, distilled water.

2.3. Seedling inoculation experiment

Seeds of each *Amaranthus* species were sown in a greenhouse into a steam-pasteurized, Torpedo sand:soil:peat (1:1:1) greenhouse mixture. The soil for the mix belonged to the Drummer/Flanagan soil series, and

the peat was Canadian sphagnum peat moss. The pH values for the sand and soil were approximately 6.1 and 5.8, respectively. When weeds reached the 2 to 4 true-leaf stage they were thinned to two seedlings per pot, and pots were arranged in a completely randomized design with 4 replications. Seedlings in the 4-leaf stage had four fully expanded true leaves with the fifth leaf beginning to expand and the sixth leaf starting to open (14 days after planting for some species).

The day following thinning, conidial suspensions were prepared as described above. Two drops of inoculum (0.04 ml) containing approximately 40,000 *M. amaranthi* conidia or 120,000 *P. amaranthicola* conidia were placed with the aid of a sterile 1-ml syringe on top of each of two fully extended leaves of two seedlings per pot. Following inoculation, seedlings were incubated for 24 h in a dark dew chamber at 21 ± 0.5 °C or 28 ± 0.5 °C ambient temperature. Following the incubation period, leaves were detached, cleared, and stained as described below. Due to space, equipment, and labor constraints, the experiments at each temperature were conducted separately. Individual experiments at each temperature were repeated.

2.4. Leaf clearing and staining techniques

After 24 h of incubation in the dew chamber, the upper most treated leaves from two plants per pot were detached and decolorized in a chromatography tank using a method similar to that of Ryan and Clare (1974). One end of a sheet of chromatography paper was placed in the bottom of a chromatography tank and draped over glass dividers that were half the height of the tank. The other end of the paper remained suspended at least 12–15 cm above the decolorizing solution (equal parts v/v of glacial acetic acid and absolute ethanol) which was in the bottom of the tank. This created a horizontal surface for placement of inoculated leaves with the inoculated leaf surface facing upwards. Depending on the size of the tank, 200–300 ml of the decolorizing solution was used. The solvent was allowed to flow up and across the sheet of chromatography paper, decolorizing the leaves for 48 h. Leaves were removed from the tank and transferred to SDW-saturated filter paper for 30 min in order to leach out the acetic acid-ethanol prior to coating the leaves with agarose (Ryan and Clare, 1974). Molten agarose (0.5%) was sprayed as a fine mist onto the leaves that had been placed on microscope slides and placed on ice.

The agarose coated leaves were stained using the periodic acid-Schiff method (Preece, 1959), modified by using 0.5% periodic acid and Lillie's Cold Schiff's Reagent (Wall, 1996). The sulphurous acid step that follows immersion was omitted because it has been shown that water (SDW) rinses are sufficient (Llewellyn, 2005). Immersion time was increased to 10 min for each step to allow penetration of the agarose covering.

Immediately after staining and prior to permanent mounting, agarose was removed to enable a clearer view of the leaf surface and improve mounting. Stained leaf specimens were mounted in 10% glycerol and viewed under bright-field microscopy (100×, 200×, and 400×) using a Nikon Eclipse E800 microscope (Nikon Instruments, Inc. Melville, NY 11747) with a CoolSNAP *fx* video camera (Photometrics, Tucson, AZ 85706) and electronic caliper capabilities of MetaMorph Imaging System v. 6.1r3 (2003, Universal Imaging Corp.). An individual location on each leaf surface was randomly selected and 100 spores were counted and observed for germination. In addition, lengths of 10 random germ tubes were measured using electronic calipers of the MetaMorph at 200×.

2.5. Statistical analysis

Data for germ tube length and percent germination were subjected to analysis of variance and covariance using the mixed models procedure of SAS (Release 9.1 2002–2003, SAS Institute, Cary, NC). Fungal treatment and weed species were fixed main effects, repeated trials were designated as random effects, and experiments at each temperature were analyzed separately. Percent germination was transformed using square-root transformation prior to analysis to equalize variance. Non-transformed means are reported with transformed *P*-values.

3. Results and discussion

3.1. Conidia germination

A significant interaction ($P < 0.0014$) for percent conidia germination between weed species and fungal organism was observed. Germination of *M. amaranthi* conidia was comparable among weed species, averaging 32.2% at 21 °C and 44.8% at 28 °C (Fig. 1). At 21 °C, germination of *M. amaranthi* conidia was higher ($P < 0.04$) than germination of *P. amaranthicola* on the leaf surface of *A. retroflexus*, yet less ($P < 0.0001$) than *P. amaranthicola* germination on the leaf surface of *A. rudis*. At 28 °C, germination of *M. amaranthi* conidia was higher ($P < 0.0001$) than germination of *P. amaranthicola* conidia on all seven weed species. Smith (2003) reported an optimum temperature of 20 °C for germination of *M. amaranthi* conidia in 1/4-strength potato dextrose broth.

Germination of *P. amaranthicola* conidia depended upon the *Amaranthus* species at both temperatures (Fig. 1). At 21 °C, *P. amaranthicola* conidia germination was highest (49%) on leaf surfaces of *A. rudis* and least (19%) on *A. retroflexus*. At 28 °C, germination of *P. amaranthicola* conidia was highest (28%) on *A. rudis* and similar to germination observed on *A. palmeri*, *A. powellii*, and *A. hybridus*. Germination of *P. amaranthicola* conidia was least (10%) on leaf surfaces of *A. spinosus* at 28 °C. Wyss and Charudattan (2000) showed host-specific responses between *A. spinosus*, and *A. viridus*, where *P. amaranthicola* conidia formed a penetration peg only on the surface of *A. viridus*. Host-specific germination of

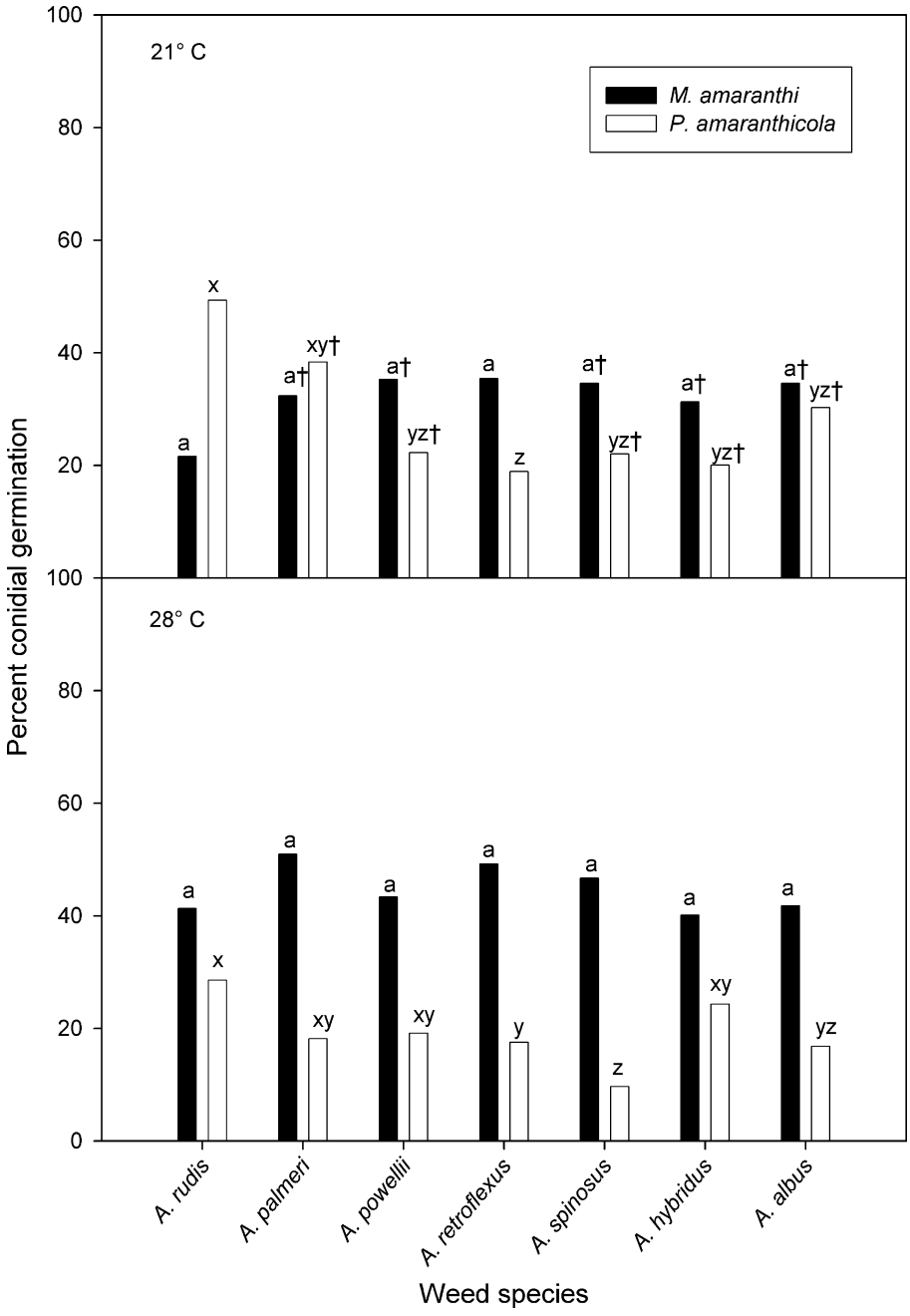

Fig. 1. Percent germination of *Microsphaeropsis amaranthi* and *Phomopsis amaranthicola* conidia on the leaf surfaces of seven *Amaranthus* species at 21 and 28 °C. Conidia were allowed to germinate for 24 h after application to leaf surfaces. Bars with the same letter or symbol across weed species or within weed species indicate no significant difference at $P < 0.05$.

P. amaranthicola conidia was also observed by Chandramohan and Charudattan (2003) on its host, *A. hybridus*, but not on two other non-host species.

Other researchers have examined conidial germination of either *M. amaranthi* or *P. amaranthicola* using in vitro techniques to evaluate compatibility of the organisms with pesticides or adjuvants (Rosskopf et al., 2005; Smith and Hallett, 2003, 2006; Wyss et al., 2004); environmental conditions favoring germination (Mintz et al., 1992; Rosskopf et al., 2005; Smith et al., 2006); or morphological characteristics (Heiny et al., 1992; Rosskopf et al., 2000a).

3.2. Germ tube length

A significant weed species-fungal treatment interaction existed for germ tube length at both 21 °C ($P < 0.0001$) and 28 °C ($P < 0.0001$). At 21 °C, the length of hyphae germinating from *M. amaranthi* conidia on the surfaces of all seven *Amaranthus* species was longer ($P < 0.0001$) than hyphae germinating from *P. amaranthicola* conidia (Fig. 2). Germ tube lengths for *M. amaranthi* on leaf surfaces of *A. palmeri* and *A. powellii* were 35.9 and 35.1 μm, respectively, and exceeded germ tube lengths on the other five *Amaranthus*

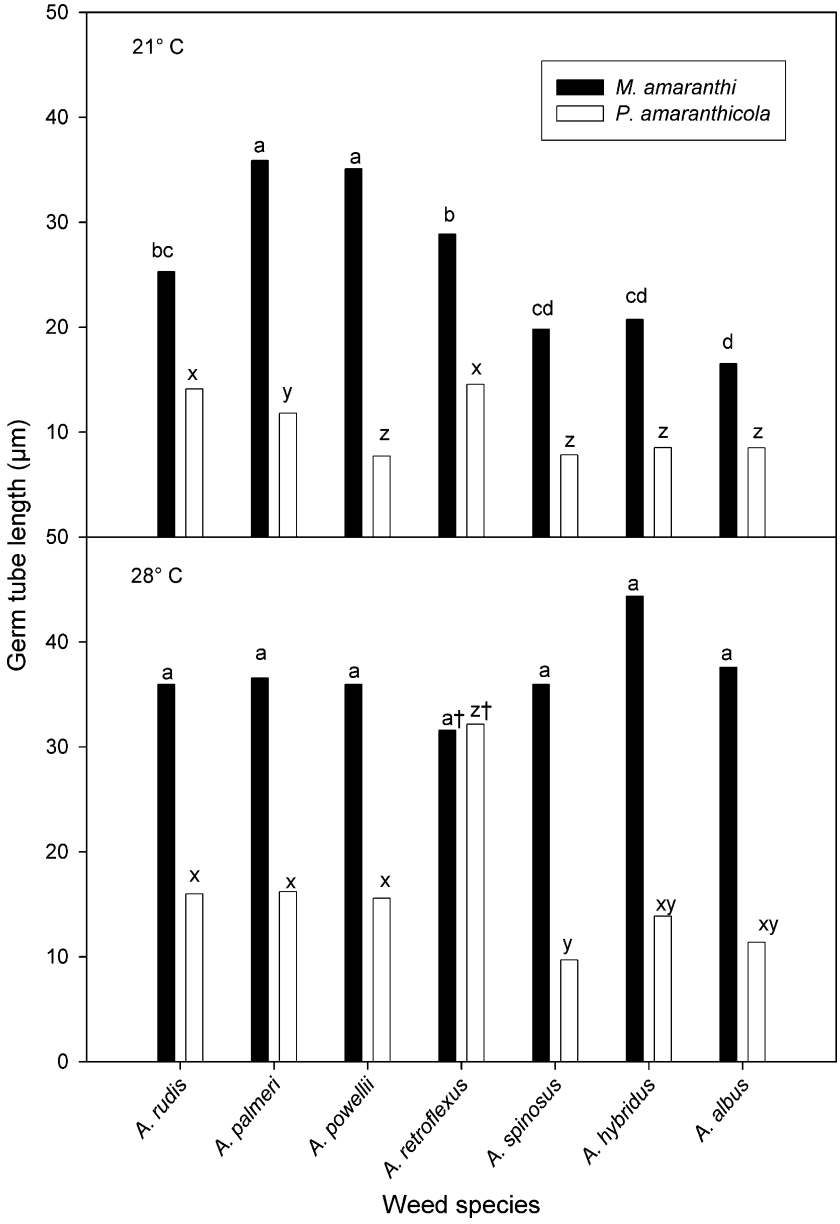

Fig. 2. Germ tube lengths of *M. amaranthi* and *P. amaranthicola* conidia at 21 and 28 °C on the surfaces of seven *Amaranthus* species. Germ tubes were allowed to grow for 24 h after application to leaf surfaces. Bars with the same letter or symbol across weed species or within weed species indicate no significant difference at $P < 0.05$.

species. Germ tubes of *P. amaranthicola* were longest (14.1 and 14.5 µm, respectively) on the leaf surfaces of *A. rudis* and *A. retroflexus*.

Lengths of *M. amaranthi* germ tubes were comparable across weed species at 28 °C, averaging 36.8 µm (Fig. 2). Furthermore, length of germ tubes from *M. amaranthi* conidia were 55–73% longer ($P < 0.0001$) than germ tubes from *P. amaranthicola* conidia for all weed species except *A. retroflexus*. Leaf surfaces of *A. spinosus* supported the least growth of *P. amaranthicola* conidia at 28 °C, limiting germ tube length to 9.7 µm.

Germ tube length tended to differ between the two temperature regimes. Germ tube lengths for *M. amaranthi* on the leaf surfaces of *A. spinosus*, *A. hybridus*, and *A. albus* averaged 19.0 µm at 21 °C and 39.3 µm at 28 °C (Fig. 2). Lengths of *M. amaranthi* germ tubes on *A. rudis* leaf surfaces were 25.3 µm at 21 °C and 35.9 µm at 28 °C. Although reduced in growth, a similar trend was observed for *P. amaranthicola* germ tubes between temperatures. Lengths of *P. amaranthicola* germ tubes on *A. powellii*, *A. retroflexus*, and *A. hybridus* were 7.7, 14.5, and 8.5 µm at 21 °C and 15.5, 32.1, and 13.8 µm at 28 °C, respectively. These results are consistent with the report by Smith (2003) that the greatest *M. amaranthi* hyphal growth on solid medium occurred at temperatures between 20 and 30 °C.

It could be argued that lower germination by *P. amaranthicola* conidia is due to self-inhibition, as shown by Sparace et al. (1991) for conidia of *P. convolvulus*, which at densities of

10^6 per ml or higher failed to germinate without dilution. In contrast, Ormeno-Nuñez et al. (1988) and Morin et al. (1990) reported the need for high inoculum concentrations (5×10^6 conidia per ml and 10^9 conidia per m^2, respectively) of *P. convolvulus* to obtain extensive disease development. In the current study, conidia suspensions for *P. amaranthicola* and *M. amaranthi* were diluted with water, and were approximately 1/3 of the concentration used in previous research where 100% host mortality was reported (Ortiz-Ribbing and Williams, 2006). In addition, Rosskopf et al. (2000b) reported the highest level of pigweed control in the field from *P. amaranthicola* at a conidial concentration of 6×10^7, with no indication of self-inhibition. There was no significant difference in *A. hybridus* mortality levels when sprayed with conidia concentrations ranging from 1.5×10^5 to 1.5×10^7 conidia per ml, illustrating higher spore concentrations are not inhibitory to disease development (Rosskopf et al., 2005). Mintz et al. (1992) used *M. amaranthi* spore concentrations up to 6×10^6 conidia per ml and obtained 96% mortality of *A. albus* without evidence of reduced weed control due to self-inhibition. For *P. amaranthicola* and *M. amaranthi*, self-inhibition does not appear to be the factor limiting germination; rather host growth stage and environmental factors, such as leaf surface moisture, dew period, and temperature have been implicated as factors limiting germination and disease development (Rosskopf et al., 2000b; Mintz et al., 1992; Morin et al., 1990; Ormeno-Nuñez et al., 1988; Smith, 2003; Smith et al., 2006).

Results on conidia germination and germ tube length of *P. amaranthicola* and *M. amaranthi* on leaf surfaces from this study contribute to the understanding of whole-plant response to these pathogens. More pronounced disease symptoms observed with *M. amaranthi* compared to *P. amaranthicola* by Ortiz-Ribbing and Williams (2006) on six of the same weed species draws a parallel to the enhanced conidial germination and growth of *M. amaranthi* reported here. Furthermore, poor germination of *P. amaranthicola* and short germ tube growth on *A. spinosus* may account for less severe disease symptoms and lack of mortality of *A. spinosus* seedlings in previous research (Ortiz-Ribbing and Williams, 2006). Moreover, Wyss and Charudattan (2000) reported conidia of *P. amaranthicola* did not germinate on *A. spinosus*.

Conidial germination and germ tube formation are prerequisites for successful infection (Nielsen et al., 2000), and the results reported herein show that differences exist between the two fungal pathogens studied. The observed host-specific responses by *M. amaranthi* and *P. amaranthicola* could be explained by host-specific recognition by the pathogens (Heath, 2000, 2001), possibly a need to recognize appropriate surfaces in order to take up materials necessary for germination (Nielsen et al., 2000). Jones and Epstein (1990) found that a particular source of carbon or nitrogen might influence attachment of *Nectria haematococca* (Berk. & Br.) macroconidia to leaf surfaces. Populations of other phylloplane microorganisms might also influence conidial germination and germ tube elongation. Fernanado et al. (1994, 1996) showed that certain bacteria isolated from the surface of the velvetleaf (*Abutilon theophrasti* Medic.) reduced germination of *Colletotrichum coccodes* (Wallr.) S.J. Hughes conidia and germ tube length but favored appressoria formation of a mycoherbicide.

4. Conclusions

Germinating conidia and germ tube elongation are initial requisites for disease development; subsequent processes including host penetration ultimately contribute to disease spread and plant death. Conidial germination and germ tube length may contribute to host responses of *P. amaranthicola* and *M. amaranthi*, but whether numerous conidia and longer germ tubes result in multiple penetrations at infection sites is unknown. Poor conidial germination and elongation of germ tubes of *P. amaranthicola* on *A. albus* in the current study compared to the high seedling mortality (up to 83%) of *A. albus* in previous studies indicates that post-germination infection processes are important (Ortiz-Ribbing and Williams, 2006).

In this study, host-specific responses among the seven *Amaranthus* species, as evidenced by conidial germination and germ tube length were greater for *P. amaranthicola* than *M. amaranthi.* While conidial suspensions of each fungus germinated on the leaf surfaces of all seven *Amaranthus* species, temperature influences conidial germination and germ tube length for these organisms on some host weed species. The extent to which post-germination infection processes such as penetration, invasion, and colonization contribute to host specificity warrants further study. Such knowledge will prove useful in further defining the role of these organisms as bioherbicides.

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