Inhibition of urediniospore germination in *Puccinia hemerocallidis* by Bacto Agar and changes in percent germination and germ-tube elongation on agarose over time

Y.H. Li, R.N. Trigiano, M.T. Windham, L.M. Vito, D.C. Fare, J.M. Spiers, and W.E. Copes

Abstract: Agar is a commonly used gelling agent and a raw material used in other gelling agents. The effects of five gelling agents and potato dextrose agar on urediniospore germination, and changes in percent urediniospore germination and germ-tube elongation in *Puccinia hemerocallidis* over time were investigated in vitro. Percent urediniospore germination differed significantly between the tested gelling agents. Urediniospores germinated well on Gelrite, agarose, Phytagar, and Oxoid No. 3 agar in decreasing order, and percent urediniospore germination was negatively correlated with the concentration of gelling agent. Urediniospores germinated poorly on the substrates containing more than 0.5% Bacto Agar. The concentration of Bacto Agar that caused 50% inhibition of urediniospore germination was 18.2 µg/mL in 1% agarose substrate. However, there were no significant differences in germ-tube elongation between the concentrations of Bacto Agar water extract tested. Unidentified inhibitory compounds from Bacto Agar water extract were adsorbed on a C18 column and the effluent water did not affect spore germination. However, the methanol-eluted solution from the C18 column completely inhibited urediniospore germination when the solution was evaporated and reconstituted with water. Changes in percent urediniospore germination and germ-tube length on 1% agarose water substrate over time fitted well with negative exponential models. The time to the half-asymptote of percent urediniospore germination and germ-tube length was 1.4 and 6.0 h, respectively, and the time to 95% of the asymptote was 6.1 and 30.9 h for spore germination and germ-tube elongation, respectively.

Key words: gelling agent, urediniospore, daylily rust, *Hemerocallis*, inhibition.

Résumé : L'agar-agar est un agent gélifiant courant qui entre également dans la composition d'autres agents gélifiants à titre de matière première. Les effets de cinq agents gélifiants et de la gélose dextrosée à la pomme de terre sur la germination des urédospores, ainsi que les variations du taux de germination et d'élongation du tube germinatif de *Puccinia hemerocallidis*, ont été étudiés in vitro. Les taux de germination des urédospores ont varié significativement selon l'agent gélifiant testé. Les urédospores ont bien germé sur, en ordre décroissant, Gelrite, agarose, Phytagar et Oxoid agar N^{o} 3, et le taux de germination des urédospores a été corrélé négativement avec la concentration des agents. Les urédospores n'ont pas très bien germé sur les substrats contenant plus de 0,5 % de Bacto Agar. La concentration d'inhibition 50 % de Bacto Agar quant à la germination des urédospores a été de 18,2 µg/mL sur un substrat composé à 1 % d'agarose. Toutefois, il n'y a pas eu de différence significative quant à l'élongation du tube germinatif en ce qui a trait aux concentrations d'extraits aqueux de Bacto Agar testées. Des composés inhibitoires non identifiés, issus de l'extrait aqueux de Bacto Agar, ont été adsorbés par une colonne C18, et l'effluent aqueux n'a pas nui à la germination des spores. Par contre, après avoir été évaporée et reconstituée avec de l'eau, la solution de méthanol éluée de la colonne C18 a complètement inhibé la germination des urédospores. Les variations relatives aux taux de germination des urédospores et d'élongation du tube germinatif qui se sont produites au fil du temps, associées

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au substrat aqueux contenant 1 % d'agarose, correspondent bien aux modèles exponentiels négatifs. Le temps pour atteindre la demi-asymptote correspondant au taux de germination des urédospores et d'élongation du tube germinatif a été de 1,4 heures et de 6,0 heures, respectivement, et celui associé à 95 % de l'asymptote a été de 6,1 heures pour la germination des spores et de 30,9 heures pour l'élongation des tubes germinatifs.

Mots-clés : agent gélifiant, urédospore, rouille de l'hémérocalle, *Hemerocallis*, inhibition.

Introduction

Evaluating the suitability of gelling agents, which are supposedly benign toward the growth functions of microorganisms and other types of organisms, is essential before they can be used in research. Many gelling agents, such as Bacto Agar (product No. 0140–01, Difco Laboratories, Detroit, Michigan), (Bonde et al. 2007; Pfister et al. 2004; Tapsoba and Wilson 1997), Oxoid No. 3 agar (Elahinia 2000; Ellison et al. 1990, 1992), agarose (Hallett et al. 1990), and potato dextrose agar (PDA) (Buck and Williams-Woodward 2003; Mueller and Buck 2003) have been used to investigate fungal-spore germination in vitro. A low proportion of spore germination (11.6%) was also reported for *Colletotrichum graminicola* (Ces.) G.W. Wilson on 4% water agar (Chaky et al. 2001). In contrast, Egley (1994) reported that percent germination of *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore conidia increased as the concentration of agar increased from 0.025% to 5%. Adverse effects of gelling agents on plant-tissue culture (Arregui 2003) and pollen germination (Karapanos et al. 2006; Kohlenbach and Wenicke 1978) have been reported.

Puccinia hemerocallidis Thüm., the causal agent of rust on daylilies (*Hemerocallis* spp.), is an introduced fungal pathogen in the United States of America (Hernández et al. 2002; Williams-Woodward et al. 2001). Urediniospores of *P. hemerocallidis* germinate within a few hours and initiate germ tubes within 24 h. Elongating germ tubes orientate to stoma on the leaf surface, and infection pegs, which originate from appressoria that form at the end of the germ tubes, penetrate the stomal openings (Li et al. 2007). Knowledge of urediniospore germination and germ-tube elongation is desirable for investigating the biology of *P. hemerocallidis* and developing strategies for managing daylily rust. Effects of temperature, light, and fungicide application on germination of *P. hemerocallidis* urediniospores on PDA substrate have been investigated in vitro (Buck and Williams-Woodward 2003; Mueller and Buck 2003). However, poor germination of *P. hemerocallidis* urediniospores on Bacto Agar and PDA substrates was observed in our preliminary studies. Understanding the inhibition of urediniospore germination on some brands of gelling agent is necessary not only for choosing a gelling agent suitable for spore-germination experiments, but also for finding an agent to control daylily rust. The objectives of this study were to determine the influences of gelling agents on urediniospore germination and to describe the temporal progression of urediniospore germination and germ-tube elongation in *P. hemerocallidis* in vitro.

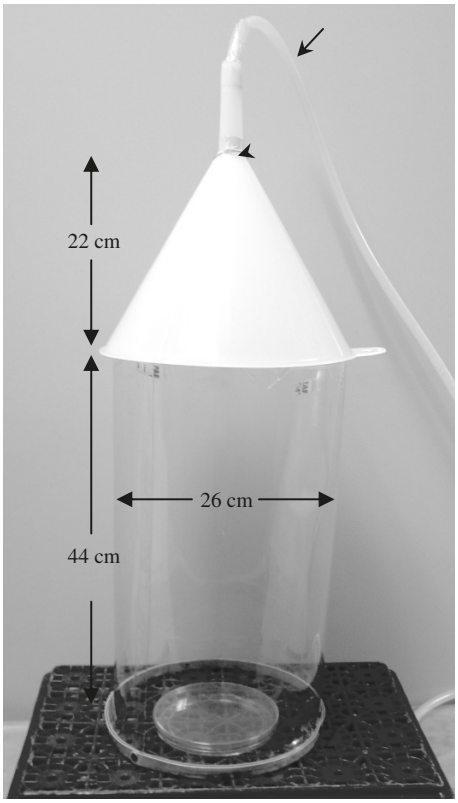
Materials and methods

A single-pustule isolate of *P. hemerocallidis* was maintained by inoculating healthy leaf segments of daylily 'Pardon Me' at 8- to 10-d intervals. Fully expanded healthy daylily leaves were collected from the greenhouse at the Plant Sciences Unit of the East Tennessee Research and Education Center at the University of Tennessee in Knoxville and washed in running distilled water for 1 min. The 6–8 cm long leaf segments were cut and placed abaxial surface up on two layers of moistened filter paper in 9 cm diameter Petri dishes. After they were misted with distilled water, the leaf segments were inoculated using infected leaves containing urediniospore pustules by means of a settling-tower inoculation method described previously (Li et al. 2007). Inoculum density on inoculated leaf segments was adjusted to 200–300 spores/cm^2 leaf area. Petri dishes with inoculated leaf segments were sealed with Parafilm and incubated in the dark for 24 h at room temperature before being incubated at 21 ± 1 °C with a continuous photoperiod. Light was provided by four 40 W residential fluorescent bulbs hung 45 cm above the Petri dishes. Urediniospores that were released from the infected leaf segments 8–10 d after inoculation were used as inoculum for all experiments.

In all experiments, gel-coated slides were inoculated with urediniospores from diseased leaf segments using the settling tower (Fig. 1), which was connected to a compressor (Gast Manufacturing Inc., Benton Harbor, Michigan). Inoculated slides were placed on two layers of moist paper towel in a metal pan and covered with aluminum foil to retain ample moisture for spore germination and avoid gel desiccation. After 5 h of incubation in the dark at 21 ± 1 °C, slides were sampled and stained with 0.05% trypan blue in lactophenol (two drops per slide), covered with a cover slip (20 mm × 50 mm), and examined microscopically for germination, using a compound microscope (Nikon Instruments Inc., Atlanta, Georgia) at 200× magnification. Urediniospores with germ-tube length greater than or equal to the radius of a urediniospore were considered germinated. Percent urediniospore germination was determined by randomly counting 100 urediniospores on each slide. In each experiment three slides per treatment were used, and the experiment was repeated three times.

To determine the effects of the gelling agents on urediniospore germination, Superfrost microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania) were coated using the following five gelling agents: Bacto Agar, Oxoid No. 3 agar (product No. LP0013, Oxoid Ltd., Basingstoke, Hampshire, England), Phytagar (product No. 10675–023, Life Technologies, Grand Island, New York), Gelrite (product No. 71010–52–1, Research Products International Corp., Prospect, Illinois), agarose (product No. BP1356–100, Fisher Scientific, Fair Lawn, New Jersey), and a PDA medium (product No. 213400, Difco, Sparks, Maryland). The gelling agents were used in five concentrations ranging from 0.5% to 6% (*m*/*v*) to coat glass slides (1 mL of each melted gelling agent per slide) on a slide-warmer (Fisher Scientific Co., Pittsburgh, Pennsylvania) at 60 °C and then

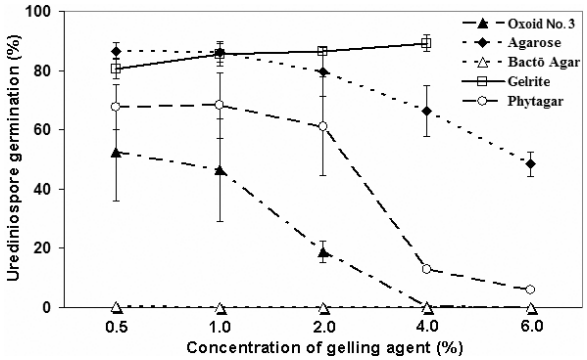
Fig. 1. Chamber used for inoculating agar-coated slides with urediniospores of *Puccinia hemerocallidis*. Diseased leaves with fresh urediniospores were hung at the top of the funnel (arrowhead). Plastic tubing (arrow) connects the tower to an air compressor. The base of the unit is perforated to vent air out from the chamber.



allowed to solidify at room temperature. Owing to the difficulty of coating the slides evenly with Gelrite because it is water-soluble, the 6% Gelrite treatment was excluded from the experiments.

To determine the concentration of Bacto Agar that caused 50% inhibition (IC_{50}) of urediniospore germination and germ-tube elongation, a 4% (*m*/*v*) aqueous suspension of Bacto Agar was stored at 4 °C overnight. From the initial supernatant of the suspension, a half-dilution series was made with melted 1% aqueous agarose (not inhibitory) in vials and used to coat the glass slides. Percent urediniospore germination was determined using the method described previously. Germ-tube length was assessed by measuring 25 germinated urediniospores on each slide using NIS-Elements software with a compound microscope 24 h after inoculation (HAI). Because of lower percent germination, germ-tube length was measured on 0.0078%–0.5% Bacto Agar. The experiment was repeated three times. An exponential-decrease model ($y = ae^{-bx}$) was fitted to curves of percent urediniospore germination versus concentration of Bacto Agar water extract using the nonlinear regression procedure of SAS (SAS Institute Inc. 2004). The IC_{50} value for urediniospore germination on Bacto Agar was estimated from the derived exponential equation. The effects of the different concentrations of Bacto Agar extract on germ-tube elongation were determined with an analysis of variance us-

Fig. 2. Effects of gelling agents at different concentrations on germination of *Puccinia hemerocallidis* urediniospores in vitro 5 h after inoculation. Vertical bars show the standard error of the mean.



ing the general linear model procedure of SAS (SAS Institute Inc. 2004).

Putative separation of the germination inhibitors in Bacto Agar was accomplished using a C18 Sep-Pak cartridge (Water Associates, Milford, Massachusetts). Two and one-half millilitres of a 4% aqueous extract (*m*/*v*) Bacto Agar was pressed through the cartridge and the water effluent collected. Some of the compounds adsorbed on the cartridge were eluted with 2 mL of 100% methanol. The methanol was evaporated in a fume hood and the residue redissolved in 1 mL of distilled water. The water effluent and the reconstituted methanol fraction were mixed with an equal volume of melted 2% agarose (not inhibitory) at 60 °C, which was used to coat glass slides for determining the effects of each dilution on urediniospore germination.

To describe the temporal progression of urediniospore germination and germ-tube elongation, slides were coated with 1% aqueous agarose and inoculated with urediniospores. Inoculated slides were randomly sampled at 1, 2, 4, 6, 12, 24, 36, 48, 72, and 96 HAI by selecting one slide in each sample point. Urediniospore germination and germ-tube length were assessed. The negative exponential model $y = a(1\ e^{-bt})$ was fitted to the curves of mean urediniospore germination and germ-tube length versus time using the nonlinear regression procedure of SAS (SAS Institute Inc. 2004). The empirically derived equations that fitted well with the curves of urediniospore germination and germ-tube elongation were used to estimate the asymptote, time to the half-asymptote ($T_{0.5a}$), and time to 95% of the asymptote ($T_{0.95a}$).

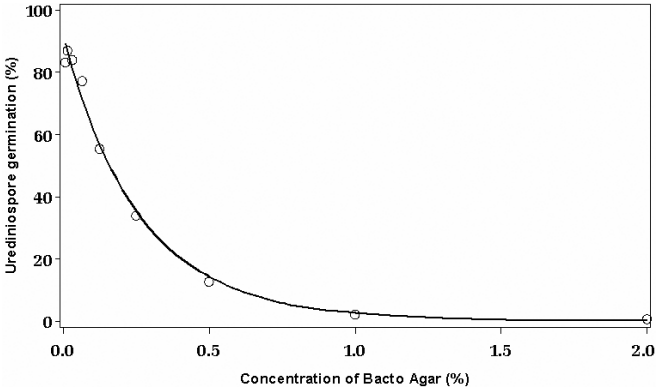
Results and discussion

In general, percent urediniospore germination decreased as the concentration of gelling agent increased, except that there were no changes on Bacto Agar and Gelrite (Fig. 2). Percent germination decreased to less than 20% when the concentration of Oxoid No. 3 agar or Phytagar exceeded 4% (*m*/*v*), and germination was almost completely inhibited on Bacto Agar at all concentrations tested. Higher percent germination was observed on Gelrite and agarose than on the other gelling agents. Percent urediniospore germination on PDA is not included in the graph because it differs in scale from the other gelling agents (15 g Bacto Agar in 40 g com-

mercial PDA). When the PDA concentration was converted to the amount of agar in the substrate, mean urediniospore germination was 79.0%, 83.4%, 72.2%, 16.4%, and 7.6% for substrates with 0.19%, 0.38%, 0.75%, 1.5%, and 2.3% Bacto Agar, respectively, in PDA substrate. The sharp decrease in urediniospore germination in the series of PDA concentrations was related to the increase in Bacto Agar concentration in the PDA substrate. The recommended PDA concentration is 39 g/L, which contains 15 g agar. Therefore, the lower rate of urediniospore germination in our preliminary experiments could have been due to the inhibitory effects of 1.5% agar in the PDA on germination of *P. hemerocallidis* urediniospores. The gelling agents and PDA tested in this study are all agar-based except Gelrite, for which the highly purified polysaccharide, a mixture of 3,6-anhydrogalactose and sulfates, is obtained from red algae through extraction and purification procedures (Sukhoverkhove et al. 2000). Of the agar-based gelling agents, agarose, which is a fraction of agar, is a highly purified form of agar. Antimicrobial activities of crude extracts and some chemical compounds obtained from red algae have been reported (Blunden 1993; Puglisi et al. 2007). The results of the present study suggest that the differences in the effects of gelling agents on germination of *P. hemerocallidis* urediniospores are a function of the amount of agar and its purity. Because of the self-gelling hydrocolloidal nature of Gelrite, agarose was used as a gelling agent in further experiments on the inhibition and temporal progression of urediniospore germination and germ-tube elongation. Water agar was reported to reduce the germination of *C. graminicola* spores (Chaky et al. 2001). However, germination of *C. truncatum* spores increased as the concentration of Bacto Agar was increased (Egley 1994), and 100% spore germination on 1% water Bacto Agar was reported for *Gigaspora albida* N.C. Schenck & G.S. Sm. (Maia and Yano-Melo 2001). The results of those studies indicate that the effects of agar on spore germination could be species-dependent.

Percent urediniospore germination decreased as the concentration of Bacto Agar in the 1% agarose substrate increased (Fig. 3). The curvilinear response of percent urediniospore germination to Bacto Agar concentration is well described by the exponential decrease equation $y = 91.59\ e^{-3.81x}$ ($R^2_{adj.}$ = 0.998), where y is percent urediniospore germination, e is the natural logarithm, and x is the Bacto Agar concentration (g/100 mL). The estimated IC_{50} value of Bacto Aagar extract for urediniospore germination was 18.2 µg/mL. The mean length of urediniospore germ tubes measured at 24 HAI in all Bacto Agar concentrations tested, including the 1% agarose control, was 810 µm, and lengths did not differ significantly among concentrations (P = 0.2637). In the present study, Bacto Agar inhibited germination of *P. hemerocallidis* urediniospores, but not germ-tube elongation. In a study of bean rust caused by *Uromyces phaseoli* L., Rajam et al. (1989) reported that both germination and germ-tube growth of urediniospores were inhibited by α-difluoromethylornithine and α-difluoromethylorginiine. However, the inhibitory effects could be reversed by polyamines. Similarly, spore germination and germ-tube elongation in *Aspergillus niger* Tiegh. and *Botryodiplodia theobromae* Pat. were inhibited by the antifungal compound β-sitosterol (Aderiye et al. 1989).

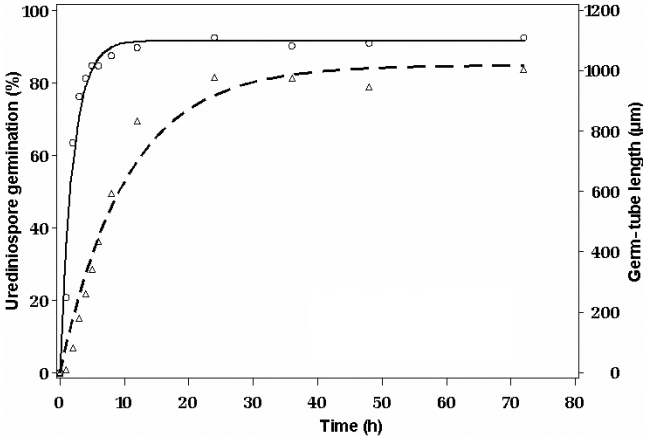
Fig. 3. Mean observed and predicted percent germination of *Puccinia hemerocallidis* urediniospores on glass slides coated with Bacto Agar extract at various concentrations in 1% agarose 5 h after inoculation. The circles represent the mean of observed values and the solid line represents values estimated using the derived exponential equation $y = 91.59\ e^{-3.81x}$ ($R^2_{adj.}$ = 0.998, P = 0.0001).



Urediniospore germination was completely inhibited on the substrate consisting of 1% agarose combined with the methanol fraction extracted from Bacto Agar, whereas no inhibition was observed in the water effluent. Complete inhibition of urediniospore germination on the substrate consisting of 1% agarose combined with the methanol fraction suggests that the inhibitory compounds in Bacto Agar are water-soluble and absorbable on a C18 column. Attempts to identify specific compounds in the methanol fraction were unsuccessful (data not shown). A nongelling and cold-water-soluble constituent of a commercial agar caused toxic symptoms and poor long-term shoot survival in pine micropropagation (Nairn et al. 1995). Inhibitory substances in Bacto Agar can be absorbed by active carbon (Kohlenbach and Wernicke 1978). These results may provide ways to extract and purify inhibitory compounds from algae, the source of Bacto Agar, for biocontrol of daylily rust in the future.

The increase in percent urediniospore germination over incubation time was estimated using the empirically derived function $y = 91.5(1 - e^{-0.494x})$ ($R^2_{adj.}$ = 0.996, $P < 0.0001$), where y is percent urediniospore germination, e is the natural logarithm, and x is time in hours (Fig. 4). From the equation, the asymptote of urediniospore germination was estimated as 91.5% and $T_{0.5a}$ was 1.4 HAI. Urediniospore germination reached 95% of the asymptote at 6.1 HAI. A similar time to reach maximum urediniospore germination was reported for *Puccinia substriata* Ellis & Barthol. var. *indica* Ramachar & Cummins, the causal agent of pear millet rust (Tapsoba and Wilson 1997). The empirically derived function of the progression of germ-tube elongation was $y = 1017.4(1 - e^{-0.097x})$ ($R^2_{adj.}$ = 0.989, P = 0.0001), where y is germ-tube length in micrometres, e is the natural logarithm, and x is time in hours. The estimated asymptote of germ-tube elongation was 1017.4 µm, $T_{0.5a}$ was 7.2 HAI, and $T_{0.95a}$ was 30.9 HAI. Germination of *P. hemerocallidis* urediniospores in vitro reached the highest percentage at about 6 HAI. In the present study we evaluated urediniospore germination among treatments in vitro at 5 HAI, when urediniospore germination was about

Fig. 4. Temporal progression of urediniospore germination (○) and germ-tube elongation (△) in *Puccinia hemerocallidis* on glass slides coated with 1% agarose. The symbols represent the mean of observed values and the lines represent values estimated using negative exponential equations.



90% on the 1% agarose substrate. In previous reports, percent germination was assessed in vitro at 16 HAI (Mueller and Buck 2003) and 24 HAI (Buck and Williams-Woodward 2003) to determine the effects of light, temperature, and fungicide use on germination of *P. hemerocallidis* urediniospores. The results of the present study suggest that germination of *P. hemerocallidis* urediniospores could be assessed as early as 5 HAI.

Inhibition of spore germination was quite evident on some agar preparations and is related to the degree of refinement of the product: the relatively unrefined Bacto Agar inhibited germination the most of any of the agar compounds, while agarose, which is highly processed, inhibited germination the least. The non-agar gelling compound Gelrite did not significantly inhibit spore germination. Interestingly, none of the gelling agents inhibited germ-tube elongation. Although we could not identify the chemical compound or combination of compounds present in Bacto Agar responsible for inhibition, they appear to be of a low molecular weight (data not shown), heat-stable, and polar so that when they are added back into a non-inhibiting medium such as agarose, they significantly impede urediniospore germination. It would be interesting to extend studies of this phenomenon to other rust species and other groups of fungi and fungus-like organisms. The inhibitory effects on spore germination could also be correlated with the general deterioration in vigor and loss of virulence in some fungi cultured for long periods on media containing Bacto Agar. The decline of some organisms in our culture collections usually can be reversed and vigorous growth restored on Bacto Agar by first culturing the fungi in liquid medium, or if a plant pathogen, by growing it on sterilized host tissue.

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