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

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**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° 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...
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 (Chakravarty 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 ‘Par-
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 ($y = a(1 - e^{-bx})$) 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-
cercial 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. hemerocalliudis_ 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 (Sukhovekhov 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. hemerocalliudis_ 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 Agar 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. hemerocalliudis_ urediniospores, but not germ-tube elongation. In a study of bean rust caused by _Uromyces phaseoli_, Rajam et al. (1989) reported that both germination and germ-tube growth of urediniospores were inhibited by \( \alpha \)-difluromethylornithine and \( \alpha \)-difluoromethylglycinine. 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 \( \beta \)-sitosterol (Aderiye et al. 1989).

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 \( y = 91.5(1 – e^{-0.097x}) \ (R^2_{adj} = 0.996, P < 0.0001) \), 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 striiformis_ Ellis & Barthol. var. _indica_ Ramachar & Cummins, the causal agent of pear millet rust (Tapsoha 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. hemerocalliudis_ 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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References


