Impact of nutritional conditions on yields, germination rate and shelf-life of Plectosporium alismatis conidia and chlamydospores as potential candidates for the development of a mycoherbicide of weeds in rice crops

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Running title: influence of nutritional conditions on *P. alismatis*

SUMMARY

The effect of nutritional conditions on spore qualities was investigated in order to select which propagules: conidia or chlamydospores, would be most suitable for mycoherbicide development. *P. alismatis* was grown in a liquid basal medium supplemented with glucose and a mineral nitrogen source (sodium nitrate) or an organic nitrogen source (casamino acids). Conidial and chlamydospore yields, germination rate and shelf-life were compared. Two growth models were developed: on one hand, sodium nitrate added as the sole nitrogen source was partially utilized (8%), resulting in poor growth (177 ± 2 mg dry weights $flask^{-1}$; 3.4 ± 1 x 10^5 conidia mg DW^{-1}). In these conditions, *P. alismatis* produced dense, melanized-like aggregates that contained chlamydospores (7 ± 0.4 x 10^5 chlamydospores mg DW^{-1}). Germination rate of chlamydospores and conidia produced in these conditions was high (80%). Twenty percent of chlamydospores were able to germinate after 4 months storage at 25°C, while survival of conidia declined rapidly (<2%). When casamino acids were added to the liquid medium as the sole nitrogen source, *P. alismatis* produced sparser pellets resulting in high dry weights (537 ± 9.2 mg DW $flask^{-1}$ and high conidia numbers (1.8 ± 0.3 x 10^6 conidia mg DW^{-1}), while no chlamydospore was observed. The germination rate of conidia produced in casamino acids was low (33 ± 13 %) and microcycle conidiation occurred. Five percent of these conidia germinated after 4 months storage. These data indicate that chlamydospores may be suitable for mycoherbicide development, provided further optimization of yields is achieved.

INTRODUCTION

Under Australian conditions, aquatic weeds in the Family *Alismataceae* have the potential to compete heavily with direct seeded rice. Of these species, *Damasonium minus* or starfruit is regarded as the most important weed. The control of starfruit is almost exclusively reliant on the use of only one herbicide (bensulfuron-methyl,Londax®) which has contributed to the emergence of herbicide-resistant weed biotypes throughout the Australian rice growing areas. This resistance and the potential for the contamination of waterways by synthetic herbicides have spurred the search for alternative weed control strategies at Charles Sturt University since the early 1990s (Ash *et al*., 2003).

The endemic fungus *Plectosporium alismatis* [synonym *Rhynchosporium alismatis*] (Pitt *et al*., 2004) is being developed as a mycoherbistat (Crump *et al*., 1999) for the control of starfruit and other closely related weed species (Cother 1999; Cother & Gilbert, 1994; Jahromi *et al*., 2001). So far, most studies have been related to the application of conidia collected on solid media (Jahromi *et al*., 1998). These conidia are able to infect host species (Lanoiselet *et al*., 2001; Jahromi *et al*., 2002; Pitt *et al*., 2004), leading to reduced biomass of the weed or to reduced seed set (Fox *et al*., 1999).

In our laboratory, a methodology for producing a liquid culture production method for the development of a *P. alismatis* mycoherbicide is currently being investigated. Previous studies showed that *P. alismatis* produced conidia and mycelium and, interestingly, chlamydospores in a liquid culture medium based on

the Czapex Dox composition, in which nitrogen and carbon are provided by sodium nitrate and malt extract (Cliquet *et al.*, 2004). These chlamydospores differentiated into three types: single-celled, double-celled, and hyphal intercalary chains. These overwintering structures make the ideal candidates to be considered for the development of a stable bioherbicide (Jackson & Schisler, 2002).

However, these chlamydospore are produced in low numbers compared with conidia that are easily and abundantly produced in complex sources (Cother & van de Ven, 1999). Moreover, recent studies show that conidia may be potential mycoherbistats provided a suitable time of application and pertinent formulations are applied to protect conidia from UV light (Ghajar *et al.*, 2006). Although chlamydospores are assumed to be desiccation tolerant structures, comparisons of survival of conidia and chlamydospores grown in the same culture conditions are scarce in the literature. Shabana *et al.* (2002) observed that survival of *Fusarium oxysporum* was more related to storage temperature and relative humidity than to the type of dry propagules considered, microconidia or chlamydospores.

In the development of a biological control agent, fungi are evaluated based on their mode of growth and differentiation, propagule yields, stability and biocontrol efficacy (Jackson & Schisler, 2002). In order to determine whether the mycoherbicide is to be based on conidia or chlamydospores, yields, desiccation tolerance and shelf-life of conidia and/or chlamydospores produced in various cultural conditions were compared in the present work.

MATERIALS AND METHODS

Isolate

Plectosporium alismatis RH 145 (DAR 73154) was isolated from *Damasonium minus*. Stock cultures were maintained in a mixture of soil:sand mixture as previously described (Jahromi *et al.*, 2002).

Inoculum production

Sub-cultures on Potato Dextrose Agar (PDA, Difco, Detroit, MI, USA) were sampled from the soil and sand mixture and renewed every year. From these sub-cultures conidia were inoculated on PDA plates and incubated at 25°C. Four-day old Petri dishes of the fungus were washed with distilled water and the conidia suspension inoculated into shake flasks for liquid culture (4×10^3 conidia mL^{-1}).

Media Composition

The basal mineral composition for *P. alismatis* growth was derived from a Czapex-Dox composition which contained: K_2HPO_4, 1.0 g; $MgSO_4.7H_20$, 1.0 g; KCl, 0.5 g; $Fe_2SO_4.7H_2O$, 0.018 g; deionized water: 1 L. For chlamydospore production, malt extract (Amyl Media, Sydney, NSW, Australia, 2.2 g L^{-1}) and sodium nitrate (Sigma Chemical, St. Louis, MO, USA, 3 g L^{-1}) were used, providing respectively 0.92 g C L^{-1} and 0.5 g N L^{-1}. The liquid culture medium for enhanced chlamydospore production contained malt extract, 8.8 g L^{-1}, and sodium nitrate, 5.74 g L^{-1}, providing respectively 3.68 g C L^{-1} and 1 g N L^{-1} (Cliquet *et al.*, 2004).

A defined medium was prepared in which malt extract 2.2 g L^{-1} was replaced by glucose (2.2 g L^{-1}, providing 0.92 g C L^{-1}). This medium contained sodium nitrate 3.12 gL^{-1} (providing 0.5 g N L^{-1}). Bacto yeast nitrogen-base without amino-acids and ammonium sulphate (Difco) was provided in the medium as a nitrogen-free, vitamin source (0.17 g L^{-1}).

In nitrogen source experiments, malt extract (8.8 g L^{-1}) was replaced by glucose (3.68 g C L^{-1}). One of the following substrate sources was added to the medium as the sole nitrogen source (1g N L^{-1} each): technical casamino acids (Difco), bacto yeast extract (Difco), tryptone, (Difco), glutamic acid, $CaNO_3$, $NaNO_3$ (Sigma). The initial pH was adjusted to 7 and maintained by daily addition of NaOH or HCl.

Growth and harvest

All cultures were placed in flasks (100 mL in 250-mL flasks), inoculated with conidia and incubated at 25°C on a rotary shaker incubator (Infors HT Bottmingen, Switzerland) at 100 rpm.

Cultures were vacuum-filtered on 110 mm ∅ cellulose filter papers (Whatman plc, Brentford, UK). Filtered cultures were rinsed with 50 ml deionized water and allowed to dry on the bench top until constant weight. Dry mats were weighed and resuspended in 21.5 ml distilled water. The suspension was fragmented in a Potter homogeneizer (Fisher Scientific Bioblock, Illkirch, France). Propagule counts were performed using a haemocytometer.

Shelf-life experiments

Cultures were grown in media containing 3.68 g C L^{-1} and 1 g N L^{-1} as previously described, and contents filtered on cellulose filters until constant dry weights (24 h). Dry biomass was stored at 25°C. Ten mg dry weights were homogenized, providing a suspension of approximately 10^6 propagules mL^{-1}. Hundred µl of the suspension were placed onto a cellophane piece for germination assay.

Chlamydospore and Conidial Germination

Drops of the propagule suspension were placed on four 2-cm square pieces of cellophane on the surface of water agar plates. Cellophane pieces were removed after 12 h at 25°C and germination evaluated microscopically after staining with lactophenol cotton blue as previously described (Cliquet *et al.*, 2004).

Nitrate measurement

Supernatant of cultures was filtered through a 0.2 µm nitrate cellulose membrane. Initially all residual nitrate was reduced to nitrite using a cadmium reduction column. Nitrate quantification was achieved with a flow injection system (Lachat Quick Chem 8000, Lachat Instruments, Milwaukee, WI, USA) based on the American Public health association method (Apha, 2001)

Glucose measurement

The method was based on the simultaneous oxidation of the aldehyde functional group present in glucose, and the reduction of 3,5-dinitrosalicylic acid (DNS) into 3-amino,5-nitrosalicylic acid

under alkaline conditions (Miller, 1959). The intensity of developed colour was determined with a spectrophotometer (UNICAM helio beta, France) at 540 nm.

Statistical Analysis

All growth experiments were performed using duplicate or triplicate flasks and all experiments were repeated at least once. Statistical analysis of variance was performed. For data not suitable for ANOVA, standard errors values were estimated as a measure of variance.

RESULTS AND DISCUSSION

Composition of media for chlamydospore production

Three liquid media were able to support chlamydospore production (2.4 to 20.3 x 10^4 chlamydospores mg DW^{-1}). In these 3 media, sodium nitrate was the sole nitrogen source (Table 1). Dry weights and conidial yields were low (respectively <400 mg $flask^{-1}$ and < 7.1 x 10^5 conidia mg DW^{-1}). This poor growth is probably the consequence of the partial utilization (8 %) of nitrate by *P. alismatis* (Figure 1). Although glucose was exhausted during growth, depletion is likely not responsible for chlamydospore formation since additional experiments showed that chlamydospores were formed in defined medium with increased glucose concentrations (data not shown).

Influence of nitrogen sources on P. alismatis growth

Increasing casamino acid concentrations in the defined medium containing sodium nitrate resulted in an increase in conidia numbers and, in contrast, to the inhibition of chlamydospore formation (Figure 2). These results indicate that organic nitrogen provided by casamino acids was probably utilized preferentially to inorganic nitrogen by *P. alismatis,* as previously reported for a majority of filamentous fungi (Garraway & Evans, 1984). In filamentous fungi, chlamydospore production may vary considerably depending on the nutritional environment (Hebbar *et al*. 1996), some fungi producing chlamydospores when submitted to a nutrient excess, while some others when submitted to starvation conditions (Gardner *et al*., 2000). In our conditions the formation of chlamydospores is likely the consequence of the addition of a non preferred nitrogen source, leading to starvation conditions.

Propagule yields produced in media supplemented with various nitrogen sources (1 g N L^{-1}) and glucose (3.68 g C L^{-1}) were compared with propagule yields produced in our sodium nitrate, malt extract medium supporting chlamydospores (Table 2). Unexpectedly, various pigmentations and morphological structures were observed in our cultures. Dense, pink or brown, melanized-like hyphal aggregates (Figure 3A, 3B) from day 2 of growth in respectively media containing sodium nitrate and glucose (Figure 3A) or sodium nitrate and malt extract (Figure 3B) were produced. These aggregates contained chlamydospores. Similarly, Gardner *et al*. (2000) observed that chlamydospore formation occurred in fungal pellets, or when agar was added to the liquid medium.

In contrast, sparse spherical, hyphal aggregates (i.e pellets, Figure 3C) were produced in media supplemented with organic nitrogen

sources. Microscopical observation revealed the presence of a few chlamydospores, although in too low numbers to be estimated. The medium composition, and particularly the type and concentration of the nitrogen source, have been recognized as particularly influential on fungal morphology in liquid culture and specifically on the pellet structure (Gibbs *et al*, 2000). The formation of chlamydospores is likely the consequence of the aggregate formation: in this complex structure, substrate and oxygen may, among other factors, become limiting, developing, therefore, stressful environmental conditions which may favor chlamydospore and melanin formation. Melanization by *Colletotrichum truncatum* was correlated to glucose concentration in the liquid medium (Jackson & Bothast, 1990). Additional experiments are required to determine in which conditions melanization occurs and whether melanin formation may modulate pathogenicity, as reported in the rice blast fungus *Magnaporthe grisea* (Money & Howard, 1996).

Growth was enhanced when organic nitrogen sources were added to the liquid media, with maximum conidial numbers reached in media supplemented with casamino acids (1.8×10^6 conidia mg DW^{-1} (Table 2).

Abundant conidial yields by *Plectosporium alismatis* have been reported in liquid culture media with a high organic nitrogen content (Cother & Van de Ven, 1999). The absence of chlamydospore formation in media containing organic nitrogen (i.e.: yeast extract, tryptone, glutamic acid) is likely the consequence of the addition of organic nitrogen, as mentioned for casamino acids.

Conidia and chlamydospores produced in nitrate-malt extract or nitrate-glucose media and conidia produced in a medium containing casamino acids were harvested for further germination and shelf-life experiments.

Germination rate of freshly harvested conidia and chlamydospores

The germination rate of conidia produced in a medium supplemented with casamino acids was low (33.7 ± 13% germination) compared with the germination rate of conidia and chlamydospores produced in media supplemented with sodium nitrate (approx. 70-90% germination, Figure 4). Microcycle conidiation through direct conidiogenesis from a conidium or a chlamydospore (Figure 5) with minimal intervening hyphal development was observed after 4 h incubation on cellophane pieces. In our experimental conditions, formation of daughter conidia may result in increased conidia numbers with, as a consequence, a decrease in the conidial germination rate based on 100 spores examined. Microcycle conidiation for several decades has been considered a survival mechanism during stress for a variety of moulds (Ahearn *et al.*, 2007). Any enhancement of fungal dissemination would be a significant advantage for *P. alismatis* to be used as a mycoherbicide and, therefore, deserves further research.

Shelf-life of conidia and chlamydospores

A significant decrease in germination rate occurred by the first 2 weeks of storage at 25°C (Figure 6), regardless of the type of propagules, or culture conditions considered. Chlamydospores

produced in sodium nitrate-glucose or sodium nitrate-malt extract were able to germinate after 4 months storage at 25°C, while conidia produced in the same culture conditions declined rapidly (0% after 2 months storage). These results agree with other work ((Elzein & Kroschel, 2004) that shows the capability of chlamydospores to maintain viability during storage. In our conditions, stability requirements may be fulfilled through a chlamydospore-based mycoherbicide, provided their effectiveness against weeds is demonstrated in further phytopathogenic bioassays.

Despite a decrease in germination rate, conidia produced in casamino acids survived significantly better than conidia produced in sodium nitrate (6% germination after 40 days storage, Figure 6). Other studies showed that desiccation tolerance of *Paecilomyces fumosoroseus* blastospores was related to the nitrogen sources and concentrations added to the liquid medium (Cliquet & Jackson, 2005; Jackson *et al.*, 2003)

As a conclusion, these studies demonstrate that *P. alismatis* mycoherbicide may be based on chlamydospores, provided higher chlamydospore numbers can be obtained in liquid culture. Since previous experimental studies showed that increasing sodium nitrate and/or malt extract concentrations had no significant effect on chlamydospore yields (Cliquet et al, 2004), further work will focus on the impact of physical and chemical factors on chlamydospore production. Moreover, modifications of fungal morphology, pigmentation and microcycle conidiation in response to stress conditions open new perspectives of research.

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