Biological control of aquatic weeds by *Plectosporium alismatis*, a potential mycoherbicide in Australian rice crops: comparison of liquid culture media for their ability to produce high yields of desiccation-tolerant propagules

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**Summary**

A methodology to develop a stable, effective *Plectosporium alismatis* (Oudem.) Pitt, Gams and Braun [syn. *Rhynchosporium alismatis* (Oudem) J.J. Davis] mycoherbicide is currently being investigated. We compared a nitrate–malt extract medium liquid culture medium to other liquid media for their ability to support high conidial and chlamydospore yields and subsequent tolerance of conidia and chlamydospores to air-drying. When grown in a casamino acids–glucose-based liquid medium, *P. alismatis* developed hyphae and produced high yields of conidia (1 × 10⁷ conidia per millilitre) and dry weights (220 mg dry weight per erlen), while no chlamydospore was formed. In a nitrate–glucose-based medium, growth was poor, *P. alismatis* producing aggregated hyphae that contained 6.5 × 10⁴ chlamydospores per millilitre. The addition of nitrate in the casamino acids–glucose-based medium restored partially chlamydospore formation (1 × 10⁴ chlamydospores per millilitre). Chlamydospores and conidia were air-dried and stored at 25°C. No conidia germinated after 40 days storage, while 50% to 20% chlamydospores, respectively, produced in a nitrate–malt extract medium or in nitrate–glucose medium, remained viable after 120 days storage.

**Keywords:** fermentation, air-drying, storage, chlamydospores, conidia.

**Introduction**

The endemic fungus, *Plectosporium alismatis* (Oudem.) Pitt, Gams and Braun [syn. *Rhynchosporium alismatis* (Oudem) J.J. Davis] (Pitt et al., 2004) is being developed as a mycoherbicide (Crump et al., 1999) for the control of starfruit and other closely related weed species (Jahromi et al., 2001). The fungus sporulates abundantly on solid media (Jahromi et al., 1998) and is able to infect host species (Lanoiselet et al., 2001), leading to reduced biomass of the weed or to reduced seed set (Fox et al., 1999). A culture production method for the development of *P. alismatis* mycoherbicide is currently being investigated. *P. alismatis* produces high numbers of conidia in most liquid media; chlamydospore formation also occurs in a liquid standard medium based on the Czapex–Dox composition, in which nitrogen and carbon are provided by sodium nitrate (3 g l⁻¹) and malt extract (2.2 g l⁻¹; Cliquet et al., 2004).

We modified the carbon and the nitrogen sources and concentrations of the nitrate–malt extract medium to investigate the impact of nitrogen and carbon sources on both conidia and chlamydospore inductions. Shelf-life of air-dried conidia and of air-dried chlamydospores produced in modified carbon and nitrogen media was determined. Conidia and chlamydospores were air-dried and stored at 25°C and under ambient conditions. To evaluate their viability, conidia and chlamydospores were germinated on water agar (WA) and screened on rice leaf disk agar (RLDA) for the development of mycelium. Conidia and chlamydospores germinated after 40 days storage.

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sources was investigated and compared to shelf-life of air-dried propagules produced in the nitrate–malt extract medium.

**Materials and methods**

**Isolate**

*P. alismatis* RH 145 (DAR 73154) was isolated from *Damasonium minus* (R.Br) Buch., which was obtained from the culture collection of the New South Wales Department of Primary Industries. Stock cultures were maintained in a soil/sand mixture.

**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 to produce conidial suspensions for liquid culture as described hereafter.

**Media composition**

The basal mineral composition of nitrate–malt extract medium and defined medium was derived from a Czapex–Dox composition containing: 1.0 g K₂HPO₄; 1.0 g MgSO₄·7H₂O; 0.5 g KCl; 0.018 g Fe₂(SO₄)·7H₂O in 1 l deionized water. The nitrate–malt extract medium contained 3 g NaNO₃ (Sigma Chemicals, St. Louis, MO, USA) and 2.2 g malt extract (Amyl Media).

In the defined medium, malt extract was replaced by 2.4 g l⁻¹ glucose at the carbon concentration (920 mg C l⁻¹) provided by malt extract. The 3.12 g l⁻¹ NaNO₃ provided the same nitrogen content (0.5 g N l⁻¹) than in the nitrate–malt extract medium. Bacto yeast nitrogen base without amino acids and (NH₄)₂SO₄ (Difco) was provided as a nitrogen-free vitamin source (0.17 g l⁻¹). As the organic nitrogen source, 0.5 g N l⁻¹ technical casamino acids (Difco) was used. In shelf-life experiments, propagules were produced in media containing 3.68 g C l⁻¹ and 1 g N l⁻¹.

**Growth and harvest**

All cultures of 100 ml were placed in 250-ml flasks (Bellco Glass, Inc, Vineland, NJ, USA), inoculated with 4 × 10⁵ conidial suspension and incubated at 25°C on a rotary shaker incubator (Certomat BS-1 Braun Biotech International, Germany) at 100 rpm for 7 days for growth experiments or for 4 days for drying and storage experiments. 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 re-suspended in 50 ml distilled water. The suspension was fragmented in a Potter homogenizer (Fisher). Spore counts were performed using a haemocytometer.

**Chlamydospore and conidial germination**

Drops of the propagule suspension were placed on four 2-cm² pieces of cellophane on the surface of water agar plates. Cellophane pieces were removed after 12 h at 25°C and germination evaluated microscopically using lactophenol cotton blue as previously described (Cliquet et al., 2004).

**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 analysis of variance, standard errors values were estimated as a measure of variance.

**Results and discussion**

*P. alismatis* produced significantly higher yields of conidia when grown in casamino acids (1 × 10⁴ conidia per millilitre) compared to conidial yields produced in sodium nitrate (2 × 10³ conidia per millilitre; Fig. 1). When nitrate was the sole nitrogen source, 6.5 × 10⁴ chlamydospores per millilitre were observed; however, the addition of sodium nitrate to the medium containing casamino acids resulted in the production of less chlamydospores (1 × 10⁴ chlamydospores per millilitre; Fig. 1).

When grown in a medium containing casamino acids, *P. alismatis* produced numerous hyphae resulting in high dry weights with a maximum of 220 mg dry weight per erlen, compared to low dry weights (80 mg per erlen) obtained when sodium nitrate was the sole nitrogen source (Fig. 2). Homogenization of cultures showed that chlamydospores were mainly formed inside these aggregates.

In our culture conditions, replacing sodium nitrate by casamino acids as the sole nitrogen source and at the same nitrogen content improved growth as expressed by the high conidial yields and dry weights. 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 and Evans, 1984). The absence of chlamydospore formation in these conditions is likely the consequence of the addition of organic nitrogen.

In filamentous fungi, chlamydospore production may vary considerably depending on the nutritional environment, i.e. a nutrient excess or starvation conditions (Gardner et al., 2000). In our culture conditions, starvation due to a lack of organic nitrogen is likely responsible for chlamydospore formation.
Figure 1. Impact of the carbon and nitrogen sources on *Plectosporium alismatis* conidial and chlamydospore production. NaNO$_3$ + malt extract: sodium nitrate: 3 g l$^{-1}$; malt extract: 2.2 g l$^{-1}$; NaNO$_3$ + GLC: sodium nitrate + glucose (1); CA+GLC: casamino acids + glucose (2); NaNO$_3$+CA+GLC: sodium nitrate + casamino acids + glucose (3); In (1), (2) and (3), glucose provides 0.92 g C l$^{-1}$; nitrogen sources provide each 0.5 g N l$^{-1}$.

Figure 2. Influence of casamino acids concentration on production of chlamydoospores and conidia by *Plectosporium alismatis* (Na nitrate: 0.5 g N l$^{-1}$; casamino acids 4.7g l$^{-1}$ = 0, 5g N l$^{-1}$).
Conidia and chlamydospores produced in nitrate–malt extract or nitrate–glucose were air-dried, and germination was estimated during storage at 25°C (Fig. 3). The type of propagules, i.e. conidia or chlamydospores, had a significant impact on survival rate (Fig. 3). No conidia germinated after 40 days storage, while 50 to 20% chlamydospores, respectively, produced in a nitrate–malt extract medium or in nitrate–glucose medium remained viable after 120 days storage (Fig. 3). Microscopic observation showed that some chlamydospores produced in nitrate–glucose medium sporulated through a microcycle conidiation.

**Figure 3.** Shelf-life at 25°C of air-dried *Plectosporium alismatis* conidia and chlamydospores. *Plectosporium alismatis* was grown in a nitrate–malt extract medium (8.8 g l⁻¹ malt extract, 5.74 g l⁻¹ sodium nitrate) or in a nitrate–glucose medium (6 g l⁻¹ sodium nitrate, 10 g l⁻¹ glucose) and harvested after 7 days growth.
Conclusion

This work shows that numerous factors are to be investigated to develop a stable mycoherbicide. Survival during storage depends upon the type of propagules produced and upon the culture conditions during growth. Moreover, the microcycle conidiation observed during germination experiments may allow the fungus to extend rapidly and colonize aquatic weeds effectively. As a conclusion, chlamydospores may be promising stable propagules compared to conidia, although the nutritional conditions impact these qualities.

More work needs to be done to consider as many parameters (physical, chemical and morphological) as possible in an experimental design for the selection of factors that impact chlamydospore formation and tolerance to drying.

References


