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Characterization of aggregates produced by the potential mycoherbistat *Plectosporium alismatis* in submerged culture: germination, UV-radiation tolerance and infectivity

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Abstract

Plectosporium alismatis, a potential mycoherbistat of *Alismatacae* spp., has been previously shown to produce aggregates which contain chlamydospores in liquid culture. In this study we evaluated the impact of medium composition on the formation and composition of aggregates. In shake flask cultures using 5.74 gL⁻¹ sodium nitrate, 8.8 gL⁻¹ malt extract or glucose and 0.1% Tween 80, *P. alismatis* formed small, uniform (diameter of 75% aggregates <720µm), dense, melanised aggregates containing 10⁴ conidia and 10³ chlamydospores, these numbers remained unchanged during growth. One hundred percent of 7-day-old aggregates exposed to desiccation or/ and UV-radiation germinated. In bioassays using leaf discs of *Alisma plantago-aquatica*, *P. alismatis* aggregates caused necrosis, regardless of whether aggregates had been exposed to desiccation and/or UV-radiations prior to application on leaf discs, whereas other propagules (10³ propagules disc⁻¹) exposed to drying and UV-radiation stress were unable to cause necrosis. This preliminary research shows the potential of aggregates to be used as part of a formulation of biocontrol agents, provided adequate conditions for optimal aggregate yields are found.

Introduction

The endemic fungus *Plectosporium alismatis* (Oudem) W. M. Pitt, W. Gams & U. Braun (synonym *Rhynchosporium alismatis*, *Spermosporina alismatis*) has the potential to be used as a biological control agent of weeds species of *Alismataceae* (Cother and Gilbert 1994;

Pitt, Goodwin, Ash, Cother and Cother 2004). The fungus reduces the biomass of the weed (Fox, Cother and Ash 1999), especially when used with low rates of the herbicide Londax (Jahromi, van de Ven, Cother and Ash 2006).

With the goal of developing a low-cost, submerged culture production process that yields high concentrations of viable, virulent *P. alismatis* propagules, we are currently evaluating how culture conditions may impact *P. alismatis*'s growth and morphology in liquid culture.

Recent studies have shown that high *P. alismatis* conidial numbers and dry weight accumulation can be easily produced in media that contain an organic nitrogen source (such as casamino-acids, yeast extract, or tryptone (Cliquet and Zeeshan 2008)). In contrast, in a liquid culture medium based on the Czapek Dox composition, in which nitrogen is provided by sodium nitrate, *P. alismatis* produces low

conidial yields and interestingly, chlamyospores (Cliquet, Ash and Cother 2004), which make ideal candidates for the development of a stable mycoherbicide (Jackson and Schisler 2002).

In our sodium nitrate, malt extract based medium, chlamyospores are found mostly inside non-uniform, dense, brown, melanized aggregates (Moulay, Cliquet, Zeeshan, Ash and Cother 2008; Cliquet and Zeeshan 2008). Homogenization of cultures is required in order to release chlamyospores. Chlamyospores are promising propagules showing viability after 4 months storage at 25°C (Moulay et al. 2008; Cliquet and Zeeshan 2008) and tolerance to UV-radiation exposure (Texier, Davy and Cliquet 2009). These findings indicate that chlamyospores may be viable from September to November, the period of rice sowing in New South Wales, Australia (Lanoiselet, Cother, Ash and van de Ven 2001).

However, homogenization of aggregate-producing cultures in order to release chlamyospores represents an additional step in the development of the mycoherbistat, which is incompatible with the development of an economical, large-scale mass production method. On the other hand, some plant pathogenic fungi of weedy plants produce conidia or other forms of propagules, including microsclerotia (Jackson and

Schisler 1995; Boyette, Jackson, Bryson, Hoagland, Connick and Daigle 2007), which can be variously formulated and applied as bioherbicides. The selection of culture conditions that favor microsclerotia production and stability after air-drying has been reported (Shearer and Jackson 2006), demonstrating the ability of microsclerotia to act as potential biocontrol agents for weeds. *P. alismatis* Aggregates may represent promising microgranules for the development of a stable and effective bioherbicide to be applied directly in rice fields.

Therefore, the objective of this study was to determine the yield, size and composition of *P. alismatis* aggregates produced in liquid culture. They were then compared to conidia and chlamydospores for their ability to survive drying or/and UV-radiation exposure, and for their ability to cause necroses on *Alisma plantago-aquatica* leaf discs.

Materials & Methods

Liquid culture experiments

Plectosporium alismatis DAR 73154 was obtained from the culture collection of the New South Wales Plant Pathology

Herbarium held by NSW Department of Primary Industries, Orange, Australia. The fungal pathogen was originally isolated from *Damasonium minus* (R.Br) Buch and maintained as a single conidium culture at -80°C as previously described (Texier et al., 2009).

Stock solutions were stored as single spore cultures grown on PDA (Difco, Detroit, MI, USA), cut into plugs and stored at -80°C . A suspension of frozen cultures was inoculated onto a PDA plate, incubated for 2-3 weeks at 25°C until profuse sporulation occurred and renewed every month. Plates were gently washed with 3 mL sterile distilled water and conidial suspension (approx. 1×10^7 conidia mL^{-1}) stored at -80°C . Frozen conidial suspensions were inoculated (4×10^3 conidia mL^{-1}) into shake flaks for liquid culture.

Liquid cultures were based on a mineral composition as follows: K_2HPO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; KCl , 0.5 g; $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g; deionized water: 1 L. For chlamydospore and aggregate production, malt extract (Difco Laboratories, Detroit, MI, USA, 8.8 g L^{-1}) or glucose. $\cdot\text{H}_2\text{O}$ (3.68 g C L^{-1}) and sodium nitrate (Sigma Chemicals, St Louis, MO, USA, 5.74 g L^{-1}) were added to the mineral medium as the sole carbon and nitrogen sources (respectively 3.68 g C L^{-1} and 1 g N L^{-1} ; Cliquet et al. 2004). Flasks containing the malt

extract, sodium nitrate medium (100 mL medium in 250 mL flasks) were inoculated with conidia (4×10^3 conidia mL⁻¹). Cultures were incubated at 25°C on a rotary shaker incubator (Infors HT Bottmingen, Switzerland). Cultures were hand-shaken daily to prevent the formation of a mycelium ring on the flask wall.

In medium composition experiments, polyoxyethylene sorbitan mono-oleate (0,1% Tween 80, Alfa aesar GmbH &Co, Karlsruhe, Germany) was added to the malt extract, sodium nitrate medium. A defined medium was prepared in which malt extract was replaced by glucose.H₂O (8.8 g L⁻¹, providing 3.68 g C L⁻¹). The initial pH was adjusted to 7 and maintained by daily addition of 1N HCl or 1N NaOH. For each treatment, 4 flasks were used: 2 flasks for biomass accumulation measurement, 2 flasks for propagule (i.e. conidia and chlamyospores) and aggregate yields determination. The malt extract, sodium nitrate medium supplemented with Tween 80 was used to produce conidia, chlamyospores and aggregates in all growth kinetics, drying, UV-radiation exposure, and infectivity studies.

In growth kinetics experiments, flasks were shaken for 7 days at 25°C and 120 rpm. Four replicate flasks were harvested after 2, 3, 5, 6 or 7 days incubation.

Evaluation of aggregate yields, diameter and circularity

Cultures from duplicate flasks were poured each into 6 Petri dishes (Ø 140 mm) containing 0.1% Tween 80 and photographed. The image analysis software Image J (version 1.41, NIH, USA) was used to evaluate aggregate concentrations, diameter and circularity. The degree of circularity was measured using an ordinal scale which ranged from 0 (irregular aggregates) to 1 (spherical aggregates).

Evaluation of propagule counts and dry weights

Liquid fungal cultures from duplicate flasks were harvested for the determination of free, total propagule (conidia and chlamyospores) counts and dry weights. Free conidia and chlamyospore concentrations were measured microscopically using a hemocytometer after appropriate dilution of whole cultures. For dry weight determination, duplicate whole cultures were vacuum-filtered on a pre-dried, weighed 110 mm Ø cellulose filter (Whatman Plc, Brentford, UK), and washed with 50 mL deionized water. Filters were allowed to

dry in a pan on the bench top until constant weight was achieved and then final weight was recorded.

For total propagule determination, dry fungal mats were suspended in 21.5 mL sterile distilled water and fragmented in a Potter homogenizer (Fisher Scientific Bioblock, Illkirch, France). Total counts in the homogenised suspension were determined microscopically. The concentration of conidia and chlamydo spores in aggregates was determined as the ratio $[(\text{total propagules L}^{-1}) - (\text{free propagules L}^{-1})] / (\text{total aggregates L}^{-1})$

Preparation of aggregates

Whole cultures were vacuum-filtered on cellulose filters in a Buchner-funnel and rinsed with 50 mL distilled water in order to remove the spent medium. Pin-head size aggregates were collected from cellulose filters for further investigations.

Drying experiments

Whole cultures (2 flasks) were homogenised to release propagules, vacuum-filtered on cellulose filters and placed in a

pan at room temperature until dry mats reached a constant weight (12 h).

Whole cultures (2 flasks) containing aggregates were vacuum-filtered on cellulose filters and placed in a pan on the bench top, at room temperature, until constant weight (12h). The moisture content of dried whole cultures measured using a halogen moisture analyzer (Ohaus, MB 45) was equal to 6.5%.

Chlamyospore, conidial and aggregate viability

Seven-day old cultures harvested from 2 duplicate flasks were homogenised. Propagule suspensions were rinsed and adjusted with sterile distilled water to 5×10^6 propagules mL^{-1} . Serial dilutions were sprayed on the surface of PDA plates. Plates were incubated at 25°C for 5 days and colony forming units (c.f.u.) were counted. For the determination of dry propagule viability, aliquots from homogenised dry mats on cellulose filters were gently rehydrated with sterile distilled water and adjusted to 5×10^6 propagules mL^{-1} . Serial dilutions were sprayed on PDA and colony forming units determined, as previously mentioned.

For the determination of aggregate viability, 100 aggregates 700 μm average diameter, which composed the majority of aggregates in 7-day old cultures, were collected from wet or dry cellulose filters. Aggregates were sprinkled on PDA and water agar plates. Germination was evaluated microscopically by observing hyphal germination. Preliminary germination experiments showed that all aggregates placed on PDA germinated after 18h incubation at 25°C whereas 46 % aggregates placed on water agar germinated after 40h incubation at the same temperature. Consequently, PDA was selected for further germination experiments.

Exposure of propagules and aggregates to UV-radiation

Irradiation experiments were conducted in a dark cabinet as previously described (Texier et al. 2009). Triplicate 200- μL droplets of freshly-harvested, homogenised suspension (5×10^6 propagules mL^{-1}), or from homogenised rehydrated dry fungal mats (5×10^6 propagules mL^{-1}) were placed in a petri dish (89 mm diameter) (Greiner Bio-one 34/15 with vents, Courtaboeuf, France) covered with plastic lid, at pre-specified position at a distance of 30 cm under the UV-lamp, and droplets of propagule suspensions were

simultaneously placed in a dark cabinet with no lamp (controls). Temperature and relative humidity in the dark cabinets remained constant (21°C, RH = 75%) during the experiment.

Duplicate hundred freshly-harvested aggregates, or duplicate hundred dry aggregates, were placed in a Petri dish and exposed to UV-radiation under the same irradiation parameters to those applied to propagules. In order to prevent dehydration, distilled water was placed beneath the plates. Preliminary experiments (data not shown) indicated that 30% propagules and 70% aggregates germinated following UV-radiation exposure of 45 min. This exposure time to UV-radiation (UV-B dose = 3.6 kJm⁻²; UV-A dose = 0.72 kJm⁻²) is compatible with doses given for full-spectrum sunlight (Ghajar, Holford, Cother and Beattie 2006) and was selected for further studies. At the end of UV-radiation exposure, 100-µL were taken from the 200-µL propagule droplet, sprayed onto water agar plates and cfu counts determined after 4 days incubation at 25°C.

Aggregates were placed on PDA plates and microscopical observation of hyphal germination performed after 18h incubation at 25°C. Duplicate flasks were used for propagule and aggregate UV-exposure experiments.

Infectivity on leaf discs

Alisma plantago-aquatica is naturally infected by *P. alismatis* (Lanoiselet et al. 2001) and has been recently found to grow naturally in La Retenue de Moulin-Neuf, moulin de Kerbenoc'h, Plonéour-Lanvern, Brittany, France.

Leaves were surface sterilized with 1% chlorine and rinsed with sterile distilled water. Leaf discs were cut with a 10 mm diameter cork borer and placed on the surface of 1.5% water granulated agar (Difco) containing $1 \mu\text{g mL}^{-1}$ of benzylaminopurine (Acros Organics N.V., Thermo Fischer Scientific, Belgium) in plastic Petri dishes. Four replicate discs were inoculated with 0.2 mL of the appropriate dilutions of a propagule suspension taken from a seven-day-old homogenised culture, ranging from 10^1 to 10^3 propagules per disc, or with aggregates collected from cellulose filters (1 or 10 aggregates per disc). Two discs were inoculated with sterile distilled water. The entire experiment was repeated once.

Leaf discs affected by necrosis and chlorosis were examined 5 d after inoculation. As different methods of inoculation were used (either with a propagule suspension or by placing an aggregate directly onto the leaf surface, the use of either the percentage of necrotic/chlorotic leaf disc or an

ordinal scale in the range 0-10 (no symptom-entire disc chlorotic/necrotic, Cother and van de Ven (1999)), was not considered relevant. Instead, a simple score (no symptom) or +1 (chlorotic/ necrotic area) was selected.

Statistical analysis

All experiments were repeated at least once.

Aggregate diameter and circularity frequency distribution were performed using Statgraphics Plus V.4. (Toulouse, France).

Anova analysis and Duncan mean separation tests ($P < 0.05$) based on mean values for propagule and aggregate yields, were performed using Statgraphics. For data not suitable for ANOVA, standard errors values were estimated as a measure of variance.

Results and discussion

In media supplemented with nitrate, (providing 100% N in glucose-based medium; providing 85% N in malt extract-based medium, 15% being provided by malt extract), *P. alismatis* produced non-uniform, melanized, dense aggregates ranging in size from a few micrometers to more than one millimeter

diameter (Table 1) whereas no hyphal suspension was observed in cultures. Nitrate has been shown to be a non-preferred nitrogen source, partially utilized by *P. alismatis* (Cliquet and Zeeshan 2008) which, in these nitrogen limiting, stressful conditions, produces low dry weight accumulation and low aggregate yields (Table 1).

Further homogenization and microscopic observation of the three 7-day-old cultures showed that all aggregates contained chlamydospores (1×10^3 chlamydospores aggregate⁻¹) as previously reported (Cliquet and Zeeshan 2008) and conidia (1×10^4 conidia aggregate⁻¹; Table 1). Although aggregate yields were low (1.9×10^5 L⁻¹), the addition of Tween 80 in the liquid culture and enhanced agitation speed resulted in approximately 10% increase in aggregate yields (Table 1). Moreover, aggregates produced under these conditions were small, spherical (circularity = 0.9, data not shown) and uniform (aggregate diameter uniformity, expressed as relative frequency distribution : 75% aggregate diameter < 720 μ m, Table 1). Fungal aggregates were observed from day 2 (Table 2), becoming little nodes visible by naked eye at seven days growth (680 μ m average diameter, Table 2) and eventually turning dark which were visible as dark pin-point to pin-head size bodies (Fig. 1).

In aggregates harvested from cultures grown in malt extract, sodium nitrate and Tween 80, dry weight accumulation, total propagule numbers, free propagule numbers and propagule density evaluated from day 3 to day 7 varied slightly, (Table 2). These results indicated that once inoculated into the liquid media, conidia probably agglomerated into aggregates and produced conidia and chlamydospores through germination and microcycle conidiation as previously reported (Cliquet and Zeeshan 2008; Table 2). This could be attributed to limited nitrogen available for growth.

Likewise, abbreviated, asexual cycles, with short hyphae becoming agglomerated into aggregates have been reported in fungi grown in stressful environments; among the number of culture conditions able to modify fungal morphology, medium composition, and specifically limitation of particular nutrients including nitrogen, appear significantly influential (Braun and Vecht-Lifshitz 1991; Gibbs, Seviour and Schmid 2000). Nitrogen starvation, limited O₂ and poor nutrient diffusion inside aggregates are likely responsible for low dry weights, low aggregate numbers and chlamydospore formation as previously mentioned (Gibbs et al. 2000).

Tween 80 added to a slurry of *Metarhizium anisopliae* cells in water has been shown to induce cell aggregation, which is the first phase in the process of developing carrier-free granulates for combating pests and treating plants (Andersch et al., 1995). The impact of Tween 80 appears to be strain-dependant, either improving the size of *Rhizopus nigricans* pellets (Zindarzac, Komel and Pavko 2000) or inhibiting pellet formation of *Trichoderma risaii* (Domingues, Queiroz, Cabral and Fonseca 2000). Tween 80 probably interacts with cell wall hydrophobic surfaces, whose properties may differ in different biological systems, resulting in propagule cohesion. Smaller aggregates may be the result of shear forces developed at higher speed (120 rpm), resulting in disintegration (Gibbs et al. 2000) when compared to aggregates formed in media shaken at 100 rpm without Tween 80.

Studies related to industrial application of enzyme-producing fungi indicate that fungal physiological state depends, among other factors, on the size of aggregates. Small, uniform aggregates may offer less of a diffusional barrier to O₂ and nutrients thus leading to increased yields and a better physiological state (Gibbs et al. 2000). Moreover, studies on *Penicillium chrysogenum* have

shown that pellets with diameters less than 400 μm consist of actively metabolizing cells (Schügerl, Wittier and Lorenz 1983). Therefore, Tween 80 and agitation speed may play a significant role in the development of a viable mycoherbistat. This is provisional on aggregate yields optimized through the identification of suitable carbon and nitrogen sources and concentrations. Additional multi-factorial studies are required to determine under which conditions aggregate yields can be increased and whether aggregate size and viability are correlated.

Microscopic observation of 7-day old cultures showed that aggregates in their final form were composed of hairy compact, microsclerotia-like masses (Fig. 2A) containing chlamydospores (Fig. 2B). Other studies related to submerged liquid culture fermentation have demonstrated that, in specific nutritional conditions, plant pathogenic fungi produce melanized aggregates that happen to be microsclerotia (Jackson and Schisler 1995; Shearer and Jackson 2003, 2006). The microsclerotia stability and effectiveness in soil and in aquatic environment have been reported as having potential for use as bioherbicides (Boyette et al. 2007; Shearer and Jackson 2006).

However, according to Ainsworth's definition (1971) a microsclerotium is a "firm, rounded, mass of hyphae with or

without the addition of host tissue or soil, normally having no spore in or on it". Comparing this definition to the way aggregates are formed and functioned is concluded that the term MS is likely inappropriate for multi-celled aggregates containing conidia and chlamydo spores observed in the present study.

Survival of *P. alismatis* to UV-exposure and /or drying was significantly influenced by the type of fungal structures (propagules, i.e.: conidia and chlamydo spores, or aggregates) exposed to stress (Table 3). Seven-day-old propagule germination expressed as [(% c.f.u. at 5d incubation/total c.f.u.) x 100] was significantly reduced (more than 80% suppression) after drying and subsequent UV-radiation exposure (Table 3). Similarly, a drastic drop in conidial germination due to drying and subsequent UV-radiation exposure has been simulated at the laboratory and previously reported (Texier et al. 2009).

In contrast, it is interesting to note that less than 5% of the 7-day old, dried aggregates exposed to UV-radiation failed to germinate after 18h on PDA (Table 3). Considering the 7-day-old propagule concentration per aggregate (approx. 10^4 conidia and 10^3 chlamydo spores aggregate⁻¹,

Table 2), we suggest that a significant number of propagules survive stress and germinate. Moreover, although the drying process at room temperature is similar for both propagules and aggregates, drying rate in the aggregate center is probably slower than drying speed of exposed, homogenised propagules, resulting in differences in viability as reported elsewhere (Cliquet and Jackson 1997). In addition, internal cells are likely protected from UV-radiations by the aggregate external layers.

While chlamydospores and mycelial structures such as microsclerotia are mentioned as overwintering structures, literature on the impact of environmental effects on fungal structures is scarce. Surprisingly, viability of dried, UV-exposed aggregates was higher ($97\pm 8\%$) compared to viability of freshly-harvested, UV-exposed aggregates ($70\pm 6\%$). Although the detrimental impact of desiccation or UV-radiation exposure experimented as one-factor at-a-time is widely reported, interactions between these environmental factors can impact significantly fungal viability (Texier and Cliquet, 2009). Because moisture level (Shabana, Baka and Abdel-Fattah 1997), and UV-radiation (Ghajar et al. 2006) are among the most constraining factors for the development of mycoherbivores, the impact of these factors

and of their interaction on the viability of potential mycoherbivores deserve more attention.

A. plantago-aquatica leaf discs inoculated with freshly-harvested propagules (conidia and chlamydospores) developed a primary infection, expressed by necrosis at inoculation site after 5 d incubation compared to healthy water controls. Similarly, freshly-harvested aggregates infected *A. plantago-aquatica* leaf discs (Table 4). These results demonstrate that the sodium nitrate, malt extract medium supplemented with Tween 80 supports production of propagules and aggregates able to incite disease in *Alisma plantago-aquatica*. However, thresholds of 10^2 freshly harvested propagules disc⁻¹, and 10^3 freshly-harvested, UV-exposed propagules disc⁻¹ were required in order to induce leaf infectivity, whereas dry propagules were not able to cause disease, regardless of whether they were exposed to UV-radiation prior to pathogenicity tests or not (Table 4). These data indicate that drying is likely more stressful than UV-radiation in the infectivity process. This contrasts with the observation that the drying process of propagules was less detrimental than UV-radiation exposure on germination (49% and 30% propagule germination, respectively, Table 3). UV-radiation may induce microcycle

conidiation or stimulate appressoria formation as reported by Ghajar et al. (2006), with as a consequence, increased *P. alismatis* infectivity. Further studies are needed to evaluate more accurately how fungal attributes may impact the processes involved in early stages of infection.

In conclusion, these studies demonstrate that in specific culture conditions, aggregates containing chlamydospores and conidia are produced. The tolerance of aggregates to drying and UV-radiation exposure shows the potential of the use of aggregates as part of a formulation of to be further investigated as biocontrol agents, provided high yields can be obtained. Further work is needed to find adequate nutritional and physical conditions as well as time of incubation that enhance aggregate yields, stability and effectiveness. In addition, the relationship between cultural age and stability, as well as possible sporogenesis from aggregates will have to be evaluated.

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References

- Ainsworth, G.C., James, P.W., and Hawkworth, D.L. (1971),
 '*Dictionary of the Fungi*', Kew, UK: Commonwealth
 Mycological Institute.
- Andersch, W, Hartwig, J, Homeyer, B, Stenzel, K, 1995.
 Self-supporting carrier-free granulates for combating
 pests and treating plants. U. S. Patent #5,418,164)
- Boyette, C., Jackson, M.A., Bryson, C., Hoagland, R.,
 Connick, W., and Daigle, D. (2007), '*Sesbania exaltata*
 biocontrol with *Colletotrichum truncatum* microsclerotia
 formulated in 'Pesta' granules', *Biocontrol*, 52, 3,
 413-426
- Braun, S., and Vecht-Lifshitz, S.E. (1991), 'Mycelial
 morphology and metabolite production', *Trends in*
 Biotechnology, 9, 63-68.
- Cliquet, S., and Jackson, M. A. (1997), 'Comparison of Air-
 Drying Methods for Evaluating the Desiccation Tolerance

of Liquid Culture Produced *Paecilomyces fumosoroseus* spores', *World J. Microbiology and Biotechnology*, 13, 299-303.

Cliquet, S., Ash, G., and Cother, E.J. (2004), 'Conidial and chlamyospore production of *Rhynchosporium alismatis* in submerged culture', *Biocontrol Science and Technology*, 14, 8, 801-810.

Cliquet, S., and Zeeshan, K. (2008), 'Impact of nutritional conditions on yields, germination rate and shelf-life of *Plectosporium alismatis* conidia and chlamyospores as potential candidates for the development of a mycoherbicide of weeds in rice crops', *Biocontrol Science and Technology*, 18, 685-695.

Cother, E.J., and Gilbert, R.L. (1994), 'Efficacy of a potential mycoherbicide for control of *Alisma lanceolatum* and *Damasonium minus* in Australian rice crops', *Australian Journal of Experimental Agriculture*, 34, 1043-1050.

Cother E.J., and van de Ven, R. (1999), 'The influence of nutrition on conidial production by *Rhynchosporium*

alismatis and on their subsequent infectivity to *Alisma lanceolatum*', *Biocontrol Science and Technology*, 9, 3, 395-407.

Domingues, F.C., Queiroz, J.A., Cabral, J.M., and Fonseca, L.P. (2000), 'The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30' *Enzyme and Microbial Technology*, 26, 394-401.

Fox, K.M., Cother, E.J., and Ash, G.J. (1999), 'Influence of *Rhynchosporium alismatis* on seed production and viability of the rice paddy weed *Damasonium minus* (starfruit)', *Australasian Plant Pathology*, 28, 197-199.

Gardner, K., Wiebe, M.G., Gillespie, A.T., and Trinci, A.P.J. (2000), 'Production of chlamydospores of the nematode-trapping *Duddingtonia flagrans* in shake flask culture', *Mycological Research*, 104, 205-209.

Ghajar, F., Holford, P., Cother, E., and Beattie, A. (2006), 'Effects of ultraviolet radiation, simulated or as natural sunlight, on conidium germination and

appressorium formation by fungi with potential as mycoherbivores', *Biocontrol Science and Technology*, 16, 451-469.

Gibbs, P.A., Seviour, R.J., and Schmid, F. (2000), "Growth of filamentous fungi in submerged culture: problems and possible solutions", *Critical Reviews in Biotechnology*, 20, 17-48.

Jackson, M.A., and Schisler, D.A. (1995). 'Liquid culture production of microsclerotia of *Colletotrichum truncatum* for use as bioherbicidal propagules', *Mycological Research*, 99, 879-884.

Jackson, M.A., and Schisler, D.A. (2002), 'Selecting Fungal Biocontrol Agents Amenable to Production by Liquid Culture Fermentation', in *Proceedings of the 7th meeting of the IOBC OILB, Kusadasi, Turkey: IOBC/WPRS Bulletin*.

Jahromi, F., Van de Ven, R., Cother, E.J., and Ash, G.J. (2006), 'The interaction between *Plectosporium alismatis* and sublethal doses of bensulfuron-methyl reduces the growth of starfruit (*Damasonium minus*) in

rice", *Biocontrol Science and Technology*, 16, 9, 929-940

Lanoiselet, V., Cother, E.J., Ash, G.J., and Vandeven, R. (2001), 'Production, Germination and Infectivity of Chlamydospores of *Rhynchosporium alismatis*', *Mycological Research*, 105, 441-446

Moulay, C., Cliquet, S., Zeeshan, K., Ash, G.J., and Cother, E.J., (2008), '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', In: *Proceedings of the XII International Symposium on Biological Control of Weeds, La Grande Motte, France*, pp. 306-310.

Pitt, W.M., Goodwin, S.B., Ash, G.J., Cother, N.J., and Cother, E.J. (2004), '*Plectosporium alismatis* comb. nov. a new placement for the *Alismataceae* pathogen *Rhynchosporium alismatis*', *Mycological Research*, 108, 7, 775-780.

Schügerl, K., Wittier R., and Lorenz, T. (1983), 'The use of molds in pellet form', *Trends Biotechnol.*, 1, 120-123.

Shabana, Y.M., Baka Z.A.M., and Abdel-Fattah G.M. (1997), '*Alternaria eichhorniae*, a biological control agent for waterhyacinth: mycoherbicide formulation and physiological and ultrastructural host responses', *European Journal of Plant Pathology*, 103, 99-111.

Shearer, J.F., and Jackson, M.A. (2003), 'Mycoherbicide compositions and methods of preparing and using the same', *US Patent 6,569,807 B1*.

Shearer, J.F., and Jackson, M.A. (2006), 'Liquid culturing of microsclerotia of *Mycoleptodiscus terrestris*, a potential biological control agent for the management of hydrilla', *Biological Control*, 38, 298-306

Texier, S., Davy, M., and Cliquet, S. (2009), 'Culture age impacts *Plectosporium alismatis* propagule yields and subsequent desiccation and UV-radiation tolerance', *Biocontrol Science and Technology*, 19, 277-288.

Zindaršič, P., Komel, R., and Pavko, A. (2000), 'Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets', *World Journal of Microbiology and Biotechnology*, 16, 589-593.

Legends of figures and tables Cliquet et al., "Characterization of *Plectosporium alismatis* aggregates"

Figure 1. Seven-day-old aggregates produced by *Plectosporium alismatis* in liquid media containing malt extract, sodium nitrate and supplemented with 0,1% Tween 80

A macroscopic observation
B,C microscopic observation

Table 1. The impact of the medium composition on propagule and aggregate production by *Plectosporium alismatis* in submerged culture

Table 2. Growth of *Plectosporium alismatis* in a malt extract, sodium nitrate medium supplemented with 0.1% Tween 80

Table 3. The impact of UV-radiation exposure on the germination rate of *Plectosporium alismatis* propagules (conidia and chlamydospores) and aggregates produced in submerged culture

Table 4. The impact of propagule or pellet UV-radiation exposure and/or dessiccation on the disease caused by *Plectosporium alismatis* on *Alisma plantago-aquatica* leaf discs

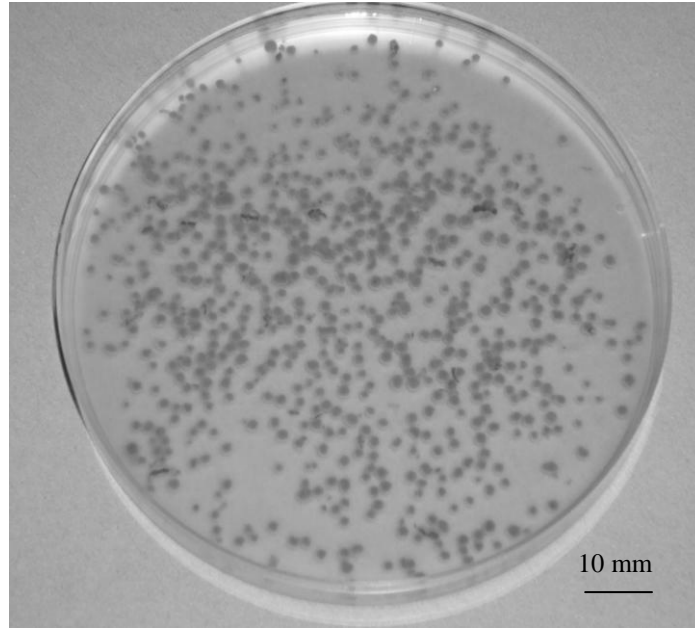


Figure 1A

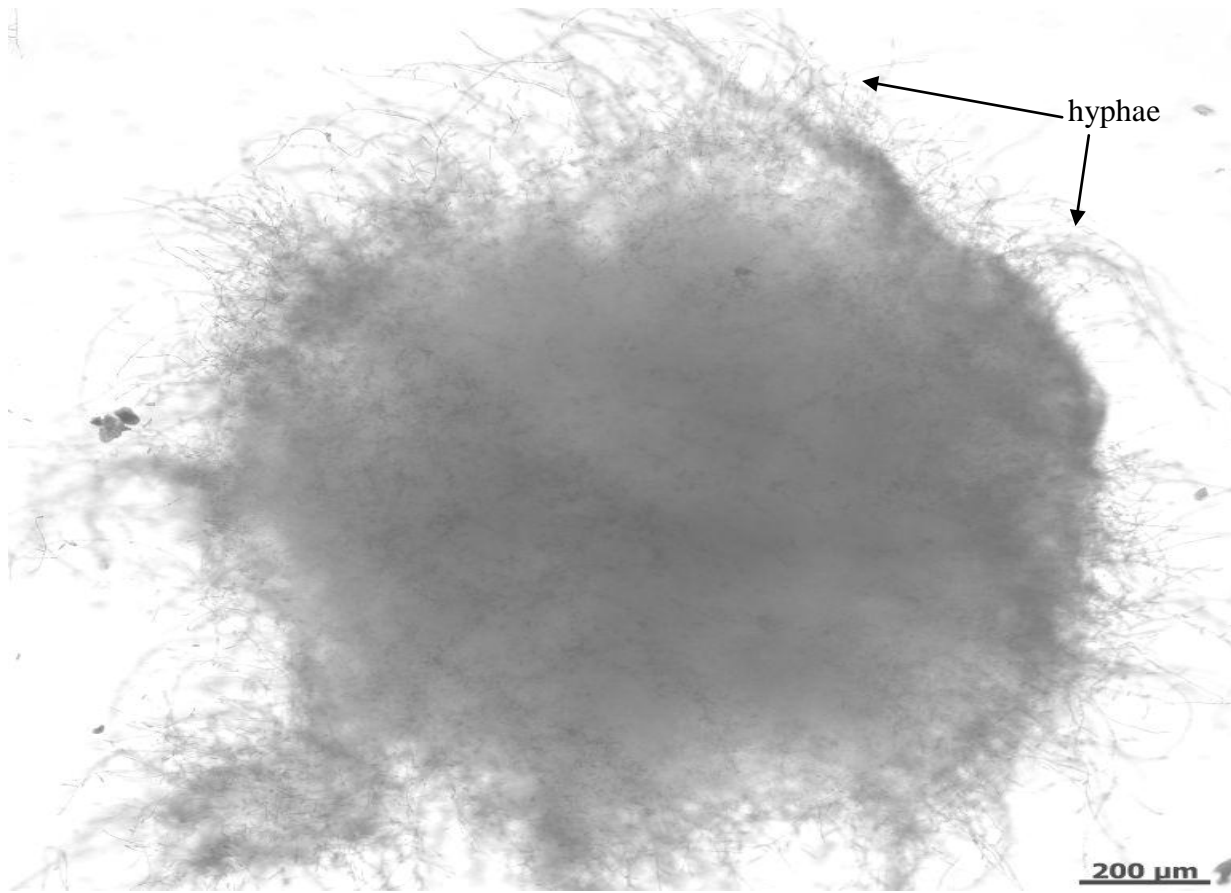


Figure 1B

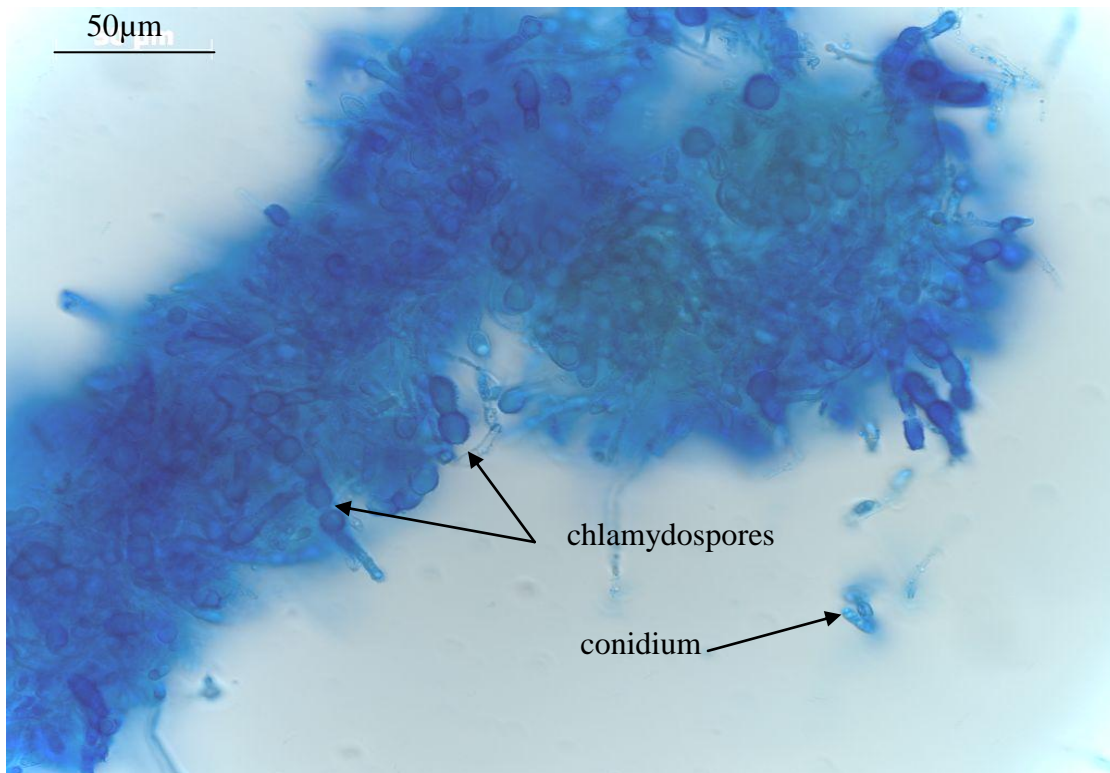


Figure 1C

Table 1. The impact of the medium composition on propagule and aggregate production by *Plectosporium alismatis* in submerged culture

Growth conditions				Propagules ^b			Aggregates					
Carbon source	Sodium	Tween	Agitation	Dry	Total	Total	Numbers	As relative frequency, %				
g L ⁻¹	nitrate	80	(rpm)	weights	Conidia	Chlamydo spores	(x10 ⁵ L ⁻¹)	(As total volume distribution (%)) ^c				
	g L ⁻¹	(%)		mg mL ⁻¹	x10 ⁵ mL ^{-1b}	x10 ⁴ mL ^{-1b}		x=diameter (µm)				
								0<x<360	360<x<720	720<x<1080	1080<x<1440	x>1440
Glucose.H ₂ O 8.8	5.74	0	100	1.4 ± 0.1	9.2 ± 1.5	3.4 ± 0.7	0.05±0.001	31 (0.01)	20(0.15)	9 (0.31)	5 (0.47)	33 (99.07)
Malt extract 8.8	5.74	0	100	3.5 ±0.08	25 ± 3.5	29.1 ± 2.4	0.15±0.02	24 (0.01)	11(0.07)	13 (0.37)	10 (0.77)	40 (98.79)
Malt extract 8.8	5.74	0.1	120	2.5 ±0.04	50.0±1.3	32.0 ± 1	1.9±0.2	25 (0.02)	50(1.18)	10 (1.1)	4.6 (1.38)	10 (96.31)

^a*P. alismatis* was grown in flasks containing 100 mL liquid medium and shaken for 7 days at 25°C, with pH daily adjusted to 7.

^bProduction of conidia and chlamydo spores during growth evaluated after homogenisation of whole culture, including aggregates.

^c Volume calculated from each class midpoint; for x>1440µm, a mean value = 2000µm was selected

Table 2. Growth of *Plectosporium alismatis* in a malt extract, sodium nitrate medium supplemented with 0.1% Tween 80

Culture age ^a (days)	Dry weights	Aggregate production		Conidia			Chlamydo spores		
	Mg mL ⁻¹	Mean diameter µm	Aggregate yields x10 ⁵ L ⁻¹	Total ^c x10 ⁹ L ⁻¹	Free ^d x10 ⁹ L ⁻¹	x10 ⁴ aggregate ⁻¹ ^e	Total ^c x10 ⁸ L ⁻¹	Free ^d x10 ⁸ L ⁻¹	x10 ³ aggregate ⁻¹ ^e
2	0.92±0.23 ^f	-	0,9±0.2	0.5	-	-	<10 ⁷	<10 ⁷	<10 ³
3	1.0±0.1	520±20	1.1±0.5	6.5±0.8	4.2±0.7	0.9±0.26	1.0±0.09	0.8±0.06	0.3±0.04
5	1.5±0.1	540±60	1.6±0.2	6.1±0.7	5.1±0.5	0.6±0.2	2.8±0.2	1.0±0.08	1.2±0.5
6	1.4±0.2	650±30	1.5±0.2	6.5±0.6	5.2±0.4	0.8±0.2	3.3±0.3	3.2±0.3	0.7±0.2
7	1.9±0.4	680±12	1.2±0.3	4.5±0.7	3.1±0.4	1.1±0.04	3.2±0.14	2.1±0.2	1.1±0.01

^a*P. alismatis* was grown in medium containing 0.1% Twin 80 and supplemented with 8.8 g L⁻¹ malt extract, 5.74 g L⁻¹ sodium nitrate. Flasks were shaken at 25⁰C and 120 rpm and pH maintained daily at 7.

^b Aggregate circularity range 0-1, 0: irregular aggregates; 1: spherical aggregates.

^c Total counts after whole culture homogenisation

^d in whole culture

^e calculated from difference between total counts and free counts and related to aggregates

^f standard error (SE)

Table 3. The impact of UV-radiation exposure on the germination rate of *Plectosporium alismatis* propagules (conidia and chlamydo spores) and aggregates produced in submerged culture

	Propagule germination (%) ^a		Aggregate Germination (%) ^b	
	Control	UV-radiation exposure ^c	Control	UV-radiation exposure
Freshly-harvested	89.5 ± 10 ^d	30 ± 3	81.5 ± 1	70.5 ± 6
After desiccation ^e	49 ± 5	16 ± 4.2	100 ± 10	97 ± 8

^a Propagule(conidia and chlamydo spores) germination: [c.f.u. / c.f.u. freshly-harvested propagules]x100

^b Hundred aggregates were sprinkled on PDA and hyphal germination evaluated microscopically after 20h at 25°C

^c transmission of UV-B and UV-A radiations

^d %germination ± s.e.

^e propagules and aggregates were dried overnight at room temperature

1 **Table 4.** The impact of propagule or pellet UV-radiation exposure and/or desiccation on the disease caused by
 2 *Plectosporium alismatis* on *Alisma plantago-aquatica* leaf discs

Treatment	Propagule numbers per leaf disc ^a				Aggregate number per leaf disc		
	0 ^b	10±3 ^c	100±30 ^d	1000±42	0	1	10
Freshly harvested	- ^f	-	+	+	-	+	+
Freshly-harvested, UV-exposed ^e	-	-	-	+	-	+	+
Dried	-	-	-	-	-	+	+
Dried, UV-exposed	-	-	-	-	-	+	+

3 ^a serial dilutions of 7 day-old homogenised cultures

4 ^b Control: sterile distilled water 200 µl

5 ^c based on appropriate dilution

6 ^d propagule numbers ± s.e

7 ^e transmission of UV-B and UV-A radiations

8 ^f no chlorosis/necrosis observed; +: chlorosis/necrosis observed

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