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ORIGINAL PAPER

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Production of the fungal biocontrol agent *Ulocladium atrum* by submerged fermentation: accumulation of endogenous reserves and shelf-life studies

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Abstract A method was developed for the induction of submerged conidiation of *Ulocladium atrum* Preuss (isolate 385) for the first time, using an oatmeal extract broth. Two inoculum types were produced by this process: spores and mycelial fragments. Spore production was stimulated by reducing the broth water potential (Ψ) to –2.1 MPa and adding 20 mM calcium chloride. In contrast, mycelial fragments were dominant at –7.0 MPa Ψ. Maximum total inoculum (mycelial fragments and conidia) yields were approximately 2×10^7 ml^{-1} after 9 days incubation at 25 °C at 100 rpm. Biomass from liquid cultures responded to water-stress by accumulating increased concentrations of endogenous sugar alcohols (polyols), particularly glycerol. Long-term shelf-life studies showed that submerged inoculum from cultures subjected to an intermediate water-stress (–2.1 MPa Ψ) and containing enhanced levels of glycerol (>300 mg g^{-1} freeze-dried material) retained viability significantly better ($P<0.05$) than that from unstressed cultures, when assessed on agar with fully available water. This level of viability was comparable to that of aerial *U. atrum* spores from a 4-week solid-substrate fermentation on oat grains. However, in contrast to aerial spores, the ability of submerged biomass to germinate in drier conditions declined significantly after 6 months.

Introduction

The phyllosphere fungus *Ulocladium atrum* Preuss (strain 385) has shown potential for controlling grey mould caused by *Botrytis cinerea* Pers. in glasshouse- and field-grown crops (Elmer et al. 1995; Kohl et al. 1995a, b, 1998; Fruit and Nicot 1999; Schoene and Kohl 1999). The antagonist out-competes the pathogen for resources in necrotic crop debris during the saprophytic stage of the pathogen's life-cycle (Kohl et al. 1995a, b). As a result, the pathogen's sporulation capacity is suppressed and disease epidemics are significantly delayed.

Mass production is one of the most important aspects for commercial development of a biocontrol product. *U. atrum* has previously been produced only by solid-substrate fermentation on oat grains (Kohl et al. 1998). However, liquid rather than solid substrate fermentations are often preferred in industry, as they tend to be more economic (Lappa 1979), shorter and more controllable. Therefore, the potential for producing *U. atrum* in this way requires investigation.

The storage of biocontrol propagules produced by liquid fermentation is often problematic, with viability declining more rapidly than in aerial spores from solid-substrate fermentation. However, stress-induced endogenous compounds, particularly polyols, have improved the storage-life of fungal biocontrol propagules such as *Penicillium oxalicum* (Pascual et al. 2000). This is the first time that such studies have been carried out with *U. atrum*.

The objectives of this study were to: (a) investigate the potential for submerged conidial production of *U. atrum* and (b) compare shelf-life over 12 months of characterised *U. atrum* biomass from liquid culture, with that of aerial spores from a solid-substrate (oat) fermentation.

Materials and methods

Fungal isolate

Fungal isolate *U. atrum* 385 was obtained from Plant Research International (Wageningen, The Netherlands).

Induction of submerged conidiation and production of *U. atrum* inoculum by liquid fermentation

Experiment 1

Various media were assessed for their potential to induce submerged conidiation in *U. atrum* cultures: (1) oatmeal extract broth

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(OEB; 30% w/v) unmodified or modified with glycerol or polyethylene glycol (PEG) 200 solutions to reduce the media water potential (Ψ), (2) Czapek Dox broth (Difco) and (3) a minimal salts medium in which the carbon:nitrogen ratios were manipulated to 80:1, 30:1 and 10:1 with glucose:casamino acids as follows: 9.2:0.6 g l^{-1}, 7.8:1.7 g l^{-1} and 3.5:4.9 g l^{-1} (Jackson and Bothast 1990).

Glycerol was added at 7.3% and 24% (w/v) to achieve final broth Ψs of –2.1 MPa and –7.0 MPa, respectively. The Ψs of liquid media were measured with a rapid response Novasina Humidat-1C-11 (Novatron, Horsham, U.K.). Sigmacote (Sigma Chemicals U.K.) was used to prevent fungal attachment to the glass culture vessels. The samples were inoculated with 1 ml of *U. atrum* spore suspension (1×10^6 ml^{-1}) in 250-ml Erlenmeyer flasks and incubated at 25 °C on a rotary shaker at 100 rpm for 7 days. After fermentation, the biomass was separated from the culture broth by homogenisation for up to 8 min and centrifugation at 3,000 rpm for 12 min.

Experiment 2

Spore production was investigated in 50-ml cultures of OEB treated in the following ways: (1) unmodified broth of –0.28 MPa Ψ, (2) unmodified broth of –0.28 MPa Ψ + 20 mM $CaCl_2$, (3) unmodified broth of –0.28 MPa Ψ + 40 mM $CaCl_2$, (4) broth modified to –2.1 MPa Ψ with glycerol/water solution, (5) broth modified to –2.1 MPa Ψ with glycerol/water solution +20 mM $CaCl_2$, (6) broth modified to –2.1 MPa Ψ with glycerol/water solution +40 mM $CaCl_2$, (7) broth modified to –7.0 MPa Ψ with glycerol/water solution, (8) broth modified to –2.1 MPa Ψ with glycerol/water solution +20 mM $CaCl_2$ and (9) broth modified to –2.1 MPa Ψ with glycerol/water solution +40 mM $CaCl_2$

After 24 h, a small amount of biomass was aseptically removed from each flask and the morphological development of cultures was monitored using light microscopy (Olympus OM BX40 microscope). This procedure was repeated every 24 h for 7 days, after which spores were harvested, re-suspended in 50 ml of water containing Tween 80 and quantified with a haemocytometer. Inoculation, incubation and culture harvesting were as described earlier.

A further experiment extended the fermentation time to 12 days. Temporal inoculum yields from unstressed (–0.28 MPa Ψ) and water-stressed (–2.1 MPa and –7.0 MPa Ψ) liquid cultures were quantified by haemocytometer counts after 5, 7, 9 and 12 days. Viability (% germination) of biomass was also assessed at different levels of water stress (–0.28, –2.1 and –7.0 MPa Ψ) on PEG-modified water agar after incubation for 24 h at 18 °C.

Production of aerial *U. atrum* spores from solid-substrate fermentation

Sterile, water-saturated oat grain substrates were inoculated with 0.2 ml of a 1.0×10^6 *U. atrum* spore suspension. The cultures were incubated in microporous spawn bags (Cuero et al. 1985) for 4 weeks at 18 °C (Kohl et al. 1998). After incubation, the spores were separated from the substrate by washing, filtering and centrifugation at 3,000 rpm for 10 min.

Analysis of endogenous reserves in *U. atrum* biomass

For characterisation of spores and biomass in terms of concentrations of endogenous reserves, samples were frozen and freeze-dried (Edwards High Vacuum International) and prepared for high-performance liquid chromatographic analysis (Gilson 715), according to the method of Hallsworth and Magan (1997). Endogenous concentrations of polyols in *U. atrum* biomass from a solid oat fermentation and from 7-day-old liquid cultures were determined.

Shelf-life studies

The shelf-life of characterised *U. atrum* inoculum from liquid cultures after 7 days was compared to that of control aerial spores from the 4-week solid-substrate fermentation described earlier. Each treatment was adjusted to 1×10^5 inocula ml^{-1} and 15 aliquots (1-ml each) were dispensed into 2-ml Eppendorf tubes. These were centrifuged for 10 min at 13,000 rpm. The resultant pellets were stored as wet pastes at 4 °C. Samples from each treatment were re-suspended in 1 ml of water +0.01% Tween 80, after 1, 2, 3, 6 and 12 months storage, before being plated onto water agar, or agar modified with PEG 200 to –3.0 MPa Ψ. The germination plates were incubated as described earlier.

Replication and statistical analysis

All treatments were carried out at least in triplicate. Data were subjected to analysis of variance. When significant differences were observed, the means were compared by Duncan's least significant difference test at $P<0.05$ probability. In some cases, the standard error of the means is shown in the figures.

Results and discussion

Submerged conidiation by the biological control agent (BCA) *U. atrum* was induced for the first time during this study and provided an initial step in the development of a liquid fermentation production process. Spore production occurred only in a nutrient-poor OEB medium, with all other media tested proving unsuitable for this purpose. A morphological study showed that cultures grew vegetatively for at least 24 h after inoculation. Melanised conidiophores were observed after 48 h, with submerged spore production occurring after 72 h. *U. atrum* cultures consisted of mycelial pellets, the relative sizes of which tended to decrease with the Ψ of the broth. Figure 1 shows that spore yields after 7 days increased from 2×10^6 to 1.4×10^7, when the medium was modified with glycerol/water solutions to –2.1 MPa Ψ. However, when PEG 200 was used to modify the broth to the same Ψ, mycelial growth was poor and sporulation did not occur. This suggests that *U. atrum* is more sensitive to solute stress by PEG 200, than by glycerol. While it is recognised that water availability is an important factor for induction of sporulation in solid-substrate fermentation, few reports exist for liquid fermentation, even though water-stress has been shown to induce and influence submerged conidiation in some fungi (Inch and Trinci 1987; Humphreys et al. 1989; Jin et al. 1991; Pascual et al. 1997). The physiological mechanism by which Ψ affects sporulation in liquid culture is not known, although the involvement of a surface-active protein has been suggested (Morton 1961).

The addition of calcium chloride (20 mM), to glycerol-modified OEB of –2.1 MPa Ψ, further increased *U. atrum* spore yields to 1.7×10^7 spores ml^{-1}. Calcium has been demonstrated previously to induce submerged conidiation in various *Penicillium* spp (Foster et al. 1945; Pitt and Poole 1981; Ugalde and Pitt 1984; Pascual et al. 1997) and appears to interact with other nutritional factors to induce spore production. For exam-

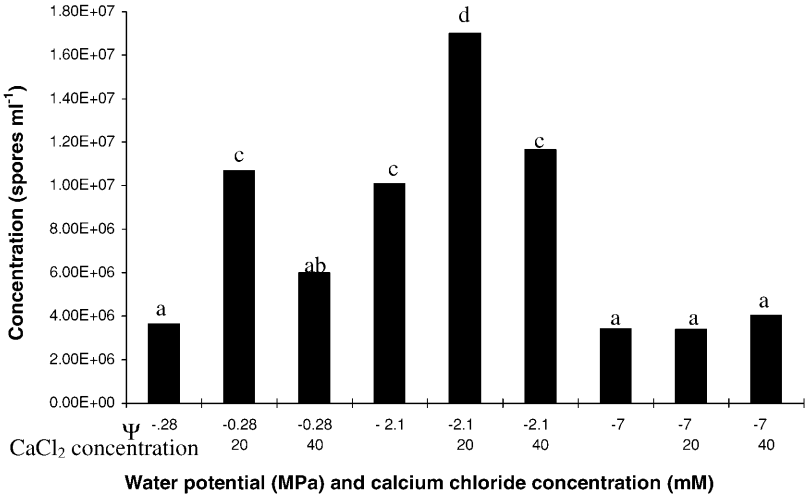

Fig. 1 Effect of water potential (Ψ) and calcium chloride ($CaCl_2$) concentration on submerged conidiation by *Ulocladium atrum* in unstressed (–0.28 MPa), and glycerol-modified (water-stressed; –2.1 MPa and –7.0 MPa) oatmeal extract broth after 7 days at 25 °C and 100 rpm (n=3). Least significant difference (LSD; $P<0.05$) is 2.57×10^6. Different letters indicate significant differences

ple, spore production by *P. oxalicum* was enhanced when calcium (20 mM) was added to liquid medium with a high C:N ratio (142:9; Pascual et al. 1997).

Temporal studies on liquid fermentation were carried out to assess optimum fermentation times for inoculum production. For practical purposes, fermentation length is important as it impacts directly on the economics of the production process. During this study, concentrations of both mycelial fragments and spores were investigated, as it was thought they may serve as an alternative inoculum form. Figure 2 shows that optimum fermentation lengths varied according to the Ψ of the broth. The time difference may be due to slower development of the fungus in water-stressed conditions. While maximum spore concentrations were obtained from the –2.1 MPa Ψ cultures, the –7.0 MPa Ψ cultures yielded the highest concentration of mycelial fragments. Thus, biomass forms as well as yields can be manipulated by altering culture conditions. Spores are generally preferred, on account of their better storage characteristics (Smith 1978). However, mycelial fragments would in theory make good biocontrol propagules, as they can produce new mycelium terminally and laterally. Furthermore, spores that remained attached to fragments were still able to germinate. Thus colonisation and establishment of *U. atrum* may be more rapid from a fragment carrying a spore, than from a single spore alone.

Table 1 shows that concentrations of the endogenous reserve, glycerol, within submerged *U. atrum* biomass could be manipulated by altering the culture conditions. Glycerol levels increased with water-stress to a maximum concentration of >300 mg g^{-1}. Polyols accumulate in stressed conditions in a number of fungal species (Brown and Simpson 1972; Hallsworth and Magan 1994) and are differentially effective in osmoregulation.

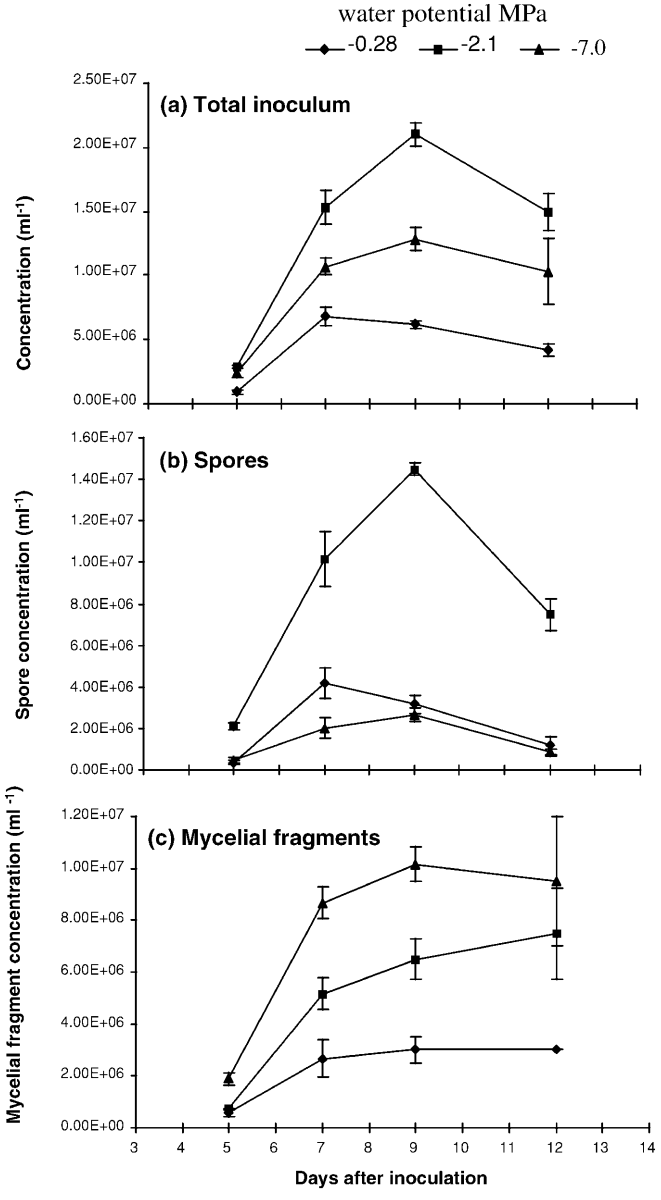

Fig. 2 Effect of water potential on temporal production of (**a**) total *U. atrum* inoculum, (**b**) spores and (**c**) mycelial fragments in liquid fermentation (oatmeal extract broth, 25 °C, 100 rpm) over 12 days, (n=3). Bars represent standard errors

Table 1 Temporal accumulation of endogenous glycerol in biomass (conidia and mycelium) of *Ulocladium atrum* produced by liquid fermentation at three levels of water potential; and glycerol content of control aerial spores from a solid oat fermentation after 4 weeks. Data are means of six replicates from two identical experiments and indicate milligrams of glycerol per gram of freeze-dried biomass. Means followed by the same letter are not significantly (P<0.05) different

Water potential (MPa)	Days after inoculation (liquid fermentation)				Aerial spores
	5	7	9	12	
–0.28	0.11 a	0.68 a	4.36 a	6.00 a	68.14 b
–2.1	54.24 b	48.30 b	63.37 b	65.62 b	–
–7.0	319.08 d	142.52 c	86.19 bc	134.55 c	–

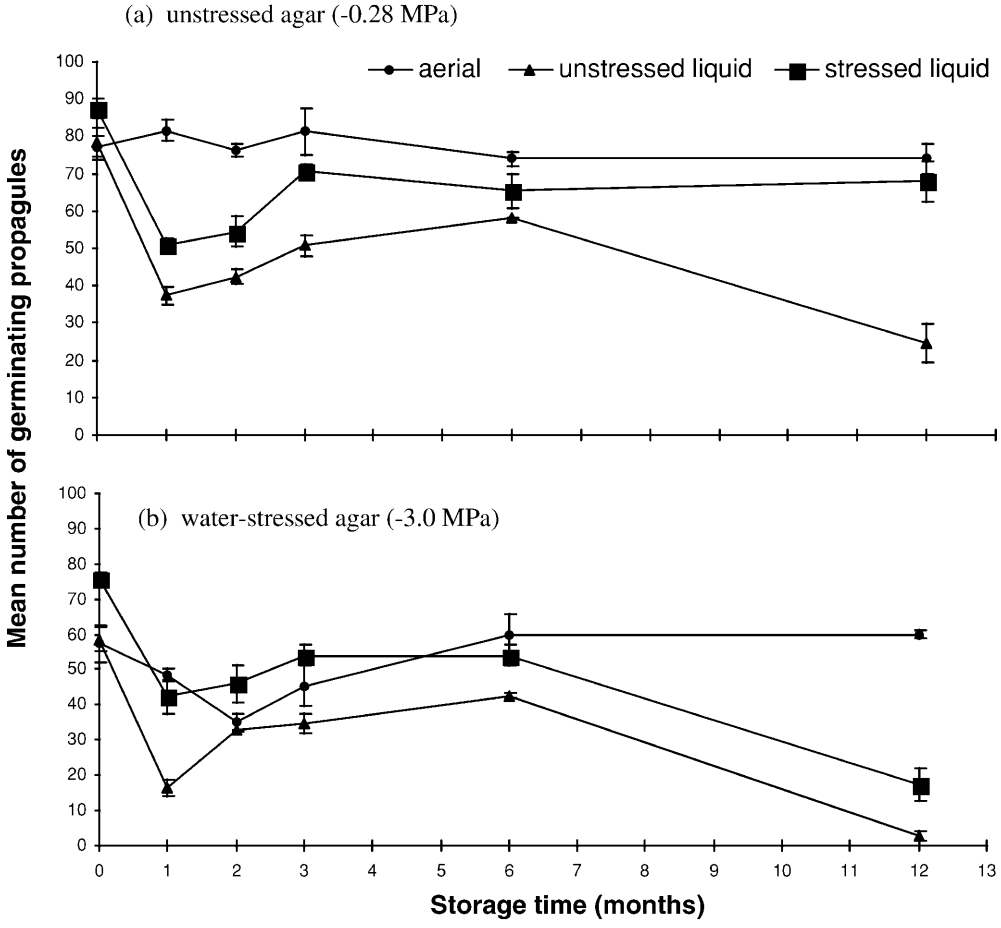

Fig. 3 Temporal viability comparison between standard *U. atrum* inoculum (wet pastes) from unmodified solid oats for 4 weeks at 18 °C and from unmodified and modified liquid culture for 7 days, on unstressed (–0.28 MPa; **a**) and stressed (–3.0 MPa; **b**) water agar. *Bars* represent standard errors (n=3×100 spores). LSD (P<0.05) is 17.36 in **a** and 10.31 in **b**

Although accumulation patterns of other polyols such as erythritol, arabitol and mannitol were investigated, glycerol was the only reserve that responded to water-stress in submerged biomass. This may reflect its efficiency as a compatible solute. It is unclear why glycerol accumulated to relatively high concentrations in the aerial spores (68.14 mg g^{-1} freeze-dried material), as the only stress imposed during fermentation was temperature-stress (18 °C). However, natural grain substrates are heterogeneous in nature and therefore nutritional; and localised environmental factors may have influenced glycerol accumulation in this case.

A particular challenge in biocontrol is the production of propagules that retain viability during storage, without compromising biocontrol efficacy. Adequate shelf-life for a BCA product requires stability over at least 1 year (Rodham et al. 1999). For this reason, long-term shelf-life studies were carried out to investigate the viability of *U. atrum* inoculum produced from unmodified and modified liquid cultures, and aerial spores from a solid-substrate (oat) fermentation. Figure 3 shows that the aerial spores and water-stressed, submerged inoculum both containing significantly (P<0.05) higher glycerol concentrations than the unstressed liquid treatment; retained similar viability after 12 months storage as wet pastes, with average germination rates on saturated-water agar of 70–74%. In contrast, the unmodified liquid treatment contained virtually no glycerol and displayed a signifi-

cant ($P<0.05$) decline in viability (<30%) after 6 months, suggesting that glycerol may be important in maintaining viability in *U. atrum* inoculum during extended periods of storage. Previous studies (Pascual 1998) with BCAs, such as *Epicoccum nigrum*, have also shown that enhanced levels of glycerol and arabitol improved spore viability after 27 weeks storage. A further contributory factor to the decline in viability of unmodified, submerged inoculum compared to the –2.1 MPa Ψ liquid treatment, may also be the lower ratio of spores to mycelial fragments within this treatment. It could be that the spores survive during storage, yet the fragments degrade (Smith 1978). This is not surprising, as spores are adapted for periods of dormancy and have a lower metabolic rate than mycelial fragments derived from metabolically active mycelium. The present study showed that the ability of *U. atrum* inoculum to germinate in drier conditions (–3.0 MPa Ψ) was lost in the biomass from liquid cultures after 6–12 months storage, yet was retained in aerial spores. This could be accounted for by physiological and physical differences between inoculum types, such as thickness (Munoz et al. 1995) and roughness (Thomas et al. 1987) of spore walls, hydrophobicity (Munoz et al. 1995) and the state of hydration of the propagule (Thomas et al. 1987). Properties of the spore surface may influence environmental resistance of the inoculum during storage. Previous studies (Munoz et al. 1995) showed a lower viability in submerged *Trichoderma harzianum* conidia (15%) when compared to aerial spores (100%) after 45 days. Pascual et al. (2000) found that aerial *P. oxalicum* spores were superior to submerged spores, in terms of viability and biocontrol efficacy.

The current study has shown that it is possible to produce viable inoculum of the BCA *U. atrum* by liquid fermentation and that yields can be increased by reducing Ψ and adding $CaCl_2$ to the medium. Endogenous glycerol concentrations can be manipulated according to culture conditions and this may have beneficial effects on the shelf-life of propagules and their ability to germinate in dry conditions. The process of producing submerged inoculum warrants further investigation, bearing in mind the economic and technical benefits associated with liquid, rather than solid fermentation. However, the process will need more detailed research and development for industrial use. For example, the development of a highly standardised liquid medium with quantitative nutritional values would be essential for the reproducibility and consistency of inoculum yields required for commercial exploitation.

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