Factors affecting mass production of *Duosporium yamadanum* in rice grains

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Summary

Duosporium yamadanum (Matsuura) Tsuda & Ueyama is a pathogenic fungus that attacks purple nutsedge, *Cyperus rotundus*, L. in Brazil. Occasionally, it is found causing severe natural epiphytotics of leaf blight on that host in the field. Exploratory studies have already indicated that this fungus has potential for the development of a mycoherbicide. To help confirm its potential, we studied mass production of inoculum of *D. yamadanum* in solid fermentation using polished rice as the substrate for cultivation of *D. yamadanum*. The effects of the following factors were investigated for their influence on conidial production: water content, length of incubation period before the opening of the plastic bags containing the substrate and addition of supplements to the substrate. Maximum production of conidia was obtained with a water content of 40–60% in the substrate (w/v), with average production at each harvest of 2.5×10^5 conidia per gram of substrate. Water contents above 60% inhibited growth and sporulation. Opening bags containing the inoculated substrate after 3 to 4 days resulted in the highest levels of sporulation; average of 3.0×10^5 conidia per gram of substrate. The supplementation of the substrate with calcium carbonate did not significantly increase the sporulation levels compared to controls, whereas the addition of urea or sucrose led to a significant reduction in sporulation; average of 9.0×10^4 conidia per gram of substrate.

Keywords: *Cyperus rotundus*, purple nutsedge, weed biological control, bioherbicide.

Introduction

Purple nutsedge, *Cyperus rotundus* L., is often considered the world's worst agricultural weed (Holm *et al.*, 1977). Infestations are extremely difficult to control through mechanical methods, and chemical control has either been ineffective or limited by cost, environmental or management problems associated with the most promising products. This weed has been a target by several biological control programmes involving insect (Frick and Quimby, 1977; Frick *et al.*, 1979; Phatak *et al.*, 1987) and fungal (Phatak *et al.*, 1982; Upadhyay *et al.*, 1991; Prakash *et al.*, 1996; Neto, 1997; Okoli *et al.*, 1997; Ribeiro *et al.*, 1997; Kadir *et al.*, 1999, 2000a,b; Rosskopf *et al.*, 1999; Kadir and Charudattan, 2000) natural enemies. Although some experimental results have been particularly promising, no commercial mycoherbicide is available, and there are no classical biological control agents in the pipeline. A combination of methods in an integrated management approach is often mentioned as ideal to minimize the problems associated with intensive chemical control (Bariuan *et al.*, 1999). Although Brazil is known to be outside the centre of origin of *C. rotundus*, a survey carried out in this country revealed a series of fungal pathogens showing clear potential for utilization for the development of a mycoherbicide (Barreto and Evans, 1994, 1996). Among these, the dematiaceous hyphomycete, *Duosporium yamadanum* (Matsuura) Tsuda & Ueyama, was selected as specially promising, as it was capable of spontaneously causing severe leaf blight epihytotics in *C. rotundus* in the field (Barreto and Evans, 1994). A series of intensive studies on the biology and management of *D. yamadanum* was initiated in 1995 by Pomella (1999) and continued by Macedo (2006) and mostly confirmed that this fungus has potential as a biological control agent. Among the most common limitations hampering the development of mycoherbicides are those related to the mass production of abundant, good quality inoculum to be used as the active ingredient. Large-scale production of fungal biological control agents for weed biological control is mainly through techniques of liquid, diphasic or solid fermentation (Jackson *et al.*, 1996). Liquid fermentation is the favoured technique because it is nor-

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mally the most economically viable method (Tebeest and Templeton, 1985). The diphasic method is usually not viable economically because it often involves one growth phase on a medium solidified with agar (Walker, 1980), but this is not necessarily true, as there are cheap alternatives to agar. Finally, solid fermentation utilizes substrates such as grains for fungal colonization and sporulation. This technique is commonly used for small-scale production of fungi that do not sporulate well in liquid media (Pandey, 2003). Pomella (1999) reported good results in mass production of *D. yamadanum* through solid fermentation. Additional investigations were undertaken aimed at perfecting the technique originally developed by Pomella (1999). Investigations on the effects of some parameters on sporulation such as use of nutritional supplements, length of period of incubation before initiation of harvesting and water content in substrate were performed.

Material and methods

General conditions for experiments

Our experiments used a selected strain of *D. yamadanum* (RWB 476). The fungus was grown for 7 days in Petri plates containing V8-juice agar, at 25°C, under a light regime of 12 h/day. Three culture discs (diameter, 20 mm) were cut from the margin of actively growing cultures and transferred to flasks containing 100 ml of sterile (autoclaved) liquid medium (200 ml V8-juice diluted with 800 ml of water). Flasks were maintained for 4 days under agitation at 140 rpm. The resulting mycelium was aseptically blended within the remaining medium, and 20 ml of the mycelial suspension was used as seed and transferred to each of a series of polypropylene bags containing 150 g of polished rice + 75 ml of water. The bags with rice were autoclaved before seeding with mycelium of *D. yamadanum*. After seeding, the bag were closed and left in a controlled temperature room at 25°C and a 12 h/day light regime (light from two fluorescent lamps and one NUV BLB 60 W lamp placed 40 cm above the bags) for either 3 days or (in one specific experiment) for a series of five periods of incubation of different lengths. The mass of grains was loosened by gently pressing the bags with the hands to allow for a uniform colonization of the substrate by the fungus. After 3 days, the bags were opened, and a first conidial harvest was performed. Colonized rice grains were then transferred to a flask containing 250 ml of sterile water supplemented with an antibiotic (chloranphenicol at 2.5 ppm) and vigorously stirred with a glass rod. The rice was sieved out of this suspension and placed on aluminium trays (19 × 28 × 2 cm) previously cleaned with 70% alcohol and held at 25 ± 3°C for further periods of conidial production. An interval of 24 h between each harvesting episode was maintained. The quantity of conidia in the remaining suspension was estimated with a haemocytometer. At 24-h intervals, the harvesting procedure was repeated, and conidial production was evaluated. The number of harvests performed varied for different experiments.

Effects of different levels of water content in the substrate on sporulation of *D. yamadanum*

Pomella (1999) used an arbitrary proportion of 80% water in the substrate with seemingly adequate results. A range of different proportions of water were tested in this experiment, namely 40%, 50%, 60%, 70% and 80% of volume of water per weight of substrate (polished rice grains). Our experimental unit consisted of one plastic bag containing 150 g of rice seeded with *D. yamadanum*. The experiment was arranged in a completely randomized scheme with four repetitions.

Influence of different supplements added to the substrate on sporulation of *D. yamadanum*

The effect of nutritional supplementation of the basic substrate (polished rice) on sporulation of *D. yamadanum* was investigated by following the general procedure described above. The treatments consisted in supplementing the water added to the basic substrate (polished rice) before autoclaving with either calcium carbonate (1.5 g/l), urea (2.0 g/l) or sucrose (20 g/l). Control consisted of a group of bags containing the basic substrate without any supplement. Evaluation of sporulation was performed as previously described. Nine harvests were done in this experiment, which was arranged in a completely randomized scheme with four repetitions.

Effects of different lengths of incubation of *D. yamadanum*, before initiation of harvesting, on sporulation

In this experiment, the general procedure described above was followed, but different periods of incubation between seeding the substrate and the first harvest were tested: 3, 4, 5, 6, 7 and 8 days of incubation at the aperture of the recipients (DIAR). The evaluation was performed as described above. The experiment was arranged in a completely randomized scheme with four repetitions.

Statistical analysis

Variance analysis were performed with the software SAS (version 8.3; Statistical Analysis System, Cary, NC, USA). Conidial production was evaluated by calculations of areas under the curve as described by Madden *et al.* (2007). The assays with an independent quantitative variable were analyzed by comparing areas under the curve of conidial production (AUCCP) obtained for each treatment. Variance analysis of the effects of treatments was performed and compared with Tukey's test at 5% of probability.

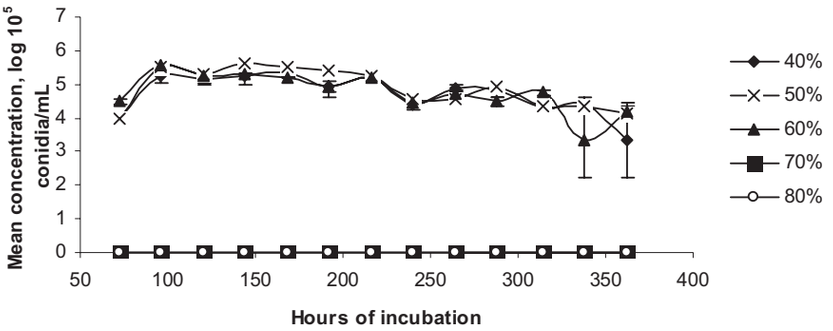

Figure 1. Mean concentration of log 10^5 conidia per millilitre of *Duosporium yamadanum* produced for treatments with different percentages of water added to the substrate (for a total of 13 harvests). Bars represent the standard error.

Results

Effects of different levels of water content in the substrate on sporulation of *D. yamadanum*

The highest sporulation levels were obtained with a water content ranging from 40% to 60%. The largest yield of conidia was obtained in the second harvest. A clear reduction in yields was observed on the tenth harvest, that is, at hour 314 (Fig. 1). No statistical difference was observed for total yield obtained for treatments with 40%, 50% and 60% of water. No colonization or sporulation was obtained for the treatments with 70% and 80% of water (Fig. 2).

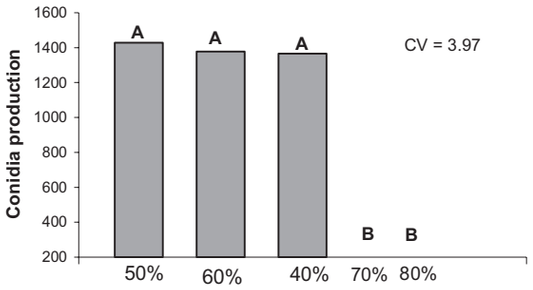

Figure 2. Conidial production (AUCCP) for different water percentage on the substrate (mean of four repetitions). Columns with the same letters did not differ statistically under Tukey's test at 5% of probability.

Influence of different supplements added to the substrate on sporulation of *D. yamadanum*

The highest levels of sporulation were attained for the first harvest in all treatments (Fig. 3). Although the highest level of sporulation was obtained for the treatment where calcium carbonate was added to the substrate, there was no statistical difference between this treatment and the control. Treatments involving supplementation with urea and sucrose led to significantly less sporulation (Fig. 4).

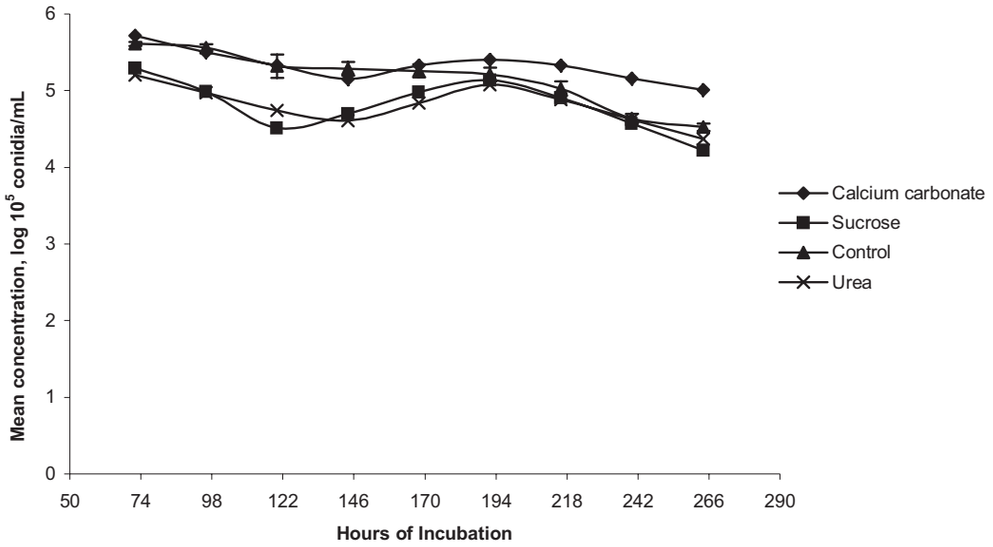

Figure 3. Mean concentration of log 10^5 conidia per millilitre of *Duosporium yamadanum* for substrates supplemented with different substances (total of nine harvests). Bars represent the standard error.

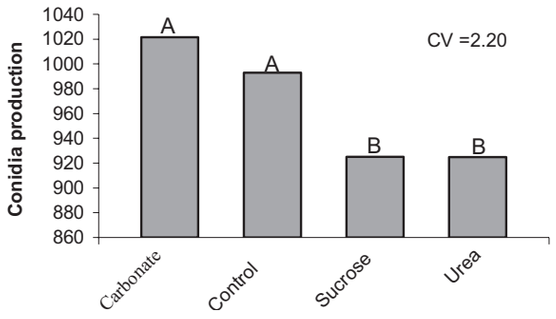

Figure 4. Conidial production (AUCCP) for substrates supplemented with different substances (mean of four repetitions). Columns with the same letters did not differ statistically under Tukey's test at 5% of probability.

Effects of different lengths of incubation of *D. yamadanum*, before initiation of harvesting, on sporulation

Allowing *D. yamadanum* to grow on rice within the bags for 3 or 4 days was shown to be significantly better, in terms of sporulation, than the other periods that were tested. Longer periods of incubation led to a significant reduction in sporulation. Sporulation was maintained for longer periods of time for treatments subjected to shorter periods of incubation and dropped to zero after eight harvests (for 8 days of incubation) and at the last harvest (for 7 days of incubation; Figs. 5 and 6).

Discussion

Viability of a mycoherbicide depends heavily on the development of an adequate methodology for large-scale production of fungal propagules (Jackson *et al.*, 1996). Each fungus has different requirements for optimal production (quantity and quality), and often minor adjustments in a mass production protocol may have significant impacts on final results. Although almost all commercial mycoherbicides have relied on liquid fermentation, mass production of entomopathogens has generally relied on solid fermentation, particularly based on grains, such as rice, as a substrate (Wyss *et al.*, 2001; Tarocco *et al.*, 2005).

Preliminary attempts by Pomella (1999) to mass produce *D. yamadanum* in liquid media failed to yield any sporulation. Later, the same author demonstrated that solid fermentation might offer an adequate alternative for mass production. The protocol described by Pomella (1999) for this purpose is in contrast to the present study in some respects. For instance, water content in the substrate of 40%, 50% and 60% gave better sporulation compared to 70% to 80% found to be best by Pomella (1999). In the present study, higher levels of water content led to an inadequate consistency of the substrate. In high water-content treatments, grains became too soft and water soaked and lack of aeration within the substrate mass probably did not allow proper colonization of the substrate and sporulation of the fungus. Other differences between the two production protocols may explain the discrepancies between observations made in this work and those made by Pomella (1999). In the latter, seeding of the medium was done with culture disks, harvests were initiated much later (13 days) and the plastic bags were not completely sealed but had a cotton plug closing the bag's openings. This might have allowed a progressive dehydration of the substrate generating conditions that were less favourable for the fungal growth than those provided by the set of conditions adopted in the present study.

Of particular interest was the persistence of sporulation on colonized rice grains observed in this study. After 13 harvesting episodes, conidia were still relatively abundant for the best treatments, despite the progressive decline in sporulation. Our utilization of blended

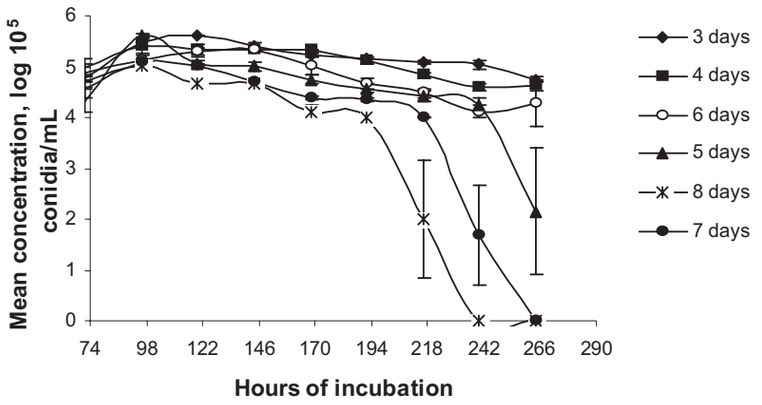

Figure 5. Mean concentration of log 10^5 conidia per millilitre of *Duosporium yamadanum* for different periods of incubation within sealed plastic bags before initiation of harvesting. Bars represent the standard error.

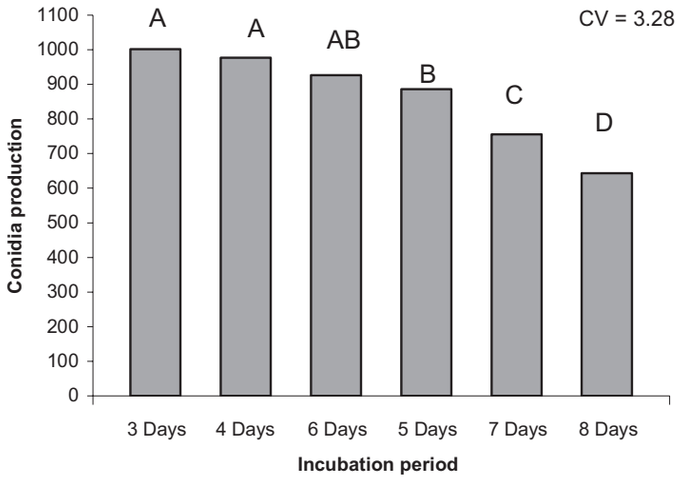

Figure 6. Conidial production (AUCCP) for different periods of incubation within sealed plastic bags before initiation of harvesting (mean of four repetitions). Columns with the same letters did not differ statistically under Tukey's test at 5% of probability.

mycelium as seed allowed for a quick colonization of the substrate, shortening considerably the process of conidium production for this fungus. The period of spore production and harvest number was increased.

The levels of moisture that are more appropriate for mass production of fungi vary from species to species. For example, the ideal moisture content in the substrate was shown to be 30% to 40 % for *Metarhizium anisopliae* (Metsch) Sorok. var. *acridum* (Arzumanov *et al.*, 2005) and also for *Penicillium oxalicum* Currie & Thom (Larena *et al.*, 2002), whereas for *Mucor bacilliformis* Hesselt, the ideal is 90% (Lareo *et al.*, 2006). The long period of incubation within the bags adopted in Pomella's protocol was shown to be unnecessary. Pomella waited for a thorough and visible colonization of the rice mass before opening the bags and starting the conidial harvest to avoid problems with contamination. However, using the techniques described in this paper, contamination was not a problem and before *D. yamadanum* colonies are visible with naked eye, colonization is well advanced. Keeping the bags closed for longer periods was shown to be harmful for sporulation, perhaps because it leads to stress such as reduced exchange of gases, lack of oxygen, poor heat dissipation or others.

Supplementing the substrate with urea or sucrose is known to increase sporulation for many fungi, but in the case of *D. yamadanum*, it was clearly harmful. The addition of calcium carbonate resulted in a statistically negligible increase in sporulation. Higher concentrations of calcium carbonate might have a more significant effect on sporulation, an aspect deserving further investigation.

The results obtained in this study provide improvement on the protocol for mass production proposed by Pomella (1999).

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