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Effect of Different Factors on Growth and Spore Production of a *Colletotrichum* sp., a Potential Biocontrol Agent for Waterhyacinth

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INTRODUCTION

Waterhyacinth (*Eichhornia crassipes* (Mart.) Solms) is one of the worst weeds in the world (Holms et al. 1997, Jiang et al. 2003). Native to South America, waterhyacinth was introduced into the USA, India and several other tropical and subtropical countries in the 1800s. It now occurs in most countries of the world lying between 40°N and 45°S latitude (Gopal 1987, Center 1994). It propagates rapidly forming dense mats that spread out across the water surface, blocking traffic, destroying natural landscapes, affecting water quality, decreasing bio-diversity, providing conditions for mosquitoes to breed and retarding agricultural development (Holms et al. 1997, Perkins 1973).

As in many parts of the world, water hyacinth causes serious problems in China (Wu 2001, Wang 2004). Physical and chemical controls have been used with some success in China, but biological means are also receiving recognition. A project was initiated in 2000 to find biocontrol agents native to mainland China, but until recently no suitable agents had been reported (Ding et al. 2001).

METHODS

In 2004, waterhyacinth plants or leaves with disease symptoms were collected from Jiaxing, Zhejiang and Huangpu River, Shanghai. The disease symptoms consisted of brown spots with yellow striations towards the tip of the leaves and petioles. Leaf pieces (2-3 mm^2) were cut from the margins of necrotic lesions on the leaves, surface disinfected in bleaching powder solution for 3-4 min. Five leaf pieces were placed on potato dextrose agar (PDA) plates and incubated at 25°C. A *Colletotrichum* sp., was isolated and a pure culture was obtained by a single-spore technique. In the laboratory, the isolate was tested to establish its pathogenicity to waterhyacinth by performing Koch's postulates. It was found to be a virulent pathogen of water hyacinth. The initial symptoms produced by the isolate were brown-grey spots, that later enlarged to form brown, rounded spots, covering most of the leaf area. Efficacy on waterhyacinth was calculated using a disease index (DI).

$$DI = [(ld \times \text{Number of leaves with the rating}) / (\text{Gross leaves} \times 4)] \times 100\%$$

where ld is a leaf damage rating of 0-4 (0 = leaf with no damage; 1= spots cover < 1/3 of leaf area; 2 = spots cover >1/3 but <2/3rds of leaf area; 3 = spots cover >2/3rds leaf area; 4= leaf dead. One month after inoculation with *Colletotrichum*, the waterhyacinth was given a DI of 65.28%.

To develop this isolate as a biological control agent for water hyacinth, optimum cultural conditions were determined for mycelial growth and spore production. The conditions tested included media, light, pH, carbon and nitrogen levels, liquid volume and shaker speed.

A representative culture of the *Colletotrichum* sp., isolated from diseased water hyacinth plants collected in Shanghai and Zhejiang provinces, China, was used. The PDA culture of the isolate was grey green in color and had mycelium that was branched and regularly septate. Under constant darkness at 25°C, slimy orange-colored sporodochia developed in the center of the culture after 7 days. Conidia of the isolate were hyaline, one-celled, having thin smooth walls. They were short and cylindrical in shape (5-7 × 14-20 µm). After 7 days, perithecia appeared as black dots submersed in the agar. The perithecia were ostiolate and contained asci 9-12.5

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× 11-23 µm in size (Figure 1). Each ascus contained eight meniscate ascospores. Working cultures of the isolate were maintained on PDA tubes.

For all agar culture studies, 5 mm-diameter discs from 7-day old PDA cultures were placed in the center of 9 cm petri dishes containing 13 ml of media. Each treatment was replicated 3 times. Colony diameter was measured 7 days post inoculation. The plate surface was then washed 3 times with 10 ml tap water to dislodge the fungal spores. Spore production was quantified using a hemacytometer. To determine effect of temperature on mycelial growth and sporulation, the isolate was incubated in a homoeothermic incubator at 5, 10, 15, 20, 25, 30, and 35°C. Light studies were conducted at 25 ± 2°C. Comparable plates were incubated under constant illumination (continuous light; CL; 10000 lux, fluorescent lamp), constant darkness (continuous darkness; CD) or a 12 h light/dark photoperiod (12:12 L:D; 10000 lux, fluorescent lamp).

Eight kinds of media were tested to determine the effect of nutrients on mycelial growth and sporulation. The media used were PDA (potatoes, 200 g; dextrose, 15 g; agar, 20 g; H_2O, 1 l); tap water agar (TWA) (agar, 20 g; H_2O, 1 l); water-hyacinth dextrose agar (WHDA) (water hyacinth leaves, 200 g; dextrose, 15 g; agar, 20 g; H_2O, 1 l); PDA plus yeast extract (PDAY) (potatoes, 200 g; dextrose, 15 g; Difco yeast extract, 5 g; agar, 20 g; H_2O, 1 l); maize soybean dextrose agar (MSDA) (maize, 15 g; soybean, 15 g; 1 dextrose, 5 g; agar, 20 g; H_2O, 1 l); maize dextrose yeast extract agar (MD-YEA) (maizes, 30 g; dextrose, 15 g; Difco yeast extract, 5 g; agar, 20 g; H_2O, 1 l); maize agar (MMA) (maizes, 30 g; agar, 20 g; H_2O, 1 l) and Czapek-Dox agar (CDA) ($NaNO_3$, 2 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4$, 0.5 g; $FeSO_4$, 0.01 g; sucrose, 30 g; agar, 20 g; H_2O, 1 l).

To evaluate pH affects on growth and sporulation, the pH of the PDA medium was adjusted before autoclaving to 5.0, 5.6, 6.0, 6.6, 7.0, 7.6, 8.0, 8.5, 9.0, 9.5 or 10.0 by buffers. A citric acid-Na_2PO_4 buffer was used to adjust the pH to 5.0-7.6 and a boric acid-potassium chloride-sodium hydroxide buffer was used to adjust the pH to 8.0-10.0.

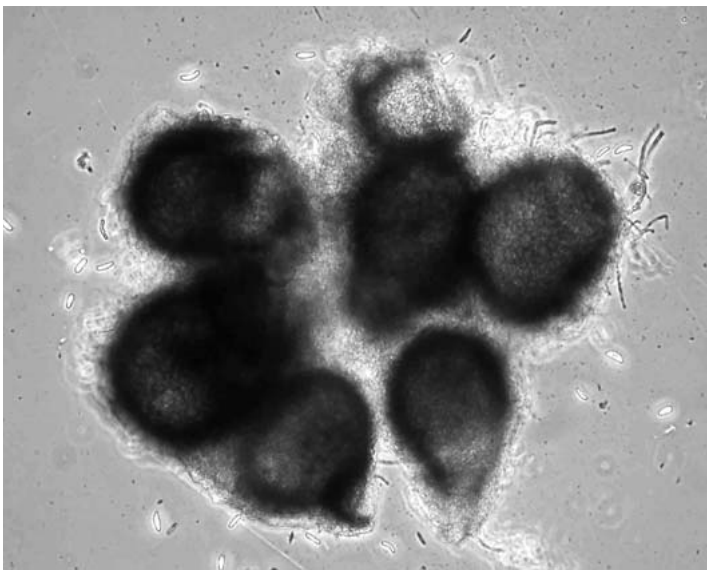

Figure 1. The perithecium of a *Colletotrichum* sp. active on waterhyacinth.

In general, different sources of carbon as well as nitrogen have effects on mycelial growth and spore production of some fungi. Selecting CDA as the basic medium, dextrose, mannitol and sodium citrate were used to replace sucrose while keeping the carbon content constant. In the same way, urea and ammonium dihydrogen phosphate were used instead of sodium nitrate.

To determine the effect of aeration on mycelial growth and sporulation, different liquid volumes and shaker speeds were tested. Liquid volumes of 40 ml, 80 ml and 120 ml were added to 250-ml Erlenmeyer flasks. They were inoculated with 5-mm-diameter discs from 7-day-old PDA cultures. The inoculated flasks were incubated on reciprocating shakers at 80, 120, and 160 strokes/min. Each treatment was replicated three times. After 7 days, the mycelium was filtered, washed using tap water, air-dried to a constant weight and weighed. Spore production was quantified using a hemacytometer.

The data were calculated using EXCEL software. In the study of individual factors, the data on growth and sporulation were analyzed by Duncan's new multiple range (SSR) test.

RESULTS AND DISCUSSION

Although the *Colletotrichum* isolate can grow and produce spores in the range of 5-35°C, temperature had a significant effect on mycelial growth. Colony diameter after 7 days of growth was significantly better at 25°C ($p < 0.05$) than any other temperature tested. It was nearly 6 fold greater than colony diameter of cultures incubated at 5, 10, or 35°C. Spore production was optimum at 25°C (1.8×10^7) but not significantly better than 20°C (1.6×10^7). As with growth, spore production was lowest at 5, 10, and 35°C. Generally speaking, waterhyacinth blooms in October and November in China, so the isolate would be applied at that time in order to decrease seed production. No significant difference was found among the three illumination levels ($p > 0.05$). Colony diameters were 5.83 cm at continuous light, 6.80 cm at continuous darkness, and 6.63 cm at 12 h light/dark photoperiod after 7 days. The spore counts were 1.2×10^6, 9.43×10^6, and 1.67×10^6 in this order. The results indicate that the isolate is not sensitive to light.

The effect of media on mycelial growth and sporulation was significant. Of the eight kinds of culture medium, WHDA and MSDA were the best for mycelial growth (9 cm after 7 days) while CDA was the least suitable with no mycelial growth. Following WHDA and MSDA, PDA (7.40 cm), MD-YEA (6.63 cm), TWA (4.9 cm), PDAY (4.77 cm), and MMA (4.47 cm) were decreasingly effective, in promoting mycelial growth. The best medium for inducing sporulation was MD-YEA (1.4×10^7), but it was not significantly different from the other media ($p > 0.01$).

The best pH for mycelial growth was between 5.6 (8.2 cm) and 6.0 (8.1 cm) which was slightly better than 6.6 (7.87 cm), 7.0 (7.90 cm) and 7.6 (7.63 cm). The least suitable were 8.0 and 9.5 ($p < 0.01$). The best spore production was obtained at 6.6 (8.7×10^6) but not significantly better than 7.6 (8.43×10^6) or 7.0 (6.4×10^6). The number of spores at pH 7.0 was nearly 8 fold and 3 fold greater than sporulation incubated at pH 5.6 and 6.0 respectively.

Dextrose was the best source of carbon for mycelial growth, followed by mannitol and sodium citrate, with significant difference in that order ($p < 0.05$). Sucrose was the least effective, as the medium containing this carbon source produced no hyphal growth. Among these sources of carbon, mannitol was the best for inducing spore production (2.7×10^5) ($p < 0.05$) followed by dextrose (1.7×10^5), while no sporulation occurred when sucrose and sodium citrate were the source of carbon.

In a series of trials with nitrogen, the maximum mycelial growth was obtained from sodium nitrate (6.27 cm), followed by urea and ammonium dihydrogen phosphate, but was only moderately effective for spore production. Urea was the best nitrogen source for sporulation while ammonium dihydrogen phosphate was the least effective.

No significant difference was found among treatments of liquid volume and shaker speed for mycelial growth respectively ($p > 0.05$). Despite differences in shaker speed, the best sporulation was obtained at 120 ml PDA per 250-ml Erlenmeyer flask (1.62×10^7), followed by 80 ml (1.18×10^7), while 40 ml was the least effective (6.67×10^6) ($p < 0.05$). Of three shaker speed levels, the best spore production occurred at 120 strokes per minute (1.63×10^7), followed by 80 strokes (1.06×10^7) and the least at 160 strokes per minute (7.79×10^6) ($p < 0.05$). It appears from the results that the optimum treatment was 120 ml PDA per 250 ml Erlenmeyer flask matching with 120 strokes ($p < 0.05$). Using these parameters, the number of spores produced was 2.32×10^7. The *Colletotrichum* sp. reported herein has not been previously reported from waterhyacinth in China. Because it is an indigenous pathogen to China, it could potentially be a candidate for bioherbicide development for waterhyacinth management. Our experimental results demonstrate that the isolate can be grown easily under a variety of conditions and that it could be mass-produced. However before a bioherbicide formulation could be manufactured on a large scale, some questions, such as scale-up, cost, and market size must to be considered. If the isolate were to be used in China or in other countries, host range and pathogenicity of this *Colletotrichum* sp. must also be investigated.

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