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The effect of nutrition on conidiation and conidial germination of *Fusarium anthophilum* obtained from barnyard grass (*Echinochloa crus-galli*)

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ABSTRACT
An isolated *Fusarium anthophilum* (A. Braun) Wollenweber (isolate 16C) has been considered as a candidate for microbial control of barnyard grass [*Echinochloa crus-galli* (L.) P. Beauv]. The effects of nutrition on conidiation, germination rate and germ-tube elongation of conidia of the fungus were evaluated. Conidia yield, conidial germination and conidial germ-tube elongation of the fungus grown in a liquid medium with a C: N ratio of 5:1 was significantly more than that in the same medium with C: N ratio of 15:1 or 40:1. Glycerol at 1, 2 or 5% (v/v) in the medium with a C: N ratio of 5:1, significantly enhanced conidiation of the fungus. The highest conidia production was obtained in the medium contained 5% glycerol. Glycerol did not enhance germination and germ-tube elongation. Harvested conidia in 0.2, 0.5, 1 or 2% Tween 40 resulted in higher germination and germ-tube elongation than those harvested in distilled water, when they were placed on top water agar for 8 h at 20 °C. The effect of Tween 40, particularly at 0.5% (v/v) was more pronounced after 9 and 35 days desiccation period at 20 °C and 15% RH.

Keywords: Microbial control, *Fusarium anthophilum*, *Echinochloa crus-galli*, Glycerol, Nutritional regimes, Tween 40, C:N ratio.

INTRODUCTION

Biological control of weeds is an alternative approach, utilizing living organisms to control or reduce the population of undesirable weed species. The classical approach with exotic plant pathogens to control weeds was developed in the beginning of the 1970s (Alan, 1991). An alternative approach to bioherbicide development is based on the idea that an endemic (i.e., native) pathogen might control its weed hosts through a massive dose of inoculum at susceptible stages of weed growth (Charudattan, 1991; Daniel *et al*., 1973). Much research on the development of new mycoherbicides has been conducted during two past decades worldwide. Several weeds have been targeted for microbial control (Boyette & Walker, 1985; TeBeest *et al*., 1992; Schroeder, 1993; Johnson *et al*., 1996; Imaizumi *et al*., 1997; Thomas *et al*., 1998; Montazeri & Greaves, 2002 a), but little attempts has been reported for barnyard grass.

Some fungal species have been reported as candidates for biological control of barnyard grass *Echinochloa crus-galli* (L.) P. Beauv. *Exserohilum monoceras* (Drechsler) Leonard & Suggs, *E. rostratum* (Drechsler) Leonard & Suggs, *Curvularia lunata* (Wakker) Boedijin, *C. aeria* (Wakker) Boedijin, *Colletotrichum graminicola*, *Pyricularia grisea* (Ces.) Wils and *Ustilago trichophora* (Link) Körn (Tsukamoto *et al*., 1997) have been shown to have some promise as microbial agents for biocontrol of barnyard grass. *Exerohilum monoceras* has been evaluated as a potential bioherbicide for the control of *Echinochloa* species (Zhang & Watson, 2000). In addition, the potential of *Cochliobolus lunatus* Nelson &. Haasis, which induces necrosis on barnyard grass resulting

in death of young seedlings, has been reported as a biological control agent against this weed (Scheepens, 1987). However, no mycoherbicide has been registered at commercial level for biocontrol of barnyard grass.

Nutritional regimes during conidiation influence conidia production, germination rate, desiccation tolerance and virulence of fungal conidia (Jackson & Bothast, 1990; Schisler *et al*., 1991; Montazeri & Greaves, 2002 a). In addition to nutrition during conidiation, harvesting the conidia in some carbohydrates can influence their germination and survival (Montazeri & Greaves 2002 b). Baily *et al*., (2004) reported that Tween 20 (1%, v/v) plus pathogen caused the most severe disease by *Pleospora papaveracea* (de Not.) Sacc. On opium poppy (*Papaver somniferum* L.) Tween series were shown to promot germination in some bioherbicidal applications (Abbas & Egley 1996; Daigle & Cotty 1991).

An indigenous isolated *Fusarium anthophilum* (A. Braun) Wollenweber obtained from Guilan province, North of Iran, has been identified as a pathogen for microbial control of barnyard grass (Mojaradi *et al*., 2006). In current investigation, the influence of C: N ratios (with constant 4 g L^{-1} carbon) and glycerol in the culture media during conidiation, and suspending the harvested conidia in Tween 40 at different concentration, on *F. anthophilum* growth was studied. The evaluated parameters were conidiation, conidial germination rate and conidial germ-tube elongation.

MATERIALS AND METHODS

Effect of nutrition

F. anthophilum (isolate 16c) obtained from barnyard grass in Guilan, the North of Iran, was stored at 4 °C on PDA (Merk, Darmstadt, Germany). Petri dishes containing PDA were inoculated centrally with 6 mm plugs taken from a stock culture of *F. anthophilum* and incubated at 20 °C in darkness. Conidia were harvested from one-week old cultures by lightly scraping into sterile distilled water using a razor blade. The suspension was filtered through two layers of muslin to remove mycelia and agar fragments. The resultant conidial suspension adjusted to 5×10^5 conidia mL^{-1}, using sterile distilled water, after counting the conidia using a haemocytometer. Conidial suspension was added to 250 mL flasks, each containing 50 mL semi-defined liquid culture medium (Schisler *et al*., 1991), to give a final conidial concentration of 5×10^4 conidia mL^{-1}. The media were prepared with C:N ratios of 5:1, 15:1 and 40:1 (calculated as described by Jackson and Schisler, 1992) using glucose (BDH Laboratory Supplies, Pool, UK) as the carbon source and casamino acids (casein, Hy-case amino; Sigma Chemical Co., St Louis, USA) as nitrogen source. The total amount of carbon in all media was kept constant at 4 g L^{-1}. The final pH of all media was adjusted to 5 with 1 M-HCl or 1 M-NaCl before autoclaving. Flasks (five replicates for each treatment) were closed with loose-fitting stainless steel caps and placed on a rotary shaker with 90 rpm at 25±2 °C. The cultures were shaken by hand once a day to remove fungal growth from the flask wall.

In a separate investigation, the semi-defined liquid medium with a C: N ratio 5:1 contained 0, 1, 2 or 5 5% (v/v) autoclaved glycerol was inoculated with conidia of the fungus as explained above. The flasks (five replicates for each treatment) were placed on a rotary shaker with 90 rpm at 25±2 °C.

In both experiments, one week after inoculation of the media with conidia, the cultures were filtered through two-layered muslin cloth. Number of conidia in each resultant suspension was determined using a haemocytometer.

To determine the effect of the treatments on germination of conidia and germ-tube elongation, the conidial suspensions were centrifuged at 300 ×g for 3 minutes and the resultant pellets were suspended in distilled water and re-centrifuged. The final pellets were suspended in the distilled water and the concentration of each suspension was adjusted to 1×10^6 conidia mL^{-1}. Conidial suspension was put on autoclaved semi-permeable discs (6 mm diam.; Viskings Dialysis Membrane; Medicell International Ltd, London, UK), which were cut using a punch. For each treatment, five replicate discs each received 3 µL of conidial suspension using a micropipette were placed on glass slides at room temperature of (25±2 °C) for 30 minutes to dry. The discs were placed on TWA (Tap Water Agar) and incubated at 20 °C for 8 h. After incubation, a 5-µL droplet of aniline blue/lactophenol [0.1

g aniline blue (Riedel-De-Haen, Seelze, Germany) + 67 mL lactophenol (BDH Laboratory Supplies, Pool, UK) + 20 mL Distilled water] was put on each disc to stop conidial growth and stain the conidia. About 50 conidia on each disc were counted under microscope, and the percentage of germination was determined. Germ-tube length of 10 germinated conidia on each disc was determined using a goniometer eyepiece in a microscope.

Effect of Tween 40

The conidia of *F. anthophilum* grown in a semi-define liquid culture medium with a C:N ratio of 5:1 were harvested after one week and filtered as described above. The resultant conidal suspension was placed in 5 tubes, each contained 5 mL of the suspension and centrifuged at 300 ×g for 3 minutes. The resultant pellets were suspended in 0.2, 0.5, 1, or 2 % (v/v) of Tween 40. Suspending the pellet in the distilled water was considered as control. Each suspension was diluted to 1×10^6 conidia mL^{-1} using appropriate solution. Conidial suspension (3-µL droplet) was put on autoclaved semi-permeable discs and left on a glass slide at room temperature (22±2 °C, 50-55% RH) for 30 minutes. The discs were stored at 20 °C with 15% RH (Montazeri and Greaves, 2002a) up to 35 days. At each interval (after 0, 9 and 35 days), 5 replicated discs were placed on TWA and incubated at 20 °C for 8 h. Germination of conidia and their germ-tube elongation were determined microscopically as explained above.

Analysis of variance

For each experiment, the data were subjected to analysis of variance in a completely randomized design using SAS™ (SAS Institute Inc., 1989). The treatment means were compared with Duncan's Multiple Range Test.

RESULTS

Conidia production by *F. anthophilum* in the semi-defined liquid culture medium with a C: N ratio of 5:1 was higher than those with C: N ratios of 40:1 or 15:1 after one week incubation at 25±2 °C on rotary shaker with 90 rpm (Fig. 1). In the medium with a C:N ratio of 5:1, the fungus yielded 9.5×10^6 conidia mL^{-1}, whereas in the medium with C:N ratios of 40:1 and 15:1, conidia production was 3.2×10^6 and 6.4×10^6 conidia mL^{-1} respectively. Parallel to conidiation, germination rate and germ-tube elongation of conidia obtained from the medium with a C:N ratio of 5:1 was higher than those obtained from the media with C:N ratios of 15:1 or 40:1 (Fig. 2 and 3).

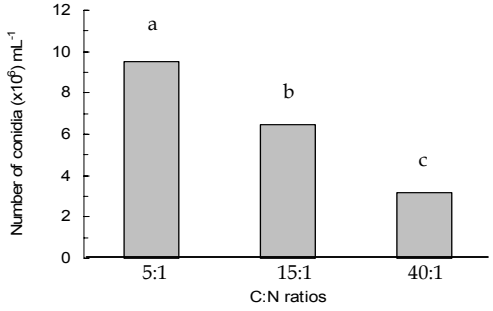

Fig 1. Conidiation of *Fusarium anthophilum* in semi-defined liquid culture media with C: N ratios of 5:1, 15:1 and 40:1 incubated on a rotary shaker at 22±2 °C for seven days. Columns with different letter have significant differences at p=0.05.

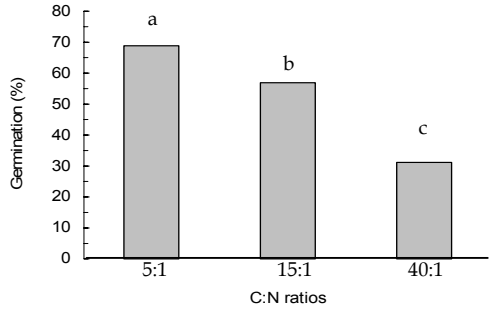

Fig. 2. Germination rate of *Fusarium anthophilum* conidia in semi-defined liquid culture media with C:N ratios of 5:1, 15:1 and 40:1, after 8 h incubation at 20 °C on PDA. Columns with different letter have significant differences at p=0.05.

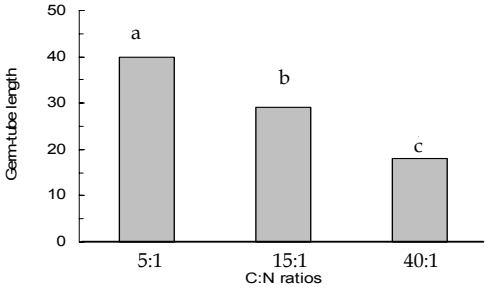

Fig. 3. Germ-tube elongation of *Fusarium anthophilum* conidia grown in a semi-defined liquid culture medium with a C:N ratios of 5:1, 15:1 or 40:1, after 8 h incubation at 20 °C on PDA. Columns with the same letter have no significant differences at p=0.05.

Glycerol in the liquid culture medium at 1, 2 or 5% (v/v) enhanced conidiation of the fungus (Fig. 4). The highest conidia production (16.7×10^6 conidia mL^{-1}) was obtained in the medium contained 5% glycerol, which was significantly higher than those contained glycerol at 0 (control; 8.93×10^6 conidia mL^{-1}) 1% (13.5×10^6 conidia mL^{-1}) or 2% (15.6×10^6 conidia mL^{-1}). Glycerol at all concentrations had no positive effect on germination and germ-tube elongation (Fig. 5 and 6).

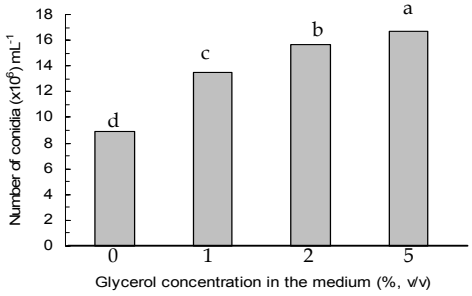

Fig 4. Conidiation of *Fusarium anthophilum* in a semi-defined liquid culture medium with a C:N ratios of 5:1 contained glycerol at 0, 1, 2 or 5% (v/v). Columns with different letter have significant differences at p=0.05.

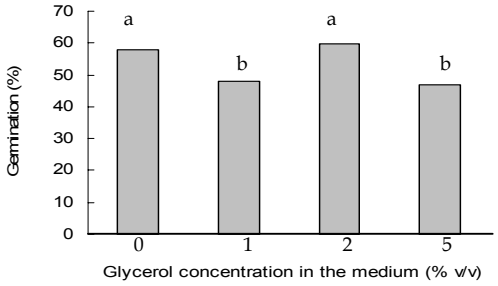

Fig 5. Germination rate of *Fusarium anthophilum* conidia grown in a semi-defined liquid culture medium with a C:N ratios of 5:1 contained glycerol at 0, 1, 2 or 5% (v/v), after 8 h incubation at 20 °C on PDA. Columns with the same letter have no significant differences at p=0.05.

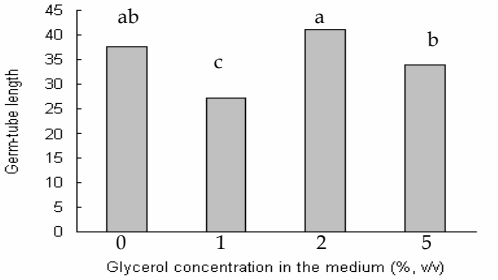

Fig 6. Germ-tube elongation of *Fusarium anthophilum* conidia grown in a semi-defined liquid culture medium with a C:N ratio of 5:1 contained glycerol at 0, 1, 2 or 5% (v/v), after 8 h incubation at 20 °C on PDA. Columns with the same letter have no significant differences at p=0.05.

Germination percentage and germ-tube elongation of conidia which were suspended in 0.2, 0.5, 1 or 2% of Tween 40, regardless of storage period, was significantly greater than those suspended in the distilled water (Fig. 7 and 8). Suspended conidia in Tween 40 at 0.5%, resulted in higher germination than those suspended at the other concentrations, when they were placed on PDA for 8 h at 20 °C after 9 or 35 days storage at 20 °C and 15% relative humidity (Fig. 7). For fresh or 9-day stored conidia, germ-tube length of suspended conidia in Tween 0.2% was significantly longer than those which were suspended at higher concentrations of Tween 40 (Fig. 8). However, after 35 days storage, there was no significant difference between suspended conidia in Tween 40 at 0.2% and 0.5% (Fig. 8).

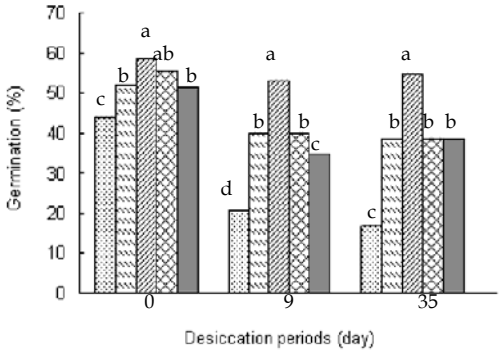

Fig 7. Germination percentage of *Fusarium anthophilum* conidia harvested in Tween 40 at 0 ( ), 0.2 ( ), 0.5 ( ), 1 ( ) or 2% ( ) (v/v). After desiccation periods, conidia on semi-permeable discs were placed on PDA at 20 °C for 8 h to allow germination. For each period, Columns with the same letter have no significant differences at p=0.05.

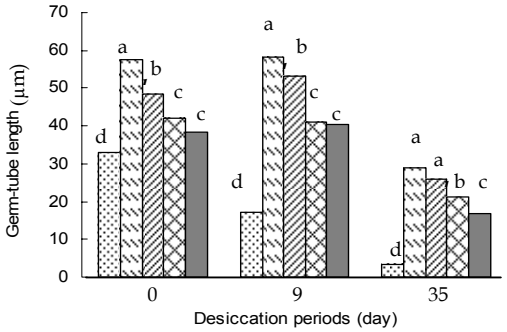

Fig. 8. Germ-tube elongation of *Fusarium anthophilum* conidia harvested in Tween 40 at 0 ( ), 0.2 ( ), 0.5 ( ), 1 ( ) or 2% ( ) (v/v). After desiccation periods, conidia on semi-permeable discs were placed on PDA at 20 °C for 8 h to allow germination. For each period, Columns with the same letter have no significant differences at p=0.05.

DISCUSSION

for a pathogen be successful as a mycoherbicide, producing abundant inoculum in an artificial culture media and maintaining its viability during storage are essential. Conidiation and conidia germnation of fungi are influenced by nutritional regimes (Jackson & Bothast, 1990), but the specific responses of different species vary. Schisler *et al*., (1991) reported that conidia of *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore produced in a medium with a C:N ratio of 10:1 germinated more rapidly, formed more appresoria and produced more severe disease in *Sesbania exaltata* than those produced in media with C:N ratios of 30:1 or 80:1. Conidia yield by *Alternaria alternata* L. (isolate 423, obtained from *Amaranthus powelii* S.Watson), on agar media with a C: N ratios of 5:1 and 15:1 was significantly higher than that of C:N ratio of 40:1 (Montazeri and Greaves, 2002 a). The result of the present work indicated that *F. anthophilum* grown in a medium with C: N ratio of 5:1, produced more conidia, higher conidia germination and longer germ-tube than in the media with C:N ratios of 15: or 40:1. Adding glycerol into the media, especially at 5% (v/v), enhanced conidiation, but had no positive effect on conidia germination and germ-tube elongation.

Various adjuvants and amendments have been used either to improve or modify spore germination, pathogen stability and virulence, environmental requirements, or host preference, all of which greatly influence the bioherbicidal potential of a candidate microorganism (Boyette *et al.,* 1996). Most weeds are covered with a waxy cuticle that prevents a water-based product from spreading evenly, which can result in unequal distribution of active ingredient (microbial agent) (McWhorter *et al.,* 1988). Surfactants, such as Tween series, help to wet the plants and aid in dispersing the fungal spores throughout the spray mix. Surfactants may affect the growth or germination of fungi, so preliminary experiments should be conducted to determine this. Direct effect of surfactant on fungal growth and development vary depending on the fungal species. Tween 20 promoted spore germination when incorporated into some bioherbicide formulations (Abbas & Egley, 1996; Daigle & Cotty, 1991), but it failed to improve bioherbicide performance in other situations (Green *et al*., 1997; Klein & Auld, 1995). In the present investigation, Tween 40 enhanced germination and germ-tube elongation of both the fresh and stored conidia of *F. anthophilum* for 9 or 35 days at 20 ^{o}C with 15% relative humidity. The effect of Tween 40 was more pronounced on conidia germination and germ-tube elongation respectively at 0.5% and 0.2% (v/v).

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