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INFLUENCE OF ADJUVANTS ON SPORE GERMINATION, DESICCATION TOLERANCE AND VIRULENCE OF *Fusariumanthophilum* ON BARNYARD GRASS (*Echinochloa crus-galli*)

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ABSTRACT

Spores of Fusarium anthophilum isolate 16c, a pathogen of barnyard grass, were harvested from 10 day old semi-defined culture medium with a C:N ratio of 5:1 and suspended in pure white coconut oil, diethanolamine, PZ, RG[22] or volk, each at 0.2, 0.5, 1 or 2% Spore suspension in distilled water was considered as the control. Droplets (3-µL) of spore suspensions were separately placed on semi-permeable membrane and stored at 25 ^{o}C with 15% RH for 0, 6, 12 or 38 days. At each interval, four replicated discs of each treatment were place on PDA for 8 h to evaluate germination rate and germ tube elongation. In glasshouse experiments, spores suspended in the above mentioned adjuvants were sprayed onto seedlings of barnyard grass to determine their influence on the virulence of the pathogen. For non-desiccated spores, the highest germination was achieved with coconut oil 2%. After 6-, 12- or 38-day desiccation period, the germination of spores suspended at each concentration of coconut oil or volk at 1% was significantly higher than those suspended in the other adjuvants or water. Germ tubes of non-desiccated spores which were suspended in each concentration of volk, 0.2 or 0.5% PZ, 0.5% diethanolamine or 0.5% coconut oil were significantly longer than other treatments. The enhancing effect of coconut oil 0.5% on germ tube elongation was more pronounced after 6, 12 and 38 day desiccation periods. All adjuvants used in this study significantly enhanced disease expression, compared with water, but the effect of coconut oil 1%, PZ 0.5% and RG 0.2% was higher than diethanolamine at 2% and volk at 1%.

Keywords:Biological control, *Echinochloa crus-galli* var. *praticola* , *Fusarium anthophilum*, adjuvants.

INTRODUCTION

Biological control of weeds, which is believed to be safe to other organisms, is an alternative approach to control or reduce the population of undesirable weed species. There are two approaches to biological weed control using plant pathogens: a) the classical approach and b) the mycoherbicide approach (Templeton & Smith, 1977). In the classical approach, an alien pathogen is introduced into susceptible weed population and allowed to spread (Templeton & Smith, 1977). In the mycoherbicide approach, a pathogen is applied to target weeds using techniques and methodologies similar to those used with chemical herbicides (Charudattan, 1991). This approach is better suited to produce the rapid and high level of weed control. At the present, several mycoherbicides have been registered in USA and Canada (Daniel *et al*., 1973; Mortensen, 1988; Ridings, 1986; Charudattan, 1991). Moreover, many researchers are investigating microbial herbicides for various weeds (Boyette*et al*., 1993; Tsukamoto *et al*., 1997; Cother *et al*., 2000; Baily *et al*., 2004). Among registered mycoherbicides, Collego (*Colletotrichum gloeosporioides* f. sp. a*eschynomene*) is used for control of northern jointvetch in rice and soybean in several states in the USA (TeBeest, & Templeton, 1985).

Several fungal species have been reported as candidates for biological control of barnyard grass. *Exerohilum monoceras*, *E. rostratum*, *Curvularia lunata*, *C. aeria*,*Colletotrichum graminicola*, *Pyricularia grisea* and *Ustilago trichophora* (Tsukamoto *et al*., 1997) have been shown to have some promise as microbial agents for biocontrol of barnyard grass. *E. monoceras* has been evaluated as a potential bioherbicide for the control of*Echinochloa* species (Zhang & Watson, 2000). In addition, the potential of *Cochliobolus lunatus*, 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 for barnyard grass.

Since mycoherbicides can be influenced by environmental factors, a wide range of adjuvants can be used to minimize the undesirable effects of these factors (Prasad, 1993; 1994), and overcome weed defenses to achieve adequate control (Hoagland, 1996). 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). Bailey *et al*. (2004) evaluated the effect of several adjuvants on spore germination, appressorium formation and virulence of *Pleospora papaveracea*, a biocontrol agent of opium poppy. In their experiment, the inclusion of Tween 20 (1% v/v) plus pathogen (1×10^6 spores mL^{-1}), greatly enhanced spore germination and disease incitement on opium poppy. The response of mycoherbicides to different adjuvants varies, with different concentration. Grant *et al*. (1990) evaluated the effects of 16 adjuvants on spore germination and production in *C. gloeosporioides* f.sp. *malva*. In their studies, many alcohol- and ether-based adjuvants inhibited spore germination.

An indigenous isolate of *Fusarium anthophilum* (A. Braun) Wollenweber obtained from Guilan, North of Iran, has been identified as a pathogen for microbial control of barnyard grass (Mojaradi *et al*., 2006). Sporulation and spore germination of the pathogen grown in a semi-defined liquid medium with a constant carbon concentration of 4 gL^{-1} and a C:N ratio of 5:1 were higher than in other nutritional regimes (Mojaradi *et al*., 2006). The purpose of this investigation was evaluate the effects of some adjuvants on germination, germ-tube elongation and desiccation tolerance of spores and disease expression by the pathogen on barnyard grass.

MATERIALS AND METHODS

Spore Production

Based on previous investigation (Mojaradi *et al*., 2006), a liquid semi-defined medium with a constant carbon concentration of 4 gL^{-1} and a C:N ratio of 5:1 was used for spore production. The final pH of the medium was adjusted to 6 with 1-M HCl or 1- M NaOH before autoclaving. The contents of flasks were inoculated under sterile conditions with two 5-mm plugs of *F. anthophilum*, obtained from 7-day old culture on potato dextrose agar (PDA) medium. Flasks were placed on an orbital shaker (90 rpm) in room temperature (25±2 ^oC). The cultures were shaken by hand daily to remove fungal growth from the flask internal wall. After 10 days, the contents of flasks were mixed and filtered through 4 layers of muslin cloth to remove mycelia and culture debris. The spore suspension was used for further studies.

Spore Germination and Desiccation Tolerance

Spore suspension in 21 tubes, each containing 15 ml of the suspension, was centrifuged at 6000 rpm for 4 minutes. The resulting pellet of each tube was separately suspended in pure white coconut oil (Sime Darby Edible Product Ltd., Jurong Town, Singapore), diethanolamine (Merk KGaA, Darmstadt, Germany), PZ (Pars Zangaran, Shahryar, Tehran, Iran), RG^{22} (Pars Zangaran, Shahryar, Tehran, Iran) or volk (Giah, Karaj, Iran), each at 0.2, 0.5, 1 or 2%. Suspending the pellet in distilled water was considered as the control. The resulting suspensions were diluted to 1×10^6 spore mL^{-1} in appropriate solution, using a haemocytometer. Droplets (3-µL) of each suspension were put separately onto autoclaved 6-mm discs of semipermeable membrane (Visking Dialysis Membrane, Medicell International Ltd, London, UK) and air dried for 30 minutes at ambient laboratory temperature (22±2˚C) and 45±2 % relative humidity. The dried discs were stored for 0 (no storage), 6, 12 or 38 days at 25 ˚C in polycarbonate boxes (17×11×4 cm) containing 100 g $CaCl_2.H_2O$ (BDH Laboratory Supplies Poole, UK) to obtain 15% relative humidity (Montazeri & Greaves, 2002). The humidity was monitored throughout the storage period using a hygrometer (Rotronic Ag, Basserdorf, Swizerland) inserted through a narrow pipe sitting in the end of the box. The experiment was arranged as completely randomized design with four replications.

At each interval of storage, four discs were taken from each treatment and placed on PDA in Petri dishes at 20˚C for 8 h, with the spores on the upper surface. Then, 5-µL droplet of aniline blue/lactophenol [0.1 g aniline blue (Riedel-de-Hean, Seelze, Germany) plus 67 mL lactophenol (BDH laboratory supplies, Poole, UK) + 20 ml distilled water)] was put on each disc to stain the spores and stop their growth. The number of germinated spores and length of germ-tube present in a sample of randomly selected spores was determined. Germination and germ-tube elongation were determined microscopically for about 40 spores on each disc. The experiment was conducted twice and the means of data (obtained from both experiments) were subjected to analysis of variance.

Virulence Assay

Seeds of barnyard grass were placed on moistened filter paper in Petri dishes and incubated at 25 ˚C for 7 days in a germinator in continuous darkness to induce seed germination. Germinated seeds were transplanted in 20 cm plastic pots (5 seedlings per pot) containing a clay loam:peat:sand mixture (6:4:2) and placed on a glasshouse bench. Temperature in the glasshouse was 23±3 ˚C with 60±3% relative humidity (RH) with 16 h day length. Plants were irrigated once a day.

The spores of *F. anthophilum* were suspended in RG^{22}, PZ, pure white coconut oil, volk, diethanolamine (respectively at 0.2%, 0.5%, 1%, 1%, and 2%) or distilled water to achieve a concentration of 1×10^6 spore mL^{-1}. Each resulting suspension was sprayed on four replicated pots, each containing five seedlings of barnyard grass at 4-5 leaf stage, using a laboratory aerosol sprayer (Airbrush, Humbrol Ltd., Hull, UK) delivering an application volume of 10 mL per pot from a distance of 30 cm from the target. Untreated pots were considered as control. The pots were kept for 24 h in a white plastic box in which the relative humidity was over 90%. The seven treatments were replicated four times in a completely randomized design.

To asses the effect of treatments on disease severity, the seedlings of each pot were removed 10 days after treatment and were dried in an oven at 75 ^oC for 72 h after washing their roots. The dried seedlings of each pot were weighed.

Analysis of Variance

In both experiments, the data obtained at each storage period were subjected to analysis of variance using SAS^{Tm} (SAS Institute Inc., 1989). The treatment means were compared with Duncan's Multiple Range Tests.

RESULTS AND DISCUSSION

There were significant variations in germination and germ tube elongation of spores treated with different adjuvants. For the non-stored spores, coconut oil at 2% enhanced their germination significantly than the control and other adjuvants (Fig. 1 A). Germination of the non-stored spores treated with coconut oil at 0.2, 0.5 and 1% and volk at 1%, was also higher than the control (Fig. 1 A). After 6 and 12 days desiccation period (when spores were placed on PDA medium for 8 hours) the effect of coconut oil at all concentrations and volk 1% in enhancing spore germination was significantly more than the other treatments (Fig. 1 B & C). At this interval, the influence of coconut oil at all concentrations was significantly higher than other adjuvants (Fig. 1 D).

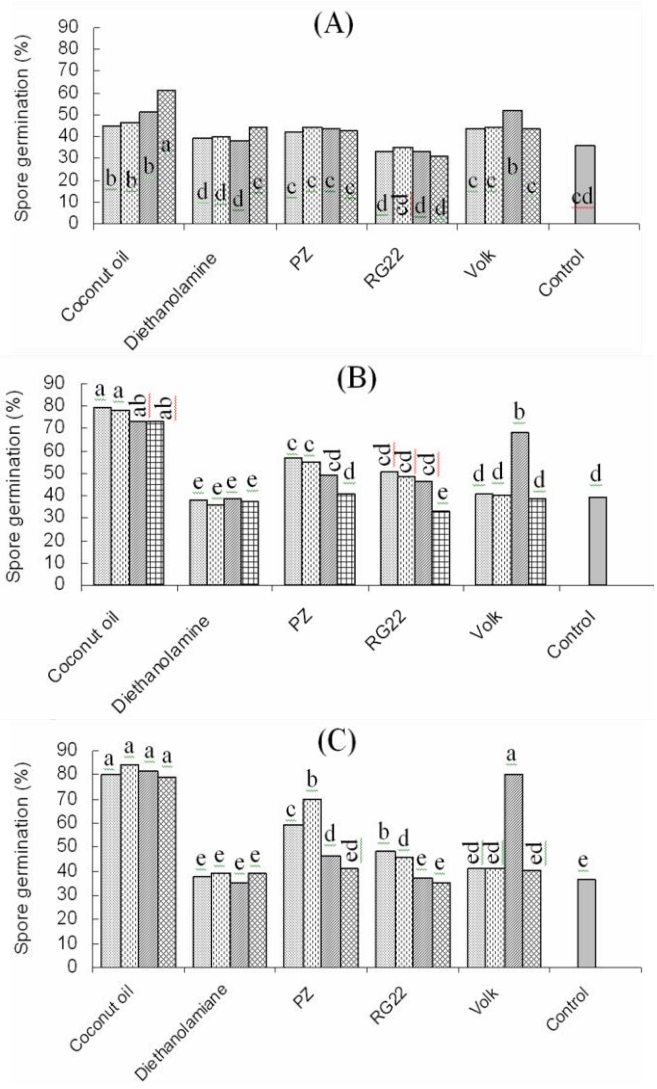
For non-stored spores, volk at all concentration, PZ at 0.2 or 0.5%, diethanolamine at 2% and coconut oil at 2% had significant effect, compared with control, on germ tube elongation (Fig. 2 A). After 6 to 38 days desiccation period, the effect of coconut oil 0.5% on germ tube elongation was significantly higher than the other treatments (Fig. 2 B-D). In addition to this treatment, the effect of coconut oil 1 or 2 %, PZ 0.2% and volk 1% on germ tube elongation of stored spores was significant (Fig. 2 B-D).

The dry weight of barnyard grass seedlings treated with spores suspended in each adjuvant was significantly lower than those of suspended in water and the control (untreated with spore suspension) (Fig. 3). Among the adjuvants, the dry weight of seedlings inoculated with spore suspensions which contained 2% RG^{22}, 1% coconut oil or 0.5% PZ was significantly lower than those treated with spore suspensions containing 2% diethanolamine or 1% volk (Fig. 3).

The simplest mycoherbicide delivery system uses water to carry the fungus. However, most weeds are covered with a wax cuticule that prevents a water-based product from spreading evenly, which can result in unequal distribution of microbial agent (McWhorter *et al*., 1988). Adjuvants help to wet the plants and aid in dispersing the fungal spores throughout the spray mix (Boyette *et al*., 1996). But, the spore germination of fungi may be affected by adjuvants (Bailey *et al*., 2004; Boyette *et al*., 1996). The reaction of various fungal pathogens to different adjuvands varies with different concentration. In the current investigation, coconut oil at all at concentrations and volk 1% significantly enhanced spore germination of non-stored spores. The effect of adjuvants on spore germination after a desiccation period is important, because mycoherbicides must retain their viability and virulence under desiccation condition in storage and delivery system. The results of this study indicated that the effect of coconut oil, especially at 0.5%,

was more pronounced on germination and germ tube elongation after desiccation periods. Thus, after 38 days storage at 20 ^{o}C and 15% relative humidity, when spores were placed on PDA medium for 8 h, germination of treated spores with coconut oil was over 80%, whereas that of control was 37%. The similar results were obtained for germ tube elongation.

Various adjuvants have been used to improve the bioherbicidal potential of a candidate microorganism. Disease severity from *Bipolaris sorghicola* on Johnsongrass was significantly increased by adding 1% Soy-Dex (Helena Chemical Co., Memphis, USA) to the fungus spray mix (Van Dyke and Winder, 1985; Winder & Van Dyke, 1990). Walker and Riley (1982) showed that non-ionic nonoxynol surfactant had no effect on spore germination of *Alternaria cassiae*, but enhanced efficacy of the pathogen in control of *Senna obtusiolia*. In the current research, all adjuvants, compared with water, significantly enhanced disease expression on barnyard grass incited by fresh spores of *F. anthophilum* isolate 16c. Among the adjuvants, the effect of coconut oil, PZ and RG^{22} was more than the others. The enhancing effect of coconut oil on the disease was expected because of its significant influence on spore germination and germ tube elongation. But, the effect of other adjuvants, especially PZ and RG^{22} which had non significant effect on germination of fresh spores, may be attributed to inducing host susceptibility to the pathogen.



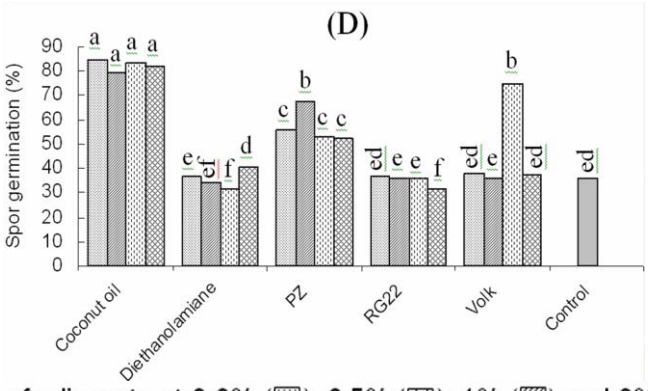
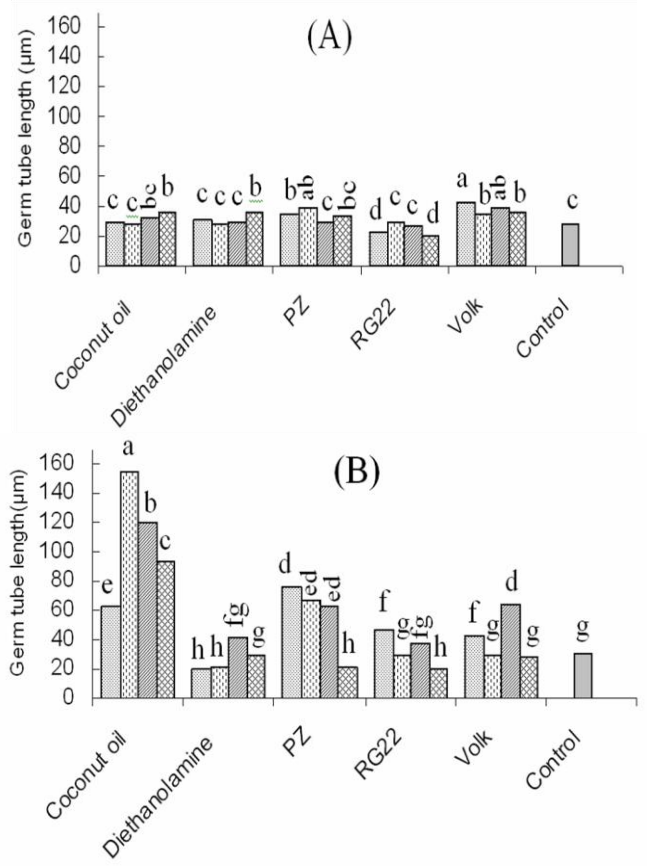


Fig. 1. The effect of adjuvants at 0.2% (▨), 0.5% (▨), 1% (▨) and 2% (▨), compared with control (▨), on spore germination of *Fusarium anthophilum* isolate 16c after 0 (A), 6 (B), 12 (C) or 38 (D) days incubating at 20 °C with 15% relative humidity. At each interval, spores were placed on PDA medium for 8 h to stimulate germination. Columns with the same letter have no significant differences at p=0.05.



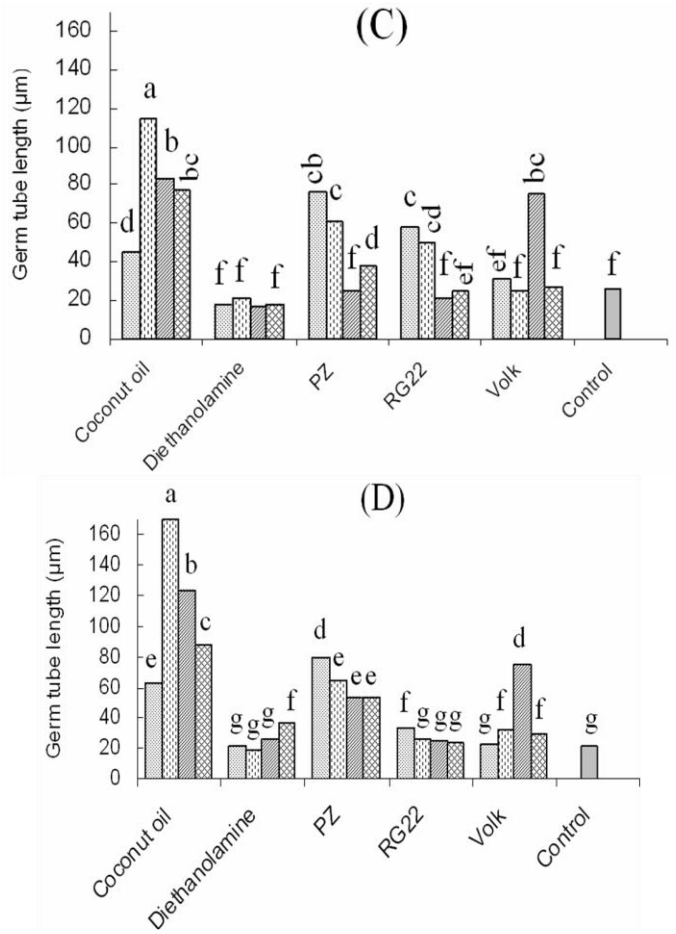


Fig. 2. The effect of adjuvants at 0.2% (▒), 0.5% (▒), 1% (▒) and 2% (▒), compared with control (▒), on spore germ tube elongation of *Fusarium anthiphilum* isolate 16c after 0 (A), 6 (B), 12 (C) or 38 (D) days incubating at 20°C with 15% relative humidity. At each interval, spores were placed on PDA for 8 h to stimulate germination. Column with the same letter have no significant differences at p=0.05.

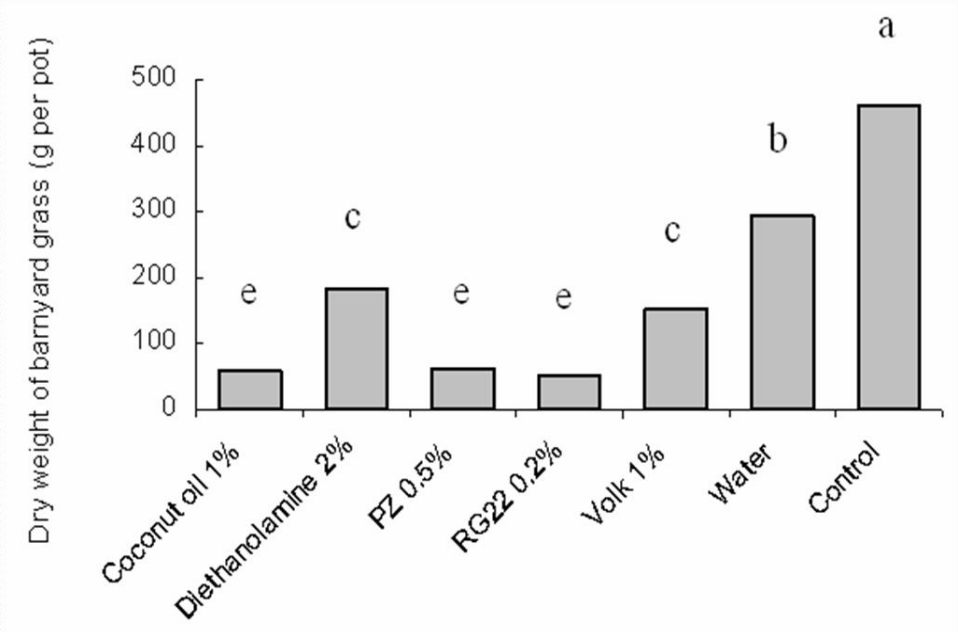


Fig. 3. Dry weight of barnyard grass seedlings 10 days after inoculations with spores suspended in the adjuvants, compared with the control (non-inoculated seedlings). Columns with the same letter have no significant differences at p=0.05.

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