ASSESSMENT OF PHYTOTOXIC SPENT BROTH AND SPORES OF *ALTERNARIA ALTERNATA* (LC#508) AS A FORMULATION WITH ENHANCED MYCOHERBICIDAL ACTIVITY TO CONTROL *LANTANA CAMARA*

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Abstract: Enhanced social concern towards the ill effects of synthetic agrochemicals, their residual toxicity and resistance development in the target pests created a necessity of exploration of alternatives. Fungal biocontrol agents have been used as herbicides over two decades and research continues to enhance their efficacy comparable to synthetic herbicides. Combinations of synthetic herbicides with the fungal biocontrol agents was an approach adopted to enhance mycoherbicidal activity but had limited acceptance due to negative effects of their synthetic components. Hence a necessity for development of the formulations having least toxicity or being benign to non-target organisms was felt. The present study brings forth a new dimension of holistic and ecofriendly bioherbicidal formulations comprising of crude phytotoxins and spores of *Alternaria alternata* LC# 508 which enhances outstandingly its mycoherbicidal potential. Spent broth of *Alternaria alternata* (LC#508) exhibited toxic activity to its spores (autotoxicity) at a concentration of 50μg/ml which was used for developing five formulations FN1 to FN5. Formulations No. 5 was the best of all the formulations when evaluated by pot trails compared to control and other formulations. Disease onset and mortality was observed in 3.5 days and 5 days respectively in case of formulation No 5 (50 μg of crude spent broth + 1x 10^6 spores/ml) when compared to spore spray alone. Thus the formulation of crude spent broth and fungal spores could be developed as a cost effective and efficacious bioherbicide formulation when compared to the spores alone.

Key words: *Alternaria alternata,* autotoxicity, fungal biocontrol agents, formulations, *Lantana,* mycoherbicides, phytotoxins

INTRODUCTION

Increasing incidences of resistance to crop protection agents and societal concern towards clean environment and health of the user necessitated the search of ecofriendly and effective formulations to replace synthetic agrochemicals. Thus strategies that could be ecofriendly, efficient and safer as compared to current crop protection agents have been extensively studied (Holt 1992; Charudattan and Dinoor 2000).

Weeds represent one of the most costly and limiting factors in crop production. *Lantana camara* (Family: *Verbenaceae*) has been recognized as a global weed infesting 14 crops across 47 countries of the world. The plant is toxic to humans and the ruminant livestock and therefore demands immediate check through ecofriendly methods. Biological control using plant pathogens is a widely accepted alterative to control weeds for over two decades (Charudattan and Dinoor 2000; Saxena and Pandey 2001), however its the environmental window of efficacy is narrow as compared to their synthetic counterparts. This necessitates the development of suitable stable formulations to enhance their efficacy under varied environmental conditions. Extending dew periods, spore dispersal and virulence are essential features sought in a mycoherbicidal formulation to be efficacious (Anderson 1983). Ionic as well as organosilicone surfactants like Tween-80 and Silwett-77 have been incorporated with the biological control agent (BCA) for the development of effective formulations and have been evaluated for their use as commercial mycoherbicides (Daigle and Connick 1990; Knudsen *et al*. 1991; Connick *et al*. 1998; Ahmed *et al*. 2003).

BCA's have also been formulated with synthetic crop protection agents to enhance their potential and simultaneously reduce the input of synthetic agrochemicals (Kulshreshtha 1986; Wymore *et al*. 1987; Watson and Gottlieb 1990). Integrating the crude phytotoxins with their producers i.e BCA's for formulation development is a new concept which has not been exploited so far.

In the present communication we assess the role of integration of crude spent broth obtained from indigenous isolate of *Alternaria alternata* LC#508 to enhance its mycoherbicidal potential for the management of *L. camara* (Saxena *et al*. 2002).

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MATERIALS AND METHODS

Production of inoculum

The original isolate of *A. alternata* LC#508 (ITCC 4896) was recovered from diseased leaves of *Parthenium hysterophorus as* well as *L. camara* from Bargi Hills, M.P. India. It was confirmed to be pathogenic according to Koch's postulates. The isolate was grown on Fresh Potato Dextrose Agar (FPDA) plates at 26±2°C for 7 days. The conidia were harvested by flooding the Petri dishes with sterile distilled water (SDW). The conidial suspension was then filtered through muslin cloth to remove the mycelial bits. The resulting suspension was stored in a refrigerator till further use.

Production of crude phytotoxin

There are phytotoxins present in the crude spent broth of *A. alternata* LC#508 (ITCC 4896) which are active in the range of 100–1000 μg/ml (Saxena 2004). To produce crude phytotoxins, 50 ml of presterilized potato dextrose broth in 250 ml Erlenmeyer flasks was inoculated with 5mm mycelial disc of 7-days old culture of *A. alternata* LC#508 under aseptic conditions and incubated in shaker at 26±2°C for 14 days in triplicates. The contents of the inoculated Erlenmeyer flasks as well as control were aseptically filtered using autoclaved pre-weighed muslin cloth. The filtrate was centrifuged at 8000 rpm for 20 min. and the supernatant passed through a dried preweighed Whatman No. 1 filter paper to make the filtrate cell free. The so obtained cell free filtrate was concentrated by half and subsequently extracted by chloroform (4x50 ml) to obtain a crude mixture of phytotoxins. This was subjected to *in vacuo* evaporation to obtain a crude residue of mixture of phytotoxins which was stored at 4°C till further use.

Food poison assay

Food poison assay was adopted to check a toxic concentration of crude phytotoxins of the fungus, *A. alternata* LC# 508 (ITCC 4896) (Nene and Thapalyal 1997; Singh *et al*. 2004). Stock solution of crude phytotoxic residue having concentration of 200 mg/ml was prepared in 5% dimethylsulphoxide (DMSO). The stock solution was added in appropriate concentrations to PDA medium in order achieve a final concentration of 10μ–100μg /20 ml of PDA which was then aseptically poured into sterile 90 mm Petri dishes in triplicates and allowed to solidify. The control plates were dispensed with only 5% DMSO. The medium in each plates was then inoculated with 5 mm disc of 7-day old culture of *A. alternata* upside down in the centre of Petri dishes. The treated as well as control plates were incubated at 26±2°C till the Petri dishes in control set were maximally covered i.e. 7 days. The mycelial growth (mm) of the test fungus was measured diametrically in different treatments and inhibition calculated using the formula:

$$I\% = [(C - T)/C] \times 100$$

where I refers to Inhibition; C refers to the diameter of fungi in the control and T refers to diameter of fungi in the test.

Formulation development

Based on the results of the food poison assay formulations of spore and crude phytotoxins were prepared for all the concentrations below ED_{50}. The five formulations prepared were designated as FN1 to FN5 (Table 1). They consisted of crude phytotoxin in concentrations below and up to ED_{50} and spores concentration of $1x10^6$ spores/ml. All formulations were stored at 4°C till further use.

Table 1. Concentrations of spores and crude spent broth for development of different formulations

Formulation Code	concentration of BCA	concentration of crude spent broth
FN1	$1x10^6$ spores/ml	10μg/ml
FN2	$1x10^6$ spores/ml	20 μg/ml
FN3	$1x10^6$ spores/ml	30 μg/ml
FN4	$1x10^6$ spores/ml	40μg/ml
FN5	$1x10^6$ spores/ml	50μg/ml

***In vivo* pot assays of the bioherbicide formulation**

Four to six week old plants having 4–8 leaves were grown in pots in a growth chamber with illumination of 12 h daily for the period of 10 days at 26±2°C for acclimatization. The test set as well as control set comprised 10 plants each. Five different test sets were set up corresponding to five formulations developed viz. FN1; FN2; FN3; FN4 FN5 which were used for spraying experimental plants. The control set for each test formulation received a spray of $1x10^6$ spores/ml and control over control (placebo) received only water. The plants were assessed for disease severity on the basis of average leaf area damaged (ALAD) every 12 h on a five-grade scale (Chaing *et al.* 1989) until all plants died. The experiment was repeated twice.

RESULTS AND DISCUSSION

Autotoxic activity

Partially purified spent broth within a concentration range of 60–100 μg/ml induced extreme inhibition in colony diameter of the test fungus ranging from 77–98.5% when compared to the control. ED_{50} of the *A. alternata* LC#508 phytotoxic residue was 50μg/ml (Fig. 1). A variety of plant essential oils and extracts have been evaluated for the biological control of plant pathogenic as well as human pathogenic fungi (Mishra and Dubey 1990; Wilson *et al*. 1997; Phongpaichit *et al*. 2005). Fungal secondary metabolites produced by one fungal species have been found to possess antifungal activity towards other species of the same or different genera (Duan *et al*. 2007; Cabras *et al*. 2006; Vincente *et al*. 2001). There are hardly any instances where the antifungal activity of fungal secondary metabolite produced has been tested against itself.

***In vivo* pot assays of the bioherbicide formulation(s)**

On the basis of *in vitro* autotoxic assay, concentrations of 10–50 μg/ml were prepared with the spores as carrier and sprayed on 4–6 weeks old *Lantana* plants. The onset of disease was greatly reduced in formulations when compared with the control i.e spore spray. FN5, FN4 and FN3 reduced the disease onset by 84 hours while FN2 reduced

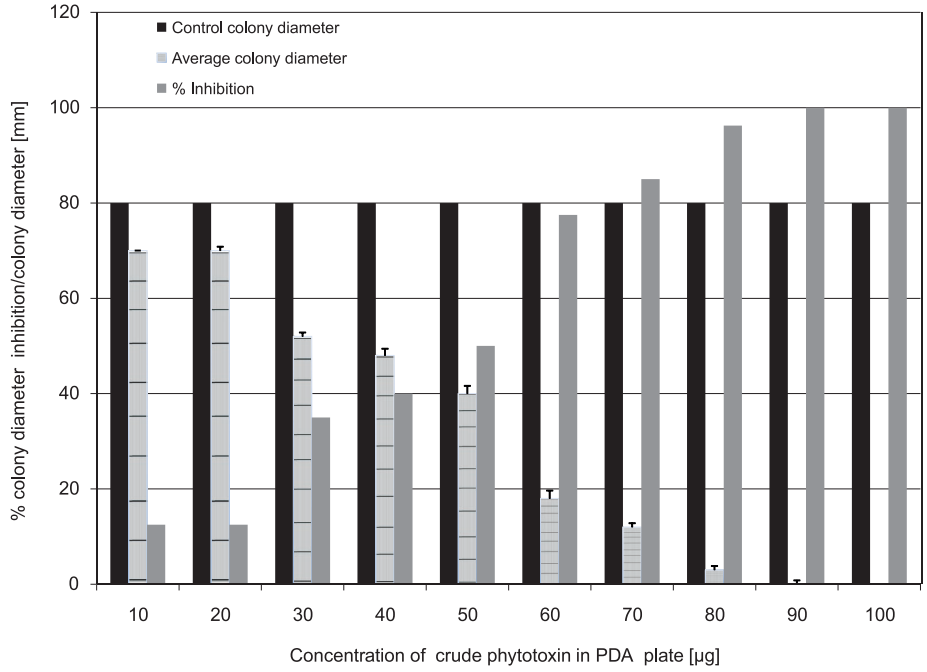

Fig. 1. Autotoxic activity of crude phytotoxins against *A. alternata* LC#508 by Food Poison Assay

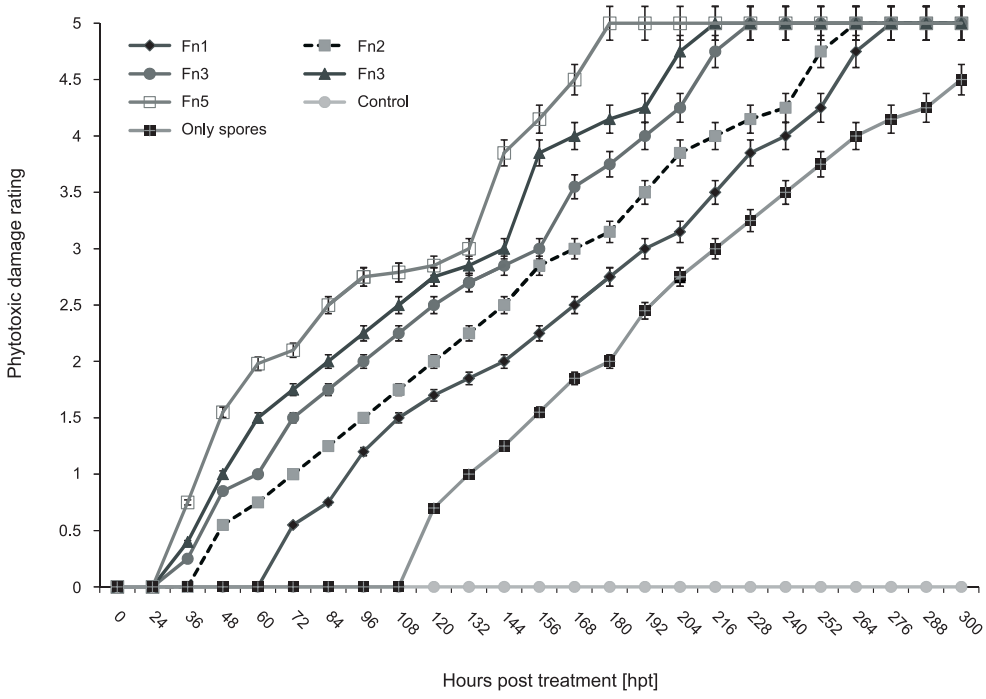

Fig. 2. Comparative mycoherbicidal efficacy of formulations (FN1-FN5) developed with spores of *A. alternata* LC#508

it by 72 hours and FN1 by 48 hours when compared to the control. Similarly, mortality was observed earlier in formulations as compared to the control. FN1 reduced the mortality of plants 24 hours earlier compared to control and FN2 earlier by 36 hours. Mortality was achieved 72 hours earlier by FN3, 108 hours by FN4 and 120 hours by FN5. Overall the best formulation which induced early onset of the disease and mortality was FN5 (Fig. 2). Synergism of chemical herbicides with fungal biocontrol agents have been tested with the intention of increasing the disease severity of the mycoherbicides (Altman *et al*. 1990; Prasad 1994). Synergistic activity of chemical herbicides with fungal biocontrol agents has been found to enhance their mycoherbicidal potential. Formulation of *Plectrosporium alismatis* with 1.5% Londax (bensulphuron–methyl) was found to be effective in field trials against starfruit (*Damasonium minus*) (Jahromi *et al*. 2006). Similarly, effect of Metribuzin enhanced the mycoherbicidal potential of *Collectotrichum truncatum* against *Matricaria perforata* i.e scentless chamomile. However they have not been very popular due to cost implications as well as for their residual toxicity to non-target organisms. Synergistic activity of crude spent broth exhibiting phytotoxicity produced by the fungal biological control agent with its spores has

not been tested so far with the idea of enhancing the mycoherbicidal potential i.e disease severity caused by the biocontrol agent. Furthermore, the spent broth can serve as osmoticum and provide relevant conditions for storage of the biocontrol agent thus preventing the use of surfactants and hydrants.

CONCLUSION

The present study indicates that crude phytotoxins produced in spent broth of the fungal biocontrol agents could be effectively combined with their propagules for the enhancement of their mycoherbicidal potential as a cost-effective replacement to synthetic chemical herbicides in integrated weed management as a novel holistic and ecofriendly approach. This would further help in dramatically reducing the input of harmful synthetic chemicals into the environment.

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POLISH SUMMARY

BADANIE PÓŁPŁYNNEGO PODŁOŻA I ZARODNIKÓW *ALTERNARIA ALTERNANA* JAKO FORMULACJI O WZMOŻONEJ AKTYWNOŚCI MYKOHERBICYDOWEJ (LC#508) DO ZWALCZANIA *LANTANA CAMARA*

Wzmożone zainteresowanie społeczne oraz niekorzystne efekty syntetycznych agrochemikaliów, toksyczność ich pozostałości i rozwój odporności u zwalczanych agrofagów stworzyły potrzebę poszukiwania alternatywnych rozwiązań. W ciągu 20 lat wykorzystano grzyby będące czynnikami biologicznego zwalczania, a badania te są kontynuowane w celu uzyskiwania aktywności porównywanej z aktywnością herbicydów syntetycznych. Kombinacja herbicydów syntetycznych z grzybowymi czynnikami biologicznego zwalczania była zaadoptowanym podejściem mającym na celu pobudzenie aktywności mykoherbicydowej, ale z powodu negatywnych efektów fungicydów syntetycznych uzyskała ona ograniczoną akceptację. Uznano więc, że istnieje potrzeba opracowania formulacji mających jak najmniejszą toksyczność w stosunku do organizmów nie będących przedmiotem zwalczania. Prezentowane badania ukazują nowy wymiar holistycznej i przyjaznej środowisku formulacji bioherbicydowych w porównaniu do surowych fitotoksyn i zarodników *A. alternanta* LC#508, znacząco pobudzających potencjał mykoherbicydowy. Wykorzystane półpłynne podłoże wzrostowe *A. alternata* LC#508 wykazywało bardziej toksyczną aktywność niż jego zarodniki (autotoksyczność) w stężeniu 50 μlg/ml, które użyto do opracowania pięciu formulacji FNl do FN5. Formulacja nr 5 była najlepsza w ocenianych doświadczeniach wazonowych, w porównaniu do kontroli oraz innych formulacji. Wystąpienie choroby i śmiertelność obserwowano odpowiednio po 3,5 i 5 dniach, w przypadku formulacji nr 5 (50 μlg surowej wykorzystanej pożywki półpłynnej + l x 106 zarodników/ml), w porównaniu do opryskiwania tylko zarodnikami. Istnieje więc możliwość opracowania ekonomicznie opłacalnej formulacji będącej skutecznym mykoherbicydem, porównywalnej do zarodników grzyba.