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Current Status of Entomopathogenic Fungi as Mycoinecticides and Their Inexpensive Development in Liquid Cultures

Abid Hussain[1,2*], Ming-Yi Tian[1], Sohail Ahmed[3] and Muhammad Shahid[4]
[1]*Department of Entomology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou,*
[2]*Department of Arid Land Agriculture, Faculty of Agriculture and Food Sciences, King Faisal University, Hofuf, Al-Hassa,*
[3]*Department of Agricultural Entomology, University of Agriculture, Faisalabad,*
[4]*Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad,*
[1]*China*
[2]*Saudi Arabia*
[3,4]*Pakistan*

1. Introduction

Synthetic chemical pesticides remained the mainstay of pest eradication for more than 50 years. However, insecticide resistance, pest resurgence, safety risks for humans and domestic animals, contamination of ground water, decrease in biodiversity, and other environmental concerns have encouraged researchers for the development of environmentally benign strategies for pest control including the use of biological control agents. Naturally occurring biological control agents are important regulatory factors in insect populations. Many species are employed as biological control agents of insect pests in glass-house and row crops, orchards, ornamentals, range, turf and lawn, stored products, and forestry and for the abatement of pest and vector insects of veterinary and medical importance (Burges, 1981; Lacey & Kaya, 2000; Tanada & Kaya, 1993).

The application of microorganisms for control of insect pests was proposed by notable early pioneers in invertebrate pathology such as Agostino Bassi, Louis Pasteur, and Elie Metchnikoff (Steinhaus, 1956, 1975). These biological control agents such as viruses, bacteria, protozoa, nematodes and most fungi exert considerable control of target populations.

Among micro-organisms, entomopathogenic fungi constitute the largest single group of insect pathogens. Generally, two groups of fungi are found to cause diseases in insects. Entomopathogenic fungi belong to the orders Entomophthorales and Hypocreales (formerly called Hyphomycetes). Several other entomopathogenic fungi from other taxonomic groups are also known. Until now, over 700 species of fungi are known to infest insects (Wraight et

*Corresponding Author

al, 2007). Such insect killing fungi present major advantages. Firstly, they are important natural enemies of arthropods (Chandler et al, 2000), capable of infecting them directly through the integument. Secondly, cultivation of those fungi and production of infective conidia are easy and fairly cheap (Roberts & Hajek, 1992). Finally, entomogenous fungi can be found under different ecological conditions (Ferron, 1978).

Unique among entomopathogens, fungi do not have to be ingested and can invade their hosts directly through the exoskeleton or cuticle. Therefore, entomopathogenic fungi can infect non-feeding stages such as eggs and pupae. The insect cuticle is the first barrier against biological insecticides. Insect cuticle mainly formed from three layers such as, epicuticle, procuticle and epidermis. Each layer has different chemical structure and properties (Juárez & Fernández, 2007). The epicuticle is very thin (0.1–3 μm) and multi-layered. The outermost surface layer of the epicuticle is the lipid layer, it is mostly resistant to enzyme degradation and exhibits characteristic such as water barrier properties (Hadley, 1981); unless physically disrupted, it can help to prevent passage of cuticle degrading fungal enzymes. The site of invasion among insects is often between the mouthparts, inter-segmental folds or through spiracles. At these sites, locally high humidity promotes conidial germination and the cuticle is non-sclerotised and more easily penetrated (Clarkson & Charnley, 1996; Hajek & St. Leger 1994).

Conidia upon landing on a potential host, initiates a series of steps that could lead to a compatible (infection) or a non-compatible (resistance) reaction. In a compatible reaction, fungal recognition and attachment proceed to germination on the host cuticle. Once the epicuticle is breached, progress by the penetration peg through the cuticle may be more or less direct via penetrant hyphae, penetrant structures may also extend laterally (Hajek & St. Leger, 1994). Fargues (1984) proposed adhesion to occur at three successive stages: (1) adsorption of the fungal propagules to the cuticular surface; (2) adhesion or consolidation of the interface between pre-germinant propagules and the epicuticle; (3) fungal germination and development at the insect cuticular surface, until appresorium is developed to start the penetration stage. Zacharuk (1970b) proposed an active adhesion process for *M. anisopliae* after detecting epicuticle dissolution and mucoid material penetrating the pore canals. Infection will proceed after a successful penetration has been achieved.

In terrestrial environment, fungal conidial germination proceeds with the formation of germ tube (Boucias & Pendland, 1991) or appressorium (Madelin et al, 1967; Zacharuk, 1970a), which forms a thin penetration peg that breaches the insect cuticle via mechanical (turgor pressure) or enzymatic means (proteases) (Zacharuk, 1970b). Exocellular mucilage, proposed to enhance binding to the host cuticle, is also secreted by several entomogenous fungi during the formation of infective structures (Boucias and Pendland, 1991). In *M. anisopliae*, appressorium formation, hydrophobins, and the expression of cuticle-degrading proteases are triggered by low nutrient levels (St. Leger et al, 1992), demonstrating that the fungus senses environmental conditions or host cues at the initiation of infection. The production of cuticle-degrading enzymes, chitinases, lipases and proteases, has long been recognized as important determinant of the infection process in various fungi, facilitating penetration as well as providing nourishment for further development (Charnley, 1984; Dean & Domnas, 1983; Hussain et al, 2010b; Samsináková et al, 1971;). Among the proteases found in entomopathogenic fungi, the spore bound Pr1 has been well characterized and its role in cuticle invasion has been established (Hussain et al, 2010b; St. Leger, 1994). Ultra-

structural studies of *M. anisopliae* penetration sites on *Manduca sexta* larvae have shown high levels of Pr1 coincident with hydrolysis of cuticular proteins (Goettel et al, 1989; St. Leger et al, 1989). Pr1 inhibition studies also showed delayed mortality in *M. sexta* larvae, resulting from delayed penetration of the cuticle (St. Leger et al, 1988). Furthermore, construction of a *M. anisopliae* strain with multiple copies of the gene encoding Pr1 and over-expressing the protease resulted in 25% reduction of time to death among *M. sexta* compared to those infected by the wild-type strain (St. Leger et al, 1996). Furthermore, it has also been reported that successive *in vivo* passage enhanced the capacity of the fungus to cause infection (Daoust et al, 1982; Hussain et al, 2010b), which ultimately increased the activity of spore bound Pr1 (Shah et al, 2007).

After penetration through the cuticle, the conidia invade into the hemocoel to form a dense mycelial growth (Zimmerman, 1993). Along with penetration, fungi also produce secondary metabolites, derivatives from various intermediates, some of which have insecticidal activities (Vey et al, 2001). It has been experimentally proved that the entomopathogens producing these toxins, infection has been shown to result in more rapid host death (McCauley et al, 1968), compared to strains that do not produce these metabolites (Kershaw et al, 1999; Samuels et al, 1988). The insecticidal properties of destruxins, cyclic depsipeptide toxins from *Metarhizium* spp, described by Kodaira, (1961) are shown to be produced in wax moth and silkworm larvae by Roberts, (1966) and Suzuki et al, (1971), Furthermore, these toxins have been tested against various insects (Roberts, 1981). Currently, over 28 different destruxins have been described, mostly from *Metarhizium* spp, with varying levels of activities against different insects (Vey et al, 2001). The level of destruxin has been correlated with virulence (Al-Aïdroos & Roberts, 1978) and host specificity (Amiri-Besheli et al, 2000). Studies on the activities of destruxins have also shown modulation of the host cellular immune system, including prevention of nodule formation (Huxham et al, 1989; Vey et al, 2001) and inhibition of phagocytosis (Vilcinskas et al, 1977) among infected insects. Destruxins are produced as the mycelium grows inside the insect. Other representative toxins produced by entomopathogenic fungi include oosporein, beauvericin, and bassianolide from *Beauveria* spp. (Eyal et al, 1994; Gupta et al, 1994; Suzuki et al, 1977), efrapeptins (Dtolypin) from *Tolypocladium* spp. (Weiser & Matha, 1988), and hirsutellin from *Hirsutella thompsonii* (Mazet & Vey, 1995). Inside the insect haemocoel, the fungus switches from filamentous hyphal growth to yeast-like hyphal bodies that circulate in the hemolymph. The proliferation of these hyphal bodies occurs through budding (Boucias & Pendland, 1982). Later the fungus switches back to a filamentous phase and invades internal tissues and organs (Mohamed et al, 1978; Prasertphon & Tanada, 1968). The fungus later erupts through the cuticle and an external mycelium covers all parts of the host and formed infective spores under appropriate environmental conditions (Boucias & Pendland, 1982; McCauley et al, 1968;). Under suboptimal conditions, some fungi form resting structures inside the cadaver as in the case of *Nomuraea_rileyi* under conditions of low relative humidity and temperature (Pendland, 1982). The life cycle of the fungus is completed when the hyphal bodies sporulate on the cadaver of the host. The external hyphae produce conidia that ripen and are released into the environment. This allows horizontal transmission of the disease within the insects (Khetan, 2001).

Among 85 genera of entomopathogenic fungi only six species are commercially available for field application (Table 1). However, comparatively few have been investigated as potential mycoinsecticides. Fungal pathogens particularly *B. bassiana, I. fumosorosea* and *M. anisopliae*

are being evaluated against numerous agricultural and urban insect pests. Several species belonging to order IIsoptera (Hussain et al., 2010a ; Hussain et al., 2011), Lepidoptera (Hussain et al., 2009), Coleoptera (Ansari et al., 2006), Hemiptera (Leite et al., 2005) and Diptera (St. Leger et al., 1987) are susceptible to various fungal infections. This has led to a number of attempts to use entomopathogenic fungi for pest control with varying degrees of success.

Fungus	**Product and Company**	**Formulation**
Aeschersorzia aleyrodis	Koppert / Holland	Wettable powder
Beauveria bassiana	Naturalis™, Troy Bio-Science, USA.	Liquid formulation
B. bassiana	Conidia, AgrEvo, germany, Columbia	Suspendible granules
B. bassiana	Brocani™, Laverlam, Columbia	Wettable powder
B. bassiana	Boverol / Czeck Republic	Wettable powder and dry pellets
B. bassiana	Mycontrol-WP / Mycotech. Corp. USA	Wettable powder
B. bassiana	Ostrinil / natural Plant Protection / France	Microgranules of mycelium
B. brongniarti	Betel / Natural Plant Protection / France	Microgranules of mycelium
B. brongniarti	Engerlingspilz / Andermatt – Biocontrol / Switerzarland	Barley kernels colonized with the fungus
M. anisopliae	Bio -path™ / Eco Science / OSA	Conidia on a medium placed in trap / chamber
M. anisopliae	Biogreen / Biocare Technology Pvt. Ltd / Australia	Conidia produced on grains
M. anisopliae	Biologic Bio 1020 / Bayer AG Germany	Granules of mycelium
Paecilomyces fumosoroseus	Pfr 21 / WR Grace USA	Wettable powder
Verticillium lecanii	Mycotal / Koppert / Netherlands	Wettable powder
V. lecanii	Vertalec / koppart / Netherlands	Wettable powder

(Bhattacharyya et al., 2004)

Table 1. Commercial formulations of entomopathogenic fungal pesticides

The majority of fungal production systems consist of two stages system in which fungal inoculum of hyphal bodies is produced in liquid culture and then transferred to a solid substrate for production of aerial spores (Devi, 1994). For practical use of entomopathogenic fungi as bio-insecticides at each stage, it is necessary to develop culture medium and method that produce high concentrations of viable and virulent propagules at low cost (Jackson, 1997). These goals can be achieved by using the most favorable inexpensive components for fungal growth at the lowest concentration that afford high yield. Most common compounds for fungal entomopathogens include agro-industrial by-products such as corn steep liquor (Zhao et al., 2010) and sugarcane molasses (Hussain et al., 2011). Our previous investigations showed that both the by-products stimulate the growth of the

propagules of entomopathogenic fungi. Corn steep solid and cotton seed flour with yeast extract and KCl, NaCl etc., optimized blastospore production under water stress conditions (Ypsilos & Magan, 2005).

Entomopathogenic fungi infect insects in an aggressive manner by secreting cuticle degrading enzymes such as esterases, lipases, N-acetylglucosaminidases and chitinases (St. Leger et al., 1986). However, the production of extracellular protease Pr1, a major virulence determinant, plays an important role in the success of entomopathogenic fungi in insect penetration, which leads to the subsequent pathogenicity in the target host (Hussain et al., 2010b; Shah and Butt 2005). Previously, agro-industrial by-products such as corn-steep liquor and molasses have been used as alternative growth substrates to produce exopolysaccharides (Fusconi et al., 2008; Sutherland, 1996). Sugarcane molasses (SM), an industrial by-product rich in fermentable sugars, was proposed as a nutritious medium to produce bacterial cellulose by *Zoogloea* sp. (Paterson-Beedle et al., 2000). While, the dried powder of corn steep liquor was used as an inexpensive substitute for beef extract in the medium, which enhanced the lipase production of the strain of *Serratia marcescens* (Zhao et al., 2010). In the past, there is no report on the activity of extracellular protease Pr1 enzyme from the spores of entomopathogenic fungi cultivated from rice, previously grown on media with different composition. The current study is initiated in order to evaluate the effects of three different sources of nitrogen and two sugar sources in different combinations and concentrations in order to determine i) production in liquid media ii) activity of extracellular protease Pr1 of the locally isolated strains of entomopathogenic fungi.

2. Materials and methods

2.1 Culturing of entomopathogenic fungi

The entomopathogenic fungi *M. anisopliae* (EBCL 02049), *B. bassiana* (EBCL 03005) and *I. fumosorosea* (EBCL 03011) were originally isolated from *C. formosanus* in China. The strains were deposited at European Biological Control Laboratory, France. The strains were successively sub-cultured on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, US) at 26 ± 0.5 ^{o}C, in complete darkness. Fungal strains maintenance was identical to our previous study, where it was extensively described (Hussain et al., 2009). In brief, 24-day-old spores of studied strains cultivated on PDA were used as inoculum in all the growth media.

2.2 Influence of liquid media composition on the *in vitro* growth of entomopathogenic fungi

The three sources of nitrogen: peptone (Sigma), yeast extract (Sigma) and corn steep liquor (Shanghai Xiwang Starch Sugar Co., Ltd.), and two sources of sugar: glucose (Sigma) and sugar molasses, were used in different combinations as shown in Table 2. In all the treatments, the following salts were used at the concentration, $CaCl_2 . 2H_2O$ (0.06%), KCl (0.28%), $MgCl_2 . 6H_20$ (0.16%), $MgSO_4 . 7H_2O$ (0.2%), $NaHCO_3$ (0.03%) and $NaH_2PO_4 . H_2O$ (0.1%). To avoid reactions among the salts, they were prepared in compatible pairs: $CaCl_2. 2H_2O$ with KCl; $MgCl_2. 6H_20$ with $NaH_2PO_4. H_2O$; $MgSO_4. 7H_2O$ with $NaHCO_3$. The other precautionary measures to avoid precipitation were adopted as described by Leite et al. (2005). Media preparations were finalized by adjusting the pH to 6.2, with filter-sterilized HCl (0.1%) and NaOH (10%).

GROWTH MEDIA	SUGAR AND NITROGEN SOURCES				
	Glucose (G) %	Sugar molasses (SM) %	Corn steep liquor (CSL) %	Peptone (PE) %	Yeast extract (YE) %
G + CSL	2.66		1		
G + PE	2.66			1	
G + YE	2.66				1
G + CSL + PE	2.66		0.50	0.50	
G + CSL + YE	2.66		0.50		0.50
G + PE + YE	2.66			0.50	0.50
G + CSL + PE + YE	2.66		0.333	0.333	0.333
SM + CSL		2.66	1		
SM + PE		2.66		1	
SM + YE		2.66			1
SM + CSL + PE		2.66	0.50	0.50	
SM + CSL + YE		2.66	0.50		0.50
SM + PE + YE		2.66		0.50	0.50
SM + CSL + PE + YE		2.66	0.333	0.333	0.333
G+SM+CSL+PE+YE	1.33	1.33	0.333	0.333	0.333

Table 2. Composition of the media of shake flask cultures in agitated liquid cultures of entomopathogenic fungi

The media were poured into 250 ml Erlenmeyer flasks and autoclaved. After cooling, all the flasks were inoculated with one milliliter of spore suspension (1×10^6 spores/ml) in 0.03 % Tween 80 (Sigma-Aldrich, St Louis, MO, US) from 24-day-old cultures of *B. bassiana*, *M. anisopliae* and *I. fumosorosea* grown on PDA. Four replicates were used for each growth medium. After inoculation, cultures (100 ml) were grown in 250 ml shake flasks at 120 rpm on a rotary shaker, 25 ± 0.5 ^{o}C and 16 h fluorescent light photophase. After 120 h of growth, fungal biomass of each flask was evaluated separately. The fungal biomass was filtered through Whatman No. 1 filter paper. After filtration, the filtrates were dried for 24 h at 70 ^{o}C and weighed.

2.3 Influence of liquid media composition on the growth of blastospores of entomopathogenic fungi

The optical densities of the inocula of all the studied fungi grown on different media after different time intervals such as 20 h, 40 h and 60 h were measured at 600 nm (OD_{600}) by using a spectrophotometer (Shimadzu UV-1800). The media without inoculation were used as control.

2.4 Production on solid substrates

The entomopathogenic fungi were grown on solid substrate (rice grains) as described in our previous study (Hussain et al., 2011). In brief, 50 % diluted 10 ml inoculum obtained from above media was added into a self-aerating bag containing 20 g of sterilized par boiled rice,

separately. Inoculated rice granules were mixed thoroughly from the outside of the bag. Inoculated rice bags were then incubated for 18 days at ambient conditions (24 ± 2 ºC, 75-85% RH). The sporulating rice in bags from each growth medium was then allowed to dry for 10 d at 30 ˚C. The spores were separated from the rice by sieving through a 300 µm mesh. A collecting vessel, such as a bucket was fitted to the plastic sheeting at the bottom of the sieve to create a funnel into the collecting vessel. The sieve was shaken until all the loose spores had been removed from the rice and had collected in the vessel below. The spores were then further sieved using a 106 µm sieve to separate the larger rice dust particles from the spores. The spores as powder were kept at 4 ºC for subsequent analysis of the activity of extracellular protease Pr1.

2.5 Influence of artificial media on the activity of extracellular protease Pr1

The activity of Pr1 protease bound to 10 mg spores harvested from the rice of above mentioned media were assayed using a modified method of St. Leger et al. (1987). Briefly, 10 mg spores were washed once in 0.3% aq Tween 80 solution and twice in distilled water, were then incubated in 1 ml of 0.1M Tris HCl (pH 7.95), containing 1mM Succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma) for 5 min at room temperature. The spores were pelleted by centrifugation for 5 min at 12000 g (Fastwin Bio-Tech Company Limited). A 200 µL of supernatant was transferred to quartz cuvette. The absorbance was measured at 405 nm by using a spectrophotometer (Shimadzu UV-1800). Buffered substrate was used as a reference. The amount of spore bound extracellular protease Pr1 is expressed as micromoles of nitroanilide (NA) released per minute.

2.6 Statistical analyses

All experiments were repeated four times except the activity of spore bound Pr1, repeated three times. Data were analyzed by analysis of variance using the ANOVA procedure of SAS (SAS Institute, 2000) for a completely randomized design. When the effect was significant ($P < 0.05$), means were separated using Duncan's Multiple Range Test.

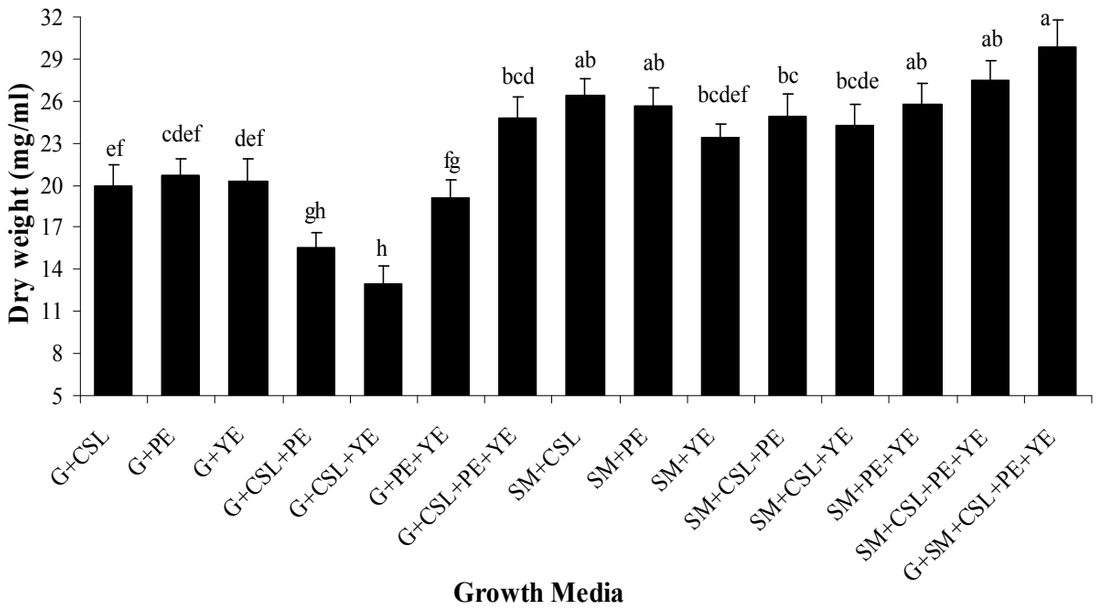
3. Results

3.1 Effect of different sources of sugar and nitrogen on the dried fungal biomass of entomopathogenic fungi

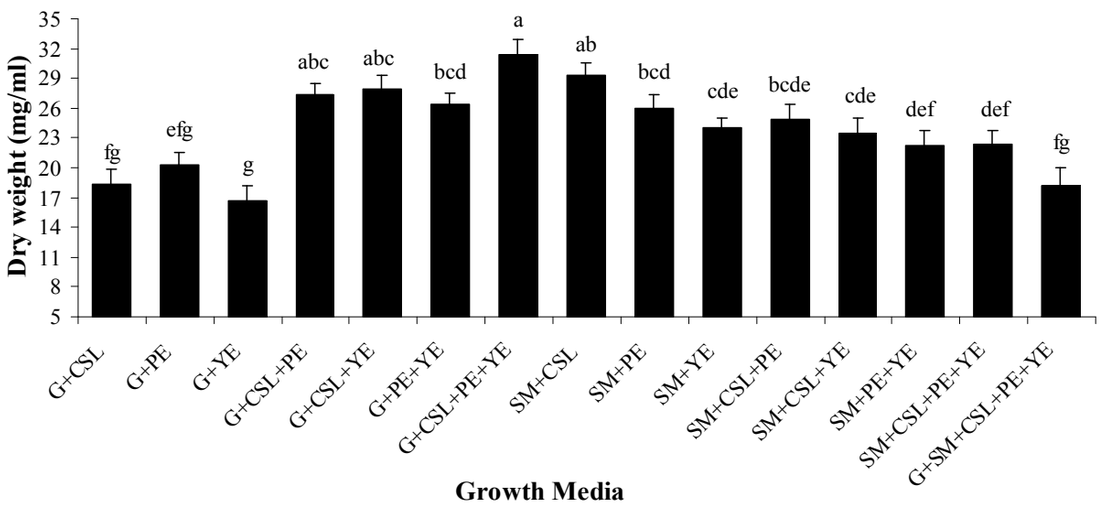
Significant differences in dried biomass of *M. anisopliae* ($F = 10.632$; df = 14, 45; $P < 0.001$), *B. bassiana* ($F = 9.286$; df = 14, 45; $P < 0.001$) and *I. fumosorosea* ($F = 9.596$; df = 14, 45; $P < 0.001$) were observed when fungi were grown on media containing different sources of nitrogen and sugar in different combinations. The media supplemented with sugarcane molasses (SM) afforded comparatively higher growth of *M. anisopliae* and *I. fumosorosea* than glucose (Fig. 1a, c). The growth medium contained G + CSL + YE, showed the lowest growth (12.95 mg/ml) of *M. anisopliae*. The glucose in combination with CSL, PE and YE showed higher growth; while their combination among them did not afford higher fungal growth (Fig. 1a). The growth medium such as G + CSL + PE, G + CSL + YE and G + PE + YE, afforded the lowest growth of *I. fumosorosea* and non significant differences were observed among them (Fig. 1c). *B. bassiana* grown on media supplemented with SM in combination with CSL, PE

and YE showed significantly higher growth compared to the media containing glucose as sugar source (Fig. 1b). In contrast, their combinations (CSL, PE and YE) with one another in the presence of glucose as sugar source produced higher growth compared with SM (Fig. 1b). The medium SM + CSL afforded higher growth ranges from 31.46-35.28 mg/ml in all the studied fungal strains (Fig. 1a-c).

(a)



(b)



(c)

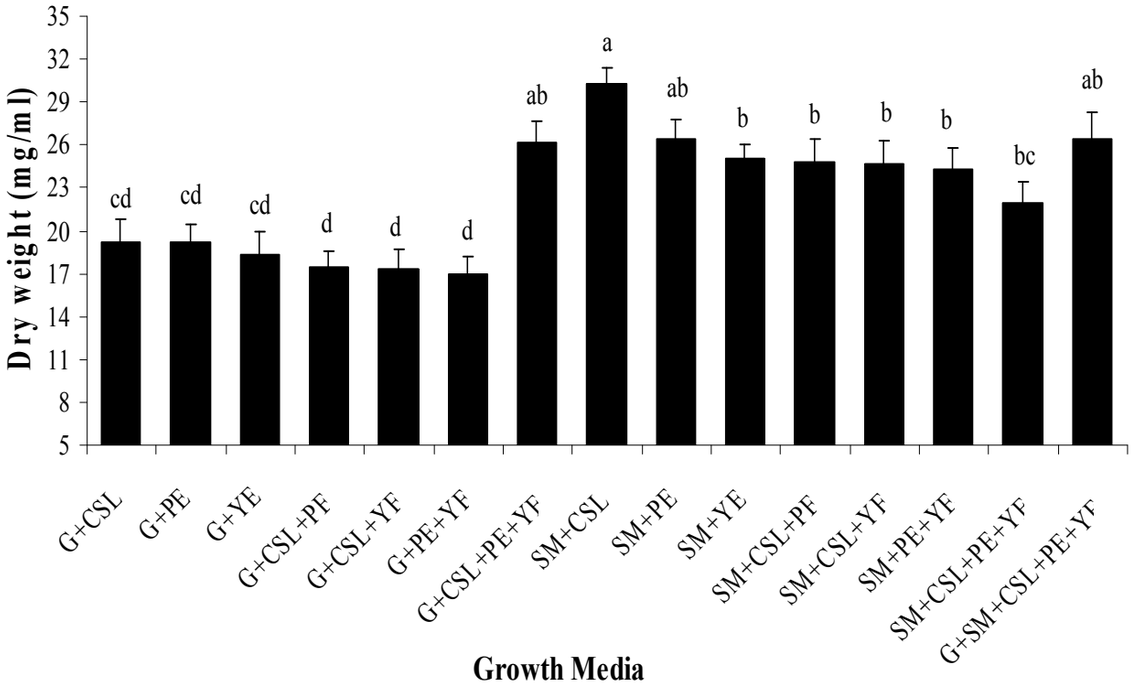


Fig. 1. Influence of media composition on the dried biomass (mg/ml) of (a) *M. anisopliae* (b) *B. bassiana* and (c) *I. fumosorosea*. Mean ± SE values with the same letter(s) along the bars of different growth media are not significantly different ($P < 0.05$). For detail of treatments see table 1.

3.2 Effect of different sources of sugar and nitrogen on the blastospores growth of entomopathogenic fungi

M. anisopliae growth observed from the shake flask cultures supported with different media differed significantly after 20 h ($F = 96.535$; df = 14, 45; $P < 0.001$), 40 h ($F = 77.536$; df = 14, 45; $P < 0.001$) and 60 h ($F = 67.381$; df = 14, 45; $P < 0.001$). As the incubation time elapsed, the concentration of the blastospores increased. After 20 h complete growth media (G + SM + CSL + PE + YE), afforded the highest growth of the yeast like hyphal bodies of the *M. anisopliae*.

While, G + PE, G + YE and G + PE + YE showed the lowest optical density of the growth media. After 40 and 60 h of incubation, the medium SM + CSL produced higher concentration of the blastospores (1.995) and remained significantly at higher level than any medium. While G + PE + YE media, showed the lowest values at both the studied time intervals (40 h and 60 h). The complete medium also afforded the growth of the fungi, which ultimately showed higher OD values of the blastospores but significantly at lower level than SM + CSL medium after 40 h and 60 h (Fig. 2a).

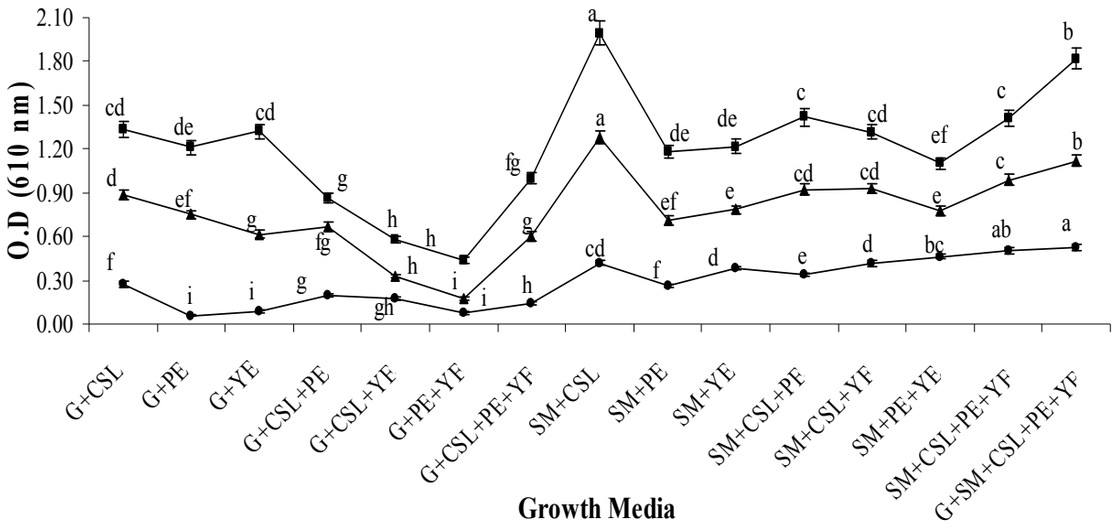


Fig. 2a. Blastospores concentration (OD) of *M. anisopliae* grown on different media supplemented with different sources of nitrogen and sugar after —●— 20 h, —▲— 40 h and —■— 60 h incubation. Mean ± SE values with the same letter along the bars of different growth media are not significantly different ($P < 0.05$). For detail of treatments see table 1.

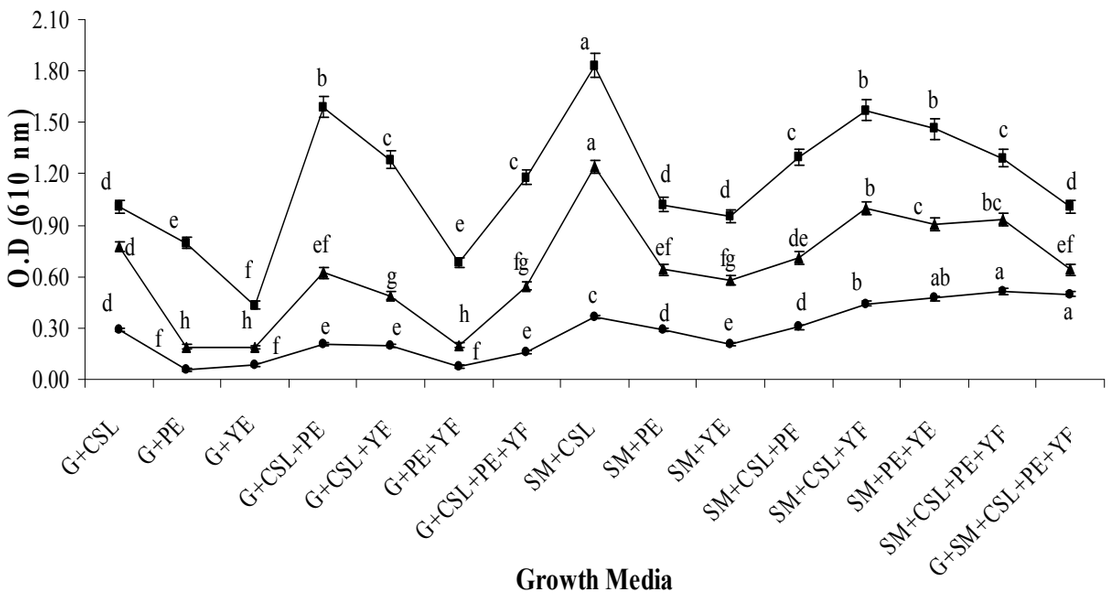


Fig. 2b. Blastospores concentration (OD) of *B. bassiana* grown on different media supplemented with different sources of nitrogen and sugar after —●— 20 h, —▲— 40 h and —■— 60 h incubation. Mean ± SE values with the same letter along the bars of different growth media are not significantly different ($P < 0.05$). For detail of treatments see table 1.

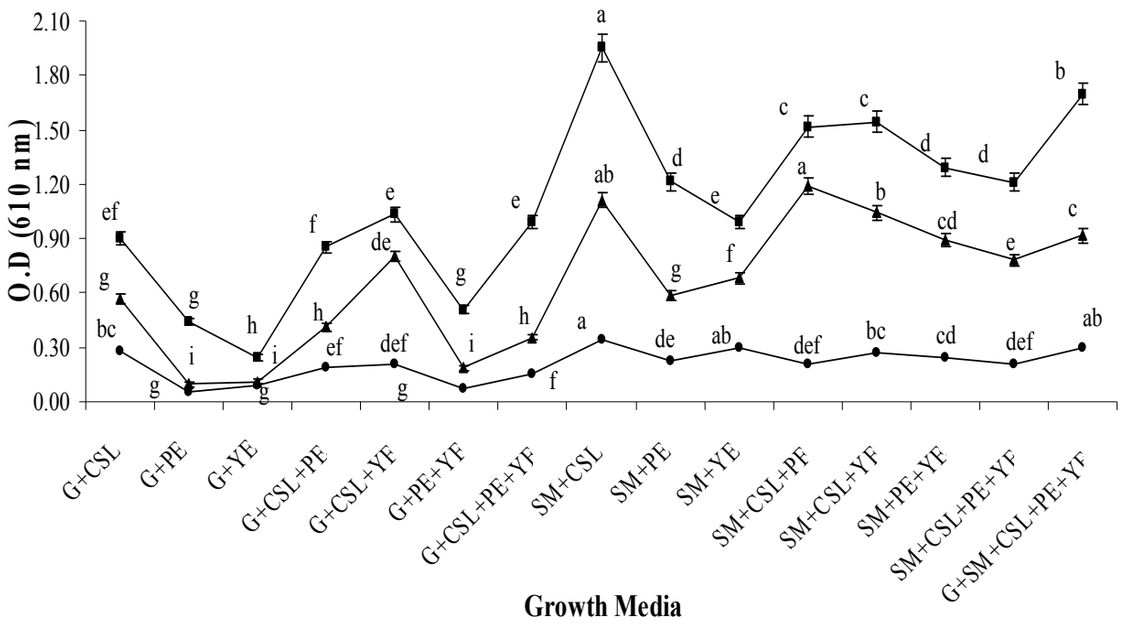
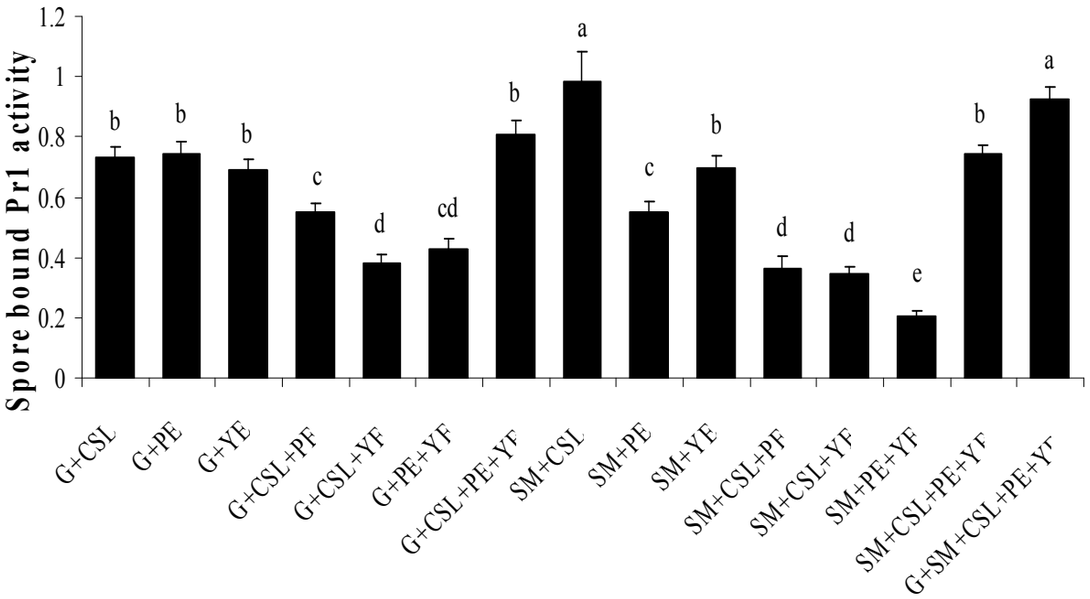


Fig. 2c. Blastospores concentration (OD) of *I. fumosorosea* grown on different media supplemented with different sources of nitrogen and sugar after 20 h, 40 h and 60 h incubation. Mean ± SE values with the same letter along the bars of different growth media are not significantly different ($P < 0.05$). For detail of treatments see table 1.

Blastospores growth was highly variable and significant differences among the growth of *B. bassiana* on all the growth media after 20 h ($F = 172.619$; df = 14, 45; $P < 0.001$), 40 h ($F = 103.584$; df = 14, 45; $P < 0.001$) and 60 h ($F = 62.153$; df = 14, 45; $P < 0.001$) were observed. After 20 h of incubation, media supplemented with SM showed higher growth compared to all the media containing glucose as sugar source in all the combinations with CSL, PE and YE. On the whole, the media SM + CSL after 40 h and 60 h showed the highest growth of blastospores. CSL in combination with PE and YE in the presence of G as sugar source enhanced the growth of the fungi. Comparatively, the media supplemented with SM found to cause stimulant effect on the growth of the blastospores (Fig. 2b).

There was a significant difference in the blastospores concentration of *I. fumosorosea* after 20 h ($F = 36.819$; df = 14, 45; $P < 0.001$), 40 h ($F = 145.915$; df = 14, 45; $P < 0.001$) and 60 h ($F = 110.554$; df = 14, 45; $P < 0.001$) grown on different media supplemented with different sources of nitrogen and sugar in all possible combinations. *I. fumosorosea* grown on SM + CSL exhibited the highest growth not only after 20 h of incubation but also after 60 h (Fig. 2c). While, SM + CSL also promoted the growth resulting higher blastospores but remained significantly lower than SM + CSL + PE, which showed the highest growth after 40 h of incubation. On the whole, media supplemented with SM as sugar source showed higher growth of the blastospores compared with the media containing glucose as sugar source (Fig. 2c).

(a)


Spore bound Prl activity
1.2
1
0.8
0.6
0.4
0.2
0
b b b c d cd b a c b d d e b a
G+CSL
G+PE
G+YE
G+CSL+PE
G+CSL+YE
G+PE+YE
G+CSL+PE+YE
SM+CSL
SM+PE
SM+YE
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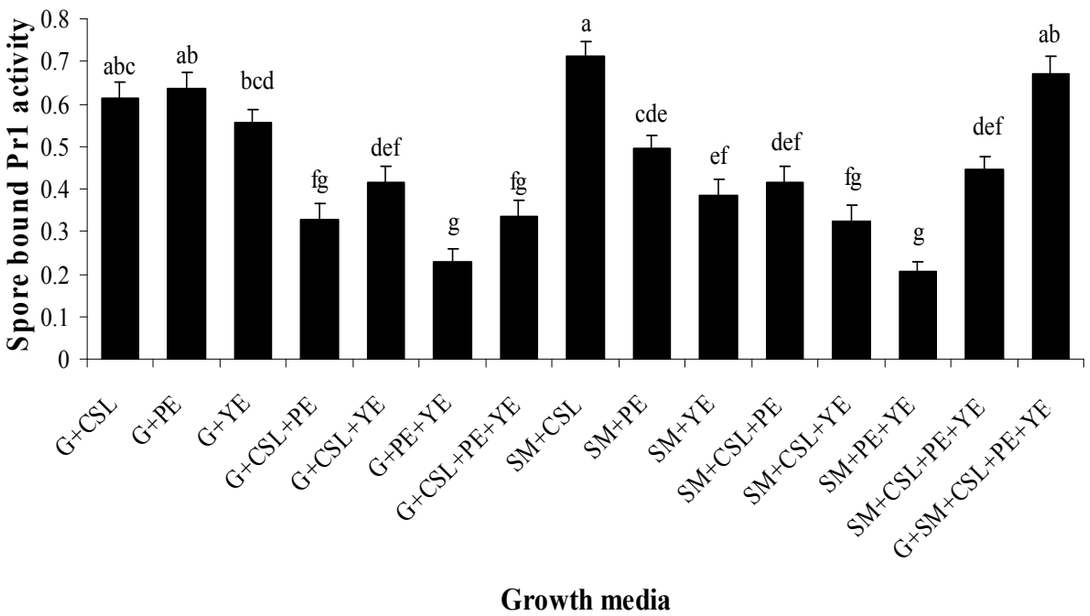
SM+CSL+PE+YE
G+SM+CSL+PE+YE
Growth media

(b)


Spore bound Prl activity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
abc ab bcd fg def g fg a cde ef def fg g def ab
G+CSL
G+PE
G+YE
G+CSL+PE
G+CSL+YE
G+PE+YE
G+CSL+PE+YE
SM+CSL
SM+PE
SM+YE
SM+CSL+PE
SM+CSL+YE
SM+PE+YE
SM+CSL+PE+YE
G+SM+CSL+PE+YE
Growth media

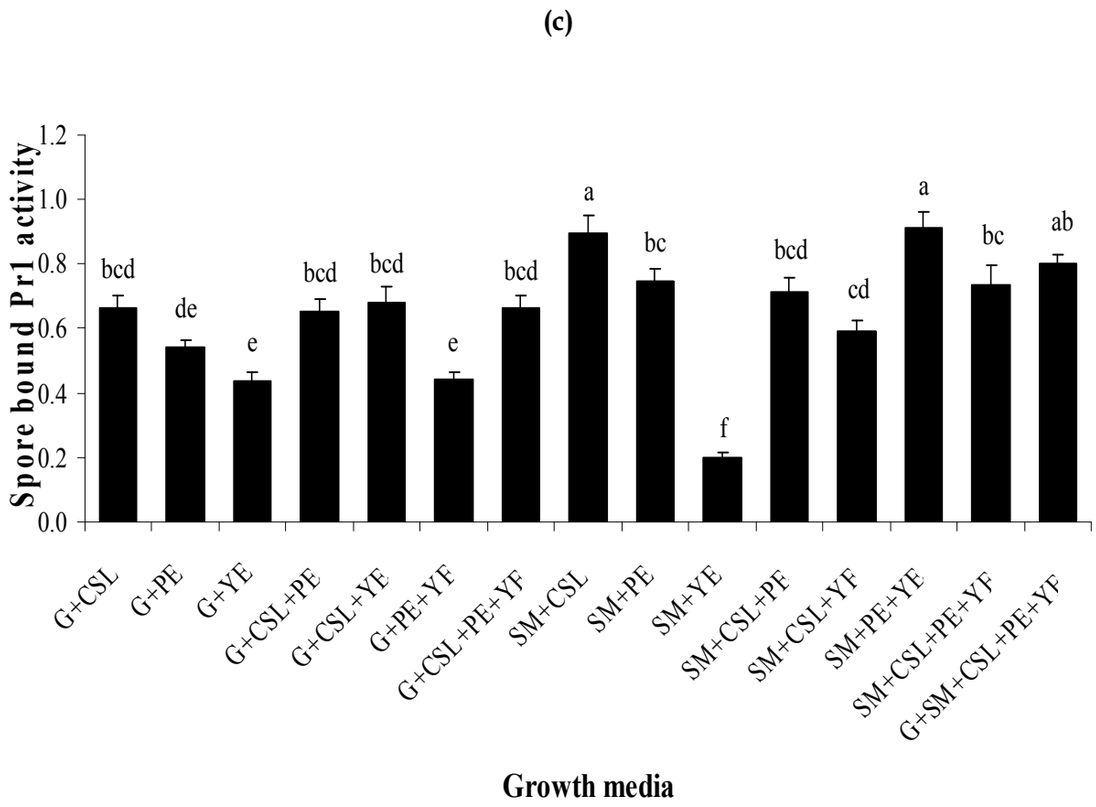


Fig. 3. Extracellular protease Pr1 activity (µmol NA ml^{-1} min^{-1}) of (a) *M. anisopliae* (b) *B. bassiana* and (c) *I. fumosorosea* grown on media supplemented with different sources of nitrogen and sugar. Mean ± SE values with the same letter along the bars of different growth media are not significantly different ($P < 0.05$). For detail of treatments see table 1.

3.3 Effect of different sources of sugar and nitrogen on the enzymatic activity (µmol NA ml-1 min-1) of spore bound Pr1 of entomopathogenic fungi

Significant differences in enzymatic activity of spore bound Pr1 were observed when different sources of sugar and nitrogen in all possible combinations were used to grow *M. anisopliae* ($F = 28.945$; df = 14, 30; $P < 0.001$), *B. bassiana* ($F = 20.302$; df = 14, 30; $P < 0.001$) and *I. fumosorosea* ($F = 21.376$; df = 14, 30; $P < 0.001$). The spores of all the studied entomopathogenic fungi cultivated from rice inoculated with medium supplemented with SM + CSL showed the highest activity of Pr1 (Fig. 3a-c). *M. anisopliae* spores grown on complete media also showed higher enzymatic activity (Fig. 3a). The growth medium SM + PE + YE in case of *M. anisopliae,* while SM + PE + YE and G + PE + YE, in case of *B. bassiana* showed the lowest enzymatic activity (Fig. 3a-b). The addition of PE and YE solely in combination with glucose produced spores of *B. bassiana* with relatively higher enzymatic activity compared to SM (Fig. 3b). In case of *I. fumosorosea*, CSL supplemented media showed comparatively higher enzymatic activity except the medium supplemented with SM + YE (0.20 µmol NA ml^{-1} min^{-1}) (Fig. 3c).

4. Discussion

These studies demonstrated that sugar and nitrogen sources significantly effect the growth of blastospores produced by cultures of *B. bassiana*, *M. anisopliae* and *I. fumosorosea*. Higher blastospores growth and dried fungal biomass was produced in cultures grown on media supplemented with sugarcane molasses (Fig. 1a, c). Previous studies with *Paecilomyces farinosus* (Hotmskiold) and *Paecilomyces lilacinus* (Thom.) showed that media supplemented with SM supported the highest growth of the studied fungi (Leena et al, 2003).

Mass production technology is an important way for improving mycoinsecticides based on the blastospores. Accelerated blastospores growth rates in the current study from the media supplemented with SM and corn steep liquor (CSL) greatly improved the dried fungal biomass production of the studied entomopathogenic fungi. While, our results disagreed with the findings of Leite et al, (2003) who concluded that replacement of CSL as nitrogen source gave relatively low yield of the three studied fungal strains. Our findings revealed that the fungal organisms directly interact with the culture conditions and strongly influence the growth of blastospores.

All the studied fungi did not have the same characteristics when cultivated in media with different complex sources of nitrogen and sugar. Sugarcane molasses, a by-product from the sugar industry, supported higher growth of dried biomass of *M. anisopliae* and *I. fumosorosea*, compared to the media supplemented with glucose. Thus, it may be speculated that SM efficiently enhanced the growth of blastospores, which ultimately led to the production of higher fungal biomass of the fungi. The complete media SM + G + CSL + PE + YE, greatly enhanced the growth of *M. anisopliae* and *I. fumosorosea*. These components when evaluated separately afforded lower growth for *M. anisopliae*. This suggests that these components differ concerning types of nutrients, and therefore provide complete nourishment when offered together.

CSL, peptone and yeast extract afforded higher growth of *B. bassiana* in fungal cultures supplemented with glucose, while these nitrogen sources did not enhance the growth of fungi in the presence of SM. On the other hand, these nitrogen sources when used in combinations (SM + CSL + PE, SM + CSL + YE, SM + PE + YE, SM + CSL + PE + YE), did not increase fungal production of *B. bassiana*. These combinations in the presence of glucose greatly enhanced the dried fungal biomass. Even, the complete medium showed significantly lower growth. On the basis of above findings, we may suggest that nutrition greatly influenced the growth of *B. bassiana*. The result of our study corroborates similar research on the effect of nutrition and propagule production in *Metarhizium* spp. and other entomopathogenic hyphomycetes (Inch et al, 1986; Rombach, 1989; Kleespies & Zimmermann, 1992; Jackson et al, 1997; Vidal et al, 1998).

CSL, a by-product from the corn industry, supported higher growth of the blastospores (Fig. 3a-c), and also ultimately led to the production of spores with higher enzymatic activity of Pr1. Since, peptone and yeast extract are expensive sources of nitrogen; CSL was chosen because of its stimulatory effect and cost economics and could be used to replace the nitrogen sources used previously. Supplementation with 1% CSL with 2.66% SM enhanced not only the growth of all the studied fungi but also enzymatic activity of spore bound Pr1 of only *M. anisopliae*. This is in agreement with the results of McCoy et al, (1988), that nutrition is one of the several factors that may determine the specificity of a fungal

pathogen. The results suggest that the growth of blastospores can be efficiently improved from inexpensive CSL and SM, making fermentation an economical and environmental friendly process.

The management of arthropod pests generally involves preventive measures and remedial control (Lewis, 1997; Su & Scheffrahn, 1998). Currently registered insecticides have undergone rigorous field-testing, efficacy results have been mixed. Some insecticides are expensive and less persistent, leading to reduced longevity and the failure of the chemical barrier (Su and Scheffrahn, 1998). In addition, large quantities of persistent insecticides are raising concerns about applicator safety, environmental contamination and possible deleterious effects on non-target animals. By keeping in mind the above mentioned drawbacks, it is the urgent requirement to standardise the microbe base products against insects for the safety of human beings, animals and environment.

5. Conclusion

In conclusion, the replacement of nitrogen and sugar sources with CSL and SM respectively, in the liquid production medium significantly improved the growth and the activity of spore bound extracellular protease Pr1 of *M. anisopliae* for the first time. A more rapid growth rate for blastospores production permitted us to select this appropriate media for large scale commercial development of entomopathogenic fungi for the safe management of insect pests, in order to avoid the deleterious effects of insecticides. Entomopathogenic fungi being component of an integrated approach can provide significant and selective insect control. In the near future, we expect to see synergistic combinations of microbial control agents with other technologies (in combination with semiochemicals, soft chemical pesticides, other natural enemies, resistant plants, chemigation, remote sensing, etc.) that will enhance the effectiveness and sustainability of integrated control strategies.

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The present book is not a classical manual on Zoology and the reader should not expect to find the usual treatment of animal groups. As a consequence, some people may feel disappointed when consulting the index, mainly if searching for something that is considered standard. But the reader, if interested in Zoology, should not be disappointed when trying to find novelties on different topics that will help to improve the knowledge on animals. This book is a compendium of contributions to some of the many different topics related to the knowledge of animals. Individual chapters represent recent contributions to Zoology illustrating the diversity of research conducted in this discipline and providing new data to be considered in future overall publications.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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