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GROWTH AND SPORULATION OF *TRICHODERMA POLYSPORUM* ON ORGANIC SUBSTRATES BY ADDITION OF CARBON AND NITROGEN SOURCES

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Abstract

During the present study nine different organic substrates *viz*., rice grains, sorghum grains, wheat grains, millet grains, wheat straw, rice husk, cow dung, sawdust and poultry manure were used for mass multiplication of *Trichoderma polysporum*. Grains, especially sorghum grains were found to be the best substrate for *T. polysporum*. Wheat straw and rice husk were less suitable, whereas, cow dung, sawdust and poultry manure were not suitable for growth of the fungus. Sucrose @ 30,000 ppm and ammonium nitrate @ 3,000 ppm were found to be the best carbon and nitrogen sources for growth and sporulation of *T. polysporum.* Amendment of the selected C and N sources to wheat straw, rice husk and millet grains resulted in significantly higher growth and conidia production by *T. polysporum* as compared to un-amended substrates. Sorghum and rice grains showed suppression in growth and sporulation of *T. polysporum* when amended with C and N sources. During studies on shelf life, populations of *T. polysporum* attained the peck at 60-135 days intervals on different substrates and declined gradually thereafter. However, even after 330 days, the populations were greater than the population at 0-day. At 345-360 days interval, populations were less than the initial populations at 0- days. Shelf life on C+N amended wheat straw and rice husk were more as compared to un-amended substrates.

Key words: *Trichoderma polysporum*, Mass production, Shelf life.

Introduction

The fungal diseases of plants especially those caused by soil-borne pathogens are among the major threats to agricultural production. It has been assessed that about 25% of the yield in western countries and nearly 50% in developing countries is lost due to plant diseases; one third of it is due to fungal pathogens (Bowyer, 1999; Vipul, 2006). The root infecting soil-borne fungi often produce root rot disease complexes that may result in the death of the plants. Since soil applied pesticides are costly and produce environmental hazards, crop resistance to root pathogens is believed to be the ideal means of controlling plant diseases (El-Katatny *et al.,* 2000; Saleem *et al.,* 2000). However, many crops have little or no resistance to certain plant pathogens. Use of microbial antagonists in the control of plant disease is an alternative method for disease management that would also protect our environment from the hazardous effect of the chemicals (Spiers *et al.,* 2005; Bennet *et al.,* 2006).

Trichoderma species are well known bio-control agents to suppress infection by soil-borne root infecting fungi like *Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium* sp., *Pythium* species and root knot nematodes (Ghaffar, 1978, 1988, 1992; Kucuk & Kivanc, 2004; Benitez *et al.,* 2004; Khan & Shahzad, 2007; Hesamedin, 2008). *Trichoderma* species suppressed *R. solani* and *M. phaseolina* infection on Banana, egg-plant, cotton, sugar beet and soybean (Tarek & Moussa; 2002; Hashem, 2004; Thangavelu, 2004; Hesamedin, 2008; Kumari *et al.,* 2012).

Most of the previous reports have emphasized on use and mass production of *T. harzianum* and *T. viride* for the control of root infecting pathogens, whereas, other *Trichoderma* species have received comparatively less attention (Saju *et al.,* 2002; Sharma & Singh, 2004). In our studies, *T. polysporum* was found to inhibit the *In vitro* growth of root infecting fungi and produced coiling around their hyphae that shows that it is a promising biocontrol agent for the control of root infecting fungi.

The main hindrance in the large-scale application of biocontrol organisms is the lack of a cheap and effective method for mass multiplication of biocontrol agents. Reports have been made where grains, especially rice and sorghum grains were found suitable for mass multiplication of *Trichoderma* species, whereas, wheat straw, rice straw or husks were found less suitable for growth and sporulation of biocontrol agents (Dawar & Ghaffar, 2003). However, use of grains as substrate for large scale production of biocontrol inoculums would be costly and uneconomical. Enhancement of growth and sporulation of *Trichoderma* species on cheap but less suitable substrates would provide an economical method for mass production of biocontrol agents. The present report describes the effect of carbon and nitrogen sources on enhancement in growth and sporulation of *T. polysporum* on wheat straw and rice husk.

Materials and Methods

Evaluation of different substrates for multiplication of *T. polysporum:* Cultures of, *T. polysporum* present in the Pest & Disease Research Lab (PDRL), Department of Agriculture & Agribusiness Management, University of Karachi were used during the present studies. Rice grains, sorghum grains, millet grains, wheat grains, wheat straw, saw dust, rice husk, poultry manure and cow dung evaluated for growth and sporulation of *T. polysporum* using method described by Ghaffar (1992). The substrates were soaked in water for two hrs in containers and 50g of a substrate was transferred in each polyethylene bag. The bags were sealed and then sterilized in an autoclave at 15psi for 20 minutes. After cooling, the substrates in bags inoculated by injecting 2

ml conidial suspension of *T. polysporum* containing $1.2x10^7$ conidia per ml. There were three replicate for each treatment. The inoculated substrates stored at 30±2°C and fungal population determined after 15 days intervals with the help of haemocytometer.

Effect of carbon sources on *In vitro* growth of *T. polysporum*: Different carbon sources *viz*., Sucrose, Maltose, Dextrose, Glucose, Starch and Cellulose @ 0, 10,000, 20,000, 30,000, 40,000 and 50,000 ppm were added to Czapek's Dox Agar (CzDA) medium without nitrogen sources before the medium was autoclaved. CzDA without carbon and nitrogen sources served as control. Media were sterilized for 15 minutes at 15 psi. Penicillin @100,000 units L^{-1} and streptomycin @0.2g L^{-1} were added to sterilized stock media just before pouring to inhibit the bacterial growth. The media were poured in 9cm diam., Petri plates @ 10 ml per plate. There were three replicate for each treatment. After solidification, a 5mm diam., inoculum disc of *T. polysporum* was placed in center of each Petri plate. The plates were incubated at 28±2°C and diameters of the growing colonies were recorded at 24 hrs intervals.

Effect of nitrogen sources on *In vitro* growth of *T. polysporum:* Different nitrogen sources viz., NPK, Urea, DAP, ammonium nitrate and sodium nitrate were used separately @ 0, 1,000, 3,000, 5,000, 7,000, 9,000 and 10,000ppm to see its effect on *In vitro* growth of *T. polysporum* by the methods described above. No carbon source was added to the medium.

Combined effect of selective carbon and nitrogen source on *In vitro* growth of *T. polysporum:* Sucrose was used @ 30,000ppm along with the selected nitrogen source ammonium nitrate @ 3,000 ppm for *T. polysporum.* Effect on *In vitro* growth of *T. polysporum* and conidia production was recorded by the methods described above.

Effect of carbon and nitrogen sources on growth and sporulation of *T. polysporum* on organic substrates: Five selected substrates *viz.*, Rice grains, sorghum grains, millet grains (suitable substrates) and wheat straw and rice husk (less suitable substrates) were used for multiplication of *T. polysporum*. The substrates were soaked in water for two hrs in containers and 50g of a substrate was transferred in a polyethylene bag. Sucrose as the selected carbon source was mixed with the substrates @ 1.5g per 50g substrate, whereas, nitrogen source ammonium nitrate was used @ 0.15g per 50g substrate. Carbon and nitrogen sources were also used separately. Substrates without carbon and nitrogen sources served as control. Growth and population of *T. polysporum* determined using methods described above.

Self-life of *T. polysporum*: The shelf-life of *T. polysporum* was evaluated on sorghum grain, millet grains, rice grains, wheat straw and rice husk. Polyethylene bags filled with 50 g of each substrate were inoculated with 2 ml conidial suspension of the test antagonist containing $1.2x10^7$ conidia per ml. Each substrate was evaluated with and without selected carbon and nitrogen sources. The bags were stored at room temperature and populations of the microbial antagonist were determined from 0 to 360 days with 15 days interval.

Results

Growth of *T. polysporum* on different substrates: Generally, cereal grains were found more appropriate for the mass production of antagonistic fungus *T. polysporum* as significantly high populations were recorded on cereal grains as compared to other substrates (Fig. 1). However, the highest population *T. polysporum* was observed on sorghum grains ($53.2x10^8$cfu g^{-1}) followed by millet grains ($47.433x10^8$ cfu g^{-1}). The poultry manure appeared to be the most unsuitable substrate and showed the lowest *T. polysporum* population ($1.06x10^8$cfu g^{-1}). Cow dung ($1.76x10^8$cfu g^{-1}) and saw dust ($2.26x10^8$cfu g^{-1}) were also not suitable (Fig. 1). Rice grains, wheat grains, wheat straw and rice husk performed moderately and produced $29.30x10^{8,}$ $23.3x10^8$, $20.33x10^8$ and $12.60x10^8$ cfu g^{-1} of substrate, respectively.

Effect of different carbon sources on *In vitro* growth of *T. polysporum*: In all carbon sources, the colony growth of *T. polysporum* increased with increasing concentrations except in case of cellulose and starch where maximum colony growth occurred at the lowest concentration. Significantly, higher colony growth was recorded on sucrose followed by dextrose, glucose and maltose amended media. However, dextrose, glucose and maltose amended media produced less conidia but supported more superficial mycelial growth as compared to sucrose amended medium that supported abundant conidial production. Use of sucrose @ 30,000 ppm was found most suitable for colony growth and sporulation of *T. polysporum* and no positive effect of further increasing concentrations of sucrose were observed (Fig. 2a,b). Sucrose @ 30,000 ppm was therefore selected as suitable carbon source for further experiments.

Effect of different nitrogen sources: Increasing concentrations of nitrogen sources *viz*., DAP, NPK and ammonium nitrate showed positive correlation with the growth of *T. polysporum*, however, NPK was less effective. Urea showed toxic effect and suppressed the growth of the fungus. Growth of *T. polysporum* in DAP amended plate was very thin whereas good mycelia growth and heavy sporulation of the fungus were observed on ammonium nitrate amended media. Use of ammonium nitrate @ 3,000 ppm was found most suitable since any increase in its concentration did not give enhancement in growth and sporulation of *T. polysporum* (Fig. 3). Ammonium nitrate @ 3,000 ppm was, therefore, selected as suitable nitrogen source for further experiments.

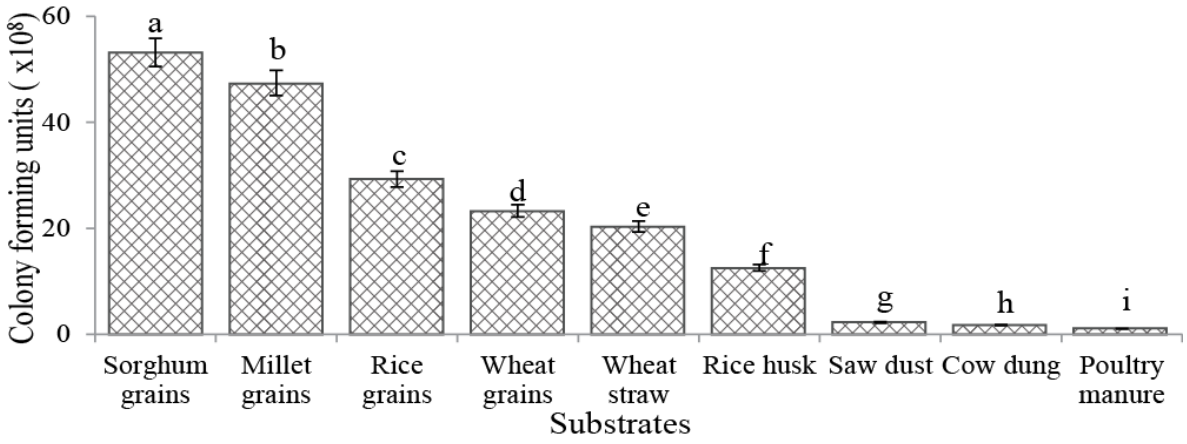

Fig. 1. Population of *Trichoderma polysporum* after15 days incubation on different substrates.
Bars with similar letters at the top are not significantly different at p<0.05 level as determined by DMRT

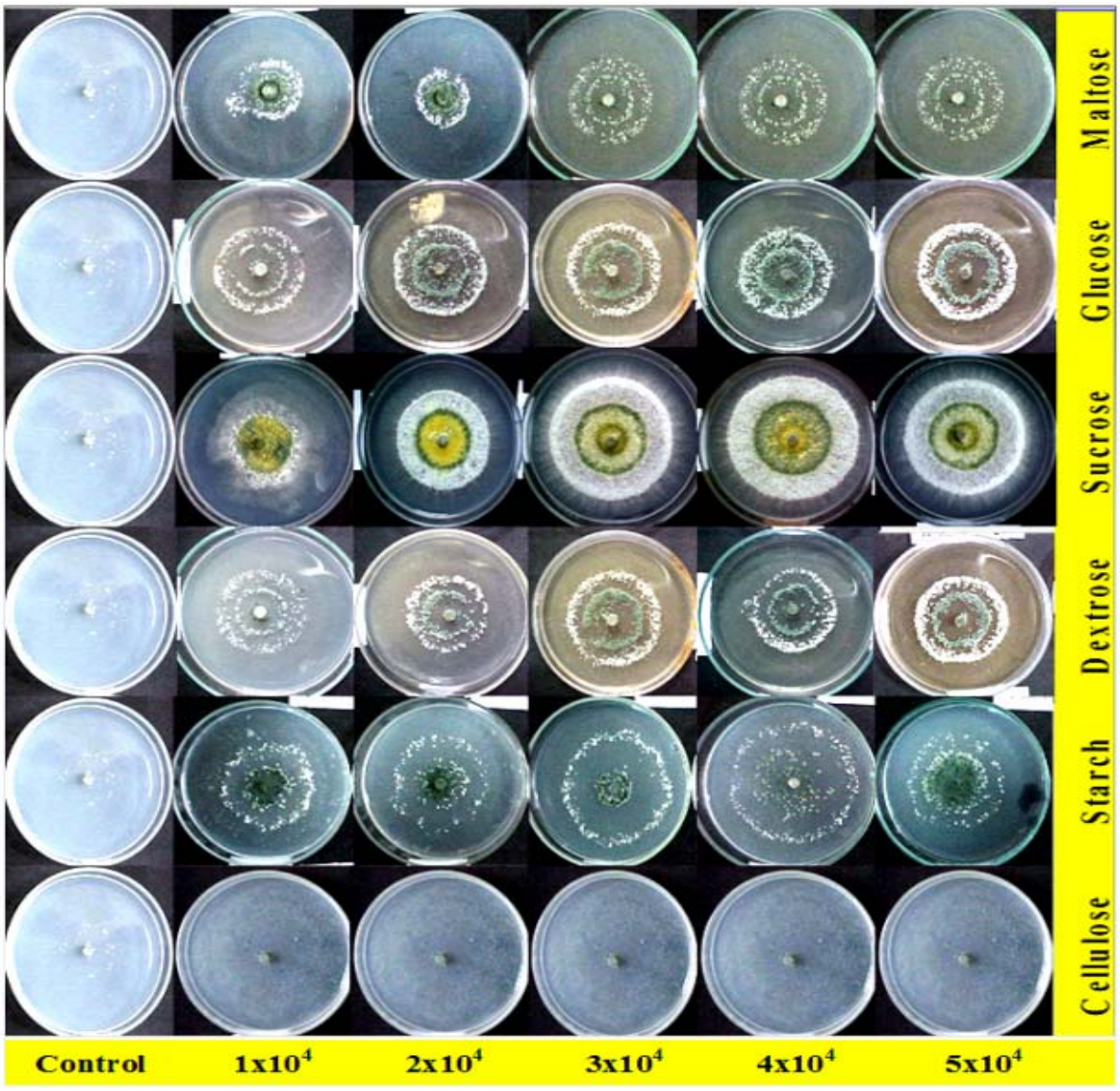

Fig. 2a. Effect of different carbon sources on *In vitro* growth of *Trichoderma polysporum.*

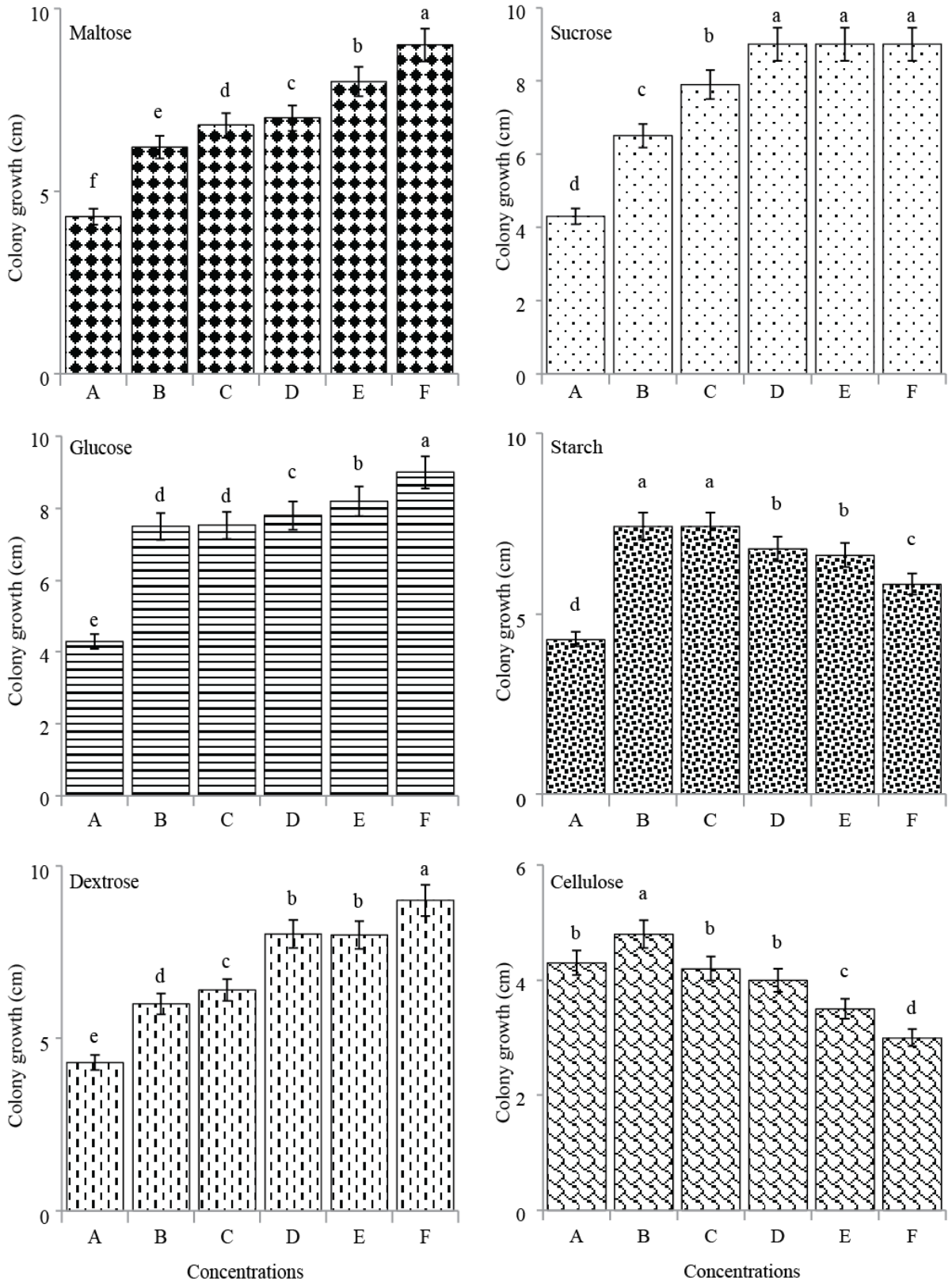

Fig. 2b. Effect of different carbon sources on *In vitro* growth of *Trichoderma polysporum.*
Treatments with similar letters at the top are not significantly different at $p<0.05$ level as determined by DMRT. Each bar shows S.E. at the top.
A= Control, **B**= $1x10^4$, **C**= $2x10^4$, **D**= $3x10^4$, **E**= $4x10^4$, **F**= $5x10^4$ ppm

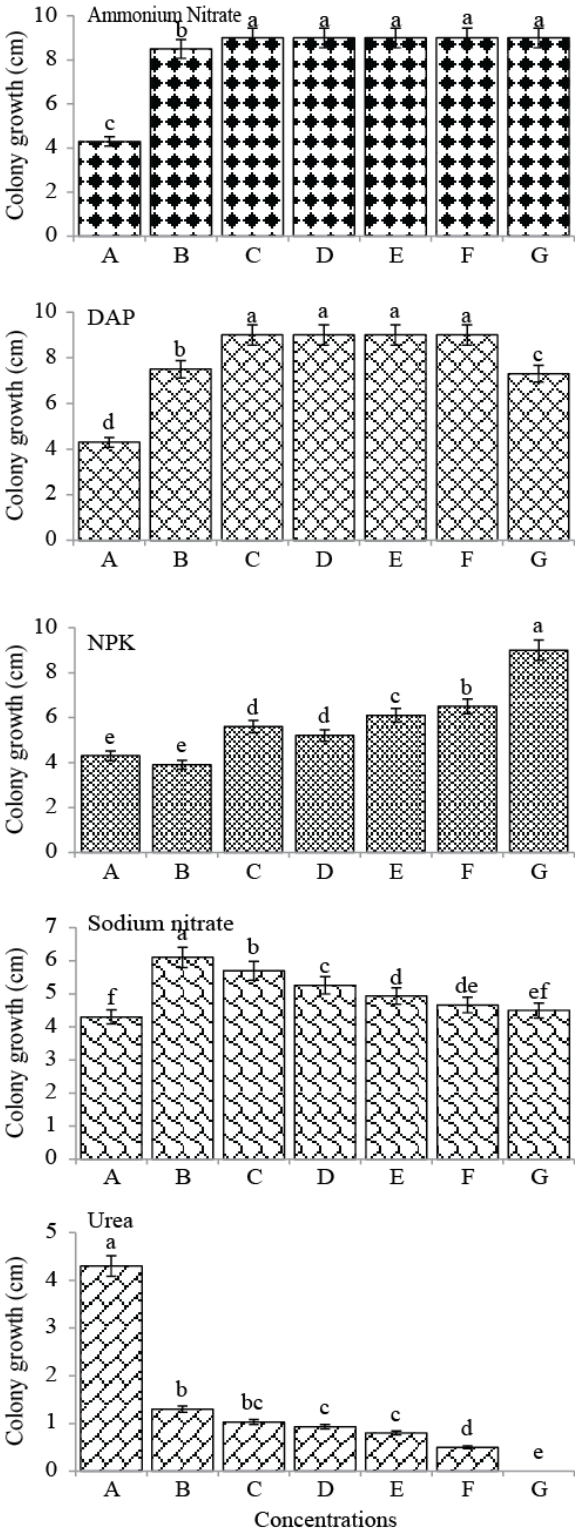

Fig. 3. Effect of different nitrogen sources on *In vitro* growth of *Trichoderma polysporum.*
Treatments with similar letters at the top are not significantly different at p<0.05 level as determined by DMRT. Each bar shows S.E. at the top.
A= Control, **B**= $1x10^3$, **C**= $3x10^3$, **D**= $5x10^3$, **E**= $7x10^3$, **F**= $9x10^3$ and **G**= $1x10^4$ ppm

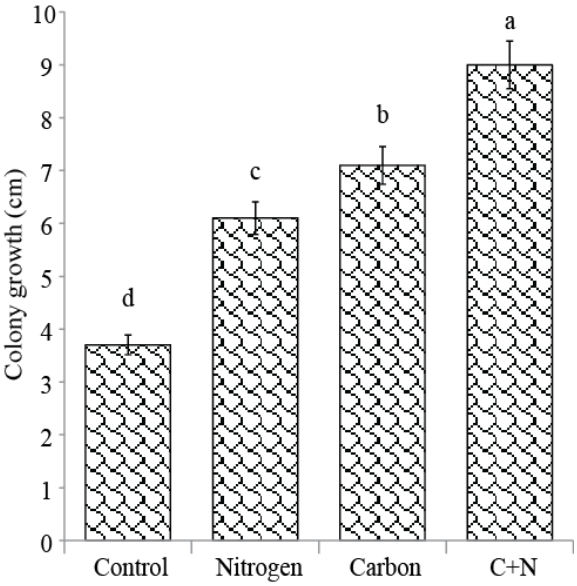

Fig. 4. Effect of different carbon and nitrogen sources on *In vitro* growth of *Trichoderma polysporum.*
Treatments with similar letters at the top are not significantly different at p<0.05 level as determined by DMRT. Each bar shows S.E. at the top.

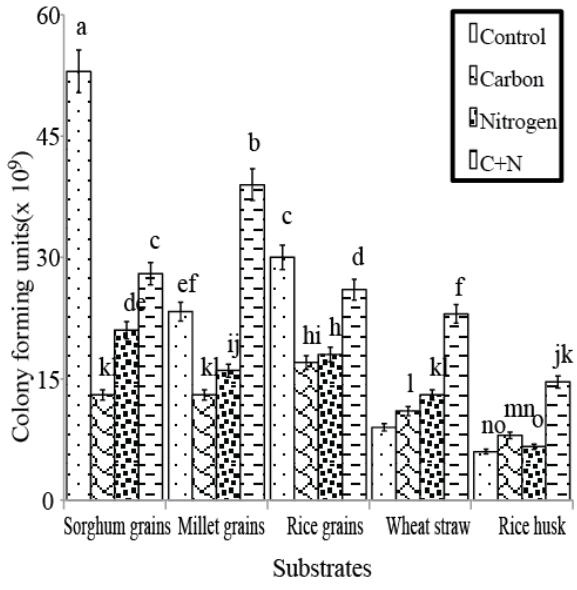

Fig. 5. Effect of selected C+N sources on the growth and sporulation of *Trichoderma polysporum* on organic substrates.
Treatments with similar letters at the top are not significantly different at p<0.05 level as determined by DMRT. Each bar shows S.E. at the top.

Combined effect of carbon and nitrogen sources: The combined use of best carbon and nitrogen sources acted positively on mycelial growth and conidial production of *T. polysporum* and significantly higher colony growth of *T. polysporum* was recorded on medium amended with sucrose @ 30,000 ppm + ammonium nitrate @ 3,000 ppm as compared to control (Fig. 4).

Effect of carbon and nitrogen sources on sporulation of *Trichoderma polysporum* on organic substrates: In case of sorghum grains and rice grains, the addition of carbon and nitrogen alone or in combination acted negatively and less sporulation of *T. polysporum* was observed as compared to un-amended substrates. Among all the treatments, highest population of *T. polysporum* was recoded on un-amended sorghum ($44x10^9$cfu g^{-1}) and rice grains ($43x10^9$cfu g^{-1}). However, the addition of carbon and nitrogen sources significantly enhanced the conidial population on wheat straw, rice husk and millet grains. In case of wheat straw, conidial population of the test fungus increased from $17x10^9$cfu g^{-1} on un-amended substrate to $33x10^9$cfu g^{-1} on C+N amended substrate. Similarly, in rice husk the number of conidia of *T. polysporum* increased from $10x10^9$cfu g^{-1} on un-amended substrate to $25x10^9$cfu g^{-1} on C+N amended substrate (Fig. 5).

Shelf life of *Trichoderma polysporum*: Conidial population of *T. polysporum* on sorghum grains the after 15 days incubation was $57x10^9$ cfu g^{-1} on un-amended and $26x10^9$ cfu g^{-1} on C+N amended substrate. The highest populations of *T. polysporum* observed after 60 days of incubation that were $77x10^9$ and $53x10^9$ cfu g^{-1} substrate in un-amended and C+N amended treatments, respectively. The conidial populations on amended and un-amended substrates declined gradually thereafter and after six month storage, the conidial populations reduced to $15x10^9$ and $7x10^9$ cfu g^{-1} on un-amended and C+N amended, respectively. The conidial populations of *T. polysporum* after 360 days of incubation reduced to $0.02x10^9$ and $0.008x10^9$ cfu g^{-1} in un-amended and C+N amended treatments, respectively. Growth and sporulation on rice grains also showed similar trend. Millet grains also showed similar trend but growth and sporulation of *T. polysporum* was greater on C+N amended grains as compared to un-amended millet grains (Fig. 6).

On wheat straw, the conidial population was $1.3x10^9$ cfu g^{-1} on C+ N amended and $1x10^9$ cfu g^{-1} on un-amended substrates after 15 days of incubation. On C+N amended wheat straw, the conidial population reached to its maximum after 105 days of incubation ($3.5x10^9$ cfu g^{-1}) whereas it took 135 days to reach to the maximum level on un-amended straw ($1.2x10^9$ cfu g^{-1}). Thereafter, *T. polysporum* populations slowly declined on both types of substrates and after 6 month of incubation $1.5x10^9$ and $0.062x10^9$ cfu g^{-1} of substrate were recorded on C+N amended and un-amended wheat straw, respectively. The conidial production after 360 days of incubation declined to $0.002x10^9$ cfu g^{-1} on C+N amended, and $0.001x10^9$ cfu g^{-1} on un-amended wheat straw. Growth and sporulation of *T. polysporum* on rice husk also showed similar trend except that the populations were slightly lower than those on wheat straw; maximum populations on C+N amended and un-amended rice husk were recorded after 105 days of incubation and no populations were recorded after 360 days of incubation (Fig. 6).

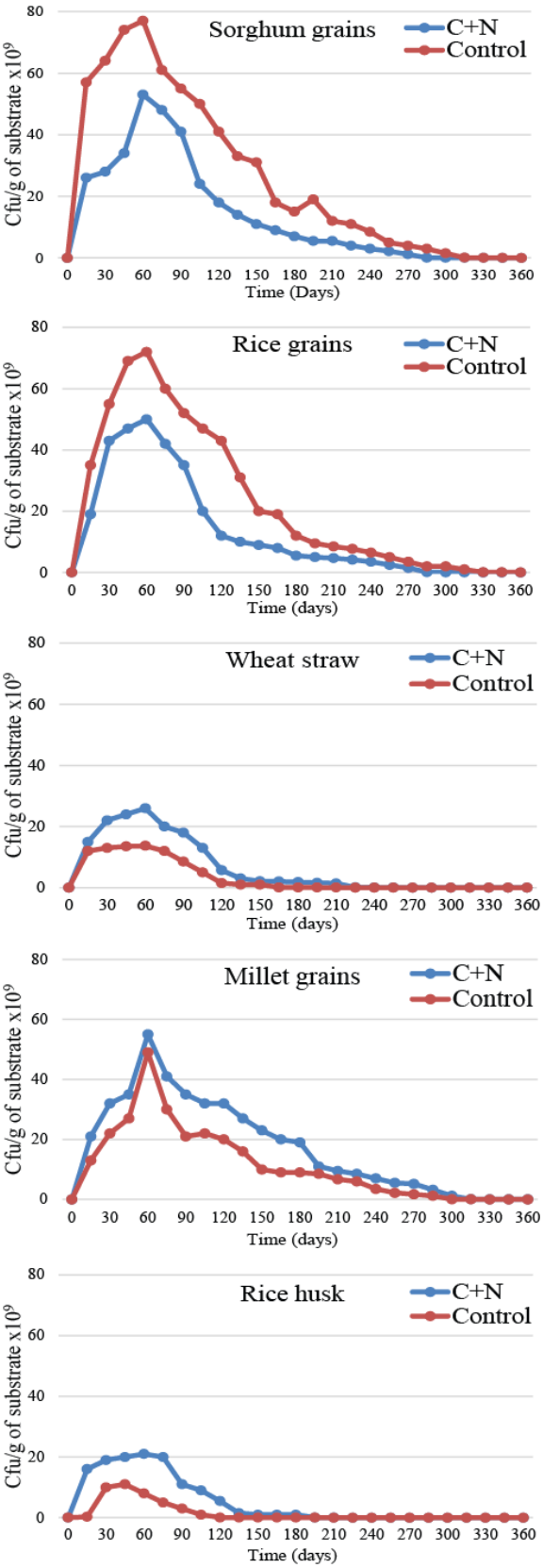

Fig. 6. Effect of C and N amendment on growth of *Trichoderma polysporum* on different substrates.

Discussion

Besides their effectiveness, the main hindrance in the widespread application of biocontrol agents like *T. polysporum* is their large scale availability for field use. Many workers have tried number of substrates such as rice grain, sorghum grain, millet grain, cotton cake, mustard cake, wheat straw, wheat bran, cotton waste, maize stover, rice straw, saw dust, sugarcane bagasse, sugarcane ash, farmyard manure (FYM) and wheat bran for mass multiplication of bio-control agents (Rettinassababady & Ramadoss, 2000; Saju *et al.*, 2002; Sharma & Chandel, 2003; Sangle & Bambawale, 2005; Panday, 2009; Azher *et al.*, 2009). During the present studies sorghum grains followed by millet grains appeared as the most effective substrates in which highest populations of *T. polysporum* were recorded. Our results are in confirmation to those reported by Dawar & Ghaffar (2003), Malik & Dawar (2003), Rini & Sulochana (2007) and Panday (2009) who also found that sorghum grains produced significantly more population of *Trichoderma* species than other substrates.

Efforts have also been made to improve the conidial yield of biocontrol agents by supplementing the nutritional supplements to growth substrates (Jackson *et al.*, 1991, Jose *et al.*, 1999; Naima *et al.*, 2004). The present studies revealed that among various carbon sources, the maximum colony growth of *T. polysporum* was obtained on sucrose followed by dextrose, glucose and maltose amended media. Ammonium nitrate at lower concentrations appeared as the best nitrogen source. The combined use of with sucrose @ 30,000 ppm and ammonium nitrate @ 3,000 ppm was more useful for the growth of *T. polysporum.*

Our results are in accordance to those reported by Abdullah *et al.* (2005) who found that mycelial yield of *T. harzianum* was significantly increased when the medium was added with sucrose or glucose as a carbon source. Monga (2001) also observed that among the different carbon sources, sucrose and glucose were the greatest for sporulation of *T. koningii* and *T. harzianum.* He also reported that these two species produced maximum biomass on maltose and glucose. Jayaswal *et al.* (2003) observed that the growth and sporulation of *T. viride* significantly influenced by different carbon and nitrogen sources. They also observed the greatest growth and sporulation of *T. viride* was observed on sucrose, peptone and trehalose supplemented medium as compared to individual carbon sources. Similarly, Jayaraj & Ramabadran (1998) investigated the effect of seven different nitrogen sources on *in-vitro* growth and sporulation of *T. harzianum* and found that ammonium nitrate, ammonium sulphate and sodium nitrate provided greatest growth and sporulation of the fungus. Jayaswal *et al.* (2003) also found that growth and sporulation of *T. viride* were ideal by ammonium form of nitrogen compared to nitrite or nitrate forms. Similarly, Aube & Gagnon (1969) observed that *T. viride* isolates grew best in media having glucose, sucrose, cellobiose and monitol as a carbon sources and asparagine and ammonium nitrate as nitrogen sources. Jakson *et al.* (1991) also observed that the addition of glucose in bacteriological peptone medium enhanced the spore germination of *G. virens* and *T. viride* up to 70%, and also observed that the usage of glucose (carbon source) and potassium nitrate or L-alanine (nitrogen source) maximized the biomass production of *G. virens*. Longa *et al.* (2008) determined that amongst the different carbon and nitrogen sources, *T. autoviride* produced greatest mycelial biomass on peptone, tryptone, nitrate, mannose, galactose and sucrose. Similarly Seyis & Aksoz (2005) reported that *T. harzianum* showed more xylanase activity in sucrose containing medium as compared to maltose and lactose containing media. Ammonium sulphate was the most appropriate nitrogen source for xylanase production whereas urea increased the xylanase activity.

Present studies also revealed that the addition of carbon and nitrogen sources significantly enhanced the conidial population in wheat straw, rice husk and millet grains, which without amendment of carbon and nitrogen showed poorer performance. Similarly, Prasad & Rangeshwaran (2000a) while making an improved medium for mass production of *T. harzianum* found that of the 3 nitrogen sources tested soya flour and sucrose as carbon source supported the highest biomass, number of viable propagules and spore production.

Studies on the shelf life and antagonistic activity of *Trichoderma* species revealed that shelf lives of these fungi varied greatly with the type of substrate or medium and temperature during storage (Sanyal *et al.*, 2003, Kolombet *et al.*, 2008, Prasad & Rangeshwaran, 2000b; Prasad *et al.*, 2002, Singh *et al.*, 2007, Agrosin *et al.*, 1997). Khan *et al.* (2011) reported that vermicompost, de-oiled caster cake and FYM reserved shelf life of *Trichoderma viride* for 220, 190 and 180 days, respectively, as compared to the gypsum and talc powder where the cfu g^{-1} declined after 80 days storage. Our studies also showed that nutritionally rich substrates maintained greater shelf life as compared to the nutritionally poor substrates.

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