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Full Length Article

Effect of Different Substrates and Carbon and Nitrogen Sources on Growth and Shelf Life of *Trichoderma pseudokoningii*

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Abstract

Trichoderma pseudokoningii is a hyperparasite on hyphae of many plant pathogens. However, no reports on its mass production are available. Nine different organic substrates *viz*., rice grains, sorghum grains, wheat grains, millet grains, wheat straw, rice husk, cow dung, sawdust and poultry manure were therefore evaluated for mass multiplication of *T. pseudokoningii.* Grains, especially sorghum grains were found more suitable for growth and sporulation than wheat straw, rice husk and cow dung. Sawdust and poultry manure were least effective. Sucrose and dextrose were found to be the most suitable carbon sources, whereas ammonium nitrate was found to be the best nitrogen source for growth and sporulation of *T. pseudokoningii* on Czapek's Agar plates. Amendment of selected C and N sources to organic substrates resulted in greater growth and conidia production by *T. pseudokoningii* on wheat straw, rice husk and millet grains but not on sorghum and rice grains. During studies on shelf life, populations of *T. pseudokoningii* on different substrates attained the peck at 60-75 d interval and declined gradually thereafter. However, even after 330 d, the populations were greater than the population at 0-day. At 345-360 d interval, populations were less than the initial populations at 0 d. © 2014 Friends Science Publishers

Keywords: *Trichoderma pseudokoningii*; Mass multiplication; Carbon and nitrogen sources; Shelf life

Introduction

The primary diseases threatening crop production are due to soil-borne plant pathogens that play a major role in the development of root rot disease complexes on many important field and horticultural crops, which often result in the death of plants. Subsequently soil applied pesticides are costly and produce environmental hazards (Cook and Baker, 1983; Saleem *et al*., 2000; El-Katatny *et al.*, 2000). Crop resistance to pathogens is the ideal means of controlling plant diseases but many crops have little or no resistance to certain plant pathogens. Use of microbial antagonists for the biological control of plant diseases is an alternative method for disease control which would also protect our environment from the use of hazardous chemicals (Larena *et al*., 2002; Harman *et al.*, 2004).

Trichoderma species are also known to produce plant growth promoting compounds that may or may not be essential for biological control (Omer and Shahzad, 2007). Clarkson *et al.*, (2004) observed that two isolates of *T. viride* and one isolate of *T. pseudokoningii* degraded up to 80% sclerotia of four isolates of *Sclerotium cepivorum* in a silty-clay soil. Ferdousi *et al.* (2010) found that *T. harzianum, T. virens* and *T. pseudokoningii* were highly effective against *Alternaria* fruit rot of chili and improved all the growth characters. Poliquit (1998) reported that *T. pseudokoningii* and *T. viride* inhibited the radial growth of *Phytophthora* by 69.6% and 63.5%, respectively. Taha and Salahuddin (1988) reported that *T. harzianum, T. pseudokoningii, Penicillium pinophilum, Bacillus cereus* and *Leuconostoc mesenteroides* were effective antagonistic microorganisms against *F. solani* root rot disease of broad bean.

There are reports where studies on mass production of *T. harzianum, T. viride, Gliocaldium virens, Paecilomyces lilacinus,* and *Rhizobium meliloti* have been made (Dawar and Ghaffar, 2003; Pramod and Palakshappa, 2009) but information on mass production of *T. pseudokoningii* is rather scanty. Fei *et al*. (2010) reported that wheat bran and corncob with a ratio of 8:2, H_2O 55%~60%, KNO_3 0.1%, calcium sulfate 1% and sucrose 1% supported good growth of *T. pseudokoningii*. The aim of present studies was to study the suitability of different substrates for mass production of *T. pseudokoningii* and effect of C+N sources on improvement in growth and sporulation of *T. pseudokoningii*.

Materials and Methods

Multiplication of *T. pseudokoningii*

Culture of *T. pseudokoningii* present in the Pest and Disease Research Lab (PDRL), Department of Agriculture and Agribusiness Management, University of Karachi was used during the present studies. Pure cultures of *T.*

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pseudokoningii were produced on potato sucrose agar (PSA) medium. Ten mL sterilized water was added to one week old culture plate of the fungus and the conidia separated from the conidiophores by gently rubbing the agar surface with a sterilized spatula. The contents were poured in a sterilized beaker and the process was repeated to most of all the conidia in suspension. Two drops of Tween twenty were added to the conidial suspension to suspend the hydrophobic conidia in water. The population of conidia per mL of suspension was determined by a haemocytometer using the following formula:

$$\text{Cfu mL}^{-1} = \text{No. of conidia in large square} \times \text{dilution factor} \times 1.25 \times 10^6$$

The population of *T. pseudokoningii* adjusted to 1.2×10^7 conidia per mL using $N_1V_1 = N_2V_2$ formula. This population was used in all the experiments.

Sorghum grains, rice grains, millet grains, wheat grains, wheat straw, saw dust, rice husk, poultry manure and cow dung were used for mass multiplication of *T. pseudokoningii*. The substrates were soaked in water for two h, squeezed with hands to remove excess moisture in containers and 50 g of a substrate was transferred in each polyethylene bag. The bags were sealed and then sterilized in an autoclave at 15 psi with 121ºC for 20 min. After cooling the substrates in each bag were inoculated by injecting 2 mL conidial suspension of *T. pseudokoningii* containing 1.2×10^7 conidia per mL. There were three replicate for each treatment. The inoculated substrates were stored at 30±2ºC and their population determined with the help of haemocytometer after 15 d intervals.

Effect of Carbon Sources on *In Vitro* Growth on *T. Pseudokoningii*

Different carbon sources *viz*., Sucrose, Maltose, Dextrose, Glucose, Starch and Cellulose were added to Czapek's Dox Agar (CzDA) medium without nitrogen sources to get final concentrations of 0, 10,000, 20,000, 30,000, 40,000 and 50,000 ppm before the medium was autoclaved. CzDA without carbon and nitrogen sources served as control. Media were sterilized for 20 min at 15 psi with 121ºC. Penicillin @100,000 U L^{-1} and streptomycin @ 0.2 g L^{-1} were added to sterilized stock media just before pouring to inhibit the bacterial growth. The media were poured in 9 cm dia., Petri plates @ 10 mL per plate. There were three replicate for each treatment. After solidification, a 5 mm dia, inoculum disc of *T. pseudokoningii* was placed in center of each Petri plate. The plates were incubated at 28±2°C and dia of the growing colonies were recorded daily.

Effect of N on *In Vitro* Growth of *T. pseudokoningii*

Different Nitrogen sources viz., NPK, Urea, DAP, ammonium nitrate and sodium nitrate were used separately to the CzDA medium to get the final concentrations of 0, 1,000, 3,000, 5,000, 7,000, 9,000 and 10,000 ppm. No carbon source was added to the medium. There were there replicates for each treatment. The effect of nitrogen sources on *in vitro* growth of *T. pseudokoningii* analyzed by the method described above for carbon source.

Combined Effect of Selective Carbon and Nitrogen Source on *In Vitro* Growth on *T. pseudokoningii*

Sucrose @ 30,000 ppm and ammonium nitrate @ 3,000 ppm were used as selected carbon and nitrogen sources. The carbon and nitrogen sources were mixed in the PSA medium, whereas, PSA without added carbon and nitrogen sources served as control. There were there replicates for each treatment. Effect of carbon and nitrogen sources on *in-vitro* growth and conidia production by *T. pseudokoningii* was recorded by the methods described above.

Effect of Carbon and Nitrogen Sources on Growth and Sporulation of *T. pseudokoningii* on Organic Substrates

Five selected substrates *viz.*, rice grains, sorghum grains, millet grains (most suitable substrates), wheat straw and rice husk (less suitable substrates) were used for multiplication of *T. pseudokoningii*. The substrates were soaked in water for two h in containers, squeezed with hands to remove excess moisture, and 50 g of a substrate was transferred in a polyethylene bag. Sucrose @ 1.5 g per 50 g substrate and ammonium nitrate @ 0.15 g per 50 g substrate were mixed with the substrates as selected carbon and nitrogen sources. Carbon and nitrogen sources were also used separately. Substrates without carbon and nitrogen sources served as control. There were there replicates for each treatment. Growth and population of *T. pseudokoningii* was determined using the methods described above.

Shelf-life of *T. pseudokoningii*

The shelf-life of *T. pseudokoningii* 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 1.2×10^7 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 d with 15 d interval. Three replicate bags of each treatment were used for determination of population at each time interval.

Experiments were carried out in complete randomized block design. Data were analyzed by ANOVA using Statistix 8.1 software. Least significant difference (LSD) were calculated at $p<0.05$ level.

Results

Growth of *T. pseudokoningii* on Different Substrates

Generally, cereal grains were found more suitable for mass production of *T. pseudokoningii* as significantly maximum

populations were recorded on cereal grains as compared to other substrates (Fig. 1). However, the highest population of *T. pseudokoningii* was observed on sorghum grains (58.7×10^8 cfu g^{-1}) followed by millet grains (54.53×10^8 cfu g^{-1}). The poultry manure was least suitable substrate and produced significantly lowest *T. pseudokoningii* population (1.06×10^8 cfu g^{-1}) followed by cow dung (2.10×10^8 cfu g^{-1}) and saw dust (2.27×10^8 cfu g^{-1}). Rice grains, wheat grains, wheat straw and rice husk performed moderately and produced 25.23×10^8, 23.06×10^8, 17.76×10^8 and 15.56×10^8 cfu g^{-1} of *T. pseudokoningii*, respectively (Fig. 1).

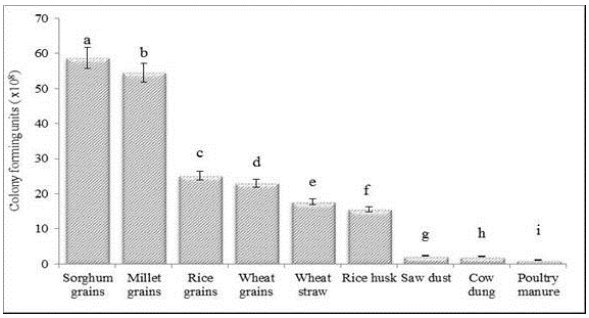

Fig. 1. Population of *T. pseudokoningii* after15 day's incubation on different substrates.

Effect of Different Carbon Sources on *In-vitro* Growth of *T. pseudokoningii*

In all carbon sources the colony growth of *T. pseudokoningii* increased with increasing concentrations except in case of cellulose and starch where maximum colony growth occurred at lowest concentration (Fig. 2). Significantly highest colony growth was recorded on sucrose followed by dextrose, glucose and maltose amended media (Fig. 2). However, Dextrose, glucose and maltose amended media produced less conidia but more superficial and fluffy mycelial growth of *T. pseudokoningii* as compared to the sucrose amended medium which support abundant conidial production of test antagonistic fungus. Sucrose was therefore used as a selected carbon source in further experiments.

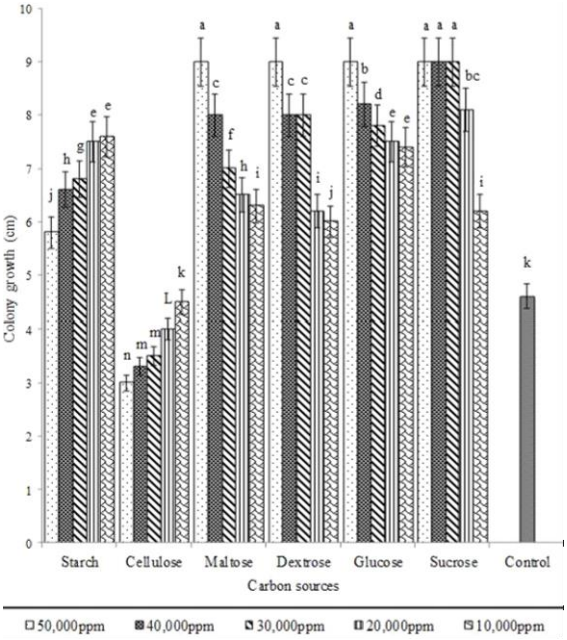

Fig. 2. Effect of different carbon sources on *in-vitro* growth of *T. pseudokoningii.*

Effect of Different Nitrogen Sources

Urea appeared to be the most toxic nitrogen source showed significant growth suppression at all concentrations. Sodium nitrate was the second least affective N-source. NPK gave good growth at 10,000 ppm and the growth declined with decrease in concentration. Petri plates containing DAP amended media were filled within 4 d but mycelial growth was very scanty and submerged with no conidia production at all. Maximum growth and conidia production was observed where the media were amended with Ammonium nitrate @ 3,000 ppm or more (Fig. 3). Ammonium nitrate @ 3000 ppm was therefore selected as the best nitrogen source for further experimentation.

Combined Effect of Carbon and Nitrogen Sources

The combined use of best carbon and nitrogen sources acted positively on the mycelial growth and conidial production of test antagonistic fungus as significantly higher colony growth of *T. pseudokoningii* 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 the Sporulation of *T. pseudokoningii* on Organic Substrates

In case of sorghum grains and rice grains the addition of carbon and nitrogen alone or in combination acted negatively on the sporulation of *T. pseudokoningii* as in both substrates significantly very high population of *T. pseudokoningii* recorded on un-amended (control) substrates as compared to carbon-nitrogen amended substrates (Fig. 5). Among all the treatments highest population of *T. pseudokoningii* was recorded on un-amended sorghum grains (50×10^9 cfu g^{-1}) and rice grains (30×10^9 cfu g^{-1}). However, the addition of carbon and nitrogen significantly enhanced the conidial population of *T. pseudokoningii* in C and N amended wheat straw, rice husk and millet grains as compared to un-amended substrates (Fig. 5). In case of wheat straw the conidial population of the test fungus increased from 13×10^9 cfu g^{-1} (in un-amended substrate) to 24×10^9 cfu g^{-1} (in C+N amended substrate). Similarly, in rice husk the number of conidia of *T. pseudokoningii* were increased from 8×10^9 cfu g^{-1} (in un-amended substrate) to 24×10^9 cfu g^{-1} (in C+N amended substrate) (Fig. 5).

Shelf-life of *T. pseudokoningii*

On sorghum grains, the conidial populations of *T. pseudokoningii* were 46×10^9 cfu g^{-1} on un-amended, and 26×10^9 cfu g^{-1} on C+N amended substrates, after 15 d of incubation and reaches to highest i.e. 76×10^9 cfu g^{-1} on un-amended, and 53×10^9 cfu g^{-1} on C+N amended substrates at 60 d. After then the conidial population of *T. pseudokoningii* gradually decreased in both the treatments. After 180 d storage, the populations of *T. pseudokoningii* declined to 18×10^9 and 14×10^9 cfu g^{-1} on un-amended and C+N amended substrates, respectively. Populations in 360 d old inocula further declined to 0.09×10^9 and 0.015×10^9 cfu g^{-1} on un-amended (control) and C+N amended sorghum grains, respectively (Fig. 6).

Populations on rice grains also showed similar trend. The conidial populations were 34×10^9 and 19×10^9 cfu g^{-1} on un-amended and C+N amended rice grains, respectively, after 15 d growth. Greatest conidial populations i.e. 73×10^9 cfu g^{-1} on un-amended and 48×10^9 cfu g^{-1} on C+N amended rice grains were observed after 60 d. The populations declined gradually thereafter and after 360 d incubation, only 0.057×10^9 and 0.006×10^9 cfu g^{-1} were recorded in un-amended (control) and C+N amended rice grains, respectively (Fig. 6).

In case of millet grains, the conidial populations of *T. pseudokoningii* were 40×10^9 cfu g^{-1} on C+N amended, and 21×10^9 cfu g^{-1} on un-amended substrates after 15 d of incubation. The maximum conidial populations was achieved after 60 d of incubation that were 65×10^9 cfu g^{-1} on C+N amended, and 50×10^9 cfu g^{-1} on un-amended millet grains. After that, the conidial populations of *T. pseudokoningii* reduced gradually and only 0.058×10^9 and 0.002×10^9 cfu g^{-1} were recorded on C+N amended and un-amended millet grains, respectively, after 360 d growth (Fig. 6).

After 15 d incubation, populations of *T. pseudokoningii* on wheat straw were 1.4×10^9 cfu g^{-1} in C+N amended and 1.1×10^9 cfu g^{-1} in un-amended treatments. The populations reached to maximum i.e. 3.7×10^9 and 1.3×10^9 cfu g^{-1} substrates in C+N amended and un-amended treatments after 105 d incubation, respectively. Thereafter, the *T. pseudokoningii* populations on both types of substrates declined gradually and after 6 month of incubation 2.5×10^9 and 0.062×10^9 cfu g^{-1} of *T. pseudokoningii* were recorded on C+N amended and un-amended substrates, respectively. The conidial populations of *T. pseudokoningii* after 360 d of incubation reduced to 0.002×10^9 cfu g^{-1} on C+N amended, and 0.0015×10^9 cfu g^{-1} on un-amended wheat straw (Fig. 6).

In case of rice husk, the conidial populations of *T. pseudokoningii* were 1.1×10^9 cfu g^{-1} on C+N amended and 0.065×10^9 cfu g^{-1} on un-amended substrates. The conidial population achieved its peaks after 105 d incubation, where 3×10^9 and 1.3×10^9 cfu g^{-1} were recorded in C+N amended and un-amended substrates, respectively. The population of

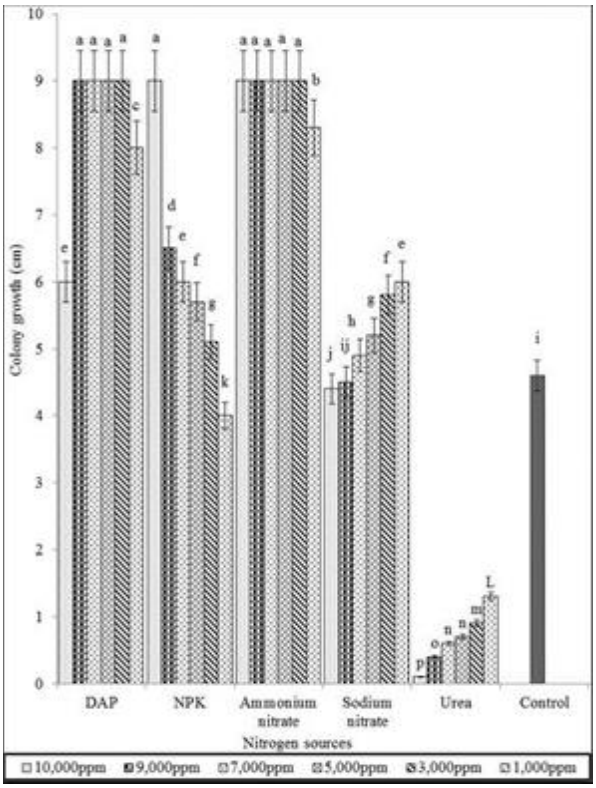

Fig. 3. Effect of low concentration of different nitrogen sources on *in- vitro* growth of *T. pseudokoningii.*

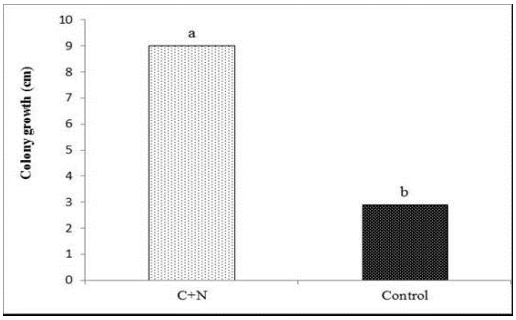

Fig. 4. Effect of selected carbon and nitrogen sources on *in- vitro* growth of *T. pseudokoningii*.

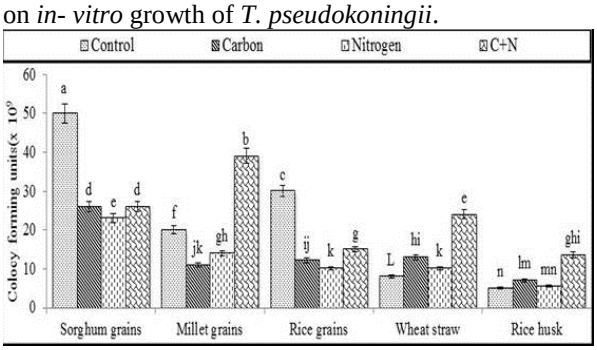

Fig. 5. Effect of selected C+N sources on the growth and sporulation of *T. pseudokoningii* on organic substrates.

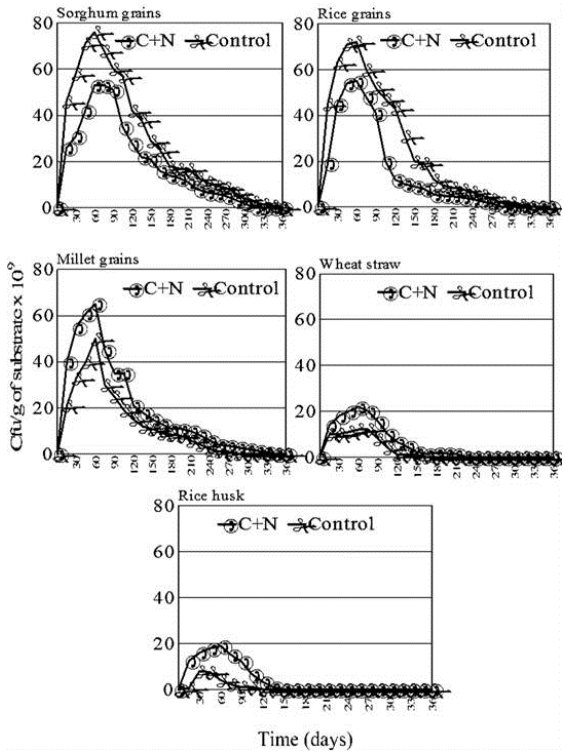

Fig.6. Effect of C and N amendment on shelf life of *T. pseudokoningii* on different substrates.

T. pseudokoningii was slowly declined on both types of substrates and after 6 month of incubation 0.045×10^9 and 0.025×10^9 cfu g^{-1} were recorded on C+N amended and un-amended rice husk, respectively. No population was recorded after 315 d incubation on C+N amended rice husk, and after 285 d on un-amended rice husk (Fig. 6).

Discussion

Despite of their effectiveness, the main difficulty in the widespread application of bio-control agent like *T. pseudokoningii* is their large-scale availability for field use. For this purpose several workers have tried different substrates such as sugarcane bagasse, sugarcane ash, rice grain, sorghum grain, millet grain, cotton cake, mustard cake, wheat straw, rice straw, saw dust, farmyard manure (FYM) and wheat bran were used for mass multiplication of bio-control agents (Sharma and Singh, 2004; Sangle and Bambawale, 2005; Sharma *et al.*, 2005). During the present studies, sorghum grains followed by millet grains appeared to be the most effective substrates in which highest population of *T. pseudokoningii* was recorded. Our findings are in confirmation to those reported by Tiwari *et al* (2004) who found millet as the greatest indigenous substrate for mass production of *T. viride*, with sporulation of 8×10^9 spores after 15 d incubation. Malik and Dawar (2003) and Dawar and Ghaffar (2003) also reported that sorghum grains produced significantly more population of *T. harzianum* than other substrates. The reports on mass multiplication of *T. pseudokoningii* are not available. It seems that present work is the first work on mass multiplication of *T. pseudokoningii.*

During the present studies also revealed that the addition of carbon and nitrogen sources significantly improved the conidial population of *T. pseudokoningii* in wheat straw, rice husk and millet grains as compared with un-amended carbon and nitrogen substrates. In this respect, Abdullah *et al.* (2005) reported that mycelial yield of *T. harzianum* was significantly enhanced when the medium was supplemented with sucrose or glucose as a carbon source. Monga (2001) also observed that sucrose and glucose as a carbon sources provided best sporulation of *T. koningii* and *T. harzianum*. He also reported that these two species produced maximum biomass on maltose and glucose.

Among the five different nitrogen sources i.e. urea, sodium nitrate, ammonium nitrate, DAP and NPK used during the present studies, maximum colony growth of *T. pseudokoningii* were observed on ammonium nitrate followed by DAP. Jayaswal *et al.* (2003) also reported that growth and sporulation of *T. viride* was favored more by ammonium form of nitrogen as compared to nitrite or nitrate forms. Jayaraj and Ramabadran (1998) also assessed the effect of seven different nitrogen sources on *in vitro* growth and sporulation of *T. harzianum* and observed that ammonium nitrate, ammonium sulphate and sodium nitrate provided maximum growth and sporulation of the antagonistic fungus. Gashe (1992) reported that nitrogen in the form of KNO_3 was better than NH_4Cl or urea for the growth of *Trichoderma* species.

On the basis of these results, different combinations of carbon and nitrogen sources were tried and it was found that culture medium amended with sucrose @ 30,000 ppm and ammonium nitrate @ 3000 ppm provided the best mycelial growth of the tested *T. pseudokoningii.* Jayaswal *et al.* (2003) found that the growth and sporulation of *T. viride* was greatly influenced by various carbon and nitrogen sources. They observed the best growth and sporulation of *T. viride* on sucrose, peptone and trehalose supplemented medium. Growth and sporulation both were favored by ammonium forms of nitrogen compared to nitrite or nitrate forms.

The results of our experiments on the effect of C and N sources on growth and sporulation of *T. pseudokoningii* on different substrates showed that amendment of carbon and/or nitrogen sources in sorghum and rice grains substrates failed to produce any positive effect on conidia production by *T. pseudokoningii*. It means sorghum and rice grains substrates performed better and produced higher population of *T. pseudokoningii* when used without the addition of carbon and/or nitrogen sources. However, in case of millet grains, wheat straw and rice husk significantly increased populations of *T. pseudokoningii*

were recorded on C+N amended substrates as compared to the un-amended substrates.

During experiments on shelf life of the *T. pseudokoningii* on different substrates with and without amendment with extra nutrients, it was observed that on sorghum, rice and millet grains (either amended with C+N or not), the population of *T. pseudokoningii* attained the peek after 60 d of incubation, then gradually decline and reached to lowest at 360 d. In case of rice husk and wheat straw, the population of *T. pseudokoningii* reached to maximum after 60 d of incubation in case of un-amended substrates and after 45 d, in case of C+N amended substrates. On wheat straw the population of these antagonistic fungi was lowest after 360 d, however, on rice husk, the conidia of *T. pseudokoningii* lost their viability after 285 d and 315 d, when multiplied on un-amended and C+N amended substrates, respectively.

These studies exposed that shelf lives of the fungus varied greatly with the type of substrate or medium and temperature during storage (Prasad *et al.,* 2002; Singh *et al.,* 2007). Khan *et al.* (2011) reported that vermi-compost, de-oiled caster cake and farm yard manure formulations reserved shelf life of *T. viride* for 220, 190 and 180 d, respectively, as compared to the gypsum and talc powder where the cfu g^{-1} declined after 80 d of storage. Our studies also indicated that nutritionally rich substrates supported greater shelf life as compared to the nutritionally poor substrates. However, in view of the greater cost of the grains, enhancement of *T. pseudokoningii* population of rice husk, what straw and millet grains by C+N amendment is quite encouraging since it could provide a cheap means for mass production of the biocontrol agent that however need further elucidation.

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