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*Full Length Research Paper*

# Assessment of endophytic fungi cultural filtrate on soybean seed germination

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**Soybean seeds have high amount of isoflavones but its germination is often confronted with a variety of environmental problems resulting in low germination rate and growth. To overcome this in eco-friendly manner, we investigated the influence of cultural filtrate (CF) of gibberellins-producing endophytic fungi on soybean seed germination. Three endophytic fungi namely: *Chrysosporium pseudomerdarium*, *Aspergillus fumigatus* and *Paecilomyces* sp. were previously isolated from the roots of soybean plants. The culture filtrate application of the three endophyte resulted in significantly higher rate of soybean seed germination, germination percentage, relative seed germination percentage, peak value, germination value, shoot and root length, germination index and vigour index. Among the endophytes, *A. fumigatus* significantly increased the rate of germination, shoot and root length and vigour index. Same trend was noted in germination percentage and relative seed germination percentage for all the endophytic fungi. However, *C. pseudomerdarium* was the only one that enhanced germination index. The enhanced soybean seed germination by endophytes can be used for seed priming and hence improved crop plant growth under extreme environmental conditions.**

**Key words:** *Chrysosporium pseudomerdarium*, *Aspergillus fumigatus*, *Paecilomyces* sp., soybean, seed germination.

## INTRODUCTION

Soybean (*Glycine max* L. Merr.) is considered as an important source of vegetable protein and oil (Moussa, 2004). High yield in annual crops depends on rapid and uniform field emergence of their seeds (Parera and Cantliffe, 1994; Subedi and Ma, 2005; Yari et al., 2010). Germination of seed start with the uptake of water by the inactive dry seed and cease with the protrusion of embryonic axis and sign of radical formation (Bewley, 1997). In agricultural ecosystem, early seed germination set trend for seedling performance and ultimately proper plant establishment (Weitbrecht et al., 2011). To obtain better seeds germination and afterward improved plant growth, studies have elucidated the seed priming with plant growth regulators as reported by Wen et al. (2009) and Weihong (2004). There are some studies suggesting the use of plant hormones such as Gibberellins for seed priming (Wallace and Elizabeth, 2000; Debeaujon and Koornneef, 2000; Grappin et al., 2000). Among phytohormones, gibberellic acid (GA) are mostly responsible for cell division and elongation, activation of embryo, weakening of endosperm layer and mobilization of endosperm food reserves are its prominent action. In most species during seed development, GA biosynthesis results in build up and storage of bioactive GAs or precursors of inactive GAs (Groot and Karssen, 1987; Toyomasu et al., 1998; Kamiya and Garcia-

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**Abbreviation: CF,** Cultural filtrate.

Martinez, 1999; Yamaguchi et al., 2001, Kucera et al., 2005). During germination, GA counteracts the effects of abscisic acid (ABA) contents activity thus releasing the dormancy which positively regulates germination (Taiz and Zeiger, 2002; Davies, 2004; Kucera et al., 2005). In seed germination process, expression of genes are induced via GA signalling thus encoding enzymes responsible for mobi-lization of food reserves including starches, lipids and protein stored in endosperm (Peng and Harberd, 2002).

Most potential producers of secondary metabolites are endophytic fungi, however little is known about their role in gibberellin biosynthesis and abiotic stress resistance (Waller et al., 2005). Endophytic fungi live inside plant tissues in symbiosis during their life cycle (Bacon and White, 2000; Maheshwari, 2006). Development of endophytic fungal association with roots can change mineral nutrient composition in plant tissues, plant hormonal balance, chemical composition of root exudates, plant-protection against biotic and abiotic stresses and also affect soil structure (Schulz and Boyle, 2005; Rodriguez et al., 2008; Redman et al., 2011). Symbiotic association of endophytic fungi in seed germination degrade cuticle cellulose and make available carbon for seedling which improves germination, vigour and establishment (Jerry, 1994).

This study was conducted to investigate the possible role of endophytic fungi for improving soybean seed germination. Soybean is sensitive to improved germination and can easily lose its viability to germinate if surrounding conditions are not conducive. Previously, 42 different strains of endophytic fungi were isolated from the roots of soybean plants. Among them, D-2-1 (*Chrysosporium pseudomerdarium*), HK-5-2 (*Aspergillus fumigates*) and S-5-1 (*Paecilomyces* sp.) were found plant growth promoting to *Waito-C* rice (gibberellin biosynthesis mutant) and Dongjin-byeo (normal gibberellins pathway) cultivars. Gas chromatography-mass spectroscopy (GC-MS) selected ion monitor (SIM) analysis showed that *C. pseudomerdarium*, *A. fumigates* and *Paecilomyces* sp. (Hamayun, 2008; Hamayun et al., 2009a, b; Khan et al., 2011) can secrete both physiologically active and inactive gibberellins in various quantities in their growing mediums. Though, there are a few examples of effect on seed germination involving microbes, however, we failed to find any report related to GA producing endophytes. In the present study, we aimed to assess the role of GA producing endophytes on seed germination and growth of soybean seeds. The findings of the present study can be adopted for an eco-friendly and nature based method of seed priming.

## MATERIALS AND METHODS

### Endophyte isolation and identification

Endophytic fungi was isolated from the roots of soybean plants and was identified through DNA extraction, PCR techniques, sequencing and phylogenetic analysis of 18S (ITS; ITS-1; 5´-TCC GTA GGT GAA CCT GCG G-3´ and ITS-4; 5´-TCC TCC GCT TAT TGA TAT GC-3´) and 28S (LSU; LR0R (F) (ACC CGC TGA ACT TA AGC) and TW13(R) (GGT CCG TGT TTC AAG ACG) with the help of methods already described in Khan et al. (2011). The endophytic fungal strains *C. pseudomerdarium*, *A. fumigates* and *Paecilomyces* sp. were selected on the basis of their bioactive role in promoting growth of *Waito-C* and Dongjinbeyo rice (data not shown). The sequence of these strains were already submitted to NCBI GenBank and were given accession no. EU823311 (*C. pseudomerdarium*), EU823312 (*A. fumigates*) and EU823315 (*Paecilomyces* sp.) respectively as reported in Hamayun et al. (2009a, b).

### Inoculation of media for bioassay

Prior to germination the selected isolated fungal strains were inoculated in a Czapek broth medium (250 ml) and afterwards incubated for seven days at 30°C and 120 rpm. The mycelia were harvested by centrifugation at 5000×*g* at 4°C for 15 min. The supernatants obtained were frozen at -20°C and lyophilized in freeze dryer. After lyophilization, the supernatants of each fungus were diluted in 1 ml of autoclaved distilled water.

### Seed germination bioassay

A static test was performed by incubating at 25°C in dark condition. Petri-dishes (90 × 15 mm) and filter papers (Whatman#1, size 90 mm) were autoclaved to ensure complete sterilized conditions, and 10 seeds per Petri-dish were maintained. For surface sterilization, seeds were kept in 2.5% sodium hypochlorite for 5 min and then washed thoroughly with autoclaved double distilled water (DDW). Petri plates lined with filter paper were moistened with 5 ml autoclaved DDW. 20 µl from diluted lyophilized cultural filtrate (CF) of each fungus was used and control with no CF. Prior to vigour index all the pots and horticulture soil were sterilized (autoclaved for 15 min at 121°C, 15 psi, soil were autoclaved three time and well mixed repeatedly before sterilization) and 10 seeds per pot were maintained in complete randomized design (CRD). At the time of sowing, same amount of diluted lyophilized CF of each fungus was applied to their respective pots and control with only autoclaved DDW.

### Experimental design and calculations

To investigate the effect of endophytic fungi CF on germination behaviour of soybean, the experiment was designed (complete randomized design with 21 replications per treatment repeated three times using excel for general calculations) with four sets of treatments, that is, the control (DDW), CF of *C. pseudomerdarium*, CF of *A. fumigates* and CF of *Paecilomyces* sp. Soybean variety *Taekwangkong* were used for seed germination bioassay. Seed germination, root elongation and germination index (GI, a factor of relative seed germination and relative root elongation) was evaluated according to Tam and Tiquia (1994).

$$\text{Relative root elongation (\%)} = \frac{\text{Mean root elongation in cultural filtrate}}{\text{Mean root elongation in the control}} \times 100$$

$$\text{Germination index} = \frac{\text{\% Seed germination} \times \text{\% Root elongation}}{100}$$

$$\text{Relative seed germination (\%)} = \frac{\text{Number of seeds germinated in the CF}}{\text{Number of seeds germinated in control}} \times 100$$

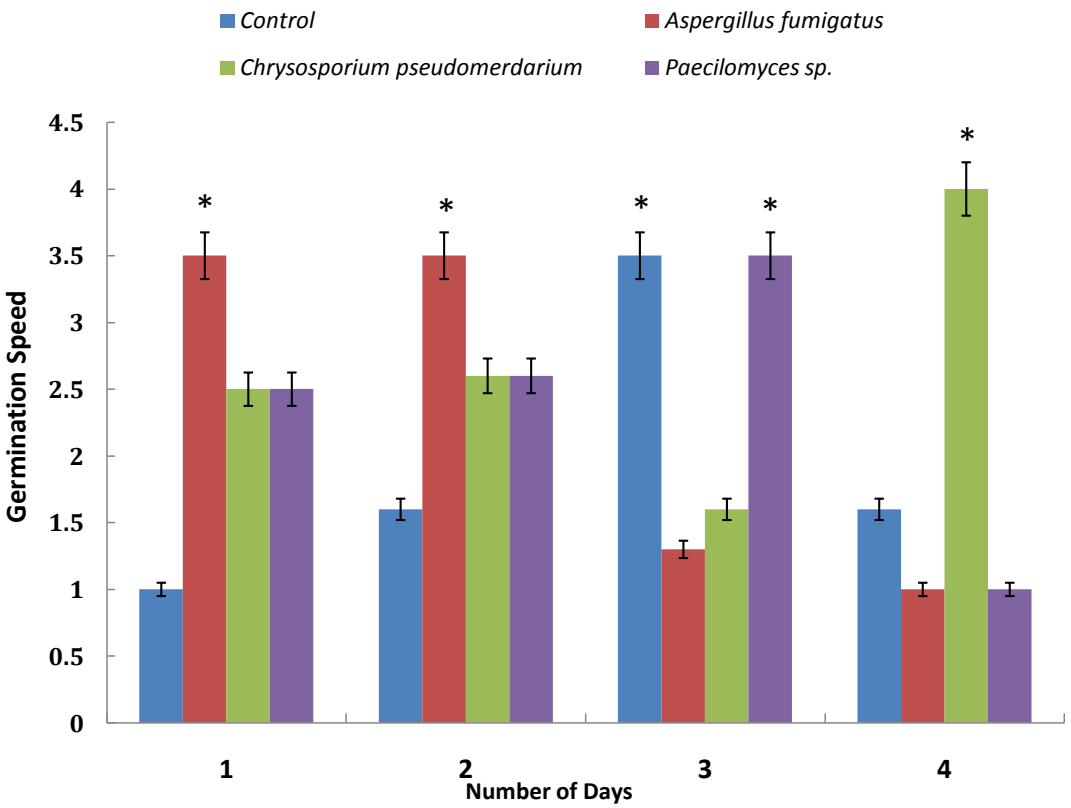



**Figure 1.** Effect of culture filtrate of different endophytic fungi on germination speed of soybean. Each value is the mean ± SE of 21 replicates per treatments. Control, without endophytic CF. *Values are significant ($P$<0.05) as compared to control.

The vigour index was calculated according to (Orchard. 1977).

Seedling vigour index (SVI) = [Seedling length (cm) × germination percentage]

Shoot and root of each seedling were carefully measured by millimetre callipers. The mean value derived from that was converted into centimetres. For seed germination, the method designed by Ramana et al. (2002) was followed. Seed parameters were recorded by following formula:

$$\text{Speed of germination} = \frac{\text{Seed germinated number}}{\text{Days of first count}} + \ldots\ldots + \frac{\text{Seed germinated number}}{\text{Days of final count}}$$

$$\text{Germination percentage} = \frac{\text{Number of seed germinated}}{\text{Total number of seed}} \times 100$$

$$\text{Peak value} = \frac{\text{Cumulative percent germination on each day}}{\text{Number of days elapsed since initial imbibitions}}$$

Germination value = Peak value × Germination (%)

**Data analysis**

The experiment was designed in complete randomized design with 21 replications per treatment repeated three times while MS office Excel (2007) was used for general calculations. The mean and standard error of the replications for each treatment were calculated using GraphPad Prism (Ver 5.1, USA). Data were compared for significant differences, based on the Student's t-test ($P$ < 0.05), between control and treatments (Gomez and Gomez, 1984) using GraphPad Prism (Ver 5.1, USA). Some values were significantly ($P$ < 0.05) different from control as evaluated by Student's t-test.

## RESULTS AND DISCUSSION

### Effect of endophytes on speed of germination

The endophyte *A. fumigatus* released significant amount of bioactive $GA_3$ (8.38 ng/ml), $GA_4$ (2.16 ng/ml) and $GA_7$ (1.56 ng/ml) and inactive $GA_7$ (0.5 ng/ml), $GA_{19}$ (1.2 ng/ml) and $GA_{24}$ (0.8 ng/ml) in its CF as reported in Hamayun et al. (2009) and hence highest speed (4.087) of soybean seed germination was revealed. The soybean seeds treated with the CF of *A. fumigatus* significantly ($P$ < 0.05) improved the speed of germination as compared to control (2.696) (Figure 1). Similar results were also observed for the CF of *C. pseudomerdarium* and *Paecilomyces* sp. which improved the speed of

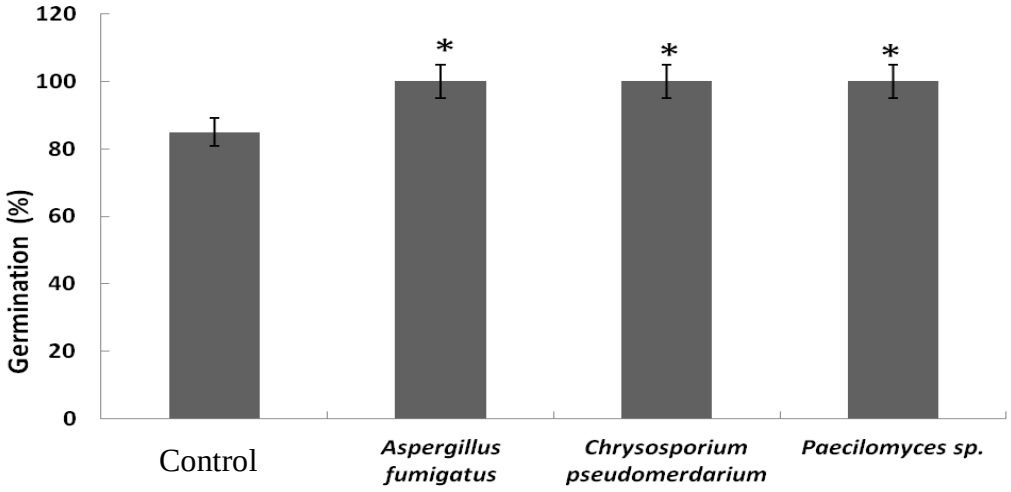



**Figure 2.** Influence of different endophytic CF on the germination percentage attributes of soybean seed, each value is the mean ± SE of 21 replicates per treatments. Control, without endophytic CF. *Values are significant ($P < 0.05$) as compared to control.

germination, however, they were at par with each other. Our results are in conformity with those of Khan et al. (2011) who also reported highest speed (4.732) of germination in soybean seed treated with CF of *Penicillium funiculosum* LHL06. One of the main reason for high speed of germination is the highest amount of bioactive $GA_3$ (8.38 ng/ml) in CF of *A. fumigatus* as observed by Hamayun et al. (2009a), which may confirm our results.

## Germination percentage

GA is the important phytohormone that promotes and maintains germination, neutralize the inhibitory effect of ABA in association with cytokinins and render the unnecessary environmental signals (Bewley and Black, 1982; 1994; Bewley, 1997; Léon-Kloosterziel et al., 1996). The CF of all the endophytic fungi had almost similar results for germination percentage. Seed treated with CF was significantly higher (100%) as evidenced from improved germination percentage as compared to control (85%) (Figure 2). Similar findings were also noted by Khan et al. (2011) who reported 100% germination in endophytic treated seeds after six days of incubation. As all these endophytic fungi produced GA in there CF as reported by Hamayun et al. (2009a, b) thus, in the present study improved germination (17.65%) was observed.

## Relative seed germination percentage

The seeds treated with CF of endophytic fungi had considerably higher (13%) relative seed germination than its control. Same value (13%) was recorded for all the three endophytic fungi (Figure 3).

## Peak and germination values

Peak value is cumulative percentage germination divided by the number of days since initial imbibitions thus predict the most vigorous components of seed lot (Czabator, 1962; Thomson and EL-kassaby, 1993) and product of peak value and germination percentage results in germination value. Mean analysis of peak and germination values shows significantly higher results for all the endophytic fungi at 95% level of probability. Similar higher response of peak value and germination value was recorded for *A. fumigatus* than the rest of endophytic fungi (Figure 4). However, the results of endophytic CF application were highly significantly different ($P < 0.05$) than control.

## Average shoot and radical length

The growth and development of seedling are affected by various internal as well as external factors that is, environmental and endogenous hormonal factors (Quail, 1998; Lin, 2000; Wang et al., 2009; Wahid et al., 2007). Significantly, higher shoot length (8 cm) was observed in seeds treated with CF of *A. fumigates* and CF of other endophytic fungi were not significantly higher ($P < 0.05$) than the control (Figure 5); while same mean values (4 cm) of the average of radical length was observed in treated seeds. Germination is terminated with radical

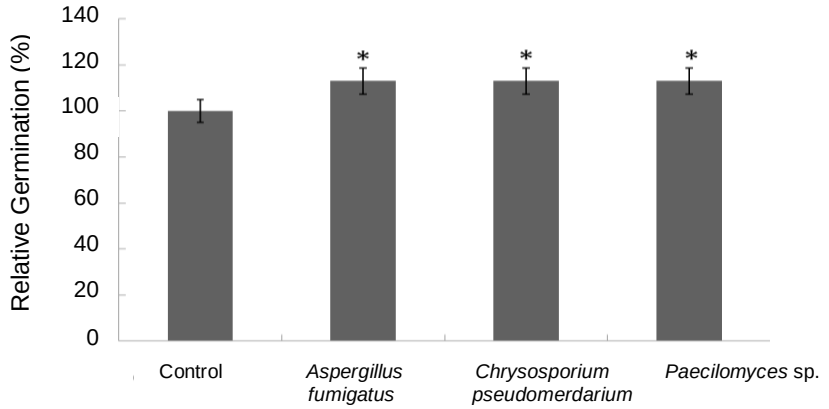
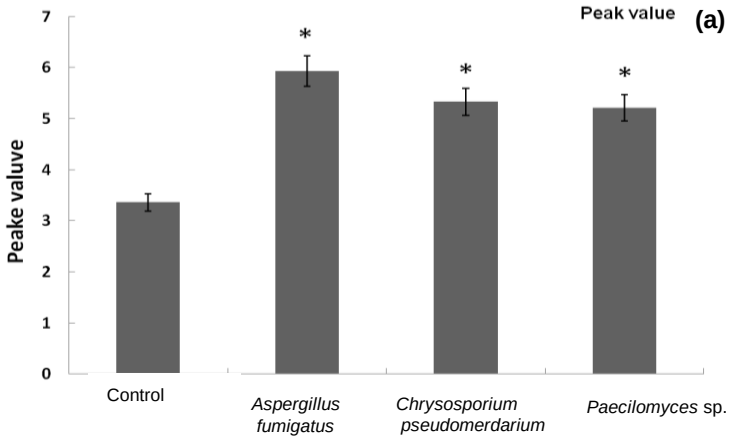



**Figure 3.** Relative germination percentage of soybean seed as triggered by different endophytic culture filtrate, each value is the mean ± of 21 replicates per treatment. Control, without endophytic CF. *Values are significant ($P < 0.05$) as compared to control.



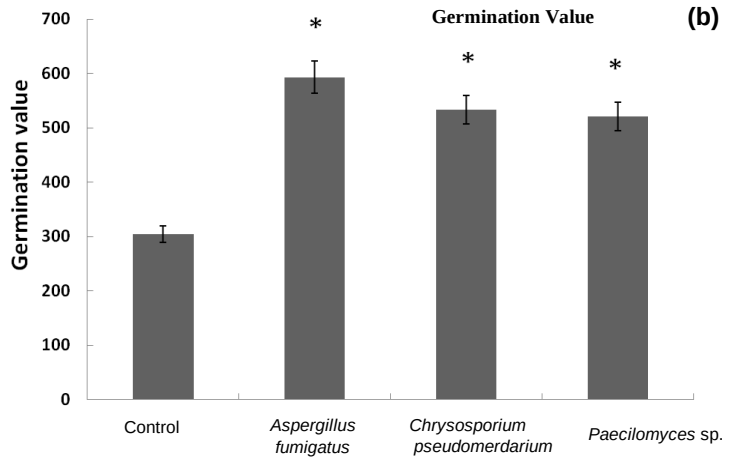




**Figure 4.** Effect of endophytic CF on peak and germination value. Each value is the mean ± of 21 replicates per treatment. Control, without endophytic CF. *Values are significant ($P < 0.05$) as compared to control.

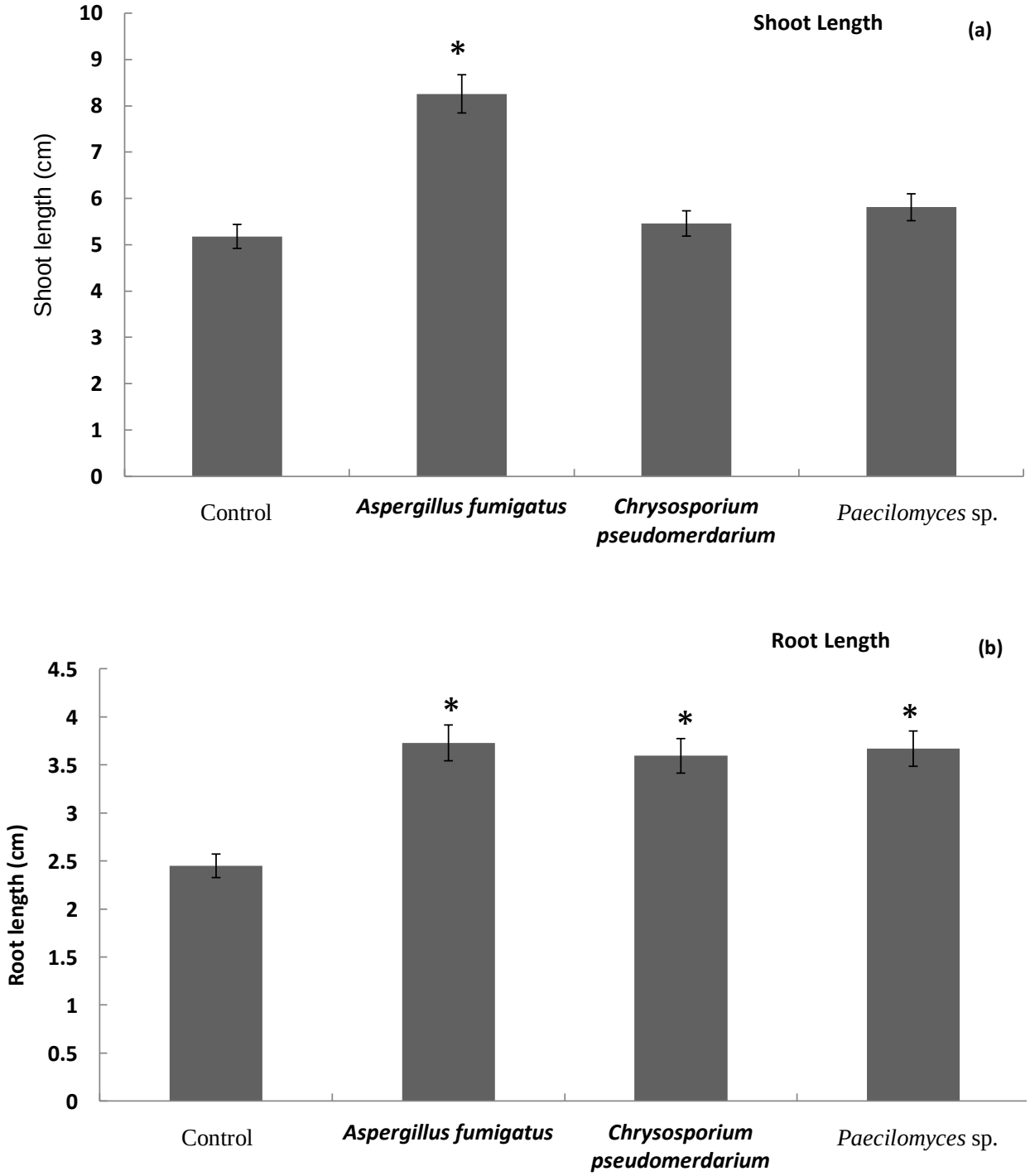



**Figure 5.** Mean shoot and root length of soybean seed as affected by endophytic CF interaction. Each value is the mean ± of 21 replicates per treatment. Control, without endophytic CF. *Values are significant ($P$<0.05) as compared to control.

extension through the structures surrounding (cotyledons and seed coat) the embryo indicating the seedling growth (Bewley, 1997). The higher radical length shows the early germination of soybean, making it more feasible for greater root length and deep soil penetration.

**Germination and vigour indices**

Germination index is generally either the speed of germination or maximum percentage of germination (Correa et al., 2000). The results show that, the soybean

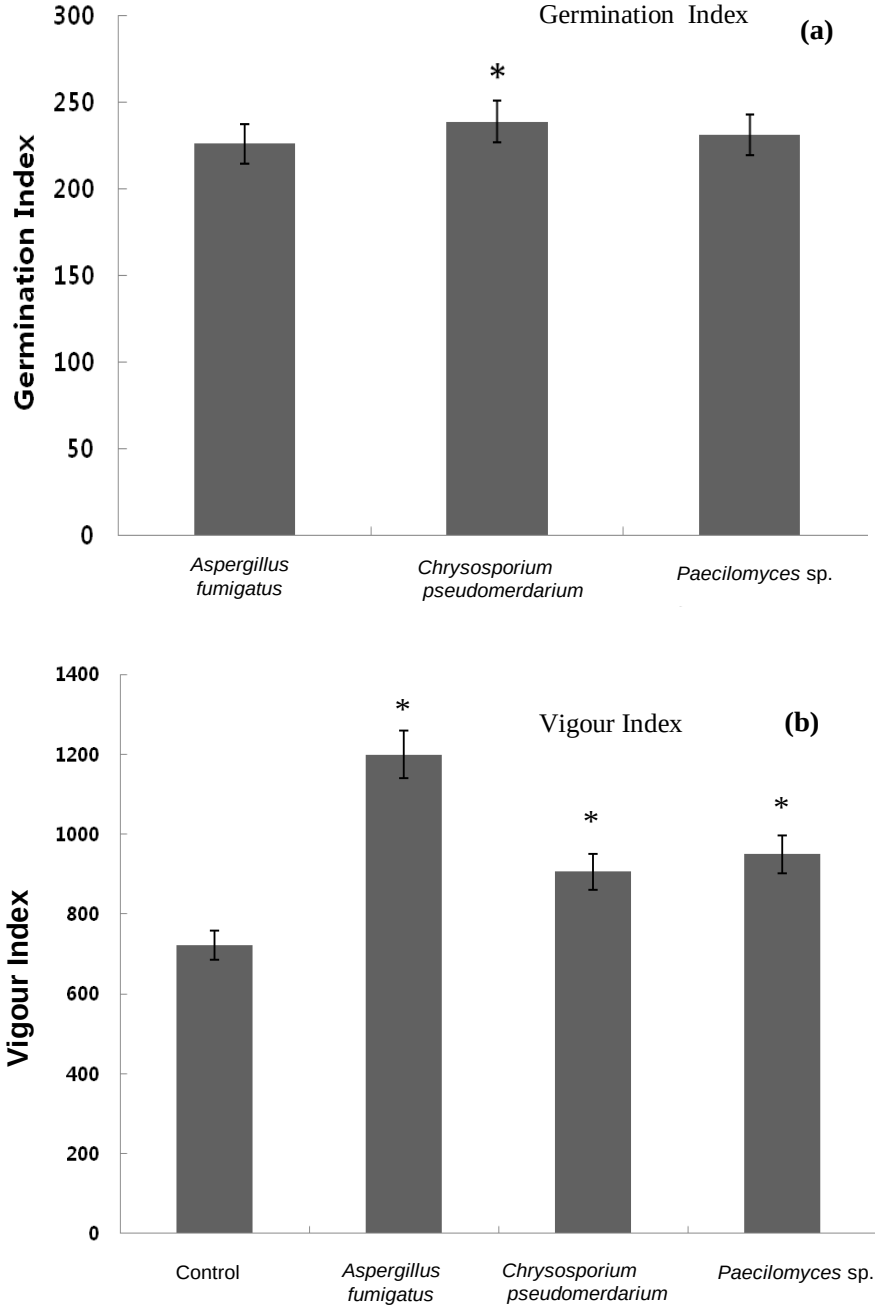



**Figure 6.** Germination index and vigour index of soybean seed as affected by application of different endophytic CF. Each value is the mean ± of 21 replicates per treatment. Control, without endophytic CF. *Values are significant ($P < 0.05$) as compared to control.

seeds treated with CF of endophytic had improved germination and vigour index ($P < 0.05$) than control. In case of germination index, *C. pseudomerdarium* has the highest (239) germination index followed by *Paecilomyces* sp. (231) (Figure 6). Different increasing trend has been observed in vigour index. *A. fumigatus* applications give the significantly higher (1199) vigour index followed by *Paecilomyces* sp. (949) (Figure 6). Maisuria and Patel (2009) reported the same results and observed that rhizospheric fungi *Trichoderma viride* increased (21863.3) the vigour index as compared to control (20503.3) in soybean, at the same time they also observed retardation of seed germination by other species (*Pythium aphanidermatum, Fusarium oxysporum,*

*Rhizoctonia* sp., *Fusarium solani, Macrophomina phaseolina and Pythium* sp.) o f fungi. The retardation was mainly attributed due to toxic metabolite (marticin, isomarticin, Zearalenone, tenanzoic acid, alternariols, tenuazonic acid) they secrete affecting directly or indirectly seed germination. Elliott (2003) reported that high vigour index resulted in higher total plant weight and yields also improved. By improving the vigour index with CF of endophytic, we can improve our yield in worst condition like salinity, drought and high temperature (Wahid et al., 2007).

## Conclusion

Among phytohormones, gibberellin is the most important and essential hormone that perhaps controls plant growth and development as well as seed germination and development (Davies, 2004; Ogawa et al., 2003; Debeaujon and Koornneef, 2000). Endophytic fungi are now the most recognized source of phytohormone such as gibberellin. Keeping in view this unique characteristic of endophytes, we conclude on the basis of our investigation to elaborate its role in seed priming for improved crop establishment and growth.

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