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Short Communication

Induction of chlamydospores to enhance the shelf-life in *Trichoderma asperellum* using phosphorus deficient medium

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Soil has biologically complex environment, where immense group of microbes habituating especially fungal and bacterial microorganisms and these soil microbes have ability to transform complex compounds into simpler that enter again the ecosystem as well as they also mobilize, biotransformation and bioaccumulation processes of nutrients, micronutrient and heavy metals in the soil (Gadd 1993). Morphological behaviour and activity of the microbes highly influenced by the environmental factors and nutrient stress such as drought, heat, salinity, cold, can have a devastating impact on microbial activities as well as on plant growth and crop yield (Suzuki et al. 2014). Multiple tip-formations near the hyphal apex in filamentous fungi especially in *Aspergillus nidulans* and *Neurospora crassa* due to environmental and nutritional stress and this phenomenon happened due to disturbance of the process of tip extension and such aberration might be potential benefits in increasing the number of exploratory branches emanating from a mycelium to tackle risk of the environmental factors (Markham 1992). Phosphorus is an important growth-limiting nutrient and a large portion of inorganic phosphates (P) applied to soil in the form of fertilizer by the farmers which is rapidly immobilized after application, nearly >80% of P becomes immobile and unavailable for plant uptake because of adsorption, precipitation or conversion to organic form (Holford 1997). Thus, the release of insoluble and fixed forms of phosphorus by P solubilizing microorganisms (PSMs) especially bacteria, fungi and actinomycetes reside in the rhizosphere of crop plants which plays an important role in solubilization of bound phosphates, making them available to plants (Beileski 1973, Schachtman et al. 1998, Nautiyal 1999, Ezawa et al. 2002, Sujatha et al. 2004). Among these microbes, fungi have been reported to possess greater ability to solubilise rock-phosphate than bacteria and actinomycetes. Application PSMs viz., Mycorrhizae, *Trichoderma*, *Aspergillus* and *Penecillium* species are known to improve solubilization of fixed soil P and applied phosphates, resulting in higher yields as compared to non-inoculated crop plants (Naik et al. 2014).

Trichoderma species are free-living, fast growing, opportunistic, avirulent, plant symbionts which are highly interactive in root, soil and foliar environments of the plants are widely bio-augmented and exploited as commercially viable biofungicides for biological management of soilborne diseases of crop plants all over the world (Harman et al. 2004). The biggest problems with *Trichoderma* formulation are the shelf-life, suitability, and persistency under adverse climatic condition. Tackling these problems, Li et al. (2005) developed a technique to induction of conidia and chlamydospores in the hyphae of *T. harzianum* and *Gliocladium roseum* by the antimicrobial metabolites of *Bacillus subtillis*. Lewis and Papavizas (1983, 1984) also reported that some species of *Trichoderma* have ability to produce chlamydopsores on natural substrates and they also emphasized their role in survival in soil under stress condition. Among the natural substrates, Molases-Corn Steep liquor, showed highest number of chlamydospores formation (10.4×10^7 chlamydopsores/ gram mycelium) followed by sucrose nitrate and glucose tartrate media. *Trichoderma* are widely used as seed priming or furrow application by mixing with compost/ vermicompost and it showed positive effect when seed exposed to physiological, biotic or abiotic stresses and thus protect seeds and seedlings. A report indicated that the physical stresses such as aeration and light inhibited the formation of sclerotia and inhibition in induction of basidiospores in *Sclerotium rolfsii* in *Cyperus rotundas* meal agar medium (Maurya et al. 2010).

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Several reports indicated that the conidia of *Trichoderma* derived from solid state fermentation are highly tolerant to abiotic stresses while liquid fermentation based has relatively shorter shelf-life. Reduction in pH and addition of starch in the formulation increased shelf life of the formulation (Kolombet et al. 2007). Report indicated that the use of colloidal chitin @ 0.1, 0.2 or 0.5% (w/v) in molasses yeast extract medium enhanced the shelf-life of the talc formulation of *T. harzianum* additional 2 months. Addition of glycerol (3 & 6%) in production medium prolonged the shelf-life (with viability of $>2 \times 10^6$ CFU g^{-1}) to 7 and 12 months as compared to 4–5 months shelf-life in formulations derived without the addition of glycerol in the medium of the talc formulation (Sriram et al. 2005, 2011). Chlaymydospres based formulations of *Trichoderma* sp. exhibited longer shelf life (80% viability for 9 months) than conidia based formulations (80% viability for 4 months) at room temperature. Chlamydospores are produced by many fungi and represent enlarged, thick-walled vegetative cells with varied forms and condensed cytoplasm that form within hyphae or at hyphal tips. Biological functions ascribed to these chlamydospores differ between species. Chlamydospores are known vegetative structures which have ability to survive under adverse condition by fungal mycelium and their role in long term survival of the fungus reported by several workers. Patterson (1991) reported that the chlamydospores of *A. solani* were responsible for long-term survival of the fungus. Environmental stress conditions such as drought, heat, salinity, cold, or pathogen infection can have a devastating impact on microbial population of the soil as well as crop plant also. The microbial adoptability in adverse environmental/ soil condition not yet fully explored. Keeping these in view, experiments were design to see the growth behaviours and adaptability of *Trichoderma* species under phosphorus stress.

In the present study, Pikovskaya's medium (PVK) was selected and is a novel defined microbiological growth medium which is highly developed for screening of phosphate solubilising microorganisms and was consistently demonstrated phosphate solubilising efficacy of the test fungi/ bacteria on the formation of visible halo/zone on agar plates. In the present study, a novel biocontrol agent, *T. asperellum*, was selected to see their growth behaviours and adaptability under phosphorus stress. Three different combinations of bound phosphate (TCP) and available phosphates (KH_2PO_4): (1) TCP @ 10 $g.l^{-1}$,; (2) TCP @ 10 g/l + KH_2PO_4 @ 0.5 g/l and (3) KH_2PO_4 @ g/l were supplemented in the medium to see their effect on growth behaviour especially colony morphology and frequency of the chlamydospores formation of *T. asperellum in vitro*. As per results, the growth of fungal mycelium of *T. asperellum* was relatively slow n very much compact which covered 100 mm Petri plates in 7-9 days while in normal course of time it covers 100 within 4 days of inoculation. When microscopically (Leica, Germany) observed, these fungal hyphae showed morphologically distinguish characters to PDA (Himedia, India) grown hyphae of *T. asperellum*. The frequency of the induction of terminal and intercalary chlamydospores in the hyphae of PVK (Himedia, India) amended with bound phosphate (TCP) were very high (28.67, 32.23 and 33.63%) as compared to control (Table 1, Figure 1). The experiments were repeated trice for counter confirmation about the induction of chlamydospores. The phosphate solubilisation index was also very high which have huge numbers of chlamydospores. These induction of the frequency of the chlamydospores might be due to enhance solubilisation of bound phosphate from the phosphate deficient medium to survives in adverse condition and

Table 1: Morphology and growth behaviours of *Trichoderma asperellum* in PDA and PVK medium

Characters	Media screened	
	PDA	PVK
Growth	Fluffy and dark green	Compact, light green
Sporulation	High	Low
Chlamydospores	Frequency low	Frequency high
Chlamydospores (mean of 10 microscopic field)	19.67	28.67, 32.23 and 33.63

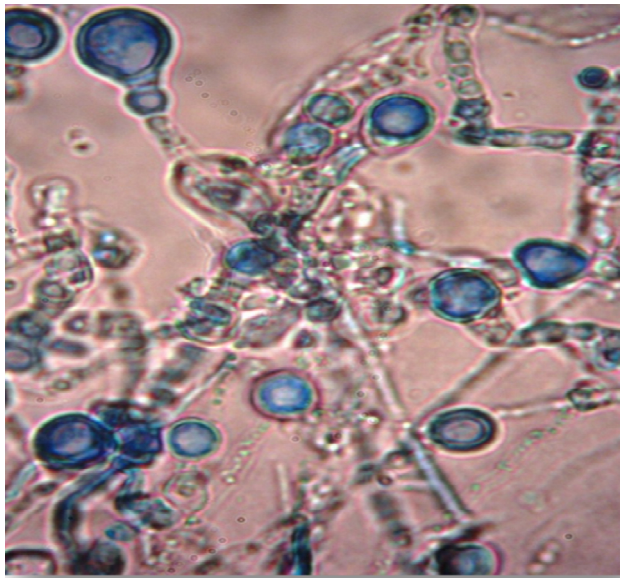

Figure 1: Chlamydospores (400X) with fungal hyphae Chlamydospores formation in *Trichoderma asperellum* under phosphorus deficient medium

that phosphorus stress can be exploit in mass multiplication of high quality formulation development of the *T. asperellum* which have ability to induce huge chlamydospores, can certainly enhanced the shelf-life of the formulation up to 12-18 months without desiccation which can certainly revolutionized the popularity of bio agents with longer storage life among the farmers.

Among the biocontrol agents, *Trichoderma* based formulations are widely used in organic vegetable and legume cultivation across the world for the management of soilborne phytopathogens but the greater drawback of *Trichoderma* based formulations are their poor shelf-life and live spore counts which leads to poor efficacy disease causing pathogen management. Keeping these in view, induction of high frequency of the chlamydospores in *Trichoderma asperellum* was observed when supply of phosphates in the form of bound phosphate (TCP) in the PVK medium *in vitro*. After supplementation of TCP, growth of *T. asperellum* was recorded relatively slower n very compact which covered 100 mm Petri plates within 7-9 days as compared to control (4 days). Microscopic observations indicated that the induction of terminal and intercalary chlamydospores in the hyphae and frequency recorded 28.67, 32.23 and 33.63% higher in phosphate deficient PVK medium than control. These chlamydospores induction in *T. asperellum* certainly may enhanced the shelf-life as well as they can survives in adverse climatic condition. Phosphate deficient PVK medium can be exploited in induction of chlamydospores in *Trichoderma* species for enhancing the shelf-life of the *Trichoderma* based formulation upto 12-18 months without desiccation which may certainly revolutionized trust and popularity of *Trichoderma* formulation among the farmers.

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