

COMBINATION OF FUNGAL AND BACTERIAL ANTAGONISTS FOR MANAGEMENT OF ROOT AND STEM ROT DISEASE OF SOYBEAN

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Abstract

Trichoderma harzianum and plant growth promotory rhizobacteria *Pseudomonas fluorescens* were tested alone and in combinations for their relative biocontrol potential against many soil-borne plant pathogens viz., *Rhizoctonia solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina* responsible for root and stem rot disease of soybean. Investigations under glass-house and field conditions revealed a general trend towards greater suppression and enhanced consistency against the pathogens by mixture of antagonists. Application of more than one antagonists of diverse origin is suggested as a reliable means of reducing the variability and increasing the reliability of biological control.

Introduction

Biological control of plant pathogens using introduced microorganisms has been investigated intensively because of lack of alternative management practices, and most of the studies deal with single biocontrol agent as antagonist to either a single or multiple pathogen(s). Considering the fact that there is some degree of host-specificity in the antagonists even at sub-species level, this may partially account for the reported inconsistent performance of biocontrol agent preparations. Single biocontrol agent is not likely to be active in all soil environments or against all pathogens that attack the host plant and control of a wide spectrum of pathogens under a wide range of environmental conditions by applied antagonists largely remains an unfulfilled goal for biological control. This could be overcome by developing strain mixtures with superior biocontrol activity. It is likely that in most cases of naturally occurring biological control results from mixtures of antagonists, rather than from high population of a single antagonist. For example, mixtures of antagonists are considered to account for protection in disease suppressive soils (Hornby, 1983; Lemanceau & Alabouvette, 1991; Chaube *et al.*, 2003; Singh *et al.*, 2004). Consequently, application of a mixture of introduced antagonists would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control, and allow the combination of various mechanisms without need for genetic engineering (Janisiewicz, 1988; Duffy & Weller, 1995; Varshney & Chaube, 2001).

With the hypothesis that the combination of two entirely different antagonists would enhance the level of disease management, this study was performed to determine the biocontrol potential of two compatible isolates of *T. harzianum* and *P. fluorescens* individually as well as in combination against root and stem rot disease of soybean.

Materials and Methods

Colonies of fluorescent pseudomonads and *Trichoderma* spp., were isolated from the rhizosphere and non-rhizosphere soil of soybean plants grown at normal soil of Crop Research Centre, G.B. Pant University of Agriculture & Technology, Pantnagar, India. King's B medium (KB) (King *et al.*, 1954) was used for isolation of fluorescent pseudomonads, while *Trichoderma* spp., were isolated on modified *Trichoderma* selective medium (TSM)

(Mukherjee, 1991). *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Macrophomina phaseolina* were isolated from diseased soybean plants. Mass culture of *Trichoderma* isolates was prepared on barnyard millet (jhangora) grains. These grains were soaked in water for 12 hrs and then filled in 250 ml Erlenmeyer flasks (@ 50g flask⁻¹). These flasks were autoclaved at 15 p.s.i. for 30 minutes. After cooling to room temperature, the flasks were inoculated with mycelial discs cut from three days old culture of biocontrol agents, and incubated at 28°C for 12 days. Jhangora grains colonized by biocontrol agents were air dried under shade conditions and ground with the help of Willy Mill to get fine powder. This powder was passed through 50 and 80 mesh size sieves, simultaneously. The commercial formulation was prepared by diluting this powder with talc powder to get desired concentration of biocontrol agents in the formulation. Bacterial biocontrol agents (fluorescent pseudomonads) were multiplied on KB broth. Each isolate was inoculated in the flask containing 100 ml KB broth and incubated on incubator shaker at 150 rpm for 48 hrs at 25±2°C. After that, bacterial suspension was added in sterilized talc powder (@ 1:2 v/w) and mixed well under sterile conditions. Commercial formulation of desired concentration was prepared by diluting it with talc powder. The powder was packed in polythene bags, sealed, and incubated at room temperature.

Pathogen isolates were multiplied on sterilized sorghum grains presoaked overnight in 2% sucrose solution. Sorghum grains were filled in flasks and sterilized at 15 p.s.i. for 30 minutes. After cooling at room temperature the flasks were inoculated with 5 mm mycelial discs of pathogens and incubated at 26±2°C for 10 days.

Development of mixed formulation and testing of efficacy: Mixed formulation was developed using compatible isolates of fluorescent *Pseudomonas* (PBAP-27) and *Trichoderma* (PBAT-43). In this mixed formulation, equal cfu = colony forming unit (1x10⁹ per g) for both the antagonists was maintained.

Mixed as well as individual formulations were evaluated to test their comparative biocontrol efficacy under glass-house and field conditions. Plastic pots containing 2 kg field soil was mixed with pathogen inoculum (@ 1g per kg soil) prepared on sorghum grains, and kept as such for one week. Pots were irrigated regularly. Soybean seeds treated with formulations (@ 5g per kg seed) were sown in these pots (@ 25 seeds per pot). Seeds sown in infested soil without any treatment

served as control. Three replications of each treatment were maintained.

Field experiment was conducted in the sick plots at Crop Research Centre, G.B. Pant University of Agriculture and Technology, Pantnagar. Seeds treated with formulations were sown in different randomized plots and observations were taken on germination and final plant stand.

Shelf life of the antagonists: Mixed as well as individual formulations were kept at room temperature (15-35°C) and in refrigerator (4°C) and deep freeze (-20°C). Viability of the antagonists in each formulation was determined after 1, 3, 5, 7, 9 and 12 months of storage. At each sampling time, 1g formulation was suspended in 100 ml sterile distilled water and diluted up to 10⁹ dilution. One ml of the suspension was poured onto the surface of the Petri plates containing growth media. For fungal isolate *Trichoderma* selective Medium (TSM) and for bacterial isolate King's 'B' Medium (KB) were used, while for mixed formulation, TSM and KB both were used simultaneously. After 24 hrs of incubation at 26±2°C, the plates were examined under a binocular microscope and the presence or absence of hyphal growth and/or bacterial colonies were noted. Viability was recorded as percentage of viable propagules (*cfu*) per g of formulation.

Soybean seeds were coated with each formulations and one lot was kept at room temperature (15-35°C), while the other in the refrigerator. At every one-month interval, 5 seeds were taken in 100 ml sterile distilled water and kept on rotary shaker for 1h. Serial dilutions were maintained up to 10⁶. One ml suspension was poured on to the surface of Petri plates containing growth media and observation on germination of propagules was recorded in the form of *cfu* after 24h of incubation at 26±2°C.

Results and Discussion

In the community of beneficial microorganisms, fluorescent pseudomonads and *Trichoderma* spp., has emerged the largest and potentially most promising group. A total of 40 isolates of fluorescent pseudomonads and 43 isolates of *Trichoderma* were isolated from rhizosphere and non-rhizosphere of soybean and were designated as PBAP (Plant Biocontrol Agent *Pseudomonas*) and PBAT (Pant Biocontrol Agent *Trichoderma*). Mixed formulation

was developed by using most efficient and compatible isolates of *Trichoderma* (PBAT-43) and *Pseudomonas* (PBAP-27). In this formulation *cfu* of both bioagents were adjusted to 1x10⁹ per g formulation. Individual formulations of the same isolates were also developed having 2x10⁹ *cfu* per g.

Biocontrol potential of mixed formulation differed than their individual ones. In greenhouse experiments, mixed formulation has given maximum seed germination by 84.01% as compared to 80.40% and 77.58% of individual isolates PBAP-27 and PBAT-43 formulations, respectively. The mortality record was least with mixed formulation (16.15%) followed by PBAT-43 (28.14%) and PBAP-27 (32.34%) against 67.30% of control (Table 1). Under field conditions application of fungal and bacterial isolates either in mixed form or alone, significantly increased seed germination over control. Highest germination (83.28%) was recorded when seed were treated with *Pseudomonas*-alone while disease control was maximum (47.68%) in the plots where mixed formulation was used. Individual formulations also exhibited significant disease control as compared to control (Table 2).

Shelf-life of formulations: Initially, virtually all propagules of the formulation retained viability, and it was estimated to be around 10⁹ *cfu/g* in all three formulations. Over 12 month of storage, viability of formulations stored at room temperature (15-35°C) dropped significantly, while viability of formulations stored at 4°C (refrigerator) and -20°C (deep freeze) decreased slightly. In PBAT-43, viability of propagules was recorded only 22.60% after 12 month of storage at room temperature; while it was 52.79% and 65.34%, when stored at 4°C and -20°C, respectively. Bacterial antagonist PBAP-27 showed slightly lower survivability than fungal one. The viability of formulation was 16.59% at room temperature and 51.77% and 60.47% in refrigerator and deep freezer, respectively after same period of storage. Viability of mixed formulation was at par with fungal bioagent (Fig. 1). Viability on soybean seeds after a period of 9 month of storage was up to 70% at low temperature (4°C), while it varied between 36-42% at room temperature. Mixed formulation was found having almost similar viability as in individuals (Fig. 2).

Table 1. Management of Soybean root-rot and wilt-complex disease by seed treatment with TH, PsF and their mixed formulation (@ 5.0 g/kg seed) under glass-house conditions.

| Treatment | Germination (%) | Increase in germination | Post emergence mortality (%) | Disease control (%) |
|-----------|-----------------|-------------------------|------------------------------|---------------------|
| T-alone | 77.58 (61.77) | 31.00 | 28.14 (32.01) | 58.18 |
| PsF-alone | 80.40 (66.45) | 35.76 | 32.34 (34.65) | 51.94 |
| Mixed | 84.01 (63.74) | 41.46 | 16.15 (23.64) | 76.00 |
| Control | 59.22 (50.32) | - | 67.30 (55.13) | - |
| CD at 5% | 3.22 | | 3.32 | |

Values written in parenthesis are in angular transformation

Table 2. Management of Soybean root-rot and wilt-complex disease by seed treatment with TH, PsF and their mixed formulation (@ 5.0 g/kg seed) under field conditions.

| Treatment | Germination (%) | Increase in germination | Post emergence mortality (%) | Disease control (%) |
|-------------|-----------------|-------------------------|------------------------------|---------------------|
| T-alone | 73.69 (59.19) | 47.14 | 24.99 (29.96) | 44.95 |
| PsF-alone | 83.28 (61.00) | 66.29 | 32.61 (34.81) | 28.17 |
| Mixed | 76.48 (65.91) | 52.71 | 23.75 (29.12) | 47.68 |
| Control | 50.08 (45.04) | - | 45.40 (42.36) | - |
| CD (p=0.05) | 2.42 | | 3.08 | |

Values written in parenthesis are in angular transformation

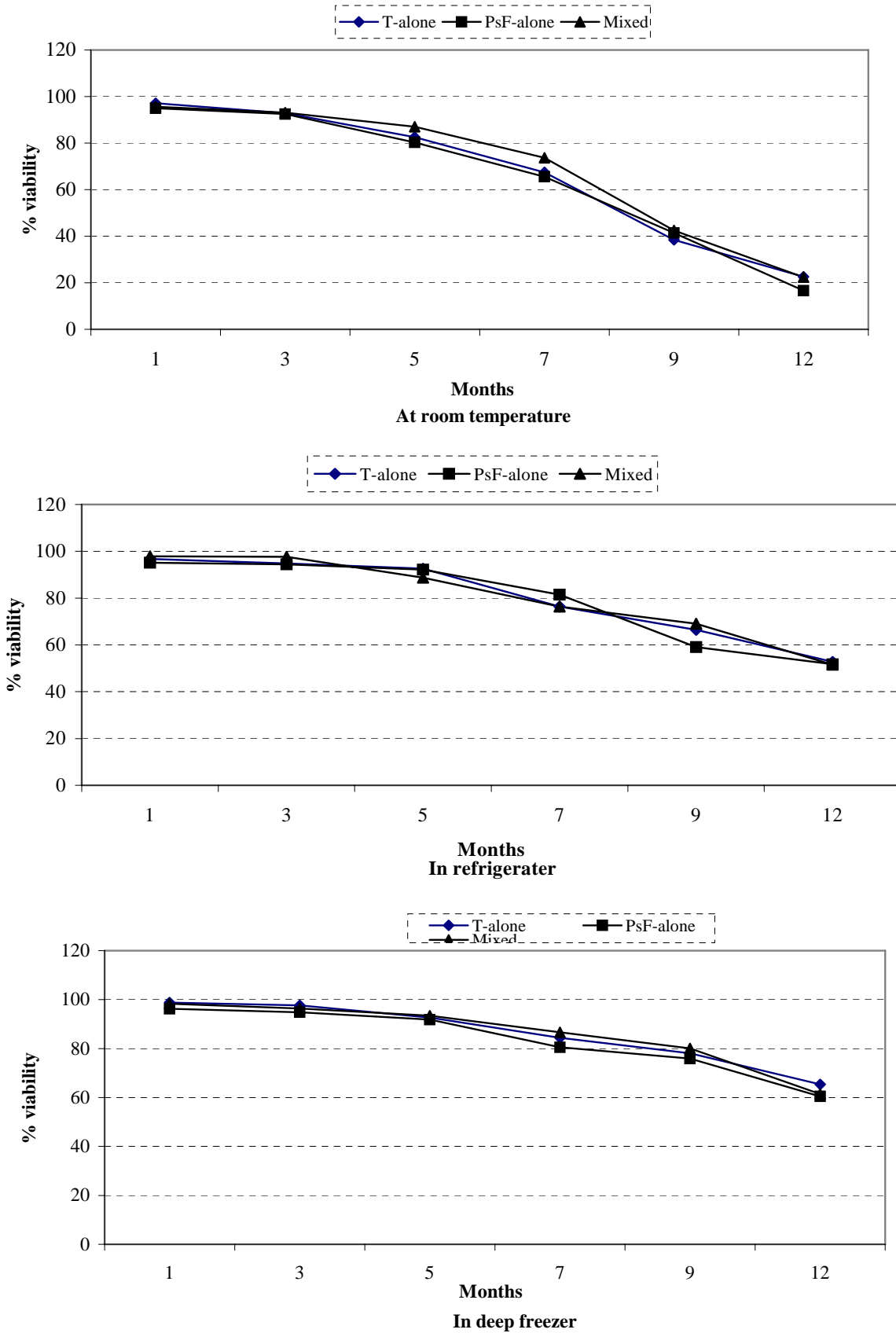


Fig. 1. Shelf-life of individual and mixed formulation.

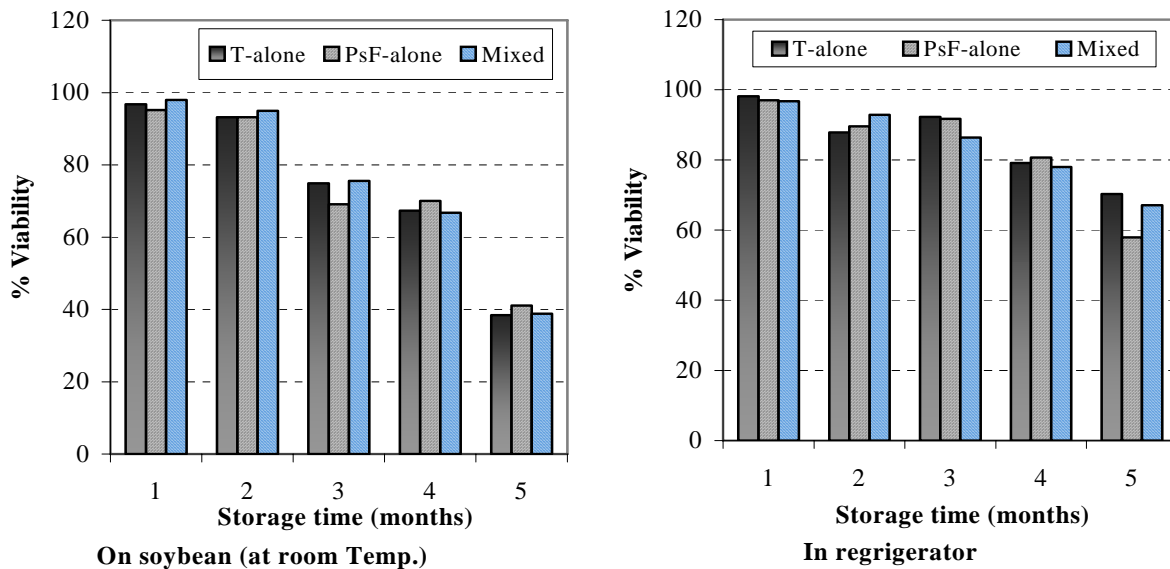


Fig. 2. Shelf-life of individual and mixed formulation on treated soybean seeds.

The success of the rhizosphere soil-microbial technologies will depend on isolating and understanding the mechanisms by which micro-organisms influence plant growth as well as basic understanding of the traits that constitute a competitive rhizosphere, and it will be useless unless the organisms can successfully compete in the field and express desired traits. There is currently a lack of information on the distribution of micro-organisms on root surface, their relative metabolic activities and how they interact to affect plant growth and function of other micro-organisms. The interactions among various microorganisms in the rhizosphere not only can affect the specific organisms of concern, but also other micro-organisms and the plant. Study of these interactions is extremely important in the ecological study of rhizosphere and correlating their specific role in the host-pathogen-other microorganisms interactions. In this view in present study, the most compatible and efficient isolates of fungal and bacterial biocontrol agents, PBAT-43 and PBAP-27 were combined for development of mixed formulation. Results presented in Tables 1 and 2 demonstrate the effectiveness of mixed formulation in the form of a general trend towards greater disease suppression and more consistent obtainment of significant disease suppression. These results are in agreement with the studies conducted by different workers earlier (Fukui *et al.*, 1999; Elad 2000; Guetsky *et al.*, 2001). However, in some reports the feasibility of combining *Trichoderma* spp. with fluorescent pseudomonads was questioned (Hubard *et al.*, 1983). According to them indigenous populations of fluorescent pseudomonads significantly reduced the biocontrol activity of *T. hamatum* applied to control *Pythium* seed rot of pea and iron competition was the primary mechanism involved. In contrast, Dandurand & Knudsen (1993) reported that the combination of *P. fluorescens* 2-79 and *T. harzianum* ThzID₁ neither inhibited nor enhanced the biocontrol activity of the latter agent against root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*. Duffy *et al.*, (1996) indicated that fluorescent *Pseudomonas* species and *T. koningii* are compatible when applied to wheat simultaneously. In growth chamber studies, none of the five individual bacterial strain or bacterial strain mixtures reduced the

suppressiveness of *T. koningii*; in fact, strain Q₃₀₋₈₄, Q₂₋₈₇, and Q₂₉₋₈₀ slightly enhanced the activity of the fungus. The performance of all the bacterial treatments was greatly enhanced by combination with *T. koningii*, suggesting that the fungus was largely responsible for the take-all suppression. Similarly in field the bacteria did not adversely affect the activity of *T. koningii*.

It is important to know the shelf-life of isolates of biocontrol agents in the form of powder of spores and other propagules, which is emerging out as feasible form of delivering the biocontrol agents. In the present investigation, the results shown in the Figures 1 and 2 demonstrate that there was no significant difference in the shelf-life of individual as well as mixed formulations. When stored at different temperatures, all exhibited significant shelf-life of 4-9 months depending upon the situation and conditions of storage. Although mixed formulation was found to be having more shelf-life than that of individual formulations when stored at room temperature, while at low temperatures (4°C and -20°C) there was no significant difference. This study seems to be novel for the comparative study of shelf-life of mixed and individual isolates of biocontrol agents. However, it was shown by different workers that commercial formulation of both fungal and bacterial biocontrol agents alone are having different levels of shelf-life. Vidhyasekharan & Muthamilam (1995) showed that talc based formulation of *P. fluorescens* Pf-1 retained up to 10⁷ cfu/g after 240 days of storage against the initial population of 10⁸ cfu/g. Prasad & Rangeswaran (2000) reported that talc and kaolin based formulations of *T. viride* retained more than 10⁶ viable propagules up to 90 days. The higher shelf-life shown by mixed formulation at room temperature might be due to the combined activity of the biocontrol agents present in the formulation. The microorganisms may release some metabolites or enzymes that could increase the activity of one or both the organisms. While at low temperatures the microorganisms may not be active. One possibility may also be there, that after a period of storage, there will be death of some propagules of both the organisms and these may serve as source of nutrition for remaining active propagules of biocontrol agents. Dandurand & Knudsen (1993) reported that there was

increase in growth and development of *T. harzianum* in the presence of *P. fluorescens* isolate 2-79RN₁₀. The presence of another strain 2-79-B46 enhanced the hyphal density of *T. harzianum* by approximately 2.5 fold. There was general decrease in bacterial population and increase in the population of *T. harzianum* suggesting that dead bacterial cells may have provided nutrients for fungal counterpart. This area is worthy of further investigation because it suggests the possibility that dying biocontrol agents may provide nutrients for plant pathogens, indicating that the application of overly large number of biocontrol agents may be counterproductive. Other possible factors may include localized change in CO₂ concentrations or pH by bacteria, both of which showed to affect the growth of *Trichoderma* spp.

Seed coating with biocontrol agents has emerged as a feasible way of delivering the antagonist for the management of plant diseases. As this technique of disease management represents a living system, the viability of the biocontrol agent may pose a limitation to its commercialization. One approach for this might be supplying the coated seeds to the farmers directly by the seed companies/agencies. A considerable time gap between coating seeds by the seed supply agencies and sowing such seeds by farmers is bound to occur and it should be ensured that sufficient propagules remain viable on coated seeds at the time of sowing. In the past, many attempts have been made to assess the viability of biocontrol agents on coated seeds. Mihuta-Grim & Rowe (1986) estimated viability of *Trichoderma* spp., on coated seeds of radish by plating the seeds on malt-extract agar every week. Mukherjee (1991) quantitatively assessed the viability of *T. virens* and found that it remained almost constant on coated soybean seeds when the seeds were stored at low temperature (5°C). Even at room temperature (15-35°C), 88% of the propagules remained viable for up to 4 months, a time sufficient for reaching the coated seeds to the farmers. In our experiment similar type of results have been observed, where formulations have shown shelf-life up to 9 months on the seed of different crops. Mixed formulation exhibited longer shelf-life as compared to individual ones when stored at room temperature, while at low temperature there was no significant difference among them. The higher shelf-life of mixed formulation at room temperature on coated seeds might be due to the reason that the biocontrol agents are reported to stimulate the exudation from the seeds (Barber & Lynch, 1977) that may serve as nutrients for the biocontrol agents (Dandurand & Knudsen, 1993). In that way there may be stimulation of some compounds from biocontrol agents also that could increase the growth and activity of biocontrol agents of mixed formulation, but at lower temperature they may remain inactive, so there will be less chance of such type of activities.

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