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An Assessment of Two Application Methods Involving the Use of *Bacillus* spp. Against *Sclerotium rolfsii* for Plant Growth-Promotion and Disease Management of Chickpea

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Abstract: *Sclerotium rolfsii* Sacc. is one of the most devastating soil-borne fungal pathogen causing collar rot disease in the chickpea crop. At present, the chemical fungicides that are used for the management of this disease not only have negative effects on the environment but also on the human health. The present study deals with an assessment of the two methods used in the environmental friendly approach involving the use of *Bacillus* spp. for the management of this disease in the chickpea plants. Two *Bacillus* species, namely, *B. valezensis* and *B. tequilensis* were applied to the seeds by the seed coating and seed biopriming methods. Treated seeds were sown in earthen pots filled with sterilized soil and infected with *S. Sclerotium rolfsii* after 25 days of sowing. The results showed that the chickpea seeds bioprimed with *B. valezensis* and *B. tequilensis* exhibited significantly higher seed germination rate, plant length and plant biomassas compared to the seed coated and the control (non-treated) plants. The bioprimed chickpea seeds challenged with *S. rolfsii* exhibited significantly decreased disease severity as compared to the seed coated and control plants. Our results also supported the fact that of the two species used in this study, *B. valezensis* exhibited better potential in plant growth-promotion (PGP) and disease suppression against *S. rolfsii* infection as compared to *B. tequilensis*. Thus, it may be concluded that seed biopriming is much more efficient approach for application of plant growth-promoting bacteria than the seed coating method to manage collar rot disease in the chickpea plants.

Keywords: *Bacillus*, Collar rot disease, Chickpea, Environmental friendly approach, Plant growth-promotion, Seed biopriming

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Introduction

Chickpea (*Cicer arietinum* L.) is not only an important legume crop but is also reported to be the second largest cereal crop cultivated in India (Nene *et al*., 2012; Tarafdar *et al*., 2018). Although several biotic factors are known to affect the production of the chickpea crop, the collar rot

disease caused by *Sclerotium rolfsii* is a major cause contributing to the mortality (55–95%) of chickpea seedlings (De Curtis, 2010). *Sclerotium rolfsii* Sacc. is the soil-borne fungal pathogen which causes collar rot or stem rot in chickpea plants (Punja, 1985; De Curtis, 2010). This disease causes approximately 30% crop loss under both field and greenhouse conditions and occasionally under conducive conditions, the loss may reach up to 95% (Suriyagamon *et al*., 2018). As the level of host plant resistance in chickpea is not substantial, the pathogenic effect of *Sclerotium rolfsii* can be minimized mainly by the use of fungicides and appropriate crop rotation methods (Kumar *et al*., 1997; Azhar *et al*., 2006). However, the continuous use of agrochemicals in various agroecosystems has drawn attention towards the need for safer alternatives, such as those involving the use of specific bacterial strains as biofertilizers and bio-pesticides in order to maintain agricultural sustainability (Shabbir *et al*., 2019). A number of mechanisms are implicated in the performance of the various biological control agents (BCAs) used to promote plant growth, such as phytohormone production, nitrogen fixation, stimulation of nutrient uptake and biocontrol of pathogenic fungi (Kumar *et al*., 1997). A variety of beneficial microorganisms, are applied as seed treatment to obtain several unique crop protection benefits (Abhilash *et al*., 2016; Singh, 2016).

In the seed coating method, the PGP (plant growth promoting) agents are combined with a suitable binder material to form a coating around the seed, whereas in the seed biopriming method, the seeds are soaked for a pre-measured time in the liquid bacterial suspension (Taylor *et al*., 1998; Reddy *et al*., 2012). *Bacillus* spp. are well-known for secreting plant beneficial secondary metabolites, antifungal metabolites (AFMs), antibiotics, and various extracellular signalling compounds and currently, they are one of the most widely studied plant growth-promoting rhizobacteria (PGPR) (Ahemad and Kibret, 2014; Agler *et al*., 2016). They also exhibit many important features, including the long shelf-life in bio-formulations, effective colonization in plant tissues, and broad-spectrum antifungal abilities (Souza *et al*., 2014). Species such as *B. valezensis* and *B. tequilensis* have been reported to promote significant growth in tomato, pepper, pumpkin and cucumber plants (Dastager *et al*., 2011; Torres *et al*., 2020; Shahid *et al*., 2021), but their efficiency in chickpea plants is least understood. In the present study, *in vitro* experiments were conducted to assess the comparative efficiencies of two seed treatment methods, i.e., seed coating and seed biopriming with *Bacillus* spp., which also exhibit antagonistic potential against the pathogenic fungus, *S. rolfsii* in chickpea plants. We addressed the following two inter-related questions: (i) What is the effect of each treatment on the plant growth parameters? and (ii) What is the protective effect of each treatment method on the pathogen challenged chickpea plants?

Materials and Methods

Collection of biocontrol agent Bacillus spp. and pathogen S. rolfsii:

Two bacterial strains, namely, *Bacillus valezensis* (MNB06; MT782282.1) and *Bacillus tequilensis* (MNB08; MT782283.1) (previously isolated from Botanical garden soil and stored at 4 C), used in the present study were obtained from the Insect Behavioural Ecology Laboratory, Department of Zoology, Institute of Sciences, Banaras Hindus University, Varanasi, Uttar Pradesh, India. The soil-borne pathogen, *S. rolfsii* was obtained from the Department of Mycology and Plant Pathology, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Preparation of cell suspension for seed biopriming:

A loopful of the isolate (i.e., each bacterial strain), from the culture stored at −80 C (containing Nutrient-broth (NB) and 20% glycerol) was streaked on the Nutrient agar (NA) and incubated at 27 C for 24 h to check for purity. The obtained single colonies were then transferred to Nutrient agar plates. After 48 h, the bacterial cells were harvested from the plates in sterile distilled water

(SDW), centrifuged at 10,000 rpm for 10 min, and the obtained pellet was re-suspended in SDW. The optical density of the suspension was adjusted using a UV-visible spectrophotometer (Thermo Scientific UV1) to obtain a final density of $2^{\times}10^{8}$ cfu/ml. The suspension was vortexed to obtain a homogeneous solution for seed biopriming.

Preparation of talc-based formulation for seed coating:

The formulations of the two strains of *Bacillus* were prepared by using the talc-based carrier material. Under aseptic conditions, 100 g of talc powder was placed in sterilized plastic tray and the pH of 7 was maintained by adding 15g/kg of calcium carbonate ($CaCO_{3)}$ to the talc powder. Further, the sticking agent, carboxymethyl cellulose (CMC) was added (10g/kg) and mixed well. The final mixture was autoclaved for 1h at 121 C (15psi). 10 ml cell suspension from the isolate was taken (2×10^{8} CFU per ml) and mixed under aseptic condition in the 100 g autoclaved talc powder. The final formulated products (from each of the two bacterial strains under consideration) were concealed (aseptically air dried) and stored separately in sterilized polybags at 4 C. Seed coating was done by using 0.2% CMC solution as adhesive agent and 4 g of the formulated product was separately used for treatment of one kg of seeds.

Seed coating and biopriming of chickpea seeds:

Chickpea seeds were surface sterilized by 1% NaOCl solution for 1 min, and rinsed thrice with SDW and further dried under a sterile air stream on pre-sterilized blotting paper (Jain *et al.*, 2012). The dried seeds were placed in sterilized Petri plates and soaked through the prepared spore suspension, where only CMC treated seeds served as the control. The excess amount of bacterial cell suspension was drained out. The treated seeds were kept in an incubator at 28±2 C and 98% relative humidity for 24 h (Singh *et al*., 2013; Jain *et al*., 2015).

Pot trial experiments:

The pot trial experiments were conducted in plastic pots where each potting mixture consisted of sterilized (autoclave at 15 lb pressure for 30 min) sand and soil (in 1:1 ratio). A total of 5 air dried seed-coated and bio-primed seeds were sown in each pot (at an approximately depth of 1.5 cm) with five sets of each treatment (n=5, per treatment) and were maintained under greenhouse conditions. After 25 days of sowing (DAS), plants were challenged with the pathogen, *S. rolfsii*. All the pot trial experiments were done using a complete randomized design (CRD) with three replications for each treatment and repeated once.

Assessment of plant growth parameters:

Five random plants from each treatment were uprooted after 25 days of sowing (DAS) and the plant growth parameters: seed germination (SG), total fresh weight (TFW) of shoot and root were recorded. The collected plants were then oven-dried at 80 C to determine the total dry weight (TDW). Shoot length (SL) and root length (RL) were estimated by using Image J software (Schneider *et al*., 2012).

Assessment of the disease severity in the pathogen challenged plants:

To evaluate the performance of bacterial suspension (for each of the two strains) in protection of plants from disease causing pathogen, mortality observations were taken after 15 days of pathogen inoculation (DPI) as per the method of Shokes *et al*. (1996). As the collar region of plants gets flaccid during the disease initiation, the disease severity was estimated on the basis of individual plants scoring on a 0-5 visual scale of increasing severity (Latunde-Dada, 1993) and the per cent disease severity was calculated according to the following formula (Erkilic *et al*., 2006):

$$\%DS = \frac{\Sigma(n \times v)}{N \times V} \; 100$$

Where; n = score of infection according to scale, v = number of seedlings per category, N = total number of seedlings which were screened, V = highest score for infection

Statistical analysis:

One-way analysis of variance (ANOVA) was performed to compare variations in plant growth characteristics and disease management exhibited by treatments. The analyses were performed using SPSS, version 25 where significant variations among the treatments were analysed using Duncan Multiple Range Test ($P \leq 0.05$), and treatments effects were represented significant at $P \leq 0.05$.

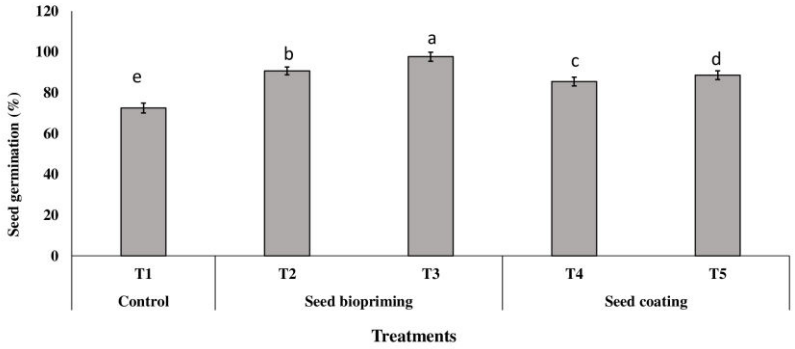
Results and Discussion

The results of our study showed that seed biopriming with *Bacillus* spp. provided a much more significant improvement in plant growth and disease resistance against *S. rolfsii* infection in chickpea plants, as compared to the seed coating method, even though the same two bacterial species were used in both the cases. Germination as well as growth of chickpea plants were found to increase significantly when treated with two species of *Bacillus* (Table 1). It was also evident that *B. valezensis* treatment by both, the seed biopriming (T3) and seed coating (T5) method, had considerably higher effect on the plant growth than that obtained by treating with *B. tequilensis*, via the T2 (seed biopriming) and T3 (seed coating) methods. All plant growth parameters, such as SG, SL, RL, and total plant biomass were significantly enhanced in treatments (T2 to T5) as compared to the control (T1). The biopriming of seeds expressed highest seed germination percentage with T3 (97.6) and T5 (88.6) as compared with seed coated treatments T2 (90.6) and T4 (85.47) and control (72.3) (Fig. 1A). Under T2, T3, T4 and T5 treatments, the host plants displayed 19.6, 24.6, 15.4 and 19% and 15.5, 18.77, 17.43 and 13.83% increase in shoot length and root length, respectively (Fig. 1C). Also, the total plant biomass from T2, T3, T4, T5 treatments was augmented by

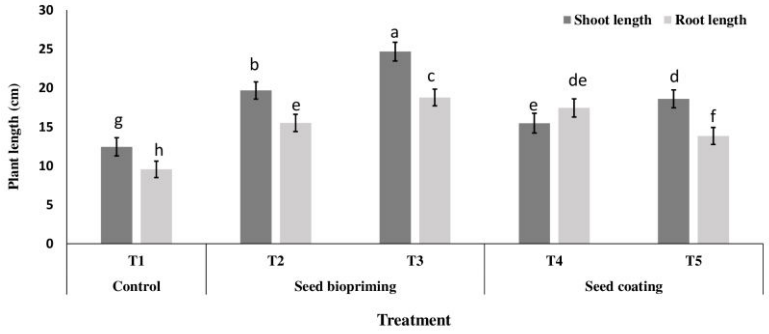
Table 1: Description of the treatments used in the plant growth-promotion and disease severity experiments. Plants under each treatment were challenged with pathogen *Sclerotium rolfsii*

Treatment	Description
T_1	Non-primed
T_2	Seeds bioprimed with *Bacillus tequilensis* (MNB06)
T_3	Seeds bioprimed with *Bacillus valezensis* (MNB08)
T_4	Seeds coated with *Bacillus tequilensis* (MNB06)
T_5	Seeds coated with *Bacillus valezensis* (MNB08)

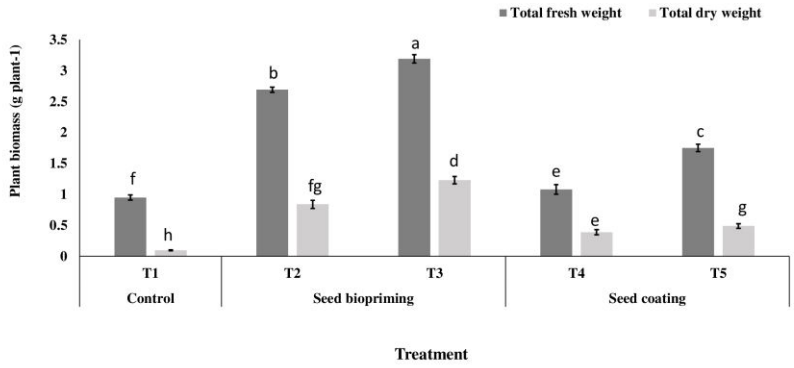
44, 60, 28.3 and 48.8 %, respectively as compared to the control. The results thus confirmed that plant growth was more prominent when the seeds were bioprimed as compared to the seeds which were only coated. As seed biopriming includes the hydration of seeds before inoculation with the microbe, it activates the early metabolism of seeds without the actual germination. Further, seed incubation with PGP bacterial inoculum increases the germination as it ensures the entrance of endophytic bacteria into the radicles of the germinating plants, while avoiding the effect of high temperature. It is reported that this further increases the speed and uniformity of germination, and ensures rapid, uniform and high establishment of crops, improving the harvest quality and yield (Mahmood *et al*., 2016). Moreover, the use of adhesives in the seed coating method hinders the further application of pesticides to the seeds (Bardin and Huang. 2003). It also reduces bacterial survival as well as nitrogen fixation (Burton and Curley, 1966) since it hinders the gaseous exchange in the leguminous seeds which causes reduction in nitrogen fixation. There may be other problems with the coating method, such as reduction in the number of



(A)



(B)



(C)

Fig. 1: Gnotobiotic plant growth promotion in chickpea plants with different treatments: T1(Control), T2 (bioprimed with *Bacillus tequilensis*), T3 (bioprimed with *B. valezensis*), T4 (coated with *B. tequilensis*), T5 (coated with *Bacillus valezensis)* after 25 days of sowing (DAS), in terms of: (A) seed germination (%), (B) plant length (shoot length; SL and root length; RL), and (C) plant biomass (total fresh weight; TFW, total dry weight; TDW). Results are expressed as mean of three replications ± SD and the significant variations among the treatments taken at same time interval are indicated by different letters according to One Way ANOVA and Duncan's multiple range *post hoc* test at $p \leq 0.05$.

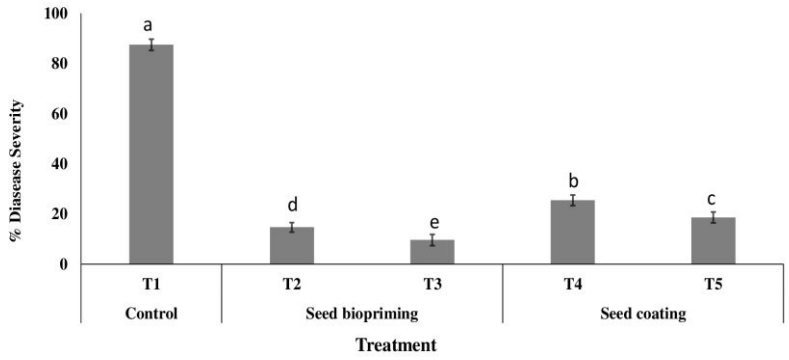


Fig. 2: Disease severity (%) in chickpea plants with different treatments: T1 (Control), T2 (bioprimed with *Bacillus tequilensis*), T3 (bioprimed with *B. valezensis*), T4 (coated with *B. tequilensis*), T5 (coated with *Bacillus valezensis)* after 15 days of inoculation (DAI) with *Sclerotium rolfsii*. Results are expressed as mean of three replications ± SD and the significant variations among the treatments taken at same time intervals are indicated by different letters according to One Way ANOVA and Duncan's multiple range *post hoc* test at $p \leq 0.05$.

bacteria on the seeds due to desiccation (Duarte *et al*., 2004).

From the present results it is clearly inferred that *B. valezensis* has higher PGP potential than the *B. tequilensis* under *in vitro* conditions. The results also derive support from earlier studies which show that *B. velezensis* possesses high genetic capacity for synthesizing cyclic lipopeptides and polyketides for highly efficient PGP (Rabbee *et al*., 2019) than *B. tequelensis*. However, further investigations need to be conducted in future studies to shed more light on the efficacy of *B. tequilensis* on PGP.

Further, our results showed the development of water-soaked lesions on the collar region of the control plants, three days after challenge inoculation of chickpea plants with *S. rolfsii*. After 15 DAI (days after inoculation) the bioprimed treatments showed a significant reduction in disease severity over the seed coating and non-treated pathogen challenged control plants (Fig. 2), Lowest disease severity was recorded in case of T3 (9.66%) followed by T2 (14.67%), T5 (18.6%) and T4 (24.56%) treatments with respect to the non-treated pathogen challenged control.It is well known that *Bacillus* spp. are potential BCA for the management of *S. rolfsii* (Sethi and Mukherjee, 2018; Sahu *et al*., 2019), due to the ability of this bacteria to produce various cell wall degrading enzymes and secondary metabolites that affect the growth of the soil pathogen. *Bacillus* spp. also use some other mechanism like antibiosis and competition to colonize with *S. rolfsii*s sclerotia and mycelium (Gholami *et al*., 2014; Kumari *et al*., 2021). Hence, the disease severity was lower in the bacteria treated chickpea plants as compared to the non-treated plants.

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