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EFFECT OF BIOPRIMING ON POPULATION OF *BACILLUS AMYLOLIQUEFACIENS* VB7 IN CHILLI SEEDS

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ABSTRACT
A study was investigated the attributes related to the movement of bacteria (*Bacillus amyloliquefaciens* VB7) from the seed to the radicle during biopriming using scanning electron microscopy (SEM) in chilli seeds. The results revealed that the bacterial cells entered into the seeds through the pore at the base, colonized the inner plane, proliferated and advanced germination through radicle emergence. The microscopic observations confirmed the importance of bacterial cells colonized inside the seed coats. Similar results were also observed by serial dilution technique using seed wash and root tip of the treated seedlings, bacterial population of 25 x 10^8 cfu ml^{-1} and 37.7 x10^4 cfu g^{-1}, respectively.

INTRODUCTION

Seeds are the most compact forms of plants, and hence less inoculum or space is needed to apply biological control agents (BCAs) at the early stage (Chao *et al.*, 1986). Seed treatment is an attractive method for introducing BCAs and plant growth-promoting rhizobacteria (PGPR) into the soil-plant environments. BCAs and PGPR are effective plant conditioners in improving plant health (Choi *et al.*, 2008; Bhattacharyya *et al.*, 2014; Liu *et al.*, 2014) and protect seeds and germinating seedlings from infection of seed or soil borne pathogens. BCA and PGPR can multiply on seed surfaces, eventually proliferating on and colonizing whole root systems (Suslow and Schroth, 1982). However, little is known on the movement of PGPR, or indeed that of many other BCAs, from the seed to the rhizosphere. For BCAs to be effective, they need to rapidly and extensively colonize seed structures to protect seeds from pathogens. This short period before pathogen infection is a critical time for seed protection (Hood *et al.*, 1998).

Heydecker (1973) defined seed priming as a presowing seed invigouration treatment in which seeds are soaked in osmotic solution that allows them to imbibe water and go through the first phase of germination, but does not permit radicle or plumule protrusion through seed coat. Seed treatment with biocontrol agents along with priming agents may serve as an important means of managing many of the soil and seed borne diseases, the process often known as "biopriming" (Rao *et al.*, 2007). The direct benefits of seed priming in all crops included: faster emergence, better, more and uniform stands, less need to re-sow, more vigorous plants, better drought tolerance, earlier flowering, earlier harvest and higher grain yield. The indirect benefits reported were: earlier sowing of crops, earlier harvesting of crops and increased willingness to use of fertilizer because of reduce risk of crop failure (Agawane & Parhe, 2015).

Many research groups have carried out studies on fluorescent Pseudomonads as a promising BCA, though the vegetative cells of Pseudomonads are sensitive to adverse environmental conditions, including dry, radiation and temperature, and hence retain a relatively short shelf-life on seeds and rhizospheres (Pierson & Weller, 1994; Shah-smith & Burns, 1996). Recent reports have provided evidence that *Bacillus* or *Paenibacillus* also elicited plant growth promotion or suppression of plant diseases (Ryu *et al.*, 2003, 2005, 2006). The major benefit of using bacilli in this respect is their endospore formations that are more stable and durable under unfavorable environmental conditions. Endospore formation can make bacilli easy to formulate and commercialize, because of their a long-term shelf-life. This characteristic has been consistently attracted the attention of major research groups attempting to develop BCAs for practical applications.

Hence a study was carried out to investigate the attributes related to the movement of bacteria from the seed to the radicle, as well as to develop biopriming techniques for consistent and effective biocontrol in chilli cv. K 2.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Bacillus amyloliquefaciens VB7 was cultured in nutrient agar broth in rotating shaker (100 rpm) for 2 days at 28 ± 2°C. Cells were removed by centrifugation at 10,000 rpm for 5 min at 4°C, the pellet was mixed with sterile carboxyl methyl cellulose (CMC) suspension. The bacterial cells were maintained at -80°C with glycerol 25 % for long-term storage (Thompson, 1996).

Biopriming with *B. amyloliquefaciens* VB7

Chilli seeds (*Capsicum annuum* cv. K2) were used for bacterial seed treatment. The cell suspension of *B. amyloliquefaciens* VB7 used for seed treatment was fixed to 10^8 cells per ml. Chilli seeds were surface sterilized with 1 per cent sodium hypochlorite solution and placed in CMC cell suspension of *B. amyloliquefaciens* VB7 for 1 h to ensure uniform coating on the seeds (Choi *et al.*, 2004).

Scanning electron microscopy (SEM)

The seed coats of the bioprimed seeds and control seeds were removed and the populations of the bacteria on the surface of seed and within the seed were observed using Scanning Electron Microscopy (SEM) at 0 h and 24 h after treatment. Endosperm and embryo were scanned at 48 h and newly emerged root tips were observed at 72 h after seed treatment by Scanning Electron Microscopy (SEM).

Population analysis of biocontrol agents by serial dilution technique

The number of colony forming unit (cfu) in seed wash and root tip was enumerated using nutrient agar medium for *B. amyloliquefaciens* VB7. One gram of bioprimed seeds were mixed with 10 ml of sterile water and shaked well at 150 rpm for 2 min. Ten fold serial dilutions were prepared and 1.0 ml aliquots from 10^{-8} dilutions were transferred to sterile Petri plates.

For colony forming unit in root tips, treated seeds were induced to germinate in paper medium (between paper). The root tip of treated chilli seedlings were collected after 14 days of sowing. The root tips were surface sterilized for one min with one per cent sodium hypochlorite solution. Root samples were macerated in sterile mortar with pestle using sterile phosphate buffer (p^H-7). Ten fold serial dilutions were prepared and 1.0 ml aliquots from 10^{-4} dilutions were transferred to sterile Petri plates. Irregular and wrinkled colonies with serrated margins appeared after 48 h of incubation was counted and the population was expressed as cfu ml^{-1} for seed wash and cfu g^{-1} for root tips (Choi *et al.*, 2004).

RESULTS AND DISCUSSION

Immediately after inoculation, the cells of *Bacillus amyloliquefaciens* VB7 were randomly scattered on the surface of the chilli seed. Also, the cells of VB7 penetrated inside the seed coat during soaking (Plate 1). After 24 h of treatment, seed coat was excised and the cells of *B. amyloliquefaciens* VB7 were observed on seed coat and surface of the endosperm. The cells of VB7 abundantly proliferated on the seed (Plate 2).

Also, a lot of bacterial cells had already moved to the seed and some bacteria were organized in micro-colonies and also entered into the embryo of the seed after 48 h of seed treatment (Plate 3). After 72 h of treatment, the radicle sprouted out of the seed and the bacteria were colonized on emerging radicle (Plate 4). Numerous cells of VB7 were aggregrated on the upper parts of the radicle and most of the bacteria were organized in micro-colonies in inner epidermal layer of the radicle.

These results coincided with those of other studies on root colonization of bacteria. Seed treatments of rhizobacteria have been generally focused on attachment and survivability of rhizobacteria on the surface of the seed (Caesar and Burr, 1991; Hood *et al.*, 1998; Shah-Smith and Burns, 1996). It was confirmed that bacterial cells (*Paenibacillus polymyxa* E681) which invade the seed coat move directly to emerging root in cucumber. After seed treatment, cells of bacteria moved from the endothelium of the seed to the emerging radicle, and increased abundantly on emerging radicle. The cells were arranged linearly toward elongation root axis (Choi *et al.*, 2004). Bacteria colonize only a small proportion of the root surface, largely the junctions between epidermal cells and the regions surrounding emerging lateral roots where carbon is secreted

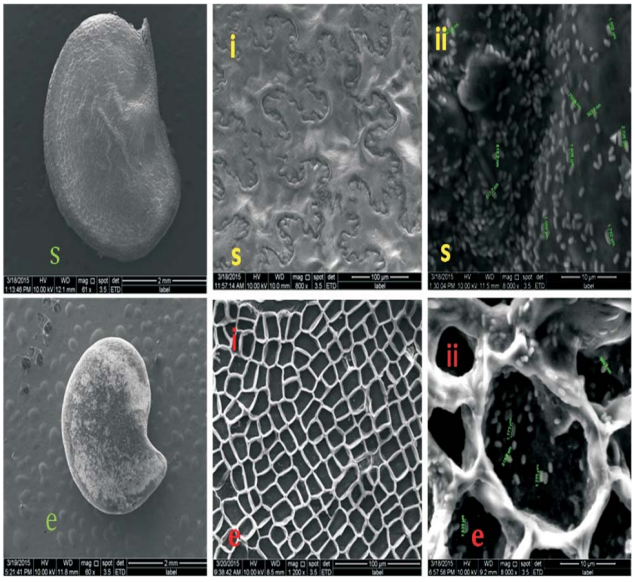
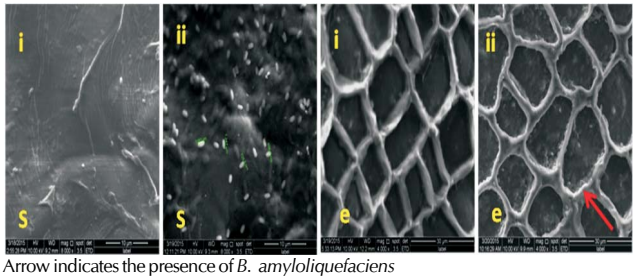


Plate 1: Scanning electron micrographs showing colonization by the *Bacillus amyloliquefaciens* VB7 on bacterized chilli seed '0' h of treatment. (i) control, (ii) Biopriming with 6 % *Bacillus amyloliquefaciens*, (s) seed coat, (e) endosperm



Arrow indicates the presence of *B. amyloliquefaciens*

Plate 2: Scanning electron micrographs showing colonization by the *Bacillus amyloliquefaciens* VB7 on bacterized chilli seed after 24 h of treatment. (i) control, (ii) Biopriming with 6 % *Bacillus amyloliquefaciens*, (s) seed coat, (e) endosperm

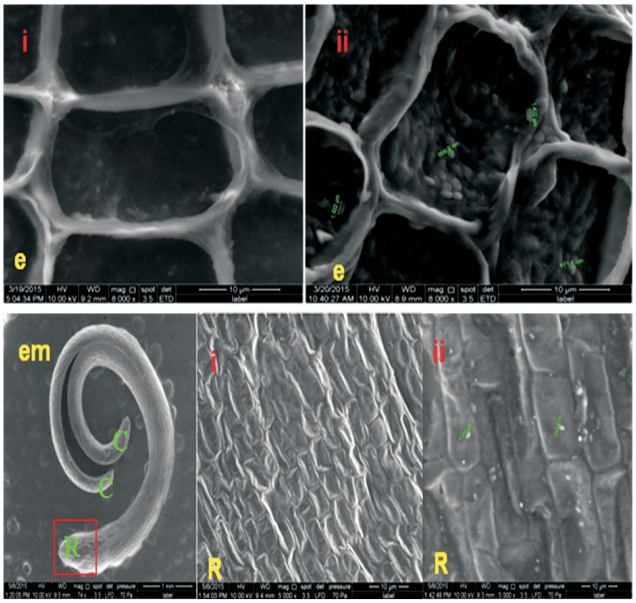


Plate 3: Scanning electron micrographs showing colonization by the *Bacillus amyloliquefaciens* VB7 on bacterized chilli seed after 48 h of treatment. (i) control, (ii) Biopriming with 6 % *Bacillus amyloliquefaciens* (e) endosperm, (em) embryo (C- Cotyledons and R- Radicle). Square box indicates the scanned portion of radicle.

(Lugtenberg and Kamilova, 2009). In addition, PGPR colonized successfully on the roots of the cucumber seeds treated with the bacteria and sustained population densities up to harvesting time (Choi *et al.,* 2013).

When chilli seeds were soaked in a bacterial suspension of VB7, bacterial cells entered the seeds through the pore at the base, colonized the inner plane, and proliferated germination advanced (Plate 4).

These results were similar to the findings of Behbahani (2010) who assessed *Bacillus subtilis* SRB28-rif1. When inoculated on seed surface, it successfully colonized the growing root of sorghum seedling forming cell-aggregates or micro-colonies. This implied that the SRB28-rif1 multiplied on the sorghum root, a property desirable for survival and functioning of a biocontrol agent.

In this study, were also analyze the population of biocontrol agents by serial dilution technique. The number of *B. amyloliquefaciens* colony forming unit ml^{-1} was observed in seed wash (25×10^8 cfu ml^{-1}) and root tip of the seedlings (37.7×10^4 cfu g^{-1}).

The PGPR strains are known to be good colonizers of plant root system owing to their competitive advantage. For effective management of any soil borne disease, the introduced antagonist should colonize the roots (Weller, 1984). The successful antagonist should colonize the rhizosphere at the time of seed germination itself and the antagonist should move from spermosphere to rhizosphere and establish (Weller and Cook, 1983). Root colonized populations of PGPR strains were significantly different between dry seed treatment and biopriming treatment. Higher populations in biopriming treatments appear to be due to the higher populations within seeds (Choi *et al.,* 2013).

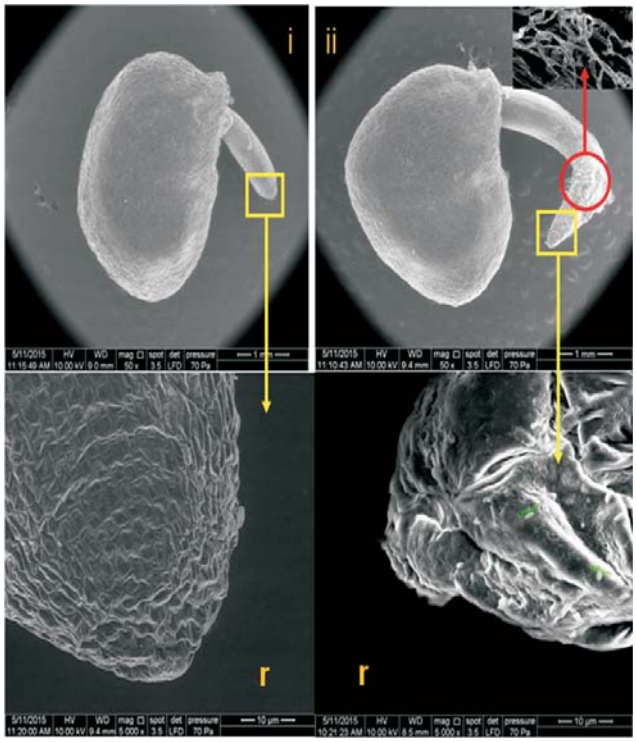
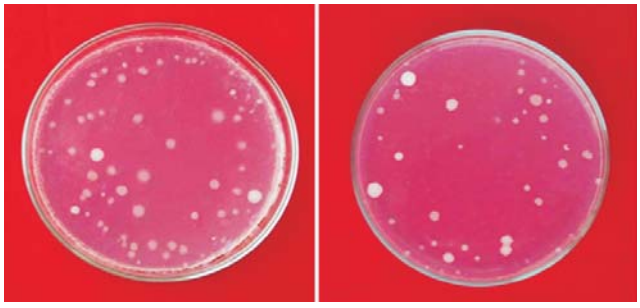


Plate 4: Scanning electron micrographs showing colonization by the *Bacillus amyloliquefaciens* VB7 on bacterized chilli seed after 72 h of treatment. (i) Control, (ii) Biopriming with 6% *Bacillus amyloliquefaciens* (r) radicle. Square box indicates the scanned portion of emerging root tip and circle indicates the scanned portion root hairs



Seed wash Root tips

Plate 5: Population of *B. amyloliquefaciens* VB7 on seed wash and root tips

However, biopriming with biocontrol agents had a dual action such as control of disease causing pathogens during germination as well as enhancement of germination and vigour by integrating the biological and physiological changes during germination (El-Mohamedy, 2004; El- Mohamedy *et al.,* 2006).

In conclusion, our results indicate that the biological control agent *B. amyloliquefaciens* VB7 were present on the surface of the seed coat, endosperm and emerging radicle. Through the observation of SEM, it was determined that bacterial cells entered seeds during the process of biopriming and its population is important in root colonization. The results obtained in this study provided a novel insight into the commercial application of biological control agents using seed

treatments.

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