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APPLICATION OF *BACILLUS* SPECIES CULTURED ON DIFFERENT LOW COST ORGANIC SUBSTRATES FOR THE CONTROL OF ROOT-KNOT NEMATODE INFECTION ON OKRA (*ABELMOSCHUS ESCULENTUS* MOENCH)

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Abstract

Low cost organic substrates such as rice grain, chickpea, wheat grains, rice husk, chickpea husk and wheat bran were used to culture *Bacillus subtilis* and *B. firmus*. Vegetative cell counts of both bacterial species generally increased on majority of substrates in initial 5 days and declined in subsequent incubation. Spore counts also increased with time but declined at 10th day. Bacterial species cultured on various substrates were inoculated in soil before sowing of okra in a pot culture experiment. The growth of okra plants was significantly increased in all substrates except chickpea husk, as compared to control. Root-knot nematode infection severity was measured in term of galls/ root system and egg masses/ root system. Both bacterial species showed varying effectiveness when grown on different organic substrates, but they controlled root-knot nematodes infection in okra in comparison to the control.

Introduction

Bacillus species are widely distributed and well known plant growth promoting bacteria, commonly present in the rhizosphere. They increase plant growth by vanishing plant pathogens by their unique antimicrobial activities, including production of antibiotics (Awais *et al*., 2007) and toxins (Mukry *et al*., 2010) to compete with pathogenic organisms. Some strains are also known to induce systemic resistance in plants against variety of pathogens. Application of *Bacillus* species as biocontrol agents against plant pathogens is increasingly interesting these days (Bargabus *et al*., 2004, Han *et al*., 2005, Liu *et al*., 2009, Yánez-Mendizábal *et al*., 2012). It is a common practice to introduce *Bacillus* species on/around plant roots by multiplying them on culture media like nutrient and Luria Bertani agar/broth. Generally a single culture type is employed for biocontrol or improvement of plant growth and yield. *Bacillus* species are ubiquitous in nature found around the diverse habitats and have ability to grow and multiply on different types of organic substrates including plant and animal materials. *Bacillus* species can multiply on a variety of substrates, including cheap agricultural byproducts (Bishop, 2002). Solid state fermentation is helpful for production of bacterial metabolites at low cost on large scale (Bora & Kalita, 2009). It is reported that growth in nutrient rich media alters the biochemical properties of *Bacillus* species (Bizzarri *et al*., 2008). The production of different hydrolytic enzymes, including α-amylase and β-galactosidase increases on different organic substrates such as corn, wheat and rice as compared to commercial medium (Konsoula & Liakopoulou-Kyriakides, 2007).

The present study report a) growth and multiplication rate of two different *Bacillus* species namely *Bacillus subtilis* and *B. firmus* on different organic substrates including Rice, Rice husk, Chickpea, Chickpea husk, Wheat grains and Wheat bran for varying incubation period and also b) the effectiveness of these *Bacillus* species grown on these organic substrates to control root-knot nematodes and the growth of okra plants.

Materials and Methods

Growth of *Bacillus* species on organic substrates: Bacteria were cultured on Luria Bertani (LB) broth for 24 hours at 37ºC in shaking incubator. Cultures were centrifuged at 4000x*g* for 10 min., and bacterial cells were re-suspended in sterilized distilled water, after washing twice with sterilized distilled water. Six different organic substrates were selected for the study viz., Rice, Rice husk, Chickpea, Chickpea husk, Wheat grains and Wheat bran. Twenty gram from an organic substrate was taken on each flask; moisture was maintained at sixty percent with distilled water and autoclaved at 121ºC at 15 psi for 15 minutes. 0.1 mL of cell suspension was added in each flask, which contained log 9.53557 CFU of *B. subtilis* and log 9.36008 CFU of *B. firmus*. Total colony forming units (CFU) and endospores /g of substrate was recorded by serial dilution technique after 5, 10 and 15 days of incubation at 28ºC. Two series of dilutions were made for each sample at the time of observation, one set of dilutions were treated with heat at 80ºC for 15 minutes in water bath (for endospore count), the other series was not heated (for total cell count). Appropriate dilution of 0.1 mL was taken in sterilized Petri plate and about 10 mL slightly warm LB agar was poured (Petras & Casida 1985). Data was transformed to $\log_{10}$ before statistical analysis.

Green house experiment: Soil was collected from the field of the Department of Botany, University of Karachi. A two factor experiment was conducted in small plastic pots under greenhouse conditions. Two *Bacillus* species (*Bacillus subtilis* and *B. firmus*) were applied in soil after growing for 10 days on different organic substrates (Rice, Rice husk, Chickpea, Chickpea husk, Wheat grains and Wheat bran). Another set of pots was also arranged that contained organic substrates without bacteria. About 3g of substrate was inoculated in 300 g soil for each plastic pot. One g of each substrate contained bacterial colony forming units (Log_{10}) as given in Table 1. The untreated pots were served as control. Five seeds were sown in each pot one day after inoculation and two seedlings per pot were

maintained after germination. After seedlings have established, freshly hatched 2000 second stage juveniles of root-knot nematodes were released in soil around the roots of each plant. The pots were randomly placed on green house bench and watered daily. The plants were carefully harvested after 45 days of nematodes inoculation. Data on fresh shoot weight and plant height was recorded immediately after harvesting. To determine the severity of root-knot nematodes infection, the roots were collected from the plants and spread on large glass Petri plates and number of galls/root system and egg masses/root system were counted under stereomicroscope (4x). Large roots were cut into two or more pieces before counting.

Table 1. Population sizes of *Bacillus* species cultured on various organic substrates (log_{10} CFU g^{-1}) at the time of their application in soil

Organic substrates	Population log_{10}. g^{-1}	
	Bacillus subtilis	***Bacillus firmus***
Rice	7.8906	8.8291
Rice husk	9.7866	9.9213
Chickpea	8.7854	8.8229
Chickpea husk	9.2997	7.9164
Wheat	9.0184	9.1940
Wheat husk	8.0918	8.8944

Results and Discussion

Bacterial populations including spore count significantly increased at 5 days of incubation. The maximum *Bacillus subtilis* population was observed in wheat bran (log_{10} CFU:10.8343), followed by chickpea (log_{10} CFU: 10.7645) on 5th day of incubation. The minimum growth of *B. subtilis* was recorded on chickpea husk. Total cell count declined at 10th day of incubation on most of the substrates except chickpea husk and rice husk. The spore count showed similar trend as we observed in total cell count. On chickpea husk and wheat grain at 15th day of incubation, total cell count and spore count was almost equal, indicating sporulation in all cells (Fig. 1). *Bacillus firmus* successfully multiplied on all substrates and population increased on all organic substrates. The maximum total spore count was observed in rice to be 11.5311 log_{10} CFU at 5 days of incubation, followed by chickpea (10.2014 log_{10} CFU) and wheat grain (10.1704 log_{10} CFU). A decline in total cell count was observed on 10th days of incubation in all organic substrates except in rice husk. In chickpea husk total cell count increased after 10 days of incubation. Similar trend of endospore count was noticed (Fig. 2). The two *Bacillus* species showed growth rates varying on different substrates. *B. subtilis* showed the maximum growth on wheat bran, while, *B. firmus* on rice. *Bacillus* species have been reported to multiply rapidly on different low cost organic substrates (Khan *et al*., 2011). The growth of *Bacillus circulans* has been reported to rapidly increase on rice straw during initial 24 hours (Dhillon *et al*., 2000). Wheat bran has been reported the best organic substrate for the growth and increased enzymatic activity of *Bacillus* species (Haq *et al*., 2003, Poopathi & Kumar, 2003). *Bacillus thuringiensis* strains was successfully multiplied and produced on rice and rice bran, exhibited insecticidal and nematicidal properties (Khan *et al*., 2011; Lee & Seleena, 1991). Chickpea and rice are rich source of Carbohydrates, proteins, minerals and vitamins, provides bacterial cells a suitable environment to multiply and produce biologically active metabolites. In *Bacillus* species sporulation takes place from 24 hours and time of sporulation depend on type of medium or substrate used; under some favorable conditions sporulation may take a week or more (Donnellan *et al*., 1964; Jinks *et al*., 1985).

All organic substrates significantly increased growth of okra plants. The maximum plant height was observed in plants treated with *Bacillus firmus* (45.7 cm) followed by that on chickpea (Fig. 3). Chickpea husk alone and after growth with bacteria and control showed least plant growth. Fresh plant weight most effectively increased on plants treated with *B. firmus* after growing on chickpea (5.2 g). However, chickpea husk found ineffective to increase plant fresh weight. Bacteria grown on different organic substrates significantly decreased the galling intensity on roots of okra. Minimum number of galls/ root system (35 galls) was recorded in plant treated with *B. firmus* and *B. subtilis* grown on rice, wheat husk and wheat grain, respectively. Most of the organic substrates when applied alone showed increased galling intensity compared to applied with bacteria except chickpea husk. Maximum infection was observed in non treated control (Fig. 4). *B. firmus* after growing on rice most significantly reduced egg mass formation (20 egg masses), followed by *B. subtilis* after growing on chickpea husk (22 egg masses). *Bacillus* species have been reported as Plant Growth Promoting Rhizobacteria (PGPR). It was observed that *B. firmus* showed improved plant growth when applied in soil as compared to *B. subtilis*. However, plant growth promotion varies with substrates used for the growth of strains. Organic amendments in soil to improve plant growth and to control variety of soil borne pathogens have been studied extensively. Further more presence of organic material continuously increase bacterial inoculum after incorporation in soil. However, growth rate and metabolite production especially proteases also vary according to presence of nutrients in the medium (Hageman *et al*., 1984). *Bacillus* species have been reported to produce antibiotics, proteases and other metabolites having nematicidal activity (Saxena *et al*., 2000; Terefe *et al*., 2009, Khan *et al*., 2010). Another important purpose was to develop *Bacillus* inoculum on low cost substrates which can be applied in soil on large scale. Wheat bran, chickpea and rice were found very good solid substrates for the development *Bacillus* inoculum. Chickpea husk yield lesser growth of *B. subtilis* during early time of inoculation.

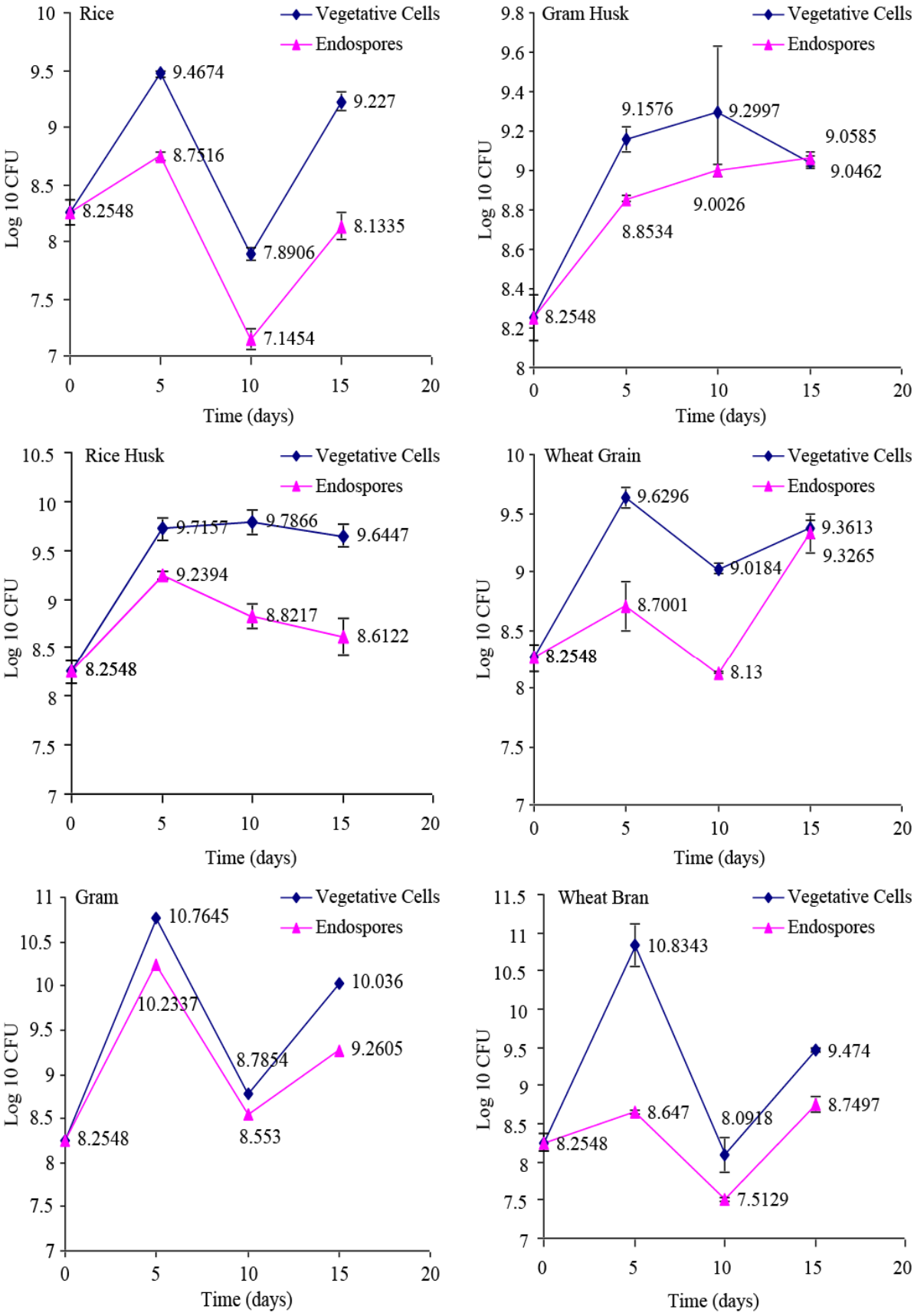

Fig. 1. Populations of *Bacillus subtilis* on different Organic substrates up till 15 days.

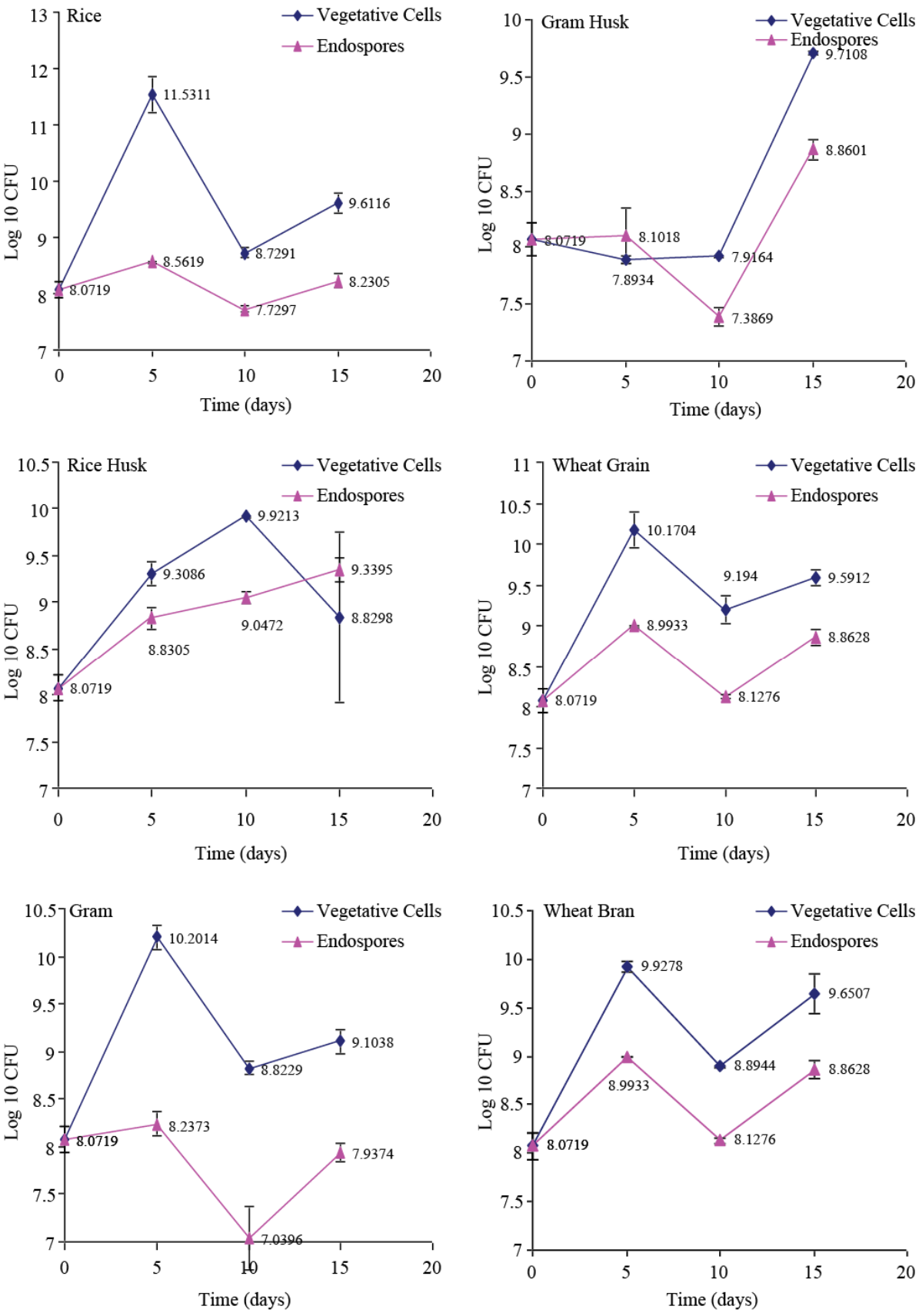

Fig. 2. Populations of *Bacillus firmus* on different Organic substrates up till 15 days.

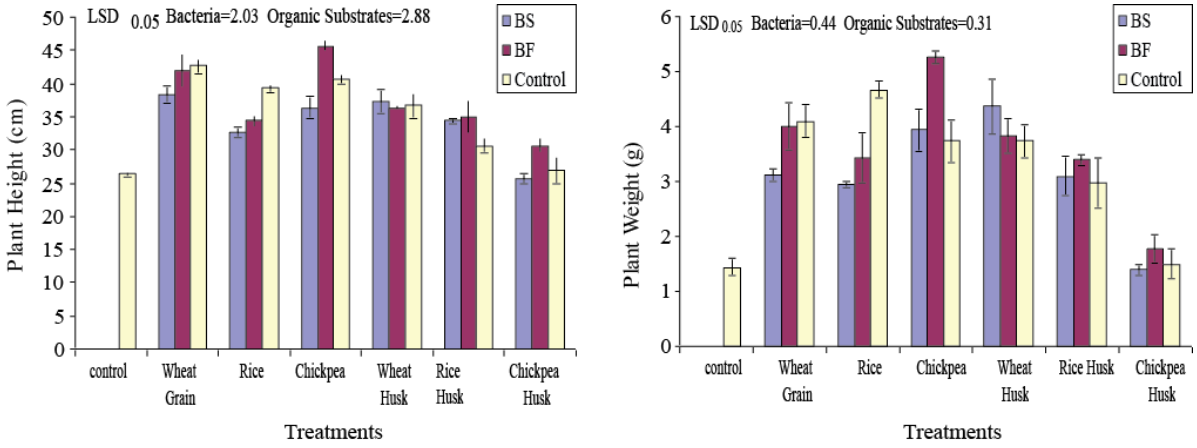

Fig. 3. Effect of *Bacillus* species on growth of Okra following application in soil after multiplication on different organic substrates.

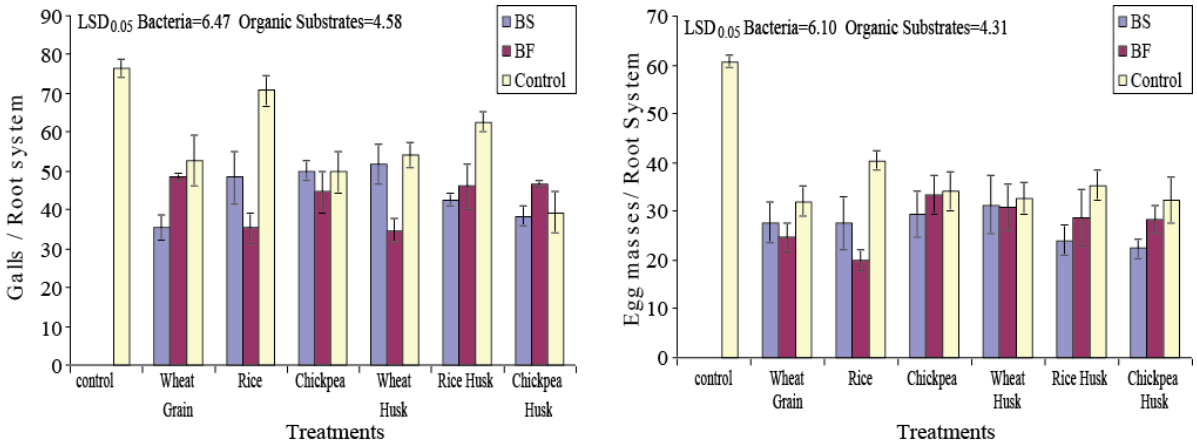

Fig 4. Effect of *Bacillus* species on root-knot nematodes in Okra following application in soil after multiplication on different organic substrates.

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