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Exploration of Chitinolytic *B. thuringiensis* Associated with Rhizosphere Soil Samples of Pulse Crops for Management of Seed and Soil Borne Pathogens

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The rhizosphere soil samples were collected during crop growth of black gram, green gram and horse gram at Regional Research Station, Paiyur, Tamil Nadu, India for isolation of *B. thuringiensis* (Bt). Thirty Bt like colonies based on colony morphology *viz*.,fried egg colonies were identified from soil samples of Black gram, green gram and horse gram. Among the 30 fried egg colonies, eleven Bt colonies viz., five from black gram, four from green gram and two from horse gram soil samples were identified based on crystal protein under microscope. All the 11 colonies were plated on 0.5% colloidal chitin agar (CCA) medium and the results revealed that among the 11 Bt colonies, six Bt colonies produced zone of clearance over 0.3cm . Out of six colonies, BG7 produced 0.8cm clearance of zone on CCA plates followed by BG10 (0.72cm). All the six colonies were further screened by chitinase activity and the results showed that among the six Bt colonies, BG7 produced highest level of (2.4 U/ml) chitinase activity followed by BG10 (1.98 U/ml). The growth conditions for isolate, BG7 which showed maximum chitinase activity were optimized under different pH, temperature and incubation time and the result showed that the strain BG7 produced maximum chitinase at pH of 7, temperature of 30 with 0.5 % colloidal chitin. This finding might be applied for the development of new *B. thuringiensis* formulations against seed and soil borne pathogens along with insecticidal toxins.

Keywords: *Bacillus thuringiensis*, chitinase, rhizosphere soil, pathogens.

Pulse crops or grain legumes are the major source of protein in the predominantly vegetarian diet of the people of India. Globally, pulses are the second most important group of crops after cereals. In 2013-14 total production was at 18.5 million tonnes, but it could not meet our demand since the overall per capita pulses availability has decreased from 1950s onwards. The growth in total production is less than growth in population. So to meet demand, India has to go for imports from countries like Canada, Russia, Myanmar, Australia etc. The key solution lies in the increment in production by increasing land under pulses and increasing productivity per hectare (http://www.simplydecoded.com/2015/10/20/indian-agriculture-examining-the-pulses/). One of the limiting factor for their production is the diseases caused by phytopathogenic fungus, affecting seeds from germination and throughout development. Consequently, yield reductions of up to 30% have been reported. There is a huge number of pathogens that attacks the pulse crops. Among them fungal pathogens particularly soil and seed borne pathogens are economically most important and play active role in their dissemination

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distribution and annual recurrence. Among the various diseases, *Pythium sp., Sclerotinia sp. Rhizoctonia sp and Fusarium sp* attacks all crops in all zones (Chaudhary *et al*., 2007).

Fungicides have commonly been used; however, pathogen resistance has developed and also harmful for man and the environment. Recently, the use of biological control has increased. Chitinolytic microorganisms have been suggested for the control of some fungi. *Serratia marcescens* chitinase reduced the incidence of disease in bean seeds caused by *Sclerotium rolfsii* (Ordentlich *et al*., 1988); *Aeromonas caviae* controlled the infection caused by *Rhizoctonia solani* and *Fusarium oxysporum* in cotton (Inbar and Chet 1991). Chitinase from different *Actinomycetes* has also produced inhibition of phytopathogenic fungi in cotton, rice, soybean, and tomato. The interest of *B. thuringiensis* as a control agent has focused on the Cry proteins acting as the most successful microbial bioinsecticide. However, Barboza-Corona *et al*., (1999) carried out the isolation of chitinolytic strains of *B. thuringiensis* using colloidal chitin as a substrate. They proposed that Cry proteins and chitinase from the same bacterial species could have synergistic effects in increasing *B. thuringiensis* insecticidal activity. However, the potential of *B. thuringiensis* chitinase might be expanded toward the control of other plant pathogenic fungi. Escudero-Abarca *et al*., (1998) pointed out the potential use of the chitinase from *Bacillus thuringiensis* var *israelensis* in the protection of bean seeds infested with phytopathogenic fungi. Several reports have described selection and characterization of chitinolytic *B. thuringiensis* strains (Barboza-Corona *et al*. 1999). Ramirez, *et al*., (2004) reported that when soybean seeds were infected with *S. rolfsii*, germination was reduced from 93% to 25%; the addition of *B. thuringiensis* chitinase (0.8 U/ mg protein) increased germination to 90%. Hence the present study is aimed to isolate and screen the *B.thuringiensis* for its chintinolytic activity against major diseases of pulses.

MATERIALS AND METHODS

The soil samples were collected from rhizosphere soil samples of black gram, Green gram and horse gram at RRS, Paiyur for isolation of *B. thuringiensis*. For each crop, the rhizosphere soil samples were collected from ten different location and pooled together. All the soil samples were collected aseptically from top to a depth of 2-3 cm after scrapping off the surface material with a sterile spatula. The soil samples are stored in sterile containers. *Bacillus* like colonies were isolated from soil samples and stained with Coomassie Brilliant Blue stain to observe the presence crystalline inclusions under microscope as per the method described by Ramalakshmi and Udayasuriyan, (2010). The isolates with crystalline inclusions were further plated on 0.5% colloidal chitin agar (CCA) medium for chitinolytic activity. The isolates showing inhibition zone on agar plates were further screened for chitinase activity based on colorimetrically by detecting the amount of N-acetyl glucosmine released from the colloidal chitin substrate. The growth conditions, *viz*., different pH, temperature and incubation time for promising isolate, BG7 which showed maximum chitinase activity were optimized for maximum chitinase production as per the method described by Shanmugaiah *et al*., (2008).

RESULTS AND DISCUSSION

Bacillus thuringiensis (Bt) like colonies are white to off white colour, slightly raised elevation, spread out and seems to fried egg on plate (Smith and Couche, 1991 and Mane *et al*., 2015). In the present study, thirty Bt like colonies were identified on agar plates from soil samples of Black gram, green gram and horse gram. Further these colonies were stained with Coomassie Brilliant Blue stain and observed under bright filed microscope for the presence of crystalline inclusions. Initial identification of Bt is mainly based on the presence of crystalline inclusions. Rampersad and Ammons, (2005) stated that bright field microscopy is more useful than phase contrast microscopy for high throughput evaluation of bacterial colonies for the presence of crystals and also for identification of small crystals. Out of thirty colonies, eleven colonies showed bipyramidal or bipyramidal with cuboidal crystalline inclusions (Fig 1). The remaining nineteen colonies were negative for the presence of crystalline inclusions.

Many strains of *Bacillus* are known to produce chitinolytic enzymes during their growth

including *B. thuringiensis* (Wen et al., 2002; Driss et al., 2005; Waldeck et al.,2006; Chang et al., 2007). In the present study. All the 11 colonies were plated on 0.5% colloidal chitin agar (CCA) medium and the results revealed that six out of 11 Bt colonies produced zone of clearance over 0.3cm . Out of six colonies, BG7 produced 0.8cm clearance of zone on CCA plates followed by BG10 (Table 1). All the six colonies were further screened by chitinase activity based on colorimetrically and the results showed that among the six Bt colonies, BG7 produced highest level of (2.4 U/ml) chitinase activity followed by BG10 (1.98 U/ml) (Table 1). The isolate, BG7 which showed maximum chitinase activity were further inoculated into broth and incubated under different pH, temperature and incubation time for maximum chitinase production. The result showed that the strain BG7 produced maximum chitinase at pH of 7 compared to other pH tested (Fig 2). Among the different temperature of 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C and 45^{0}C tested, incubation under 30^{0}C showed maximum chitinase activity of 2.5U/ml (Fig 2). Addition of colloidal chitin at 0.5% and above induced the maximum chitinase production in *Bacillus* sp. NCTV2 (Wen et al., 2002). The pH of the culture medium is playing

Table 1. Characterization of Bacillus thuringiensis for chitinase activity

Isolate name	Gram reaction	Crystalline inclusions	Diameter of the zone of clearance (cm)	Chitinase activity (U/ml)
BG3	Positive	Bipyramidal	-	-
BG6	Positive	Cuboidal	0.26	0.92
BG7	Positive	Bipyramidal + Cuboidal	0.52	2.40
BG8	Positive	Bipyramidal + Cuboidal	-	-
BG10	Positive	Bipyramidal + Cuboidal	0.24	0.88
GG1	Positive	Bipyramidal	-	-
GG2	Positive	Bipyramidal+Cuboidal	0.46	1.98
GG3	Positive	Bipyramidal	0.28	1.02
GG12	Positive	Bipyramidal+Cuboidal	0.22	0.82
HG5	Positive	Bipyramidal+Cuboidal	-	-
HG7	Positive	Cuboidal	-	-

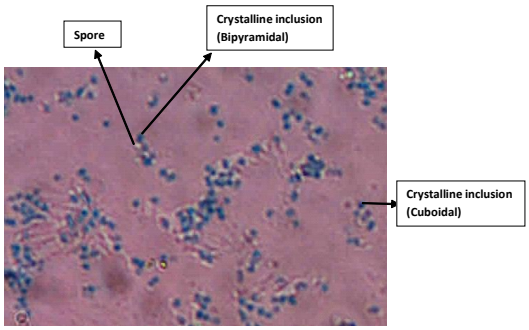

Fig. 1. Crystalline inclusions of the new isolates of Bacillus thuringiensis (BG7)

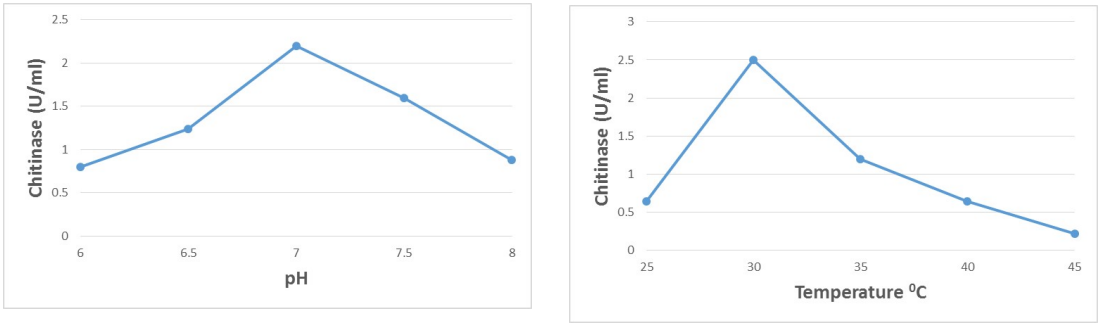

Fig. 2. Optimization of chitinase production by BG7 under different pH and temperature

important role in chitinase production. Majority of the bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH and whereas fungi mostly secret it in acidic conditions (Zhang et al., 2004; Sharaf, 2005). The maximum chitinase production was observed in yeast nitrogen based medium (YNB) amended with 0.3% colloidal chitin at pH 8.0 and 35°C after four days of inoculation (Shanmugaiah et al., 2008). The isolate, BG7 could be an ideal candidate for controlling major diseases of pulses. Hence further studies are required to find out their efficacy against different soil and plant pathogens. This result might be useful to develop new *B.thuringiensis* formulations.

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