International Journal of Agricultural and Life Sciences- IJALS (2016), Volume 2 (4) pp.54-59
http://doi.org.in/10.9379-sf.ijals-122066-009-0081-x

Available online at http://www.skyfox.co
ISSN: 2454-6127

Research Article

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Molecular characterization of chitinase and β-1, 3-glucanase gene of soybean plant growth promoting bacterium *Bacillus* sp. SJ-5

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Received: Oct 2016 / Accepted: Oct2016/ Published: Dec 2016

ABSTRACT: Plant-growth-promoting bacteria (PGPB) colonize the rhizosphere of many plant species and confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases caused by plant pathogenic fungi. The aim of the present study was to molecularly characterize the presence of biocontrol gene chitinase and β-1, 3-glucanase of the *Bacillus* sp. SJ-5 to understand its role in fungal pathogen inhibition. Genomic DNA was isolated from *Bacillus* sp. SJ-5 and chitinase and β-1, 3-glucanase gene were amplified using specific primers. On the agarose gel 402 pb and750 bp bands were detected for chitinase and β-1, 3-glucanase respectively. Upon homology analysis it confirms the presence of chitinase and β-1, 3-glucanase gene in SJ-5 and sequences submitted to Genbank. The study indicates the potential of this PGPB to inhibit fungal pathogen through cell wall degrading enzymes production.

Keywords: Plant growth promoting bacteria, Soybean, Chitinase, β-1, 3-glucanase

INTRODUCTION

Soybean, being a nutrient rich plant with a wide range of beneficial effect on human health and by product, also had a wide range of enemy in the form of different pathogens that affect it in different manner to reduce its quality as well as quantity. Several pathogens like fungi, bacteria, viruses and nematodes attack on the soybean plant and cause different disease leading to the great losses in the yield. Among the notable fungal diseases, Fusarium rot known as 'Fusarium wilt and necrosis of root and lower of soybean' and '*Rhizoctonia* root rot' are the most destructive diseases of the crop and are very common also (Hashem et al. 2009). Chitin and β-glucans are the major constituents of the fungal cell wall. Plant growth promoting bacteria (PGPB) have the ability to reduce or prevent deleterious effect of pathogen by means of production of some antibiotics, cell wall degrading enzymes, competition for the niche etc.(Pandya *et al.,* 2015; Saini *et al.,* 2015; Hayat *et al.,* 2010; Bhattacharyya and Jha 2012; Coutinho *et al.,* 2015). Compant *et al.,* (2005) reported that the biocontrol capacity of the PGPB strains is because of their capacity to produce chitinase and β-1, 3-glucanase. Chitinases are specific enzymes which can degrade the chitin by hydrolyzing β-glycosidic bonds. As chitin is the primary component of most of the fungal cell wall chitinase is directly correlated with it. Chang *et al.,* (2007) reported that *B.cereus* QQ308 which had positive activity for different hydrolytic enzymes, including chitinase, chitosanase and protease, inhibited the growth of several important soil-borne fungal plant pathogens including *F. oxysporum*, *F. solani*, and *P. ultimum* in the form of culture itself or its culture supernatant. Production of chitinase, cellulase, pectinase, amylase, β-1, 3 glucanase, protease and lipase was reported in *Pseudomonas* sp. gave protection to tomato plants from fungal pathogens (Solanki et al. 2012). β-glucanase hydrolyses β-glucans, one of another major component of fungal cell wall. Kumar *et al.,* (2012) isolated four bacterial strain namely *Bacillus* sp. BPR1, BPR3, BPR4 and BPR 7 with positive glucanase activities as it grew on laminarin azure-amended minimal medium. The aim of the present study was to molecularly characterize cell wall degrading enzymes gene β-1, 3-glucanase and chitinase of *Bacillus* sp. SJ-5 to find out these enzymes role in inhibition of fungal pathogen *F. oxysporum* and *R. solani*.

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MATERIALS AND METHODS

BACTERIAL CULTURE

Plant growth promoting bacterial strain *Bacillus* sp. SJ-5 (MCC-2609), isolated from the soybean rhizospheric soil, was used for the study and maintained on nutrient agar plates for short term while for long time, preserved on nutrient broth (NB, HiMedia, India) amended with 50 % glycerol at -80°C.

BACTERIAL GENOMIC DNA ISOLATION

Bacterial genomic DNA was isolated by Bollet *et al.,* (1991) method with little modification. For the DNA isolation, 24 h old culture, grown in NB was used after centrifugation. Quantitative estimation of the DNA was done spectrophotometrically by using biophotometer (Eppendorf, India) that is based on Beer Lamberts law while for the qualitative estimation agarose gel electrophoresis was performed and observation for the DNA band was done by visualizing gel for the DNA band in UVITECH gel doc system.

MOLECULAR CHARACTERIZATION OF BIOCONTROL GENES

PCR amplification of chitinase and β-1,3-glucanase genes responsible for biocontrol against fungal pathogens were done from genomic DNA of the bacterium isolate SJ-5 by using gene specific primer (GSP) designed online by Primer3 tool, available online at the site http://primer3.ut.ee/ and custom synthesized by Sigma Aldrich. The 25 µl of reaction mixture consisted of 100 ng of genomic DNA, 1 µl Taq polymerase (3U/ul), 2 µl of 10× buffer, 2 µl dNTP mix (10mM), 2 µl MgCl2 (25mM) and 1 µl of each primer (10 pmol/µl). Chitinase gene was amplified by using primer: *cbF* –GAATATCCTGGCGTTGAAACGAT and *cbR*-GCCACGTCCGTAAAAGGGT with PCR condition : initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Another gene β-1, 3-glucanase was amplified by using primer: *gbF*- TGGCACACCATACGAAAGAA and *gbR*- AGATGCTTGTCCATCACCTAAC with PCR condition: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 45 sec, extension at 72 °C for 1 min and a final extension at 72°C for 10 min. For the assessment of both genes PCR product was loaded in the 1.2 % agarose gel (wt/vol) containing EtBr and ran for 45 min at 85 Volts in 1X TAE buffer. Observation for the DNA band was done by visualizing gel for the DNA band in UVITECH gel doc system.

PCR PRODUCT PURIFICATION AND SEQUENCING

Obtained PCR production was purified using Promega kit 'Wizard® SV Gel and PCR Clean-Up System' as per manufacturers instruction. Purified PCR product was checked quantitatively and qualitatively through biophotometer and gel electrophoresis respectively, and Sanger sequencing was done at National Chemical Laboratory (NCL), Pune by using ABI 3730xl DNA analyzer (Applied Biosystem, USA).

SEQUENCE HOMOLOGY ANALYSIS AND SEQUENCE SUBMISSION

Homology analysis of the sequences obtained through sequencing for different PGP and biocontrol genes were done by using BLAST tool of NCBI. Based on maximum identity score first five sequences were selected for further analysis. Selected sequences were aligned using Clustal W and checked for the gap. Phylogenetic and molecular evolutionary analysis of all the genes was performed using MEGA 6 software (Tamura et al 2013). Upon confirmation of the genes; sequences were submitted to NCBI Genbank and accession number assigned by NCBI.

RESULTS

MOLECULAR CHARACTERIZATION OF BIOCONTROL GENES OF BACTERIA

Molecular characterization of biocontrol properties of bacterium SJ-5 was done by amplifying biocontrol genes, chitinase and β-1, 3-glucanase of SJ-5 by using GSP. Gene amplification using GSP showed sharp bands of the specific genes near to desired amplicon size (Fig 1). For the chitinase gene a 402 bp sharp band was detected while for the β-1, 3-glucanase gene a 750 bp band was observed on the agarose gel under the UVITECH gel doc system.

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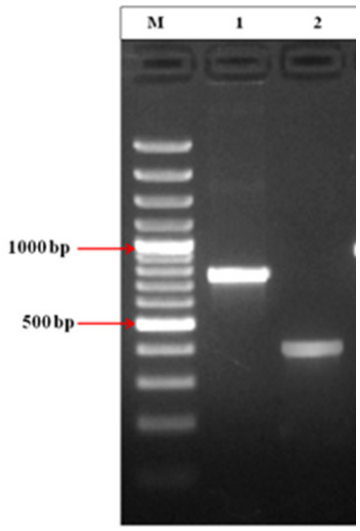

Fig 1 PCR amplification of biocontrol genes of *Bacillus* sp. SJ-5; Lanes M-Marker (100 bp plus, Thermo scientific); 1- β-1, 3-glucanase; 2- Chitinase

SEQUENCE HOMOLOGY ANALYSIS AND SEQUENCE SUBMISSION

Sequence homology analysis of the concerned gene, confirmed the amplification of the desired gene. On the BLAST analysis both gene sequences have shown similarity with the concern gene of the *Bacillus* sp. In phylogenetic analysis, chitinase and β-1, 3-glucanase genes sequence of SJ-5 were grouped with respective gene of other *Bacillus* isolates as a single cluster (Fig 2). The chitinase and β-1, 3-glucanase gene sequences were dully submitted in NCBI Genbank with accession no. KT263585 and KU183550, respectively.

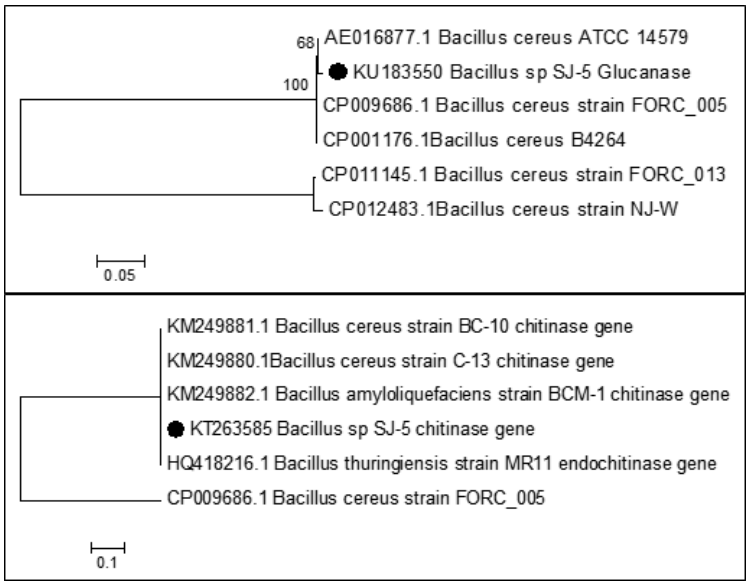

Fig.2 Phylogenetic analysis of biocontrol genes of *Bacillus* sp. SJ-5

DISCUSSION

Due to rapid growth, easy handling and aggressive colonization of the rhizosphere, antagonistic bacteria are considered ideal biological control agents against phytopathogens. Research studies have shown that use of PGPR specifically as biocontrol agents against soil borne fungal plant pathogens is the best alternative or complementary strategy to physical and chemical disease management (Saraf

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et al., 2014; Berg and Smalla 2009; Haas and Defago 2005). In our previous study we found strong antifungal activity of *Bacillus* sp. SJ-5 against both fungal pathogens as it inhibits growth of *F. oxysporum* as well as *R. solani* on dual culture plate (Jain *et al.,* 2016). Based on different research studies *Bacillus* sp. reported with potent antagonistic activity against different fungal phytopathogens to provide protection to host plants from invading pathogens and promote plant growth (Kumar et al. 2012; Walia *et al.,* 2013; Reyes-Ramirez *et al.,* 2004; Seo *et al.,* 2012). Direct antifungal activity of the *Bacillus* sp. is due to the presence of cell wall degrading enzymes such as chitinase, β-1, 3 glucanase, protease etc. and some of the antibiotic compounds such as Bacillomycin, Fengycin, Iturin A, Mycosubtilin, Zwittermicin A etc. (Leclere *et al.,* 2005; Kloepper *et al.,* 2004; Koumoutsi *et al.,* 2004). Chen *et al.,* (2004) have proven the role of chitinase in antifungal activity by transforming *chiA* gene of *B. circulans* WL-12 in to *B. subtilis* F29–3 and found enhanced antifungal activity of the culture supernatant against the *Botrytis elliptica*. Chitinases are glycosyl hydrolases that catalyze the hydrolytic degradation of chitin, an insoluble linear β-1, 4-linked polymer of N-acetylglucosamine (GlcNAc) that form one of the major constituent of the fungal cell wall (Bhattacharya et al. 2007) while the another cell wall degrading enzymes β-1, 3 glucanase catalyzes the hydrolysis of glucans, another major constituent of the fungal cell wall, with 1, 3-β-linkage and has an endolytic mode of action (Hong and Meng 2003).

Comparative studies have been done between the two chitinolytic bacteria *B. thuringiensis* NM101-19 and *B. licheniformis* NM120-17 isolated from the rhizosphere and found *B. thuringiensis* NM101-19 more effective than that of *B. licheniformis* in increasing the germination of soybean seeds infected with various phytopathogenic fungi (Gomaa 2012). Foliar spray of chitinolytic bacteria *B. circulans* GRS 243 and *S. marcescens* GPS 5 with 1% colloidal chitin on the Arachis hypogaea resulted in reduced lesion frequency by 60% compared with application of bacterial cells alone against the late leaf spot (LLS), caused by *Phaeoisariopsis personata* (Kishore *et al.,* 2005). Yan et al. (2011) have purified chitinase by sepharose fast flow ion exchange column chromatography and Sephadex G-75 gel filtration and characterized from the *B. subtilis* SL-13 which found to be promote sprouting and seedling growth of tomato and control *Rhizoctonia* rot caused by *R. solani*. β-1, 3-glucanase purified from the culture medium of a *PaeniBacillus* strain and *Streptomyces sioyaensis* were found to have the ability to damage the cell-wall structures of the growing mycelia of phytopathogenic fungi *P. aphanidermatum* and *R. solani* AG-4, but the *PaeniBacillus* enzyme reported to have a much stronger effect on inhibiting the growth of fungi tested (Hong and Meng 2003). Apart from these, *P. cepacia* having β-1,3 glucanase activity but no chitinolytic activity was also found with decreased in the incidence of diseases caused by *R. solani*, *Sclerotium rolfsii* and *P. ultimwn* by 85, 48 and 71%, respectively (Fridlender et al. 1993). *PaeniBacillus* sp. 300 found positive for the chitinase and β-1,3-glucanase enzymes activity when supplemented with colloidal chitin as carbon source, suppressed Fusarium wilt of cucumber (*Cucumis sativus*) caused by *F. oxysporum* f. sp. *cucumerinum* in nonsterile, soilless potting medium (Singh et al. 1999). Earlier He *et al.,* (1994) have purified zwittermicin A from the *B. cereus* UW85 and showed its antifungal activity against *P. medicaginis* causing agent of damping-off disease of alfalfa through reversibly reduction in elongation of germ tubes derived from cysts. In the present study we have molecularly characterize chitinase and β-1,3-glucanase gene of SJ-5 to understood mechanism behind our previous study regarding pathogen suppression by SJ-5 (Jain *et al.,* 2016).

CONCLUSION

This study has provided an insight into the role of bacterial isolate SJ-5 in the fungal pathogen suppression by production of cell wall degrading enzymes to protect soybean plant. These studies show that the bacterium SJ-5 possesses chitinase and β-1, 3-glucanase gene and by using these it can inhibit fungal pathogen and promote soybean plant growth in indirect manner. This bacterium may be used as efficient PGPB for soybean production in farmer's fields. It is an environment friendly and cost effective technology.

ACKNOWLEDGEMENT

Authors would like to acknowledge Dr. Yogesh Shouche (Scientist 'G', NCCS Pune) for providing sequencing facility. The research was supported by DBT Grant No.BT/PR1231/AGR/21/340/2011 to DKC. Some of the research has been supported by SERB-DST Grant No. SR/FT/LS-129/2012 to DKC.

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ISSN: 2454-6127

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How to cite this article
Jain, S., Kumari, S., Vaishnav, A., Choudhary, D. K., & Sharma, K. P. (2016). Molecular characterization of chitinase and β-1, 3-glucanase gene of soybean plant growth promoting bacterium Bacillus sp. SJ-5. *International Journal of Agricultural and Life Sciences*, 2(4), 54-59. doi: 10.9379/sf.ijals-122066-009-0081-x

CONFLICTS OF INTEREST
"The authors declare no conflict of interest".