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DEVELOPMENT OF DNA MARKERS FOR DETECTION OF INOCULATED BACTERIA IN THE RHIZOSPHERE OF WHEAT (*Triticum aestivum* L.)

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Plant growth-promoting rhizobacteria (PGPR) are a group of soil microorganisms that improve plant growth and yield through a number of diverse mechanisms such as phosphate solubilization, nitrogen fixation, production of phytohormones, and repression of soil borne pathogens. Due to high cost of chemical fertilizers and negative environmental effects, the use of PGPR as biofertilizer is advantageous for development of sustainable agriculture. The objective of this study was to develop DNA-based markers for five PGPR strains to detect these bacteria in the rhizosphere of inoculated wheat. The rhizobacterial strains included four phosphate solubilizer strains (*Arthrobacter* strain WP-2, *Bacillus* strain MP5, *Rhodococcus* strain M28 and *Serratia* strain 5D) and one phytohormone producer *Azospirillum* strain WS1. DNA-based markers were developed using 16S rRNA gene restriction patterns, Random Amplified Polymorphic DNA-PCR (RAPD-PCR), Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and BOX-PCR. In this study, the most differentiating DNA patterns for all strains were obtained by using the BOX primer in PCR. All the strains tested as single-strain inocula resulted in improved growth of wheat plants. The inoculated strains were successfully re-isolated from wheat rhizosphere and confirmed by BOX-based DNA markers. These results indicated that BOX-PCR is a useful and highly discriminatory fingerprinting technique for rapid detection of bacterial strains.

Keywords: Bacterial inoculation, DNA fingerprinting, growth promotion, wheat

INTRODUCTION

Synthetic fertilizers play an important role in modern agriculture as they provide essential nutrients such as nitrogen (N) and phosphorus (P) to plants. Chemical fertilizers often have low use efficiency as only a fraction of the applied nutrients are taken up by plants. For example, a significant part of P applied as fertilizer is converted into insoluble form in soil, thus becoming less available to plants (Gyaneshwar *et al*., 2002) and applied N can be lost through nitrate leaching, resulting in contamination of groundwater (Biswas *et al*., 2000). Overuse of these fertilizers can cause negative environmental impact not only on quality of water and air but also on beneficial microbes present in the soil (Babalola, 2010). These negative environmental effects resulting from continued use of chemical fertilizers can be decreased by application of biofertilizers. Biofertilizers contain plant growth promoting rhizobacteria (PGPR) including P solubilizing bacteria (PSBs), symbiotic and non-symbiotic N_2-fixing bacteria and arbuscular mycorrhizal (AM) fungi (Aseri *et al.,* 2008). PGPR are free-living bacteria present in rhizhosphere of plants that exert beneficial effects on plant growth through a number of diverse mechanisms such as P solubilization, N fixation, repression of soil borne pathogens, production of phytohormones, improving plant stress tolerance to drought, salinity and metal toxicity (Bhattacharyya and Jha, 2011). Many different genera of PGPR have been commercialized for use in agriculture as biofertilizers to reduce the use of synthetic fertilizer.

Microorganisms present in biofertilizers colonize the rhizosphere or the interior of the plant and promote growth by increasing availability of primary nutrients to the host plant when applied to seed, plant surfaces or soil. Biofertilizers consist of selected strains of beneficial soil microorganisms cultured in the laboratory and packed in a suitable carrier material. The use of biofertilizers has gained much importance for enhancing plant growth and yield. Hazardous effects and high cost of chemical fertilizers have contributed to the acceptance of biofertilizers by farmers. Biofertilizers are being promoted as an alternative to synthetic fertilizers to improve plant growth and yield in modern agriculture (Vessey, 2003).

Bacterial strain typing methods have been successfully employed for differentiation and also in detection of inoculated bacteria in the environmental samples (Tacao *et al.,* 2005). These methods include DNA-banding patterns which discriminate the strains on the basis of size and number of DNA bands (fragments) generated by PCR amplification of genomic DNA or by cleavage of DNA using restriction enzymes (Li *et al*., 2009). Due to advances in molecular biology techniques, a large numbers of highly

informative DNA markers have been developed for the identification of genetic polymorphism. PCR-Restriction fragment length polymorphism (PCR-RFLP) involves RFLP analysis of a specific locus amplified by PCR (Wichelhaus *et al.,* 2001). PCR-RFLP has been used frequently for typing a variety of bacteria including *Brucella* species (Dahouk *et al.,* 2005) and *M. tuberculosis* (Cohn and OBrien, 1998). Repetitive sequence based PCR (rep-PCR) uses the primers which bind with many of repetitive sequences present in bacterial genome and produce unique DNA fingerprints of individual microbial strain (Versalovic *et al.,* 1991). Three families of repetitive sequences *i.e*., the 35–40bp repetitive extragenic palindromic (REP) sequence (Stern *et al*., 1984), the 124–127bp enterobacterial repetitive intergenic consensus (ERIC) sequence (Lupski and Weinstock, 1992), and the 154bp BOX element sequences (Koeuth *et al.,* 1995), have frequently been used in rep-PCR. REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting can be used to differentiate bacterial isolates at species, subspecies and strain level because it is extremely reliable, reproducible, rapid and highly discriminatory (Ishii and Sadowsky, 2009). RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers and thus do not require prior knowledge of a DNA sequence (Lin *et al.,* 1996). RAPD produce a genetic profile, which is an array of amplified DNA fragments that is specific for each strain (Wang *et al.,* 1993).

The objective of the present study was the development of DNA-based markers for PGPR strains to be used as inoculants for wheat. The strain-specific DNA banding patterns (markers) were used for differentiation of PGPR at strain level and detection of inoculated PGPR in wheat rhizosphere.

MATERIALS AND METHODS

***Bacterial cultures*:** Bacterial cultures were obtained from NIBGE culture collection (NIBGE Biotech Resource Center, NBRC), Faisalabad (Table 1).

Table 1. Bacterial strains used in the present study.

Sr.	Genus Name	Strain	Host Plant	Accession
1	*Arthrobacter*	WP-2	Wheat	HE661626
2	*Azospirillum*	WS1	Wheat	HE977616
3	*Bacillus*	MP5	Maize	HE646780
4	*Rhodococcus*	M28	Maize	HE646777
5	*Serratia*	5D	Chickpea	HE804807

***Restriction analysis of 16S rRNA gene with different restriction enzymes*:** Total genomic DNA of bacterial strains was isolated by the CTAB method (Wilson, 1987) with modifications. Bacterial cultures were grown in 5 ml LB medium for 24 h and 1.5 ml cultures were centrifuged for 2 min at 13,000 rpm to get cell pellet. The cell pellet was re-suspended in 567 µl TE buffer by vortexing. To lyse the cells, sodium dodecyl sulfate (30 µl of 10% solution) and lysozyme (6 µl of 100 mg/ml solution) were added. After lysis proteinase K (6 µl of 20 mg/ml solution) was added. The samples were incubated at 37°C for 1 h. Then sodium chloride solution (100 µl of 5M solution) was added and mixed thoroughly. CTAB/NaCl solution (80 µl) was added, mixed and incubated at 65°C for 10 min. Chloroform (780 µl) was added, mixed and centrifuged for 10 min. The upper layer was transferred to new Eppendorf tube and again extracted with chloroform (780 µl). After centrifugation for 10 min the aqueous phase was transferred to a new Eppendorf tube and sodium acetate (20 µl of 3M solution) was added and mixed gently. Absolute ethanol (1 ml) was added and incubated at -20°C for 1 h. The samples were centrifuged (13,000 rpm for 10 min) to pellet the DNA. The supernatant was discarded. The pellets were washed with 70% (200 µl) ethanol and the DNA was re-suspended in 50 µl of sterilized water. To each samples RNase (1 µl of 1 mg/ml solution) was added, incubated at 37°C for 1 h and extracted again with chloroform (100 µl). To aqueous phase, sodium acetate (2 µl of 3M solution) and absolute ethanol (100 µl) were added and incubated at -20°C for 1 h. DNA pellet was obtained by centrifugation (13,000 rpm for 10 min), washed with 70% ethanol (200 µl) and re-suspended in 50 µl of sterilized water. In PCR, 16S rRNA gene was amplified using primers PH 5′ AAGGAGGTGATCCAGCCGCA 3′ and PA 5′ AGACTTTGATCCTGCTCAG 3′ (Edwards *et al.,* 1989) and PCR products were purified by using GeneJET™ Extraction Kit (Fermentas, Germany).

Three restriction enzymes (four bases cutters) *i.e*., FastDigest *Hae*III, FastDigest *Msp*I, and FastDigest *Hha*I (Fermentas, Germany) were used to get restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA gene. Each reaction mixture (20 µl) contained 10 µl of purified PCR (about 3 µg DNA) product, 1 µl of restriction enzyme (10U), 3 µl of 10X buffer and 6 µl of sterilized distilled water. The reaction mixture was incubated at 37°C for 3 h. Different fragments generated by restriction enzyme were separated on 1.2% agarose gel in 1X TAE buffer containing ethidium bromide (2 µl of 20 mg/ml for 100 ml gel). DNA ladder, 1kb (Fermentas, Germany) was used as size markers. The gels were run at 20V for 8 h, viewed under UV light and photographed using gel documentation system (Vilbour Lourmat CN-1000-26MX, France).

***Amplification of DNA by using ERIC, BOX and RAPD primers*:** For ERIC-PCR primers ERIC2 5′ AAGTAAGTGACTGGGGTGAGCG 3′ ERIC1R 5′ ATGTAAGCTCCTGGGGATTCAC 3′ (Versalovic *et al.,* 1991) and For BOX-PCR primer BOXAIR 5′ CTACGGCAAGGCGACGCTGACG 3′ (Louws *et al.,* 1994) were used separately in reaction mixtures. A reaction

mixture (25 µl) contained 15 ng template DNA, 2.5 µl of 10X *Taq* polymerase buffer, 2 µl of 5mM dNTPs, 2.5 µl of 25mM $MgCl_2$, 02 µM of each primer and 1U *Taq* DNA polymerase in a 0.25 ml thin-walled PCR tube. The reaction conditions used for PCR were: 94°C for 1 min, followed by 30 cycles of 94°C for one minute, 42°C for 1.5 min and 72°C for 1 min. Finally, the reaction mixture was incubated for 10 min at 72°C. Amplified PCR products were separated on 1.2% agarose gel in 1X TAE buffer containing ethidium bromide (2 µl of 20 mg/ml for 100 ml gel). DNA ladder, 1kb (Fermentas, Germany) was used as a size marker. The gel was run at 20V for 8 h, viewed under UV light and photographed using gel documentation system (Vilbour Lourmat CN-1000-26MX, France).

For RAPD-PCR primers OPI-6 5′ AAGGCGGCAG 3′ and OPI-11 5′ ACATGCCGTG 3′ were used separately in PCR reactions. The reaction conditions were the same as described above for ERIC-PCR and BOX-PCR.

***Effect of bacterial inoculations on wheat grown in earthen pots*:** Selected bacterial strains were used as inoculants for wheat plants grown in earthen pots filled with 10 kg non-sterile soil. There were three replicates of each treatment and two N controls, one with full recommended and other with half recommended N dose. Chemical fertilizer urea was applied as N source (for full recommended N dose control 1.52g/pot and for half recommended N dose control 0.76g/pot). Chemical fertilizer was applied in two split doses. For inoculation of plants, bacterial cultures were grown in 100 ml of LB liquid medium at 30°C for overnight. Ten seeds of wheat variety Sehar-2006 were sown in each pot. After germination, 5 seedlings were maintained in each pot. Inoculum (1 ml) was used to inoculate seeds sown at one location in the pot. Second inoculum was given after germination of seeds. Plants were irrigated when required and harvested at maturity. Data regarding straw weight and grain weight were recorded after drying plants in an oven at 65°C for 2 days.

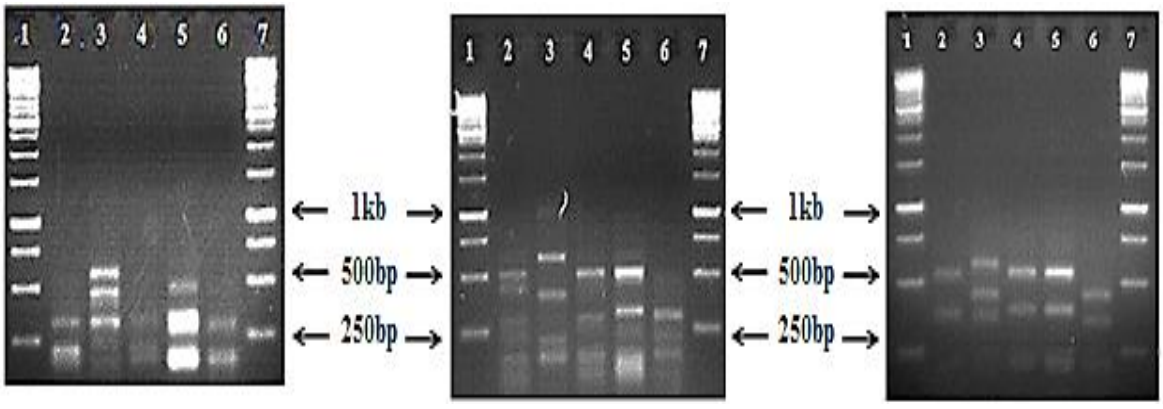
***Estimation of microbial population and re-isolation of inoculated strains in rhizosphere of wheat*:** The microbial population of all treatments in earthen pots was determined four times during the experiments. Rhizospheric soil (5g) was taken from 3 different plants of each treatment and mixed together. One gram soil was added in 9 ml dH_2O and serial dilutions (10X) up to 10^{-5} were prepared. Aliquots (100 µl) of serial dilution (10^{-3}, 10^{-4} and 10^{-5}) were spread on LB agar plates and incubated at 30°C for overnight growth (Somasegaran and Hoben, 1994). Following the standard method, only those plates were counted that contained 30-300 colonies. Colony forming unit (CFU/g soil) was calculated using following formula:

CFU/g soil = No. of colonies X dilution factor

For re-isolation, colonies resembling inoculated strains were selected and transferred to fresh LB agar plates. Desired colonies were picked from LB plates, observed under microscope and reconfirmed by BOX-PCR.

RESULTS

***Restriction analysis of 16S rRNA gene*:** With the restriction enzyme FastDigest *Hae*III the DNA banding pattern was the same for *Serratia* strain 5D, *Bacillus* strain MP5 and *Azospirillum* strain WS1 as two identical DNA bands were detected in these strains. In *Rhodococcus* strain M28 and *Arthrobacter* strain WP-2, three DNA bands were formed but the profiles were distinct due to difference in their size (Fig. 1A). The distinct patterns exhibited by *Rhodococcus* strain M28 and *Arthrobacter* strain WP-2 may be used for identification of these strains. The banding pattern with FastDigest *Msp*I enzyme was same for *Bacillus* strain MP5, *Arthrobacter* strain WP-2 (three DNA bands) and was



A **B** **C**

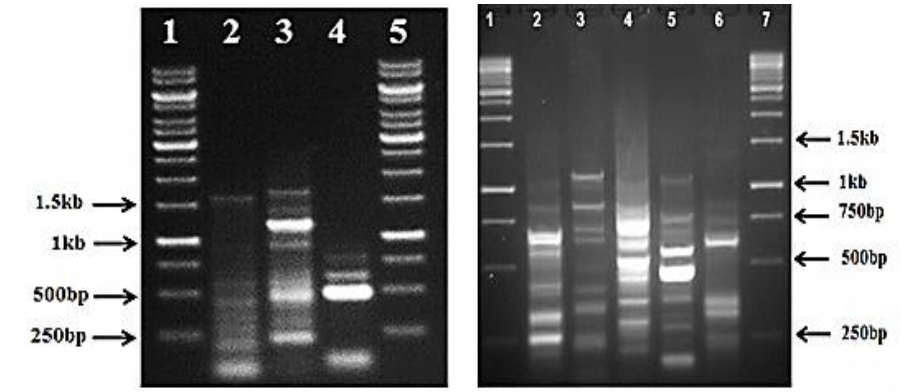
Figure 1. **Restriction pattern of 16S rRNA gene of different bacterial strains with restriction enzyme**. A) FastDigest *Hae*III, B) FastDigest *Msp*I, C) FastDigest *Hha*I. Lane 1: 1kb DNA ladder (Fermentas, Germany); Lane 2: *Serratia* strain 5D; Lane 3: *Rhodococcus* strain M28; Lane 4: *Bacillus* strain MP5; Lane 5: *Arthrobacter* strain WP-2; Lane 6: *Azospirillum* strain WS1; Lane 7: 1kb DNA ladder

different for *Rhodococcus* strain M28 (four DNA bands). The DNA banding profile of *Serratia* strain 5D and *Azospirillum* strain WS1 was also different but DNA bands were very faint (Fig. 1B). From *Bacillus* strain MP5 and *Arthrobactor* strain WP-2, identical DNA banding patterns were obtained (three DNA bands) with FastDigest *Hha*I. Three bands were also obtained from *Rhodococcus* strain M28. From *Serratia* strain 5D and *Azospirillum* strain WS1 two DNA bands were obtained but the profiles were distinct due to difference in their size (Fig. 1C).

***Amplification of DNA by using ERIC and BOX primer*:** In ERIC-PCR *Serratia* strain 5D exhibited only faint DNA bands of different sizes. *Bacillus* strain MP5 also gave specific banding pattern with many DNA bands of different sizes. A very bright band of 1.2kb was observed in *Bacillus* strain MP5. *Arthrobacter* strain WP-2 generated DNA banding pattern with a major band of 500bp (Fig. 2A).

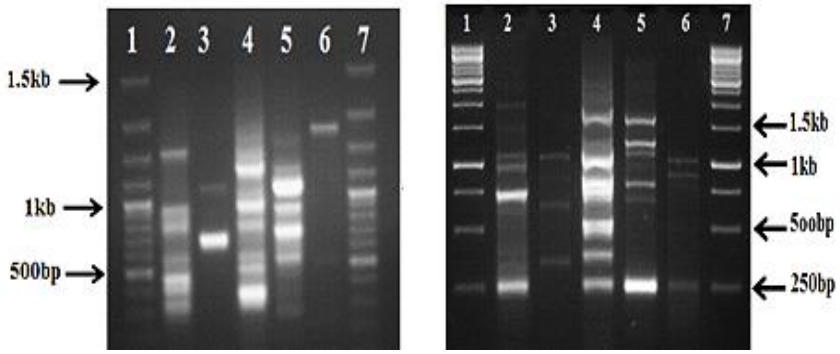
The most differentiating DNA patterns were obtained by the BOX primers (Fig. 2B). *Serratia* strain 5D produced bright as well as faint DNA bands. Three bright DNA bands were detected in the size range of 250bp to 1kb in this strain. *Rhodococcus* strain M28 produced many faint DNA bands ranging from less than 250bp to 1100bp, while *Bacillus* strain MP5 gave many bright bands with the same primer. *Arthrobacter* strain WP-2 exhibited many faint DNA bands and two bright DNA bands in the size range of 450bp to 650bp. From *Azospirillum* strain WS1, faint DNA bands (<1kb) were obtained.

***Amplification of DNA by using random primers OPI-6 and OPI-11*:** The banding patterns of all strains with both primers were totally different. Many bright DNA bands were produced with primer OPI-6 while OPI-11 generated only faint DNA bands (Fig. 3). *Serratia* strain 5D produced many bright bands with primer OPI-6 while with OPI-11 it showed



A **B**

Figure 2. Amplification of DNA of different bacterial strains in rep-PCR. A) ERIC primer; Lane 1: 1kb DNA ladder; Lane 2: *Serratia* strain 5D; Lane 3: *Bacillus* strain MP5; Lane 4: *Arthrobactor* strain WP-2; Lane 5: 1kb DNA ladder, **B)** BOX primer; Lane 1: 1kb DNA ladder; Lane 2: *Serratia* strain 5D; Lane 3: *Rhodococcus* strain M28; Lane 4: *Bacillus* strain MP5; Lane 5: *Arthrobacter* strain WP-2; Lane 6: *Azospirillum* strain WS1; Lane 7: 1kb DNA ladder



A **B**

Figure 3. Amplification of DNA of different strains by using random primers in PCR. A) OPI-6 Primer; Lane 1: 100bp DNA ladder; Lane 2: *Serratia* strain 5D; Lane 3: *Rhodococcus* strain M28; Lane 4: *Bacillus* strain MP5; Lane 5: *Arthrobacter* strain WP-2; Lane 6: *Azospirillum* strain WS1; Lane 7: 100bp DNA ladder, **B)** OPI-11 primer; Lane 1: 1kb DNA ladder; Lane 2: *Serratia* strain 5D; Lane 3: *Rhodococcus* strain M28; Lane 4: *Bacillus* strain MP5; Lane 5: *Arthrobacter* strain WP-2; Lane 6: *Azospirillum* strain WS1; Lane 7: 1kb DNA ladder

two bright bands. *Rhodococcus* strain M28 gave one bright and one faint DNA band with primer OPI-6 and 3 faint DNA bands with primer OPI-11. *Arthrobacter* strain WP-2 and *Bacillus* strain MP5 produced many bright bands with both primers OPI-6 and primer OPI-11.

***Effect of bacterial inoculations on wheat grown in earthen pots*:** Increase in straw weight and grain weight was noticed in all inoculated plants over non-inoculated (half N) control; however these values were less than non-inoculated full N control. Full N control showed maximum values for all growth parameters. After full N control, maximum increase in grain weight (14.3%) and straw weight (19.4%) was obtained in plants inoculated with *Serratia* strain 5D (Fig.4).

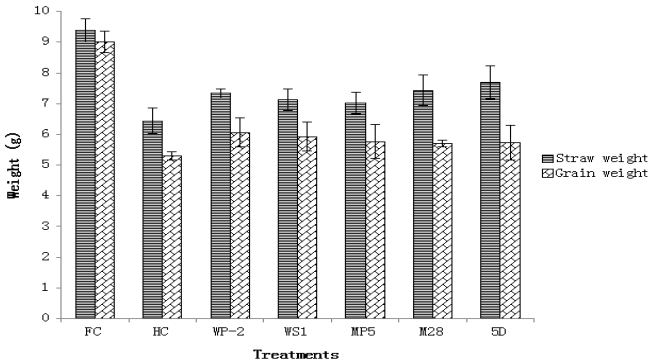


Figure 4. Effect of the bacterial inoculants on wheat grown in earthen pots. The values are average of three replicates. Error bars show standard deviation of mean within the sample. FC: Full N control (non-inoculated); HC: Half N control (non-inoculated); 5D: *Serratia* strain 5D; M28: *Rhodococcus* strain M28; MP5: *Bacillus* strain MP5; WP-2: *Arthrobacter* strain WP-2; WS1: *Azospirillum* strain WS1. All treatments were given with half recommended N dose

***Estimation of bacterial population in rhizosphere of wheat*:** In pot experiment, the microbial population in the rhizosphere of wheat inoculated with *Serratia* strain 5D, *Rhodococcus* strain M28, *Arthrobactor* strain WP-2 and *Azospirillum* strain WS1 increased up to 70 days after sowing and then started to decline while in *Bacillus* strain MP5 it increased up to 83 days after sowing (Fig. 5).

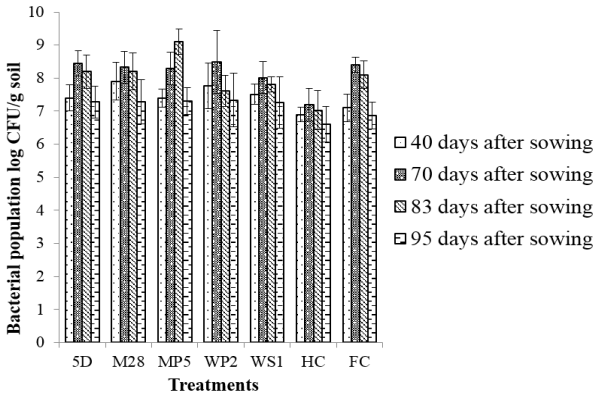


Figure 5. Bacterial population at different growth stages in the rhizosphere of wheat grown in earthen pots. The values are average of three replicates. Error bars show the standard deviation of mean within the sample. 5D: *Serratia* strain 5D; M28: *Rhodococcus* strain M28; MP5: *Bacillus* strain MP5; WP-2: *Arthrobacter* strain WP-2; WS1: *Azospirillum* strain WS1; HC: Half N control (non-inoculated); FC: Full N control (non-inoculated)

***Re-isolation of inoculated strains from wheat rhizosphere*:** *Azospirillum* strain WS1 was identified on the basis of its pinkish colored colony and specific cell motility.

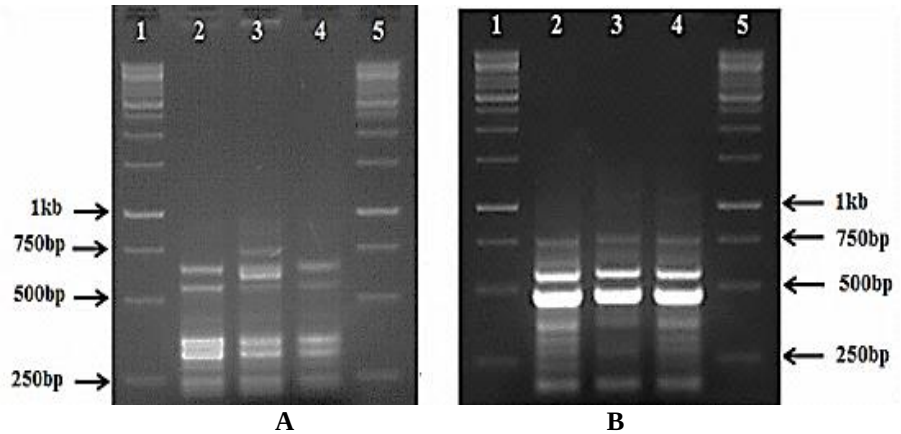


Figure 6. BOX-PCR of re-isolated colonies obtained from the rhizosphere of plants inoculated with; A) *Azospirillum* strain WS1, B) *Arthrobacter* strain WP-2. Lane 1: 1kb DNA ladder; Lane 2: Re-isolated colony; Lane 3: Re-isolated colony; Lane 4: Pure culture colony; Lane 5: 1kb DNA ladder

Arthrobacter strain WP-2 and *Bacillus* strain MP5 were identified by their yellow and white colonies, respectively. *Serratia* strain 5D and *Rhodococcus* strain M28 were easily detected by red and orange colors, respectively.

***BOX-PCR of re-isolated strains from wheat rhizosphere*:** The re-isolated colonies exhibited the same DNA banding profile as that of original pure culture except *Azospirillum* strain WS1. One colony of *Azospirillum* strain WS1 showed the same banding pattern as that of original pure culture while the other re-isolated colony exhibited the different banding pattern. Results of BOX-PCR of *Azospirillum* strain WS1 and *Arthrobacter* strain WP-2 are shown in Fig. 6.

DISCUSSION

For development of DNA markers for bacterial strains, different fingerprinting techniques (PCR-RFLP, rep-PCR and RAPD-PCR) were used in this study. RFLP of rRNA genes has provided useful data in the past with respect to bacterial species and strain description and the technique has been used extensively as a tool for taxonomic and identification purposes (Stull *et al.,* 1988; Gerner-Smidt, 1992). The RFLP analysis of PCR-amplified 16S rRNA genes was used to differentiate bacterial strains in the present study. With the restriction enzyme *Hae*III, the DNA pattern was the same for *Serratia* strain 5D, *Bacillus* strain MP5 and *Azospirillum* strain WS1. However, *Rhodococcus* strain M28 and *Arthrobacter* strain WP-2 showed specific profiles. The DNA banding pattern with *Msp*I was the same for *Bacillus* strain MP5 and *Arthrobacter* strain WP-2 and the pattern was different for *Rhodococcus* strain M28, *Serratia* strain 5D and *Azospirillum* strain WS1. In *Bacillus* strain MP5 and *Arthrobacter* strain WP-2 the profile was identical with *Hha*I, and the profiles of *Rhodococcus* strain M28, *Serratia* strain 5D and *Azospirillum* strain WS1 were unique and specific for the respective strains. Similar restriction patterns of some bacterial strains observed in the present study is due to highly conserved nature of 16S rRNA gene.

All strains were used for ERIC-PCR but the amplification of DNA from *Rhodococcus* strain M28 and *Azospirillum* strain WS1 was not successful. The results of ERIC-PCR showed specific DNA banding patterns of three strains *i.e*., *Serratia* strain 5D, *Bacillus* strain MP5 and *Arthrobacter* strain WP-2. The genetic diversity of *Paenibacillus* populations in the rhizosphere of wheat has been reported by using ERIC-PCR and the fingerprints were highly reproducible (Guemouri-Athmani *et al.,* 2000). The use of ERIC-PCR for rapid and reliable detection of *Arthrobacter* has been reported (Wang *et al*., 2009).

RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing for many microorganisms (Gurtler *et al.,* 2001). In the present study RAPD primers OPI-6 and OPI-11 were used. Strain-specific DNA banding patterns were obtained with both the primers. However, only a single DNA band was obtained from DNA of *Azospirillum* strain WS1 with primer OPI-6 and three faint DNA bands were formed with primer OPI-11. Therefore, these primers were not sufficiently discriminatory for *Azospirillum* strain WS1.

In this study, the most differentiating DNA patterns for all five PGPR strains were obtained by using BOX primers in PCR. Similar results have been reported for *Geobacillus*, *Bacillus* and *Streptomyces* strains, where the BOX-PCR was found to be of greater usefulness than REP-PCR and ERIC-PCR for strain differentiation (Lanoot *et al.,* 2004; Adiguzel *et al.,* 2009).

In plant inoculation experiment, all five bacterial strains increased the straw and grain weights of wheat plants over non-inoculated control. Beneficial effects of inoculation with *Arthrobacter* sp. on of *Pisum sativum* under salt stress has been reported where 18% increase in fresh weight was observed (Barnawal *et al.,* 2014). Qaisrani *et al*. (2014) have reported beneficial effects of *Azospirillum* sp. on seedlings of maize and recorded up to 27% increase in dry weight over non-inoculated control. Inoculation of *Bacillus* sp. has increased the grain yield of wheat over non-inoculated control by 14.8% and 15.7% in pot and field experiment, respectively (Tahir *et al*., 2013). Inoculation with *Rhodococcus* sp. improved the growth and nutrient uptake of pea plant cultivated in soil supplemented with cadmium (Safronova *et al.,* 2006). Hameeda *et al*. (2008) have reported significant increase in grain yield of field-grown maize when inoculated with *Serratia* sp. over non-inoculated treatment.

The microbial population in the rhizosphere of all treatments was determined at four different growth stages. The microbial population in the rhizosphere of all treatments increased up to 70 days after sowing and then started to decline except in *Bacillus* strain MP5. The microbial population in the rhizosphere of plants increased up to 83 days after sowing when *Bacillus* strain MP5 was used for inoculation. Cruz *et al*. (2015) have reported gradual increase in microbial population till 109 days after inoculation.

For studying survival of the inoculated bacterial strains, preliminary identification of colonies recovered from the rhizosphere of inoculated plants was made on the basis of color on LB agar medium. *Arthrobacter* strain WP-2, *Azospirillum* strain WS1, *Bacillus* strain MP5, *Rhodococcus* strain M28 and *Serratia* strain 5D formed yellow, pinkish, white, orange and red colonies, respectively. Banding pattern by BOX-PCR further confirmed the identities of inoculated and re-isolated strains used in this study. Previously Romero *et al.,* (2015) also used BOX-PCR to prove the identity of the inoculated and re-isolated bacterial strains from seeds of tomato.

***Conclusion*:** In the present study DNA-based markers were developed by using 16S rRNA gene restriction patterns, RAPD-PCR, ERIC-PCR and BOX-PCR. BOX-PCR showed most differentiating DNA patterns for all strains. All the strains tested as single-strain inocula resulted in improved growth and yield of wheat plants. The inoculated strains were successfully re-isolated from wheat rhizosphere and confirmed by BOX-based DNA markers. These results indicated that BOX-PCR is useful and highly discriminatory fingerprinting technique for rapid detection of bacterial strains.

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