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Research Paper

Inoculation with Selected Microbial Consortia Not Only Enhances Growth and Yield of *Withania somnifera* but also Reduces Fertilizer Application by 25% Under Field Conditions

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Withania somnifera also known as Ashwagandha is an important medicinal plant used in ayurveda as a rejuvenator and for treatment of various ailments. The roots are rich in withanolides, which is the active constituent of this medicinal plant. Earlier pot culture studies indicated that *Bacillus licheniformis* (Bl) to be the best PGPR and *Aculospora laevis* (Al) to be the best AM fungus and the consortium consisting of both (Bl+Al) is best for inoculating *W. somnifera* under glasshouse conditions. These pot culture experiments were followed by field experiment to validate the results and to find out the possibility of reducing the amount of chemical fertilizer application through the inoculation of selected microbial consortia. The five treatments for the field experiment were 1) Uninoculated control (UC), 2) Microbial consortia of *B. licheniformis* +*A. laevis* (MC), 3) MC+50%NPK, 4) MC+75%NPK and 5)100% NPK (recommended level of NPK fertilizers for this crop, without MC). The results brought out that the plant growth, dry weight, and root yield of *W. somnifera* when treated with MC+75% NPK was significantly higher as compared to UC and was on par with 100%NPK treatment. This suggested that the fertilizer application in *W. somnifera* can be reduced by 25% when applied along with the selected microbial consortia. Tracking of the inoculated PGPR in the rhizosphere soil during the field experiment was also done by using species specific primer for *B. licheniformis* (BL1-FP and BLI-RP) that indicated the presence of inoculated organism in the rhizosphere soil.

Keywords: ***W. somnifera; B. licheniformis; A. leavis;*** **Microbial Consortium; Fertilizer**

Introduction

The increasing need for environmental friendly agricultural practices is driving the use of fertilizers based on beneficial microorganisms. In this context, the reduced use of chemical fertilizers coupled with increased application of biofertilizers is considered to reduce the toxicity in the environment. Even though the inoculation of plants with these microorganisms is a well known practice, the formulation of inocula with a reliable and consistent effect under field conditions is still a bottle neck for their wider use (Malusa *et al*., 2012). It has been observed that although biofertilizers improve yield significantly but their combination with chemical fertilizers gives better results. Saeed *et al*., (2015) showed that combination of biofertilizer and half the quantity of chemical fertilizer treatments resulted in maximum fruit yield in ***Cucumis sativus***. Studies have shown that dual inoculation of an efficient PGPR and AM fungus reduces the application of chemical fertilizers to about 25-50% in the crop plants (Chauhan *et al*., 2015, Thilagar *et al*., 2016). Similar experiments have shown that the combination of biofertilizers with reduced chemical fertilizers have

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given better yield in some of the medicinal and aromatic plants like patchouli (Manjunath *et al*., 2002), and kalmegh (Arpana and Bagyaraj, 2007).

Medicinal plants are used to cure many ailments. Many botanicals and some dietary supplements are good sources of antioxidants and anti inflammatory compounds (Balasubramanian and Palaniappan, 2001). Fertilizer application has been reported to have an influence on the phytochemical quality of medicinal plants. Inorganic fertilizers are said to reduce the antioxidant levels, while organic and biofertilizers enhance antioxidant content in plants (Dumas *et al*., 2003). The role of biofertilizers in partial reduction of N and P fertilizers was revealed by many workers such as Safwat and Badran (2002) on Cumin, Hassan (2009) on Roselle and Soliman *et al*. (2012) on Acacia. Increase in the quality of medicinal products and also reduction in 25-50% of the recommended dose of NPK was observed by several workers when the application was complimented with mixture of biofertilizers in plants such as *Hibiscus* (Abo-Baker and Mostafa, 2011), *Nigella sativa* (Shaalan, 2005) and *Ammi visnaga* (Yuonis*et al*., 2004).Earlier we had screened several AM fungi and PGPR and selected the best organism that improved the growth of *W. somnifera* under pot culture conditions (Anuroopa and Bagyaraj, 2015; 2017a). Later dual inoculation with the selected AM fungus (*Acaulospora laevis*) and PGPR (*Bacillus licheniformis*) enhanced the growth of *W. somnifera* significantly compared to inoculation with either of them under pot culture conditions (Anuroopa and Bagyaraj, 2017b). In the present study validation of the pot culture trial under field conditions and their ability to reduce the application of chemical fertilizers on *W. somnifera* was investigated.

Microorganisms introduced into the environment undergo growth, physiological adaptation, conversion to non-culturable cells, physical spread, and gene transfer (Van Elsas *et al*., 1998). Recent developments in the techniques for studying rhizobacterial communities; detecting and tracking the inoculated bacteria are important in future application and assessment of effectiveness and consistent performance of microbial inoculants in crop production and protection.

Developments in molecular tracking techniques have greatly increased detection of inoculated microorganisms (Pickup, 1991). Techniques used for detection of inoculated microorganisms in the soil include use of DNA probes (Holben *et al*., 1988), PCR (Ruppel *et al*., 2006) and use of selective markers (Jefferson, 1989). All of the above techniques require extraction of cells and removal of humic material prior to DNA extraction.Several approaches of this type have been reported with various degrees of success (Yeates *et al*., 1998). For detection of wild-type microorganisms released in the environment, molecular monitoring methods based on PCR techniques using natural genome polymorphism have largely facilitated the discrimination at genus and strain level, thus minimizing the drawbacks of introduced markers (Mahaffee *et al*., 1997). Natural polymorphism can be detected by the use of random amplified polymorphic DNA (RAPD) procedure (Felici *et al*., 2008) or by the use of species specific primers (Vinod *et al*., 2014) in which a very small amount of total DNA is subjected to PCR using a synthetic oligonucleotide of sequence specific primer. In fact, depending on the type of primer used and on the conditions of the PCR reaction, amplification of bacterial genomes can generate DNA fragments that are diagnostic for a genus, species, or even a particular strain (Vinod *et al*., 2014).

Therefore the primary objective of this investigation was to determine the efficiency of the selected microbial consortium (*B. licheniformis+A. laevis*) in reducing the input of chemical fertilizers under field conditions and to identify the presence of inoculated PGPR *B. licheniformis* by PCR based detection method using primer specific for *Bacillus licheniformis* BL1-FP 5' GCG CGG ATG GAA TTC AAC CTG ATT 3' and BL1-RP 5' AGT TTG GGA GCT TGA AGC CGA CTA 3' (Vinod *et al*., 2014).

Material and Methods

Field Investigation

This investigation was carried out during *kharif* (monsoon season; June-December) 2015-2016 to study the effect of microbial consortia (selected from the pot culture studies) in conjunction with different concentrations of NPK fertilizers on growth, plant nutrition, biomass, total withanolide concentration of *W. somnifera* raised under field conditions at Central Institute of Medicinal and Aromatic Plants, Bengaluru.

During the cropping season Bengaluru had a temperature ranging from 30ºC (maximum) to 21ºC (minimum), humidity ranging from 65 to 74% and an average precipitation of 106 mm. The experimental area was prepared and brought to a fine tilth and arranged in a randomized block design, with four replications of the following five different treatments:

T1: Uninoculated control (UC);

T2: Microbial consortia (MC);

T3: MC + 50% NPK;

T4: MC + 75% NPK;

T5: 100% NPK (recommended level of fertilizer for cultivation of *W. somnifera*, without MC)

The soil sample from a depth of 0-20 cm was collected from the experimental site before imposing treatments and the soil was analyzed for various physico-chemical properties by adopting appropriate methods. The field soil was red sandy loam witha 6.8 pH, 0.08% organic carbon, 279.64 kg/ha available nitrogen, 7.5 kg/ha available phosphorus and 180 kg/ha available potassium. The size of each plot was 2.4 m×3.6 m. The total size of the experimental area used was 14 m×16 m including all the treatments containing 20 plots. Farmyard manure (18.5Kg/plot) and vermicompost (1.25Kg/plot) applied and mixed thoroughly with soil. The recommended level of fertilizer for *W. somnifera* cultivation is 30:30:30 kg of N:P_2O_5:K_2O ha^{-1} respectively. The fertilizers were applied to the soil as per the treatment; nitrogen in form of urea, phosphorus in the form of single super phosphate (SSP), and potassium in the form of muriate of potash (MoP). Urea contained 46% nitrogen, SSP contained 16% P_2O_5 and MoP contained 60% of K_2O. The amount of urea, SSP and MoP applied to each plot in case of 100% NPK treatment (T5) were 65.22, 187.5 and 50kg/ha^{-1} respectively. In case of T3 and T4, 50% and 75% of these fertilizers were applied respectively. Half of the recommended dose of N was added at the time of planting while remaining half of N was added after two weeks.

Microbial Consortia Inoculation

The liquid inoculum of *B. licheniformis* was produced (1.5 L) in LB broth and the liquid inoculum containing cfu 2×10^8 was mixed with *A. laevis* mycorrhizal inoculum (3 kg) with infective propagule numbers of 2.8×10^3/g determined by the method of Porter (1979). Ten grams of microbial consortia was applied to each planting hole excepting UC and 100% NPK. The most commonly used cultivar of *W. somnifera* (Poshita) was obtained from CIMAP, Bengaluru, India. Thirty day-old, uniform size seedlings were transplanted to the main field with spacing of 60 cm between rows and 30 cm between plants. Plants were irrigated whenever necessary.

Plant Parameters Studied

Twenty plants from each treatment were randomly selected to study the parameters. The plant parameters including plant height, stem girth, biovolume index (BI), dry weight of shoot and root, total plant dry weight, shoot and root NPK concentrations and total withanolide concentration in the root were determined. Plant height was measured from soil surface to the growing tip of the plant and stem girth was measured one cm above the soil surface using digital Vernier calipers during harvesting. Biovolume index (BI) (a measure of total volume of a plant) was determined using the formula 'Biovolume index = Plant height (cm) × Stem girth (mm)' given by Hatchell (1985). The plants were uprooted on 140 DAT. Dry weight of shoot and root was determined after drying in an oven at 60ºC for 48 h. The concentration of N, P and K in the shoot and root was determined by methods outlined by Jackson (1973). Total withanolide concentration in roots was determined by HPLC (Manwar *et al*., 2012).

Mycorrhizal parameters such as percent root colonization and spores in the root zone-soil were determined. The roots were cut into 1 cm bits, washed, cleared with KOH, acidified with HCl and stained with trypan blue in lacto glycerol. The stained roots were observed under microscope for determining per cent mycorrhizal colonization (Philips and Hayman, 1970). The AMF spore numbers in the root zone-soil was determined by wet sieving and decantation method (Gerdemann and Nicolson, 1963).

The root region- soil samples were collected from the various treatments for microbiological and biochemical analysis. The rhizosphere population of *Bacillus* species was enumerated by the standard dilution plating technique (Johnson and Curl, 1972) after subjecting the diluents to 80ºC in a water bath

for 10 min and plating on LB medium. The colony number and characteristics were noted and the population was expressed as cfu g^{-1} of soil. The total bacterial population in the rhizosphere soil was also enumerated using nutrient medium

Soil Parameters Studied

Soil pH and electrical conductivity were measured in a 1:2.5 (w/v) aqueous solution. Total organic carbon (Anderson and Ingram, 1989), available nitrogen, phosphorus and potassium were determined by the standard methods (Jackson, 1973). Soil enzyme activities such as dehydrogenase, acid and alkaline phosphatase were estimated using standard procedures outlined by Casida and Klein Santoro (1964) and Eivazi and Tabatabai (1977).

Statistical Analysis

The data collected in the field study was subjected to statistical analysis suitable to CRBD using the ASSISTAT (7.7 beta) software. Data were subjected to the analysis of variance at significant level (<0.05) and means were compared by Duncan's multiple range test.

Tracking of Inoculated PGPR in the Field

Collection of Soil Samples

Five samples from each plot of the same treatment were randomly collected from the rhizosphere region of field soil and pooled to form composite sample from each treatment. These samples were homogenized and spread in trays to be cleaned of extraneous materials (pieces of root, leaves, small stems, etc.) followed by drying and storing in plastic containers. When used for analysis, the samples were sieved with 2-mm mesh sieves and used.

Isolation of Bacterial DNA from Soil (Fast DNA™ SPIN Kit)

500mg of soil sample and 978 µl Sodium Phosphate Buffer were taken in a Lysing Matrix E tube and vortexed for 10-15 seconds. 122 µl MT Buffer was added and shaken vigorously to mix and then vortexed for 10-15 seconds. The mixture was then homogenized in the FastPrep@ Instrument for 40 seconds at a speed setting of 6.0. Later the sample was centrifuged at 14,000 g for 5-10 minutes and the supernatant was transferred to a clean 2.0 ml microcentrifuge tube. 250 µl PPS (Protein Precipitation Solution) was added and mixed by shaking the tube 10 times manually, then incubated at room temperature for 10 minutes without vortexing. The pellet was precipitated by centrifuging at 14,000 g for 5 minutes. Later the supernatant (600-800µl) was transferred to a clean 2.0 ml microcentrifuge tube. Equal amount of Binding Matrix was added to the microcentrifuge tube and shaken gently by hand for 3-5 minutes to allow binding of DNA to matrix. The mixture was centrifuged at 14,000 g for 2 minutes and the supernatant was discarded. 1 ml of 6 M Guanidine Isothiocyanate was added, resuspended and centrifuged twice at 14,000 g for 2 minutes. The solution was mixed by pipetting up and down several times. 800 µl of solution was transferred to a SPIN™ Filter tube and centrifuged at 14,000 g for 5 minutes. Catch tube was emptied. Mixing, transferring and centrifuging were repeated for the remaining solution to the same SPIN™ Filter tube. The flow-through was discarded. 500 µl of prepared SEWS-M was added to the SPIN™ Filter tube and mixed gently by hand and centrifuged at 14,000 g for 5 minutes. The catch tube was emptied and centrifuged again for 5 minutes to remove the residual ethanol. The SPIN™ Filter was transferred to a clean 2.0 ml catch tube and air dried for 5 minutes at room temperature. 100 µl DES was added to the SPIN™ Filter tube and gently resuspended the pellet by finger flicking. The mixture was centrifuged at 14,000 g for 2 minutes to elute the DNA into the catch tube, the SPIN filter was discarded. DNA now ready for PCR and other downstream applications was stored at –20°C for extended periods or 4°C until use.

PCR Amplification of Isolated Soil DNA Using 16S rRNA Primers for Bacterial Identification

The 16S rDNA amplification was performed with Forward primer (27f)-AGAGTTTGATCTGGC TCAG and Reverse primer (1492r)-TACGGYTACC TTGTTACGACTT (Sigma-Aldrich, India). The PCRs were performed in a 25 µl reaction volume containing 16 µl PCR grade water (Sigma), 2.5 µl PCR buffer (10×), 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 1 µl of each primer (20 pmol/µl), 1 µl (5 U/µl) ofTaq polymerase (Sigma-Aldrich) along with 20-50ng of template DNA. PCR was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg). The amplification program consisted of

an initial denaturation step at 94°C for 3 min followed by 32 cycles of denaturation at 94°C for 45 seconds, annealing for 60 seconds at 51°C and extension for 90 seconds at 72°C. A final extension step at 72°C for 10 min was included at the end of the amplification. All PCR products were electrophoresed, imaged and analyzed in a Gel Documentation System (Syngene Inc. Cambridge).

Amplification of DNA Using Species Specific Primers for Bacillus Species

Species specific primers for *Bacillus licheniformis*, BL1-FP 5' GCG CGG ATG GAA TTC AAC CTG ATT 3' and BL1-RP 5' AGT TTG GGA GCT TGA AGC CGA CTA 3' were used for amplification of total DNA obtained from inoculated soil. The reaction mixture (25 µl) contained PCR buffer, 1X (Sigma-Aldrich); 3 mM MgCl2; 0.2 mM dNTP mix; 0.5 U *Taq* DNA polymerase; 10 pmol primer, and 100 ng template DNA. The PCR was performed with initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of 94ºC for 1 min., primer annealing temperature ranging from 45ºC to 65ºC for 30 seconds followed by 72ºC for 2 minutes and final extension at 72ºC for 6 minutes. PCR product was resolved in agarose (2%) gel electrophoresis with ethidium bromide staining.

Cloning and Sequencing

A 1410 kb amplicon obtained with species specific primers was eluted from the agarose gel and purified using a Gel purification kit (Sigma Corporation, USA) according to the manufacturer's recommendations. The purified fragment was cloned in the pGEM®-T Easy Vector Systems (Promega) and transformed into competent *Escherichia coli* strain DH-5α (Sambrook *et al*., 1989). The cloning of the amplicon was confirmed by restriction with *Nco*I. The insert was sequenced using ABI PRISM Big Dye Terminators v3.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing BL1-FP primer.

Insilico Analysis

The sequenced DNA sequence (861 nucleotides) from the selected bacterial strain has been analyzed using *insilico* approach. BLAST search (Altschul *et al*., 1990) was performed using the easily accessible and publically available webpage National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) against nr database. To obtain the homologous sequences and understand its similarities with other genomes, a phylogenetic tree was constructed. To understand the domain and family of the query gene NCBI conserved domains database (CDD) (Marchler-Bauer *et al*., 2011) search was performed.

Results

Field Investigation

(a) *Plant growth parameters* : The aim of field study was to investigate the different levels of recommended chemical fertilizers along with the selected microbial consortia (*B. licheniformis+A. laevis*) in order to know the possibility of reducing the recommended level of chemical fertilizer with no adverse effect on growth, plant nutrition and total withanolide concentration of *W. somnifera*. The influence of microbial consortia and different levels of fertilizers on plant height, stem girth and BI of *W. somnifera* is given in Table 1. The results of the field experiment on plant growth parameters showed that plants inoculated with MC with reduced level of fertilizers did not differ significantly from plants with recommended dosage of fertilizers without MC. There was no significant difference seen in the height in all the treatments. However the plants treated with MC+75% NPK showed significant difference in stem girth and BI when compared to UC, but was statistically on par (which means not differing statistically) with the other treatments. Shoot, root and dry total plant dry weight were higher in MC+75% NPK and were on par with 100% NPK and MC+50%NPKdiffering significantly from the microbial consortia alone and UC treatments (Table 1).

Nutrient Parameters Studied

The shoot and root N concentration did not differ significantly among the treatments; however there was significant variation in the P and K concentration. The highest shoot P concentration was recorded in the MC treatment and differed significantly from other treatments. The highest shoot K concentration was recorded in the MC+50% NPK treatment, differing significantly from other treatments (Table 2). Root P and K concentrations were same in MC+75% NPK

Table 1: Influence of microbial consortia at varying concentrations of chemical fertilizers on growth parameters of *W. somnifera* under field conditions

Treatments	Height (cm/plant)	Girth (mm/plant)	Bio volume index	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Total dry weight (g/plant)
UC	48.5	9.97^{b}	490.21^{b}	8.78^{b}	4.53^{b}	13.31^{b}
MC	49.5	11.08^{ab}	565.64^{ab}	8.96^{b}	4.40^{b}	13.36^{b}
MC+50NPK	50.85	11.34^{ab}	593.75^{ab}	13.11^{ab}	5.30^{ab}	18.42^{ab}
MC+75NPK	53.40	13.04^{a}	704.31^{a}	14.58^{a}	7.50^{a}	22.08^{a}
100NPK	53.30	12.58^{a}	685.69^{a}	12.64^{ab}	6.09^{ab}	18.74^{ab}
	NS					

Note: Values in each column followed by the same letter are not significantly different at P = 0.05, UC = Uninoculated control; MC = *Bacillus licheniformis +Acaulospora laevis*; 100NPK=Recommended level of chemical fertilizer for *W. somnifera*, without MC

Table 2: Influence of microbial consortia at varying concentrations of chemical fertilizers on shoot and root NPK and total withanolide concentrations in *W. somnifera* under field conditions.

Treatments	Shoot			Root			Total withanolide (%)
	N%	P%	K%	N%	P%	K%	
Control	2.60	0.54^{b}	0.69^{c}	1.91	0.53^{c}	0.85^{c}	0.14^{c}
MC	2.65	0.56^{a}	0.65^{d}	1.88	0.58^{b}	1.02^{b}	0.27^{ab}
MC +50NPK	2.59	0.53^{bc}	0.81^{a}	2.02	0.58^{b}	1.14^{b}	0.20^{bc}
MC+75NPK	2.59	0.54^{b}	0.75^{b}	1.66	0.66^{a}	1.42^{a}	0.31^{a}
100NPK	1.59	0.14^{b}	0.75^{b}	1.66	0.65^{a}	1.42^{a}	0.28^{c}
	NS			NS			

Note: Values in each column followed by the same letter are not significantly different at P = 0.05, UC = Uninoculated control; MC = *Bacillus licheniformis +Acaulospora laevis*; 100NPK=Recommended level of chemical fertilizer for *W. somnifera*, without MC

and 100% NPK treatments and differed significantly from other treatments and UC plants.

The total withanolide concentration in the roots was found to be significantly high in plants treated with MC+75% NPK and on par with MC treatment, but differing significantly from the other treatments. The least withanolide concentration was recorded in UC plants (Table 2).

Mycorrhizal Parameters

Percent mycorrhizal root colonization was highest in MC(alone) treated plants but not differing significantly from MC+50%NPK and MC+75% NPK treatments respectively. Mycorrhizal colonization was significantly less in UC as well as 100% NPK treatments. Mycorrhizal spore numbers in the root zone soil was highest in MC treatments and was on par with MC+50% NPK treatments but varied significantly from other treatments (Table 3).

Soil Bacterial Population as Influenced by Different Treatments

The results of the total bacterial population in rhizosphere soil indicated that they were significantly higher in MC treatment followed by MC+75% NPK and MC+50%NPK, the three treatments not differing statistically. The least number of bacteria were encountered in 100% NPK treatment and UC. *Bacillus* species population was also significantly high in MC treatment and was statistically on par with MC+75% NPK and MC+50% NPK treatments (Table 3).

Table 3: Influence of microbial consortia at varying concentrations of chemical fertilizers on mycorrhizal root colonization and mycorrhizal spore numbers, total bacterial and *Bacillus* population in root soil of *W. somnifera* under field conditions

Treatments	Mycorrhizal root colonization(%)	Mycorrhizal spore count/50g soil	Bacterial population (10^8 cfu/g of soil)	*Bacillus* (10^5 cfu/g of soil)
UC	44.9[b]	272.66[c]	15[b]	3[b]
MC	75.5[a]	466.66[a]	63[a]	18[a]
MC+50NPK	56.1[ab]	389.66[ab]	47[a]	9.66[ab]
MC+75NPK	57.5[ab]	343.00[bc]	47.66[a]	10[ab]
100NPK	45.5b	304.66[bc]	16.66[b]	3.66[b]

Note: Values in each column followed by the same letter are not significantly different at P = 0.05, UC = Uninoculated control; MC = *Bacillus licheniformis +Acaulospora laevis*; 100NPK=Recommended level of chemical fertilizer for *W. somnifera*, without MC

Soil Enzyme Activities as Influenced by Different Treatments

The dehydrogenase activity in the soil ranged from 51.36 to 63.44 μg of 2, 3, 5-triphenyl formazan (TPF) released g^{-1} of soil h^{-1}.The activity of this enzyme was significantly affected by different treatments. Higher dehydrogenase activity was observed in the treatment MC+50%NPK which differed statistically from all other treatments; and the UC showing the least dehydrogenase activity. Higher activity of acid phosphatase was recorded in the treatmentMC+75% NPK which was on par with MC alone treatment but the two differed significantly from other treatments. The alkaline phosphatase was significantly higher in MC+75% NPK and MC+50% NPK treatments and differed significantly from MC alone and UC treatments. The least alkaline phosphatase activity was recorded in the UC treatment (Table 4).

Physico-chemical Characteristics of Rhizosphere Soil at Harvest

It was observed that the pH of the soil was not affected significantly by different treatments. The pH ranged from 6.9-7.3. Electrical conductivity which is the measure of the soluble salts concentration in the soil at a given temperature was found to be significantly higher in MC+50% NPK treatment compared to all other treatments. The soil organic carbon concentration improved significantly in all the treatments with MC compared to the UC and 100% NPK treatment. Initially it was 0.08% and later it increased up to 0.21% in the treatments MC+75% NPK and MC alone. The UC plot and 100% NPK, where microbial consortia were not applied, had 0.12% OC content at the end of the experiment (Table 5). The available N was found to be highest in 100% NPK treatment and least was recorded in UC and MC alone treated soils. There was no significant

Table 4: Influence of microbial consortia at varying concentrations of chemical fertilizers on soil enzyme activities of *W. somnifera* under field conditions

Treatments	Dehydrogenase (μg of TPF released g^{-1} of soil h^{-1})	Acid phosphatase (μg of p-nitrophenol released g^{-1} of soil h^{-1})	Alkaline phosphatase (μg of p-nitrophenol released g^{-1} of soil h^{-1})
UC	51.36[e]	27.3[c]	34.5[d]
MC	58.52[c]	32.7[ab]	37.3[c]
MC+50NPK	63.44[a]	27.4[c]	39.4[ab]
MC+75NPK	60.42[b]	33.6[a]	40.6[a]
100NPK	54.61[d]	31.3[b]	38.0[bc]

Note: Values in each column followed by the same letter are not significantly different at P = 0.05, UC = Uninoculated control; MC = *Bacillus licheniformis +Acaulospora laevis*; 100NPK=Recommended level of chemical fertilizer for *W. somnifera*, without MC

Table 5: Influence of microbial consortia at varying concentrations of chemical fertilizers on soil physicochemical properties at harvest of *W. somnifera* under field conditions

Treatments	Soil pH	EC(ds/m)	OC(%)	N(Kg/ha)	P_2O_5*(Kg/ha)	K_2O(Kg/ha)
UC	7.3	0.07^b	0.12^c	392.0^c	10.0	190^d
MC	6.9	0.08^b	0.21^a	392.0^c	12.5	200^b
MC+50NPK	6.7	0.12^a	0.18^b	470.4^b	12.5	200^b
MC+75NPK	6.9	0.08^b	0.21^a	470.4^b	12.5	235^a
100NPK	7.1	0.07^b	0.12^c	548.8^a	12.5	195^c
	NS				NS	

Note: Values in each column followed by the same letter are not significantly different at P = 0.05, UC = Uninoculated control; MC = *Bacillus licheniformis +Acaulospora laevis*; 100NPK=Recommended level of chemical fertilizer for *W. somnifera*, without MC
OC: Organic carbon; EC: Electrical conductivity, *NS = Not significant

difference in the available P in the different treatments. The K availability significantly improved in the MC+75% NPK treatment compared to other treatments at the time of harvest. Application of microbial inoculants along with fertilizers showed significant improvement in K availability as compared to UC and 100% NPK treatments (Table 5).

Tracking of Inoculated PGPR in the Field

Based on pot culture studies done earlier, a microbial consortia consisting of *A. laevis* + *B. licheniformis* was selected as the best for its growth promotion activity on *W. somnifera*. Validation of the results of pot culture study under field condition was one of the objectives of the present study. Further tracking down the inoculated *B. licheniformis* in the rhizosphere soil at harvest is another objective of the present study. Tracking of this strain would require specific analytical methods for monitoring and quantification in the environment.

DNA isolation of the rhizosphere soil sample was done (Fig. 1A) and the efficiency of this extraction protocol was confirmed by polymerase chain reaction amplification of the 16srDNA gene (Fig. 1B).

Later species specific primer for *B. licheniformis* was used for amplification. The genetic analysis by using species specific primers BL1-FP 5' GCG CGG ATG GAA TTC AAC CTG ATT 3' and BL1-RP 5' AGT TTG GGA GCT TGA AGC CGA CTA 3' led to the generation of 1410Kb fragment (Fig. 1C), which was purified and cloned in pGEMT Easy vector and transformed into *E. coli* DH5α (Fig. 1D). White colonies were selected as transformed clones and the isolation and purification of the plasmid DNA were carried out using a plasmid purification kit (Sigma Corporation, USA) according to the manufacturer's recommendations.

The plasmid containing the desired amplicon was sequenced (Fig. 2A) and using the BLAST search at NCBI, the query sequence has been predicted to belong to *B. licheniformis* group of bacteria. The BLAST hits predicted top nine *B. licheniformis* group bacteria (Fig. 2B and 3). This result indicated that the query gene belongs to the *Bacillus* group of bacteria. The CDD analysis predicted the query gene belongs to the Aldo/keto reductase superfamily, which is characteristic of *B. licheniformis* group of bacteria (Fig. 4). The phylogenetic analysis revealed that the query gene belonged to the firmicutes phyla of bacteria (Fig. 5A).

Discussion

Influence of Microbial Consortia and Fertilizers on W. somnifera Under Field Condition

The main objective of the study was to investigate the performance of the selected microbial consortia for *W. somnifera* under field conditions. To develop sustainable agricultural practice, it is necessary to validate the performance of the selected microbial consortia (through pot culture studies) under field conditions (Ahmad *et al*., 2008). The second objective was to evaluate the interaction of microbial consortia in reducing the field application of NPK fertilizer. Reduction in the fertilizer application with AM fungi alone or together with PGPR, without sacrificing yield component, has been reported by earlier workers in

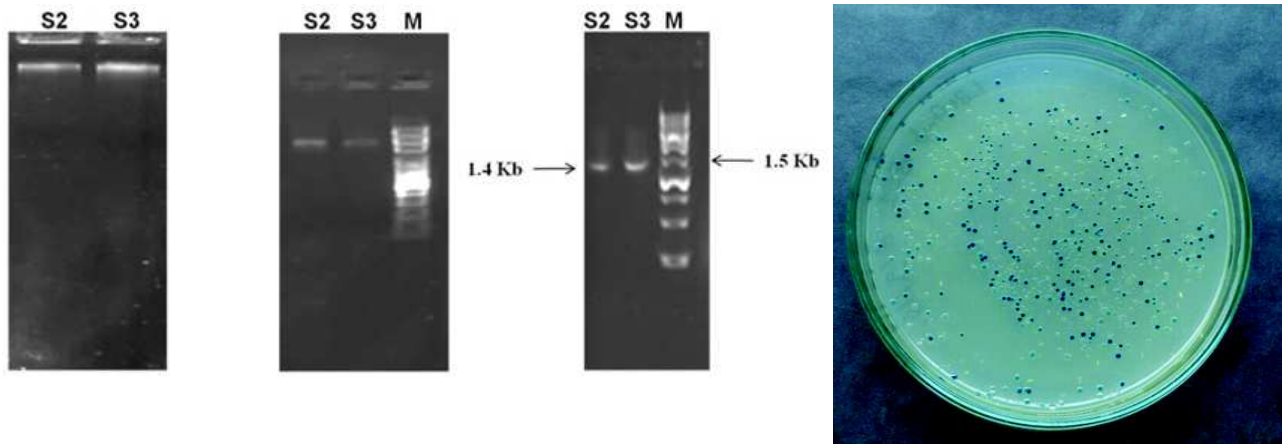


Fig. 1: A-D: (A) Total genomic DNA of representative soil sample S2 containing consortium and genomic DNA S3 of *B. licheniformis*; (B) PCR amplification of 16S rDNA region from total genomic DNA of representative soil sample S2 and genomic DNA S3 of *B. licheniformis*; (C) PCR amplified product from total genomic DNA by *B. licheniformis* specific primer of representative soil sample S2 and genomic DNA S3

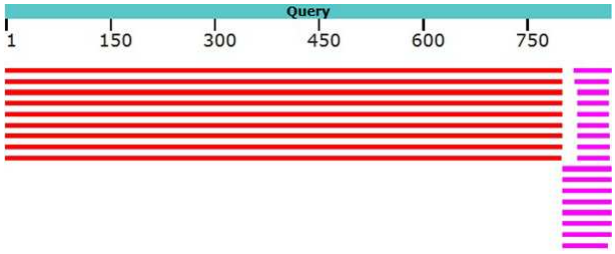
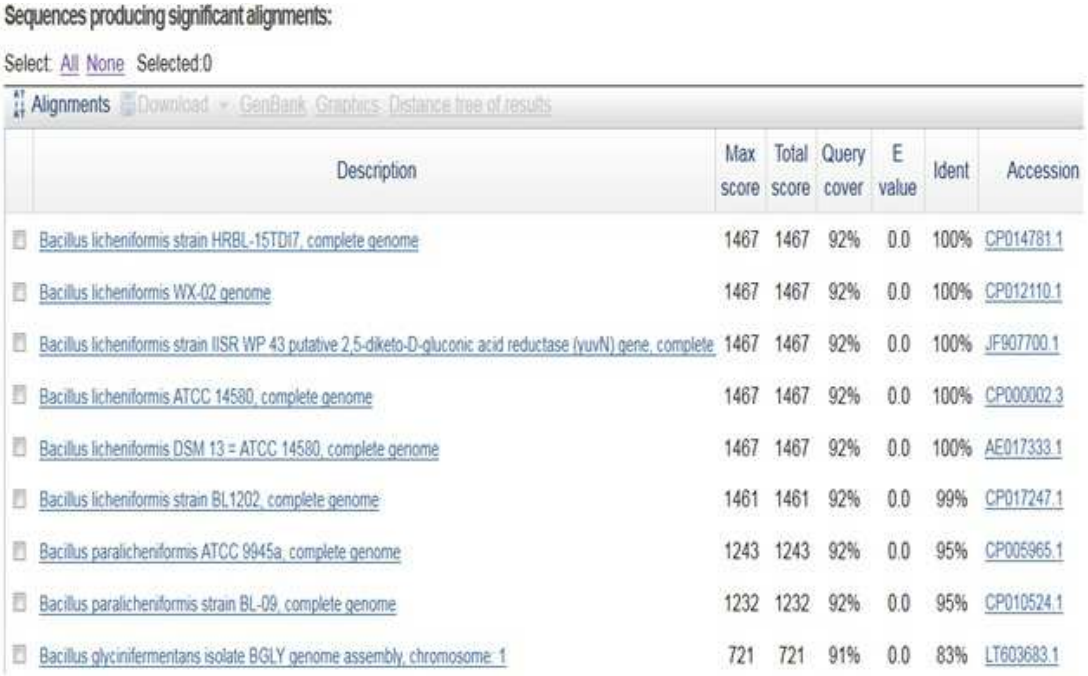


Fig. 2: (A) Sequence of amplified DNA Fragment using BL-1(FP) &BL-1(RP) Nucleotide sequence (863 letters)

>1st_BASE_2431709_Sample_4_Primer_4
>5'CTTTCCAAATGGTGGACATTGAAATTGCTGACTCCGATCGCTTTCACTCTGCCGTCTT
TGTACAGCTTTTCAAGTGCGCGCCACGTATCTTTATATTTGTCTTTTCCTGGCCAATGAAT
CAAATATAAATCAAGGTAGTCAAGGCCGAGTTTTCGAAGGCTTTCATCAAAAGCCGCCA
ATGTTTTTTCATATCCCTGGTCACTGTTCCAAACCTTTGACGTGATGAAAAGCTCTTCGC
GGGGAACGCCTGATTCCTTAATCCCTTTGCCGACGCCTTCTTCATTTTGATAGATAGCGG
CTGTGTCAATGCTCCGGTATCCGTTTTTAATGGCTGCTTTTACAGATTCAACGACTTGGC
TGCCTTCTTCCACTTTGAAAACTCCAAGTCCAAACCATGGCATTTTCACACCGTTATGTA
ATGTCACAGTGTCCTTTAAACTGCTTACCATTTTGAACGACCACCTTTCTTTTTTTCAGAT
GCTTTTAAACGCACCGCTTTTATTATCTCACACTGGGTGGATTTTACAAAACAACTTGCT
CAACCGATGGAATGAGCCAAAGCAAGCGCTCCGGTGATTCCCTGATCGTCGCCAAGGCC
CGGCGGCACGATGTATTCTTCAAGATCAGGCAGGTCGACATATCCGTTCAAATATTCGG
CCAGCTTTTTGCGAATCAGCGGGAACAGCTGCTGCTGCTTCATGACACCGCCGCCCATG
ATGATTTTTTCAGGGCACAGAATCAAGATATATTGCATGAGGGCCTGTGCTAAATAGTC
GGCTTCAAGCTCCCAAACTAAGGGCGAATTCCACATTGGGCTGCAGCCCGGGGGATCCA
CTAGTTCTAGAGCGGCCGCACCGCGGGAG3'

Fig. 2: (B) NCBI BLAST search top hits output


Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain HRBL-15TDI7, complete genome	1467	1467	92%	0.0	100%	CP014781.1
Bacillus licheniformis WX-02 genome	1467	1467	92%	0.0	100%	CP012110.1
Bacillus licheniformis strain IISR WP 43 putative 2,5-diketo-D-gluconic acid reductase (yuvN) gene, complete	1467	1467	92%	0.0	100%	JF907700.1
Bacillus licheniformis ATCC 14580, complete genome	1467	1467	92%	0.0	100%	CP000002.3
Bacillus licheniformis DSM 13 = ATCC 14580, complete genome	1467	1467	92%	0.0	100%	AE017333.1
Bacillus licheniformis strain BL1202, complete genome	1461	1461	92%	0.0	99%	CP017247.1
Bacillus paralicheniformis ATCC 9945a, complete genome	1243	1243	92%	0.0	95%	CP005965.1
Bacillus paralicheniformis strain BL-09, complete genome	1232	1232	92%	0.0	95%	CP010524.1
Bacillus glycinifermentans isolate BGLY genome assembly, chromosome: 1	721	721	91%	0.0	83%	LT603683.1

Fig. 3: NCBI BLAST predicted top hits output

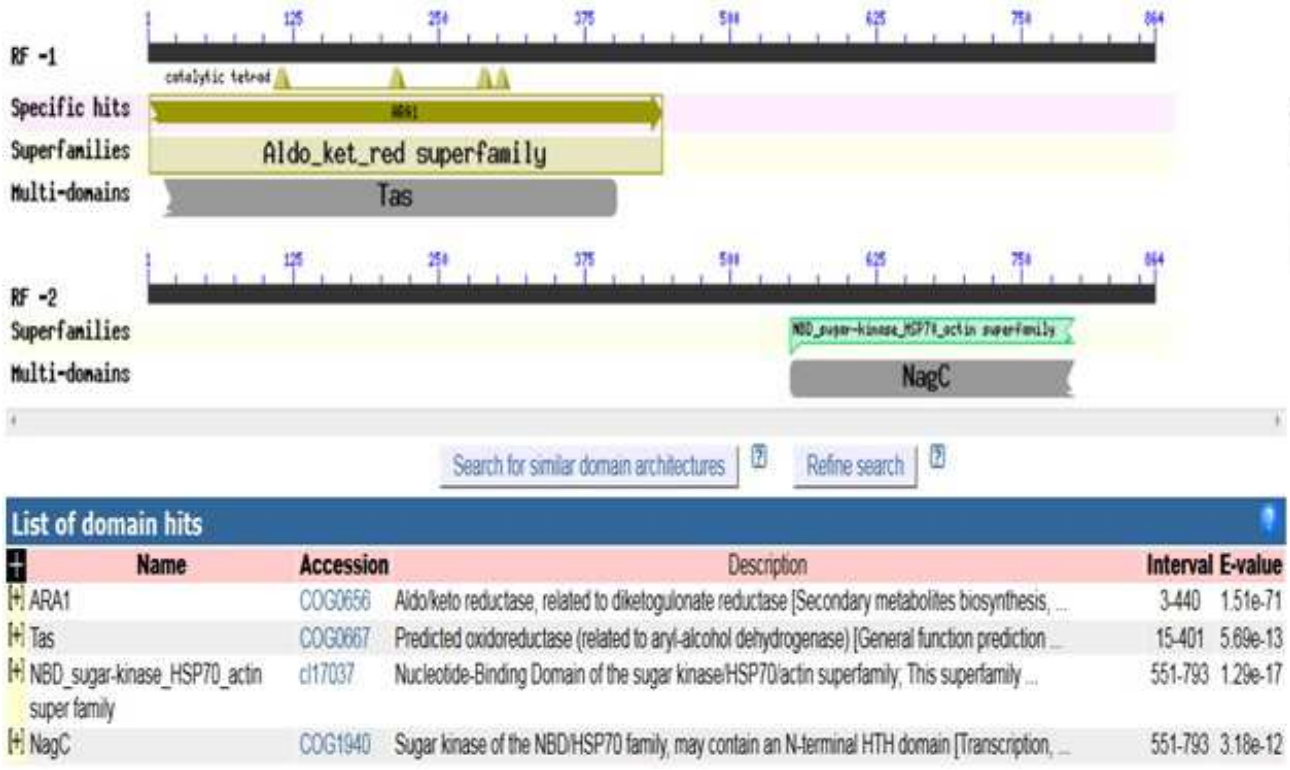


Fig. 4: NCBI-CDD for query sequencing

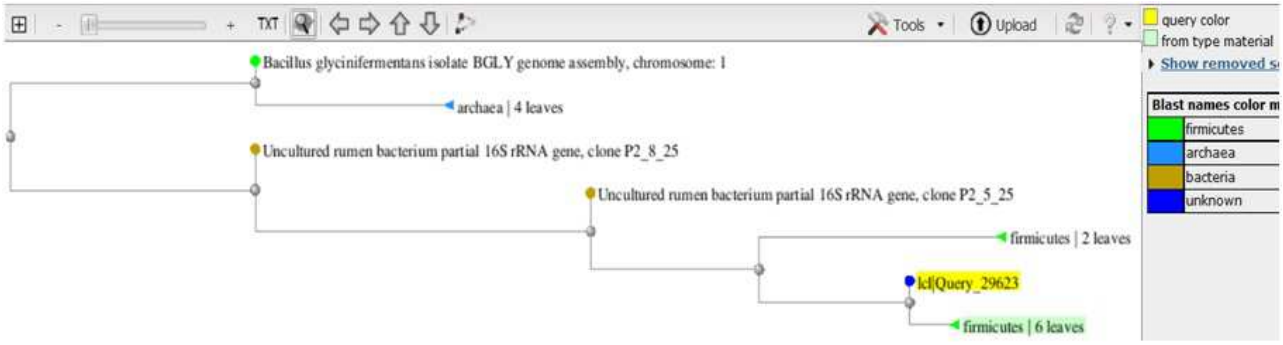


Fig. 5: A. Phylogenetic analysis using query nucleotide sequence

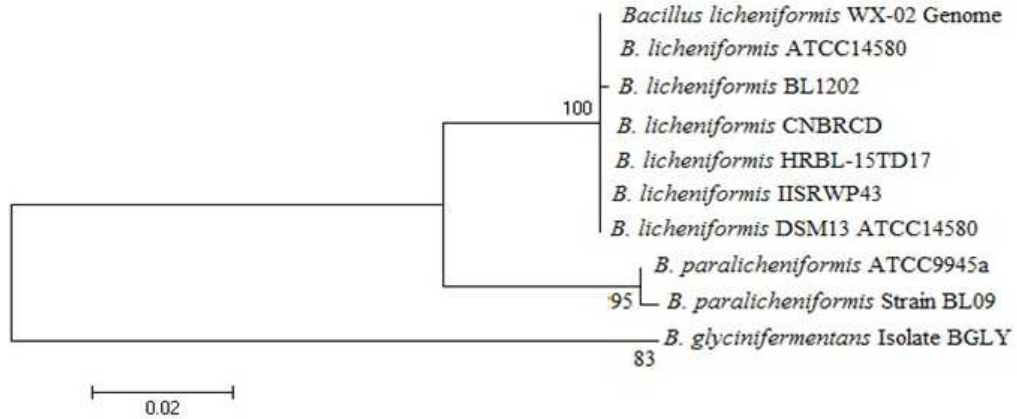


Fig. 5: B. A Tree plot was constructed with the NJ method using 863bp fragment of the Aldo/keto reductase gene showing the relationship of *B. licheniformis* with other Bacillus spp.

other crops (Rana *et al*., 2012; Balakrishna *et al*., 2013). In the present study the microbial consortia (MC) selected through pot culture studies was evaluated under field conditions along with 50%, 75% and 100% NPK without MC, with suitable controls.

Plants inoculated with MC+75% NPK had the highest plant height, stem girth and BI followed by plants receiving 100% NPK without MC. This is in agreement with the previous studies where improvement in growth and biomass of crop plants have been reported because of inoculation of microbial consortia with reduced level of chemical fertilizers (Hemavathi *et al*., 2006; Chauhan and Bagyaraj, 2015). The highest values for the shoot dry weight, root dry weight and total plant dry weight was recorded in MC+75% NPK treatment which did not differ significantly from 100% NPK without MC and MC+50% NPK treatments, but differed significantly from the UC. Similar results were obtained when *Coleus forskohlii* plants were inoculated with *Pseudomonas monteilii* and *Rhizophagus fasciculatus* (*Glomus fasciculatum*) (Singh *et al*., 2013). The shoot and root N concentration did not differ significantly among the treatments; however, there was significant variation in the P and K concentration, the highest being recorded in the 100% NPK treatment and the least in the UC treatment. Synergistic interaction between AM fungi and PGPR enhancing the uptake of macronutrients N,P and K has been reported earlier in other crop plants (Bagyaraj *et al*., 2015; Vafadar *et al*., 2014). The total withanolide concentration in the roots was found to be high in plants inoculated with MC+75%NPK which was statistically significant and was onpar with MC treatment, but differed significantly from all other treatments, the least withanolide concentration being in UC plants. These results uphold the observations made by Baker and Mostafa (2011) on Roselle plants and Hend *et al*. (2007) on peppermint.

The per cent mycorrhizal colonization was also significantly highest in the MC treatment but on par with the treatmentsMC+75% NPK and MC+50% NPK compared to 100% NPK. This is in accordance with the earlier results that AM fungi proliferate well at lower fertilizer levels (Abdullahi and Sheriff, 2013; Bagyaraj, 2014). The results of the total bacterial population and *Bacillus* spores in rhizosphere soil indicated that they were significantly higher in MC treatment followed by MC+75% NPK and MC+50%NPK respectively, the least number of bacteria were encountered in 100% NPK treatment and UC plots. Similar results were obtained in soils cropped with chilly (Thilagar *et al*., 2016) and rice (Nakhro and Dkhar, 2010).

Soil enzyme activities such as dehydrogenase, acid and alkaline phophatases are used as an indicator of microbial activity. They play a major role in depolymerization of macromolecules and thus help in decomposition and mineralization of potential soils (Schimel & Bennet, 2004). The dehydrogenase activity was significantly affected by different treatments. Higher dehydrogenase activity was observed in the treatment MC+50%NPK which differed statistically from the other treatments. Higher activity of acid phosphatase was recorded in the treatments MC+75%NPK which was on par with MC treatment and differed significantly from other treatments. The alkaline phosphatase was significantly higher in MC+75%NPK and MC+50%NPK and differed from all other treatments. This supports the earlier observations made by Hemashenpagam and Selvaraj (2011) that acid and alkaline phosphatase and dehydrogenase activities were more in the root zone soil of medicinal plant *Solanum viarum* inoculated with *Glomus aggregatum*, *Bacillus coagulans* and *Trichoderma harzianum* compared to uninoculated plants.

It is well documented that soil microorganisms contribute to the buildup of stable organic matter, rapid recycling of nutrients and also improve soil health. In the present study the soil organic carbon content improved significantly in all the treatments compared to the UC and 100% NPK treatment. Initially it was 0.08% and later it increased up to 0.21% in the treatment MC+75% NPK. This trend is in agreement with the observations made by Mengual *et al*. (2014). The available N was found to be highest in 100% NPK treatment and least in UC and MC treated soils. There was no significant difference in the available P in the different treatments. Solubilization of inorganic phosphorus and mineralization of organic phosphorus occurs due to the action of low molecular weight organic acids and synthesis of a variety of different phosphatases by various soil bacteria (Glick 2012; Zaidi *et al*., 2009). *Bacillus licheniformis* is phosphate solubilizing bacteria, the beneficial effects

of the inoculation with PSB used alone or in combination with other rhizospheric microbes have been reported (Zaidi and Khan, 2005; Vikram and Hamzehzarghani, 2008). Besides providing P to the plants, the phosphate solubilizing bacteria also augment the growth of plants by stimulating the efficiency of BNF, enhancing the availability of other trace elements by synthesizing important plant growth promoting substances (Zaidi *et al*., 2009). Application of PGPR or Consortium is effective in solubilizing the available phosphorus in soil and in turn helping the plant in P uptake. This is perhaps the reason for no significant difference in available soil phosphorus at harvest. Application of microbial inoculants along with fertilizers showed significant improvement in K availability as compared to UC and 100%NPK treatments.

Tracking of Inoculated PGPR in the Field

Based on pot culture studies done earlier, a microbial consortia consisting of *A. laevis* + *B. licheniformis* was selected as the best for its growth promoting activity on *W. somnifera*. Validation of the results of pot culture study under field condition was one of the objectives of the present study. Further tracking down the inoculated *B. licheniformis* in the rhizosphere soil at harvest was another objective. Tracking of this strain would require specific analytical methods for monitoring and quantification in the environment. Monitoring methods can be grouped into cultivation based and DNA-based techniques (Van Elsas *et al*., 1998). With cultivation, the population densities are estimated by plating and CFU counts or using the Most Probable Number procedure. These methods allow the detection of viable-culturable microbial cells, but they may lack specificity. DNA-based methods are more specific but they do not discriminate between DNA from viable, resting, or dead cells or soil bound cell-free DNA, and as a result an overestimate of viable population.

The rhizosphere environment relies on a number of factors such as the composition of soil, including differences in root exudation and rhizodeposition related to the plant species (Sørensen, 1997; Jaeger *et al*., 1999), plant growth stage, cropping practices, and other environmental factors (Grayston *et al*., 1998; Horwarth *et al*., 1998; Lupwayi *et al*., 1998). All of these variables are determinants in the establishment of soil microbial communities, either introduced or natural. With this view, future investigations should focus on a larger number of soil typologies and plant species to determine the most suitable conditions for the establishment of this bacterium in the rhizosphere.

Further, it showed that cloning and sequencing of the unique amplicons can help in designing SCAR primers for routine use with total DNA as templates for identifying specific *Bacillus* species. It is advantageous to use genus specific primers since it encompasses maximum number of species of a genus. The amplicon revealed the presence of *B. licheniformis* in the inoculated soil.

In conclusion, the SCAR primer specific to *B. licheniformis* used resulted in specific 1410-bp amplified product. The use of SCAR primers enables the unambiguous detection and quantification of the specific bacterium in the soil. A similar approach for detection and monitoring of bacterial inoculants in the environment has been used for *Pseudomonas* species (Chapon *et al*., 2003; Pujol *et al*., 2005) and *B. licheniformis* (Vinod *et al*., 2014). The monitoring tool used here allowed us to evaluate the presence of *B. licheniformis* in the rhizosphere of *W. somnifera* plants grown under field conditions. This approach is a qualitative approach; for quantification metagenomic analysis would indicate the amount of inoculated bacterium present in the soil.

Conclusions

A field study with the selected microbial consortia was validated under field conditions with the possibility of reducing the recommended fertilizer (NPK). It can be concluded that the fertilizer application for *W. somnifera* can be reduced by 25% through inoculation with the selected microbial consortia (*B. licheniformis* + *A. laevis*) with no adverse effect on growth, biomass, nutrition and total withanolide concentration of *W. somnifera*. This microbial technology can easily be followed by farmers, which will reduce the application of chemical fertilizer in cultivation of *W. somnifera*

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