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Diversity of *nifH* gene amplified from rhizosphere soil DNA

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Rhizosphere soils of chickpea (*Cicer aeritinum*) and wheat (*Triticum aestivum*) were analysed for the diversity of diazotrophic microorganisms by sequencing the *nifH* gene. A clone library of soil DNA amplified using highly degenerated *nifH* primers targeting the prokaryotic nitrogenase was constructed and subsequent RFLP analysis of *nifH* gene from clone libraries identified 28 and 25 genotypes in chickpea and wheat rhizosphere respectively. A total of 41 *nifH* genotypes were identified, out of which 12 were common in both rhizosphere soils. Sequencing of 41 *nifH* genotypes showed similarity to the corresponding genera of diazotrophs belonging to alpha, beta, gamma and delta proteobacteria. The prominent diazotrophs were *Azospirillum brasilense*, *Bradyrhizobium* spp., *Azotobacter chroococcum*, *Methylococcus capsulatus*, *M. sylvestris*, *Sinorhizobium meliloti* and *Burkholderia vietnamenesis*. *A. brasilense* was the dominant diazotroph present in the both rhizosphere soils. *S. meliloti* was only present in the chickpea rhizosphere, while *B. vietnamanesis* and *Azoarcus tolulyticus* were observed only in the wheat rhizosphere. The chickpea and wheat rhizospheres were diverse in diazotrophic bacterial community, as indicated by the occurrence of the *nifH* genotypes.

Keywords: Arid zone soils, diazotrophs, genetic diversity, *nifH* gene, soil DNA.

NITROGENOUS fertilizers are one of the most widely used chemical fertilizers, as deficiency of nitrogen in the soil often limits crop yields. Consumption of nitrogen fertilizer

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in Asia has increased from 1.5 to 47 millions tonnes (mt) during the last 35 years[1]. In general, less than 50% of the added nitrogen is available to the plants. Biological nitrogen fixation, the enzymatic reduction of nitrogen to ammonia, replenishes the loss of nitrogen from soil–plant ecosystems. Soil diazotrophs are the main source of nitrogen input in primary-production ecosystems[2]. Nitrogen fixation occurs in phyla from Archaea and Eubacteria. These microorganisms possess the enzyme nitrogenase encoded by the *nifK*, *nifD* and *nifH* genes. These genes have been used to study phylogenetic relationship among nitrogen-fixing bacteria[3].

Haryana, a state in northern India with annual rainfall of <500 mm, falls under arid and semi-arid zones. Wheat and chickpea are two major crops cultivated during winter. Wheat is grown with the addition of chemical fertilizers (NPK) under irrigated conditions, while chickpea is grown without the addition of chemical fertilizers under rainfed conditions. Very little information is available on diversity of diazotrophic bacteria in the rhizospheres of chickpea and wheat grown under these conditions.

In earlier studies, cultivation methods have been used which identify only 5–10 dominating genera of diaztrophs. Due to unculturability of many prokaryotes cultivation-based strategies have severe limitations for the description of diversity of free-living soil diazotrophs[4]. The molecular methods provide a more complete picture of the diazotrophic community than culture-based approaches[5,6]. Different techniques have been utilized to characterize the diversity of *nifH* gene pool identification from various environments, e.g. cloning of PCR-amplified products or by denaturing gradient gel electrophoresis, PCR-based restriction fragment length polymorphism (RFLP), and fluorescently labelled terminal restriction fragment length polymorphism (t-RFLP)[7–9]. These techniques have been successfully applied to describe diazotroph communities in different soil systems, including forest soils[10], pasture, agricultural soils[11], wetland soils[12] and rhizosphere soils[13]. The phylogeny of diazotrophs based on the *nifH* gene is similar to the phylogeny based on 16S rRNA gene and therefore the *nifH* gene has been widely used to study phylogeny and diversity of diazotrophic bacteria[14,15].

A large number of *nifH* primers have been designed to study the diversity of diazotrophs[7,8,10,11]. However, the use of highly degenerated primers combined with low-stringency amplification conditions may lead to biased results. In this work, we have designed some novel primers to study the diversity of *nifH* gene by directly amplifying the community soil DNA of two different rhizosphere soils.

Two rhizosphere soil samples, S14 and S25, from chickpea and wheat were collected from two fields. S14 was sandy loam soil and was under chickpea–pearl millet cropping sequence for the last five years. S25 was loamy sand soil and was under cotton–wheat cropping sequence for the last five years. Ten plants of chickpea (*Cicer aerietinum*) cv. HC-1 and wheat (*Triticum aestivum*) cv. WH147 were uprooted after 60 days of sowing at different locations from the fields. The chemical properties of the soils were analysed using standard methods[16].

DNA was extracted from 200 mg of soil samples using Fast DNA Spin Kit (Bio 101) following manufacturer's instructions. Five washings of isolated soil DNA with 1 ml of 5.5 M guanidine thiocyanate were performed to remove the PCR-inhibitor, humic acid.

The *nifH* primers used for amplification of *nifH* in this study were novel. The region from base-pair positions 25 to 48 (relative to the *nifH* sequence of *Azotobacter vinelandii*) was chosen for the forward primer, *nifH*for and the region from base-pair positions 462 to 491 was chosen for the reverse primer, *nifH*rev. The sequences of the primers were as follows: *nifH*for 5′ TAY GGN AAR GGN GGH ATY GGY ATC 3′ and *nifH*rev 5′ ATR TTR TTN GCN GCR TAV ABB GCC ATC AT 3′. In these sequences Y represents C/T, N represents A/C/G/T, R represents A/G, H represents A/C/T, B represents G/C/T and V represents A/G/C.

Touchdown PCR protocol was used for the amplification of *nifH* directly from soil DNA[8]. A total of 24 µl PCR reaction mixture comprised 12 µl Jumpstart REDTaq ReadyMix (Sigma, USA), 10 pmol each of *nifH*for and *nifH*rev primers, 1% DMSO, 1 µl template soil DNA (approximately 40 ng) and PCR-grade water to give a final volume of 24 µl. Touchdown PCR was performed in Mastercycler Personal (Eppendorf, Germany). Non-touchdown PCR was carried out in TRIO Thermoblock (Biometra, The Netherlands) for reamplification of *nifH* fragments from the clones: initial denaturation of 3 min at 94°C, 30 cycles of 45 s at 94°C, 30 s at 50°C, 60 s at 72°C, and final elongation of 5 min at 72°C. In the non-touchdown PCR, instead of Jumpstart REDTaq ReadyMix, REDTaq ReadyMix (Sigma–Aldrich, USA) was used in the PCR reaction mixture.

The amplified *nifH* PCR products from S14 and S25 were excised from the gel and purified using NucleoSpin Extract Kit (Macherey–Nagel, Germany). The purified *nifH* PCR products were cloned in the vector pCR 2.1-TOPO using TOPO® TA cloning kit (Invitrogen, USA) following the manufacturer's recommendations. The *nifH* fragments were reamplified from the clones of S14 and S25 *nifH* clone libraries using *Escherichia coli* colonies as a template in the PCR reaction mixture. The amplified *nifH* PCR products were subjected to RFLP. Next 10 µl of amplified *nifH* PCR products was digested with 10 units of restriction endonucleases (MBI Fermentas). The reaction mixtures were incubated for 6 h at 37°C. Two restriction enzymes *Msp*I and *Hae*III were used for RFLP analysis and resolved on 2% Tris borate agarose gel.

The *nifH* fragments were reamplified from *E. coli* colonies using M13 universal and M13 reverse primer[17] and purified with NucleoSpin extract kit (Macherey–Nagel). The amplified and purified PCR products from the unique RFLP patterns of the *nifH* clone libraries were

Table 1. Chemical properties of soil from chickpea and wheat fields

Soil	Crop	Location	pH	C/N ratio	N-NH_4 (μg/g)	Available P (μg/g)	Total P (μg/g)
S14	Chickpea	Bhiwani	7.9	5.0	0.64	16.2	147
S25	Wheat	Hisar	6.9	13.1	1.68	50.6	189

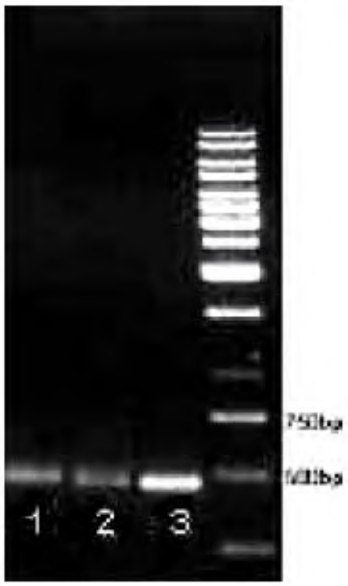

Figure 1. Amplification of community soil DNA with *nifH* primers. Lane 1, S14; lane 2, S25, and lane 3, Control genomic DNA of *Rhizobium leguminosarum* bv *viciae*.

sequenced using an ABI 3700 sequence analyzer (Applied Biosystems, USA) using M13 primers. Sequences of the clones were compared with those deposited at the National Center for Biotechnology Information (NCBI) databank, using BLAST. Alignment of DNA sequences was done with Clustal X[18]. The phylogenetic tree was constructed by neighbour-joining method with 100 replicates and was viewed using TreeView 1.6.1 software[19].

The chemical properties of the soils are given in Table 1. In wheat, higher doses of chemical fetilizers (120 kg N and 60 kg P) are applied with 4–5 irrigations during the cropping season. In chickpea, only 20 kg/ha of single super phosphate is applied and the crop is grown under rainfed conditions or one pre-sown irrigation. Therefore, wheat is grown under better management practices. The S25 field was under wheat cultivation and had 2.5 times higher C/N ratio than the S14 field which was under chickpea cultivation. The ammonical-N and available P were also higher in the S25 field in comparison to the S14 field.

Non-touchdown PCR protocol gave different non-specific bands upon amplification of the *nifH* fragment directly from soil DNA, instead of the expected size product (data not shown). The touchdown PCR protocol was successfully employed for amplification of 420 bp *nifH* fragment from both soil DNA preparations. However, for remplification of *nifH* from clone libraries, the non-touchdown PCR protocol was equally successful and gave a single band of 420 bp (Figure 1). In the present study, highly degenerated primers were designed for the diazotroph community analysis, i.e. degeneracy of *nifH*for was 768-fold and of the *nifH*rev was 3456-fold. The *nifH* primers were designed from the available *nifH* sequences of different organisms from NCBI-nr data basebank. All available *nifH* sequences originating from the following bacteria were taken to design the degenerated primers: *Frankia* sp. (M21132), *Nostoc* sp. PCC7120 (AP003583), *Paenibacillus azotofixans* (AJ299453), *Rhodobacter capsulatus* (M15270), *Gluconoacetobacter diazotrophicus* (AF030414), *Sinorhizobium meliloti* (AE007235), *Mesorhizobium loti* (AP003007), *nifH*1 and *nifH*2 of *Rhizobium* sp. NGR234 (AE00105), *Burkholderia* sp. (AJ302315), *Bradyrhizobium japonicum* (AP005941), *Clostridium acetobutylicum* (AE007538), *Klebsiella pneumoniae* (X13303), *Azotobacter chroococcum* (M73020), *Azotobacter vinelandii* (M20568), *Azoarcus* sp. BH72 (AF200742) and *Azospirillum brasilense* Sp7 (X51500). Many previous studies on diversity of diazotrophs were based on PCR amplification using universal primers designed to target *nifH* gene regions encoding highly conserved amino acid sequences which amplified different non-specific bands[7–10,20]. The PCR protocol developed by Rösch *et al.*[8] was successfully employed for the amplification of the 420 bp *nifH*-specific fragment from DNA of both soils. Widmer *et al.*[10] have developed a nested-PCR scheme to achieve specific amplification of *nifH* from bulk soil DNA with a highly degenerated universal primer set. In nested-PCR, two rounds of PCR were performed to get the expected size of the product because in first round of PCR, the expected size of the product was amplified with non-specific bands. In order to avoid two rounds of PCR, one-step touchdown PCR protocol proved better than nested-PCR.

Forty white clones with *nifH* insert clones from each soil were randomly picked up and screened for the presence of *nifH* inserts by reamplification of the *nifH* fragments from *E. coli* transformants. Different restriction patterns were recorded for the reamplified *nifH* fragments from the eighty *nifH* clones with *Msp*I and *Hae*III. RFLP analysis resulted in 11 and 16 restriction patterns respectively, from DNA of S14, and 10 and 14 restriction patterns respectively, from DNA of S25. The combined results from *Msp*I and *Hae*III gave 28 and 25 *nifH* genotypes from S14 and S25 respectively. Totally 41 *nifH* genotypes were identified from clone libraries of both soils.

Twenty-two *nifH* genotypes were represented only by a single clone. Comparison of *nifH* RFLP genotypes between S14 and S25 showed 12 common *nifH* genotypes, but the percentage of occurrence of these genotypes differed in the individual soils.

Sequencing a 420 bp *nifH* fragment from the representative clone of each *nifH* genotype group found similarity with 12 different diazotrophs of alpha, beta, gamma and delta proteobacteria (Figure 2). The clones showed 84–98% sequence similarity to *A. chroococcum* ATCC 4412 (M73020), *Bradyrhizobium* sp. (AB079620), *Methylococcus capsulatus* (AJ563958), *Methylocella silvestris* (AJ563942), *S. meliloti* (AE007235), *Burkholderia vietnamiensis* (AJ567343), *A. chroococcum* (X03916), *Azoarcus tolulyticus* (U97122), *Bradyrhizobium* sp. (AB07616), *Sinorhizobium* sp. (AJ505315), *Geobacter sulfurreducens* (AE017217) and *A. brasilense* Sp7 (X51500) diazotrophs.

In the chickpea field site (soil S14), alpha, gamma and delta proteobacteria were identified. Among alpha proteobacteria, *A. brasilense* Sp7 (X51500) was the dominant diazotroph covering 50% of the clone library. *nifH* sequences of the clones representing *nifH*5, *nifH*6, *nif*H8, *nifH*9, *nifH*12, *nifH*15, *nifH*16, *nifH*19, *nifH*20, *nifH*21, *nifH*22, *nifH*23, *nifH*24, *nifH*25, *nifH*26 and *nifH*27 genotypes were more than 90% similar to the *nifH* sequence of *A. brasilense* Sp7. Another large group of diazotrophs from alpha proteobacteria was *Sinorhizobium* (AJ505315), constituting 12.5% of the clone library. Four *nifH* genotypes, *nifH*3, *nifH*7, *nifH*17 and *nifH*18 were more than 90% similar to the *nifH* sequence of the above-mentioned *Sinorhizobium* sp. Other alpha proteobacterial diazotrophs included *Bradyrhizobium* sp. (AB079620), *M. silvestris* (AJ563942) and *S. meliloti* (AE007235) representing *nifH*2, *nifH*10 and *nifH*11 genotypes respectively. *Bradyrhizobium* sp. (AB079620) and *M. silvestris* (AJ563942) covered 2.5% each and *S. meliloti* (AE007235) covered 5% of the clone library.

The *nifH* sequence of clones representing genotypes *nifH*1 and *nifH*4 showed closer similarity to gamma proteobacteria *A. chroococcum* ATCC 4412 (M73020) and *M. capsulatus* (AJ563958) respectively. *A. chroococcum* ATCC 4412 (M73020) and *M. capsulatus* (AJ563958) were represented by 17.5 and 2.5% population in the clone library. Delta proteobacteria identified in this field site was *Geobacter sulfurreducens* (AE017217) and 5% population in clone library was representative of this genotype.

In the wheat field site (soil S25), in addition to alpha, gamma and delta proteobacteria, beta proteobacteria were also identified. *Burkholderia vietnamiensis* (AJ567343) and *Azoarcus tolulyticus* (U97122) were two beta proteobacteria recovered from the clone library. Similar to the chickpea field site, *A. brasilense* Sp7 was the dominant nitrogen fixer in the wheat field site. It constituted 47.5% of the clone library (Table 2). Eleven *nifH* genotypes showed similarity to *A. brasilense* Sp7 *nifH* sequence. *Sinorhizobium* sp. (AJ505315) and *Bradyrhizobium* sp. (AB07616) were represented by 10 and 5% population in the clone library respectively. The gamma proteobacteria *A. chroococcum* (X03916) constituted 7.5% in the clone library. Delta proteobacteria, *G. sulfurreducens* (AE017217) was represented by 15% in the clone library. Occurrence of different *nifH* genotypes represented the high diversity of free-living diazotrophs in these fields. *A. brasilense* was the dominant diazotroph identified in the *nifH* clone libraries of both soil samples. Thirty-five *nifH* sequences of the clones representing genotypes *nifH*5, *nifH*6, *nifH*8, *nifH*9, *nifH*12, *nifH*15, *nifH*16, *nifH*19, *nifH*20, *nifH*21, *nifH*22, *nifH*23, *nifH*24, *nifH*25, *nifH*26, *nifH*27, *nifH*31, *nifH*34, *nifH*38, *nifH*39 and *nifH*41 showed similarity to the *nifH* sequence of *A. brasilense*. This was probably due to a discriminative amplification of *nifH* sequence of *A. brasilense* by the newly designed primer set or the cell density of *A. brasilense* was higher in Haryana soils. MPN count of diazotrophs in the root washings of wheat was 1.7×10^6 to 13×10^6 per g of fresh roots, while in the root macerate varied between 0.6 and 5.5×10^6 per g of fresh root weight[21]. The level of colonization of *Azospirillum* was estimated as approximately 10^6–10^8 CFU per plant in Israel and Baja California, Mexico[22]. Likewise, 3.4×10^6 CFU per g *Azospirillum* was estimated in the

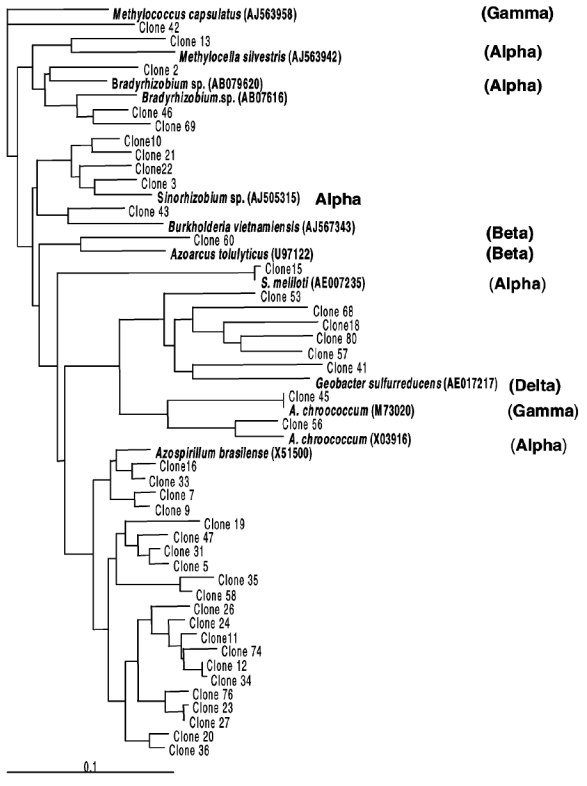

Figure 2. Neighbour-joining tree of *nifH* clones with their reference strains.

Table 2. Distribution and affiliation of *nifH* genotype groups from S14 and S25

Soil sample	Genotype	Nearest neighbours to *nifH* genotype group			
		Clone	Identity (%)	Name (accession no.)	Nearest cultivated
S14/S25	*nifH*1	45	98	*Azotobacter chroococcum* (M73020)	Gamma
S14	*nifH*2	2	90	*Bradyrhizobium* sp. (AB079620)	Alpha
S14/S25	*nifH*4	42	87	*Methylococcus capsulatus* (AJ563958)	Gamma
S14/S25	*nifH*10	13	89	*Methylocella silvestris* (AJ563942)	Alpha
S14	*nifH*11	15	98	*Sinorhizobium meliloti* (AE007235)	Alpha
S14	*nifH*28	43	91	*Burkholderia vietnamiensis* (AJ567343)	Beta
S25	*nifH*33	56	94	*Azotobacter chroococcum* (X03916)	Gamma
S25	*nifH*35	60	88	*Azoarcus tolulyticus* (U97122)	Beta
S25	*nifH*30	46	92	*Bradyrhizobium* sp. (AB07616)	Alpha
S25	*nifH*37	69	92		
S14	*nifH*3	3	94	*Sinorhizobium* sp. (AJ505315)	Alpha
S14	*nifH*7	10	93		
S14/S25	*nifH*17	21	90		
S14	*nifH*18	22	93		
S14/S25	*nifH*13	41	88	*Geobacter sulfurreducens* (AE017217)	Delta
S14	*nifH*14	18	84		
S25	*nifH*29	57	88		
S25	*nifH*32	53	84		
S25	*nifH*36	68	92		
S25	*nifH*40	80	85		
S14	*nifH*5	5	92	*Azospirillum brasilense* Sp7 (X51500)	Alpha
S14/S25	*nifH*6	7	93		
S14/S25	*nifH*8	11	90		
S14/S25	*nifH*9	12	89		
S14	*nifH*12	16	95		
S14	*nifH*15	19	90		
S14/S25	*nifH*16	20	91		
S14	*nifH*19	23	92		
S14/S25	*nifH*20	24	91		
S14/S25	*nifH*21	26	90		
S14	*nifH*22	27	92		
S14	*nifH*23	31	92		
S14	*nifH*24	33	95		
S14/S25	*nifH*25	34	90		
S14	*nifH*26	35	90		
S14	*nifH*27	36	92		
S25	*nifH*31	47	92		
S25	*nifH*34	58	90		
S25	*nifH*38	74	88		
S25	*nifH*39	76	90		
S25	*nifH*41	9	94		

rhizosphere of wheat soil from Hisar (Haryana), while *Azotobacter* population was 2×10^4 per g soil[23]. It appears that *Azospirillum* being the associate diazotroph survives and establishes better in the rhizosphere and is the dominating diazotrophs, as indicated by amplification of the *nifH* gene from the soils.

The ability to fix nitrogen is limited to the methanogens within Archeae[3,24,25]. In the *nifH* clone library of S14, *nifH* sequences of 2.5% clones belong to *M. capsulatus* (type-I methanogen, gamma proteobacteria) and 2.5% population belongs to *M. silvestris* (type-II methanogen, alpha proteobacteria). *Methylococus* is an unusual genus because it shares properties of both types[26,27]. Only type II methanogen, was identified in the S25 *nifH* clone library. The type-II methanotrophic bacteria had been identified from rice root, freshwater lake, termite gut, Douglas fir soil site and an oligotrophic ocean[10,19,20]. Mehta *et al.*[28] reported the presence of methanogen *nifH* sequences in hydrothermal vents as well as other environments. Blast homologies and positioning of the *nifH* clones in the *nifH* partial sequences-derived tree showed that the six *nifH* genotypes (*nifH*13, *nifH*14, *nifH*29, *nifH*32, *nifH*36 and *nifH*40) representing clones grouped with delta proteobacteria, *G. sulfurreducens*. Delta proteobacteria have not been identified in any soil environment. *Desulfovibrio gigas* was only delta bacteria identified in rice rhizosphere[20] and in termite (*Reticultitermes spertus*) gut[29].

Chickpea and wheat rhizosphere soils differed with respect to the occurrence of *nifH* genotypes (Figure 3). Twelve *nifH* genotypes were common in both rhizospheres.

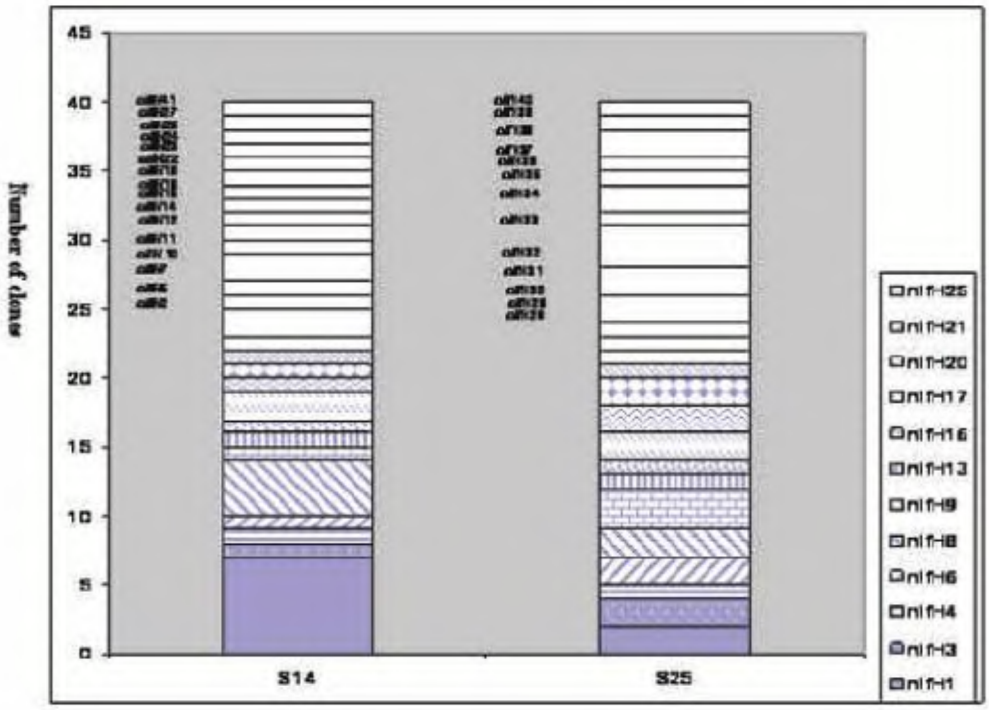

Figure 3. Distribution of *nifH* genotypes in chickpea (S14) and wheat (S25) rhizospheres. Genotypes shown in colour are common to both soils, while other genotypes are unique to S14 or S25.

Among these, six *nifH* genotypes were of *Azospirillum*, three of *Sinorhizobium*, and one each of *Geobacter*, *Azotobacter* and *Methylococcus*. Fifteen *nifH* genotypes representing nine of *Azospirillum*, three of *Sinorhizobium* and one each of *Bradyrhizobium*, *Methylcella* and *Geobacter* were only present in chickpea rhizosphere. In wheat rhizosphere, five *nifH* genotypes of *Azospirillum*, four of *Geobacter*, two of *Bradyrhizobium* and one each of *Azotobacter*, *Azoarcus* and *Burkholderia* were present. Chickpea and wheat are two different rhizospheres with different patterns of root exudation. Our earlier studies on bacterial and rhizobial diversity from the same samples confirmed that wheat rhizosphere soil was more diverse than chickpea rhizosphere soil[30,31]. Several studies have indicated that structural and functional diversity of rhizosphere populations is affected by the plant species due to difference in root exudation and rhizodeposition in different root zones[32]. Further, soil type, growth stage of plant, cropping practices and environmental factors influence the composition of the microbial community in the rhizosphere[33,34].

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Isolation and fusion of protoplasts in *Vanilla* species

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Vanilla, an important flavouring material and spice, is the fermented and cured fruit of the orchid, *Vanilla planifolia* Andrews (*syn. V. fragrans* Salisb.). Origin of much of the planting material of vanilla from limited clonal source, coupled with the threat of destruction of its natural habitats, leads to a narrow genetic base being the major bottleneck in crop improvement programmes. Isolation and fusion of protoplasts was attempted to produce hybrids with desirable traits. Protoplasts were successfully isolated from *V. planifolia* and *V. andamanica* when incubated in an enzyme solution containing macerozyme R-10 (0.5%) and cellulase Onozuka R-10 (2%) for 8 h at 30°C in dark, with good yield and viability. PEG-mediated protoplast fusion between *V. andamanica* and *V. planifolia* was successful and the fusion product (heterokaryon) could easily be identified because the protoplasts of the two species differed in size and arrangement of chlorophyll. This can be helpful in gene transfer for helpful traits, especially the natural seed set and disease tolerance observed in *V. andamanica*.

Keywords: Isolation, protoplast fusion, *Vanilla andamanica*, *V. planifolia*.

VANILLA, an important and popular flavouring material and spice, is the fermented and cured fruit of the orchid,

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