Short communication

Novel free living diazotrophs associated with black pepper in Wayanad

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Abstract

Diazotrophs are nitrogen fixing microorganisms that play a key role in maintaining soil health by converting atmospheric nitrogen into plant available form through biological nitrogen fixation (BNF). In this study, free living diazotrophic bacteria were isolated from rhizosphere soil and phylloplane of black pepper from Wayanad. Twenty predominant isolates were screened for growth and nitrogen fixation at varying levels of pH. There was a decrease in growth and N-fixation, when the pH was reduced from seven to five. Many of the isolates exhibited plant growth promoting (PGP) traits like production of indole acetic acid (IAA), ammonia and hydrogen cyanide (HCN) under *in vitro* conditions. A few isolates were found to possess antagonistic activity against the soil borne plant pathogens, *Rhizoctonia solani* and *Ralstonia solanacearum*. However, none of the isolates exhibited antagonistic activity against the pathogen, *Fusarium oxysporum*. Based on in vitro screening, four isolates were found to be promising diazotrophs, and these were identified by 16S rDNA sequencing as *Microbacterium* (NKdS and NPPV), *Cellulosimicrobium* (NPS-1) and *Brevundimonas* (NKPV-2), *Microbacterium* is an actinomycete, reported to be endophytic in certain crop species. Nitrogen fixation combined with other plant growth promoting activities makes them an attractive choice for further exploitation as biofertilizers.

Keywords: Acid tolerance, Free living diazotrophs, PGP activities.

Nitrogen is one of the important nutrients essential for crop growth. Although more than 78% of nitrogen is present in the atmosphere, it is unavailable to plants. Atmospheric nitrogen is converted into plant utilizable form by biological nitrogen fixation (BNF), which involves the conversion of nitrogen into ammonia by nitrogen fixing bacteria or diazotrophs, using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). Integrated nutrient management (INM) and organic farming are gaining importance in Kerala and biofertilizers constitute an integral component of INM. Biofertilizers capable of fixing atmospheric nitrogen can increase crop yield by 20-30% and can replace nitrogen requirement by 25% (Evans and Furlong, 2010).

Black pepper is an important spice crop of Kerala. In India, Kerala ranks first in black pepper production, contributing about 97% of the total production and Wayanad is one of the main pepper growing tracts in Kerala, with an area of 9527 ha and production of 2751 tonnes (Agricultural statistics, 2015).

Kerala, being in the high rainfall region, is predominantly an acid soil tract, with about 88% of the soils being acidic in reaction. Acidic conditions

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impair the absorption of nutrients and inhibit microbial activity. Hence, efficient biofertilizers have to be developed which are adaptable to the acidic soils of Kerala. Nitrogenous biofertilizers like *Azotobacter* and *Azospirillum* have been popularized in the state. Reports indicate that soil pH below 6.0 supports the predominance of acid tolerant strains of diazotrophs like *Azotobacter chroococcum*, *Beijerinckia indica,* and *Derxia gummosa* (Andre et al., 2007). Hence an attempt was made to isolate and characterize free-living diazotrophs from the rhizosphere and phylloplane of black pepper from different locations of Wayand district.

Diazotrophs from rhizosphere soil were isolated by serial dilution and plating technique (Johnson and Curl, 1972) and from phylloplane by leaf impression method (Lamb and Brown, 1970). Three nitrogen free media (Jensen's agar, *Beijerinckia* agar and Ashby's agar) were employed for isolation. Twenty predominant isolates were characterized by cultural and biochemical tests (Cappuccino and Sherman, 1992). These were also screened for growth and nitrogen fixation at different levels of pH (7.0, 6.5, 6.0, 5.5 and 5.0). For this, a preliminary screening was carried out to standardize the time period at which maximum population is attained in Jensen's broth at pH 7.0. Forty eight hour old culture was inoculated in 50 ml sterile Jensen's broth and incubated at 28^0 C. Population was estimated by serial dilution and plating technique at 0h, 48h, 72h, 96h, 120h and 144h after inoculation. The amount of nitrogen fixed by the isolates was estimated using microkjeldahl method (Bremner, 1960 and Jackson, 1973), after 15 days of incubation.

Twenty selected isolates were also screened *in vitro* for plant growth promoting activities including production of IAA (Brick et al., 1991), hydrogen cyanide (Bakker and Schippers, 1987), siderophore (Schwyn and Neilands, 1987) and ammonia (Cappuccino and Sherman, 1992).

The antagonistic activity of all the twenty isolates was tested against three important soil borne pathogens including, *R. solani, F. oxysporum* and *R. solanacearum*. Dual culture technique (Dennis and Webster, 1971) was used for fungal pathogens and cross streaking method (Crawford et al., 1993) for *R. solanacearum*. In dual culture method, radial growth of the fungal pathogens was measured when growth of the pathogen in control plate reached maximum. The percent growth inhibition (PGI) was calculated using the formula,

$$PGI\ (\%) = \frac{C\text{-}T}{C} \times 100$$

Where, C - Distance of fungal growth from the point of inoculation to the colony margin in control plate

T - Distance of fungal growth from the point of inoculation to the colony margin in the direction of antagonist

16S rDNA was amplified by colony PCR using universal primers 8 F and 1522 R and Mastermix (Takara, Japan) in Eppendorf Mastercycler. Amplicons of 1500 bp were sequenced at Scigenom, Kochi by Sanger sequencing in ABI Prism. Forward and reverse sequences were aligned using DNA Baser tool and homology with available sequences found using blastn tool (http: // blast.ncbi.nlm.nih.gov/Blast).

Population of diazotrophic bacteria in rhizosphere soil ranged from 9.3×10^4 to19.6×10^4 cfu g^{-1} (Table 1). In the phylloplane, population of diazotrophs on dorsal surface ranged from 0.47 to 1.23 cfu cm^{-2} and on ventral surface population ranged from 0.41 to 1.38 cfu cm^{-2}. Among the three media, maximum population was observed on Jensen's agar and hence this medium was chosen for further experiments.

A total of forty three isolates of diazotrophic bacteria were obtained in the study and based on their population, twenty predominant isolates were selected for further evaluation. Colonies of most of the isolates on Jensen's agar were colourless, raised, circular and mucoidal, with entire margin (Table 2). Fifteen isolates were identified as Gram positive

Table 1. Population of free living diazotrophs in rhizosphere of black pepper

Location	Population of free living diazotrophs (× 10^4 cfu per gram of soil)*		
	Jensen's agar	*Beijerinckia* agar	Ashby's agar
Ambalavayal	17.3[c]	15.0[c]	10.0[h]
Bathery	15.3[h]	13.3[f]	14.3[c]
Kochangod	19.6[a]	16.0[a]	15.6[a]
Kalpetta	16.3[f]	13.3[f]	12.3[f]
Malavayal	16.6[e]	14.6[d]	12.0[g]
Naikatty	15.6[g]	14.6[d]	13.0[e]
Noolpuzha	19.0[b]	15.6[b]	15.3[b]
Pazhaya Vythiri	12.6[j]	11.3[h]	9.3[i]
RARS	13.0[i]	13.3[e]	13.3[d]
Thovarimala	17.0[d]	12.3[g]	13.0[e]

a, b, c so on indicate ranking of population of free living diazotrophs in different location on the three N-free media

Table 2. Cultural and morphological characters of selected diazotrophs on Jensen's agar medium

Isolates	Colony characters				Morphological characters		
	Size	Surface	Form	Elevation	Margin	Gram staining	Shape of cell
NTS-1	Small	Watery	Circular	Raised	Entire	+ve	Rod
NTS-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NTS-3	Medium	Mucoid	Circular	Flat	Entire	+ve	Rod
NKS-1	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NKS-2	Medium	Watery	Circular	Raised	Entire	+ve	Rod
NBS	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
NKdS	Large	Mucoid	Circular	Raised	Entire	+ve	Rod
NAS	Medium	Watery	Circular	Flat	Entire	+ve	Rod
NPS-1	Large	Mucoid	Circular	Raised	Entire	-ve	Rod
NPS-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NPS-3	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NKPD	Medium	Watery	Circular	Flat	Entire	+ve	Rod
NKPV-1	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
NKPV-2	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NKdPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NNPD	Medium	Watery	Circular	Raised	Entire	+ve	Rod
NNPV	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod
NNkPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NPPV	Medium	Mucoid	Circular	Raised	Entire	+ve	Rod
NAPV	Medium	Mucoid	Circular	Raised	Entire	-ve	Rod

N: Nitrogen fixer; Second alphabet: Location code; S: Rhizosphere soil; P: Phylloplane; D: Dorsal; V: Ventral
<0.3 cm: Small , 0.3-0.5 cm: Medium, >0.5 cm: Large

rods and five were Gram negative rods. None of the isolates produced endospores.

In the preliminary screening at pH 7.0, maximum growth was observed at 96h for all the isolates and hence population was estimated in subsequent experiments after 96h of incubation (Fig. 1). All the selected isolates were found to grow and fix nitrogen at all the pH levels tested (Table 3 and 4). However, among the rhizosphere isolates, NKdS (later identified as *Microbacterium* sp.) and NPS-1 (later identified as *Cellulosimicrobium* sp.) performed better at varying levels of pH. Among the phylloplane isolates, NPPV (later identified as

Table 3 .Population of diazotrophs at different time interval at pH 7.0 (10^5 × cfu/ml of broth)

Isolate	0h	48h	96h	72h	120h	144h
NTS-1	0.23[a]	12.77[b]	78.91[c]	100.00[d]	90.27[e]	84.33[f]
NTS-2	0.26[a]	17.51[b]	83.12[c]	136.33[d]	103.92[e]	78.00[f]
NTS-3	0.29[a]	13.0[b]	47.28[c]	79.00[d]	45.67[e]	28.94[f]
NKS-1	0.31[a]	17.84[b]	72.57[c]	122.33[d]	93.55[e]	64.39[f]
NKS-2	0.42[a]	26.20[b]	97.89[c]	101.33[d]	94.21[e]	72.30[f]
NBS	0.49[a]	25.61[b]	31.10[c]	38.00[d]	18.00[e]	15.77[f]
NKdS	0.38[a]	27.81[b]	92.00[c]	200.00[d]	94.53[e]	89.54[f]
NAS	0.32[a]	17.79[b]	74.18[c]	114.66[d]	77.03[e]	71.29[f]
NPS-1	0.40[a]	27.97[b]	95.21[c]	134.33[d]	113.28[e]	95.40[f]
NPS-2	0.32[a]	20.42[b]	39.21[c]	67.00[d]	41.95[e]	35.76[f]
NPS-3	0.21[a]	18.31[b]	69.54[c]	115.00[d]	88.97[e]	63.29[f]
NKPD	0.31[a]	19.80[b]	48.27[c]	79.33[d]	43.28[e]	33.00[f]
NKPV-1	0.34[a]	24.28[b]	99.75[c]	101.00[d]	85.67[e]	40.37[f]
NKPV-2	0.33[a]	20.92[b]	86.00[c]	138.33[d]	104.56[e]	69.78[f]
NKdPV	0.31[a]	16.95[b]	92.91[c]	117.33[d]	97.48[e]	55.00[f]
NNPD	0.25[a]	32.71[b]	91.24[c]	134.00[d]	102.33[e]	88.92[f]
NNPV	0.26[a]	35.32[b]	42.64[c]	88.33[d]	64.00[e]	48.53[f]
NNkPV	0.37[a]	31.46[b]	54.69[c]	88.00[d]	37.89[e]	18.71[f]
SD	0.068	8.25	24.80	37.27	28.05	24.80

Each values are mean of three replication

a,b,c so on indicate ranking of population of diazotrophs at different time interval

Table 4. Growth of free living diazotrophs at different pH levels (× 10^5 cfu/ml) in nitrogen free broth*

Isolate	pH 7.0	pH 6.5	pH 6.0	pH 5.5	pH 5.0
NTS-1	100.00[f]	48.33[j]	8.33[jk]	3.00[jk]	0.00[k]
NTS-2	136.33[c]	62.00[i]	35.33[h]	29.33[d]	9.00[de]
NTS-3	79.00[h]	7.33[m]	5.00[k]	2.33[k]	1.66[ijk]
NKS-1	122.33[d]	67.00[ghi]	18.33[i]	9.33[hij]	7.33[ef]
NKS-2	101.33[f]	90.66[de]	43.00[fg]	26.00[de]	5.33[fg]
NBS	38.00[j]	37.00[k]	13.33[ij]	8.00[ijk]	4.00[ghi]
NKdS	200.00[a]	125.33[b]	84.00[b]	70.33[b]	13.66[c]
NAS	114.66[e]	79.33[f]	17.00[i]	5.33[jk]	4.33[gh]
NPS-1	134.33[c]	105.33[c]	72.00[c]	39.00[c]	16.66[b]
NPS-2	67.00[i]	19.33[l]	16.33[i]	1.33[k]	1.00[jk]
NPS-3	115.00[e]	79.33[f]	39.33[gh]	17.00[fg]	5.00[fg]
NKPD	79.33[h]	76.33[fg]	71.00[cd]	3.33[jk]	2.33[hijk]
NKPV-1	101.00[f]	81.33[ef]	49.00[f]	16.33[fg]	4.00[ghi]
NKPV-2	138.33[c]	118.00[b]	71.33[cd]	30.00[d]	11.00[d]
NKdPV	117.33[de]	100.00[cd]	49.33[f]	12.33[ghi]	7.00[ef]
NNPD	134.00[c]	73.33[fgh]	65.33[de]	19.00[fg]	10.00[d]
NNPV	88.33[g]	79.33[f]	61.00[e]	22.00[ef]	3.00[ghij]
NNkPV	88.00[g]	63.66[hi]	59.00[e]	6.00[ijk]	0.33[k]
SD	37.27	35.24	31.24	21.75	7.51

* 96h after inoculation. Each values are mean of three replication

a,b,c and so on indicate ranking of growth of free living diazotrophs at different pH levels

Table 5. Amount of nitrogen fixed by the free living diazotrophs at different pH levels* (mg of N g^{-1} of sucrose utilized)

Isolate	pH 7.0	pH 6.5	pH 6.0	pH 5.5	pH 5.0
NTS-1	5.60^{n}	4.70^{k}	3.27^{i}	4.66^{ij}	1.40^{h}
NTS-2	11.66^{l}	11.20^{j}	6.06^{i}	4.20^{ij}	3.26^{gh}
NTS-3	30.80^{g}	20.06^{g}	16.80^{c}	11.66^{g}	7.46^{de}
NKS-1	16.80^{j}	8.86^{j}	3.26^{j}	1.86^{k}	1.40^{gh}
NKS-2	35.00^{de}	28.00^{bcd}	15.40^{cd}	14.00^{ef}	6.06^{ef}
NBS	14.46^{k}	11.20^{j}	8.86^{h}	7.00^{h}	4.66^{fg}
NKdS	36.86^{cd}	28.46^{bcd}	22.86^{b}	21.00^{a}	16.00^{a}
NAS	22.86^{i}	14.46^{i}	9.80^{gh}	6.06^{hi}	1.86^{h}
NPS-1	40.60^{a}	35.00^{a}	24.26^{ab}	17.26^{cd}	11.66^{b}
NPS-2	21.00^{i}	18.66^{gh}	14.46^{de}	7.50^{jk}	3.30^{de}
NPS-3	32.66^{fg}	22.86^{f}	14.46^{de}	12.60^{fg}	8.40^{cd}
NKPD	34.06^{ef}	26.60^{de}	15.86^{cd}	13.06^{fg}	9.33^{cd}
NKPV-1	18.66^{j}	17.30^{gh}	13.06^{ef}	7.50^{h}	2.80^{gh}
NKPV-2	37.80^{bc}	29.86^{b}	23.80^{ab}	18.66^{bc}	14.60^{a}
NKdPV	26.60^{h}	14.46^{i}	12.60^{ef}	5.60^{hi}	1.40^{h}
NNPD	34.06^{ef}	27.06^{cde}	17.26^{c}	15.86^{de}	10.26^{bc}
NNPV	18.66^{j}	16.80^{hi}	11.66^{fg}	7.46^{h}	4.66^{fg}
NNkPV	8.86^{m}	9.80^{j}	2.80^{j}	1.86^{k}	1.33^{gh}
NPPV	39.66^{ab}	29.40^{bc}	25.66^{a}	20.06^{ab}	14.73^{a}
SD	10.94	8.49	7.27	5.98	4.83

Each values are mean of three replication

a,b,c and so on indicate ranking of nitrogen fixation of free living diazotrophs at different pH levels

Microbacterium sp.) and NKPV-2 (later identified as *Brevundimonas* sp.) were found to grow and fix nitrogen efficiently (Fig. 2 - 5). For all the isolates, maximum growth and nitrogen fixation were observed at pH 7.0 and there was a gradual decline as pH was lowered. A similar trend was earlier reported by Ninawe and Paulraj (1997) in *Azotobacter* sp. They observed, with the increasing level of pH, a gradual increasing trend of growth up to neutral pH. Similarly, nitrogen fixation was also found to decrease at acidic pH and complete inhibition was noticed below pH 5.0.

Indole-3-acetic acid (IAA) is an important plant growth regulator and growth stimulator produced by the plants as well as bacteria. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik, 1986). Among the twenty isolates, four isolates (NTS-3, NKdS, NKPV-1 and NPS-1) were found to be positive for IAA production. Among these, NPS-1 (*Cellulosimicrobium* sp.) was found to be an excellent producer of IAA while NKdS (*Microbacterium* sp.) was moderate. In a similar study by Egamberdieva (2008), IAA production was detected in some strains of *Microbacterium* and *Cellulosimicrobium*.

Volatile compounds such as HCN are produced by many bacterial strains and have been considered as important metabolites in disease control (Pal and Jalali, 1998). Out of twenty isolates screened, HCN production was recorded in five isolates (NBS, NTS-1, NPS-1, NPPV, and NAS). In a similar study conducted by Raval and Desai (2012), HCN production was detected in some isolates of PGPR from rhizosphere of sunflower.

Sixteen isolates were observed to be positive for ammonia production. Eight isolates NPS-1

Sequence analysis of isolates

CAGTGGCGACGGGTGAGTAACACGTGAGCAACCTGCCCTGGACTCTGGGATAAGCGCTGGAAACGGTGTCTAATACTGGATATGAGCTCTTCCGCATGGTGGGGGTTGGA
AAGATTTTTCGGTCTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTA
AACCTCTTTTAGCAGGGAAGAAGCTTTTGTGACGGTACTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAAT
TATTGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTG
GAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAA
ACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGACCATTCCACGGTTTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGAGTAC
GGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTGACATACACCA
GAACACCGTAGAAATACGGGACTCTTTGGACACTGGTGAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCT
TCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCCTGAAGTC
GGAGTCGCTAGTAA

A. NPS-1

ACGGTGAGTAACACGTGAGCAATCTGCCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACCGGATACGAGCTGCGAAGGCATCTTCAGCAGTTGGAAAGAATTT
CGGTCAGGGATGAGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGACGACGGCCTTCGGGTTGTAAACCTCTTTA
GCAGGGAAGAAGCGAAAGTGACGGTACTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGATTATTGGGCGTAA
AGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGG
TGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGA
TACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAG
GCTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATAGAGGAAACTTCTG
GAAACAGTCGCCCCGCAAGGTCTCTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGC
CAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACA
ATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAG

D. NKdS

CAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCTGGACTCTGGGATAAGCGCTGGAAACGGTGTCTAATACTGGATATGAGCTCCTTCCGCATGGTGGGGGTTG
GAAAGATTTTTCGGTCTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGG
TTGTAAACCTCTTTAGCAGGGAAGAAGCTTTTGTGACGGTACTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCG
GAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGG
GAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGG
GGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGACCATTCCACGGTTTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCC
TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTT
GACATACACCAGAACACCGTAGAAATACGGGACTCTTTGGACACTGGTGAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTC
GACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGAT

C. NPPV

AGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTGACTTCGGGATAACTCCGGGAAACCGGGGCTAATACCGGATATGAGCTACCCTCGCATGGGGGTGGTTGGAAA
GTTTTTCGGTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAAGGCCTTCGGGTTGTAAACCTC
TTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGGTGTGAAAACTCGAGGCTCAACCTCGAGCTTGCATCGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGACTGGAATTCCT
GGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGCAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGCTCATTCCACGAGTTCCGTGCCGCAGCAAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGC
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGCACGGGAAGCCACCAGAG
ATGGTGGTCTCTTTGGACACTCGTGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCCATGTTGCCAGC
GGGTTATGCCGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCC
GGTACAAAGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA

(*Cellulosimicrobium* sp.), NKdS (*Microbacterium* sp.), NTS-1, NKPV-2 (*Brevundimonas* sp.) NNPV, NPPV (*Microbacterium* sp.), NNkPV, and NNPD were found to be good ammonia producers, developing brown colour when Nessler's reagent was added. Kumar et al. (2012) reported the production of ammonia by some strains of PGPR isolated from rhizosphere soil of french bean. Ammonia production in *Cellulosimicrobium* sp. has been reported earlier (Singh et al., 2014).

Siderophore production is another important attribute of PGPR. However, none of the isolates obtained in the study produced siderophores.

Table 6. PGPR traits of selected isolates

Isolates	IAA	HCN	Ammonia	Antagonistic activity	
				R. solani	R. solanacearum
NKdS (Microbacterium)	+	-	+	+	-
NPS-1 (Cellulosimicrobium)	+	+	+	-	-
NPPV (Microbacterium)	-	+	+	+	-
NKPV-2 (Brevundimonas)	-	-	+	-	+

Antagonistic activity is an indirect way of promoting plant growth, as this would help plants fight against phytopathogens (Jetiyanon and Kloepper., 2002). Three isolates, viz. NkdS (*Microbacterium*), NPPV (*Microbacterium*) and NKS-1, were found to exhibit antagonistic activity against *R. solani.* Maximum per cent inhibition of 56.94 was recorded in NKS-1. Under *in vitro* screening NPPV was found to be a moderate HCN producer and ammonia production was detected in isolates NKdS and NKS-1. Hence, production of HCN and ammonia could be one of the mechanisms behind the antagonistic activity of the isolates. Antagonistic activity of *Microbacterium* sp. against *R. solani* has been reported earlier (Ji et al., 2014). None of the isolates exhibited antagonism against *F. oxysporum*. Egamberdieva (2008) also observed that isolates of *Microbacterium* and *Cellulosimicrobium* did not exhibit antagonistic activity against *F. culmorum.*

Six isolates (NPS-2, NPS-3, NNPV, NNkPV, NKPV-2 and NKPD) exhibited antagonistic activity against *R. solanacearum.* All these six isolates were found to be positive for ammonia production under *in vitro* condition and this could be one of the mechanisms behind their antagonistic activity against *R. solanacearum.*

Out of twenty isolates, based on growth, nitrogen fixation, tolerance to acidic pH and PGPR activities under *in vitro* condition, four promising diazotrophs, including two rhizosphere isolates (NPS-1 and NKdS) and two phylloplane isolates (NPPV and NKPV-2) were selected as the promising isolates (Table 5). These were identified by 16S rDNA sequence analysis (Table 6). Based on the accession in NCBI database, showing maximum homology with query sequence, isolate NKdS and NPPV showed maximum homology with *Microbacterium* sp., NPS-1 with *Cellulosimicrobium* sp. and NKPV-2 with *Brevundimonas* sp. *Microbacterium* is a Gram positive, non-sporulating rod-shaped endophytic actinobacteria, reported to be present in a broad range of environmental habitats (Costa et al., 2012). *Cellulosimicrobium* is also a Gram positive, non-endospore forming bacterium reported to promote plant growth (Chatterjee et al., 2009). The results of the study revealed that, apart from already commercialized nitrogenous biofertilizers like *Azotobacter* and *Azospirillum,* novel, free living diazotrophs suitable for acidic soils of Kerala could be exploited as biofertilizers. Since these are free living and non-specific, these could be used for any crop.

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