academicJournals

Vol. 8(26), pp. 3436-3443, 11 July, 2013
DOI: 10.5897/AJAR2013.6762
ISSN 1991-637X ©2013 Academic Journals
http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Studies on heterotrophic bacteria with special reference to *Azospirillum* from rhizosphere and root of different crops

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Accepted 9 July, 2013

Availability of heterotrophic soil bacteria and an associative micro-aerophilic nitrogen fixing *Azospirillum*, from the rhizosphere of different crop plants, was studied. Microorganisms were reported from root and rhizospheric soil (from twenty-seven different samples collected from Chhattisgarh, India) of different crop plants. The amount of total heterotrophic bacteria, in different samples, varied significantly from 3.01×10^8 to 4.90×10^8 in rhizosphere and from 1.11×10^8 to 2.91×10^8 in root tissues. *Azospirillum* population significantly varied from 3.81×10^8 to 4.90×10^8 in rhizosphere and from 2.09×10^8 to 2.88×10^8 in root tissues. Rhizospheric soils were found to harbor the highest population of heterotrophic soil bacteria and *Azospirillum* in comparison with plant tissues. Nitrogen, phosphorus, potash, organic matter (OM) and pH value have significant positive correlation but electrical conductivity (EC) have non-significant effect with total heterotrophic bacterial population. Based on the correlation co-efficient (r) value, all significant positive correlation has been grouped in three categories viz; strong (r = >0.5), medium (r = >0.4 but <0.5) and weak (r = <0.4). Thus, in the present study, N, OM and rhizospheric pH have strong significant positive correlation with total heterotrophic bacterial population while P and K content have medium correlation with bacterial population.

Key words: Heterotrophic bacteria, rhizosphere, *Azospirillum,* nitrogen fixing bacteria, correlation, regression.

INTRODUCTION

Soil microorganisms play a major role in processing all ecosystems, by acting as, the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, soil carbon sequestration, modifying soil physical structure and enhancing the efficiency of nutrient acquisition by the vegetation and enhancing plant health (Singh et al., 2011; Glick, 2012). They are the biological components of soil that constitute less than 0.5% (w/w) only of the soil mass (Tate, 1995). Among them, heterotrophic bacteria are one of the main components of microbial community. The population of these bacteria gives an indication about the soil sustainability for agriculture (Jolly et al., 2010). These heterotrophic bacteria play an important role in nutritional changes that determines plant productivity. Without heterotrophic bacteria, soil could not be fertile and organic matter, such as straws and leaves, would accumulate in a short time (Kummerer, 2004).

Among heterotrophic bacteria, *Azospirillum*, the most important bacterium among all the nitrogen-fixer, fixes

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nitrogen in many monocotyledons including maize, rice, sugarcane, sorghum and forage grasses viz; *Digitaria, Kallar* grass and *Brachiaria* (Dobereiner et al., 1976; Baldani and Dobereiner, 1980; Baldani et al., 1996, 1997; Hartmann et al., 1995; Olivares et al., 1996; Krol, 1999; Reis Junior et al., 2003; Lin et al., 2012). A large number of power plants including world second largest and India's largest sponge iron and steel plant, with the production capacity of 23720 MW power per day, are situated at Raigarh with consumption of 20,000 t coal per day that emit huge quantity of pollutant(s) leading to extensive pollution of atmosphere as well as agricultural land. With all these background information, this study was conducted with the aim to quantify the present level of heterotrophic bacteria, in general, and *Azospirillum,* in particular, in the soil of district-Raigarh, Chhattisgarh, irrespective to the level of pollution. Correlation and regression study was carried out to find the effect of different general parameters of soil on the heterotrophic bacteria, if any.

MATERIALS AND METHODS

Present study was conducted on the agricultural land of district Raigarh, also known as industrial capital of state Chhattisgarh (India), situated at coordinates of 21.9°N 83.4°E on 705 ft msl. Area of the district is 6530 km^2 and it has been divided in 09 agricultural development revenue areas called as blocks.

The most common and economical method, for sampling in an area, is the composite sampling (Rohde, 1976) that was used during the samples collection for the present investigation and, accordingly, samples were collected from November 15th to December 07th, 2008. During sampling process, sub samples were collected from randomly selected location in a field and the sub samples were mixed for analysis. Out of 09 blocks of the district, each block was divided in to 03 parts on the basis of area. Total 05 fields, from each part, were randomly selected for the sub sampling. Thus, after compositing the sub samples, total 03 representative (one from each part) samples (each 500 g) were collected, from each block, that led consequently the collection of 27 representative samples throughout the district.

Moist and well-mixed representative samples were transferred to paper bags and kept in the room equipped with exhaust fan until the loss of moisture. From these samples, 100 g soil (from each sample) was used for soil analysis at biotech laboratory, collectorate campus, Ambikapur (Surguja) and another 100 g for the isolation of *Azospirillum*. The remaining quantity, of each sample, was stored in dry and wet condition for future necessity, if needed.

Total heterotrophic bacterial population

Isolation of total heterotrophic bacteria was done through the soil dilution technique by the using Nutrient Agar Medium at Plant Pathology Laboratory, RMD College of Agriculture and Research Station, Ambikapur (Chhattisgarh), India. After removing adhering soil, root samples were thoroughly washed for several times with distilled water. These root samples were macerated through pestle and mortar and sterile water added to each macerated sample to prepare suspension (1:10). Dilutions (10^{-1} to 10^{-7}) of the samples were prepared using sterile water. The dilution plate count method, using nutrient agar medium, was used to estimate the population of total heterotrophic bacteria in soil and root samples. In present study; data, on comparative population of total heterotrophic bacteria and *Azospirillum* in rhizosphere and root tissues, were analyzed through student pair t-test.

Isolation, enumeration and purification of *Azospirillum*

Isolation of *Azospirillum* was done through the soil dilution technique and root bit cutting at Plant Pathology Laboratory, RMD College of Agriculture and Research Station, Ambikapur (Chhattisgarh), India.

By soil dilution technique

Total 1.0 g rhizospheric soil was suspended in 10 ml of sterile water and prepared serial dilutions (from 10^{-1} to 10^{-9}) of suspension in to sterile water blanks. Appropriate dilutions were poured on Nfb medium containing NH_4Cl as source of nutrient for initial growth of bacteria (Okon et al., 1977). After that, these test tubes were incubated for four to five days in incubator at 28 to 30°C.

Root bit cuttings

The roots were surface sterilized by 0.1% acidified $HgCl_2$ for three minutes and then in 70% alcohol for one minute. The roots were then subsequently washed, to free from these chemicals, with five to six washings of sterile water. The root segments were further cut in to small bits (2 to 3 cm size) aseptically and placed in semi solid Nfb medium in sterile Petri-dishes using sterile forceps and then incubated for four to five days in incubator at 28 to 30°C (Okon et al., 1977). *Azospirillum* population was calculated on basis of per gram of soil using following formula (Schmidt and Caldwell, 1967). Pure culture of each isolate was prepared on the slant.

$$\text{Number of bacteria per gram soil} = \frac{\text{No. of colony forming units} \times \text{dilution}}{\text{Dry weight of 1 g soil} \times \text{aliquot taken}}$$

Identification of *Azospirillum*

Physiological markers tests like growth behavior in medium, color change of nitrogen free broth medium, motility test, shape of the bacterium and poly-hydroxy-butarate granules test (Tarrand et al., 1978) along with biochemical markers like gram staining reaction, starch hydrolysis test (Attitalla et al., 2010) were carried out for the primary identification of the bacterium *Azospirillum*. After primary identification, cultures were sent to Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa campus, New Delhi, India - 12, for there confirmation through molecular marker (16s rRNA and 16s rDNA sequencing (Kanimozhi and Panneerselvam, 2010).

RESULTS

Total heterotrophic bacterial population (THB)

Data on heterotrophic bacterial population revealed that bacterial population varied from 3.01×10^8 to 4.90×10^8 in rhizosphere (Table 1) and from 1.11×10^8 to 2.91×10^8 in root tissues. Maximum heterotrophic bacterial population (4.90×10^8 CFUs/g soil) was recorded from *Lycopersicon*

Table 1. Different parameters of soil analysis.

S/N	Block	Crop	N	P	K	EC	OM (%)	pH		THB(10^8)	
								RH	Root	RH	Root
01	Sarangarh	*Lycopersicon esculentum*	255.5	44.1	330.1	0.30	0.80	7.0	6.9	4.06	2.15
02	Sarangarh	*Oryza sativa* (Mid- land)	126.3	42.0	303.2	0.11	0.79	6.2	7.2	4.01	2.14
03	Sarangarh	*Capsicum annum*	144.5	48.1	291.0	0.30	0.69	6.9	6.8	4.02	2.80
04	Pusour	*Coriandrum sativum*	274.6	41.4	332.4	0.40	0.86	7.1	7.0	4.21	2.11
05	Pusour	*L. esculentum*	161.1	47.0	297.2	0.19	0.61	6.9	6.9	4.80	2.91
06	Pusour	*L. esculentum*	253.2	40.5	325.5	0.80	0.89	7.2	6.7	4.90	2.09
07	Gharghoda	*O. sativa* (Mid- land)	171.5	31.0	266.1	0.40	0.44	6.6	6.1	3.09	1.70
08	Gharghoda	*Zea mays*	144.2	39.6	257.2	0.90	0.64	6.5	6.5	3.57	1.99
09	Gharghoda	*C. annum*	160.1	53.1	277.3	0.70	0.67	6.5	6.7	3.16	2.55
10	Lailunga	*C. annum*	129.5	44.8	250.2	0.50	0.66	6.8	6.5	3.83	2.49
11	Lailunga	*O. sativa* (Mid- land)	166.6	34.9	255.9	0.18	0.67	6.8	6.5	3.58	1.19
12	Lailunga	*O. sativa* (Mid- land)	162.2	48.9	259.4	0.70	0.45	6.9	6.5	3.01	2.01
13	Tamnar	*O. sativa* (Mid-land)	148.1	33.5	260.5	0.10	0.49	6.6	6.5	3.87	2.14
14	Tamnar	*C. annum*	126.4	37.1	255.2	0.40	0.86	6.4	6.3	3.03	1.79
15	Tamnar	*C. annum*	156.6	41.0	250.4	0.80	0.68	6.8	6.5	4.01	1.99
16	Sariya	*T. dioica*	163.2	30.0	248.1	0.13	0.36	6.9	6.8	3.59	2.29
17	Sariya	*C. sativum*	153.2	23.1	243.1	0.12	0.39	6.4	6.1	3.69	1.89
18	Sariya	*O. sativa* (Mid- land)	169.1	33.9	231.4	0.12	0.32	6.7	6.6	3.07	2.02
19	Raigarh	*O. sativa* (Mid- land)	146.5	24.0	226.2	0.70	0.49	6.5	6.4	3.06	1.77
20	Raigarh	*C. annum*	123.2	14.9	216.5	0.30	0.28	6.5	6.5	3.08	1.11
21	Raigarh	*L. esculentum*	254.7	33.7	336.2	0.50	0.88	7.1	6.8	3.81	2.88
22	Kharsia	*Z. mays*	175.6	37.2	297.1	0.18	0.73	6.7	6.1	3.87	1.38
23	Kharsia	*C. annum*	177.8	40.9	207.5	0.40	0.76	6.7	6.3	3.89	1.58
24	Kharsia	*O. sativa* (Mid- land)	178.3	43.5	211.2	0.21	0.78	6.8	6.4	3.99	1.36
25	Dharamjaigarh	*O. sativa* (Mid- land)	129.1	32.9	303.5	0.11	0.64	6.7	6.5	3.01	1.62
26	Dharamjaigarh	*Zingiber officinales*	126.2	37.1	277.6	0.70	0.67	6.6	6.3	3.69	1.49
27	Dharamjaigarh	*O. sativa* (Mid- land)	120.2	31.0	250.2	0.30	0.66	6.7	6.5	3.07	1.19

N = Nitrogen (Kg/ha); P = phosphorus (Kg/ha); K = potash (Kg/ha); EC, (mmol/cm); OM, Organic matter; THB, total heterotrophic Bacteria; RH, rhizosphere.

esculentum rhizosphere (Pusour) closely followed by 4.80×10^8 from *Lycopersicon esculentum* (Pusour), 4.21×10^8 from *Coriandrum sativum* (Pusour), *L. esculentum* (4.06×10^8) from Sarangarh and *Capsicum annum* (4.02×10^8) from Sarangarh blocks, respectively. Lowest heterotrophic bacterial population (3.01×10^8) was recorded from *Oryza sativa* (Lailunga) and *O. sativa (*Dharamjaigarh*),* respectively. In root tissues, the maximum of bacterial population was found in *L. esculentum* (2.91×10^8) in Pusour followed by Raigarh (2.88×10^8) and Sarangarh (2.80×10^8). While the lowest population (1.11×10^8), in plant tissues, was observed in *Capsicum annum* in Raigarh followed by *O. sativa* (1.19×10^8) in Lailunga and Dharamjaigarh blocks of the district.

Developmental block-wise distribution of the total heterotrophic bacteria (Figure 1) indicated that maximum population (average) was present in Pusour (4.63×10^8) followed by Sarangarh (4.03×10^8), Kharasia (3.91×10^8) and Tamnar (3.63×10^8), respectively. Lowest population of heterotrophic bacteria existed in Dharamjaigarh (3.25×10^8) block followed by Gharghoda (3.27×10^8) and Raigarh (3.31×10^8), respectively. Population in Sariya and Lailunga blocks stood same (3.4×10^8) thus, on the basis of total heterotrophic bacterial population, whole district is divided in to three groups viz; (1) total heterotrophic bacterial population (> 3.5×10^8) comprising of blocks Pusour, Sarangarh, Kharasia and Tamnar, (2) total bacterial population (3.5×10^8) comprising of the blocks Sariya and Lailunga and (3) total bacterial population (< 3.5×10^8) comprising of the blocks Dharamjaigarh, Raigarh and Gharghoda. Data, on comparative population of total heterotrophic bacteria in rhizosphere and root tissues, were analyzed through student pair t-test and depicted in Figure 2. Results indicated that rhizospheric bacterial population was significantly higher (3.70×10^8) over population of root tissues (1.95×10^8) indicating that bacteria remained present in rhizosphere as well as in plant tissues but more preferably in rhizosphere.

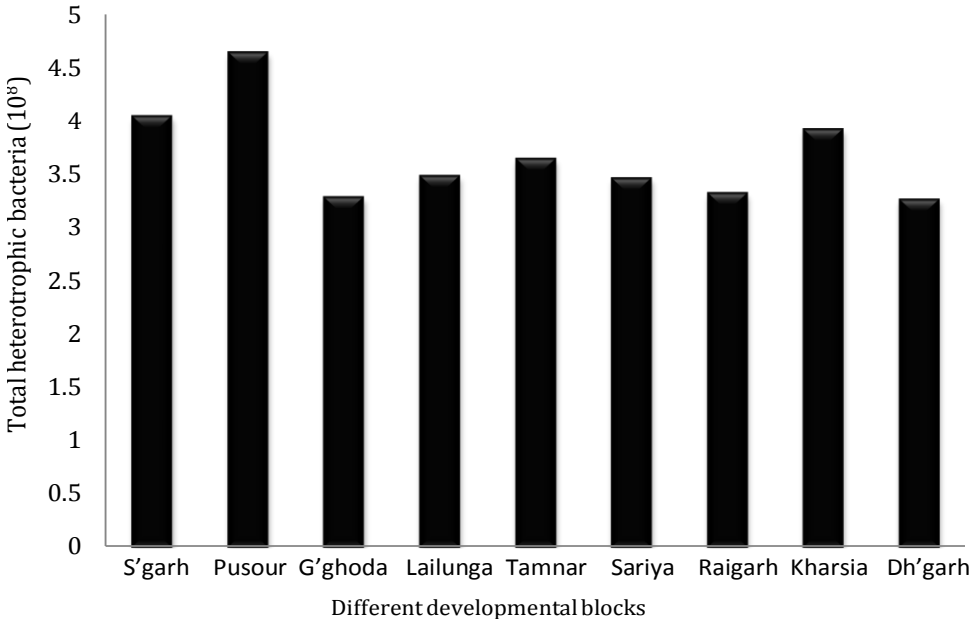

Figure 1. Block wise distribution of total heterotrophic bacterial population in (Chhattisgarh), India.

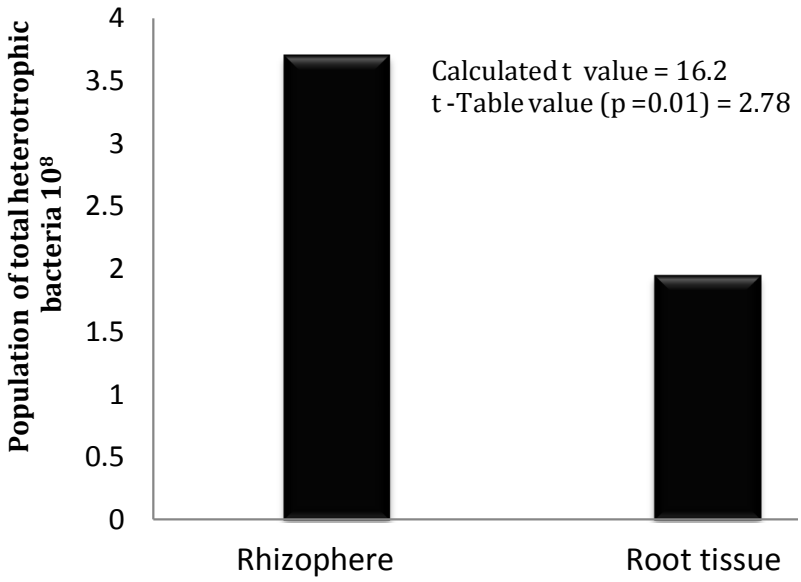

Figure 2. Comparative distribution of heterotrophic bacteria in rhizosphere and root tissues.

Comparative population of THB and *Azospirillum* in rhizosphere and root tissues

Along with the location wise variation in the distribution, population of *Azospirillum* was also significantly variable in the different crop rhizosphere (Table 2) and plant tissues. Student pair t-test analyzed data, depicted in Figure 3, revealed that the population of *Azospirillum* was significantly higher in rhizosphere (1.08×10^8), in comparison to plant tissues (0.93×10^8),indicating rhizosphere as preferable site for inhabitation.

Correlation between different soil parameters and THB

Present study, on the effect of different physio-chemical properties on the total heterotrophic bacteria (Table 3),

Table 2. Presence and absence of *Azospirillum* in rhizospheric soil and plant tissues.

SN	Block	Crop	*Azospirillum* (Present = +/ Absent = -)	
			Rhizosphere	Root
01	Sarangarh	*L. esculentum*	+	+
02	Sarangarh	*O. sativa* (Mid- land)	-	-
03	Sarangarh	*C. annum*	-	-
04	Pusour	*C. sativum*	+	+
05	Pusour	*L. esculentum*	-	-
06	Pusour	*L. esculentum*	+	+
07	Gharghoda	*O. sativa* (Mid-land)	-	-
08	Gharghoda	*Z. mays*	-	-
09	Gharghoda	*C. annum*	-	-
10	Lailunga	*C. annum*	-	-
11	Lailunga	*O. sativa* (Mid- land)	-	-
12	Lailunga	*O. sativa* (Mid- land)	-	-
13	Tamnar	*O. sativa* (Mid-land)	-	-
14	Tamnar	*C. annum*	-	-
15	Tamnar	*C. annum*	-	-
16	Sariya	*Trichosanthes dioica*	-	-
17	Sariya	*C. sativum*	-	-
18	Sariya	*O. sativa* (Mid- land)	-	-
19	Raigarh	*O. sativa* (Mid- land)	-	-
20	Raigarh	*C. annum*	-	-
21	Raigarh	*L. esculentum*	+	+
22	Kharsia	*Z. mays*	-	-
23	Kharsia	*C. annum*	-	-
24	Kharsia	*O. sativa* (Mid- land)	-	-
25	Dharamjaigarh	*O. sativa* (Mid- land)	-	-
26	Dharamjaigarh	*Z. officinales*	-	-
27	Dharamjaigarh	*O. sativa* (Mid- land)	-	-

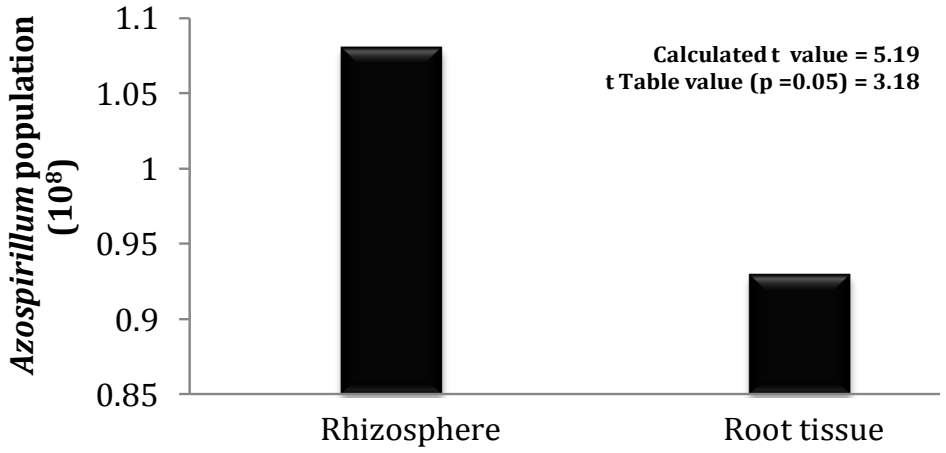

Figure 3. Distribution of *Azospirillum* in rhizosphere and plant tissues.

indicated that the heterotrophic bacterial population was greatly affected by different soil parameters like nitrogen, phosphorus, potash, EC, OM and pH. All the studied parameters have positive correlation with total bacterial population. Nitrogen, phosphorus, potash, organic matter and pH value have significant positive correlation but EC have non-significant with total heterotrophic bacterial population. Based on the correlation co-efficient (r) value,

Table 3. Correlation and regression studies between different parameters of soils with total hetero-trophic bacterial population in rhizosphere Chhattisgarh.

SN	Independent character	Dependent character	Correlation	Calculated T - value	Regression equation
01	Nitrogen	THB (Rhizosphere)	0.502	2.906*	Y=0.0061X+2.6505
02	Phosphorus	THB (Rhizosphere)	0.406	2.225*	Y=0.025082X+2.728039
03	Potash	THB (Rhizosphere)	0.461	2.603*	Y=0.006534X+1.90861
04	EC	THB (Rhizosphere)	0.005	0.026**	Y=0.011127X+3.661208
05	OM	THB (Rhizosphere)	0.503	2.917*	Y=1.504038X+2.709656
06	pH	THB (Rhizosphere)	0.504	2.925*	Y=1.119318X +3.85875

THB , Total heterotrophic bacteria; *Significant (p=0.05); **Non-significant (p=0.05).

all significant positive correlation has been grouped in three categories viz; strong (r = > 0.5), medium(r = >0.4 but <0.5) and weak (r = <0.4). Thus, in the present study, N, OM and rhizospheric pH have strongsignificant positive correlation with total heterotrophic bacterial population while P and K content have medium correlation with bacterial population.

DISCUSSION

Total heterotrophic bacterial population (THB)

Soil contains a variety of microorganisms including heterotrophic bacteria as major microorganism component. Present results, on the heterotrophic bacterial population in rhizosphere and root tissues, indicated that heterotrophic bacterial population in the rhizosphere varied from minimum (3.01×10^8) in *Oryza sativa* to maximum (4.90×10^8) in *Lycopersicum esculentum* in the Chhattisgarh. On the other hand, heterotrophic bacterial population varied in root tissues with minimum (1.11×10^8) in *C. annum* and maximum (2.91×10^8) in *L. esculentum*. Such higher variation in the population of THB (total heterotrophic bacteria) depends upon some factor such as rhizospheric positive interaction (due to release of fatty acid, protein, amino acids) and negative interaction (due to release of antimicrobial compounds, lack of suitable substrate as nutrition for microbes) between plant exudates and microbes (Bais et al., 2004). Variation in heterotrophic population has also been reported by Khan et al. (2003) in coastal areas of Bangladesh (1.33×10^7 to 24.67×10^7 CFUs/g), Sri Ramkumar and Kannapiran (2011) in coastal area of Tamil Nadu (4.25-8.25×10^6 cells/ml) and Jolly *et al* (2010) in *Taro* rhizospheric soil of Bangladesh (9.66×10^6 to 9.80×10^6 CFUs/g).

Comparative population of THB and Azospirillum in rhizosphere and root tissues

Results on the comparative distribution of heterotrophic bacterial population in rhizosphere and plant tissues indicated that rhizospheric bacterial population was significantly higher (3.70×10^8) over population of root tissues (1.95×10^8). It showed that heterotrophic bacterial population was present in rhizosphere as well as in plant tissues but more preferably in rhizosphere. Higher population of total heterotrophic population in the rhizosphere may be attributed to the chemical attraction of soil microbes to plant roots or chemotaxism may involved in initiating cross talk between plant roots and microbes (Bais et al., 2004) while low population in plant root tissues may be due to susceptibility and resistance with particular group or species of microbes.

Azospirillum species are frequent inhabitant of the rhizosphere of a wide variety of plants with three-carbon and four-carbon photosynthesis in diverse climatic regions of the world (Krieg and Dobereiner, 1984; Rao and Venkateswarlu, 1982). Results, on the comparative distribution of *Azospirillum* population in rhizosphere and plant tissues, indicated that rhizospheric bacterial population was significantly higher (1.08×10^8) over population of root tissues (0.93×10^8). It showed that population was present in rhizosphere as well as in plant tissues but more preferably in the rhizosphere. Higher population of *Azospirillum* population in the rhizosphere may be attributed to root exudates that primarily contain organic acids, sugars and amino acids that contribute major source of nutrients for the micro-flora in the rhizosphere. Krieg and Dobereiner (1984) indicated that organic acids, present in the rhizosphere, supports vigorous growth and nitrogen fixation of all *Azospirillum* species in rhizosphere. Our results also support the finding of Jolly et al. (2010) who reported that, in *Taro*, *Azospirillum* population was higher (11×10^6 MPN/g soil dry weight) in rhizosphere compared to root tissues (0.1×10^6 MPN/g root tissues dry weight). Contradictory to our findings, Ravikumar et al. (2002) showed that population of *Azospirillum* was higher in the root tissues of *Avicennia marina*, a mangrove plant.

Correlation between weather parameters and THB

Number of environmental factors (modulators) as

temperature, pH, water holding capacity etc affect the bacterial community in a particular environment (Balser et al., 2001). In the present study, results on correlation between different soil abiotic factors and population of *Azospirillum* species (heterotrophic bacterium) indicated that three factors as nitrogen, pH and organic matter strongly affected (strong correlation) the population and were positively correlated with the bacterial population. Other factors as Phosphorus and Potash also affected the population but correlation was medium while other factor as soil EC had non-significant effect on the population (week correlation).

In similar study, Bashan et al. (1995) observed that percentages of clay, nitrogen, organic matter and water-holding capacity were positively correlated with *Azospirillum brasilense* viability while percentages of $CaCO_3$ and fine or rough sand was highly negative correlation on viability.

Other factors like percentage of silt, phosphorus or potassium, and pH, electrical conductivity, and C/N ratio had no apparent effect on viability of *Azospirillum* in the soil. Thus, our results are in the affirmation with the findings of Bashan et al. (1995) in respect to respective parameters studied except pH.

As the field of study regarding the soil heterotrophic population in relation to pH is still virgin and needs thorough study. Bashan et al. (1995), in relation to *Azospirillum* species, reported that soil pH had no apparent effect on the viability of *Azospirillum*. However, in contrast to above, our study indicated that soil pH was strongly positively correlated with the heterotrophic bacterial population though the variation in soil pH was limited from 6.2 to 7.2 (data not given). Working with forest and silt loam soil; Matthies et al. (1997) and Rousk et al. (2009), described pH, as one of the most important environmental factor, has determining role in the type of microorganism(s) that predominate in different soil and they found that population of culturable bacteria decreased sharply in the acidic condition.

Thus, present study receives strong support from the above observation (Matthies et al., 1997; Rousk et al., 2009) and ascribe that soil pH is important factor for the growth and survival of heterotrophic bacterial population. The present information, generated through this study, will help for the study in future on the effect of soil pollution through pollutants, emitted by power and steel plants, in Chhattisgarh (India) and consequently for the maintenance such important microorganisms in the soil through the correction of above studied soil parameters for maintaining the soil fertility of the area.

ACKNOWLEDGEMENT

The authors are thankful to Professor and Head, Department of Soil Science, IGKV, Raipur and Dean, RMDCARS, Ambikapur, for permitting to avail the laboratory facilities, and also Professor and Head, Division of Plant Pathology, IARI, Pusa Campus, New Delhi-12, for identifying the bacterial cultures.

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