Ammonia Production and Promotion of Seed Germination by *Azotobacter* Isolated from Rhizospheric Soils of Some Vegetable Crops

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are considered to promote plant growth directly or indirectly. Various rhizospheric soil samples of five common vegetable crops revealed a significant viable plate count of aerobic heterotrophic bacteria as well as aerobic asymbiotic nitrogen fixing *Azotobacter* spp. A total of 20 isolates of *Azotobacter* recovered from these soils fairly utilised carbohydrates like glucose, fructose, lactose, adonitol and rhamnose. All isolates produced ammonia, and showed varying degrees of positive influence on germination of moong seeds *in vitro*. Four isolates A-4, A-6, A-15 and A-16 acquired high germination (100%) than control (60%). However, only one isolate A-12 was found to show inhibitory effect on seed germination. These isolates also showed higher tolerance to salt (2.5-3.5% NaCl conc.). It is expected that isolates exhibiting multi-PGP activities and elevated tolerance to environmental conditions may be suited for further assessment and development as the effective PGPR-inoculants.

Key words: Ammonia production, seed germination, *Azotobacter*, rhizospheric soils

INTRODUCTION

Soil bacteria play a major role in organic matter decomposition and are therefore central to soil ecosystem processes maintaining plant primary productivity (Griffiths *et al*, 2003). The rhizospheric soil is densely populated with microorganisms because it is rich in microbial nutrients as a result of root exudation (Ahmad *et al.*, 2005). The microbial populations interact with plants as well as among themselves. The rhizospheric bacteria may release various plant growth promoting substances as secondary metabolites, which may promote plant growth directly or indirectly. Such beneficial rhizospheric bacteria are often referred to as plant growth promoting rhizobacteria (PGPR). *Pseudomonas* is one of the extensively studied PGPR which promotes the growth of plants by protecting the plant health from soil borne phytopathogens and their toxins. The PGP activities associated with this bacterium include production of plant growth regulators (auxin, gibberellin, ethylene,

etc.), siderophores, ammonia, HCN and antibiotics. On the other hand, several other bacteria may promote plant growth directly by producing plant growth hormones and other substances in close contact to plant roots. The list of such bacteria is long, however, potential ones include asymbiotic nitrogen fixers and phosphate solubilizers in addition to well studied organisms like fluorescent *Pseudomonas* and certain species of *Bacillus* (Kloepper *et al.*, 1991).

Azotobacter chroococcum is a well known free living nitrogen fixing bacterium capable of synthesizing and secreting plant growth promoting substances like thiamin, riboflavin, IAA, gibberellin etc., and is frequently used as nitrogenous biofertilizer for a number of crops (Subba Rao, 1995). Regulatory mutants of *Azotobacter vinelandii* capable of excreting ammonia have been reported earlier but their agronomic utility is not yet known fully (Singh *et al.*, 1982). The incidence and diversity of free living asymbiotic nitrogen fixing bacteria including *A. chroococcum* in terms of their PGP properties and tolerance traits to environmental factors are poorly studied (Hayat *et al.*, 2002). Thus, it is expected that mapping of a particular area for indigenous *Azotobacter* and related bacteria for their diversity and above desirable characteristics may yield more effective and adaptive strains to be developed as eco-friendly bioinoculants. Such strains, if needed, may be further improved in their performance by routine genetic manipulations like mutation and gene transfer methods.

With this background, in the present investigation, an attempt has been made to explore the diversity of free living *Azotobacter* population in the rhizospheric soils of some selected vegetable crops and examine them for their PGP activities like ammonia excretion and promotion of seed germination.

MATERIAL AND METHODS

Composite soil samples from the rhizospheres of different vegetable crops (tomato, cabbage, pea, kale and cauliflower) growing at Rajpora in district Pulwama, Kashmir were collected from July to September, 2005, in sterile polythene bags. *Azotobacter* isolates were isolated from the soil on nitrogen free Ashby's medium. Each isolate showing characteristic growth, pigmentation and biochemical reactions as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*,1985) for *Azotobacter chroococcum* and related species was purified and given an isolate number. Similarly, heterotrophic bacteria were also isolated on nutrient agar medium. Both the microbiological media used were from Hi-Media Lab. Pvt. Mumbai, India.

The samples were cultured through spread plate technique (Taylor *et al*, 1983; Cappuccino *et al*, 1992). Five dilutions were made (i.e., 1:10, 1:100, 1:1000, 1:10000 and 1:100000) and two replicates were prepared for each dilution. The plates were incubated at a constant temperature of 28±2°C and the colonies were counted after 3rd and 5th day. Bacteria so isolated were also maintained in tube slants on suitable media.

A complete record of all the bacterial colonies appearing in each plate was kept, and the bacterial content per gram of soil was calculated (Waksman, 1952).

RESULTS AND DISCUSSION

In the present investigation, heterotrophic bacteria and specific soil bacterium *Azotobacter* were isolated and enumerated in rhizospheric soils of five vegetable crops (Table 1). The findings clearly indicated that the distribution and occurrence of bacteria did not differ significantly among various sources of rhizospheric soil samples taken from Pulwama, Kashmir. *Azotobacter* population was found in the range of $5.6x10^2$ to $7.3x10^3$ cfu /gm of these soils, whereas heterotrophic bacterial population ranged from $1.8x10^6$ to $6.5x10^7$ cfu /gm of these soils.

A total of 20 strains of *Azotobacter* were isolated, purified and designated (as A-1, A-2, A-3,....., A-20) on the basis of colony morphology, pigmentation and growth characteristics. All the isolated strains were characterised by microscopy (Gram-staining), and were found to be gram-ve, bacillary to ovoid cells. These isolates showed good growth on nitrogen-free Ashby's medium, and on prolonged incubation, black-brown pigmentation was evident which is characteristic of *Azotobacter* (Sheikh, 2002). The isolates were further characterised for their carbohydrate utilization pattern. All the isolates utilised glucose, whereas fructose, adonitol, rhamnose and lactose were utilised by 90%, 85%, 80% and 50% of the strains respectively. Thus, further differentiation of strains was achieved and these biochemical reports were similar with the results published in Bergey's Manual of Determinative Bacteriology (1985) and general characters of *Azotobacter* as described by Subba Rao (1995).

Table 1. Bacterial diversity of rhizospheric soils

S.No.	Rhizospheric Soil Samples	Microbial Diversity (CFU/gm of soil)*	
		Aerobic hertrotrophic bacteria	Azotobacter SSP.
1.	Tomato	3.8×10^7	6.5×10^3
2.	Cabbage	4.4×10^7	5.8×10^3
3.	Pea	6.5×10^7	7.3×10^3
4.	Kale	2.6×10^7	6.0×10^3
5.	Cauliflower	1.8×10^7	5.6×10^3

* **Values are of the mean of 3-5 samples**
CFU = colony forming units

On the basis of colony morphology, microscopic examination, carbohydrate utilization, pigmentation, etc., these 20 strains (from A-1 to A-20) were identified as *Azotobacter* spp. The growth behaviour of these diazotrophs was also evaluated against different salt concentrations (NaCl 0.5-3.5%). The concentration of 0.02% (present in Ashbys medium) was treated as control. Among all 20 strains, no inhibition of growth was observed at 0.5-1.5% salt concentration. At 2.5% salt concentration, growth was only slightly inhibited. At 3.0% salt concentration, the percentage of tolerant strains was 60%. However, all the strains were found to be less tolerant with increasing salt concentrations of the medium, viz., at 3.5% conc, only 30% were found to show growth. Effects of different concentrations of sodium chloride on *Azotobacter* strains were earlier also reported by Raj Kumar (1993) and are thus in confirmity with his study.

Azotobacter isolates of the present study were further evaluated for their plant growth promoting (PGP) activities viz., production of ammonia and promotion of seed germination (Table 2). All the strains produced ammonia, while only one A-12 showed inhibitory effect on the germination of the seeds of *Vigna radiata* under *in vitro* conditions. The strains A-3, A-4, A-15 and A-18 were found to be best for both ammonia production and promotion of seed germination. The strains A-4, A-6, A-15 and A-16 acquired high germination (100%) than control (60%) (Table 3). These PGP activities of *Azotobacter chroococcum* have also been reported by Narula *et al.* (1981) and Ahmad *et al.* (2005), but under different environmental conditions.

Table 2. Plant growth promoting rhizobacterial activities of isolated strains

S.No.	Strain Code	PGPR Activities	
		Ammonia Prod.	Seed Germ
1.	A-3, A-4, A-15, 1-18	+++	+++
2.	A-5, A-6, A-7, A-9, A-10, A-16	++	+++
3.	A-8, A-11, A-19	+	+++
4.	A-1, A-2, A-14, A17, A-20	+	++
5.	A-13	+	+
6.	A-12	+	-
	Control	-	+

For NH_3 Production:

+++ High production
++ Moderate production
+ Low production
- No production

For Seed Germination (%):

+++ 81 100% (High)
++ 61 80% (Moderate)
+ ≤60% (Low)

Table 3. ***In vitro*** **effect of inoculation of isolated strains on the germination of seeds of** ***Vigna radiata***

S.No.	Strain Code	Germination (%)	Root/shoot length (ratio)	
1.	A-1	70	<1	(L)
2.	A-2	65	<1	(L)
3.	A-3	85	<1	
4.	A-4	100	<1	(L)
5.	A-5	90	>1	
6.	A-6	100	<1	(L)
7.	A-7	85	<1	
8.	A-8	95	<1	(L)
9.	A-9	85	<1	(L)
10.	A-10	95	<1	
11.	A-11	85	<1	(L)
12.	A-12	--		
13.	A-13	60	>1	
14.	A-14	70	<1	
15.	A-15	100	<1	(L)
16.	A-16	100	>1	
17.	A-17	75	<1	
18.	A-18	85	<1	(L)
19.	A-19	90	<1	(L)
20.	A-20	65	>1	
	Control	60	=1	

81-100% = High 61 80% = Moderate 60% = Low

- Inoculum dose = 10^7 CFU/ml
- L is with leaves

(Root /shoot lengths taken after 3 days of sowing).

On the basis of this preliminary investigation, it may be concluded that soil of Rajpora in district Pulwama, Kashmir has a rich diversity of asymbiotic diazotrophs and predominant forms of *Azotobacter* are found. Some of these predominant forms exhibit promising plant growth promoting activities and relatively higher tolerance to salt. Further characterization at genetic level and their relative efficiency in nitrogen fixation are needed to uncover their potential application as a biofertilizer for local use.

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