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Resistance and susceptibility pattern of chickpea (*Cicer arietinum* L) endophytic bacteria to antibiotics

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ABSTRACT: Five chickpea (*Cicer arietinum*) endophytic bacteria, identified as *Erwinia herbicola*, *Enterobacter agglomerans*, *Bacillus megaterium* and *Bacillus* sp. and *Bacillus circulans* were tested for intrinsic antibiotic resistance in order to see if endophytes showed variation in resistance to antibiotics. The resistance pattern was compared with two rhizospheric bacteria *viz. Pseudomonas fluorescens* and *B. subtilis* in order to see if the susceptibility of endophytes differed with that of bacteria isolated from rhizosphere. The endophytes seemed to be less resistant to antibiotics. *B. circulans* was susceptible to all antibiotics tested except amoxycillin (10µg). However *B. megaterium* and *Bacillus* sp. and *E. agglomerans* showed some resistance. *P. fluorescens* and *B. subtilis* showed resistance to a wide range of antibiotics indicating that they could be better competitors in the rhizosphere. Preliminary screening was done to monitor *B. megaterium* and *Bacillus* sp. by using the observed antibiotic resistance. Out of the 25 root/stem/leaf tissues tested, 10 tested positive for the presence of *B. megaterium* and 11 for *Bacillus* sp. However, they could not be reisolated from the stem tissue. 3 and 2 of the leaf samples showed presence of *B. megaterium* and *Bacillus* sp., respectively.

KEY WORDS: Antibiotics, endophytic bacteria, resistance pattern

INTRODUCTION

Endophytic bacteria are those bacteria that colonize the plant internally without doing any harm to the plant but are involved in improving plant health. Endophytic bacteria are ubiquitous and colonize a broad spectrum of plant species and can move systemically throughout the plant. Most endophytic bacteria are probably found in the intercellular spaces of the root cortex or stem (Sturz and Matheson, 1996; Chen *et al.*, 1995; Hallmann, 2001).

Resistance against antibiotics is one of the parameter used to look for effective biological control agents (Siddiqui *et al.*, 2005). Studies show that intrinsic antibiotic resistance pattern could be used to distinguish bacterial strains after introduction in the rhizosphere (Chanway and Hall, 1986). Very few reports are available on the pattern of antibiotic resistance by plant endophytic bacteria.

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However, most of the reports are of those endophytes encountered during plant tissue culture (Tanprasert and Reed, 1997; Reed *et al.*, 1998). Patricia *et al.* (1995) isolated 22 endophytic bacteria from mint shoot cultures and showed that the minimal inhibitory concentration and minimal bactericidal concentration of gentamycin, rifampicin, streptomycin and timentin varied with genotype. Limited information is available on the comparison of antibiotic resistance pattern of endophytes with that of rhizosphere bacteria. In the present study an attempt was made to develop antibiotic resistance pattern for endophytic bacteria and compare it with the resistance pattern of two rhizosphere isolates. Preliminary investigation was also undertaken to see if the resistance to antibiotics could be used for identification.

MATERIALS AND METHODS

Endophytic bacteria isolated from healthy chickpea plants were previously identified based on Gram's reaction, morphological, physiological and biochemical tests (Rangeshwaran *et al.*, 2008). The endophytes included *Erwinia herbicola* (MTCC 6720), *Enterobacter agglomerans* (MTCC 6536), *Bacillus megaterium* (MTCC 6533) and *Bacillus* sp. (MTCC 6534) and *Bacillus circulans* (MTCC 6535). Two rhizosphere isolates *viz.*, *Pseudomonas fluorescens* (PDBCAB2) and *Bacillus subtilis* (PDBCABN22) which were obtained from culture collection of Project Directorate of Biological Control (PDBC), Bangalore were also used in the study.

Testing for antibiotic resistance

Antibiotic sensitivity tests were done for all the test bacteria by using the octodisks that were procured from Himedia Laboratories, India. The following octodisks were selected based on the Gram's reaction.

Octodisks for Gram positive bacteria

For Gram positives the following octiodisks were used; 1. Combi 1 [cephalothin (30 μg), clindamycin (2 μg), co-trimaxazole (25 μg), erythromycin (15 μg), gentamicin (10 μg), ofloxacin (1 μg), penicillin-g (10 units), vancomycin (30 μg)]. 2. Combi VII [amoxycillin (10 μg), cloaxcillin (5 μg), erythromycin (15 μg), tetracycline (10 μg), penicillin (2 units), co-trimaxazole (25 μg), penicillin-v (10 units), cephalexin (30 μg)]. 3. Combi XIII [penicillin-g (2 μg), tetracycline (10 μg), co-trimaxazole (25 μg), cloaxcillin (5 μg), cephradine (30 μg), erythromycin (10 μg), lincomycin (10 μg), cefuroxime (30 μg)]. 4 Gx Plus [chloramphenicol (25 μg), erythromycin (5 μg), fusidic acid (10 μg), methicillin (10 μg), novobiocin (5 μg), penicillin-g (1units), streptomycin (10 μg), tetracycline (25 μg)]. 5. G-V-Plus [amoxycillin (10 μg), tetracycline (30 μg), co-trimaxazole (25 μg), ciproflaxacin (5 μg), gentamicin (10 μg), erythromycin (15 μg), chloramphenicol (30 μg), cephalexin (30 μg)]. 6. Combi – 69 [ciproflaxacin (5 μg), ofloxacin (5 μg), sparfloxacin (5 μg), gatifloxacin (5 μg), aztreonam (30 μg), azithromycin (15 μg), vancomycin (30 μg), doxycycline hydrochloride (30 μg)].

Octodisks for Gram negatives

For Gram positives the following octiodisks were used; 1. G-1-minus [ampicillin (10 μg), ciprofloxacin (10 μg), colistin (10 μg), co-trimaxazole (25 μg), gentamicin (10 μg), nitrofurantoin (300 μg), streptomycin (10 μg), tetracycline (30 μg)]. 2. G III minus [amikacin (10 μg), carbenicillin (100 μg), ciprofoxacin (10 μg), co-trimazine (25 μg), kanamycin (30 μg), nitrofurantoin (300 μg), streptomycin (10 μg), tetracycline (30μg)]. 3. Combi 60 [amoxyclav (10 μg), ceftriaxone (30 μg), ceftizoxime (30 μg), ceftazidime (30 μg), cefpodoxime (30 μg), gentamicin (10 μg), amikacin (30 μg), cefoperazone/sulbactam (75/30 μg)]. 4. Pseudo [amikacin (30 μg), carbenicillin (100 μg), chloramphenicol (30 μg), ciproflaxacin (10 μg), cephotaxime (30 μg), gentamicin (10 μg), norfloxacin (10 μg), tobramycin (10 μg)]. 5. Combi 59 [ampicillin/sulbactam (10/10 μg), piperacillin/tazobactam (100/10 μg), ticarcillin/clavulanic acid (75/10 μg), carbenicillin (100 μg), cephalothin (30 μg), cefuroxime (30 μg), cephotaxime (30 μg), cefoperazone (75 μg)]. 6. g ii minus [cephotaxime (30 μg), cephalexin (30 μg), co-trimaxazole (25 μg), chloramphenicol (30 μg), nalidixic acid (30 μg),

furazolidone (50 μg), norfloxacin (10 μg), oxytetracycline (30 μg)].

Fresh cultures (24 to 36h) of the test bacteria were used for spread plating on tryptic soya agar (TSA) and the selected octodisks were placed on the inoculated media. The plates were incubated at 28° C for 72 h and observations recorded. The scores used to develop the resistance pattern were as follows; 1. – (not resistant: >3mm inhibition). 2. + (poorly resistant: 2 to 3mm inhibition). 3. ++ (moderately resistant: <2mm inhibition). 4. +++ (resistant: no inhibition zone seen).

The endophytes were compared with two proven plant growth promoting rhizobacteria (PGPR), *viz. P. fluorescens* (PDBCAB2) and *B. subtilis* (PDBCABN22) which were obtained from culture collection of Project Directorate of Biological Control (PDBC), Bangalore.

Inoculation and identification of introduced endophytic bacteria

A preliminary approach was undertaken to use antibiotic resistance pattern as a marker tool to identify introduced endophytic bacteria. The seeds were treated with the test bacteria by cultures that were multiplied in 100mL tryptic soya broth (TSB) on a shaker at 150rpm for 48h. The cells were harvested by centrifuging at 7000rpm for 15 minutes and re-suspended in phosphate buffer (100ml). Surface sterilized (0.1% mercuric chloride) seeds were washed five times in sterile water, treated with 0.1% carboxy methyl cellulose, air dried and then dipped in the culture suspension. Foliar spray was done seven days after germination (1 ml of the suspension was mixed with 1 L of water containing 0.1% Triton X, before spraying) and repeated at 20 and 50 days.

Resistance markers used in the study

Two endophytic bacteria namely *B. megaterium* and *Bacillus* sp. were selected for the study. The resistance markers used were penicillin G (2 μg) and co-trimaxazole (25μg) for *B. megaterium* and Penicillin G (2 μg), co-trimaxazole (25μg), cloaxcillin (5μg) and cefuroxime (30 μg) for *Bacillus* sp. (Table 5). The above-mentioned markers were used for reisolation of inoculated bacteria from 30-day-old seed treated chickpea plants. A total of 25 samples from each of root, stem and leaf tissue were analyzed for the presence of the introduced bacterium under sterile conditions. Isolation was done as per the surface disinfestation method (McInroy and Kloepper, 1995). Stem samples were surface sterilized with 20% hydrogen peroxide for 10 minutes and rinsed four times with 0.02 M potassium phosphate buffer (pH 7.0). Root samples were surface disinfected with 1.05% sodium hypochlorite and washed in four changes of buffer. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred in 9.9ml tryptic soya broth to serve as sterility check. Samples were discarded; if growth was detected in the sterility check within 48 h. Intact samples were triturated in 9.9 ml of buffer in a sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer. Dilutions were spread plated tryptic soya agar (TSA). Representative colonies (based on colony morphology) were transferred to fresh TSA plates to establish pure cultures. The pure cultures were Gram stained and then tested for resistance using the antibiotic marker disks.

RESULTS AND DISCUSSION

The endophyte *B. megaterium* was resistant to co-trimaxazole (25μg), penicillin-v (10 units), amoxycillin (10 μg) and penicillin-g (2 μg) (table 1). *Bacillus* sp. was resisitant to cephalothin (30 μg), co-trimaxazole (25μg), penicillin-g (10 units), amoxycillin (10 μg), cloaxcillin (5 μg), co-trimaxazole (25μg) and cefuroxime (30 μg) (Table 1, Plate 1). *B. circulans* was resistant only to amoxycillin (10 μg) (Table 2). The Gram negative endophyte *E. herbicola* was resistant to ampicillin (10 μg) and co-trimaxazole (25 μg). The other Gram negative *E. agglomerans* was resistant to co-trimaxazole (25 μg), gentamicin (10 μg), carbenicillin (100 μg), cefuroxime (30 μg) and cephotaxime (30 μg) (Table 3).

The rhizospheric bacterium *B. subtilis* showed resistance to cephalothin (30 μg), penicillin-

Table 1. Antibiotic resistance pattern observed with octodisks (HiMedia) for *Bacillus megaterium*.

Bacterium	Combi 1							
Bacillus megaterium (UASEBCH1)	Cephalothin (30 µg) +	Clindamycin (2 µg) -	Co-Trimaxazole (25µg) +++	Erythromycin (15 µg) -	Gentamicin (10 µg) -	Ofloxacin (1 µg) -	Penicillin-G (10 units) ++	Vancomycin (30 µg) -
				Combi VII				
	Amoxycillin (10 µg) +++	Cloaxcillin (5 µg) +	Erythromycin (15 µg) -	Tetracycline (10 µg) -	Penicillin (2 units) +	Co-Trimaxazole (25µg) +++	Penicillin-V (10 units) +++	Cephalexin (30 µg) -
				Combi XIII				
	Penicillin-G (2 µg) +++	Tetracycline (10 µg) -	Co-trimaxazole (25µg) +++	Cloaxcillin (5 µg) -	Cephradine (30 µg) -	Erythromycin (10 µg) +	Lincomycin (10 µg) -	Cefuroxime (30 µg) +
				Gx plus				
	Chloramphenicol (25 µg) -	Erythromycin (5 µg) -	Fusidic acid (10 µg) ++	Methicillin (10 µg) ++	Novobiocin (5 µg) -	Penicillin-G (1 units) ++	Streptomycin (10 µg) -	Tetracycline (25 µg) -
				G-V-Plus				
	Amoxycillin (10 µg) +++	Tetracycline (30 µg) -	Co-trimaxazole (25µg) +++	Ciproflaxacin (5 µg) -	Gentamicin (10 µg) -	Erythromycin(15 µg) -	Chloramphenicol (30 µg) -	Cephalexin (30 µg) -
				Combi 69				
	Ciproflaxacin (5 µg) -	Ofloxacin (5 µg) —	Sparfloxacin (5 µg) -	Gatifloxacin (5 µg) -	Aztreonam (30 µg) -	Azithromycin (15 µg) -	Vancomycin (30 µg) -	Doxycycline Hydrochloride (30 µg) ++

- = Not Resistant
+ = Poorly resistant
++ = Moderately resistant
+++ = Resistant

Table 2. Antibiotic resistance pattern observed with octodisks (HiMedia) for *Bacillus circulans*.

Bacterium	Combi 1							
Bacillus megaterium (UASEBCH1)	Cephalothin (30 µg) +	Clindamycin (2 µg) -	Co-Trimaxazole (25µg) -	Erythromycin (15 µg) -	Gentamicin (10 µg) -	Ofloxacin (1 µg) -	Penicillin-G (10 units) -	Vancomycin (30 µg) +
				Combi VII				
	Amoxycillin (10 µg) +++	Cloaxcillin (5 µg) -	Erythromycin (15 µg) -	Tetracycline (10 µg) -	Penicillin (2 units) -	Co-Trimaxazole (25µg) -	Penicillin-V (10 units) +	Cephalexin (30 µg) -
				Combi XIII				
	Penicillin-G (2 µg) +	Tetracycline (10 µg) -	Co-trimaxazole (25µg) -	Cloaxcillin (5 µg) -	Cephradine (30 µg) -	Erythromycin (10 µg) -	Lincomycin (10 µg) -	Cefuroxime (30 µg) -
				Gx plus				
	Chloramphenicol (25 µg) -	Erythromycin (5 µg) -	Fusidic acid (10 µg) -	Methicillin (10 µg) +	Novobiocin (5 µg) +	Penicillin-G (1 units) -	Streptomycin (10 µg) -	Tetracycline (25 µg) -
				G-V-Plus				
	Amoxycillin (10 µg) +++	Tetracycline (30 µg) -	Co-trimaxazole (25µg) -	Ciproflaxacin (5 µg) -	Gentamicin (10 µg) -	Erythromycin(15 µg) -	Chloramphenicol (30 µg) -	Cephalexin (30 µg) -
				Combi 69				
	Ciproflaxacin (5 µg) -	Ofloxacin (5 µg) —	Sparfloxacin (5 µg) -	Gatifloxacin (5 µg) -	Aztreonam (30 µg) -	Azithromycin (15 µg) +	Vancomycin (30 µg) -	Doxycycline Hydrochloride (30 µg) +

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+ = Poorly resistant
++ = Moderately resistant
+++ = Resistant

Table 3. Antibiotic resistance pattern observed with octodisks (HiMedia) for *Enterobacter agglomerans.*

Bacterium	**G I Minus**							
Enterobacter agglomerans (UASEBCH5)	Ampicillin (10 µg) +++	Ciprofloxacin (10 µg) -	Colistin (10 µg) -	Co-trimaxazole (25 µg) +++	Gentamicin (10 µg) +++	Nitrofurantoin (300 µg) +	Streptomycin (10 µg) +	Tetracycline (30 µg) -
				G III Minus				
	Amikacin (10 µg) +	Carbenicillin (100 µg) -	Ciprofoxacin (10 µg) -	Co-trimazine (25 µg) +	Kanamycin (30 µg) +	Nitrofurantoin (300 µg) +	Streptomycin (10 µg) +	Tetracycline (30 µg) -
		-		**Combi 60**				
	Amoxyclav (10 µg) +	Ceftriaxone (30 µg) +	Ceftizoxime (30µg) +	Ceftazidime (30 µg) -	Cefpodoxime (30 µg) ++	Gentamicin (10 µg) +++	Amikacin (30 µg) +	Cefoperazone/ Sulbactam (75/30 µg) -
				Pseudo				
	Amikacin (30 µg) +	Carbenicillin (100 µg) +++	Chloramphenicol (30 µg) +	Ciproflaxacin (10 µg) -	Cephotaxime (30 µg) +	Gentamicin (10 µg) +++	Norfloxacin (10 µg) -	Tobramycin (10 µg) +++
				Combi 59				
	Ampicillin/ Sulbactam (10/10 µg) +	Piperacillin/ Tazobactam (100/10 µg) ++	Ticarcillin/ clavulanic acid (75/10 µg) +	Carbenicillin (100 µg) +++	Cephalothin (30 µg) +	Cefuroxime (30 µg) +++	Cephotaxime (30 µg) +++	Cefoperazone (75 µg) -
				G II Minus				
	Cephotaxime (30 µg) +++	Cephalexin (30 µg) +	Co-trimaxazole (25 µg) +++	Chloramphenicol (30 µg) +	Nalidixic acid (30 µg) -	Furazolidone (50 µg) +	Norfloxacin (10 µg) -	Oxytetracycline (30 µg) -

- = Not Resistant
+ = Poorly resistant
++ = Moderately resistant
+++ = Resistant

Table 4. Antibiotic resistance pattern observed with octodisks (HiMedia) for *Pseudomonas fluorescens*.

Bacterium	**G I Minus**							
Enterobacter agglomerans (UASEBCH5)	Ampicillin (10 µg) +++	Ciprofloxacin (10 µg) -	Colistin (10 µg) +	Co-trimaxazole (25 µg) +++	Gentamicin (10 µg) -	Nitrofurantoin (300 µg) +++	Streptomycin (10 µg) ++	Tetracycline (30 µg) +
				G III Minus				
	Amikacin (10 µg) -	Carbenicillin (100 µg) ++	Ciprofoxacin (10 µg) -	Co-trimazine (25 µg) +	Kanamycin (30 µg) +	Nitrofurantoin (300 µg) +++	Streptomycin (10 µg) +++	Tetracycline (30 µg) ++
				Combi 60				
	Amoxyclav (10 µg) -	Ceftriaxone (30 µg) +++	Ceftizoxime (30µg) +++	Ceftazidime (30 µg) +++	Cefpodoxime (30 µg) +++	Gentamicin (10 µg) -	Amikacin (30 µg) -	Cefoperazone/ Sulbactam (75/30 µg) -
				Pseudo				
	Amikacin (30 µg) -	Carbenicillin (100 µg) +++	Chloramphenicol (30 µg) +++	Ciproflaxacin (10 µg) -	Cephotaxime (30 µg) +++	Gentamicin (10 µg) -	Norfloxacin (10 µg) -	Tobramycin (10 µg) -
				Combi 59				
	Ampicillin/ Sulbactam (10/10 µg) +++	Piperacillin/ Tazobactam (100/10 µg) +	Ticarcillin/ clavulanic acid (75/10 µg) -	Carbenicillin (100 µg) -	Cephalothin (30 µg) +++	Cefuroxime (30 µg) +++	Cephotaxime (30 µg) +++	Cefoperazone (75 µg) +++
				G II Minus				
	Cephotaxime (30 µg) +++	Cephalexin (30 µg) +++	Co-trimaxazole (25 µg) +++	Chloramphenicol (30 µg) +++	Nalidixic acid (30 µg) +++	Furazolidone (50 µg) +++	Norfloxacin (10 µg) -	Oxytetracycline (30 µg) +++

- = Not Resistant
+ = Poorly resistant
++ = Moderately resistant
+++ = Resistant

Table 5. Antibiotic resistance pattern used as selection marker for selected endophytes.

Endophyte	Antibiotic Resistance Marker			
	Octodisk Combi XIII			
	Penicillin G (2 μg)	Co-Trimaxazole (25μg)	Cloaxcillin (5μg)	Cefuroxime (30 μg)
Bacillus megaterium	+++	+++	-	+
Bacillus sp.	+++	+++	+++	+++

Table 6. Reisolation of two endophytic bacteria from seed treated as well foliar sprayed chickpea plants (30 days) under sterile conditions by using the antibiotic resistance marker.

Endophyte	Type of samples collected for endophytic isolation					
	Root tissue		Stem tissue		Leaf tissue	
	No. of samples tested	No. of positive reisolation	No. of samples tested	No. of positive reisolation	No. of samples tested	No. of positive reisolation
Bacillus megaterium	25	10	25	0	25	3
Bacillus sp.	25	11	25	0	25	2

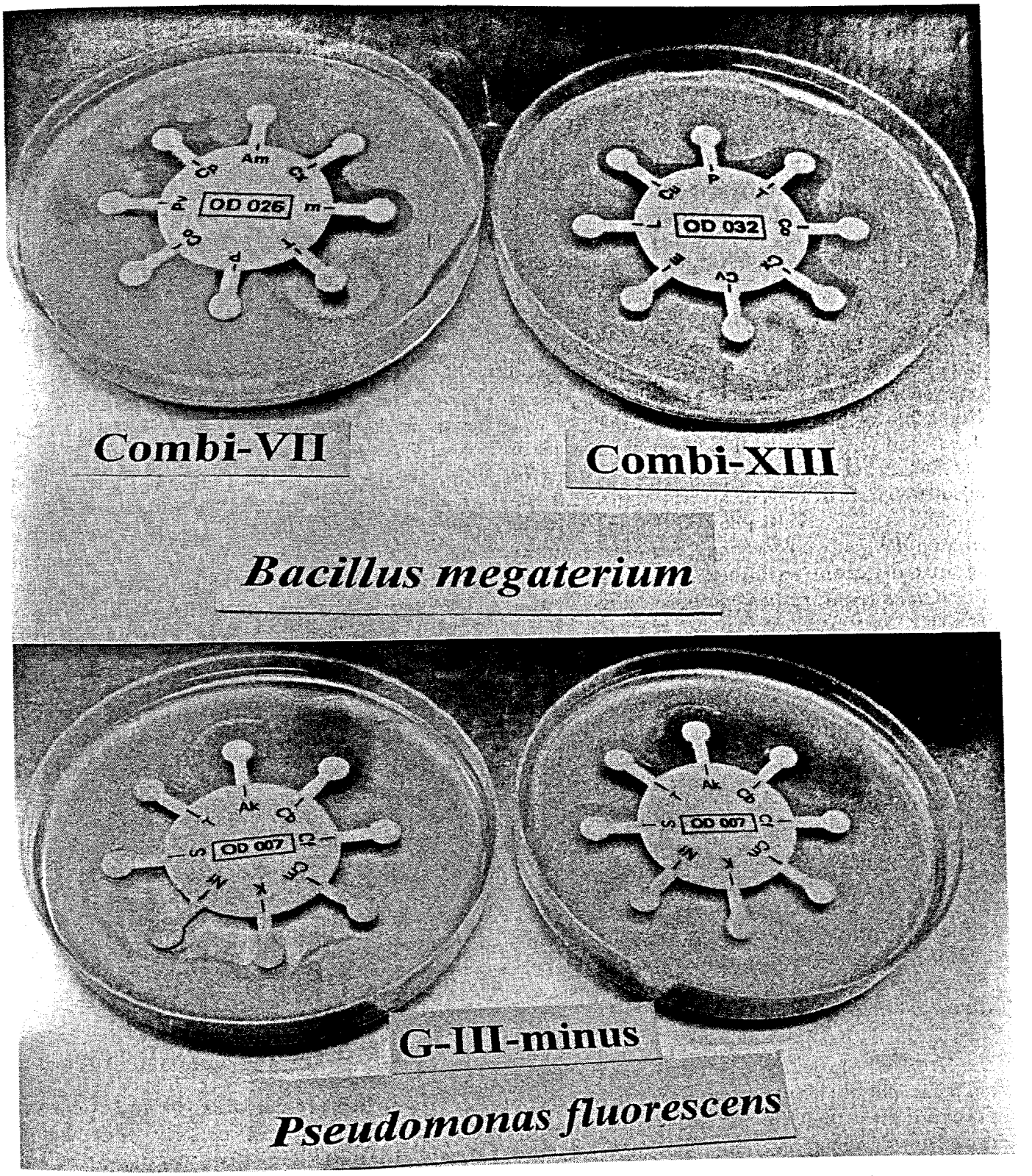

Plate 1. Antimicrobial susceptibility test of bacteria with octodisks (Himedia)

g (10 units), amoxycillin (10 µg), cloxacillin (5 µg) and cefuroxime (30 µg). *P. fluorescens* showed resistance to ampicillin (10 µg), co-trimaxazole (25 µg), nitrofurantoin (300 µg), streptomycin (10 µg), amoxyclav (10 µg), ceftriaxone (30 µg), cefoperazone/sulbactam (75/30 µg), carbenicillin (100 µg), chloramphenicol (30 µg), ampicillin/ sulbactam (10/10 µg), cephalothin (30 µg), cefuroxime (30 µg), cephotaxime (30 µg), cefoperazone (75 µg), cephalexin (30 µg) nalidixic acid (30 µg), furazolidone (50 µg) and oxytetracycline (30 µg) (Table 4, Plate 1).

Resistance against antibiotics is one of the parameter used to look for effective biological control agents (Siddiqui *et al.*, 2005). In the present study, intrinsic antibiotic resistance pattern was developed for the endophytic bacteria in order to see the resistance pattern and also to use them for reisolation from treated chickpea plants under sterile conditions. The results showed that most of the endophytes showed varying resistance patterns against different antibiotics. Surprisingly *B. circulans* was only resistant to amoxycillin (10 µg). The rhizobacteria showed better resistance to the tested antibiotics. The results show that some of the endophytes showed less resistance to the tested antibiotics, which could mean that they may not be effective competitors in a natural environment like the rhizosphere. The endophytes may need the protective environment of the internal plant tissue for survival. Patricia *et al.* (1995) isolated 22 endophytic bacteria from mint shoot cultures. They subjected the bacteria to sensitivity tests with antibiotics and found that minimal inhibitory concentration and minimal bactericidal concentration of gentamycin, rifampicin, streptomycin and timentin varied with genotype. The present study also showed that resistance to different antibiotics varied with genotype.

Studies show that intrinsic antibiotic resistance pattern could be used to distinguish bacterial strains after introduction in the rhizosphere (Chanway and Hall, 1986). In the present preliminary study, two endophytic bacteria, namely, *B. megaterium* and *Bacillus* sp. were selected for the study. Based on the resistance pattern, the markers used were penicillin G (2 µg) and co-trimaxazole (25µg) for *B. megaterium* and penicillin G (2 µg), co-trimaxazole (25µg), cloxacillin (5µg) and cefuroxime (30 µg) for *Bacillus* sp. It was evident that both the bacteria were able to colonize both the root and leaf tissue of chickpea plants. Out of the 25 root tissues tested 10 tested positive for the presence of *B. megaterium* and eleven for *Bacillus* sp. However, they could not be detected in the stem tissue. But in leaf tissue, 3 and 2 samples (out of 25 samples tested) showed presence of *B. megaterium* and *Bacillus* sp., respectively (Tables 5 and 6).

Vidhyasakeran *et al.* (1997) developed spontaneous resistant strains of *P. fluorescens* by growing the isolates on media containing 190 µg/ ml of rifampicin for reisolation from field. Song and Zhu (1998) isolated endophytic bacteria from solanum crops and developed antibiotic resistance pattern for marking the strains. Wu *et al.* (2001) obtained an endophytic bacterium 73a mutant resistant to 100µg rifampicin/ml by continuous screening on a rifampicin medium with a series of concentrations and used the resistance as a marker. In the present study the resistance pattern of each endophyte was compared with each other and a suitable marker that was unique to the isolate was identified. The preliminary study to monitor the endophyte was done under sterile conditions in order to confirm that the identified endophytes are able to colonize the internal tissues of chickpea. The results indicated that the two endophytes namely, *Bacillus* sp. and *B. megaterium* were able to colonize that root and leaf tissue of chickpea. Further studies using suitable molecular markers or immunological tools are needed to show the distribution pattern of the endophytes in different tissues of the plant.

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