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Polymorphism in Intrinsic Antibiotic Resistance of *Azospirillum* Isolates from Ornamental Plants

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Abstract : The polymorphism of *Azospirillum* isolates from ornamental rhizosphere and two reference strains were examined with respect to intrinsic antibiotic resistance (IAR) profile. All the isolates showed different intrinsic resistances to different antibiotics *viz.*, tetracycline, kanamycin, nalidixic acid, streptomycin, ampicillin, spectinomycin and chloramphenicol. All the strains demonstrated susceptibility to high concentration of all antibiotics used in the present experiment. In addition to these general patterns, we also obseved the multiple antibiotic resistances of *Azospirillum* strains. The *Azospirillum* sp. OAD-11 was resistant to tetracycline, streptomycin and ampicillin, and *Azospirillum* sp. OAD-57 was resistant to tetracycline and streptomycin. Conversely, *Azospirillum* sp. OAD-9 possessed the dual susceptibility to tetracycline and spectinomycin, whereas *Azospirillum* sp. OAD-37 was dual susceptible to streptomycin and kanamycin. Such multiple antibiotic resistant/susceptible traits could be useful for the identification of the strains in field experiments or in molecular genetic transfer experiments.

Key words: *Azospirillum*, polymorphism, intrinsic antibiotic resistance, ornamental plants

INTRODUCTION

Polymorphism or diversity is essential to life, as it permits adaptation through the creation of new organisms by genetic transfer and mutations[1]. Existence of intensive microbial diversity in soil has been previously studied. These studies estimated that there are approximately 4x10^3 species per gram of soil[2]. The attempts to determine the taxonomic identification of this enormous diversity have been accomplished only by using traditional culturing methods. Since microscopic analysis has demonstrated that only 1 to 10% of soil organisms can be isolated by these techniques[3], other methods must be employed to accurately determine diversity. Balestra and Misaghic[4] isolated four to seven times more bacteria from rye grass and alfalfa roots, and soil using 25 different media supplemented with different antibiotics.

The evolution and spread of antibiotic resistance depends on the antibiotic pressure exerted in the microbial environment. Selective effects occur in selective compartments, where partcular antibiotics concentrations result in a differential growth rate of resistant bacterial variants. This may happen even at very low antibiotic concentrations to select low-level resistant bacteria. When more than one antibiotic is present in the environment, the multiple and fluctuating pressure produces the selection of bacterial variants that use multiple or multipurpose mechanisms, or optimize a single mechanism of resistance to survive under the variable environmental conditions. Antibiotics themselves may promote bacterial diversity with mediating by the random drift effect or triggering the increase of mutational events under bacterial stress. Analysis of selective environment related antibiotic host bacteria interaction is essential to understanding the biology of antibiotic resistance[5].

The genetic diversity of soil bacteria has been demonstrated using the patterns of intrinsic antibiotic resistance[6]. Dobereiner and Baldani[7] found diversity among *Azospirillum* strains with respect to their resistance to streptomycin, tetracycline, gentamycin, erythromycin and chloramphenicol. Similar-

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ly, among the species of *Azospirillum*, differences have been found between strains isolated from comparable habitats or even from the same habitat[8]. Most of the *Azospirillum* spp. isolated from rhizosphere of grasses and soil in Argentina, were susceptible to tetracycline, kanamycin, chloramphenicol and nalidixic acid and resistant to ampicillin and carbenicillin. Diversity of *Azospirillum* was also reported by several workers with respect to antibiotic resistance[7,9-12]. These intrinsic antibiotic resistance properties (antibiotype) of *Azospirillum* could be used to examine their establishment in rhizosphere and molecular experiments. Hence, in the present study, we used intrinsic antibiotic resistance (IAR) as a tool to examine the polymorphism of *Azospirillum* isolates from ornamental plants and to obtain the antibiotypes.

MATERIALS AND METHODS

The *Azospirillum* strains were isolated from the ornamental rhizosphere soils from different geographic locations of Karnataka, India (Table 1)[13]. Two references, *Azospirillum brasilense* BR-11001 and *Azospirillum lipoferum* BR-11080, were obtained from Dr. J. Dobereiner, in Brazil. All the *Azospirillum* strains were maintained on N-free malate semisolid media. A loop-full of each *Azospirillum* was inoculated to 100 mL Luria broth and was incubated over night at 28℃ for mass cultivation.

The IAR of the isolates was examined to mark the wild type strains using with seven antibiotics with different concentrations (Table 2) on Luria agar. Fresh solutions of filtered sterilized antibiotic were added to melted Luria medium to give required concentration (Table 2). The overnight grown each culture (20 μL of a 10^{-6} CFU/mL) of isolates were spotted carefully on Luria agar plates containing the different concentration of antibiotics. The plates without antibiotics served as control to all the examined *Azospirillum* strains. Each bacterial cultures was replicated twice per antibiotic concentration. The plates were incubated at 28℃ for 24 h. Comparing with control plates, the growth of each *Azospirillum* strains was qualitatively assessed. The scoring (+/-) was done according to their growth rate.

RESULTS AND DISCUSSION

Nine *Azospirillum* isolates from different locations and ornamental plants demonstrated fairly good nitrogen fixation besides producing good amount of growth hormones[13]. These nine isolates and two reference strains were examined for their polymorphism of IAR and mark the antibiotype. This antibiotype could be useful in tracking the establishment of target bacteria in the rhizosphere and several molecular experiments[14-17].

All examined *Azospirillum* strains except OAD-9 were tolerant to tetracycline at 5 mg/L but as the concentration was increased to 15 mg/L none of the strains demonstrated the resistance. At 10 mg/L of tetracycline, only *Azospirillum* strains OAD-3, OAD-11 and OAD-57 were resistant (Table 3).

Azospirillum strains OAD-2, OAD-11, OAD-37, OAD-20 and *Azospirillum lipoferum* BR-11080 were found to be susceptible to

Table 1. The *Azospirillum* isolates from ornamental plants and different places of Karnataka province, India

Isolate Number	Plant species	Place	Soil type
OAD-2	*Gaillardia pulchella*	Dharwad	Red
OAD-3	*Gaillardia pulchella*	Halag	Red
OAD-9	*Gaillardia pulchella*	Kadampur	Black
OAD-11	*Chrysanthemum sp.*	Davenahalli	Red
OAD-15	*Chrysanthemum sp.*	Dambal	Black
OAD-20	*Chrysanthemum sp.*	Dharwad	Red
OAD-29	*Gladiolus grandiflorus*	Belgaum	Black
OAD-37	*Rosa sp.*	Lakkundi	Black
OAD-57	*Caunduala oofficinalis*	Huvinasigli	Red

Table 2. Antibiotic and their concentrations used in the present study

Antibiotic	Concentrations			Sources
	----- mg/L -----			
Tetracyclin	5	10	15	Sea Gull, India
Kanamycin	50	100	150	Hi media, Mumbai
Nalidixic acid	10	20	50	Hi media, Mumbai
Streptomycin	50	100	150	Sarabai chemicals, Mumbai
Ampicillin	50	100	150	KA.Pharmaceticals, Banglore
Spectinomycin	50	100	150	Sigma Chemicals Co, USA
Chloromphenicol	5	10	15	Hi-Media, Mumbai

kanamycin (50 mg/L), but *Azospirillum* strains OAD-3, OAD-9, OAD-29, OAD-15 and *Azospirillum brasilense* BR-11001 were found to be resistant to kanamycin (50 mg/L). Higher concentration of kanamycin (100 mg/L) completely inhibited the growth of all strains. The kanamycin susceptible *Azospirillum* strains can be explored for the molecular genetics and cloning experiments[18,19].

The nalidixic acid resistance was observed in *Azospirillum* strains OAD-2, OAD-3, OAD-9, OAD-11 and OAD-29 at 10 mg/L, but they were susceptible at higher concentrations of 20 mg/L. *Azospirillum* strains OAD-37, OAD-57, OAD-15, OAD -20, *Azospirillum brasilense* BR-11001 and *Azospirillum lipoferum* BR-11080 were found to be susceptible to 10 mg/L of nalidixic acid.

All *Azospirillum* strains, except OAD-20 and OAD-37 were susceptible to 50 mg/L of strptomycin. Further, *Azospirillum* strains OAD-11 and OAD-57 were found to be resistant to streptomycin at 100 mg/L, but all other strains were found to be susceptible at higher concentration of 100 mg/L. The percentage of low level streptomycin resistant *Azospirillum lipferum* in rhizoplane was thousand times higher than that in rhizosphere populations, while most soil isolates from rhizosphere were sensitive to streptomycin[7]. This indicates that antibiotic resistance might play a role in plant bacteria affinities.

All the examined *Azospirillum* strains were found to be susceptible at higher concentration of ampicillin (100 mg/L) except *Azospirillum* sp. OAD-11. The *Azospirillum* sp. OAD-11 was susceptible at 150 mg/L. However, all the *Azospirillum* strains resistant to low level of ampicillin.

Higher concentrations of spectinomycin (100 and 150 mg /L) inhibited the growth of all *Azospirillum* strains. However, two strains, *Azospirillum* spp. OAD-9 and OAD-29, were susceptible to spectinomycin at 50 mg/L, while rest of the strains were resistant.

Azospirillum strains OAD-3, OAD-11, OAD-57, OAD-15 and OAD-20 were found to be resistant to 5 mg/L, further, *Azospirillum* strains OAD-2, OAD-29, OAD-37 and *Azospirillum brasilense* BR-11001 were found to be resistant at 10 mg/L of chloramphenicol. However, *Azospirillum* sp. OAD-9 was exclusively susceptible to chloramphenicol even at low level.

Most of the *Azospirillum* strains were susceptible to higher concentration of kanamycin, streptomycin, ampicillin, streptomycin, spectinomycin, nalidixic acid and chloramphenicol. Similarly, 32 strains of *Azospirillum* from grasses of Argentina showed the susceptibility to 9 antimicrobial agents[19]. However, the resistance of 10 antibiotics was high in the *Azotobacter*. The *Azotobacter* also showed wide diversity with respect to

Table 3. Intrinsic antibiotic resistance profile of *Azospirillum* isolates

Strains	Concentration of Antibiotics (mg/L) in Luria Agar																					
	Tetracycline				Kanamycin			Nalidixic acid			Streptomycin			Ampicillin			Spectinomycin			Chloramphenicol		
	0	5	10	15	50	100	150	10	20	50	50	100	150	50	100	150	50	100	150	5	10	15
OAD-2	++	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	++	+	-
OAD-3	++	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-
OAD-9	++	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
OAD-11	++	++	+	-	-	-	-	+	-	-	++	+	-	++	+	-	+	-	-	+	-	-
OAD-29	++	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	++	+	-
OAD-37	++	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	++	+	-
OAD-57	++	++	+	-	+	-	-	-	-	-	++	+	-	+	-	-	+	-	-	+	-	-
OAD-15	++	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-
OAD-20	++	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-
BR-11001	++	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	++	+	-
BR-11080	++	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-

Note : '+' Growth, '-' No growth.

antibiotic resistance. Over 95% of the strains were resistant to 10 mg/L of ampicillin, chloramphenicol, erythromycin and tetracycline and 70% or more were resistant to kanamycin and nalidixic acid streptomycin and trimethoprim[20].

In spite of general pattern of IAR, it was possible to obtain an dual/multi antibiotype for most of the strains. Some of the antibiotypes in this study were as followed, *Azospirillum* sp. OAD-11 was resistance to tetracycline, streptomycin and ampicillin at higher concentration. Similarly *Azospirillum* sp. OAD-57 resulted in resistance to higher concentration tetracycline and streptomycin. Conversely, *Azospirillum* sp. OAD-9 susceptible to tetracycline and specetinomycin, whereas *Azospirillum* sp. OAD-37 were susceptible to streptomycin and kanamycin even at low concentration. Such antibiotype could be useful for the identification of the strains in the field experiments or in genetic transfer experiments[17].

Wide diversity of *Azospirillum* isolates from the ornamental plants was recorded with respect to IAR. Similar antibiotic resistance pattern has been used to classify several nitrogen-fixing bacteria[7,9-12]. Similarly, other nitrogen-fixing bacteria also recorded the diversity with respect to antibiotic resistance. *Rhizobium* strains that are able to nodulate beans were classified based on their intrinsic antibiotic resistance[21]. Thirty three isolates of *Bradyrhizobium* from *Acacia mangium* tree were also classified based on the IAR[22]. These antibiotic resistant determinants may also spread to a diverse array of strains via horizontal exchange. This might have created the intrinsic antibiotic polymorphisms among the *Azospirillum* strains in the present study.

The polymorphism is rich sources of inference that can be used to develop a model of the evolution of *Azospirillum* and beneficial activity. Although a plethora of immunological and molecular techniques is available for the discrimination of isolates, many aspects of diversity were remained poorly described and incompletely understood. Studies in this area have been hindered by many difficulties including a paucity of suitable isolate collections and techniques for accurate characterization of variants as well as theoretical framework for the interpretation of data. In this regards intrinsic antibiotic resistance pattern may help to elucidate the *Azospirillum* diversity to some extent.

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