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Isolation and Symbiotic Characterization of Azide Resistant Mutants of Different Species of *Rhizobia*

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Spontaneous azide resistant mutants were isolated from the WT strains of *Sinorhizobium fredii*, *Mesorhizobium ciceri* and *Rhizobium leguminosarum* bv. *trifolii* and their symbiotic effectivity was tested on respective host plants either in large test tubes or in Leonard jars. A total of 12 mutants were isolated from the parent strain 54D of *Sinorhizobium fredii*, 11 mutants from parent strain USDA3383 of *Mesorhizobium ciceri* and 38 from parent strain ARC100 of *Rhizobium leguminosarum* bv. *trifolii*. The host plants inoculated with a few of the mutants of all the three species of rhizobia resistant to low doses of azide had significantly higher shoot dry weight and ARA in the nodules than the plants inoculated with the parent strains.

Key words: Azide resistance, *Sinorhizobium fredii*, *Mesorhizobium ciceri*, *Rhizobium leguminosarum* bv. *trifolii*, Symbiotic effectivity, Acetylene reducing activity (ARA).

Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium, and *Allorhizobium* are the six genera of rhizobia which fix atmospheric nitrogen in the root nodules of leguminous plants (Yadav, 2007). All the strains of rhizobia are not efficient nitrogen fixers with their respective host plants. To get maximum nitrogen fixation of rhizobial strains in symbiotic association with legumes, a 'number of selective methods for screening efficient strains of rhizobia are in vogue (Yadav *et al*, 1999). The traditional way of testing each strain of *Rhizobium* on its host plant is lengthy and cumbersome. Sodium azide is the alternative substrate for nitrogenase enzyme. It was shown that induced resistance to azide in *Rhizobium leguminosarum* bv. *trifolii* L4 causes hyper N2-fixing ability in symbiotic relationship with *Pisum sativum* (Ram *et al*, 1978). Induced as well as intrinsic resistance to low doses of azide offers a promising selective method for isolation of effective (Yadav and Vashishat, 1986; Vashishat *et al*, 1986; Yadav *et al*, 1996; Yadav *et al*, 1999; Yadav *et al*, 2000) and competent (Yadav *et al*, 1992) strains of different species of rhizobia. Azide resistance marker (*azi*) in *R. leguminosarum* Rld 164 was inferred to be

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correlated with *nod* genes on the pSym (Singh and Kumar, 1989). It has also been reported that *fix* ABC genes are required for expression of azide resistance in *Sinorhizobium meliloti* and it is independent of nitrogenase activity.

The present study was aimed at isolating spontaneous azide resistant mutants of *Sinorhizobium fredii, R. leguminosarum* bv. *trifolii* and *Mesorhizobium ciceri* and characterize these for symbiotic properties. The main purpose of the investigation was to find out whether there is a definite correlation between resistance to azide and symbiotic effectivity of the rhizobial strains.

M ATERIAL AND METHODS

Bacterial strains

The wild type (WT) strains used in this study were 54D (a fast growing strain of *Sinorhizobium fredii*), ARC100 (*R. leguminosarum* bv. *trifolii*), and USDA3383 (*Mesorhizobium ciceri*).

Media and culture conditions

The medium used for isolation of azide-resistant mutants was TY (Beringer, 1974) supplemented with different concentrations of sodium azide, which was dissolved in sterile distilled water, added to the TY medium before solidification and then poured into the Petri plates.

Isolation of azide-resistant mutants

The WT strain 54D was intrinsically resistant to 5µg/ml azide and mutants were isolated at 10 (M3 to M12) and 20 µg/ml of azide (M1 & M2). Strain ARC100 was resistant up to 15 µg/ml azide and its azide resistant mutants were isolated at 20, 30 and 50 µg/ml azide. The strain USDA3383 was resistant to 20 µg/ml azide and its mutants were scored at 25 and 30 µg/ml azide. About 10^6 to 10^7 log phase culture cells/ml of the parent strains were spread on to TY plates. The plates were incubated at 30°C for 7-10 days. Spontaneous mutant clones with clear background were picked up and purified by growing these again on same concentration of azide at which these were initially isolated. These purified azide-resistant mutants were stored at -20 °C in 50 per cent glycerol as well as on YEMA slants, which were stored at 4°C in refrigerator.

Symbiotic properties of azide resistant mutants

The symbiotic properties of the WT strains and their azide resistant mutants were tested in Leonard jars and in large test tubes. The solid Plant Nutrient Solution (PNS) was used in the tubes as slants (for testing the symbiotic effectivity of *Rhizobium leguminosarum* bv. *trifolii* WT strain and its mutants), whereas the liquid PNS was used for growing the soybean or chickpea plants in Leonard jar assemblies. The concentration of various constituents of PNS in g/l was: CaSO4 0.75; K_2SO_4 0.175; $MgSO_4$ 0.197; KH_2PO_4 0.136; FeEDTA 2 ml; 10XMicronutrient solution 1 ml. Half the concentration of PNS was used for soybean plants.

The seeds of soybean (Williams Beltsville), chickpea or berseem (clover) were surface sterilized in 90 % ethanol or in 1:10 bleach solution for 1 min, and then 3-4 washings of sterile distilled water were given. Then these sterilized seeds were sown in Leonard jars with perlite or vermiculite or on solid PNS medium slants in tubes. The Leonard jar assemblies were sterilized in an autoclave for 2h. Three seeds were sown per Leonard jar and 0.5 ml of thick culture was added to each seed and seeds were covered with perlite. The Leonard jars were put in Growth Chamber at 30°C (day) and 24°C (night) temperature, with 16 h light and 8 h dark periods. The plants of soybean, berseem and chickpea were uprooted after 5 weeks of growth. The roots were removed and the upper shoots dried at 70°C for 48 h for shoot dry weight. The roots with intact nodules were put in 200 ml glass jars and ARA was measured in a GLC. After recording the ARA, the nodule fresh and dry weights were taken after drying these at 70°C for 48 h.

RESULTS AND DISCUSSION

The data on symbiotic properties (Table 1) of WT strain 54D of *Sinorhizobium fredii* and its mutants (M1-M12) indicate that the soybean (*Glycine max*) plants inoculated with the mutant strain M7 had the highest and significantly higher nodule fresh weight (0.884 g/plant), dry weight (0.158 g/plant) and shoot dry weight (1.058 g/plant) than that of plants infected with the parent strain, which were 0.491, 0.107 and 1.058 g/plant, respectively. The shoot dry weight of the soybean plants inoculated with the mutants M2 (0.903 g/plant), M6 (0.870 g/plant), M7

(1.058 g/plant), M9 (0.876 g/plant) and M11 (0.834 g/plant) was significantly higher than that of plants inoculated with the parent strain 54D (0.657 g/plant). The ARA activity in the nodules of plants inoculated with mutants was in the range of 179-326 n mol C_2H_4/hr/plant and were not significantly higher than that of the plants inoculated with the parent strain 54D (223 n mol C_2H_4/hr/plant). The soybean plants inoculated with mutant strain M7 had the highest nodule fresh weight, dry weight and shoot dry weight as compared to the plants infected with parent strain and the mutants.

The symbiotic properties of *Mesorhizobium ciceri* parent strain USDA3383 and its *azi*^{r} mutants (M1-M11) (Table 2) indicate that the host plants inoculated with the mutant M10 had the maximum and significantly higher shoot dry weight (0.461g/plant) than the chickpea plants inoculated with the parent strain (0.354 g/plant), whereas the plants inoculated with mutant M9 had the highest and significantly higher ARA (9.34 n mol C_2H_4/hr/plant) than that of host plants inoculated with parent strain (1.49 n mol C_2H_4/hr/plant). The plants inoculated with other mutants had non-significant shoot dry weight and ARA over the plants inoculated with the parent strain.

The observations on shoot dry weight and ARA (in the nodules) of berseem-clover plants inoculated with the parent strain ARC 100 and its azide resistant mutants (Table 3) indicate that the host plants inoculated with the 16 mutant strains viz; M1 (20,30,50), M2 (50), M3 (30,50), M4 (20), M5 (20), M6 (20), M8 (30), M12 (30), M13 (20), M14 (20), M15 (20), M16 (20), M18 (30) were having significantly higher shoot dry weight than the parent strain ARC100. The ARA (n mol C_2H_4/hr/plant) in the nodules of plants inoculated with 5 mutant strains i.e. M3 (20), M4 (20), M5 (20), M8 (20), M9 (20) was significantly higher than that of plants inoculated with the parent strain. The highest shoot dry weight was observed in berseem plants inoculated with the mutant M8 (30) with a value of 40.2 g/plant, whereas the highest ARA (5.64 n mol C_2H_4/hr/plant) was seen in nodules of plants inoculated with mutant strain M3 (20). The significantly higher shoot dry weight and ARA was found in host plants inoculated with the mutants, a majority of which were resistant to 20 µg/ml azide.

Sodium azide is a potent mutagen (Owais

Table 1. Symbiotic properties of WT strain 54D of *Sinorhizobium fredii* and its *azi*^{r} mutants on soybean

Strain/mutant	Nodule fresh wt. (g/plant)	Nodule dry weight (g/plant)	Shoot dry wt. (g/plant)	ARA (n mol C_2H_4/hr/plant)
UI	0.079	0.029	0.322	69
54D(WT) (5*)	0.491	0.107	0.657	223
M1 (20)	0.473	0.105	0.760	212
M2 (20)	0.639	0.128	0.903	326
M3 (10)	0.536	0.105	0.740	274
M4 (10)	0.503	0.105	0.761	310
M5 (10)	0.432	0.084	0.719	195
M6 (10)	0.641	0.111	0.870	179
M7 (10)	0.884	0.158	1.058	236
M8 (10)	0.632	0.111	0.831	265
M9 (10)	0.569	0.106	0.876	274
M10 (10)	0.495	0.093	0.753	249
M11 (10)	0.564	0.100	0.834	236
M12 (10)	0.508	0.103	0.759	243
Maximal LSD_{95}	0.208	0.034	0.176	105

ARA = Acetylene reducing activity UI = Uninoculated control
* = the values in parentheses are resistance to azide in µg/ml

& Kleinhofs, 1988) and it inhibits the terminal oxidase, cytochrome c-oxidase of electron transport chain (Linnett & Beechey, 1979; Trumpower & Gennis, 1994) and can be reduced to ammonia and dinitrogen by the nitrogenase enzyme (Hardy and Knight, 1967). In the present investigation, the host plants inoculated with the mutants of the WT strain 54D of *Sinorhizobium fredii* resistant to 10 µg/ml azide showed the maximum shoot dry weight and ARA in the nodules, as compared to the plants inoculated with the mutants resistant to 20 µg/ml azide. In case of mutants of WT strain USDA 3383, the chickpea plants inoculated with the mutants resistant to 25 µg/ml azide had maximum shoot dry weight than the plants inoculated with the mutant resistant to 30 µg/ml azide. For the mutants of WT strain ARC 100, the berseem plants inoculated with the mutants resistant to 20 µg/ml azide showed the maximum shoot dry weight and ARA compared to the plants infected with the mutants resistant to 30 or 50 µg/ml azide. The results of present investigation are in conformity with our earlier reports on various species of rhizobia in which the main observation emerged was that host plants inoculated with the strains/mutants resistant to

Table 2. Shoot dry weight and acetylene reducing activity (ARA) in the nodules of plants inoculated with WT strain USDA 3383 and its *azi*^{r} mutants

Strain/mutant	Shoot dry weight (g/plant)	ARA (nmol C_2H_4/h/plant)
UI	0.247	0.36
USDA 3383 (WT) (20*)	0.354	1.49
M1 (25)	0.418	1.76
M2 (25)	0.420	1.22
M3 (25)	0.378	1.90
M4 (25)	0.440	2.06
M5 (25)	0.425	1.39
M6 (25)	0.420	0.83
M7 (25)	0.441	0.49
M8 (25)	0.368	1.16
M9 (25)	0.431	9.34
M10 (25)	0.461	2.89
M11 (30)	0.444	0.81
Maximal LSD_{95}	0.090	1.17

* = The values in parentheses are resistance to azide in µg/ml
UI = Uninoculated control

Table 2. Shoot dry weight and ARA in nodules of berseem (clover) inoculated with the WT strain ARC100 and its azide resistant mutants

Strain/mutant	Shoot dry weight (g/plant)	ARA (nmol C_2H_4/h/plant)
UI	4.2	0.17
ARC100 (WT) (15)	13.4	1.76
M1(20)	37.8	2.17
M1(30)	33.0	2.37
M1(50)	24.4	1.37
M2(20)	18.8	2.73
M2(30)	18.4	2.10
M2(50)	24.0	1.78
M3(20)	18.8	5.64
M3(30)	28.8	1.66
M3(50)	26.6	1.93
M4(20)	25.6	3.13
M4(30)	19.6	2.19
M5(20)	26.0	3.29
M5(30)	18.4	1.68
M6(20)	24.4	2.04
M6(30)	15.0	2.42
M7(20)	17.4	2.10
M7(30)	14.4	2.56
M8(20)	18.2	2.87
M8(30)	40.2	2.54
M9(20)	14.6	5.07
M9(30)	18.0	2.04
M10(20)	13.8	2.11
M10(30)	16.4	1.36
M11(20)	17.6	2.42
M11(30)	18.0	1.53
M12(20)	20.6	1.56
M12(30)	24.0	2.22
M13(20)	24.0	2.19
M13(30)	18.0	1.59
M14(20)	34.2	1.73
M14(30)	15.6	0.51
M15(20)	34.8	0.87
M15(30)	15.6	1.42
M16(20)	25.6	0.72
M16(30)	20.0	1.61
M17(30)	18.2	1.37
M18(30)	25.0	1.40
M19(30)	17.0	1.35
Maximal LSD95	7.6	1.00

ARA = Acetylene reducing activity
UI = Uninoculated control

low doses of sodium azide (positive correlation between low doses of azide and symbiotic parameters) had more efficient symbiotic properties than the plants inoculated with strains resistant to higher doses of azide (Yadav and Vashishat, 1986; Yadav *et al*, 1996; Yadav *et al*, 1999; Minakshi *et al*, 2004).

The exact mechanism by which the induced resistance to low doses of azide provides hyper nitrogen fixing efficiency to the strains/ mutants is not clear. But, it is opined that some alternate route in place of normal electron transport chain pathway is followed by these strains for generating higher amounts of ATP, the quantity of which is a major constraint in symbiotic nitrogen fixation. In this respect, it is noteworthy that there is found a positive correlation between the rate of respiration and cytochrome c-oxidase in cultured cells and the shoot dry weight and % shoot nitrogen in the host plants (Minakshi *et al*, 2004).

CONCLUSION

Hence, from the present investigation it is concluded that mutants of WT strains of *Sinorhizobium fredii*, *Mesorhizobium ciceri* and *Rhizobium leguminosarum bv. trifolii* resistant to 10, 25 and 20 µg/ml azide, respectively induced maximum shoot dry weight and ARA in their respective host plants than the mutants resistant to higher doses of azide. So, the induced resistance to low doses of azide may be used as a potential method for isolating the efficient nitrogen fixing strains of different species of rhizobia.

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