Stimulatory effect of adonitol on redifferentiation potential of soybean root nodule bacteroids

During root-nodule development, bacteria of the family Rhizobiaceae differentiate into their endosymbiotic form and are known as bacteroids[1,2]. Bacteroids synthesize nitrogenase and accomplish the reduction of atmospheric nitrogen[3–5]. Then the reduced nitrogen is exported into the host plant[6]. In a symbiotic state the carbon and energy sources of rhizobia may be different than those cultured *in vitro*[7]. Polyols (sugar alcohols) are one of the preferred carbon sources for inclusion in rhizobial growth media[8–11], which may act as a source of energy for redifferentiating bacteroids. Earlier, most of the work related to isolation of bradyrhizobia from the root nodules of *Glycine max* has been reported by utilizing yeast extract mannitol (YEM) agar medium described by Vincent[12]. In our laboratory, invariably 20 E medium[13] is being used for the isolation of *Bradyrhizobium* from root nodules of soybean. Besides, five modifications have also been made in 20 E medium utilizing different molar concentrations of single sugar alcohol, mannitol or adonitol, and or in combination with constant concentration of glycerol to investigate the effect of these modified media on the redifferentiation potential of soybean bacteroids to free-living colony forming units (CFUs).

The ingredients of 20 E medium (control) are: 68.00 mg KH_2PO_4, 87.00 mg K_2HPO_4, 370.00 mg $MgSO_4.7H_2O$, 73.50 mg $CaCl_2$ $2H_2O$, 506.00 mg KNO_3, 6.95 mg $FeSO_4.7H_2O$, 9.30 mg Titriplex III, 4.84 mg $NaMoO_4 \cdot 2H_2O$, 4.60 g or 0.05 M glycerol, 2.00 g yeast extract, 38.25 g or 0.21 M of mannitol, 15.00 g agar, pH (6.8) per litre of distilled water. Eight bradyrhizobial isolates were obtained from soybean root nodules collected from soybean-cultivated region of Rajasthan (Kota) and Madhya Pradesh (Jabalpur and Indore) belonging to four cultivars, namely JS 335, JS 71-05, NRC 7 and NRC 12. After authentication of eight bradyrhizobial isolates in plant infection test[14], bradyrhizobial cultures were stored in glycerol vials at –40°C using 20 E medium. Bradyrhizobial isolates were nomenclatured as IJ 335-1, IJ 335-3, KJ 335-5, JJ 335-3, JJ 71-3, JN 7-5, JN 12-1, JN 12-5. First letter 'I' or 'K' or 'J' indicates the region of origin (Indore, Kota, Jabalpur), 'J 335, J 71-05, N 7 and N 12' represents name of the parent cultivar (JS 335; JJ 71-05; NRC 7 and NRC 12) and the numeric figures represent the strain assign number. One strain of *Bradyrhizobium japonicum* (USDA-110) was obtained from Dietrich Werner, Philipps-Universitat, Marburg, Germany. Thus nine strains of *B. japonicum* were used for the present investigation. Experiments were performed in quadruplicate.

Pregerminated (1–1.5 cm) soybean seedlings (cultivar JS 335) grown on 1% water agar plates were inoculated with mid log phase cultures of bradyrhizobia (10^9 cells/ml). After bacterization, two soybean seedlings for each strain were transferred in growth pouches (procured from Mega International, USA), while growth pouches with uninoculated seedlings served as control. Growth pouches were maintained in growth chamber under controlled conditions (photo period 12 h, light intesity 12000 lux, temperature 28 ± 2°C and humidity 70–80%) and received 5 ml per growth pouch, sterile N free Jenson's plant nutrient solution[14] twice in a week. After four weeks, soybean nodules developed on infected plant roots were harvested, crushed (1 g : 1 ml sterile blank) and homogenized to prepare dilution series for nine individual bradyrhizobial strains.

To quantify the number of redifferentiating bacteroides to free-living, CFUs, Miles and Mishra drop plate method as described by Somasegaran and Hoben[14] was applied. One drop (0.03 ml) nodular suspension (10^{-6}) from each dilution series was dropped with the help of sterilized glass pipettes (0.1 ml with the same tip diameter) in plates containing normal 20 E medium (control) as well as on five modified 20 E media.

After eight days of incubation (28°C), growing CFUs were registered. Standard error and LSD ($P = 0.05$) were calculated for the number of CFUs that appeared in the plates of normal 20E medium and modified 20 E media.

Table 1. Redifferentiation potential of soybean bacteroids in five modified 20 E media with different carbon combinations

Normal/ modified 20E medium	Bradyrhizobial strain: CFUs* × 33** × 10^6 g^{-1} nodule FW								
	IJ 335–1 SE	IJ 335–3 SE	KJ 335–5 SE	JJ 335–3 SE	JJ 71–3 SE	JN 7–5 SE	JN 12–1 SE	JN 12–5 SE	USDA-110
Control	49 d ± 1.58	50 d ± 1.29	65 d ± 1.29	48 c ± 0.91	57 c ± 0.91	65 c ± 1.08	74 d ± 1.58	42 d ± 0.41	41de ± 0.41
MM 1	50 d ± 1.29	57 c ± 1.71	76 c ± 1.78	52 bc ± 0.41	58 c ± 1.47	69 b ± 1.08	90 c ± 1.29	43 d ± 0.71	45 d ± 0.91
MM 2	47 d ± 1.38	53 d ± 1.83	69 d ± 1.31	50 c ± 1.08	54 cd ± 1.58	67 bc ± 1.18	77 d ± 1.31	45 d ± 1.47	43 d ± 1.08
MM 3	61 c ± 1.29	57 c ± 0.91	80 c ± 1.58	55 b ± 1.29	58 c ± 1.47	70 b ± 0.41	91 c ± 1.87	53 c ± 1.08	49 c ± 1.47
MM 4	66 b ± 1.08	64 b ± 1.08	88 b ± 1.47	57 b ± 1.78	68 b ± 1.08	70 b ± 0.91	101b ± 1.08	57 b ± 0.91	53 b ± 1.29
MM 5	97 a ± 1.08	105a ± 1.29	98 a ± 0.71	99 a ± 1.58	103 a ± 1.08	107 a ± 1.83	112 a ± 0.71	69 a ± 1.58	61a ± 1.08

Data are mean of four replicates ± SE.

Values without common letters differ significantly at LSD $P = 0.05$.

Control, Normal 20 E medium containing 0.21 M mannitol and 0.05 M glycerol; MM 1, Modified 20 E medium 1 containing 0.11 M mannitol and 0.05 M glycerol; MM 2, Modified 20 E medium 2 containing only 0.16 M mannitol without glycerol; MM 3, Modified 20 E medium 3 containing only 0.26 M mannitol without glycerol; MM 4, Modified 20 E medium 4 containing 0.11 M adonitol and 0.05 M glycerol without mannitol; MM 5, Modified 20 E medium 5 containing 0.16 M adonitol without mannitol and glycerol.

*CFUs are considered here as total number of viable bacteroids per g fresh weight of root nodules; **One drop (0.03 ml) from dilution 10^{-6} was plated on each plate of 20 E combinations. 33 (1\0.03 = 33) is conversion factor to convert CFU count in ml.

It was assessed that suspensions from nine different bradyrhizobial infected root nodules, developed highly significant enhancement in the number of CFUs ranging from 48.78 to 110.0% in MM 5 medium followed by less significant enhancement of 7.69–36.49% in MM 4 medium, 1.75–26.19% in MM 3 medium, 1.75–21.62% in MM 1 medium and 3.08–7.14% in MM 2 medium compared to number of CFUs formed in the control plates (Table 1).

Data revealed that maximum (110.0%) and minimum (48.78%) enhancement in the number of CFUs over control was observed in plates containing MM 5 medium, plated with suspension of bradyrhizobial strains IJ 335-3 and USDA-110 infected roots nodules respectively.

MM 1 medium exhibited influence on the redifferentiation potential of soybean bacteroids and improved the number of CFUs in spite of the lower molar concentration of mannitol (0.11 M) over the control (0.21 M). Highest increase (21.62%) and lowest increase (1.75%) in CFU number over control were observed in plates suspended with extracts of bradyrhizobial strains JN 12-1 and JJ 71-3-infected root nodules respectively.

Although different varieties of medium, viz. YEM agar medium[12], modified Bergensen's medium[15], cheese whey medium[16], BJSM medium[17], HM medium[18], AG medium[19,20], modified AG medium[21] and 20E medium[13,22] have been used by several workers for isolation/culturing of *Bradyrhizobium*, so far none has used a medium having adonitol as a single carbon source or in different combinations with an extra carbon of glycerol.

Thus, our study suggests that MM 5 medium in which adonitol (0.16 M) is the only carbon source, enhanced the potential of bacteroids to redifferentiate into substantial number of growing bacteria compared to carbon amendment of mannitol singly or in combinations with glycerol. The benefit of this modified 20 E medium is the isolation of *Bradyrhizobium*, even if less number of root nodules are formed due to the stress conditions that invariably exist in arid/semi-arid regions of Rajasthan. MM 1 medium containing half concentration of mannitol (0.11 M) compared to that of control (0.21 M) shows more or less similar CFU number, indicating that normal 20 E medium could be replaced with MM 1 medium for the isolation of *Bradyrhizobium* from soybean root nodules.

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