

## Role of endophytic rhizobial plasmids in legume nodulation and plant growth promotion in rice

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### ABSTRACT

The plasmid curing experiment was carried out using the strains RREM36 and RREM25 conferring resistance to Chloramphenicol (75 µg/ml) and for Kanamycin (65 µg/ml) respectively. 0.1 ml aliquot of exponentially growing cultures (approximately 10<sup>6</sup> cells/ml) was spread on TY plates and incubated at 35 and 37°C. From these plates 100 colonies were picked up and streaked on TY and TY + Kanamycin (65 µg/ml) for RREM25 and TY + Chloramphenicol (75 µg/ml) for RREM36 and incubated at 28°C for 3 days. Number of cells in TY alone and TY plates supplemented with appropriate antibiotics was counted. TY broth with graded concentration of acrydine orange (0, 5, 10, 40, 45, 50, 55 µg/ml) was prepared and 0.1 ml of exponentially growing rhizobial cells were inoculated. After 7 days of growth cell suspension (100 µl) was spread on TY plates and incubated at 28°C for 3 days. 100 colonies from each type of plate were selected and re-streaked in TY and TY plate with respective antibiotic. It was observed that plasmids of cells of RREM 25 and RREM 36 were lost at 37°C. Gradual loss of plasmids starting from 35 to 60 per cent was observed with increase in incubation period and 85 per cent loss was recorded after 7 days of incubation. Gradual decrease in growth of these two strains was observed with increase in concentration of acrydine orange 45 µg/ml and at 55 µg/ml the plasmids were lost completely in RREM25 and RREM36.

**Keywords:** Plasmids; *Rhizobium*; rice; plant growth promotion

### INTRODUCTION

*Rhizobium* species generally contain one to four large plasmids (>100 kb) in addition to the symbiotic plasmid (Thurman et al 1985). Inoculation with plasmid cured and deleted derivatives of *Sinorhizobium*

*meliloti* showed that the plasmids were largely responsible for rice growth inhibition (Perrine et al 2004, Perrine et al 2005). It has been proved that the observed inhibition is the result of the complex interaction between rhizobia, the growth medium and rice plants. Strains of *Rhizobium* species

harbor indigenous large plasmids on which the gene(s) responsible for nodulation (*nod*) and nitrogen fixation (*fix*) were located. Plasmid of *Rhizobium* can be cured or deleted by applying physical or chemical treatment. *Rhizobium* species generally lost their symbiotic properties when exposed to elevated temperature for long period (Balachander et al 2003). The eradication of the symbiotic capability is attributed to the loss/internal deletion of large endogenous plasmid. However in *Burkholderia* species experiment related to plasmid curing and the role of plasmid cured derivatives in symbiosis and endophytic colonization has not been reported so far. The present study was undertaken to determine the role of plasmid(s) of endophytic *Burkholderia* and *Rhizobium* for nodulation in legume and plant growth promotion of rice.

## MATERIAL and METHODS

Two antibiotics resistant derivatives of endophytic rice rhizobia viz *Rhizobium undicola* RREM36<sup>CmpR</sup> (resistant to Chloramphenicol (75 µg/ml) and *Burkholderia cepacia* RREM 25<sup>KamR</sup> (resistant to 65 µg/ml Kanamycin) were used in this study. Cells of endophytic rhizobial isolates and its plasmid cured derivatives were cultivated on YEM agar and broth medium at 28°C. Tryptone Yeast (TY) medium was used in place of YEM in plasmid curing experiments. Two hosts viz rice (*Oryza sativa* L) for endophytic

colonization and common bean (*Phaseolus vulgaris* L) for symbiotic properties were grown on nitrogen free Fahraeus medium containing agar slants made in Gibson tubes (38 x 200 mm). The plasmid curing experiment was carried out using the strains RREM36 and RREM25 conferring resistance to Chloramphenicol (75 µg/ml) and for Kanamycin (65 µg/ml) respectively. 0.1 ml aliquot of exponentially growing cultures (approximately 10<sup>6</sup> cells/ml) were spread on TY plates and incubated at 35°C and 37°C. From these plates 100 colonies were picked up and streaked on TY and TY + Kanamycin (65 µg/ml) for RREM25 and TY + Chloramphenicol (75 µg/ml) for RREM36 and incubated at 28°C for 3 days. Number of cells in TY alone and TY plates supplemented with appropriate antibiotics was counted. TY broth with graded concentration of acrydine orange (0, 5, 10, 40, 45, 50, 55 µg/ml) was prepared and 0.1 ml of exponentially growing rhizobial cells were inoculated. The tubes were wrapped with black paper to avoid light and incubated at 28°C. After 7 days of growth cell suspension (100 µl) was spread on TY plates and incubated at 28°C for 3 days. 100 colonies from each type of plate were selected and re-streaked in TY and TY plate with respective antibiotic. These plates were incubated at 28°C for 3 days. Plasmid cured derivatives were confirmed by comparing the growth on both type of plate. Healthy seeds of rice (*O. sativa* L cv Sarjoo-52) were surface sterilized with 96 per cent ethanol for 10 min followed by six

washings with sterilized distilled water. A second sterilization of seeds was done using 0.1 per cent acidified  $\text{HgCl}_2$  for 10 min. Finally the seeds were washed six times with sterilized distilled water. Rice seeds were incubated in BOD incubator at  $28^\circ\text{C}$  for germination. Same protocol was followed for sterilization of common bean seeds. Five days old seedlings of rice with root length ranging from 1.5 to 3.0 cm were inoculated with 1 ml bacterial culture in each tube having cell population of  $10^6$  cells/ml. The bacterial culture was diluted 1:20 in sterile distilled water. This dilution was used for inoculation of rice seedlings. Plants inoculated with sterile distilled water served as control. The germinated seedlings were transferred aseptically to culture tube (20 x 150 mm) and plastic pot containing sterilized sand. The plants were incubated in plant growth chamber with the same conditions as described earlier. Plants were harvested at 35 days after inoculation. Experiment was done in randomized block design (RBD) and data were analyzed and tested at 0.05 and 0.01 level of significance.

## RESULTS and DISCUSSION

Two procedures of curing plasmid viz high temperature and acrydine orange were used in this study. It was observed that plasmids of culture of RREM25 and RREM36 were lost at  $37^\circ\text{C}$ . Gradual loss of plasmid starting from 35 to 60 per cent was observed with increase in incubation period and 85 per cent loss was recorded

after 7 days of incubation. Gradual decrease in growth of these two strains was observed with increase in concentration of acrydine orange at 45 and at  $55 \mu\text{g/ml}$ . No growth was observed in RREM25 and RREM36. *B. cepacia* RREM25 and *R. undicola* RREM36 were significantly effective on common bean (*P. vulgaris* L) plants. Inoculation either of the two strains was able to develop nodules which were effective in symbiotic nitrogen fixation. However when common bean plants were inoculated with plasmid cured derivatives RREM25<sup>PC</sup> and RREM36<sup>PC</sup> no nodulation was observed. Effect of wild type strains RREM25 and RREM36 and their plasmid cured derivatives (RREM25<sup>PC</sup> and RREM36<sup>PC</sup>) was studied on a cultivar (Sarjoo-52) of rice. Uninoculated plants served as control to compare the effect of inoculation. Observations recorded on various plant growth parameters are presented in Table 1. Chlorophyll content of rice plants inoculated with wild type RREM25 and RREM36 was significantly higher over control. Similarly number of lateral rootlets, plant dry weight and plant height of inoculated plants were significantly higher than control. Both strains were highly effective on growth promotion of rice plants. However when these plants were inoculated with cured derivatives of RREM25 and RREM36 the inoculation effect was non-significant and values were even less than that of control (uninoculated plants) (Table 1). In general strains of *Rhizobium* of  $\alpha$ -subclass of proteobacteria

Table 1. Effect of plasmid cured derivatives on plant growth of rice

Bacterial strain	Plant height (cm)	Cumulative root length (cm)	Total # rootlets/plant	Total # lateral rootlets/plant	Fresh shoot weight/plant (mg)	Fresh root weight/plant (mg)	Dry shoot weight/plant (mg)	Dry root weight/plant (mg)	Chlorophyll content
Control	8.26	4.91	5.12	108.75	0.05	0.07	0.03	0.004	11.56
RREM25 <sup>w</sup>	15.61**	12.07**	9.00**	281.00**	0.12**	0.13**	0.07**	0.008**	14.72**
RREM36 <sup>w</sup>	15.13**	11.60**	8.65**	240.50**	0.10**	0.12**	0.06**	0.008**	13.69**
RREM25 <sup>pc</sup> (TM)	7.26ns	4.64ns	4.25ns	90.50ns	0.06ns	0.05ns	0.04ns	0.004ns	11.43ns
RREM36 <sup>pc</sup> (TM)	7.22ns	4.25ns	4.75ns	86.75ns	0.04ns	0.05ns	0.03ns	0.003ns	11.37ns
RREM25 <sup>pc</sup> (AC)	7.56ns	4.25ns	4.50ns	78.25ns	0.05ns	0.05ns	0.02ns	0.003ns	11.52ns
RREM36 <sup>pc</sup> (AC)	6.86ns	4.34ns	3.50ns	78.75ns	0.05ns	0.05ns	0.03ns	0.004ns	11.51ns
SEM±	0.3809	0.3251	0.9831	21.063	0.006	0.0057	0.0106	0.0005	0.3674
CD <sub>0.05</sub>	0.992	0.97	1.36	58.05	0.02	0.013	0.012	0.0013	0.98
CD <sub>0.01</sub>	0.810	0.99	2.07	91.43	0.04	0.019	0.023	0.0015	1.02

W= Wild type strains of endophytic rhizobia

PC= Plasmid cured derivatives of endophytic rhizobia

TM= Plasmid cured by temperature

AC= Plasmid cured by acrydine orange

are known to develop a symbiotic association with legumes. Recently it has been reported that some genera of  $\beta$ -subclass of proteobacteria such as *Burkholderia* can also develop nodules on common bean plants that are significantly effective for symbiotic nitrogen fixation (Singh 2008). Members of both groups were found to have another ecological niche of establishing endophytic association with rice and certain other cereal crops. These bacteria perform the role of PGPR and increase plant growth significantly. Perrine et al (2005) found that rice plants inoculated with *S meliloti* 1021 and 2011 showed growth inhibition under certain rice growing conditions. With the help of a series of plasmid cured and plasmid deleted derivatives of the wild type *S meliloti* 1021 and 2011 they came to a conclusion that pSymA plays a vital role in observed growth inhibition of the host plant. Since the observations recorded in this study was altogether opposite to what observed by Perrine et al (2007) it was interesting to find out the location of genes involved in plant growth promotion caused by these two groups of legume nodulating bacteria. Plasmid cured derivatives of both *B cepacia* (RREM25) and *R undicola* (RREM36) were used as the source of inoculation. It was found that these strains were non-nodulating on common bean plants. Thus the *nod* genes responsible for nodulation in legumes of both groups of bacteria used in this work were located on plasmids and not on main chromosomes. In addition these plasmids harbored the genes involved in

plant growth by these isolates. Result of plant growth promotion of rice experiment clearly indicates that the growth of rice plants was severely reduced in terms of plant height, shoot and root dry weight and total nitrogen content. Since increase in plant growth on inoculation with RREM25 and RREM36 was because of high IAA production increase phosphate solubilization and/or associative nitrogen fixation it is quite possible that the genes involved in some of the possible mechanism of plant growth promotion were located on the plasmid which is lost in the plasmid cured derivatives resulting in the loss of PGP effect on rice.

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