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Practical use of CMC-amended rhizobial inoculant for *Mucuna pruriens* cultivation to enhance the growth and protection against *Macrophomina phaseolina*

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In many parts of the world *Mucuna pruriens* is used as an important medicinal, forage and green manure crop. In the present investigation the effect of the addition of CMC in carrier during development of bioformulation on shelflife, plant growth promotive and biocontrol activity against *Macrophomina phaseolina* was screened taking *M. pruriens* as a test crop. *Ensifer meliloti* RMP6$^{Ery+Kan+}$ and *Bradyrhizobium* sp. BMP7$^{Tet+Kan+}$ (kanamycin resistance engineered by *Tn5* transposon mutagenesis) used in the study showed production of siderophore, IAA, solubilizing phosphate and biocontrol of *M. phaseolina*. RMP6$^{Ery+Kan+}$ also showed ACC deaminase activity. The survival of both the strains in sawdust-based bioformulation was enhanced with an increase in the concentration of CMC from 0 to 1%. At 0% CMC *Bradyrhizobium* sp. BMP7$^{Tet+Kan+}$ showed more increase in nodule number/plant (500.00%) than *E. meliloti* RMP6$^{Ery+Kan+}$ (52.38%), over the control in *M. phaseolina*-infested soil. There was 185.94% and 59.52% enhancement in nodule number/plant by RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ with an increase in the concentration of CMC from 0% to 1% in the bioformulations. However further increase in concentration of CMC did not result in enhancement in survival of either the strains or nodule number/plant.

Key Words——*Bradyrhizobium*; carboxymethyl cellulose; *Ensifer meliloti*; *Macrophomina phaseolina*; *Mucuna pruriens*; rhizobia; sawdust

Introduction

The edible variety of *Mucuna pruriens* (Kaunch) is an annual herbaceous legume growing wild in the Himalayan foothills, and is easily available within the local rural market. The flowering starts 54 days after planting (Hartkamp et al., 2002). It has immense medicinal value in the Ayurvedic form of medicine (Sharma, 1996). Its soil-improving effects have also been reported in India and South Asia (Buckles, 1994). *Macrophomina phaseolina* (Tassi) Goid is one of the most destructive plant pathogens causing charcoal rot, dry root rot, wilt, leaf blight, stem blight and damping off diseases in a wide range of host plants including velvet bean. It is widely accepted that chemical management of *M. phaseolina* is often uneconomical and not feasible because it is both seed and soil borne (Singh et al., 1990), and hence biocontrol offers an effective and economical alternate for its management. Application of rhizobia is a well established strategy because of their ability for symbiotic nitrogen fixation with host plants, and also due to their role in fungal disease suppression (Deshwal et al., 2003).

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For inoculation the foremost objective is to find and select the most suitable bacterial strain (Validov et al., 2007). Legume seed inoculation with rhizobia is an old agricultural practice used since the end of the 19th century (Catroux et al., 2001). *Rhizobium* is metabolically and phylogenetically separated into two broad groups, rapid and slow growers (Allen and Allen, 1950; Sylla et al., 2002). It is interesting to note that there are certain legumes wherein both fast and slow growing rhizobia nodulate the host plants, such as soybean (Broughton et al., 1984), hyacinth bean (Trinick, 1980), lupin (Jordan et al., 1984) and *M. pruriens* (Arora et al., 2000). Physiological and biochemical studies of strains that infect soybean show that these two types differ greatly in carbon nutrition and various enzymes of the central catabolic pathways (Sadowsky et al., 1983). A similar situation exists in the *M. pruriens,* where both types of rhizobia infecting the same host differ greatly in carbon source utilization pattern, but information about their symbiotic efficiency is lacking.

Many potentially useful bacteria reported in the scientific literature never appear on the commercial market, perhaps because of inappropriate formulation. The carrier is the delivery vehicle of live microorganisms from the factory to the field and should have the capacity to deliver the right number of viable cells in good physiological condition at the right time (Arora et al., 2008). The microbial inoculant is not merely a suitable carrier containing the bacteria. Other materials might be involved in the final formulations. Evidence suggests that the addition of nutrients to seed pellets may be a useful strategy for improving inoculant survival (Moënne-Loccoz et al., 1999). The aim of the study is to screen the symbiotic efficiency of a sawdust-based inoculant of fast- and slow- growing rhizobia. Another aim is to check the effect of carboxymethyl cellulose amendment (CMC) on inoculum viability and in vivo biocontrol potential against *M. phaseolina,* taking *M. pruriens* as a test crop.

Materials and Methods

Bacterial and fungal strains. Fast-growing *Ensifer meliloti* RMP6^{Ery+} indigenously resistant to erythromycin and slow-growing *Bradyrhizobium* BMP7^{Tet+} spontaneously resistant to tetracycline were taken from collection of Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, Uttarakhand, India (Arora et al., 2000). The strains were grown and maintained on Yeast Extract Mannitol Agar (YEMA) (Hi-Media, Mumbai) at 28°C and 4°C, respectively.

The fungal pathogen *Macrophomina phaseolina* ARIFCC257 was procured from Agharkar Research Institute, Pune. The fungal culture was grown and maintained on Potato Dextrose Agar (PDA) at 28°C and 4°C.

Tn5 transposon mutagenesis for kanamycin resistance. Escherichia coli WA803 having suicidal plasmid (pGS9) integrated into a transposon *Tn5* with a kanamycin-resistant marker gene was used to confer kanamycin resistance to RMP6^{Ery+} and BMP7^{Tet+}. Bacterial conjugations were performed to introduce *Tn5* into RMP6^{Ery+} and BMP7^{Tet+} following the method of Kumar et al. (2003).

Antagonistic activity. Both RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ were screened for their ability to inhibit *M. phaseolina* by a dual culture technique. Briefly, a 6 mm mycelial disc of fungal culture grown on PDA was centrally placed on YEMA plates and 0.5 µl of log phase rhizobial culture (24 h) was spotted 1 cm away from the edge of the plate. The plates were incubated at 28°C for 7 days and percentage inhibition was determined by measuring the inhibition of radial growth as a clear zone between the fungal and bacterial colony (Arora et al., 2001). The experiment was conducted in triplicate.

Plant growth promotory and biocontrol characteristics. Phosphate solubilization activity was checked by spot inoculation of isolates on Pikovskaya's medium (Pikovskaya, 1948). IAA in the culture filtrates was detected by the chromogenic method of Bric et al. (1991) using Salkowski's reagent. Utilization of 1-aminocyclopropane-1-carboxylate (ACC) as the sole source of nitrogen indicating ACC deaminase activity was screened by spot inoculation of log phase harvested pellets on modified Dworkin and Foster (DF) minimal medium (DF salts per liter, 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements, 1 mg $FeSO_4 \cdot 7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4 \cdot H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, 10 mg MoO_3, pH 7.2) with 3.0 mmol L^{-1} ACC as the sole source of nitrogen (Penrose and Glick, 2003). Siderophore production by the rhizobial isolates was tested by chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). Production of HCN was analyzed according to Castric and Castric (1983).

Sawdust-based bioformulations. For the develop-

ment of bioformulations log phase inoculum (10^9 cells/ml) of both the strains was added (35%, w/v) separately to sterilized sawdust (Somasegaran and Hoben, 1994) followed by amendment with CMC (0%, 0.1%, 0.5%, 1% and 2%, w/v). The mixture was kept for 7 days at 28°C for curing. After curing the inoculants were sealed in low density and 0.6 mm-thick polythene bags leaving a two-thirds vacant space. The bags were then stored at ambient temperature in the dark (Somasegaran and Hoben, 1994).

Shelf life. At regular time-intervals samples were processed for measuring colony forming units (CFU) up to 360 days, following Somasegran and Hoben (1984). Average cell number was calculated by estimating CFU/g of formulation on YEM agar supplemented with erythromycin (50 mg/L) and kanamycin (50 mg/L) for RMP6$^{Ery+Kan+}$ and with tetracycline (50 mg/L) and kanamycin (50 mg/L) for BMP7$^{Tet+Kan+}$.

In vivo study. *M*. *pruriens* seeds obtained from Forest Seed Centre, Dehradun, India, were presoaked for half an hour in lukewarm water and the surface sterilized using sodium hypochlorite (3.5%) followed by washing (three times) with sterilized distilled water. The field experiments were conducted in Haridwar, Uttarakhand, India (29°58′0″N, 78°10′0″E), during May to October in the years 2006–2007 and repeated in 2007–2008. Seeds were sown in sandy loam soil (74% sand, 14% silt and 12% clay, 0.035% total organic matter, pH 7.4, water holding capacity 35%) infested with *M. phaseolina* (10^3 CFU/g soil). A uniform plant population was maintained with an intra-row spacing of 15 cm with triplicate arrangement in a randomized block design in following sets of treatments for both the strains (RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$): (i) seeds + inoculum + 0% CMC, (ii) seeds + inoculum + 0.1% CMC, (iii) seeds + inoculum + 0.5% CMC, (iv) seeds + inoculum + 1% CMC, (v) seeds + inoculum + 2% CMC. For bacterization seeds were dipped in a slurry of sawdust-based inocula (25%) of RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ separately for 10 min, then dried under sterilized conditions. Seeds without any treatment were used as a control. Plots were irrigated routinely and symbiotic parameters were evaluated after a period of 60 days. Symbiotic nitrogenase activity was measured in situ as C_2H_4 evolution according to Minchin et al. (1983).

Results

Antagonistic activity and PGP characteristics

E. meliloti RMP6$^{Ery+Kan+}$ showed better inhibition of *M. phaseolina* than *Bradyrhizobium* sp. BMP7$^{Tet+Kan+}$. RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ showed 78.01% and 71.98% inhibition of *M. phaseolina*, respectively. Both *E. meliloti* RMP6$^{Ery+Kan+}$ and *Bradyrhizobium* sp. BMP7$^{Tet+Kan+}$ showed production of siderophore, IAA and phosphate solubilization activity. Only *E. meliloti* RMP6$^{Ery+Kan+}$ showed production of ACC deaminase. Cyanogenic activity was completely absent in both the strains.

Shelf life

The surviving ability of both the strains in sawdust-based bioformulation enhanced with an increase in the concentration of CMC amendment (Fig. 1a, b). An increase in the concentration of CMC from 0% to 1% showed an enhancement of CFU/g of the strains in bioformulations. Increase in the concentration of CMC to 2% did not show any significant difference in CFU/g for either RMP6$^{Ery+Kan+}$ or BMP7$^{Tet+Kan+}$ in bioformulations in comparison to that in the case of 1% CMC (Fig. 1a, b).

In vivo study

Treatment with RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ showed an increase in germination percentage and symbiotic parameters in comparison to the control (without any treatment). Higher nitrogenase activity was observed in the plants nodulated with *Bradyrhizobium* BMP7$^{Tet+Kan+}$ than *E. meliloti* RMP6$^{Ery+Kan+}$. Slow-growing BMP7$^{Tet+Kan+}$ showed better PGP and symbiotic ability than fast-growing RMP6$^{Ery+Kan+}$ in all the tested concentrations of CMC (Table 1, Fig. 2). An increase in plant growth parameters was observed with an increase in the concentration of CMC. However, enhancement in CMC concentration from 1% to 2% showed no significant increase in growth parameters. There was 15.76%, 23.96%, 185.94% and 252.63% increase (average of 2 years) in seed germination, plant biomass, number of nodules/plant and nodule weight/plant with an increase in concentration of CMC from 0% to 1% in sawdust-based inoculant of RMP6$^{Ery+Kan+}$. Strain BMP7$^{Tet+Kan+}$ showed on average 40.53%, 32.04%, 59.52% and 41.18% enhancement in seed germination, plant biomass, number of nodules/plant and nodule weight/plant with an increase in the con-

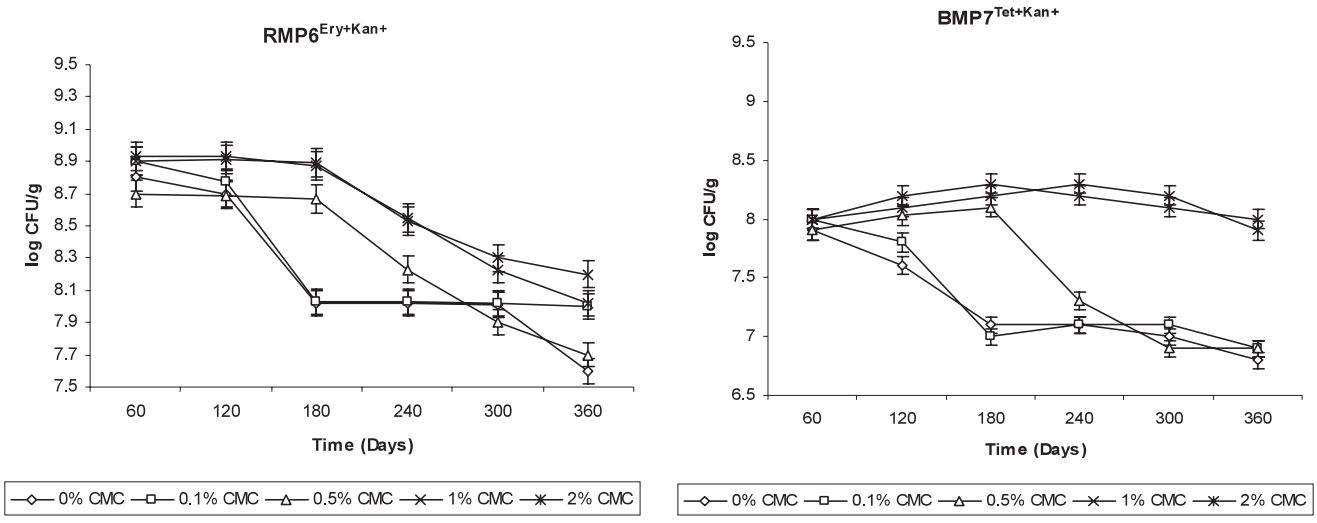

Fig. 1. Effect of different concentrations of CMC on survival of rhizobia in sawdust-based bioformulations. (a) RMP6$^{Ery+Kan+}$. (b) BMP7$^{Tet+Kan+}$.

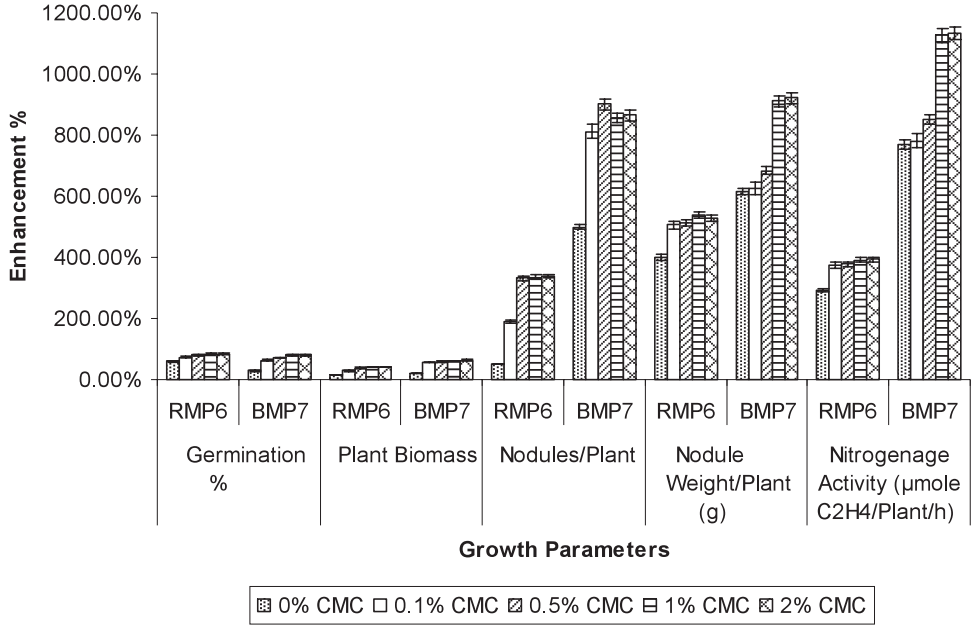

Fig. 2. Average enhancement in seed germination and plant growth parameters by bioformulations of RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ mixed with different concentrations of CMC.

centration of CMC from 0% to 1% (Table 1, Fig. 2). The nitrogenase activity per plant followed the trend of nodule weight and increased with the concentration of CMC, in bioformulations of both the strains (Table 1, Fig. 2). The nitrogenase activity per plant increased from 291.3% to 394.20% and 768.12% to 1,133.33% with RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ respectively, with an increase in the concentration of CMC from 0% to 2% over the untreated control.

Discussion

The rhizobial strains used in the study, *E. meliloti* RMP6$^{Ery+Kan+}$ and *Bradyrhizobium* BMP7$^{Tet+Kan+}$ having inhibitory activity against *M. phaseolina* showed production of IAA, siderophore and phosphate solubilizing activities. Only RMP6$^{Ery+Kan+}$ showed ACC deaminase activity. Both the strains were isolated from the root nodules of the medicinal legume *M. pruriens*

Table 1. Effect of CMC (in formulations of fast-growing RMP6$^{Ery+Kan+}$ and slow-growing BMP7$^{Tet+Kan+}$) on growth and nodulation of *M. pruriens*.

Parameters	Control	RMP6$^{Ery+Kan+}$					BMP7$^{Tet+Kan+}$				
		0%	0.1%	0.5%	1.0%	2%	0%	0.1%	0.5%	1.0%	2%
Germination %	49.8±0.03^{a}	79.3±0.03^{c}	86.5±0.04^{d}	89.9±0.05^{e}	91.8±0.04^{e}	91.7±0.02^{e}	63.9±0.04^{b}	81.1±0.05^{c}	85.4±0.03^{d}	89.8±0.03^{e}	89.7±0.04^{e}
Plant biomass (g)	8.4±0.04^{a}	9.6±0.05^{b}	10.8±0.03^{c}	11.6±0.04^{d}	11.9±0.03^{d}	11.8±0.04^{d}	10.3±0.03^{bc}	13.2±0.03^{e}	13.3±0.02^{e}	13.6±0.05^{e}	13.7±0.05^{e}
Nodules/Plant	4.2±0.03^{a}	6.4±0.02^{b}	12.1±0.04^{b}	18.1±0.03^{d}	18.3±0.05^{d}	18.4±0.05^{d}	25.2±0.02^{e}	38.3±0.03^{f}	42.1±0.04^{g}	40.2±0.05^{fg}	40.5±0.05^{fg}
Nodule weight (g)/Plant	0.19±0.04^{a}	0.95±0.03^{a}	1.15±0.05^{b}	1.16±0.02^{b}	1.19±0.02^{b}	1.21±0.05^{b}	1.36±0.05^{c}	1.38±0.02^{c}	1.49±0.04^{c}	1.92±0.03^{d}	1.94±0.03^{d}
Symbiotic nitrogenase activity (μmol C_2H_4/Plant/h)	0.69±0.05^{a}	2.70±0.04^{b}	3.27±0.02^{bc}	3.30±0.04^{bc}	3.38±0.03^{c}	3.41±0.04^{c}	5.99±0.05^{d}	6.08±0.03^{d}	6.57±0.04^{d}	8.46±0.04^{e}	8.51±0.04^{e}

Data presented are mean ± SD of two field trials each with 5 replicates. Means in the rows followed by same letters indicate no significant difference (p = 0.05) by Duncan's Multiple Range Test.

producing a large amount of biomass with more than 90% active nodules, hence improving the soil fertility (Muinga et al., 2003). Both the strains were used to develop sawdust-based formulations. A bioformulation must be assessed with regard to the protection it affords during drying and storage, and the simplicity and definability of its composition. The rhizobial bioformulations were amended with up to 2% CMC as an adjuvant. The study reports that at up to 1% CMC concentration, the population of rhizobial strains was better conserved. This clearly indicated the biostimulatory role of CMC. The population density of RMP6$^{Ery+Kan+}$ and BMP7$^{Tet+Kan+}$ was enhanced by 75% and 94% respectively, after 6 months of storage on the addition of 1% CMC in comparison to the control (0% CMC). However, there was no significant change in population density on the further increase of CMC concentration. Dubey et al. (2009) also reported the long shelf life of bioformulations containing CMC. Colloid-stabilizing properties of adjuvants were found to be important for survival. Moisture sorption by polymers affects the water available to microorganisms at different relative humidities (Deaker et al., 2007). Vidhyasekaran and Muthamilan (1995) reported that addition of xanthum gum in a talc-based bioformulation protects the population of fluorescent pseudomonads from desiccation during storage. Improvement of bacterial count (during storage) by the addition of CMC is explained by their methyl groups that allow entry of water, providing improved desiccation tolerance to rhizobia (Deaker et al., 2007).

An in vivo study showed enhancement of *M. pruriens* seed germination and growth by both the strains in *M. phaseolina*-infested soil. Inhibition of a pathogen means a role in enhancement of seed germination. Production of siderophore, IAA and solubilization of phosphate together with N_2 fixation ability have a role in plant growth promotion. Rhizobia are known to produce plant growth regulators and solubilize organic and inorganic phosphates that would have a role in their PGP activities (Antoun et al., 1998). Siderophores also contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Loper and Henkels, 1997). In comparison to *E. meliloti* RMP$_6$, *Bradyrhizobium* BMP7$^{Tet+Kan+}$ showed better PGP and symbiotic potential. Similar results were reported in earlier studies by Arora et al. (2000, 2010). Production of ACC deaminase by RMP6$^{Ery+Kan+}$

may contribute to the nodulation ability. The phytohormone ethylene has been known to inhibit nodulation in various legumes (Nukui et al., 2000). ACC deaminase converts ACC to ammonia and ß-ketobutyrate, making it unavailable for the ethylene biosynthesis pathway (Penrose and Glick, 2001); this mechanism effectively reduces the amount of ethylene evolved by the plant. Above a certain threshold, a higher level of ACC deaminase activity does not enhance nodulation further. The plants use a regulatory mechanism other than ethylene, such as a cytokinin-mediated regulatory process (Wopereis et al., 2000), to control the number of infections by *Rhizobium* to reach an optimal number of nodules, so that the host plants can obtain enough fixed nitrogen and at the same time withstand the energy cost of nitrogen fixation.

This study also showed the enhancement of PGP efficiency of rhizobial strains with an increase in the concentration of CMC up to 1%. There was improvement in seed germination percentage and nodule number/plant consequently enhancing the nitrogenase activity. Nakkeeran et al. (2005) mentioned that the performance of biocontrol agents in the formulations can be increased by the incorporation of water-soluble adjuvants, oils, stickers and emulsions. Incorporation of CMC in formulations serves as a sticker for uniform seed coating of microbes, allowing better colonization of rhizobia and competitive removal of pathogens leading to enhancement of germination of the seeds. Colonization of the root surface by rhizobia is an important prerequisite for nodule formation (Fuji shige et al., 2006). The better root colonization of rhizobial strains on the root surface on CMC addition resulted in enhancement of the nodule number/plant. The present work recommends the addition of CMC to the carrier during the development of a bioformulation to protect the inoculant from desiccation throughout storage and to promote the PGP and biocontrol ability by early colonization of roots.

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