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"Intergeneric Microbial Coaggregates"- Bioinoculation effect of different bioformulations of PGPR cells on the enhancement of plant growth stimulation and biocontrol against *Sclerotium rolfsii* in rainfed groundnut (*Arachis hypogaea*)

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ABSTRACT

The bionoculation effect of different bioformulations viz.,single strain application, coinoculation and cofloc application, of PGPR cells viz.,Methylobacterium and Rhizobium, on the enhancement of plant growth stimulation and biocontrol against Sclerotium rolfsii in rainfed groundnut was studied under in vitro condition. It was observed that the application effect of different bioformulations of Methylobacterium and Rhizobium augmented the plant growth stimulation namely, plant height, dry weight, chlorophyll content, leghaemoglobin content, nodulation, seed yield and reduced the incidence of Sclerotium rolfsii to a higher level when compared to control. Between the two single strain application, the application of Rhizobium cells recorded the higher values for PGPR characteristics and biocontrol against Sclerotium rolfsii than Methylobacterium cell application. Among the different bioformulations, the application of PGPR cells viz., Methylobacterium and Rhizobium coflocs enhanced the above said parameters to the highest level followed by coinoculation of PGPR strains and single strain application PGPR cells. It was concluded that the application of PGPR cells viz., Methylobacterium and Rhizobium, as coflocs, augmented the PGPR characteristics and bio control against the Sclerotium rolfsii to a higher level in rainfed groundnut when compared to other bioformulations.

Keywords: PGPR, Rainfed Groundnut, Microbial Cofloc, Biocontrol, *Sclerotium rolfsii*

INTRODUCTION

Groundnut (*Arachis hypogoea* .L.) is one of the most important oil seed crop produced in the world. It is generally distributed in the tropical, sub tropical and warm temperature zones of the world covering in over 80 countries. India has the largest area under groundnut cultivation in the world but it ranks second in production, next to china. In India, the rainfed groundnut ecology is the largest one in terms of area (8.0 Mha) and production (7.4 MT) but with least productivity (0.93 MT) [1]. Of the several biotic and abiotic

constraints, rainfall, low soil fertility and incidence of diseases are considered to be the major constraint that ultimately leads to low productivity in rainfed groundnut.

Nitrogen is one of the key nutrient and expensive input that frequently limits the groundnut production under rainfed condition [2]. Moreover, the incidence of collar rot disease, caused by *Sclerotium rolfsii*, is one of the most destructive fungal disease of rainfed groundnut, causing an yield loss up to 90 percent. In this connection, the use of "Plant Growth Promoting Rhizobacteria (PGPR), as biological approach, might be an alternative strategy to overcome the biological and environmental hazards posed by the consistent use of synthetic chemicals. Rhizospheric bacteria that favorably affect the plant growth and yield of commercially important crops are denominated as "Plant growth promoting Rhizobacteria (PGPR)"[3]. Several mechanisms of plant- microbe interaction may participate in the association and affect the plant growth, including, N-fixation, hormonal interaction, improvement in root growth, solubilisation of nutrients and biocontrol against phytopathogens. Thus, the PGPR affect the plant growth directly by producing and secreting plant growth promoting substances or by eliciting root metabolic activities by supplying biologically fixed nitrogen and indirectly by acting against phytopathogenic microorganisms [4].The well known PGPR include, bacteria belonging to the genera, namely, *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Azoarcus*, *Klebsiella*, *Arthrobacter*, *Enterobacter*, *Serratia* and *Rhizobium* on non-legumes.

The positive effect of *Rhizobium* – groundnut symbiosis has been reported by many authors [5-7].The helper role of *Methylobacterium* on the augmentation of establishment, nodulation and nitrogen fixation of *Rhizobium* with legume crop plants has been reported by Madhaiyan *et al.,* [5].The positive, coinoculation effect of *Rhizobium* and *Methylobacterium* on the enhancement of nodulation and nitrogen fixation in soyabean has been reported by Menakashi [8] *Rhizobium* and *Methylobacterium* are widely used as agricultural bioinoculants for the enhancement of growth and yield in rainfed groundnut[5].

However, the introduced bioinoculant exhibited poor performance in natural environments and in the rhizosphere of host plants due to lack of stress tolerance and poor survivability in soils. Okon and Lanbandera –Gonzalez[9] stressed the importance of the physiological status of microorganisms in any agricultural bioinoculant production rather than their cell numbers to ensure more stress tolerance and survival in soil. van-Veen *et al.*[10] suggested that instead of trying single strain with single trait, as agricultural bioinoculant, trying to use microbial consortia for harnessing multiple benefits. Neyra *et al.*,[11] proposed the concept of " Intergeneric Microbial Coaggregates'' for the purpose of multipurpose bioinoculant with multiple benefits.

Hence, the present research work has been undertaken with an aim to compare and evaluate the performance of different bioformulations *viz.,* single strain inoculation, coinoculation and cofloc applications of PGPR cells *viz*., *Rhizobium* and *Methylobacterium* on the enhancement of growth and yield in rainfed groundnut in order to determine the superior formulation of the bioinoculant for increasing the rainfed groundnut productivity.

MATERIALS AND METHODS

Strains of *Methylobacterium* and Rhizobium *viz., Methylobacterium. extorquens* (MB-5) and *Rhizobium* sp. (RM-5), were isolated from the phyllosphere and rhizosphere of rainfed groundnut respectively grown at different locations of cuddalore district and maintained in methanol mineral salt medium(MMS) and yeast extract mannitol agar (YEMA), respectively at 35°C with monthly transfer and used throughout the study. *Sclerotium rolfsii* AU-1 (provided by Department of plant pathology, Annamalai University) was used as reference strain for the biocontrol study and the same was maintained in oat meal agar (OMA) medium and examined periodically for its virulence.

Preparation of inoculum: All the two isolates *viz*., *Rhizobium* sp. (RM-5) and *Methylobacterium extorquens* (MB-5) were grown in YEMA and MMS medium respectively, under shaking culture condition at 30ºC ± 2ºC for 24 h. Then, the media was centrifuged at 5000 × g for 10 min, separately, to harvest the log phase cells and the pellets were washed three times with 0.1 M phosphate buffer (pH 6.8).

Finally, the cells were resuspended in the same buffer to a cell concentration of 1×10^7 CFU mL^{-1} by measuring the OD at 420 nm and used as inoculum.

Preparation of cofloc of PGPR cells: All the two isolates *viz*., *Methylobacterium extorquens* (MB-5) and *Rhizobium* sp. (RM-5), were grown in MMS and YEMA broth, respectively, duly supplemented with 0.05% yeast extract in a shaking bath at 30±2°C for 5 days. Then, the media were centrifuged, separately, at 5000x g for 10 min to get stationary phase cells and the pellets, obtained after centrifugation, washed three times in phosphate buffer (pH 6.8) and finally the cells were resuspended in the same buffer to a cell concentration of 1 x 10^7 CFU mL^{-1} by measuring the absorbance at 420nm.The preparation of Co-AG buffer was done according to the specification of Grimaudo and Nesbitt [12].The co-aggregates was prepared according to Jabara-Rizk *et al*.[13] as stated herewith. One ml aliquot of each PGPR isolate was mixed together *viz*., *Methylobacterium extorquens* (MB-5) and *Rhizobium* sp. (RM-5) was mixed together with 10 ml Co-AG buffer, vortexed for 10s, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for 24 h. After the incubation period, the coaggregates, settled at bottom of the tube were obtained after decanting the buffer.

Effect of different bioformulations of PGPR cells on seed vigour index: Groundnut **(***Arachis hypogaea* L.) seeds (*cv*. JL- 24) were surface sterilized by immersion in 95% ethanol for 1min, followed by 20 min in 1 per cent NaOCl. After rinsing three times with sterile distilled water, the sterilized seeds were placed on the surface of 1 percent water agar in petriplates (9cm dia. at the rate of five seeds per plate). Then, they were incubated in an inverted position for 3 days at room temperature to allow germination. The plates were sealed with wax to avoid agar dryness during germination.
Then, the germinated groundnut seeds were subjected to the following treatments *viz*.,T_1-control, T_2-*Methylobacterium* vegetative cells, T_3-*Rhizobium* vegetative cells, T_4-*Rhizobium*+*Methylobacterium* coinoculation and T_5-*Rhizobium*+*Methylobacterium* coaggregates, dried and shade for 30 min. After the bioinoculation, the groundnut seeds were arranged in two rows on a sheet of blotting paper dipped in sterile water. Then, they were covered with another blotting paper dipped in sterile water, rolled and placed vertically in a moist chamber at 20°C. After the incubation for 5 days, each roll was opened and the vigour indices of germinated groundnut seeds, were calculated by the method of Abdul-Baki and Anderson [14]. The experiment was arranged in Randomized block design with three replications.

Vigour index = Germination% x Total length of seedling (mm) (Root and shoot length)

Effect of different bioformulations PGPR cells on adhesion to groundnut root: The preparation of different bioformulations of PGPR cells *viz*., *Rhizobium* and *Methylobacterium* was prepared as already mentioned above. Groundnut cv. JL-24 seeds were surface sterilized and germinated as detailed earlier. After the germination, the three day old sterile seedlings were transferred to slopes of Fahraeu's solution [15], solidified with 1.5 per cent agar in test tubes. Sterile Fahraeu's solution was added to fill the empty portion of the agar slopes and the tubes were incubated for three more days (24°C day / 22°C night). After the incubation period, the roots were collected from each tube separately, washed first with sterile water and later three times in 0.1M phosphate buffer (pH 6.8), cut into 5 cm pieces and used in the adsorption experiments according to Gafni *et al.* [16] and the experiment was arranged in Randomized block design (RBD) with three replication.

Effect of different bioformulations of PGPR cells on the enhancement of growth and yield in rainfed groundnut : A pot culture experiment was conducted to study the bioinoiculation effect of different bioformulations of *viz*., single strain inoculation, co-inoculation and co-aggregates application of PGPR cells *viz*., *Rhizobium* and *Methylobacterium* together with challenge inoculation of *Sclerotium rolfsii* on the enhancement of growth and yield in rainfed groundnut with special emphasis to biocontrol against collar rot disease (*Sclerotium rolfsii*). The study was conducted during winter season (Aug to Nov, 2011) with groundnut cv. (JL-24), at the polyhouse of Department of Microbiology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India. Rectangular cement pots with 18"x12"x12" size were

filled with 45 kg of groundnut field soil flooded with water for 2 days and brought to fine puddle condition. After draining the excess, water, groundnut seeds were sown in rows in the pots, separately. The age of the seedlings were counted from the time of sowing. The experiment was arranged in randomized block design (RBD) with three replications and the following were the treatments *viz* ,Control, *Rhizobium* alone, *Methylobactrium* alone, *Rhizobium* + *Methylobacterium* co-inoculation and *Rhizobium* + *Methylobacterium* co-aggregates application. During the experimental period, the annual mean minimum and the maximum temperature of experimental area was 25ºC and 30ºC, respectively and the mean highest and lowest humidity were 96 and 78 percent, respectively. The mean annual rain fall of this area was 1500 mm. A fertilizer schedule of 25: 50: 75 NPK ha^{-1} was followed. Regarding the 'N' fertilization, 50 per cent of the same was given as basal dose, while the other 50 per cent was given as top dressing in two split doses. The entire dose of P_2O_5 and K_2O has been applied basally as super phosphate and muriate of potash, respectively. Groundnut plants were challenge inoculated by spraying *S.rolfsii* spore suspension (50,000 spore mL^{-1} inoculum level) on 10th DAS with an atomizer and the control plant was sprayed with sterile water. High humidity was created by sprinkling water frequently in the polyhouse. The crop was given a hand weeding on 30th DAS and well protected against pests and diseases. The experiment was maintained under limited water supply as per the conditions prevailing in rainfed ecosystem. Five representative samples of plant hills in each pot were pegmarked for periodical observations. The plant height, shoot dry weight, root dry weight, chlorophyll content [16,17],were recorded on 45 DAS 'N' content of the plant was estimated using the Microkjeldhal method according by Bremner, [18] and expressed to $kg^{-1}ha^{-1}$ and the Leghaemoglobin content [19] of rainfed groundnut root nodules recorded on 45th DAS. While the seed yield was recorded during the time of harvest.

Statistical Analysis: The experimental results were statistically analyzed in Randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez [20].

RESULTS AND DISCUSSION

The application effect of different bioformulations, *viz*., single strain inoculation, coinoculation and coaggregates application, of PGPR cells viz., *Rhizobium* and *Methylobacterium* on the enhancement of groundnut root adhesion and seed vigour index of groundnut cv.JL-24 was studied under *in vitro* condition and the results presented in table-1.

Table 1. Effect of different bioformulations of PGPR cells on the enhancement of seed vigour index and adhesion in rainfed groundnut cv.JL-24

Treatment	Seed vigour index	Statistics	No.of adhered cells(10^4/g dry wt. of root/h)	Statistics
Control	2439 ± 65.52	e	178.4 ± 0.71	e
Methylobacterium alone (MB-5)	3968 ± 93.34	d	238.8 ± 1.02	d
Rhizobium alone (RB-5)*	3998 ± 126.33	c	265.9 ± 2.98	c
Co-I- MB-5 + RB-5**	4200 ± 188.56	b	293.7 ± 3.46	b
Co-F- MB-5 + RB-5***	4586 ± 181.96	a	302.8 ± 6.48	a

a - Average of three replication

b - Values followed by different letters are significantly differed at 5 % according to student 't' test

* -Individual application of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum

** -Coinoculation of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum
***- Coflocs of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum

Between the two single strain inoculation treatments, the application of *Rhizobium* vegetative cells augmented the groundnut root adhesion and seed vigour index of groundnut to a higher level than the application of and *Methylobacterium* vegetative cells. But, the effect was more pronounced when the PGPR strains (both *Rhizobium* and *Methylobacterium*) were applied as coinoculation. However, the highest groundnut root adhesion and seed vigour index of groundnut was recorded when the PGPR strains (*Rhizobium* and *Methylobacterium*) were applied as coaggregates. The highest seed vigour index of groundnut (4586) and highest groundnut root adhesion (302.8 x 10^4/g dry wt of root/h) was recorded the during the application of PGPR strains *viz., Rhizobium* and *Methylobacterium,* strains, as coaggregates, followed by coinoculation and single strain inoculation. The nodulation, nitrogen fixation and the enhancement of growth and yield of different legume crops by *Rhizobium* sp. bioinoculation have been reported by many authors [21-25].The coinoculation effect of *Rhizobium* and *Methylobacterium* on plant growth stimulation and induction of systemic resistance in groundnut has been reported by many authors [26-28,5]. The phytostimulatory and adhesion mechanism of *Methylobacterium* cells with various crop plants have been already reported [29-31] . Katupitiya *et al*.,[32], Levanony and Bashan [33] and Michels *et al*. [34] reported the poor adhesion of floc negative mutant strain of *Azospirillum* to wheat roots and emphasized the positive role of Exopolysacharides (EPS) in the adhesion processes. Sadasivan and Neyra [35] reported the improved adhesiveness of *Azospirillum* bioflocs with plant roots and suggested the positive role of EPS in the early events of adhesion to plant root. Neyra *et al.,* reported the positive effect of *Azospirillum* and *Rhizobium* coflocs on the enhancement of growth and yield in Faba bean. Moreover, the phytostimulatory effect of bacterial EPS has been demonstrated in *Azospirillum* [36].In the present study the application of EPS rich, PGPR coaggregates, consisting of strains of *Rhizobium* and *Methylobacterium,* augmented the groundnut root adhesion and seed vigour index of groundnut to a higher level when compared to other treatments. The results of the present study are in conformity with the above earlier findings . The effect of different bioformulations *viz*., single strain inoculation, co-inoculation and co-aggregates application, of PGPR cells *viz*., *Rhizobium* and *Methylobacterium* on the growth and yield parameters *viz*., plant height, dry weight of root and shoot, chlorophyll content, nodule number, leghaemoglobin content, seed yield and biocontrol activity against *Sclerotium rolfsii,* causative agent of collar rot disease, in rainfed groundnut cv.JL24,was studied under pot culture condition and the results presented in Table 2.

The effect of *Rhizobium* inoculation in augmenting the growth and yield parameters of rainfed groundnut has been reported by many authors The positive role of *Methylobacterium,* as helper bacterium in augmenting the growth and yield parameters of groundnut has been well established .The positive coinoculation effect of *Rhizobium* and *Methylobacterium* in rainfed groundnut has already been reported [26-28,5].In the present study, single strain inoculation of PGPR cells *viz., Rhizobium* and *Methylobacterium* could augment the growth and yield parameters of rainfed groundnut to a higher level when compared to control. But, the effect was more pronounced when the two PGPR strains *viz*., *Rhizobium* and *Methylobacterium* were coinoculated. However, the effect was found to be the highest when the PGPR strains *viz*., *Rhizobium* and *Methylobacterium* were applied as coaggregates. Between the two single strain application, the application of *Rhizobium* was found to enhance the growth and yield parameters of groundnut to a higher level than *Methylobacterium*. Interestingly, the coinoculation of PGPR strains *viz., Rhizobium* and *Methylobacterium* recorded the highest response than single strain inoculation but not with coaggregates application. However, the response was found to be the maximum.

Table-2 Effect of different bioformulations of PGPR cells and challenge inoculation of *Sclerotium rolfsii* and biocontrol against *Sclerotium rolfsii* on the enhancement of growth and yield parameters in the rainfed groundnut cv.JL-24

Treatment	Plant height (cm)	Dry weight (g plant-1)	Chlorophyll content (mg/g of leaf)	Nodule/plant	Nodule dry (wt mg/plant)	'N' content (%)	Leghaemoglobin content (mg/g nodule	Seed weight (g plant-1)	Disease Incidence (%)
Control	28.40^{e}	11.0± 0.67^{e}	1.048± 0.052^{e}	42.5 ±1.9^{e}	34.46±0.2^{e}	0.95^{e}	**1.2**	6.52	90.50^{e}
Methylobacterium alone (MB-5)	30.42^{d}	14.3± 0.84^{d}	1.147± 0.057^{d}	52.3±0.79^{d}	38.56±0.3^{d}	1.30^{d}	**1.4**	7.37 ± 0.4d	22.62^{c}
Rhizobium alone (RB-5)*	33.10^{c}	17.2± 0.91^{c}	1.155±0.057^{c}	60.3 ±2.2^{c}	40.69±0.1^{c}	1.43^{c}	**1.5**	8.24 ± 0.54c	30.78^{d}
Co-I-MB-5+RB-5**	36.36^{b}	20.1± 0.88^{b}	1.194± 0.059^{b}	68.6±2.1^{b}	44.78±0.4^{b}	1.67^{b}	**1.7**	8.96 ± 0.69b	17.52^{b}
Co-F-MB-5+ RB-5***	39.86^{a}	23.1± 1.48^{a}	1.313± 0.065^{a}	75.5±2.3^{a}	48.95±0.2^{a}	1.85^{a}	1.9	9.94 ± 0.40a	11.00^{a}
LSD (p=0.05)	0.45	0.10	0.07	1.40	0.85	0.14	0.10	0.14	-

a - Average of three replication
b - Values followed by different letters are significantly differed at 5 % according to student 't' test
* -Individual application of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum
** -Coinoculation of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum
***- Coflocs of *Rhizobium* (RB-5) and *Methylobacterium* (MB-5) isolates at $1x10^{-7}$ $CFUmL^{-1}$ inoculum

APPLICATIONS

Application of PGPR cells *viz*., *Methylobacterium* and *Rhizobium* as natural coaggregates bioformulation, augmented the plant growth stimulation and biocontrol against *Sclerotium rolfsii* to a higher level than other formulations in rainfed groundnut in order to enhance the crop productivity.

CONCLUSIONS

The application effect of PGPR cells viz., Methylobacterium and Rhizobium, as natural coaggregates bioformulation, augmented the plant growth promoting characterstics viz., plant height, dry weight, chlorophyll content, grain yield and reduced the incidence of Sclerotium rolfsii which ultimately lead to the enhancement of groundnut productivity grown under rainfed conclusion.

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