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Co-inoculant Response of Microbial Consortia on Physiology of Blackgram [*Vigna mungo* (L.) Hepper] Seed Germination

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ABSTRACT

Background: Blackgram (*Vigna mungo* (L.) Hepper) belonging to the Leguminoceae family is rich in protein. In this crop, biological seed treatment is an environmentally sound approach for improving the vigour of seeds. Besides legume-*Rhizobium* symbiosis, several other beneficial microbes play a crucial role in vigour enhancement in blackgram.

Methods: The surface sterilised seeds were first coated with 20% gum acacia followed by the coating with bioinoculants *viz*., T_0 - Control (Dry seed), T_1 - *Rhizobium* sp. BMBS + Arbuscular Mycorrhizal Fungi (AMF) + *Methylobacterium extorquens* AM1 and T_2 - *Rhizobium* sp. BMBS + AMF + *Bacillus velezensis*.

Result: Inoculation of blackgram seeds with the *Rhizobium* sp. BMBS and AMF with *Methylobacterium extorquens* AM1 resulted in a significant increase in germination (97%), dry matter production (0.237 g 10 seedlings^{-1}), vigour index (22.99) and seed mobilization efficiency (1.11) as compared to control seeds. The biochemical parameters such as á-amylase and proteases were found to be significantly higher in the *Rhizobium* sp. BMBS + AMF + *Methylobacterium extorquens* AM1 inoculated seeds. Changes in root exudates composition due to co-inoculation assessed through GC-MS, indicated compounds with antioxidant and antimicrobial activities. Our results confirmed that the positive interaction of rhizobial strain BMBS and AMF with *Methylobacterium extorquens* AM1 may emerge as a novel bio-inoculant for sustainable pulse productivity.

Key words: Blackgram, Co-inoculation, Film coating, GCMS, Seed germination, Vigour.

INTRODUCTION

Pulses belonging to the Leguminoceae family are the chief source of protein in the Indian vegetarian diet. Among the pulses, blackgram contains 25-26% protein (Amuthaselvi *et al*., 2019). India is the largest producer as well as consumer of blackgram covering an area of 41.4 lakh ha with a production and productivity of about 22.3 lakh tonnes and 538 kg/ha respectively during 2020-21 (Indiastat, 2022). The reason for low productivity in pulses may be due to the fact that they are normally grown in marginal lands of low fertility status with inadequate soil moisture.

The use of quality seed alone could increase the productivity by 15-20%. Biological seed treatment with beneficial microbes is an environmentally sound approach for improving the vigour of seeds. Film coating is one of the methods of microbial inoculation in which a thin even coating of microbes with a binder material are coated onto the seeds.

The legume-*Rhizobium* seed inoculation has been known long back due to its role in nitrogen fixation in the root nodules. The majority of plant-microbe interactions research concentrate on a single plant-microbe association at a time and clear laboratory demonstration of co-inoculation of *Rhizobium* with other beneficial microbes are still very few.

The rhizobia-bean symbiosis, when in association with Arbuscular Mycorrhizal Fungi (AMF) is known for its benefit of better supply of phosphorus (Sanginga *et al*., 2000). Pink Pigmented Facultative Methylotrophic bacteria (PPFM) were known to influence seed germination and seedling establishment (Ivanova *et al*., 2001). Recently, a novel bacterial species named *Bacillus velezensis*, exhibits growth promotion and antagonistic activity against major plant fungal pathogens (Myo *et al*., 2019).

Positive influence of microbes on co-inoculation have been reported for *Rhizobium* - PPFM interaction in groundnut (Priya *et al*., 2019) and in pigeonpea (Raja *et al*., 2019a), *Rhizobium* - AMF in common bean (Tajini *et al*., 2011) and blackgram (Choudhury and Azad, 2004). Microbes interact with the plant system and alter the production of metabolites

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resulting in distinct root exudation pattern. In line with this, present work was hypothesized that a quadritrophic interaction is established between *Rhizobium sp*. BMBS, AMF, PPFM (*Methylobacterium extorquens* AM1) or *B. velezensis* VB7 and blackgram for seed germination and seedling vigour improvement.

MATERIALS AND METHODS

The laboratory experiment was carried out at the Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore during 2021-22. Freshly harvested blackgram variety VBN 8 seeds were obtained from Krishi Vigyan Kendra, Vamban, Tamil Nadu. The bio inoculants *viz*., *Rhizobium* sp. BMBS, *Methylobacterium extorquens* AM1 (PPFM) used in the experiment were obtained from the Department of Microbiology and *Bacillus velezensis* VB7 was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The bacterial cultures were maintained at a population of 10^8 cfu mL^{-1} while the Arbuscular Mycorrhizal Fungi (AMF) in the form of liquid formulation was procured from Uyir Organic Farmers Market, Coimbatore with a spore count of 1×10^3 spores ml^{-1}.

The surface sterilized seeds were first coated with 20 per cent gum arabic followed by the coating with bioinoculants as per the treatments *viz*., T_0 - Control (Dry seed), T_1 - *Rhizobium* sp. BMBS + AMF + *Methylobacterium extorquens* AM1 and T_2 - *Rhizobium sp*. BMBS + AMF + *Bacillus velezensis* VB7. For co-inoculation of bioinoculants in the treatment T_1 and T_2, a cocktail of the above mentioned bioinoculants were taken in equal ratios. The seeds after shade drying were used for the physiological and biochemical analysis. The experiment was conducted by adopting completely randomized block design (CRD) with seven replications.

Determination of physiological parameters

The blackgram seeds were tested for different physiological parameters *viz*., germination, root length, shoot length, dry matter production, vigour index (ISTA, 2015) and seed metabolic efficiency (Srivastava and Sareen, 1974).

Determination of biochemical parameters

The dehydrogenase activity was estimated according to Kittock and Law (1968) and α-amylase activity as per the procedure described by Paul *et al*. (1970). The protease activity was analysed according to the method described by Li *et al*. (2011) and total free amino acid according to Ching and Ching (1964).

Metabolite profiling

Treated and untreated seeds were kept for germination using paper medium (between paper). The seven days old seedlings of uniform size were transplanted into glass test tubes containing Hoagland's nutrient solution (Hoagland and Arnon, 1950). Root exudates were collected on 15th day with ethyl acetate in equal volume (1:1, v/v) and then concentrated by natural evaporation. The elute was then dissolved in one ml of MS grade methanol and subjected to identification of metabolites through Gas Chromatography-Mass Spectroscopy using Shimadzu GCMS-TQ8040 NX. One µL aliquots of the reaction mixture was injected into the gas chromatograph in 1:10 split mode. The separation was performed in Rtx-5MS column, which has a 30 m length, 0.25 mm ID and 0.25 m film thickness and was made up of 5 percent diphenyl dimethyl polysiloxane. The quadrupole mass spectrometer was operated in an electron ionization mode at 70 eV. The scan range was set to 50-650 *m/z*. Mass-spectrum interpretation was done utilizing the NIST Standard Reference Database 1A in NIST V2 data version.

Statistical analysis

Statistical analysis was performed by subjecting the data to one way analysis of variance (ANOVA) and analysing them by Least Significant Difference test for statistical significance at pd”0.05 using AGRESS software (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The co-inoculation of microbes through film coating performed better than the control seeds. Highly significant difference in germination (97%) was found in seeds coated with combination of *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 followed by *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7 (95%) (Table 1). Initially, inoculated seeds showed higher germination which might be due to the production of phytohormone, as phytohormone influences seed germination (Mia *et al*., 2012). *Methylobacterium* produce phytohormones such as cytokinins and auxins, which were known to stimulate seed germination (Lee *et al*., 2006).

Seeds coated with *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 recorded the longer root (20.40 cm) and shoot length (21.52 cm) which was on par with *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7 (20.11 cm and 21.39 cm) respectively (Table 1). Similar findings of inoculation of *Rhizobium* or PPFM on germination and seedling vigour improvement was studied earlier in blackgram (Raja *et al*., 2019) and pigeonpea (Raja *et al*., 2019a) seeds. Induction of longer roots was a growth response that might be attributed due to the production of Indole Acetic Acid (IAA) by *Rhizobium* sp. (Mohite, 2013), PPFM (Pattnaik *et al*., 2017), *B. velezensis* (Meng *et al*., 2016). The increase in seed germination and seedling length were considered typical gibberellins-like responses. Microbes were known to modulates the level of ROS at the time of germination (Gomes and Garcia, 2013).

Dry matter production, vigour index and seed mobilisation efficiency were higher in *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 (0.237 g 10 $seedlings^{-1}$, 22.99, 1.11) followed by *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7 (0.224, 21.28, 1.06), respectively (Table 1). Microbes through phytohormone and hydrolysing enzymes production interact with the seedlings and facilitates the nutrient mobilization from endosperm to embryo, that could reflect in the dry matter production and seed mobilisation efficiency. Reactive oxygen species (ROS) roles have been recognised in weakening of endosperm and mobilisation of food reserve during seed germination (El-Maarouf-Bouteau and Bailly 2008).

No significant difference was found in dehydrogenase activity (Fig 1A) among the treatments. The changes in enzymatic activities such as α-amylase activity (22.6 mg maltose min) (Fig 1B), protease activity (0.269 units/mg of protein) (Fig 1C) and total free amino acids (0.005 µg 25 seeds^{-1} 25ml^{-1}) (Fig 1D) were higher in *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 followed by *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7. Previous studies showed the ability of *Rhizobium* strains for the production of α-amylase (Oliveira *et al*., 2007) and protease enzymes (Dhole and Shelat, 2022). Besides *Rhizobium*, PPFM was also found to produce protease enzyme (Jayshree *et al*., 2014). It was clear from the physiological and biochemical studies, that co-inoculation of *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 through film coating exhibited a profound effect on vigorous seedling production of blackgram.

GC-MS based untargeted metabolomic analysis was launched to compare the metabolic difference that occurred in primary and secondary metabolism of control and bio-inoculant coated seeds in the root exudates pattern of hydroponically grown blackgram seedlings. The compounds identified in root exudates of seedlings with their potential uses were shown in Table 2. This study revealed that, more number of compounds responsible for antioxidant and antimicrobial activity were released by seeds coated with *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1 and *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7 than the root exudates of control seedlings. In line with this, changes

Table 1: Microbe mediated physiological changes during germination in blackgram seeds.

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Dry matter production (g 10 seedlings^{-1})	Vigour index	Seed mobilization efficiency
T_0 - Control	90 (71.56)	17.62	20.28	0.200	18.00	0.95
T_1 - Film coating of *Rhizobium* sp. BMBS + AMF+ *Methylobacterium extorquens*	97 (80.02)	20.40	21.52	0.237	22.99	1.11
T_2 - Film coating of *Rhizobium* sp. BMBS + AMF+ *Bacillus velezensis* VB7	95 (77.08)	20.11	21.39	0.224	21.28	1.06
SEd	1.06	0.24	0.41	0.003	0.28	0.02
CD (P=0.05)	2.40	0.54	0.94	0.007	0.64	0.03

(Figures in parenthesis indicate arcsine transformed values).

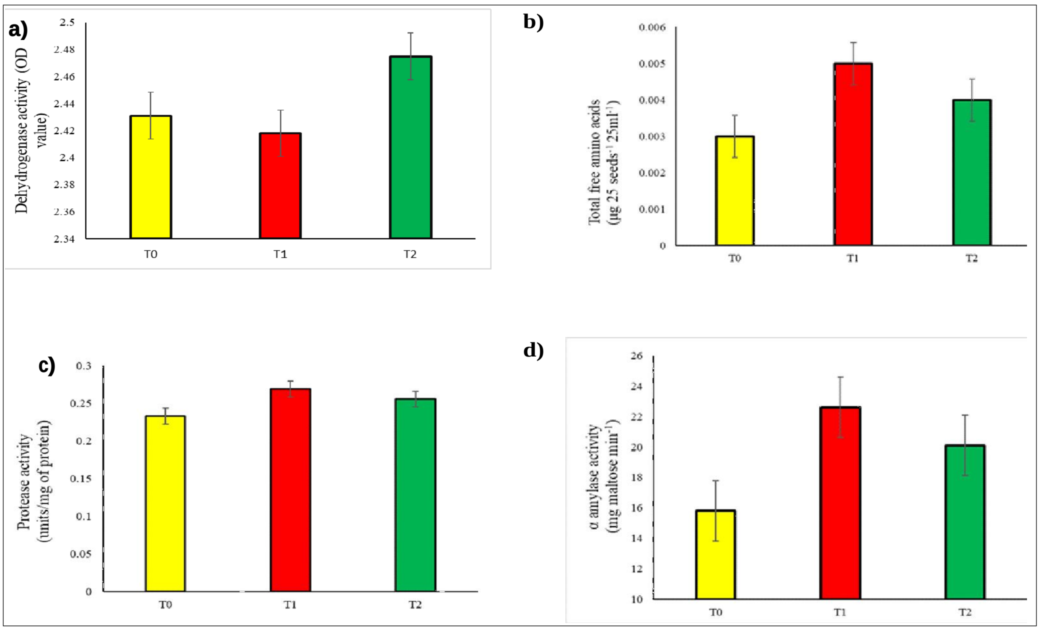

Fig 1: Effect of bio-inoculants on enzyme activity in blackgram seed A) Dehydrogenase activity B) α-amylase activity C) Protease activity D) Total free amino acid (T_0 - Control, T_1 - Film coating of *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1, T_2 - Film coating of *Rhizobium* sp. BMBS + AMF + *B. velezensis* VB7).

Table 2: Co-inoculant response of bio-inoculants on root exudates of blackgram seeds.

Retention time (Min)	Name of the compounds	Reported activity	Reference	Area (%)		
				T_0	T_1	T_2
3.35	2, 2-Dimethoxybutane	Antimicrobial	Sani *et al.* (2015)	0.51	4.58	2.05
5.19	Phenyl (2-phenyl-1,3-dioxolan-2-yl) methanol	Antifungal	Van Gestel *et al.* (1980)	0.00	6.21	6.08
8.53	sabinene	Antioxidant	Sharma *et al.* (2019)	0.00	0.95	0.00
15.46	2,4-Di-tert-butylphenol	Bacterial metabolite and antioxidant	Choi *et al.* (2013)	0.41	2.59	1.64
17.13	Phloroglucinol, O,O' -bis (trimethylsilyl)-	Antioxidant	Nakamura *et al.* (1996)	0.00	0.76	0.00
19.29	Myristic acid, TMS derivative	Antimicrobial	Sama *et al.* (2021)	0.00	1.09	0.00
21.25	Palmitic acid-TMS	Antifungal	Ma *et al.* (2021)	0.65	2.23	2.01
22.64	1-Nonadecene	Antifungal, antibacterial, antioxidant	Ferdosi *et al.* (2021)	1.33	4.07	0.00
24.35	1-Heptacosanol	Antimicrobial and antioxidant	Al-Abd *et al.* (2015)	1.33	4.07	0.00
25.46	Bis (2-ethylhexyl) phthalate	Antibacterial and antimicrobial	Qi *et al.* (2009)	8.84	2.07	9.91
27.55	Squalene	Antibacterial, Antioxidant	Beulah *et al.* (2018)	0.00	1.15	0.00

in root exudates compound upon *Pseudomonas fluorescens* inoculation in tomato was reported by Kamilova *et al*. (2006). Similarly, volatile organic compounds (VOC) produced by rhizobacteria were involved in their interaction with plant-pathogenic microorganisms and host plants by elucidating antimicrobial and plant-growth modulating activities (Vespermann *et al*., 2007).

During microbial interaction of *Rhizobium* sp. BMBS + AMF + *M. extorquens* AM1, it produced distinct metabolites such as phenyl (2-phenyl-1, 3-dioxolan-2-yl) methanol, sabinene, squalene. Phenyl (2-phenyl-1,3-dioxolan-2-yl)methanol had highest peak area percent of 6.21 possessing antifungal activity (Van Gestel *et al*., 1980). Sabinene which is a monoterpene has been found to involve in starch and sucrose metabolism and plant growth regulation (Grulova *et al*., 2022). Squalene belonging to triterpene is an antioxidant (Huang *et al*., 2009) and scavenges the free radical damage (Micera *et al*., 2020).

In nutshell, interaction between seed and bioinoculants *viz., Rhizobium* sp. BMBS + AMF + *Methylobacterium extorquens* AM1 released plant growth promoting substance, which played a role in increasing the seed germination, seedling length and dry matter production ultimately resulted in increased seedling vigour.

CONCLUSION

This investigation evaluated the responses of seed germination, physiological, biochemical parameters and root exudates pattern to co-inoculation of *Rhizobium* sp. BMBS and AMF with *Methylobacterium extorquens* AM1 and *Bacillus velezensis* in blackgram. We found that the response pattern to inoculation was highly influenced by *Rhizobium* sp. BMBS, AMF and *Methylobacterium extorquens* AM1 co-inoculation through coating enhanced seed germination, vigour, production of enzymes and useful metabolites.

Conflict of interest: None.

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