



RESEARCH ARTICLE

Non-rhizobial Root Nodule Endophytic Yeast, *Candida tropicalis* VYW1 Impacts Germination, Nodulation behavior and Metabolic flux in Blackgram (*Vigna mungo* L.)

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ABSTRACT

Nodule associated endophytic yeasts have attracted much attention due to its potential plant growth-promoting activities and protecting legume crops from both biotic and abiotic stress. The present study evaluated the potential role of an endophytic yeast *Candida tropicalis* VYW1 in modulating host fitness (blackgram) both as a single inoculant and co-inoculant with *Rhizobium* sp. VRE1. The results showed that nodule associated endophytic yeast *C. tropicalis* VYW1 when co-inoculated with *Rhizobium* sp. VRE1 significantly increased the germination percent (100%), root (34.6 cm), and shoot length (32.8 cm). The vigor index was also maximum (4250) in the treatment that received the co-inoculation of *Rhizobium* sp. VRE1 and *C. tropicalis* VYW1 besides registering enhanced hydrolytic enzyme, α - amylase, and protein content in seeds. Metabolite profiling of bacterial primed seed exudates revealed 30 diverse compounds belonging to the classes viz., sugars, amino acids, organic acids, flavones, prenol lipids, and fatty acids. The blackgram seeds primed with co - inoculants released hydroxyl benzoic acid and galactopyranose, which act as potential chemo-attractants and involves in the lectin complement pathway regulating fructose mannose metabolism. These metabolites lead to a significant increase in germination and nodulation in blackgram seedlings. Hence, the study suggests the scope of enhancing the fitness of blackgram using a co-inoculant comprising of *C. tropicalis* VYW1 and *Rhizobium* sp. VRE1.

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INTRODUCTION

Leguminous plants are symbiotically associated with rhizobia, involved in active nitrogen fixation, and this interaction plays a vital role in the production of legume crops. Blackgram (*Vigna mungo* L.) is an important pulse crop that occupies a unique place in Indian agriculture, where it has been grown since ancient times. Blackgram, also known as urdbean, is rich in protein (26%) and contributes a large proportion of protein requirements to the Indian population. It is taken as split and also as whole grain and is crucial to supplement cereal-based diets. In India, blackgram is cultivated approximately 3.25 million ha with 1.45 million tonnes of annual production. It has been estimated that roughly 40-60 million tonnes of atmospheric nitrogen are fixed annually by cultivated legume crops, which is essential not only for agriculture but also for the ecosystem functioning (Smil, 1999). Besides, nitrogen fixation could complement the use of synthetic nitrogen fertilizers that require

a considerable quantity of energy input during production and causes enormous environmental pollution. In legume - *Rhizobium* symbiosis, the rod-shaped soil bacterium, *Rhizobium*, induces nitrogen-fixing nodules on the roots of leguminous plants.

According to Tariq et al., (2012), ineffective rhizobial strains are also successful in legume - *rhizobia* symbiosis due to two major reasons. i) *Rhizobia* are acquired by plants from the soil via an intricate signalling mechanism and are not transmitted directly from plant to offspring. ii) Plant is usually infected by multiple rhizobial strains leaving the chance for non-fixing strains inside the nodule. In order to prevent resource loss to ineffective rhizobial strains, plants adopt a strategy of *partner choice and sanctions*. To overcome limitations in legume-rhizobia symbiosis, the interaction of rhizobia with other endophytic plant growth-promoting microbes (PGPM) holds a successful option that has to be explored.

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Co-inoculation of rhizobia with other plant growth-promoting rhizobacteria (PGPR) is one of the incredible resources to enhance nutrient availability and crop fitness for sustainable organic farming. PGPR are known to promote plant growth directly by the production of plant growth regulators, nutrient uptake, defense priming directly or indirectly by the production of metabolites like antibiotics, siderophore, and antagonistic against phytopathogens. Moreover, simultaneous infection with rhizobia and rhizobiome increases nodulation and growth in a wide variety of legumes (Rajendran et al., 2012). In the past, it was believed that nodule encounters only rhizobia, and now there is an introgression that unique signal attracts other microbial communities. The co-existence of other microbes inside the root nodules were termed as non-rhizobial root nodule endophytes (NRE). Some of the NRE including *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Enterobacter*, *Pseudomonas*, *Stenotrophomonas*, *Streptomyces*, *Micromonospora*, *Microbispora*, and *Candida* sp. have been reported in different legumes (Velázquez et al., 2013; Le et al., 2016; Raja et al., 2019; Thanuja et al., 2020).

The presence of endophytic *Chryseobacterium* impacts the qualitative changes in the pattern of flavonoids exudation in the legumes (Ollero, 2010). The existence of countless interactions between NRE and *Rhizobium* sp. inside nodules benefiting each partners mediated by distinct chemical signalling contributes nodulation and nitrogen fixation (Schulz and Dickschat 2007). Furthermore, the mechanism by which NRE colonizes the root nodules or the conduit supports the nodulation process, or the plant growth promotion remains unknown. Whilst the NRE microbes were unveiled within the nodule niche, the functional role of this interaction is not yet unveiled. Nevertheless, there are several proposed hypothesis that NRE might enter infection threads along with *Rhizobium* (Zgadzaj et al., 2015), and type of soil, the exact interaction between the legumes and NRE is quite complicated. Though the plant growth-promoting activities of endophytic yeasts remain obscure, phyllosphere and rhizosphere yeasts from *Drosera spatula* with Indole acetic acid activity (IAA), 1-aminocyclopropane-1- carboxylic acid (ACC), siderophore, ammonia, and polyamine production has been reported (Aserse et al., 2013; Fu et al., 2016). This, plant growth-promoting traits of yeast strains are tolerant to saline stress (Rosenblueth and Martínez-Romero 2006). Previously, we have isolated the NRE yeast, *C. tropicalis* VRE1 from root nodules of blackgram (Thanuja et al., 2017). The NRE Yeasts may also assist *Rhizobium* and other NRE counterparts by unique signalling compounds (Thanuja et al., 2020). This interaction may pose overall beneficial effects

to legume - *Rhizobium* symbiosis compared to the presence of individual entity inside nodules.

With this perspective, the present study envisages the co-operative interaction of a nodule associated yeast strain *Candida tropicalis* VYW1 and *Rhizobium* sp. VRE1, both as a single culture and co-inoculant. The effect of this combination was elucidated during the early growth phase of blackgram in terms of germination, vigor index, nodulation efficiency, and metabolic changes.

MATERIAL AND METHODS

Microbial strains and culture conditions

C. tropicalis VYW1, and *Rhizobium* sp. VRE1 obtained from root nodules of *Vigna mungo* L., (Thanuja, et al., 2017; Thanuja et al., 2020) were grown in 100 mL of Tryptone yeast extract (TY) broth separately. The inoculated flasks were incubated at 28°C in a rotary shaker for 2-3 days until the logarithmic phase ($\sim 1 \times 10^9$ viable cells mL⁻¹) (Woomer et al., 2011). For co-inoculum preparation, a cocktail of the two strains was prepared in the ratio of 1:1, and the desired population (10⁹ cfu mL⁻¹) was ensured prior to seed treatment.

Seed biotization

The rhizobial and non-rhizobial endophytic yeast isolates (*C. tropicalis* VYW1 and *Rhizobium* sp. VRE1) were bio-assayed for their effect on seedling growth using roll towel method described by Ju et al., (2019). The seeds of *Vigna mungo* L., (var. VBN 6) were surface - sterilized by soaking in 3.5 % sodium hypochlorite solution for 5 min and then washed thoroughly with sterile distilled water. The surface-sterilized healthy seeds were soaked with 2.0 mL of the log-phase culture of *C. tropicalis* VYW1 and *Rhizobium* sp. VRE1 for 1 h. The seeds were biotized (1 h) with the prepared co-inoculum. In order to maintain the cell load, enumeration of the population on the seed surface was carried out in TY medium separately. The inoculated seeds were kept on sterilized germination paper and incubated at 30°C. Seed germination percentage and seedling vigor was recorded on 8th day of incubation. Seedling vigor was calculated on the seventh day using a formula as described below (Baki et al., 1973). Similarly, root and shoot lengths were recorded at different time intervals, such as 15, 30, and 45 days after sowing.

Seed germination

Seed germination percentage is the average number of seeds germinated over the period. In this study, germination percentage was calculated on the fifth day using the standard formulae:

Germination % - Number of seeds germinated / Number of seeds placed x 100

Seed Vigour index

Seed vigour index was examined after five days of incubation according to the formula based on the product of germination (%) and seedling length (cm).

Vigour Index = Germination (%) x Seedling Length (Cm)

α - amylase activity and protein content

The α -amylase activity of *Rhizobium* sp. VRE1 and *C. tropicalis* VYW1 treated blackgram pre-germinated seeds was determined (Muscolo *et al.*, 2014). The protein content of germinated seeds was also determined (Ali Khan *et al.*, 1972).

Metabolic profiling of seed exudates using GC-MS

Extraction of seed exudates

Exactly 50 g of healthy blackgram seeds (variety VBN 6) without any cracks and injuries were used for in vitro bio-assay studies. Surface sterilized blackgram seeds were divided into 12 portions, comprising three portions for each treatment. Each part consists of 20 seeds. Three parts of seeds were selected and soaked separately in *Rhizobium* sp. VRE1, *C. tropicalis* VYW1, and the respective co-inoculum cultures for 48 h in the dark. Cultures were prepared in saline or phosphate buffer, and the population maintained at $\sim 10^9$ cfu.mL⁻¹. Final portions soaked in sterile water served as control. After 48 h of soaking, the seeds were removed from the suspension and placed for germination in Petri plates with pre-sterilized Whatman No1 filter paper. The sprouted seeds (5DAI) transferred aseptically into sterile water were kept in a shaker for 24 h to collect the exudates. The collected exudates were extracted by using equal volume of methanol as an extractant in a separating funnel. The set-up was kept under shaking condition (100 rpm) at 30 °C overnight. The solvent layer was collected and allowed to air-dry under vacuum, and the air-dried residue was then dissolved in 1 mL of methanol. The concentrated seed exudates was

filtered through 0.22 μ m pore filter membranes and checked for eventual contamination by plating an aliquot of 50 μ l in TY medium and incubated at 28 °C for 24 h for microbial growth (Martins *et al.*, 2018). The filtered extract was then injected into the sample port of GC-MS (Akila, 2019). The solvent fraction with the seeds metabolite compounds was combined and concentrated by evaporation in the rotary flask evaporator maintained at 60 °C at 80 rpm. The concentrated crude metabolites obtained and dissolved in 1 mL HPLC grade methanol, filter sterilized and analyzed in GC/MS (Perkin Elmer®, USA). The analysis conditions included: initial oven temperature at 35 °C and increased to 140 °C at a rate of 2 °C min⁻¹; the final temperature was 280 °C that increased at the rate of 10 °C min⁻¹. The spectral bins obtained in GC/MS were analyzed, and the compounds were identified against the NIST mass spectral library (2014) based on most probable hits (Dheepa *et al.*, 2016).

Statistical analysis

All data were statistically analyzed in Microsoft Excel and add-in with XLSTAT Version 2016.04.325250 (XLSTAT, 2010). Significant differences among the treatments were statistically analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at $p < 0.05$ significance level.

RESULTS AND DISCUSSION

NREY *C. tropicalis* VYW1 influenced the germination and nodulation behavior in blackgram

Endophytes colonizing inside the plant tissues contribute to the fitness of the host, and in return, they gain nutrients and protection from the host (Rosenbleuth and Martinez Romero, 2006). In the present study, an investigation was conducted to evaluate the potential role of endophytic yeast *C. tropicalis* VYW1 in modulating the health of the blackgram both as an individual inoculant and co-inoculant with *Rhizobium* sp. VRE1.

Table 1. Effect of biotization of blackgram seeds with co-inoculant of *Rhizobium* sp. and yeast strains

Treatment	Germination %	Vigour Index	Nodules	Root length (cm)	Shoot length (cm)
Control	82	1409	7	27.2 \pm 0.01 ^d	26.2 \pm 0.06 ^d
<i>Rhizobium</i> sp. VRE1	94	2934	27	30.8 \pm 0.02 ^c	29.5 \pm 0.03 ^c
<i>C. tropicalis</i> VYW1	94	3570	9	31.2 \pm 0.04 ^b	30.2 \pm 0.05 ^b
<i>Rhizobium</i> sp. VRE1 + <i>C. tropicalis</i> VYW1	100	4250	32	34.6 \pm 0.01 ^a	32.8 \pm 0.04 ^a

T1 - Absolute control without any treatments; T2 - *Rhizobium* sp. VRE1 alone; T3 - *C. tropicalis* VYW1 alone; T4 - *Rhizobium* sp. VRE1 + *C. tropicalis* VYW1; Values are mean (\pm standard error) (n=5), and values followed by the same letter in each column are not significantly different from each other on the observation day as determined by DMRT ($p \leq 0.05$).

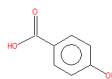


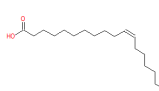
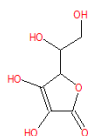
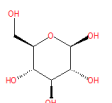
The growth parameters of blackgram such as germination percentage, root and shoot length, and the number of root nodules per plant were

significantly increased by plant growth-promoting *rhizobium* and NREY *C. tropicalis* VYW1 and their combination when compared to control. However,

the study revealed that the combined inoculation showed maximum seed germination (100%), number of nodules (32 plant⁻¹), and vigor index (4250) followed by *C. tropicalis* VYW1 alone. More vividly, the individual culture of yeast treated seedlings augmented a drastic reduction in nodule counts as compared to the co-inoculant (*Rhizobium* sp. + *C. tropicalis* VYW1). While comparing with the

individual inoculant of *Rhizobium* sp., the NREY *C. tropicalis* VYW1 performed significantly for the growth attributes viz., germination (94%), and vigor index (3570) (Table 1). As perceived earlier, the individual inoculum with (*Rhizobium* sp.) showed the least performance than co-inoculated cultures (*Rhizobium* sp. VRE1 + *C. tropicalis* VYW1) in terms of germination percentage.

Table 2. NRE endophytic yeast and *Rhizobium* treated blackgram Seed exudates metabolites

Seed exudates metabolites	Treatments				Chemical structure	Functional role	References
	T1	T2	T3	T4			
β - hydroxybenzoic acid	0.109	1.29	0.987	2.318		Chemo-attractants, influence the host range of the interaction; Present in soluble fraction of young nodules	Aguilar et al., 1988
Decane	0	0.946	1.386	2.314		Induce plant systemic resistance (ISR) against pathogens	Lee et al., 2012
Phenol	0	3.489	3.21	4.912		Signal exchange between the legumes and rhizobia during nodulation; Contribute to transcriptional activities of <i>nod</i> D dependent <i>nod</i> genes of <i>Rhizobium</i>	Mandal et al., 2010
Cis - Vaccenic acid	0	0.453	0	2.486		Plant growth regulation	Tariq et al., 2014
L(+)-Ascorbic acid	0	0.447	9.618	12.293		Functions as an antioxidant, an enzyme cofactor and as a precursor for oxalate and tartrate synthesis	Smirnov and Wheeler, 2000
α -D- Glucopyranose	0	0	0.879	2.521		Activates the lectin complement pathway	Hubbell, 1975, Thanuja et al., 2017,2020

T1 - Un inoculated control; T2 - *Rhizobium* sp. VRE1; T3 - *C. tropicalis* VYW1; T4 - *Rhizobium* sp. VRE1 + *C. tropicalis* VYW1

The significant influence of NREY *C. tropicalis* VYW1 on germination of blackgram seeds might be attributed due to its plant-growth promoting traits and secondary metabolites (Thanuja et al., 2020; Brundha et al., 2020 Unpublished). Korir et al., (2017) found that co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum* in soybean plants provided the most significant increases in shoot and root length, nodule number and total biomass, total nitrogen and grain yield (Bai et al., 2003). Similar to our findings, previous works also confirmed that the benefits bestowed by co-inoculation on plant health, especially in legumes on nodule development, are consistent (Walpola and Yoon, 2013; Morel et al., 2012; Korir et al., 2017). Further, the yeast strain, *C. tropicalis* VYW1 showed indirect PGP characters such as polyamine production and high ACC deaminase activity, siderophore, polyamines, and ammonia production (Thanuja et al., 2020). Besides, PGP yeasts exhibit antagonistic action against plant pathogens (Ferraz et al., 2019). Thus the present investigation suggests that the co-operative interaction between rhizobia and non- rhizobial

endophytic yeast (NREY) is of relevance in the enhancement of nodulation efficiency, vigor index, shoot, and root length and total biomass in blackgram.

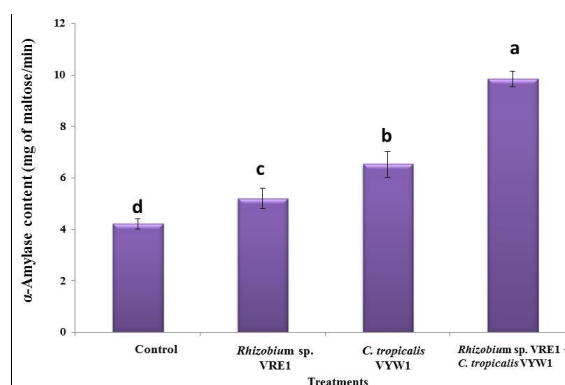


Figure 1. α - Amylase content of germinating blackgram seeds treated with *Rhizobium* sp. and NRE yeast strain. Bars with the same letter are not significantly different from each other on the observation, as determined by DMRT ($p \leq 0.05$).

NREY *C. tropicalis* VYW1 triggered hydrolytic enzymes during seed germination

Amylases are the important hydrolytic enzymes that increase sugars in germination seeds of rice (Kaur *et al.*, 2005). Starch is hydrolyzed by the amylolytic enzymes to provide substrate and energy sources for the embryo during germination, and derived monosaccharide sugars provide energy for the shoot and root development. Furthermore, while working with wheat seeds, Das and mandi (1992) observed a considerable increase in α -amylase activity during imbibition and the onset of the germination process.

Accordingly, in the present investigation, the α -amylase content (mg of maltose min^{-1}) of the blackgram seeds biotized with *C. tropicalis* VYW1 and *Rhizobium* sp. VRE1 was found to be significantly increased. However, increased α -amylase activity (9.78 mg of maltose min^{-1}) was observed in seeds treated with co-inoculum, followed by *C. tropicalis* VYW1 (6.24 mg of maltose min^{-1}), and the *Rhizobium* sp. VRE1 treated blackgram germinated seeds recorded the lowest value of (5.44 mg of maltose min^{-1}) (Figure1).

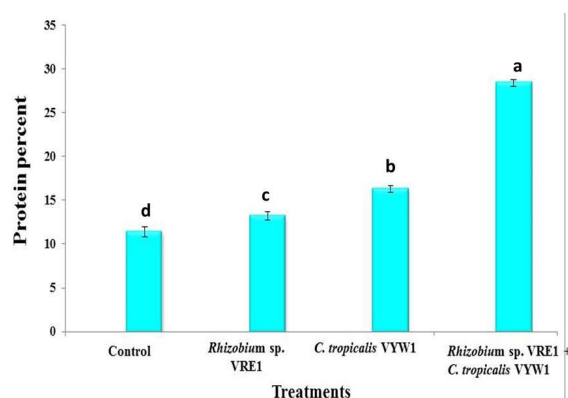


Figure 2. Protein percent of germinating blackgram seeds treated with *Rhizobium* sp. and NRE yeast strain. Bars with the same letter are not significantly different from each other on the observation, as determined by DMRT ($p \leq 0.05$).

The simultaneous increase in soluble sugars and amylase activity and decrease in starch could be due to a faster breakdown of starch. The results also suggest that *C. tropicalis* VYW1 triggered the germination process via hydrolytic enzyme activities.

During seed germination, starch hydrolysis is mediated by the enzyme α -amylase, which produces soluble oligosaccharides essential for other metabolic functions (Kaur *et al.*, 2005). Accordingly, the enhanced α -amylase activity due to bacterial priming suggests better bioconversion of starch into other oligo-sugars.

C. tropicalis VYW1 remobilizes seed reserves during seed germination

Storage proteins are mobilized to provide nutrients for seedling growth during seed germination (Wang *et al.*, 2017). The present investigation witnessed the highest average protein content (28.4%) in blackgram seeds biotized with combined inoculum (*C. tropicalis* VYW1 and *Rhizobium* sp. VRE1), followed by single inoculant of *C. tropicalis* VYW1 (16.3%) and *Rhizobium* (13.2%) (Figure2). Similar to growth attributes, here also, the interaction between *rhizobium* and non- rhizobial endophytic yeast acted synergistically and altered the metabolic flux. However, none of the research has shown non-rhizobial endophytic yeast as a component of co-inoculum to date. This research opened an insight on growth-promoting endophytic yeasts of the nodule and its prospect as a novel bio-inoculant for enhancing pulse productivity.

Metabolite profiling of co-inoculant primed seed exudates revealed chemo-attractants and antioxidants

Diverse class of metabolites (approximately 25 classes) was observed in the seeds treated with *C. tropicalis* VYW1 and *Rhizobium* sp.VRE1, as well as their respective co-inoculant (Figure 3). The seed exudates contained sugars such as glucose and xylose. However, the exudates from the untreated blackgram seeds composed of derivatives of benzoic acid, succinic acid, coumarin, ascorbic acid, and carotenoids (Table 2). Fatty acyls, carboxylic acid derivatives, benzene and substituted derivatives, phenylpropanoids, prenol lipids, and steroids are predominant in the seeds treated with co-inoculant as compared to the respective mono-culture.

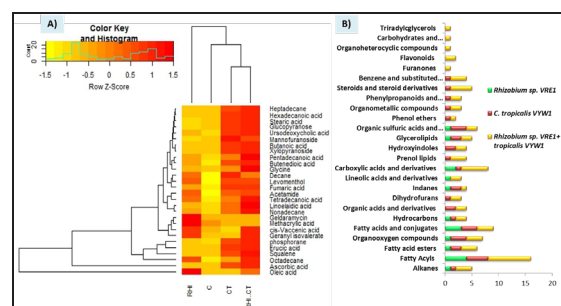


Figure 3. A) Distribution pattern of metabolites in seed exudates mediated by blackgram endophytes; B) Metabolites detected in seed exudates of biotized blackgram seeds (VBN 6) based on their differences in relative abundance; C- Control; RHI - *Rhizobium* sp. VRE 1; CT - *C. tropicalis* VYW 1; RHI+CT - *Rhizobium* sp. VRE 1 + *C. tropicalis* VYW 1.

The Z score value > 1.0 and deep intensity of red color in the heat map affirms the increase in the area percent of compounds such as ascorbic

acid, squalene, glucopyranose, mannofuranoside, hexadecanoic acids and other fatty acid derivatives in co-inoculant and *C. tropicalis* VYW1 treated seeds. The functional role of critical metabolites is elucidated in Table 2. Further, the results suggest that the exudates of NREY, as well as co-inoculant treated seeds, showed more chemo-attractants, which aids in more colonization of the inoculant on the seed surface (Aguilar *et al.*, 1988). The antioxidants would have prevented dehydration of seeds, and the sugars played a crucial role in mitigating the C requirement of the augmented bio-inoculant (Smirnov and Wheeler, 2000). The metabolite candidates serving for C assimilation provided the energy demand of the co-inoculant (*C. tropicalis* VYW1 and *Rhizobium* sp.VRE1), which might be a successful means for their survival. The signalling factors of *C. tropicalis* VYW1 that elicited the secretion of such sugar derivatives and fatty acyls need to be explored. In general, the seed exudates are comprised of a mixture of sugars and acids. Crop seeds exudates contain sugars such as glucose, fructose, and maltose, which act as a source of nutrients for the rhizosphere microflora. Along with those sugars and acids, certain other compounds such as volatiles that could alter the metabolism of plant microbiome may be present. Identification of these compounds would lead to the discovery of newer pathways by which the host plants are benefited from their endophytic microbiota (Lugtenberg *et al.*, 1999).

CONCLUSION

The present study clearly demonstrated the co-operative interaction of non- rhizobial endophytic yeast strain *C. tropicalis* VYW1 and *Rhizobium* sp. VRE1. Co-inoculation of promising non- rhizobial endophytic yeast with the symbiotic *Rhizobium* increased the germination attributes, vigour index, nodulation behavior, and metabolic flux of blackgram seedlings. Hence, the results paved a way to develop a potential yeast-based co- inoculum for sustainable pulse productivity.

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