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Enhancing Non-enzymatic Antioxidants and Yield in Summer Green Gram Through Rhizobium, Putrescine and Calcium Application

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ABSTRACT

Background: Green gram (*Vigna radiata* (L.) is a significant pulse crop in India, renowned for its protein content and nitrogen-fixing ability. This research, which explores the impact of rhizobium, putrescine and calcium chloride on green gram's biochemical properties, yield and yield attributes, provides valuable insights for the agricultural community, enlightening them on potential strategies to enhance green gram production.

Methods: The present study was conducted during the summer season for two consecutive years (2022-23). The experiment was conducted in a randomized block design with eight treatments and three replications. Treatments included individual and combined biopriming with rhizobium, foliar application of putrescine and calcium chloride.

Result: Results demonstrated that all treatments significantly enhanced the measured biochemical properties and yield parameters compared to the control. Among all the treatments, the best results were found in the treatment with the combination of rhizobium, putrescine and calcium chloride (T_7), showing statistically significant ($p<0.05$) improvements in most of the parameters at 30 and 60 DAS. The data analysis revealed the substantial increase in total flavanol content (0.541 mg g^{-1}), total flavonoid content (0.816 mg g^{-1}), PAL activity (0.078 mg g^{-1}), bound phenol content (0.230 mg g^{-1}), test weight (45.93 g), seed weight pod^{-1} (0.49 g), seed weight $plant^{-1}$ (16.99g), no. of seed pod^{-1} (9.55), no. of seed $plant^{-1}$ (34.90), biological yield (3858.77 kg/ha), economic yield (1261.50 kg/ha) and harvest index (32.70%). These findings suggest integrating microbial inoculants, growth regulators and essential nutrients can effectively enhance green gram yield and nutritional quality in summer cultivation. This study provides valuable insights for developing sustainable agricultural practices to maximize green gram production.

Key words: Calcium chloride, Flavanol, Green gram, Phenol, Putrescine, Rhizobium.

INTRODUCTION

The mung bean, scientifically known as (*Vigna radiata* (L), is a leguminous crop that has been extensively growing throughout Asia for decades. This small green legume is a fundamental element in diverse culinary traditions and a substantial resource in agricultural systems owing to its nutritional advantages and adaptability (Pareek *et al.,* 2024). Mung beans are abundant in protein, dietary fiber, vitamins and minerals, making them a valuable diet constituent in numerous impoverished nations. Mung beans are widely recognized for their excellent nutritional composition. They provide high-quality plant-based protein, essential for individuals following vegetarian and vegan diets (Barkha *et al.,* 2020). In addition, they supply crucial amino acids, vitamins, including folate and minerals such as iron, magnesium and potassium. Mung bean's abundant fiber content promotes digestive health and regulates blood sugar levels, rendering them advantageous for individuals with diabetes (Ali *et al.,* 2021). The health-promoting effects of mung beans are attributed to their bioactive components, including antioxidants, flavonoids and phenolic acids. These chemicals have various health advantages, such as reducing inflammation, fighting against microorganisms and preventing cancer. Studies have demonstrated that mung beans can lower cholesterol levels and enhance cardiovascular well-being.

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Biopriming, a seed treatment method that involves the application of helpful microorganisms before sowing, has proven to be a successful approach for improving crop production. Rhizobium, an important bacterium that engages in symbiotic nitrogen fixation, establishes nodules on the roots of legumes and provides the host plant with nitrogen in a readily usable form (Arya *et al.,* 2024). Rhizobium-based bio-priming helps improve legume production by promoting nitrogen fixation, enhancing nutrient uptake and mitigating abiotic and biotic stresses (Joharika *et al.,* 2023). This innovative approach aligns with the principles of eco-friendly agriculture, offering a viable alternative to conventional chemical seed treatments while reducing environmental impacts. Despite its potential benefits, the mechanisms underlying rhizobium-mediated bio-priming and its practical implications across different crops and ecological conditions (Yadav *et al.,* 2024).

Applying calcium chloride to the leaves of mung bean plants (*Vigna radiata*) is an essential agricultural technique that aims to improve plant health and increase crop yield. Calcium, a necessary macronutrient, is crucial in multiple physiological processes, such as cell wall formation, regulation of enzyme activity and transmission of signals (Islam *et al.,* 2024). Although calcium is crucial, its movement inside plants is restricted, frequently resulting in shortages that can negatively impact growth and development. Calcium also acts as a cementing agent in the form of calcium pectate (Shani *et al.,* 2024). Mung bean, a leguminous crop highly esteemed for its elevated protein content and capacity to fix nitrogen exhibits heightened susceptibility to calcium chloride availability during crucial developmental phases, including flowering and pod development. Foliar application, which involves directly spraying nutrient solutions onto plant leaves, provides a focused method to alleviate calcium deficiency (Srivastava *et al.,* 2024).

The foliar application of putrescine is a new and potentially useful method for improving mung bean (*Vigna radiata*) plant growth, stress tolerance and yield. A naturally occurring organic substance known as putrescine, a form of polyamine, is involved in numerous biochemical and physiological activities within plants (Shahidi and Hossain, 2023). In addition to regulating stress responses, it is essential for cell division, differentiation and maintaining membrane integrity (Hussein *et al.,* 2023). The leguminous crop mung bean's nutritional value and nitrogen-fixing capabilities are highly regarded. However, the crop is susceptible to environmental stresses like drought, salt and extreme heat, all of which reduce its yield (Prajapati *et al.,* 2024). Plants can rapidly use putrescine to mitigate the effects of stress because foliar application involves spraying nutrients directly onto the leaves, facilitating its efficient uptake (Virk *et al.,* 2024). According to studies, applying putrescine to mung bean plants improves their growth indices, enzymatic activity and yield outcomes (El-Beltagi *et al.,* 2023). Researchers and farmers may contribute to agricultural sustainability and food security by developing more robust mung bean production practices. One way to do this is by researching the advantages and mechanisms of foliar-applied putrescine (Mitra and Kumar, 2024).

MATERIALS AND METHODS

An investigation was conducted to estimate the biochemical, yield-attributing characters and yield of green gram influenced by rhizobium, Putrescine and Calcium chloride at the research farm of Lovely Professional University in the summer seasons of 2022 and 2023.

The green gram variety (SML 668) seeds were obtained from Punjab Agriculture University, Ludhiana. The experiment was conducted twice while pooled for statistical analysis using a randomised block design (RBD) with 8 treatments. T_0= control, T_1= calcium chloride (10 mM), T_2= bio priming with rhizobium (10 ml), T_3= putrescine (3 mM), T_4= calcium chloride (10 mM) + bio priming with rhizobium (10 ml), T_5= calcium chloride (10 mM) + putrescine (3 mM), T_6= bio priming with rhizobium (10 ml) + putrescine (3 mM), T_7= calcium chloride(10 mM) + bio priming with rhizobium (10 ml) + putrescine (3 mM) and 3 replications. Biopriming with rhizobium is done during sowing, whereas calcium chloride and putrescine are applied as foliar.

Total phenylalanine ammonia-lyase (PAL) activity was analyzed as per (Kalghatgi and Subba, 1975) wherein 1g leaf sample was homogenized in 2 ml sodium borate and centrifuged at 7000 g for 10 minutes. Place 1 ml of a solution containing 0.05M Tris HCl, 0.01M L-phenylalanine and 0.4 ml of water in an incubator at 30°C for 5 minutes. Inject 0.1 ml of enzyme and incubate it for 60 minutes at 30°C. Stop the reaction with 0.5 ml of 1N hydrochloric acid. Eliminate the phage, combine with 3 ml of 0.05N NaOH, retain 1 ml of supernatant with 1 ml of Folin's reagent and measure OD at 650 nm.

Total flavanol content was estimated as per (Akkol *et al.,* 2008) wherein 0.05 g of dried plant sample was taken and boiled with 5 ml 80% methanol. Mix 1 ml of methanol extract with 3 ml sodium acetate and 1 ml of aluminum chloride. Note the absorbance at 445 nm after 2.5 h.

Total flavonoid content was analyzed as per (Akkol *et al.,* 2008) wherein 1g of dehydrated plant sample was homogenized with 10 ml of ethanol. Agitate the mixture for 30 minutes and centrifuge at 3000 rpm for 3 minutes. Separate the supernatant and transfer 1 ml of plant extract, 1 ml of quercetin, 0.1 ml of 5% $AlCl_3$ and 0.1 ml of potassium acetate solution with 2.8 ml of distilled water and incubate for 30 minutes. Observe the wavelength at 415 nm with the spectrophotometer.

Bound phenols were analyzed as per (Chattopadhyay and Samaddar, 1980) wherein a 100 mg plant sample was ground with 5 ml of 3% sodium lauryl sulphate solution and centrifuged at 2000 g for 5 min. Separate the remaining substance and cleanse it with 5 ml of SDS solution, 5 ml of water, 5 ml of ethanol and 10 ml of diethyl ether. Evaporate the remaining substance and dissolve it in 3 ml of a 0.5 M NaOH solution. Combine 1 ml of supernatant with 1 ml of the Folins ciocalteau reagent (FCR). Heat the mixture for 1 minute and record the absorbance at 650 nm.

The assessment of crop productivity and the factors contributing to it, such as the test weight, seed weight per pod, seed weight per plant, number of seeds per pod and number of pods per plant, were measured at the maturity stage by considering the tagged plants. Biological yield and economic yield were assessed at the maturity stage by evaluating the tagged plants in each plot. The HI% was calculated using the formula provided by (Donald and

Hamblin, 1976), with a sample size of 1000 seeds used to measure the test weight.

$$\text{Harvest Index (\%)} = \frac{\text{Economical yield (kg/ha)}}{\text{Biological yield (ka/ha)}} \times 100$$

The software used for analysis is SPSS and OPSTAT at a significance of variance (p=0.05%).

RESULTS AND DISCUSSION

Total flavanol, flavanoid, bound phenol and PAL activity

The results showed significant differences in total flavanol, total flavonoid content, bound phenol and PAL activity across different treatments. The substantial increase was found in the treatment T_7 (calcium chloride (10 mM)+bio priming (rhizobium) (10 ml)+putrescine (3 mM) with total flavanol content 0.509 mg g^{-1} (11.81%) and 0.541 mg g^{-1} (6.07%), total flavonoid content 0.742 mg g^{-1} (10.10%) and 0.816 mg g^{-1} (6.95%), PAL activity 0.073 mg g^{-1} (4.72%) and 0.078 mg g^{-1} (5.02%), bound phenol content 0.224 mg g^{-1} (10.79%) and 0.230 mg g^{-1} (5.02%) at 30 and 60DAS as compared to control shown in (Table 1 and 2 and Fig 1 and 2). Applying rhizobium, putrescine and calcium chloride together produces the highest levels of bound phenols, PAL activity, flavanol and flavonoids, which is evidence of the synergistic effect of these treatments on one another. The activities of flavonoids include acting as chemical messengers, regulating physiological processes and inhibiting the cell cycle. In the symbiotic relationship between Rhizobia and legumes like peas, beans, clover and soy, flavonoids secreted by the host plant's roots play an essential role during the infection stage. Rhizobia that live in the soil can discern these flavonoids, which in turn trigger the release of Nod factors. After that, the host plant can recognize these Nod factors, which lead to the deformation of the root hair and various cellular responses, such as the formation of root nodules and ion fluxes. The findings of this study have significant implications for agricultural practices, as they suggest that the combination of Rhizobium, putrescine and calcium chloride can

Table 1: Total flavanol content and total flavonoid content.

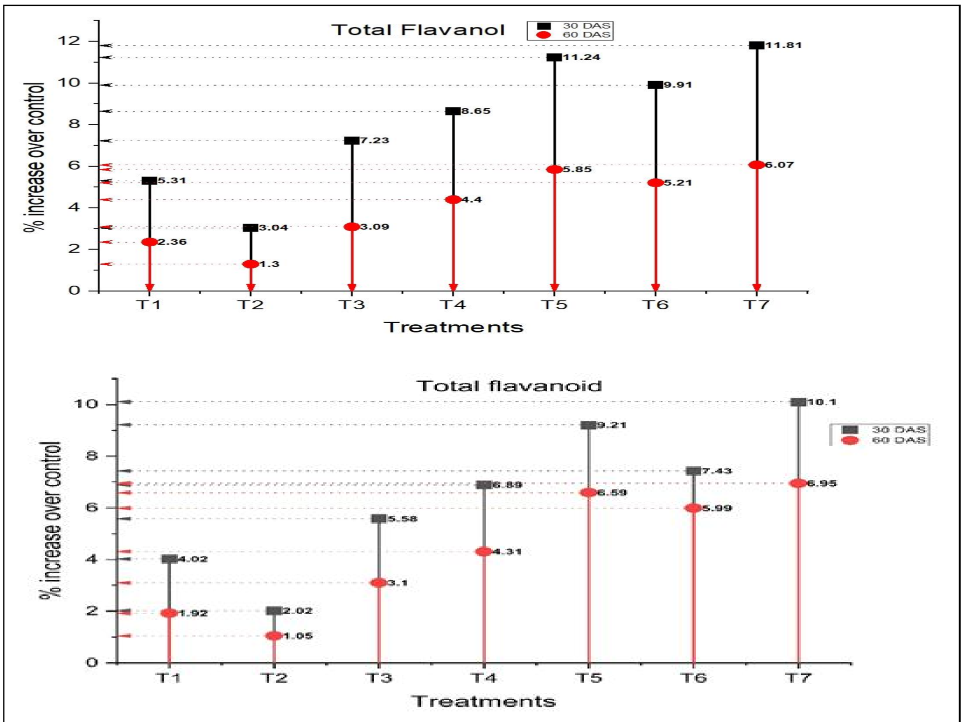
Treatments	Total flavanol content (mg g^{-1})		Total flavanoid content (mg g^{-1})	
	30 DAS	60 DAS	30 DAS	60 DAS
T_0	0.449±0.006	0.508±0.004	0.667±0.006	0.759±0.004
T_1	0.474±0.009	0.520±0.004	0.695±0.003	0.774±0.008
T_2	0.463±0.007	0.515±0.002	0.681±0.008	0.767±0002
T_3	0.484±0.004	0.524±0.002	0.706±0.005	0.783±0.005
T_4	0.493±0.002	0.531±0.000	0.716±0.006	0.793±0.006
T_5	0.506±0.004	0.540±0.004	0.735±0.006	0.813±0.006
T_6	0.498±0.004	0.536±0.004	0.721±0.007	0.80±0.006
T_7	0.509±0.005	0.541±0.005	0.742±0.003	0.816±0.003
C.D at (p<0.05%)	0.010	0.006	0.010	0.009

Note: T_0= Control, T_1= Calcium chloride, T_2= Bio priming with rhizobium, T_3= Putrescine, T_4= Calcium chloride+bio priming with rhizobium, T_5= Calcium chloride+putrescine, T_6= Bio priming with rhizobium+putrescine T_7= Calcium chloride+bio priming with rhizobium+putrescine. Pooled data from 2022 and 2023 are used to conclude the results.

Table 2: Total phenylalanine-lyase content and bound phenol.

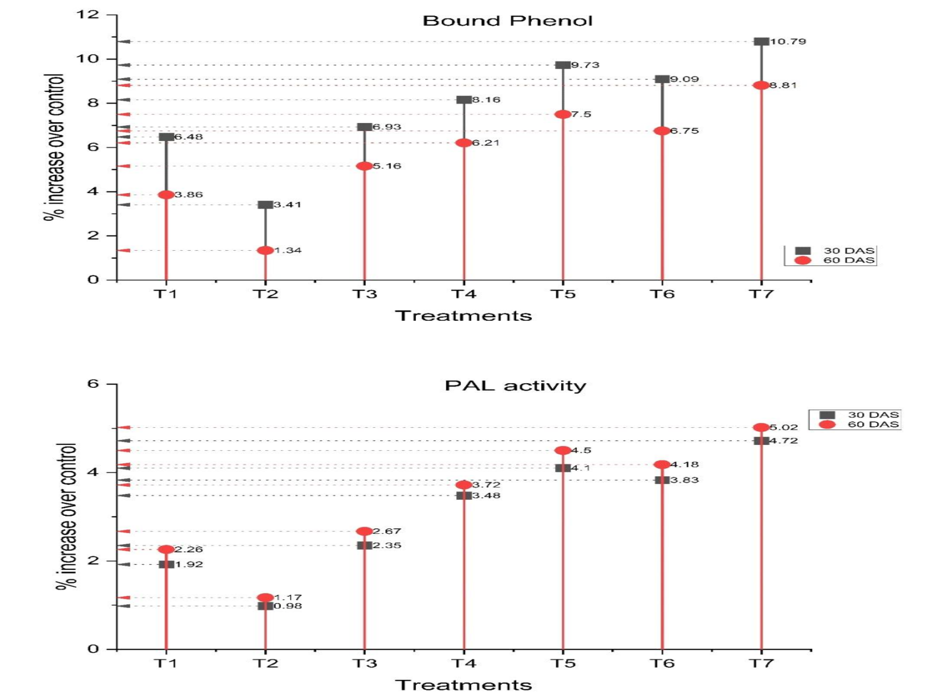
Treatments	Total phenylalanine ammonia-lyase (PAL) activity (mg g^{-1})		Bound phenol (mg g^{-1})	
	30 DAS	60 DAS	30 DAS	60 DAS
T_0	0.070±0.004	0.074±0.002	0.200±0.000	0.211±0.003
T_1	0.071±0.003	0.076±0.003	0.214±0.002	0.219±0.003
T_2	0.071±0.005	0.075±0.001	0.217±0.002	0.214±0.001
T_3	0.072±0.003	0.076±0.005	0.215±0.001	0.222±0.000
T_4	0.073±0.003	0.077±0.002	0.218±0.002	0.225±0.003
T_5	0.073±0.005	0.077±0.004	0.222±0.002	0.228±0.002
T_6	0.073±0.004	0.077±0.004	0.220±0.001	0.226±0.004
T_7	0.073±0.004	0.078±0.002	0.224±0.000	0230±0.001
C.D at (p<0.05%)	0.001	0.001	0.003	0.005

Note: T_0= Control, T_1= Calcium chloride, T_2= Bio priming with rhizobium, T_3= Putrescine, T_4= Calcium chloride+bio priming with rhizobium, T_5= Calcium chloride+putrescine, T_6= Bio priming with rhizobium+putrescine T_7= Calcium chloride+bio priming with rhizobium+putrescine. Pooled data from 2022 and 2023 are used to conclude the results.



Note: T_1= calcium chloride, T_2= bio priming with rhizobium, T_3= putrescine, T_4= calcium chloride + bio priming with rhizobium, T_5= calcium chloride + putrescine, T_6= bio priming with rhizobium + putrescine T_7= calcium chloride + bio priming with rhizobium + putrescine.

Fig 1: Treatments impact on percent increase of flavanol and flavonoid over the control.



Note: T_1= calcium chloride, T_2= bio priming with rhizobium, T_3= putrescine, T_4= calcium chloride + bio priming with rhizobium, T_5= calcium chloride + putrescine, T_6= bio priming with rhizobium + putrescine T_7= calcium chloride + bio priming with rhizobium + putrescine.

Fig 2: Treatments impact on percent increase of bound phenol and PAL over the control.

potentially improve plant growth and the production of compounds that are beneficial to the plant ecosystem. The utilization of this knowledge can result in the development of crop cultivation strategies that are more efficient and environmentally friendly, which in turn leads to increased crop yields and improved crop quality (Hussein *et al.,* 2023). The increased levels of putrescine in crops allow them to resist disease, drought and other environmental stressors during their growth and development. Additionally, they improve the plants' ability to absorb nutrients from the soil, leading to increased yields and improved crop quality. Plant cells use flavonoids and phenols as non-enzymatic antioxidants to eliminate reactive oxygen species formed due to stress and improve plant tolerance (Talaat and Shawky, 2016). One example is the observation made by Mittler (2002) that the levels of flavonoids in marigold plants increased when they were subjected to water stress. Similarly, treatments with putrescine increased the total phenol content of cotton plants and Thymus vulgaris plants (Shallan *et al.,* 2012). Additionally, the application of polyamines increased the total phenolic compounds and flavonoids in maize plants compared to the control group (Feiz *et al.,* 2019). Flavonoids and phenols are essential to improve a plant's resistance to various stresses. These compounds, which function as antioxidants that are not enzymatic, contribute to eliminating reactive oxygen species produced under stressful conditions. In this way, flavonoids and phenols shield plant cells from oxidative stress's damaging effects, enhancing plants' overall health and resilience. Because of their ability to regulate gene expression and activate defense mechanisms, these compounds also contribute to the adaptation and survival of plants in environments that are difficult to thrive in.

Yield and yield attributes

The experimental results showed that the applied treatments positively affected the yield and its contributing factors in mung bean plants. Data presented in (Table 3 and 4 and Fig 3 and 4) indicate that these factors and overall yield were significantly improved during the summer season ($p<0.05$). Treatment T_7 (which included calcium chloride, bio-priming with rhizobium and putrescine) gave the best results among the different treatments. This treatment resulted in a test weight of 45.93 g, seed weight

Table 3: Test weight, seed weight per pod, seed weight per plant, no. of seeds per pod and number of pods per plant.

Treatments	Test weight (g)	Seed weight pod^{-1} (g)	Seed weight plant^{-1} (g)	No. of seeds pod^{-1}	No. of pods plant^{-1}
T_0	32.72±1.29	0.34±0.01	9.69±0.36	7.55±0.48	28.60±0.22
T_1	35.88±1.83	0.37±0.01	11.40±0.29	8.45±0.18	30.83±0.45
T_2	35.01±1.33	0.36±0.01	10.64±0.29	7.88±0.68	29.43±0.40
T_3	38.10±1.93	0.42±0.01	13.35±0.33	9.03±0.86	32.15±0.35
T_4	39.88±1.73	0.44±0.01	14.64±0.22	9.08±0.68	33.13±0.29
T_5	44.70±1.26	0.47±0.01	16.03±0.13	9.45±0.33	34.33±0.55
T_6	42.33±1.30	0.46±0.01	15.65±0.61	9.37±0.76	33.88±0.40
T_7	45.93±1.76	0.49±0.01	16.99±0.64	9.55±0.22	34.90±0.48
C.D at ($p<0.05\%$)	2.917	0.012	0.600	0.963	0.753

Note: T_0= Control, T_1= Calcium chloride, T_2= Bio priming with rhizobium, T_3= Putrescine, T_4= Calcium chloride+bio priming with rhizobium, T_5= Calcium chloride+putrescine, T_6= Bio priming with rhizobium+putrescine T_7= Calcium chloride+bio priming with rhizobium+putrescine. Pooled data from 2022 and 2023 are used to conclude the results.

Table 4: Biological yield, economic yield and harvest index of crop.

Treatments	Biological yield (kg ha^{-1})	Economical yield (kg ha^{-1})	Harvest index (%)
T_0	3163.57±3.04	894.92±2.49	28.29±0.10
T_1	3385.10±3.27	975.48±2.74	28.82±0.11
T_2	3282.77±3.98	929.02±0.28	28.30±0.04
T_3	3485.62±1.04	995.87±5.07	28.57±0.15
T_4	3585.15±4.94	1036.03±1.11	28.90±0.07
T_5	3787.22±3.91	1196.13±3.07	31.58±0.05
T_6	3688.67±0.93	1055.07±1.28	28.60±0.04
T_7	3858.77±2.62	1261.50±5.07	32.70±0.12
C.D at ($p<0.05\%$)	4.842	5.695	0.167

Note: T_0= Control, T_1= Calcium chloride, T_2= Bio priming with rhizobium, T_3= Putrescine, T_4= Calcium chloride+bio priming with rhizobium, T_5= Calcium chloride+putrescine, T_6= Bio priming with rhizobium+putrescine T_7= Calcium chloride+bio priming with rhizobium+putrescine. Pooled data from 2022 and 2023 are used to conclude the results.

per pod of 0.49 g, seed weight per plant of 16.99 g, 9.55 seeds per pod, 34.90 pods per plant, a biological yield of 3858.77 kg ha^{-1}, an economic yield of 1261.50 kg ha^{-1} and a harvest index of 32.70%. The percent increase/ decrease and correlation are shown in Fig 3 and 4. Compared to the other treatments, treatment T_7 showed increases of 28.77% in test weight, 30.1% in seed weight per pod, 43.0% in seed weight per plant, 20.9% in the number of seeds per pod, 18.1% in the number of pods per plant, 18.02% in biological yield, 29.06% in economic yield and 13.47% in harvest index, as detailed in Table 3. The application of biological nitrogen fixation through Rhizobium (Arya *et al.,* 2024), increased cell development and stress tolerance through putrescine (Gill and Tuteja, 2010) and improved structural and physiological functions through calcium chloride (Kazemi, 2013) resulted in a significant improvement in plant growth and productivity. This was accomplished through the combination of these three methods. Putrescine has the potential to bring about revolutionary changes in agriculture and crop production due to its ability to facilitate cell development and stress tolerance. Putrescine has the potential to result in the development of plant varieties that are more resistant to adverse environmental conditions, such as drought and high temperatures and that are also more productive. In the face of the challenges posed by climate change, this enhancement can potentially improve crop yield and quality, thereby ensuring food security and sustainability. Jadhav *et al.* (2019) research shows that putrescine significantly increases pods, test weight and seeds produced. Putrescine can increase the stress

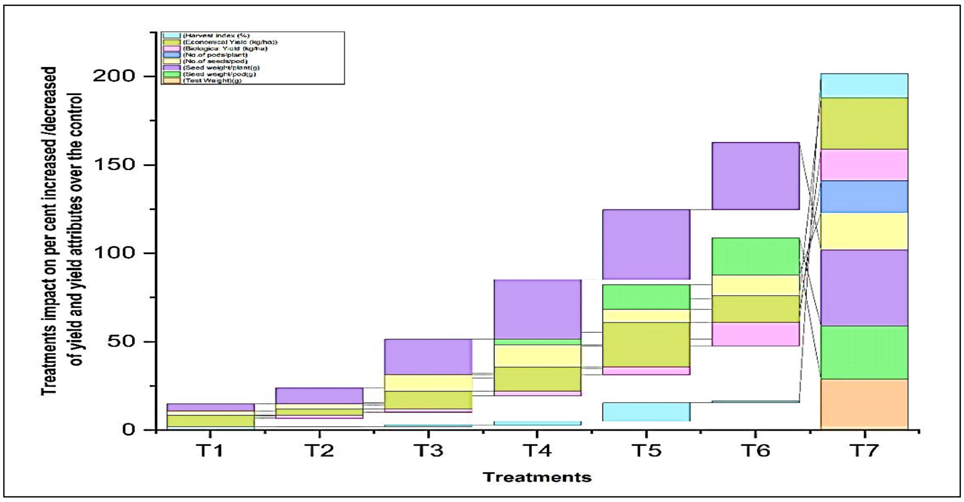


Fig 3: Treatments impact on percent increased /decreased of yield and yield attributes over the control.

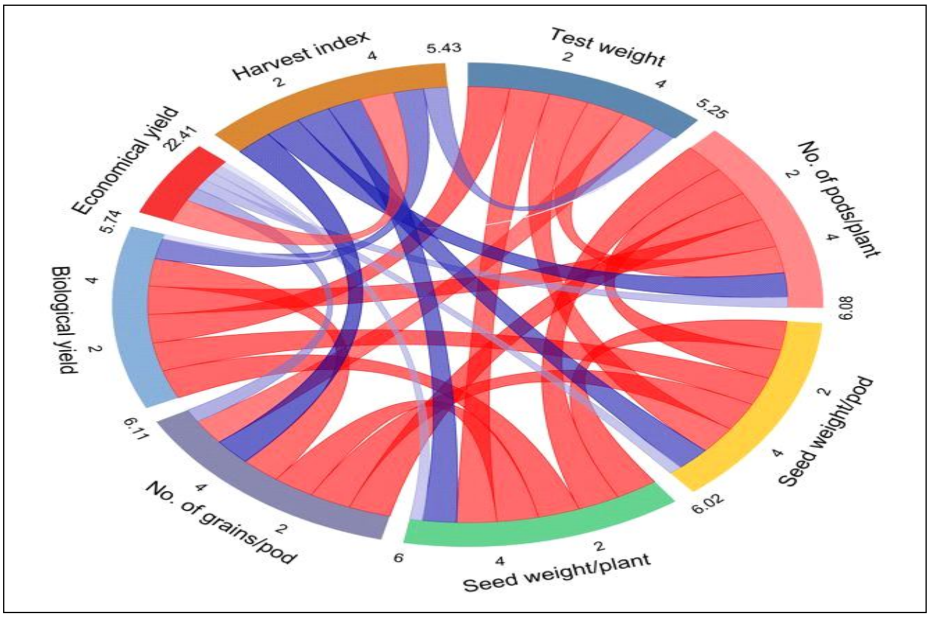


Fig 4: Correlation graph of yield attributes and yield of mung bean.

tolerance of plants through several different mechanisms, one of which is the regulation of stress-responsive gene expression. To improve a plant's ability to deal with adverse environmental conditions, putrescine activates particular genes involved in stress signaling pathways. Furthermore, putrescine assists in the preservation of cellular homeostasis by performing the function of a free radical scavenger, thereby lowering oxidative stress and serving to prevent cellular damage. According to Kumar *et al.* (2024), this treatment method has demonstrated the potential to maximize the yield of mung beans. This is evidenced by the fact that all tested parameters, such as test weight, seed weight per pod, seed weight per plant, number of seeds per pod, number of pods per plant and overall seed yield, exhibited statistically significant increases. The putrescine compound can act as a free radical scavenger and this potential is not limited to mung beans; it can also be applied to other plant species. Putrescine can improve plant overall health and longevity by lowering oxidative stress and preventing cellular damage. This, in turn, leads to increased growth, productivity and resilience in various agricultural settings. This opens up promising possibilities for developing new strategies to mitigate the adverse effects of environmental stressors on crops, which will ultimately sustainably promote food production. Putrescine has a wide range of applications in various other plant species. Using putrescine to develop resilient varieties of a wide range of crops, such as wheat, rice, fruits and vegetables, is possible by capitalizing on its capacity to improve adaptation to stress and regulate gene expression. Maintaining stable and productive agricultural systems in fluctuating climatic conditions can contribute to global food security. Putrescine, for example, has been successfully used to increase drought tolerance in wheat plants, leading to increased yields in regions prone to drought.

CONCLUSION

This study conclusively demonstrates that applying Rhizobium, putrescine and calcium chloride significantly enhances the biochemical properties, yield and yield attributes of green gram (*Vigna radiata* L.) grown during the summer season. The individual treatments contribute uniquely to improving plant growth and productivity. Rhizobium application enhanced nitrogen fixation and vegetative growth, putrescine application improved stress tolerance and secondary metabolite synthesis and calcium chloride supplementation strengthened structural integrity and metabolic regulation. The best results were observed in the combined application of Rhizobium, putrescine and calcium chloride, indicating a synergistic interaction among these treatments. This integrated approach resulted in increased bound phenol content, PAL activity, flavanol and flavonoid content, as well as significant enhancements in test weight, seed weight per pod, seed weight per plant, number of seeds per pod, number of pod per plant, biological and economical yield. These findings highlight the possibility of using multiple treatment approaches to improve the biochemical and agronomic characteristics of green gram in the summer season. Combining rhizobium, growth regulators and nutrients creates an adequate basis for sustainable agriculture techniques that maximize green gram production through synergistic effects.

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Conflict of interest

All the authors declare that there is no conflict of interest regarding the publication of this article.

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