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Nodule Associated Plant Probiotics on Seed Quality of Greengram (*Vigna radiata* L.) under Ambient Storage Condition

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ABSTRACT

Background: India is the leading producer and importer of pulses in the world. Post-harvest storage loss ranging from 5-10% which is very high in India. This situation demands the development of storage guidelines for pulses to prevent the storage losses without deterioration. The liquid-based formulations of nodule associated plant probiotics are known for increasing the plant growth and yield related parameters in field conditions. The present study was undertaken to assess the effect of seed treatment of liquid based formulation of NAPP to maintain seed viability, vigour during storage period and also to assess the shelf life of the formulation.

Methods: Population of nodule associated plant probiotics spore count was assessed in stored seeds at bimonthly intervals, SEM analysis was taken to confirm the presence of probiotics initially and effect of NAPP on seed quality during storage period was assessed.

Result: The results revealed that (T_3) seeds coated with microbial consortia (NAPP) of *Rhizobium* sp VRE1 + *Candida tropicalis* VYW1+ *Paenibacillus taichungensis* TNEB6+ AMF have recorded maximum germination (71%), germination energy (7.2) and seedling vigour index (1690). Also, coated seeds recorded highest protein content, antioxidant enzymes and lowest level of EC, free amino acids compared to control seeds after 12 months of storage under ambient condition. Microbial population was maintained at 3.2 log cfu/g of coated seeds when compared to 1.0 log cfu/g of primed seeds after one year of storage.

Key words: NAPP, Germination, SEM analysis, Protein, Antioxidant enzymes.

INTRODUCTION

Pulses are grown in temperate and tropical regions which is quite popular amongst the resource-challenged farmers due to its low input requirement and quick growing habit (Patil and Tiwari, 2021). It is an expensive source of high-quality dietary proteins in South Asia; hence popularly known as poor man meat (Hou *et al.,* 2019). Because of its nitrogen fixation ability in the root zone, pulse crops enrich soil health and restore soil fertility upon cultivation. The protein composition in pulses helps to overcome deficiency of essential amino acids in cereals and millets (Kumbhare *et al.,* 2014). Green gram (*Vigna radiata* L.) belongs to the family Leguminosae, is a seasonal crop cultivated during the *Kharif*/summer season and consumed throughout the year (Yewle *et al.,* 2020).

Seed is a vital input in agricultural production. The use of quality seeds ensures better germination and vigour, which can alone increase the crop yield upto 20-25 per cent. Seeds need to be stored from the day of harvest till the time of next sowing. They are the genetic diversity's bearer and act as vector for spread and regeneration of agricultural crops, proper storage of them is essential in order to ensure the food supply. However, issues with pest pathogen and environmental variation have plagued the quality of stored seeds. As per Food and Agricultural Organisation of United Nation about 17% of worlds food production is destroyed under storage due to insect infestation (10%) and rodents mites and diseases infection (7%) (Bouchelos, 2018). Furthermore, inappropriate and inefficient storage methods lead to a substantial quantitative loss of 7.5% during the storage (Nath *et al.,* 2015). To overcome the obstacle, seed treatment is the most effective technology. Biological seed treatments absorb more interest as a substitute over chemical seed treatment as the latter imposes numerous negative impacts on non-target animals including human beings, besides affecting the seed quality. The application of bioinoculants through biopriming and coating, where seed hydration and seed inoculation with plant beneficial microorganisms takes place to improve seed germination, enhancing seedling vigour, vegetative growth and protection against soil borne pathogens (Meena *et al.,* 2017). Very

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few studies have reported that microbial consortia during the inoculation process enhance plant productivity compared to using a single microbe (Kumar *et al*., 2016; Sarkar *et al*., 2021).

Earlier studies reported that non-rhizobial endophytes (NRE) are capable of producing biologically active metabolites and phytohormones such as indole-3 acetic acids, gibberellins, cytokinins and ACC deaminase (Rakholiya *et al.,* 2017). Furthermore, nodule endophytes provide significant benefits to legume crops by obtaining nutrients through nitrogen fixation, phosphate, Zn and K solubilization (Dudeja *et al.,* 2012). Hence the study was undertaken to investigate the effect of seed treatment with liquid-based formulations of nodule associated plant probiotics to maintain the greengram seed quality during storage.

MATERIALS AND METHODS

The storage experiment was carried out to study the effect of microbial consortia on seed quality of green gram (*Vigna radiata* L.) at Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore, India during the year 2023-2024. The methods employed are enumerated hereunder.

Bioinoculant source and seed treatments

The greengram cultivar VBN4 seeds were procured from National Pulses Research Centre, Vamban, Tamil Nadu, India. The seeds were cleaned free from foreign matter before storage and used for testing the efficacy of Nodule Associated Plant Probiotic (NAPP) *in vitro*. Microbial consortia used for the study *viz., Rhizobium* sp VRE1 + *C. tropicalis*VYW1+ *P. staichungensis* TNEB6+ AMF (Thanuja *et al.,* 2020; Raja *et al.,* 2019) were obtained from the Department of Microbiology, Tamil Nadu Agricultural University, Coimbatore, India. The germination percentage of greengram seed was 75±2% and microbial consortia (Nodule Associated Plant Probiotics) were utilized for this study containing 10^9 CFU mL^{-1}. Seeds were subjected to priming and coating with a liquid-based formulation of NAPPs. The following seed treatments were tested: Control dry seeds (T_0), Hydro priming (T_1), 6 ml priming (T_2), 4 ml coating (T_3), 6 ml priming + 4ml coating (T_4). Priming was done for 3 hours with 1:0.35 (w/v) seed to solution ratio and shade dried for 24 hrs to maintain the original moisture content. The presence of microbes over the primed and coated seeds was accomplished with Scanning electron microscopy (SEM). The SEM examination was carried out in bioprimed and coated seeds. After that, treated seeds were stored under ambient conditions.

Effect of microbial consortia on seed germination and seedling growth

The stored greengram samples were tested for germination (%), germination energy, shoot length (cm), root length (cm), vigour index and dry matter production as per the ISTA (2019) protocol.

$$\text{Germination percentage (GP)} = \frac{\sum N}{T}$$

Where:

N= Number of germinated seeds.

T= Total number of seeds.

Germination energy =

[N1/D1] + [N2-N1/D2] + [N3-N2/D3] + [N4-N3/D4] + [N5-N4/D5] + [N6-N5/D6] + [N7-N6/D7] + [N8-N7/D8]

Here:

N1, N2, … N8= Number of emerged seedlings.

D1, D2, … D8= Number of days after sowing.

Determination of biochemical parameters

Stored seeds of greengram were estimated for protein content (Ali-Khan and Youngs, 1973) and antioxidant enzymes such as catalase (Aebi, 1984) peroxidase (Malik and Singh, 1980) and superoxide dismutase (Giannopolitis and Ries, 1977).

Colony forming units of bioprimed and coated seeds under storage condition

Stored seeds of 1 g were randomly selected and soaked in 9 ml of deionized water followed by shaker at 150 rpm for one hour. Then, 1 ml of the diluted suspension was poured on NA media plates and incubated at 26± 2°C for 48 h. The shelf-life efficacy was assessed based on number of colonies appearing from each treatment.

Statistical analysis

The whole experiment was designated in triplicates and mean±SE (Standard errors) were determined. The mean values were evaluated by using DMRT analysis (Duncan multiple range test) at P≤0.05. Microsoft Excel (2019) and GraphPad Prism version 5.8 were used to generate graphs.

RESULTS AND DISCUSSION

Seed deterioration is an inevitable process that occurs due to natural ageing with time or due to improper storage that manipulates the cytological, physiological and biochemical traits of seeds, leads to loss of vigour and viability ultimately lowering the seed quality (Jyoti and Malik, 2013). Appropriate seed treatment, storage containers and conditions can maintain the seed quality and viability from deterioration during storage period (Islam *et al.,* 2013). In many gaps, seed coating with bioinoculants reduced insect infestation and maintained the seed quality. The bioinoculants and sticking agents used for coating are act as a physical barrier against insect egg laying that maintains the seed quality (Sene *et al.,* 2021).

Findings from the current experiment suggest that NAPP had a significant influence on green gram seed germination during the storage period. Among the various seed treatment with NAPP, highest germination per cent (86%) was recorded in T_4 followed by T_2 (82%) and T_3 (85%) over untreated control seeds (76%) irrespective of storage period (Table 1). Nonetheless, the seed germination per cent started declining considerably during storage period which might be potentially due to age induced seed deterioration. Although T_2 and T_4 recorded greater germination per cent T_3 maintained the seed germination per cent (71%) by the end of 12 months of storage period over other treatments (Table 1). According to Sharon *et al.* (2015), seed germination is the prime factor that is influenced by storage conditions. During storage, seed loses vigour and viability due membrane integrity damage caused by free radicals generation which eventually results in the loss of biochemical potential of the seed, namely antioxidant scavenging enzyme activity. Loss of enzyme functions might have attributed to the deterioration of lipids, carbohydrate and protein which results in lower seed germination (McDonald, 1999). According to Rakholiya *et al.* (2017), NAPP are capable of producing biologically active metabolites and phytohormones such as indole-3 acetic acids, gibberellins, cytokinins and ACC deaminase which help the seeds to overcome the deleterious effect of ROS, maintains the seed germination and seedling vigour during storage. Moreover, NAPP also aids in seed germination by synthesising IAA that promotes the upregulation of favourable endogenous phytohormones synthesis (GA and cytokinin) and downregulation of unfavourable phytohormones (ABA- ABI3 transcription factor) (Zhao *et al.,* 2020). The findings of present study clearly demonstrates that seed coating with 4 ml NAPP maintains the VBN4 seed quality upto 12 months under ambient storage condition by achieving the minimum germination per cent. Similar findings were reported by Kumutha *et al.* (2023) where seed coating with bioinoculants of (*Rhizobium* and *Arbuscular Mycorrhizal Fungus*) maintains the viability of blackgram and greengram for prolonged conditions. Seed coating with plant beneficial microorganisms

Table 1: Microbial consortia on seed germination and germination energy of greengram VBN4 under ambient condition.

Seed Treatments	Germination (%)							Germination energy						
	Storage duration (months)							Storage duration (months)						
	0	2	4	6	8	10	12	0	2	4	6	8	10	12
T_0	76^c	74^b	72^b	70^c	69^b	66^b	60^b	8.2^c	7.8^b	7.5^c	7.0^b	6.8^b	6.5^b	6.2^b
T_1	80b^c	77^b	75^b	73^{bc}	70^b	63^b	55^c	9.9^b	9.0^a	8.0^b	7.0^b	6.4^{bc}	5.5^c	4.9^c
T_2	85^a	82^a	80 ^{a}	75^{ab}	70^b	62^b	57^{bc}	10.9^a	9.2^a	8.2^b	7.1^b	6.2^c	5.5^c	5.0^c
T_3	83^{ab}	82^a	81^a	78^a	75^a	73^a	71^a	9.6^b	9.4^a	9.0^a	8.6^a	8.4^a	8.0^a	7.6^a
T_4	86^a	83^a	80 ^{a}	74^{abc}	71^b	64^b	55^c	11.4^a	9.1^a	8.0^b	6.9^b	6.5^{bc}	5.7^c	4.8^c

*The table indicated with the same superscript alphabets in the same columns were not significantly different ($p \leq 0.05$).

Table 2: Microbial consortia on dry matter production and vigour index of greengram VBN4 during storage.

Seed treatments	Dry matter production (g 10 seedlings)							Vigour index						
	Storage duration (months)							Storage duration (months)						
	0	2	4	6	8	10	12	0	2	4	6	8	10	12
T_0	0.231^c	0.227^c	0.209^c	0.188^{bc}	0.176^b	0.162^b	0.151^b	2212^e	2109^b	1922^c	1813^{bc}	1697^b	1544^b	1242^b
T_1	0.250^b	0.240^b	0.210^{bc}	0.185^c	0.169^c	0.146^c	0.140^c	2464^d	2225^b	1958^c	1752^c	1589^c	1210^c	979^d
T_2	0.286^a	0.271^a	0.213^{bc}	0.190^b	0.172^{bc}	0.150^c	0.144^{bc}	2967^b	2509^a	2152^b	1823^b	1582^c	1221^c	1066^c
T_3	0.249^b	0.240^b	0.226^a	0.212^a	0.193^a	0.177^a	0.169^a	2714^c	2624^a	2365^a	2215^a	2085^a	1891^a	1690^a
T_4	0.281^a	0.275^a	0.220^{ab}	0.190^b	0.170^{bc}	0.147^c	0.142^c	3070^a	2573^a	2152^b	1857^b	1633^{bc}	1267^c	1007^{cd}

*The table indicated with the same superscript alphabets in the same columns were not significantly different ($p \leq 0.05$)

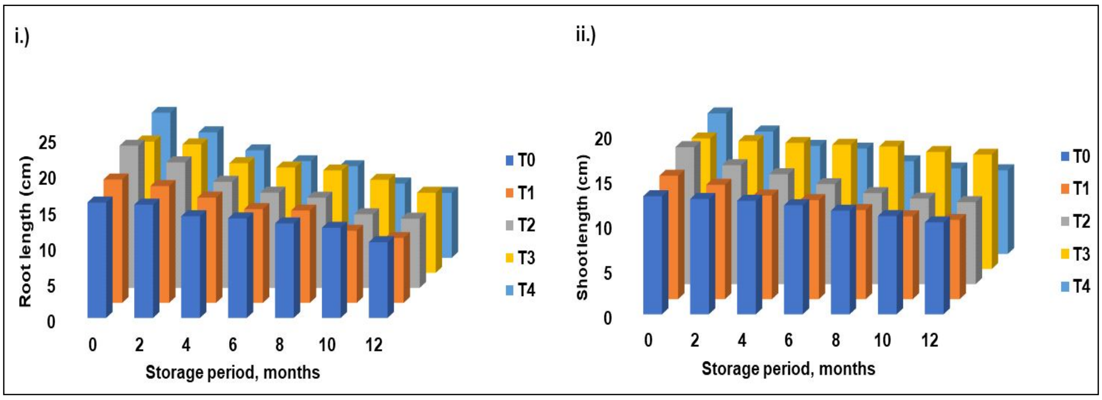

Fig 1: Microbial consortia on (i) root length (cm) and (ii) shoot length (cm) of greengram VBN4 under ambient condition.

enable the precise application of inoculum at the seed-soil interface and ensuring the ready accessibility of microbes during germination and early stages of plant development, promoting rapid establishment and subsequently maximizing crop production (Colla *et al.,* 2015). Likewise, initially maximum germination energy was recorded with T_4 (11.4) followed by T_2 (10.9), thereafter begins to decline upon storage period (Table 1). However maximum germination energy was observed in T_3 after 12 months of natural ageing. Root length, shoot length and dry matter of all the treatment and untreated seeds showed a decreasing trend with advancement of storage period and highest decline of these parameters observed at twelveth month of storage (Fig 1) (Table 2). The observations from the experiment reveals that highest root length (11.1 cm), shoot length (12.7 cm) and dry matter (0.169 g seedlings^{-1}) after 12 months of storage period was recorded with (T_3) seed coating with 4 ml NAPP. The enhanced physiological attributes of seed might be due to increased availability of nutrient to the developing seedling aided by the microbial consortia that further increases the seed constituents like carbohydrate, proteins further conversion of these macromolecules to micromolecules with the release of certain enzymes (Yadav and Khurana, 2005). A decrease in root length, shoot length and seedling dry weight during natural ageing might attributed by reduction in mobilization of reserves of stored seeds during germination (Dhakal and Pandey, 2001). The results are in line with the earlier findings of Kumar and Verma (2008) and Nagarajan *et al*. (2004). IAA produced by plant probiotics promotes seedling growth by regulating the sucrose metabolism pathway, in addition to its mediated role in the synthesis of endogenous phytohormones (Zhao *et al*., 2020). Vigor index declined significantly irrespective of treatment upon natural ageing. However, highest vigour index was recorded at the end of twelve month of storage in T_3 (1690) 4 ml NAPP coated seeds. The results are in consistent with the findings of Peerzada *et al*. (2021) in fenugreek seeds.

Successful delivery of a product under field condition determines the success of new formulation. Considering this different seed treatment with NAPP were imposed on VBN 4 greengram seed. It was observed that, the colonies forming units (cfu) gradually decreased with the advancement of the storage period irrespective of seed treatment. The maximum colonies were obtained during initial period of primed seeds (11.6 log CFU g^{-1}) compared to coated seed (11.4 log CFU g^{-1}) and SEM analysis reveals the presence of microbes over the seed coat and cotyledon region of bioprimed seeds (Fig 2). After 12 months of storage period, coated seeds recorded the maximum number of colonies (4.2 log CFU g^{-1}) compared to primed seeds (2.0 log CFU g^{-1}) (Table 3). Raja *et al.* (2019) reported similar findings that sorghum seed coated with microbial consortium maintained the germination and vigour of seeds after three months of storage but the microbial population decreased from 15 $\times$ 10^5 cfu g^{-1} of seed to 10 $\times$ 10^5 cfu g^{-1} of seed. The viability of the microorganisms in the seed might

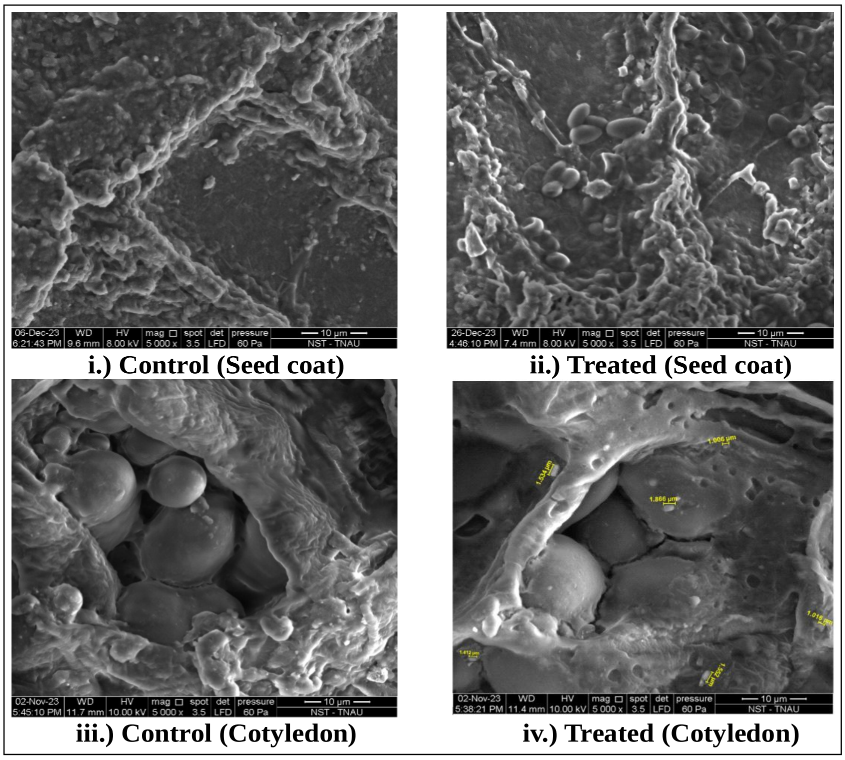

Fig 2: Scanning electron microscopy (SEM) analysis of Nodule associated plant probiotics of stored seeds.

have been supported by available seed moisture which leads to maintenance of germination and seedling vigour during storage. Throughout 12 months of the storage period, significant physiological quality loss was observed in control compared to coated seeds. Similar observations were recorded by Preethi (2022) in blackgram seeds with decreased microbial population, seed quality parameters were also reduced after 3-month storage period. These results are in accordance with those of Kumar *et al.* (2023) and Peerzada *et al*. (2021) in cowpea and fenugreek, respectively.

Seed treatment with bioinoculants exhibited a significant effect on the protein and antioxidant enzymes of greengram seeds after 12 months of storage. Coated seeds recorded maximum protein content (17.9%), catalase (0.945 µg H_2O_2 reduced g^{-1} min^{-1}), peroxidase (0.175 units mg $protein^{-1}$ min^{-1}) and superoxide dismutase (0.40 units mg $protein^{-1}$ min^{-1}) compared to control (Fig 3). Oxygen is an essential element for seeds but it may become toxic at higher concentrations. Oxygen molecule in its ground state is relatively inactive but it becomes ROS upon partial reduction. ROS consists of free radicals, an atom or molecule having an unpaired electron that is extremely reactive, starting chain reactions and producing more free radicals, that are capable of attacking healthy cells, causing them to lose their structure and function (Halliwell and Gutteridge, 2007). In general, CAT, POD and SOD are protective mechanisms of seed viability by scavenging free radicals and peroxide, which facilitate the oxide reduction cycle in the living system. The inoculated bacteria produced several antioxidant enzymes including catalase and superoxide dismutase in relation to ROS production in seeds which was in line with the findings of Preethi (2022) and Monisha (2023) in black gram.

In summary, nodule associated plant probiotics maintained seed germination and vigour during storage through improved production of metabolites and phytohormones such as indole-3 acetic acids (IAA), gibberellins (GA), cytokinins and ACC deaminase and maintained membrane integrity of seeds through quenching of free radicals by increased antioxidant activity of coated seeds.

Table 3: Influence of storage period on microbial population (Colony Forming Units/g of seeds) of greengram VBN4 under ambient condition.

Seed treatments	Colonies forming unit (log cfu/g)						
	Storage duration (months)						
	0	2	4	6	8	10	12
T_0 - Priming (6 ml NAPP)	10.6^b	9.0^b	8.2^b	7.0^c	5.1^b	3.6^b	2.0^c
T_1 - Coating (4 ml NAPP)	11.4^a	10.2^a	9.1^a	8.0^b	6.6^a	5.2^a	4.2^a
T_2 - Priming (6 ml NAPP) + coating (4 ml NAPP)	11.6^a	10.6^a	9.3^a	8.6^a	6.2^a	5.0^a	3.9^b

*The table indicated with the same superscript alphabets in the same columns were not significantly different ($p \leq 0.05$).

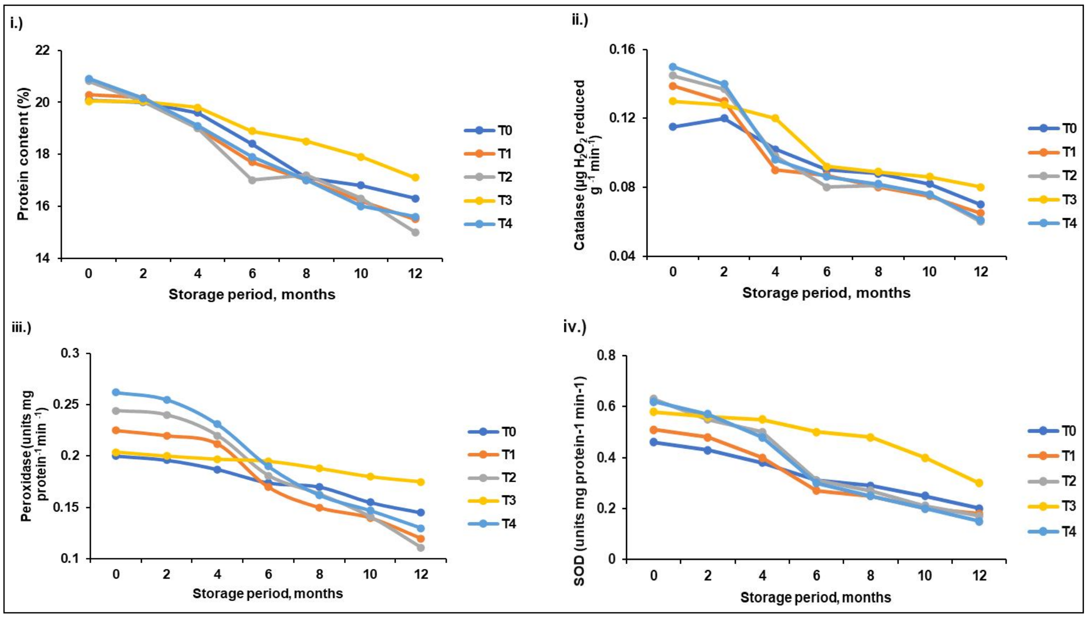

Fig 3: Microbial consortia on (i) Protein content (%), (ii) Catalase (µg H_2O_2 reduced g^{-1} min^{-1}), (iii) Peroxidase (units mg $protein^{-1}$ min^{-1}) and (iv) Superoxide dismutase (units mg $protein^{-1}$ min^{-1}) of greengram VBN4 under ambient condition.

CONCLUSION

The current study on the effect of liquid based microbial consortia on storability of greengram seeds elucidate that 4 ml Nodule Associated Plant Probiotics (NAPP) coated seed performed better as compared to primed and control seeds in terms of seed germination and seedling vigour, electrical conductivity, free amino acids, protein content (%) and antioxidants enzymes. This study concludes that microbial consortia could enhance the longevity of green gram seeds by increasing GA and IAA levels and protecting membrane integrity through increased antioxidant enzymes. Development of such microbial consortia serves as a forward milestone in bio inoculants seed treatment technology. Hence the result suggest the promising role of NAPP as an applied strategies to maintain the seed quality also as an alternative to chemical-based treatments to improve seed storability for the sustainable environment.

Conflict of interest

The authors declare that they have no competing interests.

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