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Berseem-Rhizobium symbiosis boosted growth and yield in the presence of rhizobacteria

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Abstract

Legume-Rhizobium symbiotic relationships produced is beneficial and provides a natural mode of N_2-fixation via the mini nitrogen factories i.e., nodules. The species of *Rhizobium* has an extraordinary ability to fix nitrogen to plants species and resultantly less mineral nitrogen is required for crop growth. Filed study was designed on berseem to assess the symbiotic and rhizobacteria on berseem growth and yield under varied levels of nitrogen. The screening of isolates of symbionts and non-symbionts (04 of each) has been carried out for different biochemical functions. The bacterial isolates showed promising results for IAA equivalents, solubilization index, siderophore unit (quantitively & qualitatively) and other tests were done in field experiment at varied nitrogen levels i.e., 15 & 30 kg N ha^{-1}. Results revealed that bacterial inoculation either applied separately or in combined form demonstrated significant results at each N level in comparison to control. Results of yield contributing components suggested that co-inoculation produced higher berseem fodder & dry matter yield i.e., 76.7, 16.3 t ha^{-1} at 30 kg N ha^{-1} in comparison to 76.2, 15.7 t ha^{-1} at 15 kg N ha^{-1}, respectively. Co-inoculation also improved the nodule no. and nodular mass i.e., 28.5 and 0.254 g plant^{-1} as compared to the remaining inoculation levels at higher level of N. Co-inoculation (Br_4 & Pg_4) produced the maximum seed yield i.e., 368.7 kg ha^{-1} than control i.e., 331.7 kg ha^{-1}. Higher N-P content was observed in seed and plant matter in different cuttings of berseem due to co-inoculation. The synergism between *Rhizobium* sp and rhizobacteria could be used after thorough screening and more valued approach as compared to separate application of microbes. Studies should be carried out to various legumes to accomplish beneficial effects of rhizobacteria along with symbionts to improve yield contributing factors and other quality indicators.

Keywords: Berseem, Co-inoculation, N Levels, Rhizobacteria, *Rhizobium* sp

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Introduction

Berseem (*Trifolium alexandrinum* L.) is rabi fodder and fulfils most of its nitrogen requirements by symbiotic nitrogen fixation (SNF). The green fodders like berseem, lucerne etc provides fine quality carbohydrate and protein for rearing the animals especially the livestock's and improve/restore fertility status of soil by symbiotic nitrogen fixation (Jan et al., 2014; Muir et al., 2014; Bondaruk et al., 2020). Introduction of leguminous plants in cropping pattern/schemes can enhance the soil fertility, provides fine quality proteinaceous forages to livestock and also restore the fertility status of soils for the successive crops (Cosentino et al., 2014; Goyal et al., 2021). Berseem, the Egyptian clover have the tremendous nitrogen fixing capacity by symbiotic relationships i.e., ranged between 115-400 kg N ha^{-1} year^{-1} keeping in view the prevailing conditions like adverse climate, carbon level in soil, crop cultivar and symbionts virulence (Tufail et al., 2018; Vocciante et al., 2022). Berseem is a multi-cut crop that ranged from 3-6 cutting depending upon the variety and environemntal conditions (Oushy, 2008). The symbiotic relationships between berseem and microsymbiont i.e., *Rhizobium leguminosarum* bv. *trifolii* makes the berseem crop fertilizer independent and only starter dose of N is required for initiation of plant vegetative parts and up to the onset of nodulation (Cosentino et al., 2014; Wei et al., 2018). This symbiosis causes N_2-fixation that benefits and raises succeeding crop yields (Agarwal & Ahmad, 2010; Naveed et al., 2015; Vocciante et al., 2022).

The nitrogen fixing capacity of symbionts i.e., *Rhizobium* sp is largely relied on many factors i.e., soil conditions, root colonizing capacity of symbionts, and establishment of chemical signals that necessary to formulate nodules (Qureshi et al., 2013; Jan et al., 2014; Egamberdieva et al., 2019). The phenomenon of symbiotic nitrogen fixation by legume-rhizobia relationships is cost effective, eco-friendly and vital for intensive cropping system. The symbiotic relationships between legume and its microsymbiont are largely depends on host plant chemical signals like flavonoids/iso-flavonoids and stimulated the LCOs (lipo-chito-oligosaccharide) signals termed as Nod factors by specific *Rhizobium* sp (Liu & Murray, 2016; Gouda et al., 2017; Backer et al., 2018; Wheatley et al., 2020). The production of specific Nod factors stimulated series of signals from host plant and caused the infection thread formation. The specific *Rhizobium* sp after matching the chemical signals approached the inner cortical cells of roots via infection thread to formulate nodules (Oldroyd et al., 2011; Liu et al., 2016; Carciochi et al., 2019; Egamberdieva et al., 2019; Liu et al., 2022). The nodules are the seats to fix atmospheric nitrogen by using the nitrogenase enzyme and provided the natural supply of nitrogen to plants (Berger et al., 2013; Egamberdieva et al., 2017; Wheatley et al., 2020).

The plant growth promoting rhizobacteria (PGPR) have potential of different functions viz. solubilization/mineralization of nutrients, syntheses of phyto-hormones/ siderophores/antibiotics, releasing of metabolites (primary/secondary), and inducing systemic resistance against biotic/abiotic factors etc (Appelbaum, 2018; Backer et al., 2018; Egamberdieva et al., 2019). The bacterial secretions or volatiles improved the tolerance mechanisms and ultimately increased the plant growth (Kumar et al., 2020; Liu et al., 2022). The production of polyamines considered vital for plant physiology/protection. The secretion of polyamine i.e., spermidine improved the biomass and root system architecture and higher photosynthetic rates (Naveed et al., 2015). The rhizobacteria imposed resistance to drought and abscisic acid (ABA) formation in artificially imposed drought (Massalha et al., 2017; Egamberdieva et al., 2019). Role of PGPR in combating plant pathogens by producing HCN in the rhizosphere is proved by researchers (Tufail et al., 2018). The PGPR produced volatile organic compounds (VOC) that stimulated plant biomass and induce stress tolerance (Bailly & Weisskopf, 2012; Ruzzi & Aroca, 2015; dos Santos et al., 2019). The chemical signal molecules either flavonoids/iso-flavonoids or LCOs for plant-to-microbes and microbes-to-plants appeared to be increased with introduction of PGPR in root-zones especially under sub-optimal conditions **(**Carotenuto et al., 2017; Zipfel & Oldroyd, 2017; Backer et al., 2018; Tufail et al., 2018; Soares et al., 2020).

Co-inoculation of *Rhizobium* sp and rhizobacteria to legumes had influenced positively on crop growth and ultimately yield than their alone application (Qureshi et al., 2012; Ruzzi & Aroca, 2015; Qureshi et al., 2022). The interaction of *Rhizobium* with PGPR exerted positively on the legume ontogeny (Tufail et al., 2018; Ju et al., 2019). Co-inoculation produced more efficient results by forming more nodules and improved chemical signals and finally more nitrogen fixation (Lin et al., 2019; Matse et al., 2020; Bergmann et al., 2021). The PGPR has its own impact on the legume plants by different mechanisms (direct or indirect) and resultantly improved the growth and yield (Dazzo & Yannim, 2006; Korir et al., 2017; Jabborova et al., 2021). Co-inoculation of legume plants has gained attention of researchers in coming days. The PGPR by producing hormones developed better root system by bearing more roots/lateral roots enhanced the root density and provided more bonding sites for *Rhizobium* sp that enhanced nitrogen fixation capacity (Pacheco-Villalobos *et al.*, 2016; Przygocka-Cyna & Grzebisz, 2018; Harman et al., 2021). The field trial was designed to assess the *Rhizobium*-berseem symbiosis with rhizobacteria at varied levels of N on berseem yield attributes.

Materials and Methods

Collection of microbial cultures

The pre-isolated isolates of *Rhizobium* sp and rhizobacteria (PGPR) (04 of each) were collected from Soil Bacteriology Section, Faisalabad. The pure cultures of isolates preserved at 5 ± 1 ºC in eppendorf tubes having 0.2 mL glycerol were screened for different biochemical characterization.

Biochemical characterization of isolates

The biochemical characterization for auxin biosynthesis as IAA equivalents, solubilization index (SI), siderophore unit% (SU), Chrome azurole S (CAS) assay, Exopolysaccharide (EPS) production and congo red tests of both type of isolates was carried out. Isolates of *Rhizobium* sp of berseem and PGPR (04 of each) labelled as (Br_1, Br_2, Br_3 & Br_4) and (Pg_1, Pg_2, Pg_3 & Pg_4) were screened for auxin biosynthesis (IAA equivalents) with and without L-tryptophan (L-TRP). The IAA equivalents was determined by Sarwar et al. (1992). Isolates having higher IAA values were multiplied in broth for field experimentation (Table 1).

The siderophores unit using Chrome azurole S (CAS) was evaluated qualitatively. The CAS assay was conducted by preparing King's medium and CAS in 1:15 ratio. The spot inoculation on King's medium was carried out and incubated for 28±2ºC and halos formation revealed the formation of siderophores (Schwyn & Neilands, 1987; Kotasthane et al., 2017).

The phosphate solubilization index was determined on NBRIP (Nautiyal, 1999). After incubation at 28±2 ºC for 48-72 hours, the growth and solubilization diameter revealed the P-solubilization index (SI) (Nguyen *et al.*,1992; Vazquez *et al.*, 2000). Isolates were also illustrated for exopolysaccharide (EPS), bromothymol blue (BTB) and congo-red tests by the standard methods (Krieg and Holt, 1984).

Inoculum preparation

The isolates were selected on the basis of biochemical screening/characterization. The broths were prepared and after inoculation, the bottles containing broths were incubated to obtain the optimum growth i.e., 0.5 optical density at 535 nm. The berseem seeds (cv. Super-late Faisalabad) were inoculated with the respective culture according to the treatment plan and control was kept without inoculation.

Field experiment

Field trial was conducted at Fodder sub-station, Ayub Agri. Research Institute, Faisalabad in $5x3m^2$ plot. The N and P fertilizer as urea and SSP were applied according to the treatment plan in randomized-complete-block-design (RCBD). The pre-sowing soil samples were analysed for different parameters. The soil was sandy clay loam with pH: 8.10; EC_e: 1.42 dS m^{-1}; soil N: 0.032%; organic matter: 0.60%; and available P: 6.72 mg kg^{-1}. There were two N levels i.e., 15 and 30 kg N ha^{-1} and uniform P level i.e., 60 kg P ha^{-1}. There were two N levels 15 kg N ha^{-1} (F_1), 30 kg N ha^{-1} (F_2) and four inoculation levels viz. Un-inoculated control, *Rhizobium* sp inoculation, PGPR inoculation and co-inoculation. The study has eight treatments including i.e., 15 kg N ha^{-1} (T_1), 30 kg N ha^{-1} (T_2), *Rhizobium* inoculation + F_1 (T_3), *Rhizobium* inoculation + F_2 (T_4), PGPR inoculation + F_1(T_5), PGPR inoculation + F_2 (T_6), co-inoculation +F_1 (T_7), co-inoculation +F_2 (T_8). The nodulation was checked at flowering stage and two plants from each treatment was removed and nodules per plant and nodular mass was assessed. Data regarding each cutting of berseem fodder and their dry matter was recorded. After three cuttings, the crop was left for the seed yield and seed yield was recorded. Data regarding plant/seed N & P content, soil status for N and Olsen P at harvest were recorded using standard procedures (Bremner & Mulvany, 1982; Olsen & Sommers, 1982). The statistical analysis was performed using ANOVA and significance was estimated using least significance difference (LSD) (Steel *et al.*, 1997; Duncan, 1955).

Results and Discussion

Lab screening of isolates

The *Rhizobium* sp isolates of berseem and rhizobacteria (4 of each) tagged as (Br_1, Br_2, Br_3 & Br_4) and (Pg_1, Pg_2, Pg_3 & Pg_4) were characterized/screened. The biochemical testing of isolates qualitatively & quantitatively was carried out i.e., IAA content (with/without L-TRP), solubilization index (SI), siderophore unit (SU), CAS assay, EPS production and congo-red were performed. Results presented in Table 1 clearly depicted that isolate of each type produced IAA equivalents with/without L-TRP. The *Rhizobium* sp and rhizobacteria isolates produced IAA content i.e., 2.25-3.20 and 2.30-3.35 µg mL^{-1} and bacterial cultures augmented with L-TRP, higher IAA values were obtained i.e., 3.55-4.15 and 3.25-4.25 µg mL^{-1}, respectively. Higher values of *Rhizobium* sp (Br_4) of IAA content, SI, SU, CAS assay and EPS production were obtained with i.e., 4.15 µg mL^{-1}, 2.80, 36.0%, respectively while values for rhizobacterial isolates i.e., 4.25 µg mL^{-1}, 2.85, 35.5%, respectively. The biochemical testing/evaluation of other isolates also exhibited encouraging results (Table 1).

Table 1 Some featured traits of isolates during biochemical screening

Isolates	IAA equivalents (µg mL^{-1}) L-TRP [-]	IAA equivalents (µg mL^{-1}) L-TRP [+]	Congo red test	Solubilization index (SI)	Siderophore unit (%)	*CAS-assay	EPS production
Br_1	2.25	3.55	+	3.25	30.5	+	++
Br_2	2.30	3.85	+	2.35	34.0	++	++
Br_3	3.10	3.55	+	2.55	34.5	+++	++
Br_4	3.20	4.15	+	2.80	36.0	+++	+
Pg_1	2.30	3.25	-	2.30	28.0	+	++
Pg_2	2.45	3.35	-	2.50	34.5	+++	++
Pg_3	2.50	3.65	-	2.65	35.0	++	++
Pg_4	3.35	4.25	-	2.85	35.5	+++	++

*L-TRP [-]: without L-tryptophan; L-TRP [+]: with L-tryptophan; CAS: Chrome Azurole S

Measurement of yield parameters & nodulation

Results regarding fodder and dry matter yield (sum of 03 cuttings) of berseem are presented in Table 2 clearly demonstrated that berseem fodder yield was significantly increased at both N levels and maximum value was observed in co-inoculation in comparison to untreated control. Maximum berseem fodder yield was produced by co-inoculation (Br_4 & Pg_4) i.e., 76.7 t ha^{-1} at 30 kg N ha^{-1} as compared to 76.2 t ha^{-1} at 15 kg N ha^{-1}, respectively. Co-inoculation improved the fodder yield at both N levels followed by *Rhizobium* inoculation and least values were obtained with control i.e., 68.0 and 71.1 t ha^{-1}. Results regarding nodulation presented in Table 2 revealed that maximum number of nodules and mass was observed and effect was pronounced with co-inoculation as compared to alone bacterial inoculation at both N levels. Co-inoculation also exhibited the maximum nodules and mass i.e., 28.5, 0.254 (g $plant^{-1}$) followed by *Rhizobium* inoculation i.e., 23, 0.247 as compared to control i.e., 17, 0.203 at higher level of N, respectively. There was 7.88% and 20.74% percent increase in fodder and dry matter yield due to co-inoculation at higher level of N. The berseem fodder and dry matter yield of individual cutting are presented in Fig. 1&2 clearly demonstrated that co-inoculation proved to be the best as compared to the separate bacterial inoculation and control. In other way, the berseem fodder and dry matter yield translated the similar trend of individual cuttings.

Table 2 Inoculation effect of the yield components of berseem (Average of 3 repeats)

Treatments	Fresh fodder yield (t ha^{-1})		Dry matter yield (t ha^{-1})		Nodule no. $plant^{-1}$		Nodular mass (g $plant^{-1}$)	
	15 kg (N ha^{-1})	30 kg (N ha^{-1})	15 kg (N ha^{-1})	30 kg (N ha^{-1})	15 kg (N ha^{-1})	30 kg (N ha^{-1})	15 kg (N ha^{-1})	30 kg (N ha^{-1})
Control	68.0^{b}	71.1^{ab}	12.7^{e}	13.5^{de}	13.0^{f}	17.0^{e}	0.197^{f}	0.203^{e}
Rhizobial inoculation	75.1^{a}	76.2^{a}	14.6^{bcd}	15.0^{bc}	23.0^{cd}	25.5^{b}	0.236^{c}	0.247^{b}
PGPR inoculation	72.9^{ab}	74.4^{a}	14.0^{cd}	14.6^{bcd}	18.5^{e}	22.0^{d}	0.227^{d}	0.233^{c}
Co-inoculation	76.2^{a}	76.7^{a}	15.7^{ab}	16.3^{a}	24.5^{bc}	28.5^{a}	0.249^{b}	0.254^{a}
LSD	5.8434		1.2172		1.7307		0.0031	

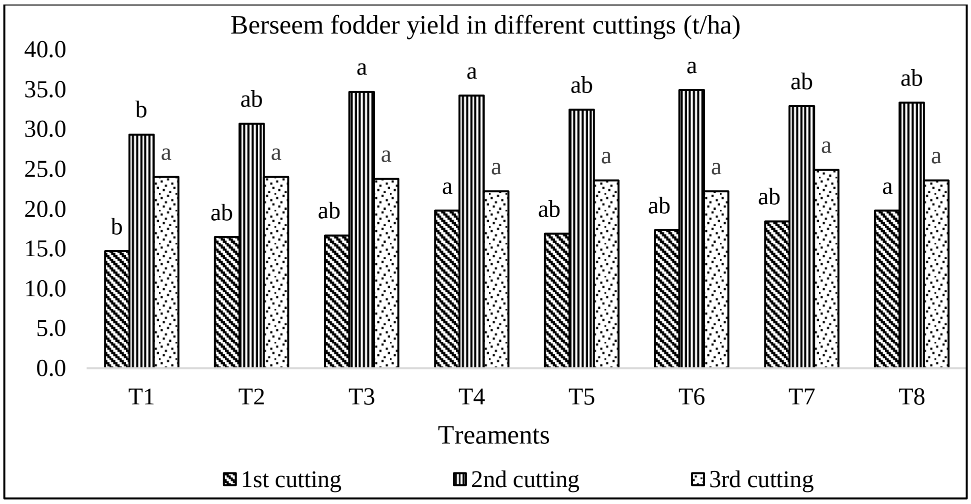

Fig. 1 Co-inoculation effect on berseem fodder cutting yield

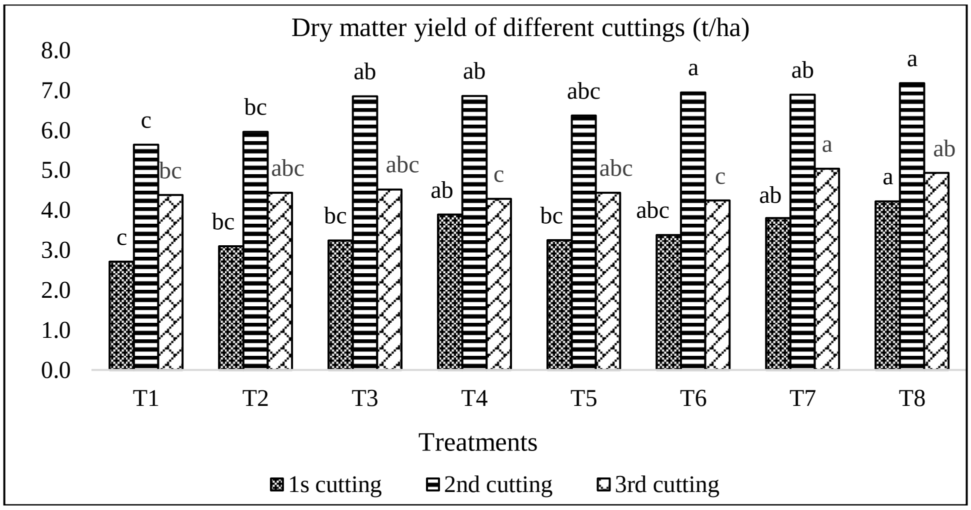

Fig. 2 Co-inoculation effect on berseem dry matter cutting yield

Results regarding berseem seed yield are presented in Fig. 3. revealed that berseem yield was significantly influenced at both N levels by bacterial inoculation and highest effect was observed by co-inoculation in comparison to un-inoculated control. Co-inoculation (Br_4 & Pg_4) also produced the maximum berseem seed yield i.e., 368.7 in comparison to control i.e., 331.7 kg ha^{-1}. High level of N i.e., 30 kg N ha^{-1} affected the seed yield more as compared to lower level of N 15 kg N ha^{-1}. Increase in seed yield due to co-inoculation was 11% as compared to separate bacterial inoculation.

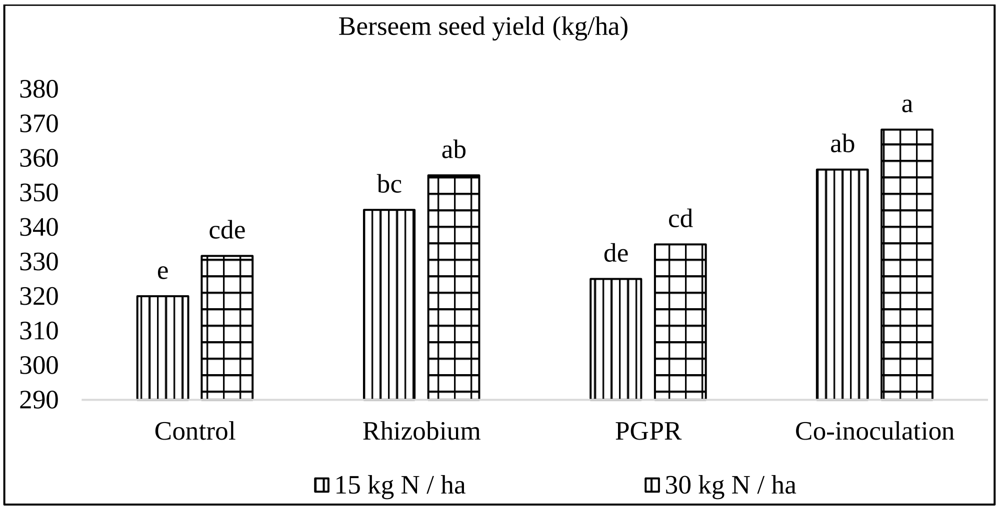

Fig. 3 Co-inoculation effect on berseem seed yield

Evaluation of N&P content and soil N and available P

Results regarding berseem seed N & P content are presented in Table 3. The seed nitrogen (N) and phosphorus (P) content were much improved by co-inoculation followed by *Rhizobium* sp inoculation and minimum values are obtained in uninoculated ones. Cumulative effect of *Rhizobium* sp and rhizobacteria (Br_4 & Pg_4) produced the highest seed N-P content i.e., 3.55, 0.258% followed by *Rhizobium* sp inoculation i.e., 3.54 and 0.251% than the control i.e., 3.46 and 0.245%, respectively at higher levels of N (30 kg N ha^{-1}). Percent increase in seed N and P was much higher in co-inoculation than the separate bacterial inoculation and control. Results regarding soil parameters at harvest validated the effect of bacterial inoculation and pronounced effect was observed co-inoculation (Table 3). Co-inoculation of symbiont and free living diazotroph at higher level of N exhibited the maximum soil N i.e., 0.048 followed by *Rhizobium* inoculation i.e., 0.047 as compared to control i.e., 0.044%. Co-inoculation showed higher soil N and available P by 9.09 and 24.6%, followed by *Rhizobium* inoculation i.e., 6.82 and 20.47%, respectively at higher N level. The rhizobacteria also influenced slight increase in the soil parameters compared to control.

Table 3 Inoculation effect on seed analysis and post-harvest soil analysis of berseem (Average of 3 repeats)

Treatments	Seed N (%)		Seed P (%)		Soil N (%)		Available P (mg kg^{-1})	
	15 kg N ha^{-1}	30 kg N ha^{-1}	15 kg N ha^{-1}	30 kg N ha^{-1}	15 kg N ha^{-1}	30 kg N ha^{-1}	15 kg N ha^{-1}	30 kg N ha^{-1}
Control	3.42^{e}	3.46^{d}	0.243^{g}	0.245^{f}	0.042^{d}	0.044^{cd}	9.77^{e}	11.14^{d}
Rhizobial inoculation	3.52^{b}	3.54^{ab}	0.247^{e}	0.251^{d}	0.045^{bc}	0.047^{a}	12.05^{cd}	13.42^{ab}
PGPR inoculation	3.47^{cd}	3.49^{c}	0.252^{d}	0.254^{c}	0.043^{d}	0.046^{b}	11.14^{d}	12.51^{bc}
Co-inoculation	3.53^{ab}	3.55^{a}	0.256^{b}	0.258^{a}	0.045^{bc}	0.048^{a}	12.05^{cd}	13.88^{a}
LSD	0.0293		0.0019		0.00166		1.111	

Results regarding Plant N-P content in different cuttings are showed in Fig. 5 & 6. Results presented in figures clearly showed higher values of N & P at t high N level and bacterial inoculation exhibited significant increase than control. The *Rhizobium* isolate (Br_4) or rhizobacteria (Pg_4) alone application or in combined form improved the plant N & P-content in comparison to un-inoculated ones at both N levels and the result was more pronounced at higher N level. Co-inoculation results in the highest plant N in the three cuttings i.e., 2.82, 2.88 and 3.03% and plant P i.e., 0.258, 0.237 and 0.223% than the separate bacterial inoculation and untreated control.

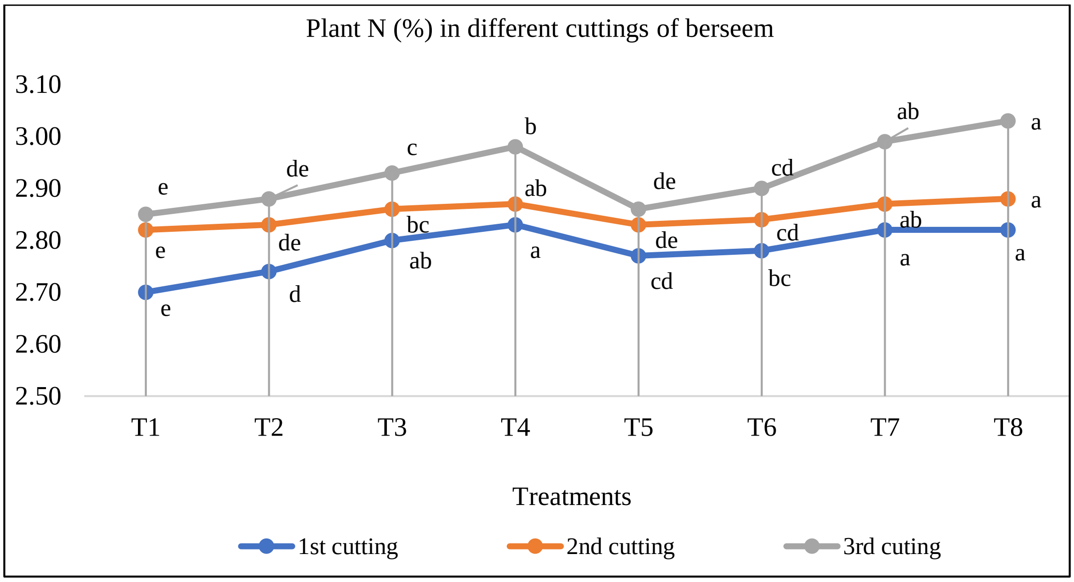

Fig. 4 Co-inoculation effect on plant N in different cuttings of berseem

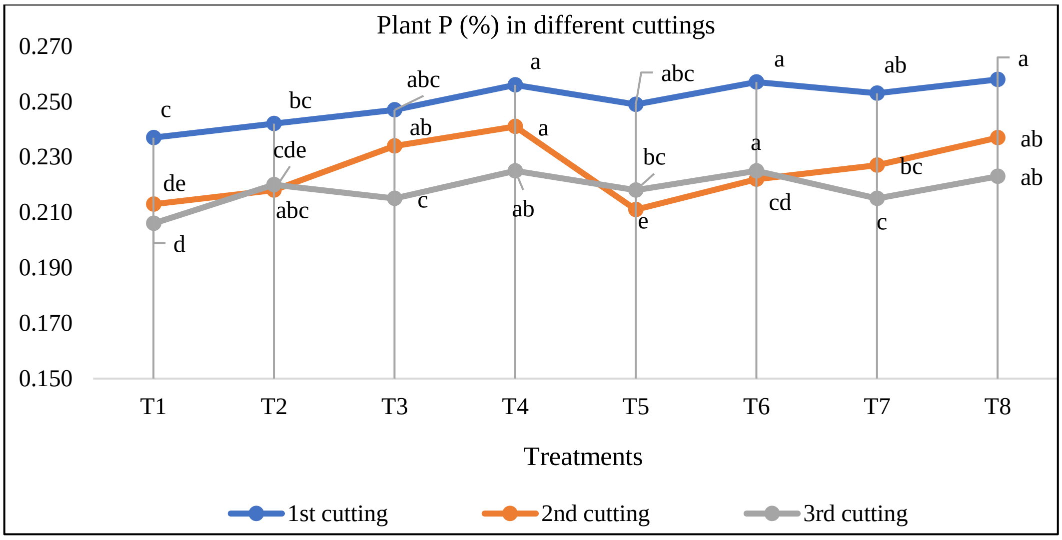

Fig. 5 Co-inoculation effect on plant P in different cuttings of berseem

Discussion

The association of specific *Rhizobium* sp with legumes in the presence of enzymatic complex 'nitrogenase' for nitrogen fixation. The species of *Rhizobium* besides fixing nitrogen, solubilize fix P, produce hormones, and functioned in the improvement of crop yields (Mehboob et al., 2011; Qureshi et al., 2012; Benjelloun et al., 2021). The rhizobacteria (free living microbes) have characteristic features of producing biological volatiles, mobilizing insoluble nutrients and perform several other functions (Ullah et al., 2017a; Soares et al., 2020; Vocciante et al., 2022). The combined inoculation or co-inoculation of legumes with specific specie of *Rhizobium* and rhizobacteria is an efficient strategy for improvement of legume yield (Parthiban et al., 2016; Kebede, 2021; Liu et al., 2022). The microbial inoculants in the form of co-inoculation improved the crop yields by various mechanisms viz. nutrients fixation/ oxidation, insoluble nutrient mobilization, hormonal regulation, acquisition of nutrient and stress tolerance (Benjelloun et al., 2021).

The isolates under study were screened for their biochemical potential in lab conditions for auxin biosynthesis (IAA equivalents), solubilization indices for phosphorus, siderophore production through quantitatively and qualitatively with CAS assay and siderophore unit (SU), formation of EPS and other biochemical tests. Isolates under study have varied degree of IAA equivalents (Table 1) and values were much improved with the auxin precursor L-TRP. Literature endorsed the proof of bacterial role for production of bacterial volatiles and metabolites, and their increased values with their precursors and other activities that eventually enhanced the crop yields (Gopalakrishnan et al., 2015; Dumsane et al., 2020). The *Rhizobium* sp and Rhizobacteria produced IAA equivalents without L-TRP (2.25-3.20; 2.30-3.35 µg mL^{-1}) and with L-TRP (3.55-4.15, 3.25-4.25), solubilization index (2.35-3.25, 2.30-2.80), siderophore unit (30.5-36.0, 28-35.5%), respectively and described by numerous workers (Pacheco-Villalobos et al., 2016; Parthiban et al., 2016; Abd El-Mageed et al., 2022). The *Rhizobium* sp (Br_4) produced the higher values of IAA content, SI, SU, CAS assay and EPS production were obtained with i.e., 4.15 µg mL^{-1}, 2.80, 36.0%, respectively while values for rhizobacterial isolate (Pg_4) i.e., and 4.25 µg mL^{-1}, 2.85, 35.5%, respectively. The microbial screening of isolates either symbiotic or non-symbiotic was verified by numerous researchers (Qureshi et al., 2013; Dumsane et al., 2020; Liu et al., 2022). The isolates of *Rhizobium* sp (Br_4) and Rhizobacteria (Pg_4) produced higher values during initial screening for growth and yield of berseem and were evaluated with two N levels i.e., 15 & 30 kg N ha^{-1} and uniform dose 60 kg P ha^{-1}.

The bacterial inoculation of symbiotic and non-symbiotic bacteria either applied separately or co-inoculation affected the fresh fodder and dry matter yield significantly at given N levels than control. The effect was more prominent at higher level of N as compared to lower level due to more vegetative growth. Increase in berseem yield parameters (fresh fodder & dry matter) might be featured to microbial biosynthesis of primary & secondary metabolites, biological active substances in the root-zone that altered the root system, more N_2-fixation by introduced symbionts, and results in improved growth and yield of legumes (Ullah et al., 2017a; Liu et al., 2022; Vocciante et al., 2022). The introduced bacterial inoculants are *Rhizobium* and free living rhizobacteria have great root colonizing power lifted the growth and yield of legumes owed to production of growth stimulants, siderophore and exopolysaccharide (EPS), better root system improved nutrient uptake and eventually improved yield of crops (Qureshi et al., 2013; Ullah et al., 2017b; Kebede, 2021). The promotion of crop growth parameters due to combined inoculation might be ascribed to numerous means viz. production of bacterial volatiles, siderophores, organic acids/phosphatases, biocontrol of plant pathogens and inducing systemic resistance (Ullah et al., 2017a; Ju et al., 2019; Liu et al., 2022). The bacterial inoculants enhanced the lant growth and development and ultimately yield might be accredited to better nutrient availability, regulation of hormones especially in sub-optimal conditions reported by numerous researchers (Korir et al., 2017; Ullah et al., 2017b; Matse et al., 2020).

The introduction of beneficial microbes by symbiotic and rhizobacteria in the root zone applied singly or in combined form improved the nodulation status of plants and significant higher values were obtained with co-inoculation in contrast to control. The better nodulation status (nodule number & mass) owed to better root system architecture, configuration of high lateral roots provided more attaching sites of microsymbionts resultantly better nodulation (Korir et al., 2017; Liu et al., 2022). The bacterial treatment resulted in curling of root hairs and development of infection thread for better nodulation. The microbial inoculation expanded the root density, better root proliferation resulted in more root surface area and more sites for invasion of microbes and enhanced nutrient uptake (Wei et al., 2018; Dumsane et al., 2020). Increase in root surface and enhance nodules due to co-inoculation reported by numerous researchers (Gouda et al., 2017; Bergmann et al., 2021).

Results revealed that bacterial inoculation promoted the berseem fodder, seed yield and other components in comparison to un-inoculated ones. The bacterial inoculation effect was significant at both nitrogen levels and higher values were obtained at higher N level implied that inoculation effect was fertilizer dependent. Results confirmed that role of bacterial inoculation in enhancement of legume growth and yields (Korir et al., 2017; Dumsane et al., 2020; Benjelloun et al., 2021). The improved nodulation and forage production expressed the seed yield enhancement (Jan et al., 2014; Tufail et al., 2018; Wei et al., 2018). Increase in seed yield might be ascribed to enhance availability of carbohydrates during reproductive growth stage resultantly improved seed formation and increased root activities for N-P uptake (Jan et al., 2014; Tufail et al., 2018; Bergmann et al., 2021).

The bacterial inoculation also augmented the seed and plant N-P content in comparison to other inoculation treatments and profound effect was observed in co-inoculation. The bacterial inoculation applied either separately or in the form of co-inoculation exerted their positive effect on legume yields and better nutrient acquisition due to improved root system development (Ruzzi & Aroca, 2015; Liu et al., 2017; Vargas et al., 2017; Backer et al., 2018; Lin et al., 2019). Improved N-P content in seed/plants in different cuttings might be attributed to more nutrient's bioavailability in the root-zone/rhizosphere (Jan et al., 2014; Parthiban et al., 2016; Przygocka-Cyna & Grzebisz, 2018; dos Santos et al., 2019; Qureshi et al., 2022). Co-inoculation improved the N-P level in the plant/seed owed due to enhance root systems, lateral hairs, more fixation of N and phosphatases solubilizes more P in the root environment (Benjelloun et al., 2021; Qureshi et al., 2022). The bacterial fixing of nitrogen, solubilizing insoluble nutrients, producing hormones and other metabolites results in better nutrient availability, better growth and eventually improved yield and yield components (Datta & Chakrabartty, 2014; Przygocka-Cyna & Grzebisz, 2018; Dumsane et al., 2020). The introduction of beneficial microbes in the root-zone and production of growth stimulants results in improved cell division and hence increased plant root-shoot growth (Berger et al., 2013; Qureshi et al., 2013; Abd El-Mageed et al., 2022; Vocciante et al., 2022).

Conclusion

Results suggested that co-inoculation affected the berseem yield components in significant manner. The bacterial inoculation of berseem specific symbiont and free living and rhizobacteria demonstrated beneficial effects on berseem growth and yield but their combined effect was proved to be more amazing. The present study clearly ascribed the bacterial inoculation as co-inoculant depicted positive response on yield components and nutrient acquisition of berseem.

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