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Phytobeneficial bacterial inoculants for common bean growth and productivity in nitrogen and phosphorus deficient soils

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Most of the soils are N and P-deficient and require high applications of chemical fertilizers to ensure optimal crop production. However, this practice poses in most cases serious environmental issues. In recent years, inoculation with beneficial bacteria has emerged as a safe and sustainable alternative to chemical fertilizers. In this context, we investigated the effect of two symbiotic *Agrobacterium radiobacter* strains (LMR670 and LMR676) and two plant growth-promoting rhizobacteria *Bacillus* sp. (M131) and *Enterobacter* sp. (P1S6), as single or combined inoculants, on common bean growth and yield under N and P-deficient conditions. In a first trial, *Agrobacterium* strains' symbiotic efficiency with common bean was evaluated in a low phosphorus and nitrogen soil under greenhouse conditions. Strain LMR670 recorded the highest nodules number (53 nodules per plant) and shoot dry weight (0.553 g plant^{-1}). This strain was then used in combination with the PGP rhizobacteria in a common bean co-inoculation assay under sufficient and deficient P levels (80 kg ha^{-1}of P and No P added). Single inoculation with LMR670 recorded the highest shoot dry weight (82% increase compared to non-fertilized control) compared to combined inoculants (46-47% increase). To corroborate the obtained results, a field experiment was conducted using the same treatments. LMR670 as a single inoculant or mixed with M131 was consistently effective leading to common bean yields comparable to N and P fertilized plants (13.07 t ha^{-1} and 12.35 t ha^{-1} respectively). In addition, single inoculation with the multi-PGP strain M131 showed positive effects on all common bean growth parameters and yield value (14 t ha^{-1}); exceeding even N and P fertilized control plants. These results suggest that the strains LMR670 and M131 can be used, in a single or combined inoculation, as effective biological fertilizers for common bean cultivation to replace phosphorus and nitrogen fertilizers. Globally our results highlight the potential of native phytobeneficial strains for successful nodulation, growth and yield of common bean under N and P-deficient conditions.

Keywords: *Agrobacterium*, co-inoculation, nutrients scarcity, PGPR, *Phaseolus vulgaris* L.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is currently estimated to be one of the most important food legumes in the world. Unlike other legumes, common bean is considered as a weak nitrogen fixer. This is due in particular to the genetic characteristics of symbiotic partners as well as soil and environmental conditions (Assefa *et al*., 2019). To address this issue, intensive efforts are made worldwide to increase the natural process of nitrogen fixation through cultivar selection (Barbosa *et al*., 2018) and promote bean growth through inoculation with selected bacteria (e.g. Pohajda *et al*., 2016).

During the past decades, considerable interests in nitrogen fixing bacteria and Plant Growth Promoting Rhizobacteria (PGPR) have emerged for sustainable agricultural practices and for crop yield increase at a low cost (O'Callaghan, 2016). PGPR have been reported to exhibit various beneficial properties such as phytohormone production (Backer *et al*., 2018), and inorganic P solubilization (Oteino *et al*., 2015). They are also reported to provide iron to iron-starved plants through the production of siderophores (Maldonado *et al*., 2020) and enhance plant stress tolerance such as salinity, drought and heavy metals (Enebe and Babalola, 2018). Thus, micro-organisms possessing PGP properties can be used as bio-inoculants to promote plant growth and development

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under various stress and mineral deficiencies. In this context and considering the importance of nitrogen and phosphorus in plant growth and development, the combination of P solubilizers with N fixing bacteria could be useful in bean production improvement by increasing available P forms in the soil and thereby enhancing nodulation and N fixation (Korir *et al*., 2017).

Despite the intensive efforts of researchers to provide efficient bacterial mixed inoculants to crop plants, variable effects on plant growth parameters have been observed either stimulatory (Tajini *et al*., 2012), negative (Grobelak *et al.*, 2015) or showing no effect (Kalozoumis *et al*., 2021). Therefore, a careful evaluation and selection of appropriate combinations of nitrogen fixing bacteria and PGP strains exhibiting high synergy and efficiency on plant growth is a key step.

To promote the use of phytobeneficial bacteria as inoculants for common bean cultivated under low fertility soils, the present study designed a multi-step protocol for selecting the best performing strains. In particular, three specific objectives were addressed: 1) To determine the ability of multi-trait PGP bacteria selected *in-vitro* to promote common bean growth and nodulation in natural conditions; 2) To evaluate the potential of P-solubilizing bacteria to assist common bean growing under P-limiting conditions; 3) To investigate the effect of single and mixed inoculants of native rhizobia and selected PGPR on the growth and productivity of common bean in N and P limiting soils.

MATERIALS AND METHODS

***Bacterial strains*:** All the strains used in the current work are part of a collection held by the Microbiology and Molecular Biology Unit (LMBM) at the faculty of sciences of Rabat, Morocco. *Agrobacterium* strains LMR670 and LMR676 were obtained from a previous screening program based on their symbiotic performance with their host plant *Phaseolus vulgaris* under greenhouse conditions (El Attar *et al*., 2019). Non symbiotic strains *Bacillus* sp M131 and *Enterobacter* sp. P1S6 were originally isolated respectively from root nodules and rhizosphere of grain legumes collected from two different locations in Morocco (Table 1).

***PGP traits of the bacterial strains*:** All 4 strains were checked for their ability to solubilize rock phosphate in liquid Pikovskaya's broth (Pikovskaya, 1948) and soluble phosphorus produced was determined by the Vanadate-molybdate method (Tandon*et al*., 1968). Auxin production was determined after growing the bacteria in Yeast Extract Mannitol (YEM) broth (Vincent, 1970) supplemented with 0.5 mg/ml of L-tryptophan following the Salkowski's colorimetric method (Ehmann, 1977).

To estimate siderophores production, strains were cultivated on an iron free medium and levels of siderophores production were estimated using Chrome Azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators (Schwyn and Neilands, 1987). A semi quantitative estimation of siderophores production was also performed as described by Machuca and Milagres (2003). The ACC deaminase activity of elite strains was determined by evaluating their ability to utilize ACC as a sole nitrogen source in DF minimal salt medium following the method described by Dworkin and Foster (1958). Plates containing DF minimal medium without ACC served as negative control and with $(NH_4)_2SO_4$ (2.0 g L^{-1}) as a nitrogen source served as a positive control.

PCR amplification and DNA sequencing of the full 16S rRNA genes of the PGPR strains were performed as described previously (El Attar *et al*., 2019).

***Effect of agrobacterial inoculation on common bean grown in soil under greenhouse conditions*:** In this first assay, the two best performing agrobacterial strains selected earlier (Table 1) were tested. The soil used was collected from the surface layer (0-20 cm) of an abandoned field of sandy soil with no history of bean cultivation. Its chemical properties were as follows: pH_{H2O} 6.72, pH_{KCl} 5.12, organic matter 0.63%, total N 0.03%, total P 2.97 mg kg^{-1}, total K 39 mg/kg and electrical conductivity 0.283 dS m^{-1}. Soil P deficiency was corrected by an amendment with superphosphate at the rate of 80 kg ha^{-1} of P.

Seeds of the commercial locally available common bean variety *Tania* were surface-sterilized with 90% of ethanol followed by 0.2% sodium hypochlorite. They were rinsed abundantly with sterilized distilled water then germinated on 1% agar plates (Vincent, 1970). Five healthy uniform seedlings were transplanted into pots (17 cm diameter and 20 cm deep) that were previously filled with five kilograms of sieved non-sterilized soil then thinned to 3 plants per pot few days after sowing. Each common bean seedling was

Table 1. **Origin and main characteristics of bacterial isolates.**

Strains	Sites of origin	Plant organ	**P (µg ml^{-1})**	**Auxin (µg ml^{-1})**	**Sid (%)**	**ACC**	**RE%**	Species (based on 16S rRNA gene sequence)	Accession numbers
LMR676	Tifelt	Nodules of common bean	16.72d	122.20a	50a	+++	89	*Agrobacterium radiobacter*	MG388318
LMR670	Delalha	Nodules of common bean	22.55c	124.23a	27b	++	74	*Agrobacterium radiobacter*	MG388314
P1S6	Settat	Rhizosphere of lentil	36.89b	-	49a	+++	-	*Enterobacter* sp.	MT560199
M131	Merchouch	Nodules of chickpea	54.74a	21.201b	30b	+++	-	*Bacillus* sp.	MT560200

P: Amount of solubilized phosphate, Auxin: Amount of auxin produced, Sid: Siderophores production, ACC: ACC deaminase activity, RE: Relative Efficiency (inoculated Shoot Dry Weight/ Shoot Dry Weight of +N) x 100, Means in the same column followed by the same letter are not significantly different at the 5% probability level by LSD's test.

inoculated with 1 mL of log phase bacterial culture (1×10^9 CFU mL^{-1}) applied at sowing stage on top of each seedling. Bacterial strains were previously grown in liquid YEM broth at 28 °C under shaking conditions. Two sets of control plants were settled: non-inoculated non fertilized control (N0) and non-inoculated fertilized control (N120) that received 120 kg ha^{-1} nitrogen in the form of urea (46% N) applied in two equal portions, one at the time of planting and the other before flowering. For each treatment, pots in triplicate were installed in a greenhouse following a completely randomized design for at least 45 days. Average day/night greenhouse conditions were 27/21°C for temperature and 16h/8h for photoperiod. Average relative humidity was 70% during the day.

***Common bean co-inoculation assay in a P-deficient soil*:** Based on the results obtained from the precedent pot trial, strain LMR670 was selected for studying its effectiveness in combination with M131 and P1S6 rhizobacteria. To investigate *in vitro* the compatibility between strains used in mixed inocula, antagonism between strains was verified following Mikiciński *et al.* (2016) procedure. The same soil from the first trial was used with two P treatments: single superphosphate at the rate of 80 kg ha^{-1} of P and no P addition (Table 2). Plants inoculated only with PGP rhizobacteria were supplied with 120 kg ha^{-1} as described above. General experimental conditions were similar to the first assay and equal volumes of *Agrobacterium* and PGPR cultures were used.

Table 2. Summary of the treatments applied in the three inoculation trials.

	Soluble P	**P0**
1st soil pot experiment	Control N120	Control N120
	Control N0	Control N0
	LMR670	
	LMR676	
2nd soil pot experiment	Control N120	ControlN120
	Control N0	Control N0
	LMR670	P1S6+N120
		M131+N120
		LMR670+M131
		LMR670+P1S6
Inoculation field trial	Control N120	ControlN120
	Control N0	Control N0
	LMR670	P1S6+N120
		M131+N120
		LMR670+M131
		LMR670+P1S6

N120: Nitrogen fertilization at a rate of 120 kg ha^{-1} of N, N0: No nitrogen fertilization, P: Single superphosphate fertilization at a rate of 80 kg ha^{-1} of P, P0: No phosphate fertilizer added

***Field co-inoculation trial*:** This experiment was carried out in a poorly fertile sandy soil and without any inoculation background. The same co-inoculation treatments previously described were used (Table 2) and laid out in a randomized complete block design with three replicates. Each plot measured 2 × 2 m and was divided into 4 rows. Inter and intra row spacing were 0.5 m and 0.2 m respectively. Soil characteristics were as follows: pH_{H2O} 7.3, pH_{KCl} 6.62 organic matter 0.89%, total N 0.03%, total P 0.14 mg kg^{-1}, total K 3.3 mg kg^{-1} and electrical conductivity 0.14 dS m^{-1}. Single superphosphate and potassium sulfate were added at the rate of 80 kg ha^{-1} of P and K. Nitrogen-fertilized treatments received 120 kg ha^{-1} N fertilizer in the form of urea (46% N) divided into three equal portions at planting, 20 days after sowing and at flowering.

Peat-based inoculants were used in this field trial. After growing strains as described above, bacterial cultures containing 10^8 CFU mL^{-1} were injected into pre-packaged 100 g sterilized peat and incubated for 6 days at 30° C. The final moisture content of the peat-based inoculants was adjusted to 30%. To ensure an optimal symbiotic performance and avoid limiting factors, plants inoculated only with PGPR strains received N fertilization while those inoculated with LMR670 received P fertilization. Non specified experimental conditions were as described before.

***Measured parameters*:** In the case of pot trials, plants were harvested 45 days after sowing and were separated into root and shoot tissues. Roots were washed then nodules were carefully removed, counted and dried to determine nodule dry weight. Shoot and root dry weights were recorded after drying at 60 °C for 48h. For the field experiment, plants sampling was done within a 1 m × 1 m quadrate in each plot during the mid-flowering stage to measure the different parameters listed above. To determine grain yield, plants were harvested manually at physiological maturity 2.5 months after sowing followed by a second harvest one week later. The grain yield reported here is a sum of both harvests.

***Statistical analysis*:** Data were checked for normality of errors and homogeneity of variances prior to the statistical analyses. One-way ANOVA was employed to determine differences among treatments. When the effects of treatment were significant at the 0.05 probability level, means comparisons were conducted using Tukey LSD as a post hoc significance test at $\alpha = 0.05$ using XLstat 2014 software

RESULTS

***PGP traits of the bacterial strains*:** Phosphate solubilization ability was observed in all tested strains. However, PGP strains showed higher amounts than *Agrobacterium* strains with M131 achieving maximum production (54.74 µg mL^{-1}). Unlike P solubilization, PGP strains produced less amounts of auxin than *Agrobacterium* strains. All tested strains produced siderophores with the highest amount achieved by the *Agrobacterium* strain LMR676 (50%). Moreover, all tested strains showed moderate to high ACC deaminase activity (Table 1).

***Agrobacterium inoculation of common bean grown in soil under greenhouse conditions*:** Common bean inoculation with *Agrobacterium* strains demonstrated a significant improvement in nodules number in comparison to the un-inoculated controls (Table 3). There was a low level of nodulation of the unfertilized un-inoculated control by the indigenous rhizobia but this nodulation did not lead to any appreciable shoot dry weight. However, LMR676 produced a higher number of nodules, plants' shoot dry weight was statistically similar to the non-fertilized plants. On the opposite, nodules dry weight and shoot dry weight obtained with LMR670 were significantly higher. Moreover, the value of the shoot dry weight represented 48% of the SDW recorded for the control plants that received nitrogen fertilization. Based on these results the best strain (LMR670) was chosen for a co-inoculation trial in combination with PGP strains under greenhouse conditions.

***Common bean co-inoculation assay under P deficient conditions*:** All control plants were weakly nodulated by the indigenous rhizobial population of the soil, but the nodules were very small as indicated by their dry weight. In the case of P-fertilized control plants, a 59% decrease of shoot dry weight was observed in comparison with plants that received both nitrogen and phosphate fertilizers (Table 4).

Table 3. Effect of *Agrobacterium* inoculation on common bean growing under greenhouse conditions.

Treatment	Nodules number /plant	Nodules dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})
Control N120+ P	0.0c	0.000d	1.144a	0.701a
Control N0/ P0	22.0b	0.011c	0.187c	0.167b
LMR670	53.0a	1.517a	0.553b	0.355b
LMR676	75.2a	0.038b	0.273c	0.271b

N120: Nitrogen fertilization at a rate of 120 kg ha^{-1} of N, N0: No nitrogen fertilization, P: Single superphosphate fertilization at a rate of 80 kg ha^{-1} of P, P0: No phosphate fertilizer added, Means in the same column followed by the same letter are not significantly different at the 5% probability level by LSD's test.

Table 4. Effect of combined inoculation on common bean growing under greenhouse conditions.

Treatment	Nodules number /plant	Nodules dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})
Control N120+ P	4.16h	0.000e	1.446a	0.828a
Control N120/ P0	11.33f	0.002de	0.879c	0.781b
Control N0+ P	16.16e	0.002de	0.852c	0.480e
Control N0/P0	28.00c	0.005c	0.508e	0.679c
LMR670	81.16a	0.065a	1.196b	0.719bc
M131	6.16gh	0.000e	0.455f	0.298f
P1S6	9.66fg	0.003d	0.556e	0.574d
LMR670+M131	21.33d	0.005c	0.691d	0.497e
LMR670+P1S6	37.83b	0.010b	0.675d	0.592d

N120: Nitrogen fertilization at a rate of 120 kg ha^{-1} of N, N0: No nitrogen fertilization, P: Single superphosphate fertilization at a rate of 80 kg ha^{-1}of P, P0: No phosphate fertilizer added, Means in the same column followed by the same letter are not significantly different at the 5% probability level by LSD's test.

Table 5. Effect of combined inoculation on common bean growing in field conditions.

Treatment	Nodules number/plant	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Yield (t ha^{-1})
Control N120+ P	2.00d	0.047bcd	52.76bc	5.803bc	12.24abc
Control N0+ P	2.00d	0.021d	46.69cd	1.970f	12.63abc
Control N120/P0	5.66cd	0.09abc	41.49cd	4.770cde	10.02cd
Control N0/ P0	3.66cd	0.023d	37.48d	3.653def	8.93d
LMR670	33.00a	0.095ab	69.78a	7.653ab	13.07ab
M131	18.66abc	0.042cd	64.06ab	9.283a	14.02a
P1S6	4.66cd	0.002d	52.94bc	5.203cd	12.35abc
LMR670+M131	23.00ab	0.128a	52.21bc	7.320ab	11.43abc
LMR670+P1S6	16.00bc	0.083abc	40.62cd	3.063ef	10.70cd

N120: Nitrogen fertilization at a rate of 120 kg ha^{-1} of N, N0: No nitrogen fertilization, P: Single superphosphate fertilization at a rate of 80 kg ha^{-1}of P, P0: No phosphate fertilizer added, Means in the same column followed by the same letter are not significantly different at the 5% probability level by LSD's test.

The strain LMR670 induced abundant nodulation of common bean plants either as a single inoculant or mixed with PGPR strains in comparison with the un-inoculated controls. Moreover, single inoculation of LMR670 produced the highest nodules number and dry weight when plants were fertilized with 80 kg ha^{-1} of P (Table 4). This treatment has a significant effect on the common bean growth that reached 82% of the SDW recorded for plants with N and P fertilization. However, number of nodules per plants inoculated with mixed inocula was significantly lower than that obtained when LMR670 was inoculated alone. Both combinations produced an average of 57% aerial biomass compared to plants inoculated with LMR670 alone under optimal P conditions and 45% aerial biomass compared to optimal fertilization conditions (Table 4).

***Field co-inoculation trial*:** Similar to previous results, bacterial inoculation generally improved common bean nodulation over the un-inoculated controls. Interestingly, the inoculation of common bean plants with the PGP strain M131 alone had positive effects on all common bean growth parameters measured with the highest yield value (14.02 T ha^{-1}). This level is even superior to the control treatment that received N and P fertilizers. Similar results were also observed in plants inoculated with the strain LMR 670 alone. This strain showed significantly higher nodules number and shoot dry weight when co-inoculated with M131 over P1S6 (Table 5).

DISCUSSION

The evaluation under greenhouse conditions of *Agrobacterium* strains effect on common bean growth in soil pots showed that LMR670 performed better than LMR676 as it produced significantly higher shoot biomass (Table 3). It is noteworthy that LMR 676 was previously found to have higher relative efficiency with common bean grown in perlite (El Attar *et al*., 2019). However, in the present assay, this strain was unable to fully express its higher relative symbiotic efficiency potential under soil conditions even though it produced an important number of nodules. As a matter of fact, introduced strains in the field face challenges such as changes in the rhizosphere environment or the strains' genetic instability which may affect both their growth and saprophytic performance (Tkacz *et al*., 2015; Vuong *et al*., 2017). In addition, a recent study conducted on the formulation of a highly effective inoculant for common bean showed that the symbiotic superior performance of an autochthonous strain might be due to its genetic versatility as it harbors a large assortment of genes contributing to its fitness and competitiveness (Pastor-Bueis *et al*., 2019). Thus, the inconsistency in performance of our strains when moving from perlite to soil conditions can be attributed to the ability of LMR670 to adapt to the soil environment and its good competitiveness for nodule occupancy. Therefore, our results support the importance of the physiological adaptation of the inoculated strains to the whole environment for a successful establishment and the expression of their full potential.

Under field conditions, combinations LMR670+M131 (*Bacillus* sp.) and LMR 670+P1S6 (*Enterobacter* sp.) produced a significant positive effect on growth parameters compared to negative control plants (N0/P0) (Table 4). This result indicates that in a P deficient soil tested PGPR strains can replace partially soluble P amounts necessary for nodulation, nitrogen fixation and plant growth. Similarly, Matse *et al.* (2020) also demonstrated in a recent study that the combination of *Rhizobium* and PGPR enhanced nodulation, nitrogen fixation, growth and macronutrient contents in white clover under low P condition.

It was also observed that the application of nitrogen to plants inoculated with PGPR and phosphorus to plants inoculated with LMR670 both had comparable effects on common bean yield to that observed in nitrogen and P fertilized plants. Therefore, it can be hypothesized that under the prevailing soil conditions, PGPR strains M131 and P1S6 can satisfy the P needs of common bean plants while the symbiosis with LMR670 can ensure a good level of their N nutrition.

The efficiency of LMR670 as a single inoculant was consistently high in all tests conducted including field conditions (Table 5). Similar results were reported by Youseif *et al*. (2017) who demonstrated the highly symbiotic stability of *A. tumefaciens* strains to nodulate faba bean roots under both greenhouse and field experiments. It could be stated that LMR670 is an efficient bean nodulating strain, which is able to insure common bean growth and optimal yield under low fertility soil conditions.

Interestingly, single inoculation with the PGP strain M131 produced the highest grain yield exceeding even the N and P fertilized control (Table 5). Given that the field soil is very poor in phosphorus, it seems that the ability of M131 to solubilize inorganic phosphate *in-vitro* may be sufficient to overcome the soil P deficiency and promote plants growth. In addition, this strain can produce siderophores and auxin, two molecules that can impact P availability for inoculated plants. Furthermore, in a comparison of siderophore-producing mutants impaired in siderophores production with the wild type strains, Ghosh *et al.* (2015) found that the mutant solubilized 10-times less P than the wild type strain. On the other hand, auxin is a signalling molecule involved in the adaptive response of the root system architecture to phosphate deprivation (Huang and Zhang, 2020). Auxin may also enhance P uptake by promoting root hair elongation of roots exposed to low external P conditions (Bhosale *et al*., 2018). Hence, the potential role of siderophores and auxin produced by PGPR strains in enhancing P availability should be also considered.

It is worth mentioning that the ability of *Agrobacterium* to form effective nodules has been reported in several research studies starting with Cummings *et al*. (2009) who were the

first to confirm a legume nodulating symbiont from *Agrobacterium* clade. The *Agrobacterium* strain *IRBG74* was isolated from root nodules of aquatic legume *Sesbania cannabina* and could effectively nodulate *S. Cannabina* and seven other *Sesbania spp.* Subsequently, Yan *et al*. (2017) also reported the isolation of two strains of *Agrobacterium salinitolerans* sp. nov. from root nodules of *Sesbania cannabina* which were also able to nodulate S. *Cannabina* plants. In another study conducted by Youseif *et al.* (2017) native nodulating faba bean *Agrobacterium* strains showed a high nitrogen-fixing capacity under greenhouse and field conditions when inoculated to their host plant.

The efficacy of inoculant strains in greenhouse experiments does not always translate to field success. This is the case of the strain P1S6 co-inoculated with LMR670 that improved common bean growth parameters in greenhouse conditions but not in field trial. Under natural conditions, the interaction between inoculant strains and plants may be affected by their interactions with indigenous bacteria, soil characteristics and weather conditions.

***Conclusion*:** The present findings suggest that the response of common bean to bacterial inoculants varies greatly depending on the experimental conditions, the inoculum composition, and the strains potential. Hence, all these aspects need to be carefully studied for the success of this promising biotechnological approach. It also stands to reason that more field experiments are necessary to confirm the positive results reported in this present study and to generalize the use of these bacterial biofertilizers as an environmentally friendly agricultural practice.

Conflict of interest: The authors declare no conflicts of interest.

Author's contributions statement: The authors declare that they have contributed to the article at a similar rate.

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