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Development of liquid inoculants for strains of *Rhizobium tropici* group using response surface methodology

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Common bean (*Phaseolus vulgaris* L.) is the most important legume for human consumption in many countries of South and Central America, Asia and Africa. The crop can benefit from the biological nitrogen fixation process, especially when inoculated with elite rhizobial strains. Strains belonging to the "*Rhizobium tropici* group" are preferred because they show high tolerance to abiotic stresses, but their survival in liquid formulations is poor, limiting their use by farmers. In this study, response surface methodology (RSM) was used to develop liquid formulations for the commercial strains CIAT 899 (*R. tropici*) and PRF 81 (*Rhizobium freirei*). A significant interaction between dibasic potassium phosphate (K_2HPO_4) and yeast extract was observed, and to reach higher cell concentration, one should employ low concentrations of yeast extract and high concentrations of K_2HPO_4. A basic formulation which may represent the basis for the development of liquid inoculants for the common bean crop was developed.

Key words: Biological nitrogen fixation, inoculation, common bean, *Phaseolus vulgaris*, *Rhizobium freirei*.

INTRODUCTION

Brazil is today the world's largest common bean (*Phaseolus vulgaris* L.) producer and consumer and the crop has great economic and social importance not only to the country, but also other South and Central America countries, African and Asian countries. In Brazil, the grain production increased by 36% in 2016/17, with 3,418.3 thousand tons produced mostly by small farmers (CONAB, 2017). Nitrogen (N) is the nutrient most required

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by the common bean crop, with about 50% being exported by the grains (Araujo et al., 1996; Fageria and Baligar, 2005). N-fertilizers are traditionally applied to the crop, resulting in high cost and environmental pollution; however, the legume can take advantage of the biological nitrogen fixation (BNF) process. Rhizobia selection programs performed in Brazil have identified elite strains that can fully supply the plant's N needs (Hungria et al., 2000, 2003). The strains used in commercial inoculants in the country belong to the "*Rhizobium tropici* group", which includes rhizobial species that share a very similar symbiotic plasmid (Ormeño-Orrillo et al., 2012; Gomes et al., 2015). These species are highly adapted to typical tropical conditions, such as tolerance to high temperature and acidic soils; therefore, the strains are employed in inoculants for the common bean crop not only in Brazil, but also in other countries of South America and Africa (Gomes et al., 2015).

Peat has been known as "the golden" pattern carrier for rhizobial inoculants, due to its properties of richness in organic matter and nutrients, capacity of retaining moisture and to offer some protection to the bacteria against high temperatures and desiccation, enabling bacterial survival for longer periods (Singleton et al., 2002; Hungria et al., 2005; Lupwayi et al., 2005; Fernandes Júnior et al., 2009; Deaker et al., 2016). Nevertheless, peat presents great variability in its physical and chemical properties, besides being a non-renewable natural resource. On the contrary, liquid formulations allow high cell concentration void of contaminants and are preferred by farmers due to ease in application of seeds or in-furrow. However, cell viability both in the inoculant and at the field may be reduced in liquid formulations due to the lower protection against abiotic stresses (Singleton et al., 2002; Hungria et al., 2005; Lupwayi et al., 2005; Tittabutr et al., 2007; Fernandes Júnior et al., 2009; Yates et al., 2016). Particularly, for the *R. tropici* group, cell survival in liquid formulations has been critical; therefore, inoculants for the common bean crop are practically entirely peat-based, but an increased adoption by the farmers would be achieved if liquid inoculants are available.

The yeast extract-mannitol (YM) culture medium, developed almost a century ago (Fred and Waksman, 1928), has been broadly used to grow rhizobia, and slight modifications have been proposed through the decades (Vincent, 1970; Somasegaran and Halliday, 1982; Hungria et al., 2016). Basically, the medium has few components, of which the two main ones are mannitol as carbon (C) source and yeast extract. At industrial level, as mannitol is an expensive C source, it is usually replaced by glycerol or sucrose (Balatti and Freire, 1996; Menéndez et al., 2014).

An increase in the adoption of common bean inoculation with elite strains can highly affect grain production and soil fertility, as well as contribute to the mitigation of greenhouse gases (Hungria et al., 2013); the availability of liquid inoculants for the crop is critical to achieve this goal. The objective of this study was to develop liquid formulations for strains of the *R. tropici* group, to be used as inoculants for common bean crops.

MATERIALS AND METHODS

Culture conditions

Stock cultures of *R. tropici* strain CIAT 899 (=SEMIA 4077, =CNPSo 142, =USDA 9030, =ATCC 49672, =BR 322) and *Rhizobium freirei* strain PRF 81 (=SEMIA 4080, =CNPSo 122, =IPR-Pv81) were grown in YM medium (Vincent, 1970) modified for mannitol content (Hungria et al., 2016) for the preparation of pre-inocula. The composition of the modified YM medium consisted of (g L^{-1}): mannitol (5 g) (Synth®), yeast extract (0.4 g) (Acumedia®), dibasic potassium phosphate- K_2HPO_4 (0.5 g) (Anidrol), magnesium sulfate heptahydrate- $MgSO_4.7H_2O$ (0.2 g) (Vetec), sodium chloride-NaCl (0.1 g) (Vetec), and pH adjusted to ~6.8. Cultivation was carried out with shaking at 180 rpm and 28°C for 24 h until a concentration of 10^6 CFU mL^{-1} was reached. The cultures were diluted with the medium culture for the proper concentrations of the assays, based on the OD_{600} of the culture, previously obtained and adjusted according to the cell counting number. The experiments were carried out from the pre-inocula with an initial concentration of 10^4 CFU mL^{-1}.

Evaluation of different sources of carbon

Sucrose (Anidrol), glycerol (Invitrogen®) and glucose (Biotec®) were evaluated as alternatives C sources to mannitol, as they are also used by strains of the *R. tropici* group (Dall'Agnol et al., 2013). Although, sucrose is used in a lower extent by some species of the *R. tropici* group, including *R. freirei* (Dall'Agnol et al., 2013), both sucrose and glycerol were chosen because of their low cost. Glucose was evaluated because it is one of the two monosaccharides constituents of sucrose, representing a C-source promptly available for *Rhizobium*, not requiring hydrolysis to be consumed by the microorganism. The culture media were used for the cultivation of both strains.

All C sources were used at the concentration of 5 g L^{-1}. The tests were performed in triplicate in a volume of 100 mL culture medium. The pH of the media was adjusted to ~6.8 and growth was performed on an orbital shaker at 180 rpm and 28°C for 96 h. In order to avoid the caramelization process and the Maillard reaction, a concentrated glucose solution of 50 g L^{-1} was filtered on a nitrocellulose membrane (Merck Millipore™) with a porosity of 0.22 µm and added under aseptic conditions, to the flasks where glucose represent the C source.

Viable cell concentration of *R. tropici* strain CIAT 899 and *R. freirei* strain PRF 81 was obtained by serial dilution in modified YM medium (Hungria et al., 2016), with the drop-plate method (Miles et al., 1938), adapted as described by O'Hara et al. (2016). The results are expressed in CFU mL^{-1}.

Response surface methodology (RSM)

The data obtained in the carbon source experiments informed the decision to use sucrose as the main carbon source in the subsequent RSM studies. From the initial data obtained, a basic

Table 1. *Rhizobium tropici* CIAT 899 and *Rhizobium freirei* PRF 81 cellular concentration when growth was evaluated in medium with different carbon sources. Evaluation was performed after 96 h of growth.

Carbon source	*R. tropici* CIAT 899 (CFU mL$^{-1)}$	*R. freirei* PRF 81 (cfu mL^{-1})
Sucrose	1.50 10^9 ± 2.01 10^{8a1}	7.78E+08 ± 9.07 10^{7a}
Mannitol	1.33 10^9 ± 7.93 10^{7a}	7.33E+08 ± 1.67 10^{8a}
Glucose	8.15 10^8 ± 1.39 10^{8b}	1.16E+09 ± 1.91 10^{8a}
Glycerol	7.17 10^8 ± 6.11 10^{7b}	7.78E+08 ± 1.05 10^{8a}
CV (%)	15.20	20.38

[1]Means of three replicates, and when followed by the same letter, for each strain, are not statistically different by the Tukey test ($p \leq 0.05$).

liquid formulation was defined for the response surface methodology (RSM) experiments. A factorial planning of 2^3 and three central points was established. The evaluated response variable was the cell concentration in CFU mL^{-1}. The linear model was evaluated with the data of 11 experimental points. Three factors (independent variables) were tested, with two levels each. In this way, C, N and P sources were analyzed simultaneously. The formulations tested varied in sucrose, yeast extract and K_2HPO_4. The pH was adjusted to ~6.8. Bacteria were grown at 180 rpm and 28°C for 72 h. The evaluation of rhizobial concentrations was performed in Petri dishes by the drop-plate method modified as described by O'Hara et al. (2016). Three experiments (A, B and C) were performed with different concentrations of the analyzed variables, as shown in Supplementary Tables S1 to S6.

Statistical analysis

The Statistica 7.0 program (Statsoft®) was used to analyze the results obtained by the RSM. The test of the model's lack of fit was evaluated through analysis of variance (ANOVA) at $p \leq 0.05$.

RESULTS

Rhizobium spp. growth with different carbon sources

Considering the YM medium with 5 g L^{-1} of mannitol (Hungria et al., 2016), when the C source was replaced by sucrose, cell concentration of *R. tropici* CIAT 899 was estimated at 1.5 × 10^9 CFU mL^{-1} after 96 h, similar to the concentration of 1.33 × 10^9 CFU mL^{-1} reached with mannitol (Table 1). However, regarding glycerol and glucose as C sources, significantly lower growth was observed (p≤0.05). *R. freirei* strain PRF 81 was used and grew with the four carbon sources tested, not showing statistic difference after 96 h of growth (Table 1). Therefore, the authors decided to continue studies on the viability of using the surface response methodology to design inoculant formulation only with strain CIAT 899.

Development of liquid formulations

Experiment A

According to the conditions established for the development of inoculant's liquid formulations for *R. tropici* CIAT 899 (Supplementary Tables S1 and S2), the cell concentration obtained ranged from 1.56 × 10^9 to 3.56 × 10^9 CFU mL^{-1} (Table 2). The response of variable presented small variation in the central points, inferring a good repeatability of the process. The response of surface analysis started by assuming that the investigated region was a linear function of the factors and estimated by the equation:

$$y = \beta_0 + \beta x_1 + \beta x_2 + \beta x_3 + \beta x_1 x_2 + \beta x_1 x_3 + \beta x_2 x_3$$

It was possible to gauge the coefficients of the model and none of the parameters (sucrose, yeast extract, K_2HPO_4 or their interactions) were significant (Table 3). The first-order model, adjusted for the coded variables could not be described. Analysis of variance (ANOVA) suggested that none of the factors had major effects; there was no interaction or evidence of the lack of fitness for the curvature in the response of the explored region. The explained percentage of variation (R^2) was 67%. Therefore, in Experiment A, no effects of the variables were observed, when the concentrations of sucrose, yeast extract and K_2HPO_4 present in the basic medium were analyzed. Based on these results, the studies were continued with a formulation in which the C source was represented by sucrose, denominated as YSac medium.

Experiment B

In the second factorial plan with the addition of central points (Supplementary Tables S3 and S4), the cellular concentration of *R. tropici* CIAT 899 ranged from 1.00 × 10^9 to 3.11 × 10^9 CFU mL^{-1} (Table 4). The repeatability of the process was considered good due to the small variation presented by the central points. The regression coefficients of the model were determined and none of the parameters evaluated (sucrose, yeast extract, K_2HPO_4 or their interactions) was significant (Table 5). The first-order model, adjusted for the coded variables, could not be described. No major effects were observed

Table 2. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment A) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (cfu mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[a]	-1	-1	-1	5	0.4	0.5	1.56 10^9
2[a]	1	-1	-1	25	0.4	0.5	1.78 10^9
3[a]	-1	1	-1	5	2	0.5	3.56 10^9
4[a]	1	1	-1	25	2	0.5	2.67 10^9
5[a]	-1	-1	1	5	0.4	2.5	1.81 10^9
6[a]	1	-1	1	25	0.4	2.5	3.11 10^9
7[a]	-1	1	1	5	2	2.5	2.78 10^9
8[a]	1	1	1	25	2	2.5	2.22 10^9
9[a]	0	0	0	15	1.2	1.5	3.11 10^9
10[a]	0	0	0	15	1.2	1.5	2.11 10^9
11[a]	0	0	0	15	1.2	1.5	2.00 10^9

Table 3. Regression coefficient and *p*-values obtained for the factorial planning with central points (Experiment A).

Factor	Regression coefficient	*p*-values
Mean	2.43 10^9	0.0001
Sucrose	8.75 10^6	0.9590
Yeast extract	3.71 10^8	0.0812
K_2HPO_4	4.38 10^7	0.7982
Sucrose x Yeast extract	-3.71 10^8	0.0812
Sucrose x K_2HPO_4	1.76 10^8	0.3328
Yeast extract x K_2HPO_4	-3.51 10^8	0.0933

with any of the factors, interaction between them, or no evidence of lack of fitness for the curvature, according to the analysis of variance. The explained percentage of variation (R^2) was close to 67% (Table 5).

In Experiment B, the concentrations of the C, N and P sources present in the YSac medium were defined as low (-1), five times higher in the central point (0) and 10 times higher in the high level (1) (Supplementary Tables S3 and S4). The proportions of C : N : P present in the YSac medium were maintained as the basis for optimization, but again, no main effects or interactions was observed.

Experiment C

Cellular concentrations of *R. tropici* CIAT 899 ranged from 1.56 × 10^9 to 4.56 × 10^9 CFU mL^{-1} after 72 h of growth (Table 6). The small variation in the central points of the variable's response also indicated a good repeatability of the process (Table 6). The regression coefficients of the model were measured and both K_2HPO_4 and the interaction of K_2HPO_4 and yeast extract were significant. The first-order model, adjusted for the coded variables, was described as follows:

$$y = 2.74*10^9 + 5.96*10^8 x_3 - 5.14*10^8 x_2 x_3$$

Results of the analysis of variance indicated that the factor K_2HPO_4 (p = 0.0239) represented the main effect, and statistically significant interaction between K_2HPO_4 and yeast extract (p = 0.0379) was verified (Table 7). There was no evidence of lack of fitness for the curvature in the response of the explored region, indicating that the response surface was satisfactorily described by the model. The explained percentage of variation (R^2) was close to 77%.

The response surface and the level curves obtained are shown in Figures 1 and 2. The plot of experimental values against predicted values showed that points were randomly distributed near the line, indicating good agreement and that the model did not show lack of significant adjustment (Figure 3).

Table 4. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment B) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (CFU mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[b]	-1	-1	-1	5	0.4	0.5	1.78 10^9
2[b]	1	-1	-1	50	0.4	0.5	1.00 10^9
3[b]	-1	1	-1	5	4	0.5	3.11 10^9
4[b]	1	1	-1	50	4	0.5	2.11 10^9
5[b]	-1	-1	1	5	0.4	5	1.89 10^9
6[b]	1	-1	1	50	0.4	5	1.89 10^9
7[b]	-1	1	1	5	4	5	1.89 10^9
8[b]	1	1	1	50	4	5	2.00 10^9
9[b]	0	0	0	25	2	2.5	2.67 10^9
10[b]	0	0	0	25	2	2.5	2.22 10^9
11[b]	0	0	0	25	2	2.5	1.89 10^9

Table 5. Regression coefficient and *p*-values obtained for factorial planning with central points (Experiment B).

Factor	Regression coefficient	*p*-values
Mean	2.04 10^9	0.0000
Sucrose	-2.09 10^8	0.1763
Yeast extract	3.19 10^8	0.0664
K_2HPO_4	-4.12 10^7	0.7621
Sucrose x yeast extract	-1.38 10^7	0.9192
Sucrose x K_2HPO_4	2.36 10^8	0.1370
Yeast extract x K_2HPO_4	-2.91 10^8	0.0840

DISCUSSION

Studies carried out by Ormeño and Zúñiga (1998) with the purpose of evaluating economic alternatives for YM medium, aiming at enabling the commercial production of inoculants, reported no significant difference in the growth of *Rhizobium* sp. PLC213, isolated from *Phaseolus lunatus*, when sucrose and mannitol were compared, while glycerol resulted in significantly lower growth. Similar results were obtained in the current study for *R. tropici* CIAT 899. However, *R. freirei* PRF 81 did not present significant differences in the comparison of the evaluated C sources.

It is worth mentioning that Castellane et al. (2014) observed higher growth and production of exopolysaccharides (EPS) when strain PRF 81 was grown in culture medium containing sucrose as the C source. Therefore, besides being a C source for *Rhizobium*, sucrose can favor the production of EPS, contributing to the protection against desiccation, by forming a layer with high water content around the cell, and favoring biofilm formation (Donot et al., 2012), altogether, leading to an increase in microorganisms survival in liquid formulations (Singleton et al., 2002; Taurian et al., 2010; Herrmann and Lesueur, 2013), and improving the inoculum quality. Other cellular components related to the C metabolism, such as polyhydroxybutyrate (PHB), can also help bacterial cell survival, improving inoculant quality (Tal and Okon, 1985; Santos et al., 2017).

The response surface methodology (RSM) consists of a combination of mathematical and statistical tools to delineate and analyze experiments for mathematical modeling of the responses (Box et al., 1978). The methodology allows the responses optimization, contributing to the improvement of products and processes, saving time and costs (Bas and Boyaci, 2007; Hao et al., 2011; Sütoa et al., 2015). When the RSM was applied in Experiment C, the response surface was shown as an inclined plane with respect to the right-to-left ascending axes and indicated that higher values of CFU mL^{-1} were obtained by moving the experimental region to

Table 6. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment C) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (CFU mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[c]	-1	-1	-1	25	2	2	1.56 10^9
2[c]	1	-1	-1	45	2	2	1.56 10^9
3[c]	-1	1	-1	25	4.4	2	2.89 10^9
4[c]	1	1	-1	45	4.4	2	2.00 10^9
5[c]	-1	-1	1	25	2	5	3.00 10^9
6[c]	1	-1	1	45	2	5	4.56 10^9
7[c]	-1	1	1	25	4.4	5	3.00 10^9
8[c]	1	1	1	45	4.4	5	2.22 10^9
9[c]	0	0	0	35	3.2	3.5	3.00 10^9
10[c]	0	0	0	35	3.2	3.5	3.00 10^9
11[c]	0	0	0	35	3.2	3.5	3.33 10^9

Table 7. Regression coefficient and *p*-values obtained for factorial planning with central points (Experiment C).

Factor	Regression coefficient	*p*-values
Mean	2.74 10^9	0.0000
Sucrose	-1.38 10^7	0.9388
Yeast extract	-7.12 10^7	0.6937
K_2HPO_4	5.96 10^8	0.0239
Sucrose x yeast extract	-4.04 10^8	0.0744
Sucrose x K_2HPO_4	2.09 10^8	0.2825
Yeast extract x K_2HPO_4	-5.14 10^8	0.0379

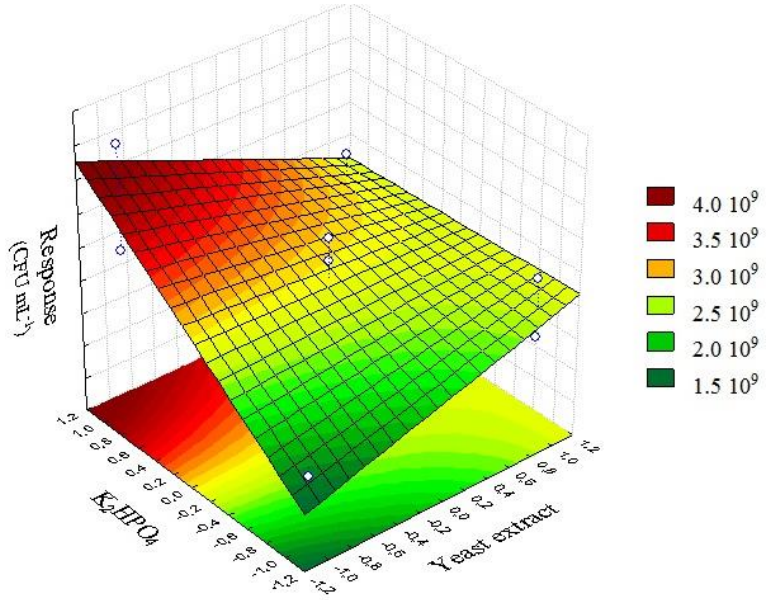

Figure 1. Response surface obtained for the factorial planning of 2^3 with three central points in Experiment C.

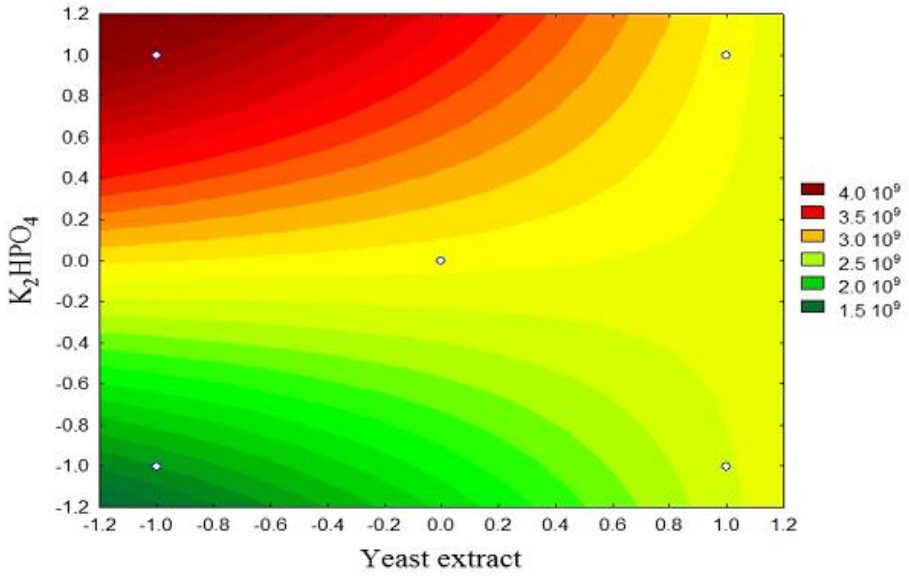

Figure 2. Level curves of the third factorial planning of 2^3 with three central points obtained in Experiment C.

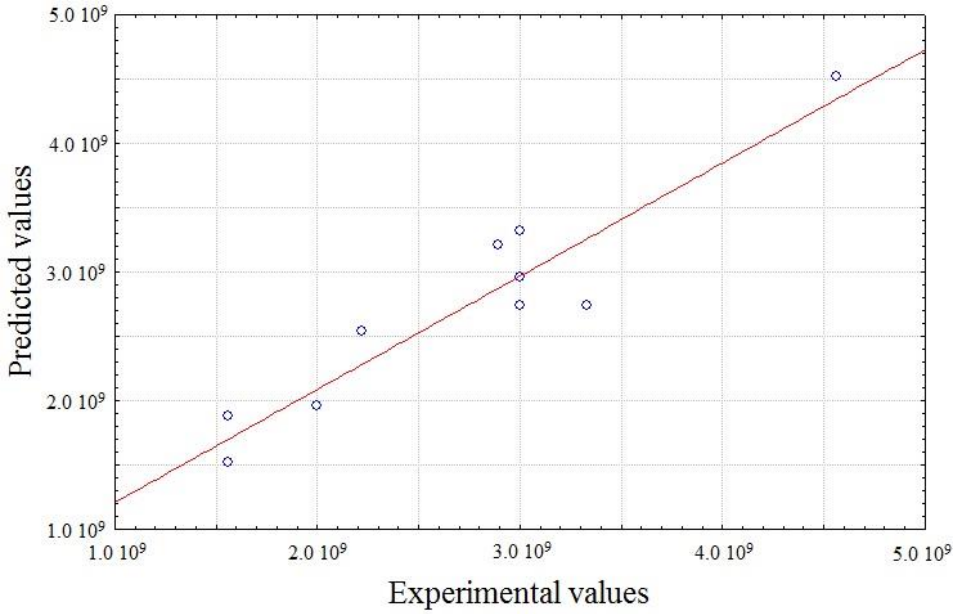

Figure 3. Experimental values plotted against predicted values for the model in factorial design 2^3 with three central points obtained in Experiment C.

lower values of yeast extract and higher values of K_2HPO_4 (antagonistic effect of factors). The results obtained in the present study have impact on the industry because the use of low concentration of yeast extract to obtain high cell growth rate is economically important, considering the high cost of this compound. In addition, there might be other benefits for the bacterium, as it has been observed for some species that the presence of high concentration of yeast extract (above 0.35%) may result in deformed cells and low viability (Skinner et al., 1977; Ben Rebah et al., 2007).

In Experiment C, higher concentrations of the three

factors were evaluated at lower level, in order to increase the cell concentration and to look for significant effects. Considering the RSM, the model $y = 2.74*10^9 + 5.96*10^8x_3 - 5.14*10^8x_2x_3$ would be used for the displacement. As factor 3 (variable K_2HPO_4) was the only one showing a significant effect according to the analysis of variance, the changes in the sucrose and yeast extract concentrations had no effect. The optimum values in the central point were determined. Recently, RSM was shown to be applicable in developing an inoculum from the plant-growth promoting bacterium, *Azospirillum brasilense* (Oliveira et al., 2017). From the current results, it can be concluded that the RSM can also be applied for the development of a basic liquid formulation for strains of the *R. tropici* group. The optimum values in the central point indicated the following concentrations: sucrose, 35 g L^{-1}; yeast extract, 3.2 g L^{-1} and K_2HPO_4, 3.5 g L^{-1}. This formulation may now be tested in the industry, validated at the field, have high impact on agriculture sustainability and increase the adoption of inoculation with elite strains by farmers growing common bean.

Conclusion

The response surface methodology (RSM) was used for the development of a liquid formulation for strains of *R. tropici* group, and to show that bacterial growth optimization required low concentrations of yeast extract and high concentrations of K_2HPO_4. A basic formulation that can impact the adoption of inoculation of the common bean crop was obtained.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Response surface methodology: Experiment A, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	5	15	25
Yeast extract (g L^{-1})	X_2	0.4	1.2	2
K_2HPO_4 (g L^{-1})	X_3	0.5	1.5	2.5

Table S2. Factorial planning of 2^3 (experiment A) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment A. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1a	-1	-1	-1	5	0.4	0.5
2a	1	-1	-1	25	0.4	0.5
3a	-1	1	-1	5	2	0.5
4a	1	1	-1	25	2	0.5
5a	-1	-1	1	5	0.4	2.5
6a	1	-1	1	25	0.4	2.5
7a	-1	1	1	5	2	2.5
8a	1	1	1	25	2	2.5
9a	0	0	0	15	1.2	1.5
10a	0	0	0	15	1.2	1.5
11a	0	0	0	15	1.2	1.5

Table S3. Response surface methodology: Experiment B, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	5	25	50
Yeast extract (g L^{-1})	X_2	0.4	2	4
K_2HPO_4 (g L^{-1})	X_3	0.5	2.5	5

Table S4. Factorial planning of 2^3 (experiment B) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment B. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1b	-1	-1	-1	5	0.4	0.5
2b	1	-1	-1	50	0.4	0.5
3b	-1	1	-1	5	4	0.5
4b	1	1	-1	50	4	0.5
5b	-1	-1	1	5	0.4	5
6b	1	-1	1	50	0.4	5

Table S4. Contd

7b	-1	1	1	5	4	5
8b	1	1	1	50	4	5
9b	0	0	0	25	2	2.5
10b	0	0	0	25	2	2.5
11b	0	0	0	25	2	2.5

Table S5. Response surface methodology: Experiment C, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	25	35	45
Yeast extract (g L^{-1})	X_2	2	3.2	4.4
K_2HPO_4 (g L^{-1})	X_3	2	3.5	5

Table S6. Factorial planning of 2^3 (experiment C) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment C. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1c	-1	-1	-1	25	2	2
2c	1	-1	-1	45	2	2
3c	-1	1	-1	25	4.4	2
4c	1	1	-1	45	4.4	2
5c	-1	-1	1	25	2	5
6c	1	-1	1	45	2	5
7c	-1	1	1	25	4.4	5
8c	1	1	1	45	4.4	5
9c	0	0	0	35	3.2	3.5
10c	0	0	0	35	3.2	3.5
11c	0	0	0	35	3.2	3.5