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Halomonas desertis G11, *Pseudomonas rhizophila S211* and *Oceanobacillus iheyensis E9* as biological control agents against wheat fungal pathogens: PGPB consorcia optimization through mixture design and response surface analysis

Abstract

Chemical agents are widely used to control agricultural pests and pathogens. However, in the last two decades, there is a strong demand for safe, effective and environmentally responsible strategies and techniques to control agricultural pathogens, and an increasing social pressure to progressively replace them with biological control agents. In this context, the application of Plant Growth Promoting Bacteria (PGPB) as biopesticides is recognized as one of the most promising tools to control plant pathogens. Therefore, the present study aimed to examine the role of three PGPB, selected from the Tunisian BVBGR bacterial collection and namely *Halomonas desertis G11*, *Pseudomonas rhizophila S211*, et *Oceanobacillus iheyensis E9* in promoting the wheat plant growth and in enhancing the defense response in wheat against four fungal pathogens identified as *Alternaria terricola*, *Chaetomium elatum*, *Fusarium tricinctum* and *Lewia infectoria*. The selected strains possessed PGP traits including ACC deaminase, indole acetic acid, inorganic phosphate solubilization. On the other hand, the strains produced siderophores, hydrogen cyanide and cell wall degrading enzymes, which can protect plants from the infection of pathogens. Using a mixture design and response surface methodology, an optimisation strategy was performed to find optimum strain combinations for maximum growth inhibition of wheat fungal pathogen. High regression coefficients R^2, between the variables and the responses indicated excellent evaluation of experimental data by the polynomial regression models. A remarkable fungal growth inhibition was observed using mono and mixed cultures. The antifungal activity of selected PGPB seems to be efficient against wheat fungal pathogens with the exception of *A. terricola*. These results indicate that extremophilic PGPB biocontrol agents have potential to promote the wheat growth under biotic stressors and can be further tested at field level for exploitation as bioinoculants.

Keywords: mixture design, RSM, PGPB consortia, biocontrol agents, wheat fungal pathogens, sustainable agriculture

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Introduction

Plant fungal pathogens cause significant damage to agricultural ecosystems and increasingly threaten global food security.[1] Cereal crops provide a greet proportion of the human population's caloric needs.[2] These crops are threatened by many pathogenic fungi that develop at different stages, resulting in huge yield losses and mycotoxin production.[3,4] Chemical control of plant pathogenic fungi is a strategy that seems to be effective in combating various biotic stresses. However, the inappropriate and inappropriate use of chemical pesticides has adverse effects on bio-flora, wildlife and natural enemies.

In response to environmental and regulatory pressures, the use of plant-growth-promoting bacteria (PGPBs) as biopesticides is an emerging and environmentally compatible technology that is considered a promising alternative to synthetic pesticides.[5–8] The PGPB strains can promote plant growth through the production of various metabolites that directly or indirectly promote plant growth.[9–11] The different modes of action of microbial biopesticides include antibiosis, production of siderophores, hydrogen cyanide, cell wall degrading enzymes, bio-surfactants and volatiles, and also induces systemic resistance in plants.[7,9,12,13]

Design of experiments is a powerful mathematical and statistical tool for obtaining more economical and profitable bioprocesses.[4,11,14,15] By using this experimental methodology, it is possible to optimize the process with a reduced number of experiments, by analyzing the interaction between the variables involved.[11] Mixing designs is a well-developed experimental approach to optimizing mixtures, in which the final product depends on the relative proportion of its constituents.[16,17] The formulation and optimization of effective biological inoculants from PGPB consortia requires a thorough understanding of interaction modes, bacterial adhesion to seeds and colonization of plant roots. In addition, antagonistic relationship studies should be conducted prior to the design and application of formulations containing microbial consortia, as some antagonistic effects may occur in plant-associated microbial consortia.[18–20]

Keeping this in view and the growing importance of PGPB control agents, this study aimed to investigate and evaluate the biocontrol potential of three newly isolated extremophilic PGPB strains against different wheat pathogenic fungi.[19] Specifically, we apply a simplex-centroid mixture design and response surface methodology to optimize the proportions of each strain for the protection of wheat against wheat fungal pathogens. Relationships between the different

proportion combinations and their effect on fungal growth inhibition were analyzed through a specialized experimental design software to select the optimal bacterial mixture.

Materials and methods

Isolation and identification of wheat pathogenic fungi

The samples of infected wheat leaves (cultivars Karim) were collected from an agricultural field situated in the region of Beja (North-East of Tunisia) and subjected to fungi isolation. Small pieces of the infected tissues were placed on Potato Dextrose Agar medium amended with streptomycin (1 mg mL-1) to inhibit bacterial growth and incubated for 7 days at 28°C. Emerging fungal hyphal tips were removed using a sterile scalpel, deposited in the center of new PDA-streptomycin plates and incubated at 28°C. Purified fungi were characterized based on the fungal culture and hyphal morphologies. Fungal identification was performed according to Sendi et al.[21] In brief, fungal DNA extraction consists of cell lysis using a TES buffer (100 mM TrisHCl, 10 mM EDTA, SDS 2%) and proteinase K (1 mg mL^{-1}) followed by sodium acetate purification. Obtained DNA was subjected to the amplification of the ribosomal ITS (Internal transcribed Spacer) region using universal primers ITS1 (5'- TCGGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′). DNA amplification reaction cycling and conditions were performed according to Sendi et al.[21] The sequences obtained were compared to reference sequences in the NCBI GenBank database using the BLASTN search option.

In vitro direct and indirect plant growth promoting traits of selected PGPB

In this study, three PGP bacterial strains were selected from the bacterial collection of the BVBGR laboratory (ISBST, University of Manouba, Tunisia). They include *Pseudomonas rhizophila* S211, *Halomonas desertis* G11 and *Oceanobacillus iheyensis* E9.[10,11,19] The selected PGPB were screened for multiple plant growth promoting and biocontrol activities including (i) nitrogen fixation revealed on Jensen's medium, (ii) phosphate solubilization revealed on Pikovaskya medium, (iii) siderophore production detected by chromeazurol S (CAS) shuttle solution, (iv) ammonia (NH_3) production revealed by addition of Nessler's reagent, (v) indoleacetic acid (IAA) production detected using the Salkowski's reagent method and (vi) hydrogen cyanide release detected using the picrate filter paper method.[10,20] The production of extracellular hydrolytic enzymes was revealed in agar plate assays using carboxymethylcellulose (CMC), skim milk and chitin as inducer substrates for cellulase, protease and chitinase activities, respectively.[10,11]

Biocontrol measurement and biocompatibility evaluation of selected PGPB strains

In order to test the antifungal activity of the selected PGPB against the phytopathogenic fungi, the dual culture technique was applied.[22] After plate incubation at 28°C for 7 days, the fungal inhibition growth percentage was estimated according to the formula:

$$\%\ inhibition = \frac{R_1 - R_2}{R_1 \times 100} \times 100$$

, where R_1 = radial growth of fungi mycelia (control); R_2 = radial growth of fungi mycelia in the presence of the PGP bacteria.

To screen the PGPB antibacterial activities, the agar overlay method was applied.[23] In brief, a volume of 1 mL of an overnight PGPB culture was mixed with 10 mL of molten agar medium and poured into a Petri dish. After cooling, plates were spotted with the other PGP bacterial suspensions and incubated. The presence of inhibition zone indicates a positive antagonistic activity.

To study the antagonistic properties of single characterized strains, a single bacterial strain was streaked as a straight line in the center of nutrient agar plate. Cultures to be tested were streaked perpendicularly across the initial culture and incubated at 28 °C for 48–96 h. Lack of microbial growth (zone of inhibition) at the intersections was indicative of the antagonism of the cultures[24] but the cultures growing in close proximity were compatible to each other. Further confirmation of mutual compatibility was done by spectrophotometric method by growing the strains in nutrient broth.

Optimization of biocontrol agent formula against wheat pathogenic fungi using a mixture design

The mixture design concept was firstly introduced by Scheffe in 1958 and after that modified mixture design methods have been developed such as the simplex centroid design method. Mixture designs are commonly applied in industrial product formulations in particular in food, pharmaceutical and chemical formulations.[25] In this paper, a simplex-centroid mixture design was applied to find optimum PGPB strain combinations for maximum growth inhibition of wheat fungal pathogens (Figure 1).[26] Three combinations are in the corners of the ternary plot experimental domain and represent the use of only a single bacterial strain specified in that corner. Three binary consortia are in the middle of the axis between two corners and represent a 50-50% combination of two strains named at those corners. The last point is located in the center of graphical experimental domain, and represent the mixture containing the same concentration for all three strains.

The simplex centroid design was applied in this study to fit the following polynomial model:

Fungal growth inhibition

$$(\%) = b_1 S211 + b_2 E9 + b_3 G11 + b_{12} S211E9 + b_{13} S211G11 + b_{23} E9G11$$

; where, S211, E9 and G11 are the code of selected PGPB strains *Pseudomonas rhizophila*, et *Oceanobacillus iheyensis* and *Halomonas desertis*, respectively; b_1, b_2 and b_3 are the linear coefficients and b_{12}, b_{13} and b_{23} are the interaction coefficients.

Design points (runs n° 1 to 7) were replicated twice in order to estimate the variance of the experimental error and to check the adequacy of the fitted models. The least squares method, a standard approach in regression analysis, was used to estimate the model's coefficients.[25] The statistical significance F, the ratio of the mean square variation due to regression and mean square residual error was tested using analysis of variance (ANOVA).[27]

Statistical analysis

NemrodW software was used to build the experimental design, conduct data calculations, plot the contours of the predicted responses and determine the optimal settings of the component proportions.[28]

Results and discussions

The socioeconomic importance of cereals makes control of cereal diseases a priority. To address this problem, farmers usually use synthetic chemicals, with major negative consequences for the environment and human health.[29] Recently, there are many examples of effective control of soilborne diseases by means of PGPB, and many strains have been shown to have potential for development

as biocontrol agents on cereals.[30–32] Although many PGPR based bioproducts are available as soil inoculants for cereals, the majority of them has marketed as biofertilizers and not as biocontrol agents. The lack of both stable formulations and knowledge of microbial adaptation to various environments means that the potential of biocontrol agents to control disease under field conditions has not been fully exploited. In this respect, formulations based on extremophilic PGPB have advantages over mesophilic bacteria, as they can withstand various biotic and abiotic stresses.[33] This present study investigated the biocontrol abilities of individual species of three extremophilic PGPB and their binary and ternary consortia against wheat phytopathogenic fungi. The isolation of fungal pathogens was carried out from symptomatic wheat plants. Four different fungi representing distinct phenotypic traits were selected. The isolates were characterized macroscopically and microscopically. A noticeable variability of the fungal mycelium color and colony diameter was observed and presented in Figure 2. After macro- and microscopic characterizations, fungal isolates were identified by fungal DNA extraction, followed by amplification and sequencing of the intergenic region (ITS) of nuclear rDNA. The sequence similarity search was performed using the BLASTn software in the NCBI genebank. Molecular analysis revealed that fungal isolates belong to the following species: *Alternaria terricola* (C2), *Chaetomium elatum* (C3), *Fusarium tricinctum* (C5) and *Lewia infectoria* (C6) (Table 1). These strains showed wheat seed germination inhibition rates varying between 60 and 80%. These pathogenic fungi especially *Fusarium* spp. and *Alternaria* spp. are common colonizers of the wheat phyllosphere and they can be pathogenic and produce mycotoxins that are harmful to consumers. Their in-field infection dynamics have been a focus for the development of new control strategies.[34]

Table 1 Molecular identification of fungal strains based on ITS rDNA sequence analysis

Strain code	Closet match	GenBank accession number[a]	Similarity (%)
C2	*Alternaria terricola*	MF480416.1	99
C3	*Chaetomium elatum*	MN249735.1	90
C5	*Fusarium tricinctum*	JX119038.1	99
C6	*Lewia infectoria*	FR648346.1	100

[a]Accession number of the most related organism

The screening of PGPB strains from the BVBGR bacterial collection allowed the selection of three wheat growth stimulating strains namely *Halomonas desertis* G11, *Pseudomonas rhizophila* S211 and *Oceanobacillus iheyensis* E9. Results from the *in-vitro* bio-control assay (HCN, NH_3, siderophore, cellulase, chitinase, protease and IAA) revealed that three strains (S211, G11 and E9) exhibited consistent bio-control characteristics (Table2). The production of HCN by PGPB played an important role in the biological control of several soil-borne pathogenic fungi as reported by Azeem et al.[35] HCN is a toxic chemical produced by some bacteria and acts as a metabolic inhibitor. It is synthesized, excreted and metabolized by these bacteria to avoid predation or competition.[36] G11, S211 and E9 strains were able to produce IAA in varying quantities. G11 strain was the highest producer, followed by S211 strain while isolate E9 was the lowest. Recent studies have shown that IAA biosynthesis is highly influenced by L-tryptophan which is believed to be the primary precursor for the production of IAA.[37] L-tryptophan is a phytohormone which affects many physiological activities of plant such as cell enlargement, cell division, root initiation, and growth rate. The selected biocontrol PGPB could exert their antagonistic activity against plant pathogens by means of secretion of siderophores. These low molecular weight compounds preferentially chelate iron and transport it into the cell across the cell membrane. The siderophores bind most of the Fe^{3+} and effectively prevent the proliferation of fungal pathogens by depriving them of available iron. Suppression of the pathogens arises because iron deficiency causes growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation, and causes changes in cell morphology.[12] An interesting mechanism used by biocontrol agents against soilborne pathogens is the production of cell wall degrading enzymes.[35,38] Cell wall degrading enzymes such as β -1, 3-glucanase, chitinase, cellulase, and protease secreted by biocontrol agents exert a direct inhibitory effect on hyphal growth of fungal pathogens. All potential biocontrol agents used in this study were found to be compatible with each other. Dual culture studies revealed no mutual growth inhibition. Growth rate of individual strains was measured by spectrophotometric method. The individual growth of strains was not affected significantly as evidenced by the optical density measurements of each the bacterial strains.

Table 2 *In vitro* plant growth promoting and biocontrol traits of selected PGPB

Strain	HCN	NH_3	IAA	siderophore	N fixation	P-solubilization	chitinase	cellulase	protease	Osmotic stress resistance (15% NaCl)
S211	+	+	+	+	+	+	+	+	+	-
G11	+	+	+	+	-	+	+	-	+	+
E9	+	+	+	+	-	+	+	+	+	+

+ presence; - absence

xAll the thre strains were tested as biocontrol agents against *C. elatum*, *A. terricola*, *C. globosum*, *F. tricinctum* and *L. infectoria*. PGPB consorcia optimization was carried out using a mixture design and response surface methodology. The three PGPB were used as individual or mixture cultures, ranging from 0 to 100% (Figure 1). Fourteen experiments were carried out according to the experimental conditions indicated in Table 3. The measured values were reported in the last columns. The analysis of variance, ANOVA, of mixture design for pathogenic fungal inhibition using PGPB consortium is presented in Table 4 which shows that the lack of fits of the regression models are significant and fisher F test demonstrate high significant ($P< 0.05$) for the regression. All the coefficient of determination (R^2)

and the adjusted coefficient of determination (R^2A) values were near to 1. Thus, we can conclude that the simplex-centroid mixture design models are adequate to describe the four-response surfaces and can be used as prediction equations as follows:

A. terricola growth inhibition

$$(\%) = 53{,}365\,S211 + 41{,}300\,E9 + 33{,}000\,G11 - 119{,}159\,S211E9 - 20{,}539S211G11 + 156{,}831E9G11$$

C. elatum growth inhibition

$$(\%) = 49{,}576\,S211 + 35{,}346\,E9 + 2{,}391\,G11 + 214{,}676S211E9 + 283{,}406\,S211G11 + 52{,}986\,E9G11$$

F. tricinctum growth inhibition

$$(\%) = 32{,}099\,S211 + 34{,}659\,E9 + 3{,}594\,G11 - 67{,}757\,S211E9 - 49{,}847\,S211G11 + 76{,}613\,E9G11$$

L. infectoria growth inhibition

$$(\%) = 92{,}942\,S211 + 56{,}792\,E9 + 20{,}392\,G11 + 49{,}629\,S211E9 - 34{,}731\,S211G11 - 94{,}471\,E9G11$$

Table 3 Mixture design of biopesticides PGPB based consortia and the corresponding observed and predicted responses.

N exp.	*P. rhizophila* S211 (%)	*O. iheyensis* E9 (%)	*H. desertis* G11 (%)	Growth inhibition of *A. terricola* (%)		Growth inhibition of *C. elatum* (%)		Growth inhibition of *F.tricinctum* (%)		Growth inhibition of *L. infectoria* (%)	
				Yexp.	Ycalc.	Yexp.	Ycalc.	Yexp.	Ycalc.	Yexp.	Ycalc.
1	100	0	0	29.72	32.099	90.9	92.942	52.17	53.365	54.05	49.576
2	100	0	0	34.29	32.099	94.2	92.942	54.36	53.365	46.32	49.576
3	0	100	0	33.33	34.659	53.6	56.792	40.1	41.3	39	35.346
4	0	100	0	35.8	34.659	59.2	56.792	42.3	41.3	32.91	35.346
5	0	0	100	0	3.594	16.6	20.392	34.6	33	0	2.391
6	0	0	100	7	3.594	23.4	20.392	31.2	33	6	2.391
7	50	50	0	20.33	16.44	86.36	87.275	16.66	17.543	90.06	96.13
8	50	50	0	13.3	16.44	91.33	87.275	19.23	17.543	97.33	96.13
9	50	0	50	2.12	5.385	46.51	47.985	40	38.048	90.2	96.835
10	50	0	50	9.4	5.385	52.6	47.985	36.9	38.048	98.6	96.835
11	0	50	50	41.17	38.28	18.86	14.975	75.02	76.358	26.66	32.115
12	0	50	50	36.14	38.28	14.23	14.975	78.5	76.358	32.7	32.115
13	33.33	33.33	33.33	20.5	18.895	46.51	47.864	45.8	44.454	100	90.319
14	33.33	33.33	33.33	15.6	18.895	42.15	47.864	41.3	44.454	91.6	90.319

Table 4 Analysis of variance (ANOVA) of pathogenic fungal inhibition by selected PGPB based on mixture design

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
A. terricola growth inhibition (%); R^2=0.953; R^2A=0.924					
Regression	2359.18	5	471.836	32,489	***
Residues	116.182	8	14.523		
Validity	2,325	1	2,325	0,143	NS
Error	113.858	7	16.265		
Total	2475.37	13			
C. elatum growth inhibition (%); R^2=0.987; R^2A=0.980					
Regression	10688.3	5	2137.67	1,256,929	***
Residues	136.057	8	17.007		
Validity	40.694	1	40.694	29,871	NS
Error	95.363	7	13.623		
Total	10824.4	13			
F. tricinctum growth inhibition (%); R^2=0.991; R^2A=0.985					
Regression	3992.87	5	798.575	1,701,309	***
Residues	37.551	8	4,694		
Validity	2,665	1	2,665	0,5348	NS
Error	34.886	7	4,984		

Table Continued..

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Total	4030.42	13			
L. infectoria growth inhibition (%); R^2=0.984; R^2A=0.973					
Regression	10688.3	5	2137.67	1,256,929	***
Residues	136.057	8	17.007		
Validity	40.694	1	40.694	29,871	NS
Error	95.363	7	13.623		
Total	10824.4	13			

***: significant at the level of 99.9 %; NS: non-significant

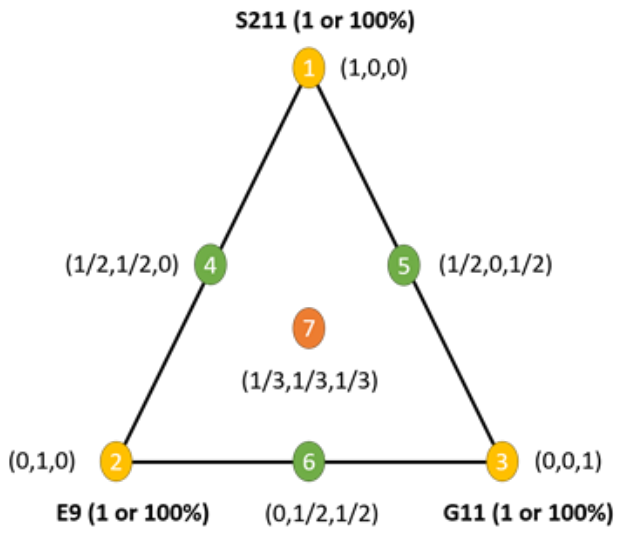

Figure 1 Seven experimental points of a simplex centroid design for ternary mixtures used to optimize biopesticide formula.

In the model equations, the model coefficients values were calculated from observed values of the responses (fungal growth inhibition, %). Coefficients with one variable displays the antifungal activity of a specific strain and with two variables exhibit interaction between bacterial cultures. The magnitude of coefficient indicates its contribution to the response (Table 5). The negative sign of the coefficient indicates that the factor will reduce the value of response while the positive sign in front of the terms indicates increase in the magnitude of the response (Table 5). For an example, the coefficient of interaction terms had very high magnitude indicating that interaction of the individual strains plays a major role in *C. elatum* growth inhibition (Table 5). The variation of the different antifungal responses according to the proportions of the three PGPB strains is represented by equilateral triangles whose vertices correspond to monocultures (Figure 3). The information we are looking for in the ternary representation is the direction of the evolution of the antifungal activity according to the proportions in bacterial biopesticides (Figure 3). The dominant strain is easily identified in the diagram as well as its influence on the response. The realization of experimental mixture design and the statistical and graphical exploitation clearly show the biological control potential of selected *PGPB* against wheat fungal pathogens. The mixed culture of extremely halophilic strains *P. rhizophila* S211 and *O. iheyensis* E9 has been demonstrated as highly effective consortium for the growth inhibition of *C. elatum* and *L. infectoria* (> 90%) (Table 6). The monoculture of *P. rhizophila* S211 and the binary consortium S211-G11 could also be considered as efficient biocontrol agents against wheat fungal pathogens *C. elatum* and *L. infectoria*, respectively (Table 6). The maximal growth inhibitions of *F. tricinctum* and *A. terricola* have reached about 80% and 40% respectively, after applying a binary consorcium (50/50%) of *O. iheyensis* E9 and *H. desertis* G11 (Table 6). In general, this study shows that mixture design and response surface methodology were appropriate methods to optimize the best bacterial consortia for obtaining maximum growth inhibition of each wheat fungal pathogen. The experimental and the predicted values were very close which reflected the accuracy and the applicability of RSM. By applying mixture designs and RSM to the optimization experiments, we can propose and develop novel biocontrol PGPB consortia against crop pathogens.[39–42]

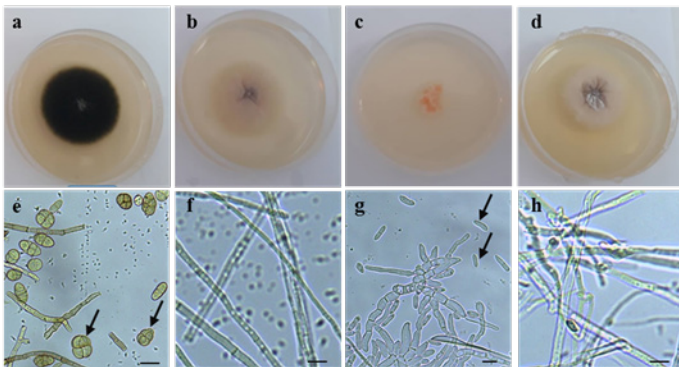

Figure 2 Colony morphology of isolated fungi on PDA: (a) *Alternaria terricola*, (b) *Chaetomium elatum*, (c) *Fusarium tricinctum*, (d) *Lewia infectoria*; Microscopic view (100 x magnification) of (e) Conidia of *Alternaria terricola* indicated with black arrows, (f) hyphal morphology of *Chaetomium elatum*, (g) microconidia of *Fusarium tricinctum* indicated with black arrows, (h) hyphal morphology of *Lewia infectoria*. Scale bars: 20 µm (e, f, h), 5 µm (g).

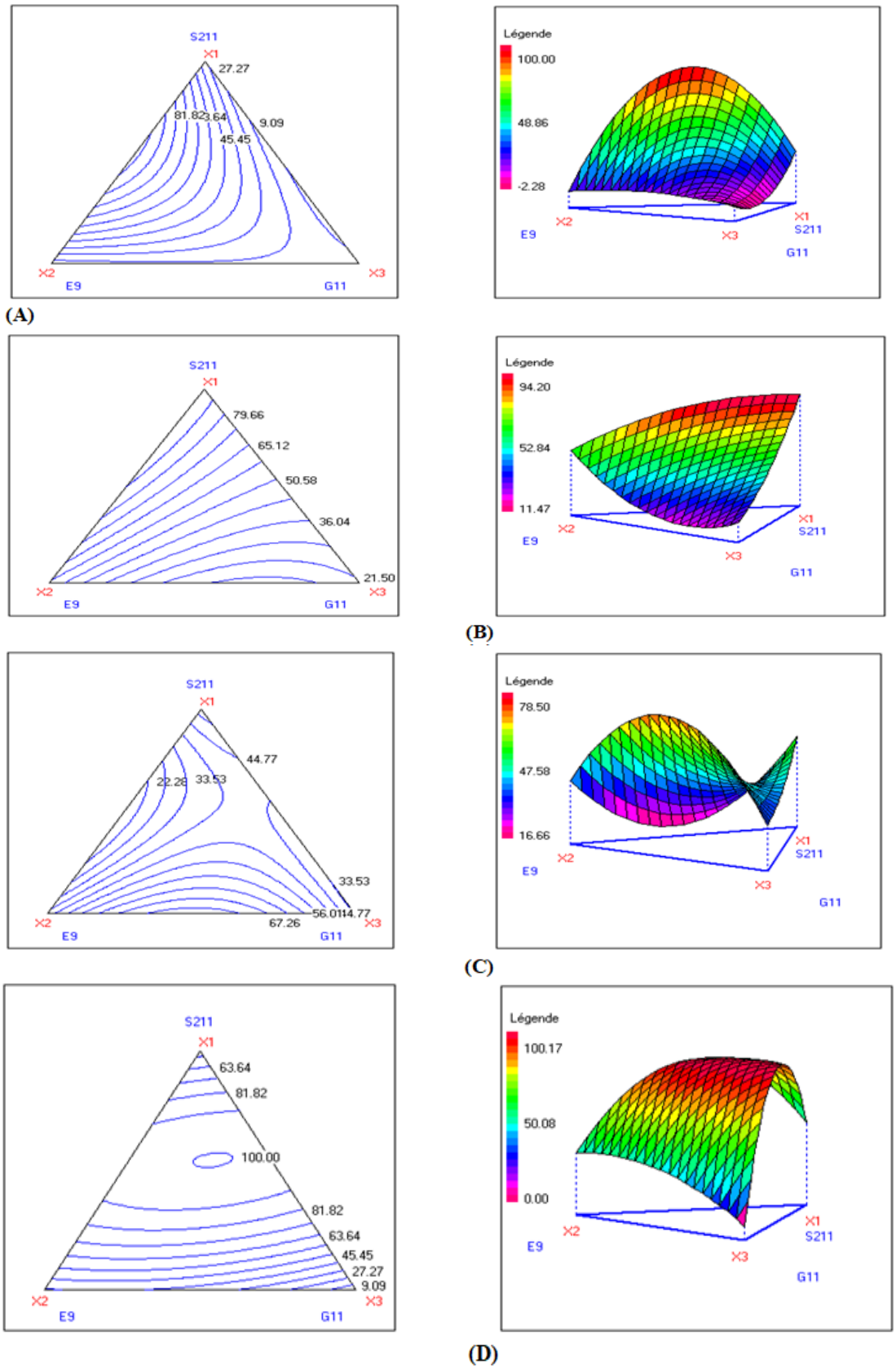

Figure 3 Mixture ontour plots and response surfaces curves showing interactive effect of three PGPB (*H. desertis* G11, *P. rhizophila* S211, *O. iheyensis* E9) on pathogenic fungal growth inhibition of **(A)** *Alternaria terricola*, **(B)** *Chaetomium elatum*, **(C)** *Fusarium tricinctum* and **(D)** *Lewia infectoria.*

Table 5 Estimated effect, regression coefficient, and corresponding t and P values in central composite design experiments

Name	Coefficient	F. Inflation	Stand. Dev	t.exp.	Significance
A. terricola growth inhibition (%)					
b_1	32,099	1,60	2,684	11,96	***
b_2	34,659	1,60	2,684	12,91	***
b_3	3,594	1,60	2,684	1,34	NS
b_{12}	-67,757	1,57	12,340	-5,49	***
b_{13}	-49,847	1,57	12,340	-4,04	**
b_{23}	76,613	1,57	12,340	6,21	***
C. elatum growth inhibition (%)					
b_1	92,942	1,60	2,905	31,99	***
b_2	56,792	1,60	2,905	19,55	***
b_3	20,392	1,60	2,905	7,02	***
b_{12}	49,629	1,57	13,354	3,72	**
b_{13}	-34,731	1,57	13,354	-2,60	*
b_{23}	-94,471	1,57	13,354	-7,07	***
F. tricinctum growth inhibition (%)					
b_1	53,365	1,60	1,526	34,97	***
b_2	41,300	1,60	1,526	27,06	***
b_3	33,000	1,60	1,526	21,62	***
b_{12}	-119,159	1,57	7,015	-16,99	***
b_{13}	-20,539	1,57	7,015	-2,93	*
b_{23}	156,831	1,57	7,015	22,35	***
L. infectoria growth inhibition (%)					
b_1	49,576	1,60	4,164	11,91	***
b_2	35,346	1,60	4,164	8,49	***
b_3	2,391	1,60	4,164	0,57	NS
b_{12}	214,676	1,57	19,141	11,22	***
b_{13}	283,406	1,57	19,141	14,81	***
b_{23}	52,986	1,57	19,141	2,77	*

Table 6 Optimal biopesticide consorcia for maximum growth inhibition of selected pathogenic wheat fungi

Pathogenic fungi	Optimal consorcia composition	Theoretical fungal growth inhibition (%)	Experimental fungal growth inhibition (%)
Alternaria terricola	E9-G11 (50/50%)	38.28	41.17
Chaetomium elatum	S211 (100%) or S211-E9 (50/50%)	92.94	94.2
		87.27	91.33
Fusarium tricinctum	E9-G11(50/50%)	76.35	78.5
Lewia infectoria	S211-E9 or S211-G11 (50/50%)	96.13	97.33
		96.83	98.6

Conclusion

Sustainable agriculture is the solution to the problems resulting from the excessive and uncontrolled use of agronomic techniques based on chemical fertilizers and pesticides to increase crop yield. Therefore, an ecological substitute to chemicals has become a necessity. Due to their diverse and unique direct and indirect traits, PGPB-based microbial agents represent an attractive and realistic option to replace chemicals. This study demonstrated that simplex-centroid mixture design and response surface methodologies are very appropriate tools to optimize the proportions of synergistic PGPB strains in terms of growth inhibiting of wheat pathogenic fungi, *A. terricola*, *C. globosum*, *F. tricinctum* and *L. infectoria*. To the best of our knowledge, this is the first study illustrating the biological control potential of individual and mixed cultures of PGPB strains *H. desertis*, *P. rhizophila* and *O. iheyensis*. To validate these results, we need to perform these experiments under greenhouse and field conditions using free and immobilized biocontrol agents. Further studies were also needed to better understand biocontrol mechanisms of selected PGPB and to purify and characterize their bioactive compounds against fungal and insecticidal pathogens.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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