Effects of nutritional and environmental conditions on *Burkholderia* spp. ecophysiological requirements related to grapevine rhizosphere

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Abstract: *A better understanding of growth and viability of PGPR may provide valuable pathways into the utilization of beneficial bacteria as biofertilizer and plant growth stimulator in sustainable agriculture in whole Mediterranean area. This investigation focused on the use of the response surface methodology to study the effects of mannitol, aromatic organic matter, and osmotic pressure on growth and viability of Burkholderia spp. strain IF25, a PGPR isolated from Vitis spp. Experiments were carried out in vitro according to Box-Behnken matrix. Salt and L-tryptophan concentrations reduce Acridine Orange total biomass. Moreover cell viability was also affected.*

Key words: Plant growth promoting rhizobacteria, low input agriculture, inoculant, multiple regression, biocontrol.

INTRODUCTION

Consumer demand of natural and chemical free agricultural products is increasing at present in Europe. Plant Growth Promoting Rhizobacteria (PGPR) are the largest group of soil free living microorganisms, that play an important role in the growth of plants and in environmental conservation [6,11].

PGPR participate in different eco-physiological processes of plant and soil and therefore different species of PGPR are important for sustainable agriculture because of being used as bacterial inoculant in low input cropping system. *Burkholderia* spp. is a group of PGPR that can be often isolated from agricultural soils. Several studies have been lead to the discovery that these bacteria have relevant PGPR phenotypic traits: they are capable to solubilize insoluble phosphate, produce auxin–like compounds and siderophores [11].

It is well documentated that stress adaptations reduce PGPR success of inoculation and bacterial performance in arid and semi-arid agricultural soils [3, 9].

Therefore a better understanding of growth and viability of tolerant PGPR may provide valuable pathways into the utilization of these beneficial bacteria as biofertilizer for phytostimulation in sustainable agriculture and forestry in whole Mediterranean area [7].

This investigation focused on the use of the response surface methodology [1] to evaluate *Burkholderia* spp. ecophysiological responses in arid soils and the effects of salt, soil organic matter (SO) and mannitol, as relevant carbon source in stressed grapevine. Growth and viability of *Burkholderia* spp. were monitored *in vitro* experiments according to Box-Behnken matrix [2].

APPLICATION OF BOX-BEHNKEN DESIGN FOR ECOLOGICAL INVESTIGATION OF BACTERIAL SALT ADAPTATION

Bacterial strain

Burkholderia spp. IF25 was used throughout this study. Cells were grown in Luria-Bertani (LB) broth [8] at 32°C with shaking for 144h.

Preparation and inoculation of YMB-modified media

The basal medium used for bacterial growth was modified-YMB (g l^{-1}: K_2HPO_4-0,5; $MgSO_4 \cdot 7H_2O$-0.2; NaCl-0,1; Yeast extract-0.5) according to the 13 experiments of the Box-Behnken Design (Table 1). Aliquots of 12 ml were dispensed in 100 ml Erlenmeyer flasks and autoclaved at 121°C for 20 min.

The different *L*-tryptophan levels were adjusted using 50-100-150 μg ml^{-1} of tryptophan solution, salt concentration in this media was adjusted by 0.3-0.6-0.9% NaCl solution, mannitol concentration was adjusted by 3.33-6.66-9.99 gl^{-1}.

Flasks were inoculated with 0.5 ml of overnight bacterial suspension, that was grown in LB and incubated with shaking at 32 °C. All experiments of the matrix were performed three times.

Cell counts

Cell suspension were sampled for culturable and total cell numbers. Viable counts were determined at late stationary phase. Samples from each experiment were diluted 10-fold in 9% NaCl solution and 100 µl of suitable dilution were plated on LB agar.

The population was determined after 48 h incubation at 30^0C. All cell enumerations was repeated and CFU ml^{-1} were calculated. Culturable cells were counted on LB plates incubated at 30^oC for 2 days. The culturability was expressed as the percentage of total Acridine Orange cell counts.

Total (Acridine Orange direct count AODC) cell counts were determined by epifluorescent microscopy - acridine orange staining method [4, 12].

Formalin-fixed cells were filtered onto a GTBP-type black filter (pore size 0.2µm; millipore), and stained with 0.01% Acridine Orange for 1 min. The filters were examined under a Dialux E22EB Leitz microscope equipped with an epifluorescence apparatus: 50-watt UV mercury lamp (Osram) and Leitz filter block H3 (excitation, 420-490nm; dichroic mirror, 510nm; suppression, 515nm). A minimum of 500 cells in more than ten fields of vision, or in the case of law counts, at least 50 fields of vision, were counted for each sample.

Experimental design and interpretation of the results

The experimental design chosen for the study was a Box-Behnken design [2]. This design was preferred because relatively few experimental combinations of the variables are adequate to estimate complex response functions.

The Box-Behnken design contains k^2+k+1 points for k variables. Each experiment could be located by its three coded values. The coded matrix and experimental values of the three environmental factors (concentration of mannitol, tryptophan and salt) are listed in Table 1. After experimental data acquisitions multiple regression analysis based on the least square method was performed.

Statistical examination of results and generation of response surface were acheaved using the software package Modde 5.0 (Umetrics AB, Umeå, Sweden) [11].

The analysis concerned the linear and quadratic effects of the three factors and their interactions. Thus, according to [1], the equations giving biomass and culturability were second-order polynomial models with 10 coefficients (b_0, b_1, b_2,...b_{23}). The significance of the coefficients was evaluated by multiple regression analysis based upon the *F*-test with unequal variance, $P<0.05$, $P<0.01$ and $P<0.001$.

Results and Discussion

Estimates of growth (expressed as total AO cells ml^{-1} in stationary phases) and culturability of batch cultures were determined for each experiment of the matrix (Table 1 referred to a total of 39 experiments).

The lower *lag* time (λ = 18 h) associated with higher specific growth rates was obtained in trial 6 with mannitol at higher level, NaCl at lower level and intermediate value for *L*-tryptophan concentration. NaCl resulted as main environmental factor affecting cell counts.

A square regression model was fitted to the biomass data and Table 2 shows the results of multiple regression analysis which provided the estimates of model coefficients. The model was significant at the 95% confidence level and the coefficients of determination, i.e., r^2, was 0.98. Moreover prediction coefficient (Q^2 = 0.82) was statistically reliable.

Concerning culturability variations in late stationary phases, the negative value of prediction coefficients (Q^2 = - 1.25) suggested a poor model for fitting. In the present case the r^2 and Q^2 value obtained for biomass data sets reflects a good fit between the observed and predicted responses [5].

Therefore about 98 % of the variation about the mean could be explained by the model and it was reasonable to use this regression model to analyse the trends in the responses [1].

Model coefficients showed significant inhibition activity of salt on bacterial biomass expressed as AO cell counts.

In addition, the analysis suggested that the factors that affected the response where the squares of concentrations of mannitol, *L*-tryptophan and NaCl (b_{11}, b_{22} , b_{33} respectively). All these factors except the square of the concentration of salt has a negative effect on the growth responses. Instead, the positive value for b_{33} suggested the occurrence of some salt positive influences in whole experimental domain.

Table 1. Experimental matrix obtained by applying the Box-Behnken design methodology for three factors and the response means for AO cells ml^{-1} and culturability.

Coded values				Experimental values			Response means	
Trials	X_1	X_2	X_3	M ($g\ l^{-1}$)	Try ($g\ ml^{-1}$)	S (%)	AO (10^9cells ml^{-1})	Cult. * %
1	-1	-1	0	3.33	50	0.6	0.56	4.5
2	+1	-1	0	9.99	50	0.6	0.40	2.2
3	-1	+1	0	3.33	150	0.6	0.41	7.8
4	+1	+1	0	9.99	150	0.6	0.30	3.3
5	-1	0	-1	3.33	100	0.3	5.20	0.5
6	+1	0	-1	9.99	100	0.3	4.40	0.9
7	-1	0	+1	3.33	100	0.9	1.60	0.07
8	+1	0	+1	9.99	100	0.9	1.80	0.08
9	0	-1	-1	6.66	50	0.3	4.90	0.82
10	0	+1	-1	6.66	150	0.3	4.61	0.56
11	0	-1	+1	6.66	50	0.9	2.13	0.06
12	0	+1	+1	6.66	150	0.9	2.35	0.08
13	0	0	0	6.66	100	0.6	3.04	0.04

Legend: * culturability. The experimental values (Ui) were calculated from the coded values (Xi) using the formula: $U_i=U_{oi}+X_i \cdot \Delta U_i$, where U_{oi} is the centred value and ΔU_i the range. Mannitol for U_{oi} =6.66 g l^{-1} and ΔU_i =3.33 g l^{-1}; for Tryptophan, U_{o2} = 100g ml^{-1} and ΔU_2 = 50g ml^{-1} ; for NaCl, U_{o3} = 0.6% and $\cdot \Delta U_3$ = 0.3%.

The models representing *Burkholderia* spp. growth in presence of combined salt, mannitol, and aromatic nitrogen sources, were second-order polynomial equations. Therefore, in order to determine minimal and optimal bacterial cells development, surface contour plots were drawn (contour plot no showed).

More specifically, NaCl concentration was fixed arbitrarily at the centre of the matrix, while levels of the two other factor varied.

At low and high salt concentrations the intermediate levels of mannitol and *L*-tryptophan (corresponding to 6.66 g l^{-1} and 100 mg l^{-1} respectively) represented the optimal environmental factors for reducing NaCl constrains.

Hence, mannitol and *L*-tryptophan, representing rhizospheric carbon sources related to stressed grapevine in arid soils managed with sustainable agricultural practices, were identified as factors favorable to *Burkholderia* spp. persistence.

Surprisingly the model highlights, that cell biomass resulted more affected at intermediate NaCl concentration (100 mM), that at same time appeared to be the level that increased cell viability expressed as culturability percentage (Table 1). Thus, at 100 mM NaCl, despite of growth reduction, the effect of salt on viability was inversely related to general trend.

This finding indicate cell capacity to retain active metabolism and potentially plant infectiousness with the occurrence of a physiological adaptation to low salinity [9].

Salt ecophysiological adaptation may be a common phenomenon in arid soil bacteria [7] and incidentally the strain examined in this work, exhibiting plant growth promotion activities, was isolated from rhizospheric grapevine soils during the dry summer of Mediterranean season. In arid soil environment plant-microbe interactions are subjected to salt modulation and physiological adaptation of PGPR to salt often improve rhizosphere competence as well as physiological traits relevant for biocontrol and phytostimolation [3].

Table 2. Model coefficients of the experimental design obtained for AO cells ml^{-1} and culturability.

		Regression coefficients	
Factors	Coefficients	AO cells ml^{-1}	Culturability (%)
Response means	b_0	9.48***	0.04^{NS}
M	b_1	-0.03^{NS}	-0.80^{NS}
Try	b_2	-0.04^{NS}	0.52^{NS}
S	b_3	-0.20**	-0.31^{NS}
M^2	b_{11}	-0.45***	2.21^{NS}
Try^2	b_{22}	-0.40***	2.20^{NS}
S^2	b_{33}	0.43***	-1.86^{NS}
M x Try	b_{12}	-0.01^{NS}	-0.55^{NS}
M x S	b_{13}	0.03^{NS}	-0.10^{NS}
Try x S	b_{23}	0.02^{NS}	-0.07^{NS}

***P<0.001; **P<0.05; NS, not significant. (M – Mannitol; Try – Tryptophan; S- Salt).

CONCLUSIONS

In this work multiple regression analysis were performed in order to evaluate linear or quadratic effects of the environmental factors ecologically relevant for *Burkholderia* spp. as PGPR in grapevine rhizosphere. Results highlighted that the target strains was able to growth under all the conditions tested. Specifically high salt and *L*-tryptophan concentrations reduce Acridine Orange total biomass. Moreover cell viability were also affected by salt. Factors analysis showed that moderate levels of mannitol, as rhizospheric carbon source, and aromatic root exudates improve growth responses and salt adaptation.

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Докладът е рецензиран.