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Promoting of Abiotic Stress–Induced Resistance Using Poly-β-Hydroxybutyrate (PHB) By *Rhizobium phaseoli* In Common Bean Plants

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Abstract

In total, 50 *Rhizobium* isolates were isolated from the mature root nodules of common beans plants (*Phaseolus vulgaris*) grown in different nine governorates of Egypt. PHB was optimized by the identified strain using response surface methodology. A total of 11 parameters (pH, incubation period, inoculum size, temperature, agitation speed, mannitol, sucrose, yeast extract, glycine, K_2HPO_4, and $MgSO_4$) were analyzed for their significant effects on PHB production by the Plackett–Burman design (PBD). Sucrose, yeast extract, glycine, and $MgSO_4$ were the main significant factors affecting PHB accumulation. Central composite design (CCD) of the response surface methodology was used to determine the optimum levels of the selected factors. *Rhizobium phaseoli* reached the maximum production (4.997 g/L) at run 36 in the presence of 25 g/L of sucrose, 0.0 g/L of yeast extract, 0.87 g/L of glycine, 0.3 g/L of $MgSO_4$, and 5% of inoculation size. Invitro experiments were carried out to test the effect of different stress conditions (pH: 6–11, temperature: 5°C–50°C, salinity: 0.01%–7%, and drought: 0%–5% w/v) on the growth of *Rhizobium phaseoli*. The results showed that *Rhizobium phaseoli* can withstand 3% – 5% NaCl, high temperature of 30°C– 45°C, alkalinity at pH value of 8 – 10, and drought stress at 3% – 5% w/v polyethylene glycol with growth loss of 50% when grown on modified medium and 75% when grown on the basal one. In vivo experiments were done to study the effect of drought stress levels on the growth parameters of common bean plants. In general, all the treatments with *Rhizobium phaseoli* grown on the modified medium were superior to *Rhizobium phaseoli* grown on the basal medium. Also, they showed high tolerance of drought conditions.

Keywords: *Rhizobium phaseoli*; Poly-β-Hydroxybutyrate (PHB); Response surface methodology; Abiotic stress; Drought; Salinity

1 Introduction

Egypt is one of the Middle Eastern countries that is known for its wide cultivation of common bean in the newly reclaimed soils. However, due to its high salinity percentage, most newly reclaimed soils employ a negative effect on the plant crop yield. In Egypt, especially in the Northern central section of the Nile Delta and on its Eastern and Western sides, many salinization problems affect about

900,000 ha of these cultivated irrigated soil areas (FAO 2016).

Basically, the soil's chemical and biological characteristics can be enhanced by the use of biofertilizers. this enhancement will lead to the use of low doses of chemical fertilizers; hence, agricultural production will be free from contaminants (Selim and Zayed 2017). Crop productivity can be raised by the use of biofertilizers via plant hormone productivity, nitrogen fixation and phosphate solubilization. Also, biofrtilizers are recommended for the improvement of soil fertility. Endophytes are defined as microbes that colonize living, internal tissues of plants without exhibiting any negative effects (Selim and Zayed 2017). Rhizobia improves the defense mechanisms of plants against the stressed conditions via inducing the production of 1-aminocyclopropane-1-carboxylate deaminase (enzyme), which facilitates plant growth by decreasing the ethylene levels, inducing salt tolerance as well as decreasing drought stress in plants (Armada et al 2015).

As reported by Ali et al (2014), rhizobia accumulates PHB inside their cells as carbon storage polymers which can support their survival and reproduction in adverse conditions (soil salinization) and improve tolerance to osmotic stress; hence, these bacteria could increase the soil fertility, improve growth, and the yield of crops. Moreover, PHB can be a significant alternative to chemical fertilizers in agriculture. Tantawy (2009) proved that the endophytic strains of *Enterobacter aerogenens* and *Enterobacter gergoviae* that were isolated from the rice tissue had the ability to tolerate the high salinity of soil and stimulated plant growth–promoting bacteria. Heat and Drought stresses significantly decrease the symbiotic nitrogen fixation (SNF) by legumes in all semiarid, arid and tropic areas. Effectiveness of *Rhizobium* is adversely affected by the heat and drought stresses which leads to a reduction in both host legume and symbiont's growth and development (Hungria and Kaschuk 2014). In Eastern and Southern Africa, drought is the major production problem in addition to diseases, and it reduces grain yield by more than 50%. Drought restricts the flow of CO_2 into the mesophyll tissue and impairs photosynthesis, alters photosynthetic pigments and carbohydrates, causes the poor diffusion of CO_2 into leaves (Mathobo et al 2017) and reduces photosynthetic assimilation rates in addition to, acceleration of the reactive oxygen species production (Zlatev and Lidon 2012).
This work aims to the isolation of a novel high PHB-producing rhizobial strain and improving its PHB production strategies by more further studies to promote the abiotic stress-induced resistance in the common bean plants.

2 Materials and Methods

2.1 Media and solution used

A yeast extract mannitol (YEM) medium (Somasegaran and Hoben, 1994) was used to cultivate, purify, and maintain the *Rhizobium* sp. In addition to, Congo red yeast extract mannitol agar medium was used to purify *Rhizobium* isolates (Somasegaran and Hoben 1994). Importantly, N-free nutrient solution was prepared to be further used in plant nutrition as described by Broughton and Dillworth (1970).

2.2 Isolation, purification, and maintenance of *Rhizobium* isolates

Fifty common bean (*Phaseolus vulgaris*) seedlings having well developed nodules were collected as described by Vincent (1970). For the isolation of rhizobial isolates, the nodules of *Phaseolus vulgaris* were washed, sterilized, crushed, and then cultured on the YMA agar for 48 hours at 28°C according to Somasegaran and Hoben (1994). All the isolates were purified by subculturing the YEM medium at monthly intervals and maintained at 4°C for further studies according to Vincent (1970). The standard inoculum comprising $3.8 – 4.1 \times 10^8$ CFU ml^{-1} was prepared as described by (Girgis and Traore 2000) for further use in the shake flask experiments.

2.3 Seeds and plant inoculation technique

For inoculation, the common bean seeds (*Phaseolus vulgaris*) were sterilized as described by Ahmad et al (2016) and sown individually in the plastic pots having a diameter of 15 cm. All the pots were inoculated by 1 mL containing 10^9 CFU/mL of *Rhizobium* cells and irrigated once daily using the free N nutrient solution according to the water requirements (WR) recommended by Allen et al (1998). 45-day-old plants were harvested and growth parameters with respect to the total number of nodules and nitrogenase activity were determined (Hardy et al 1973). Subsequently, the PHB content was measured.

2.4 PHB production by selected isolates

The purified *Rhizobium* isolates were cultured on YEM broth medium and incubated at 28°C for 48 hours. PHB was extracted and determined as described by Law and Slepecky (1961). The standard curve of PHB was obtained by using different concentrations of crotonic acid (Sigma Aldrich) and O.D. was measured at 235 nm.

2.5 Effect of carbon and nitrogen sources on PHB production

The screening and selection for the most efficient carbon and nitrogen sources on growth and PHB production by the tested isolate were conducted as described by Friesen and Friel (2019). Additionally, the rhizobial biomass and PHB were determined as the above described methods.

2.6 PHB extraction and determination

PHB was determined according to the method recommended by Hahn (1994). Then, the percentage (w/w) of thePHB against the cell's dry weight was measured.

2.7 Molecular identification of the *Rhizobium* isolate PCR amplification by *16S rRNA* gene

The nucleotide sequences of 16S rRNA gene were determined and identified in the presence of two specific universal primers (P1: 5′- TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3′ and P6: 5′-TTG ATC CTG GCT CAG AAC GAA CGC T -3′) by using the protocol of Lane (1991). The BLAST database, which was described by Altschul et al (1997) and reported in the National Center for Biotechnology Information, was used to determine the phylogenetic tree of the obtained sequences compared to ten overseas strains for each strain documented in GenBank.

2.8 Screening of most significant production parameters using Plackett–Burman design

The most significant production parameters that affected PHB synthesis were screened by PBD using Design-Expert software 11.0.0 (Stat-Ease, Inc., Minneapolis, MN 55413, USA 2018). A total of 11 variables with high and low levels were studied in 24 experiments as follows: A: mannitol, 10–20 g/lL; B: sucrose, 10–20 g/lL; C: yeast extract, 1–2 g/lL; D: glycine, 0.5–1 g/lL; E: K_2HPO_4, 0.5–1; F: $MgSO_4$, 0.2–0.4; G: pH, 6–8; H: temperature, 25°C–30°C); J: inoculum size, 3%–7%; K: incubation period, 2–5 days; and L: agitation speed, 150–200 rpm. All the trials were duplicated and PHB% was calculated as described previously.

2.9 Statistical experimental designs for the evaluation of factors affecting PHB production

2.9.1 Central composite design (CCD) and response surface methodology

CCD was used as a second optimization step for assessing PHB production by the selected rhizobial strain using the five main significant variables obtained from the PBD design in high-, middle- and low-level variable

values. A 50-run experiment was conducted including A: Sucrose, 15–20 - 25 g/l; B: yeast extract, 0.5–1–1.5 g/l; (C: Glycine 0.25–0.87, 1.5 g/l; D: $MgSO_4$, 0.2–0.3 - 0.4 g/l; and D: incubation time, 3–5–7 days. All the trials were duplicated and PHB% was calculated as described before.

2.9.2 The effect of stress conditions on the selected Rhizobial strain growth and plant growth parameters

The basal and modified YEM media were prepared with different concentrations of NaCl (0.01%–7%) for examining the salinity effects. For measuring the effect of pH, the basal and modified YEM media were adjusted at the pH levels from 6 to 11. For drought stress, different concentrations of polyethylene glycol (PEG) 6000 in a range of 0.0%–5.0% (w/v) were added to the basal and modified YEM. To test temperature effects on the experiment, the basal and modified media were incubated at different temperatures (5°C - 50°C). The media were inoculated with 1 ml of tested *Rhizobium* (10^9 CFU/mL) and incubated at 30°C at 150 rpm for 5 days (except media that tested for different temperature). After incubation, 10 ml of culture was collected and O.D was measured at 600 nm. All the treatments were triplicated for the application of different water regimes. To study the effects of drought on the plant growth parameters, the plants were cultivated as described before in the plant infection technique and a three-step experiment was performed. Three different irrigation levels, namely 100%, 80%, and 60%, were used as calculated by Allen et al (1998). The values of crop water consumptive (ET_{crop}) were calculated. The WR for each plant were calculated using the method described by Doorenbos and Pruitt (1977).

2.9.3 Statistical analysis

The obtained data were statistically analyzed using one-way ANOVA and the Tukey's multiple range tests at a significant level of $P < 0.05$ using CoStat program (Version 6.400) according to Snedecor and Cochran (1967).

3 Results and Discussion

3.1 Isolation and authentication of rhizobial isolates

Out of 50 *Rhizobium* isolates, 5 isolates (P04, P12, P25, P32, and P46) were selected based on the highest number of nodules/plant and nitrogenase activity. The selection parameters of the *Rhizobium* isolates with respect to their highest yields of PHB accumulation are presented in **Table 1**. The *Rhizobium* isolate P32 showed the most efficient number of nodules/plant (39), nitrogenase activity (0.959 µ/mol), and PHB production (0.963 g/L) **(Table 1**).

Table 1. Selection for the most active isolate to produce PHB, nitrogenase activity and number of nodules/plants

Isolates No.	No. nodules/ plant	Nitrogenase (µ/mol)	PHB (g/l)
P04	32^{c}	0.824^{d}	0.745^{b}
P12	35^{b}	0.790^{e}	0.684^{c}
P25	29^{d}	0.854^{c}	0.697^{c}
P32	39^{a}	0.959^{a}	0.963^{a}
P46	33^{c}	0.895^{b}	0.745^{b}

The small letters represent the highly significant factors. *Means that within the same column followed by the same letters are not considerably totally different ($P < 0.05$), according to Duncan's test.

3.2 Molecular identification of the *Rhizobium* isolate by 16S rRNA gene

The *Rhizobium* P32 isolate was identified as *Rhizobium phaseoli* using the phylogenetic analysis of 16S rRNA gene sequencing. Upon the amplification of 16S rRNA sequence, using universal primer, an amplified outcome of 1,405 bp was obtained, sequenced, and compared with the GenBank databases using the BLASTN software by the Finch TV program **(http://www.geospiza.com/Products/finchtv.sht ml).** The 16S rRNA sequence of the isolate P32 revealed a close relatedness to *Rhizobium phaseoli* with 99.93% similarity. The phylogenetic analysis of nucleotide sequences on the grounds of 16S rRNA were revealed to be most closely associated to *Rhizobium phaseoli* **(Fig 1).**

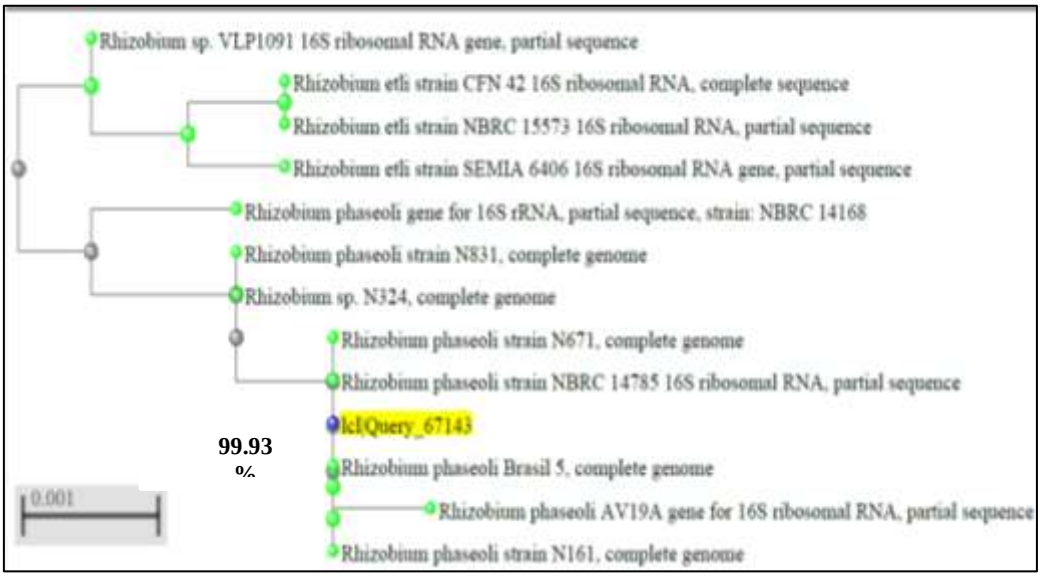

Fig 1. Phylogenetic tree of partial sequence of *16SrRNA* of *Rhizobium* P32 isolate as compared to 13 *Rhizobium* strains recorded in GenBank

3.3 Carbon and nitrogen sources for *Rhizobium* isolate P32

The different carbon and nitrogen sources were tested for PHB production by *Rhizobium phaseoli* isolate. **Fig 2** indicates that mannitol (control) was the best significant carbon source followed by sucrose and mannose. Importantly, the cell dry weight and PHB content were 2.55 g/l and 0.96 g/l, respectively. Glycine was the highest significant nitrogen source followed by yeast extract and peptone. The cell dry weight and PHB content were 2.785 g/l and 1.097g/l, respectively. Similar results by Belal (2013) showed that sucrose promotes the accumulation of PHB in the high-level contents of rhizobial isolates.

3.4 Plackett–Burman design for screening variables affecting PHB production

PBD design was conducted in 24 trial runs at high and low levels for each variable for the detection of the main significant factors on PHB production. The one-way ANOVA revealed the significance of the model with *F*-value of 6.14. **Fig 3** shows the main effects plot, which is conjugated with the ANOVA analysis to determine the mean-level differences for all the factors. The produced mean PHB for each factor level was connected by a line. Factors with horizontal line parallel to the X axis do indicate the main effects. However, factors with non horizontal line are considered as the main affecting factors. Hence, sucrose (B), yeast extract (C), glycine (D), $MgSO_4$ (F), and inoculum size (J) are significant main effects for the PHB production. The coded equation is as follows:

$\mathbf{Y_{PHB}}$= 47.49 + 2.48 (Mannitol) + 4.69 (Sucrose) + 3.07 (Yeast extract) + 3.85 (Glycine)– 0.875 (K_2HPO_4)–5.62($MgSO_4$)–1.16 (pH) + 0.959 (Temperature) + 2.77 (Inoculum size) – 2.44 (Incubation time) –0.66 (Agitation) –1.58 (Mannitol × Glycine) –1.73 (Mannitol × $MgSO_4$) –0.168 (Mannitol × pH) –0.784 (Mannitol × Inoculum size) + 3.36 (Sucrose × Yeast extract).

In this study, as a source of phosphorus and potassium, KH_2PO_4 positively affected PHB production at respective high levels of 0.05 g/100 mL for phosphorus and 0.1g/100 mL for potassium. KH_2PO_4 is an important factor for PHB production as recorded by Gundi et al (2018).

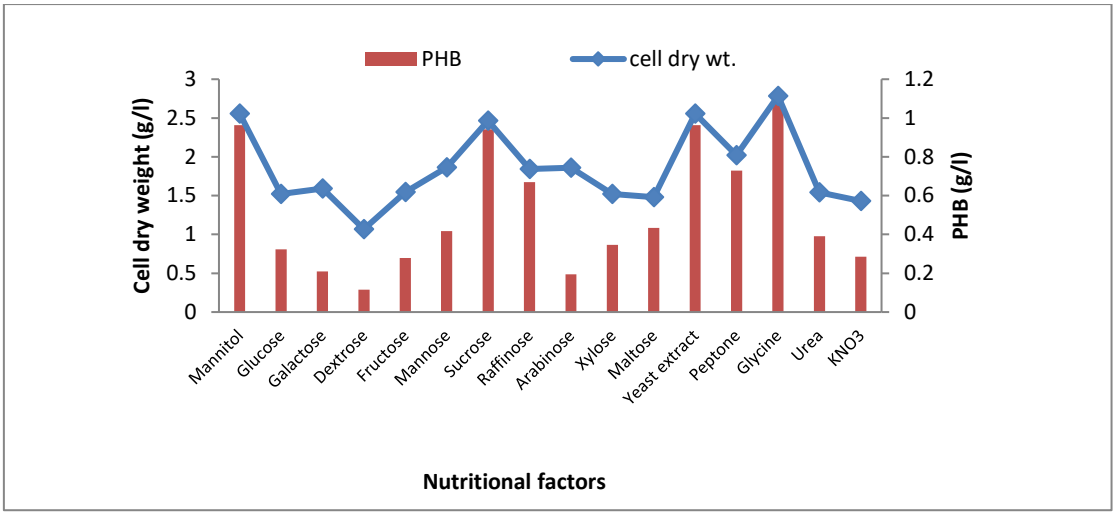

Fig 2. Effect of different carbon and nitrogen sources on the cell dry weight and PHB production by *Rhizobium phaseoli* isolate grown on the YEM basal medium at 28°C for 48 hours

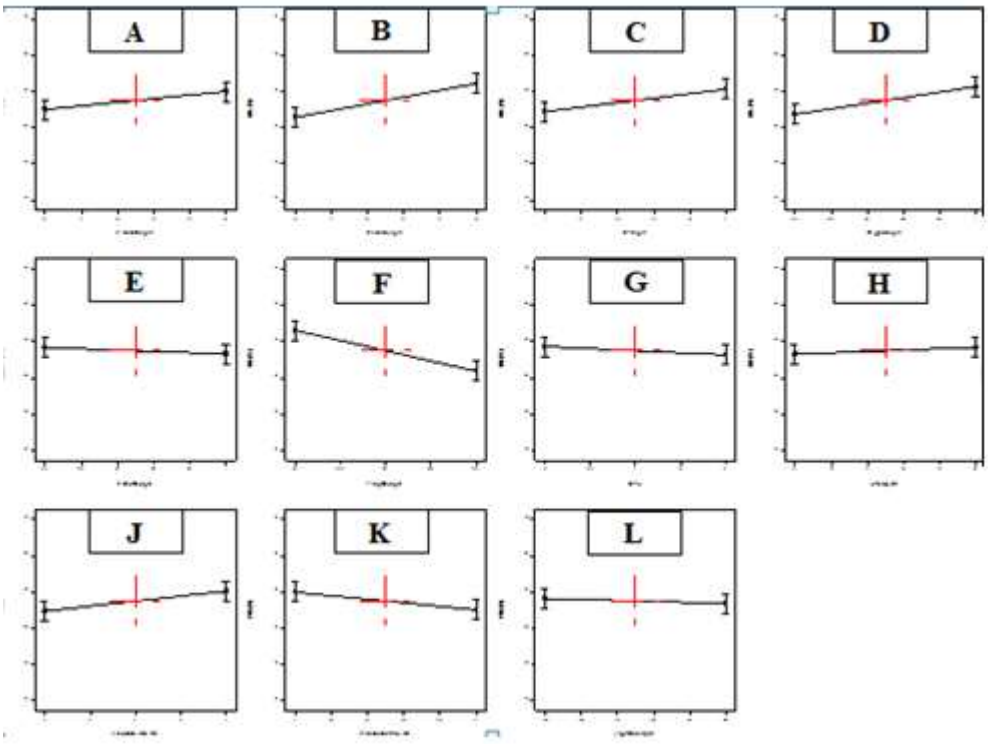

Fig 3. Main effects plot of PBD indicating that sucrose (B), yeast extract (C), glycine (D), $MgSO_4$ (F), and inoculum size (J) are the main significant medium components that affected PHB production by *Rhizobium phaseoli*

3.5 Central Composite Design (CCD) for the optimization of PHB production

In this study, 50 experiments with different combinations of sucrose concentration (A), yeast extract concentration (B), glycine (C), $MgSO_4$ (D), and inoculation size (E) were performed at three different levels coded as −1, 0, and +1 are presented along with the actual and predicted responses**.** Additionally, the software suggested higher and lower factor levels than the coded levels to decrease the noise ratio. **Table 2** shows that the maximum production (4.997 g/L) was achieved at run 36 in the presence of 25 g/L of sucrose, 0.0 g/L of yeast extract, 0.87 g/L of glycine, 0.3 g/L of $MgSO_4$, and 5% of incubation size**. Fig 4** illustrates the relationship between sucrose and lysine on PHB production that led to a sharp increase in PHB accumulated by *Rhizobium* cells to reach 4.997 g/L (5.2 times) as compared to the basal medium as plotted in **Fig 5.** The cells with high content of PHAs may survive longer than those that lack PHA or have a low PHA content, because PHB acts as a reserve material that helps the cells to live longer than those bacteria that produce low contents of PHA (Muller and Denison 2018). F-test and ANOVA were conducted. The F-value model of 2.53 implied that the model was significant. The determination coefficient R^2 of the model was 0.96 and indicated that 96% of the total variations were explained by the model and revealed a good agreement between the experimental results and the predicted values calculated from the model. The final equation in terms of actual factors:

$$*Y_{PHB} = 91.46 - 1.05\,(\text{Sucrose}) - 44.46\,(\text{yeast extract}) + 8.98\,(\text{glycine}) - 155.05\,(MgSO_4) + 1.54\,(\text{incubation size}) - 0.028\,(\text{sucrose} \times \text{yeast extract}) - 0.434\,(\text{sucrose} \times \text{glycine}) + 2.334\,(\text{sucrose} \times MgSO_4) + 0.0032\,(\text{Sucrose} \times \text{incubation size}) + 3.573\,(\text{yeast extract} \times \text{glycine}) + 27.23\,(\text{yeast extract} \times MgSO_4) + 1.707\,(\text{Yeast extract} \times \text{incubation size}) + 16.50\,(\text{glycine} \times MgSO_4) + 1.128\,(\text{glycine} \times \text{incubation size}) + 2.06\,(MgSO_4 \times \text{incubation size}) + 0.027\,(\text{sucrose}^2) + 8.69\,(\text{yeast extract}^2) - 8.43\,(\text{glycine}^2) + 159.48\,(MgSO_4^2) - 0.393\,(\text{incubation size}^2)$$

where Y is the predicted response.

3.6 *In vitro* stress endurance efficiency under different created stress conditions

3.6.1 Osmotic, thermal, alkalinity, and drought stress

The survivability of *Rhizobium phaseoli* was compared under different NaCl concentrations, high temperatures, pH values, and PEG% concentrations (drought) on both basal and modified YEM media. For osmotic stress, **Fig (6-A)** shows that *Rhizobium phaseoli* strain could withstand high NaCl concentrations in the modified medium to reach O.D of 0.983 at 4% NaCl concentration as compared with a significant weak growth at 4% NaCl concentration on the basal medium with a growth loss of 50%. For thermal stress, **Fig (6-B)** illustrates that *Rhizobium phaseoli* can withstand high temperatures when grown in the modified medium to reach O.D of 1.535 at 35°C, whereas it has a significantly weak growth at 35°C on the basal medium with a growth loss of 56.4%. For alkalinity stress, **Fig (6-C)** demonstrates that *Rhizobium phaseoli* can withstand high pH values in the modified YEM medium to reach O.D of 0.684 at a pH of 10, whereas it has a significant weak growth at a pH value of 10 on the basal medium with a growth loss of 30%. For drought stress, **Fig (6-D)** indicates that *Rh. phaseoli* can withstand high PEG concentration in the modified medium to reach O.D of 0.750 at 3.5% PEG concentration, whereas it has a significant weak growth on 3.5% PEG concentration the basal medium with a growth loss of 65%. Arora et al (2013) suggested that PHB plays a definite role in the protection of the bacterial cells from osmotic stress, where the minimum PHB content was accumulated at low or zero salinity and the maximum content was observed by the salt-tolerant strains at higher salt concentrations.

Table 2. Central composite design (CCD) of independent variables for PHB production by *Rhizobium phaseoli* strain

Run	Factor 1 A. Sucrose (g/l)	Factor 2 B. Yeast extract (g/l)	Factor 3 C. Glycine (g/l)	Factor 4 D. $MgSO_4$ (g/l)	Factor5 E. Incubation size (%)	Response 1 PHB (%)
1	15	1.0	-0.60	0.3	5.0	26.11
2	25	0.5	1.50	0.4	3.0	44.53
3	15	0.5	0.25	0.2	3.0	46.07
4	25	0.5	0.25	0.2	3.0	56.39
5	15	1.0	0.87	0.3	0.2	36.07
6	15	1.5	1.50	0.4	3.0	55.89
7	20	1.0	0.87	0.3	5.0	61.72
8	15	0.5	0.25	0.2	7.0	54.87
9	25	0.5	0.25	0.2	7.0	64.17
10	15	1.5	1.50	0.2	7.0	51.87
11	25	1.5	0.25	0.2	7.0	48.71
12	15	0.5	1.50	0.2	3.0	54.11
13	8.1	0.5	1.50	0.4	7.0	61.52
14	25	1.0	0.87	0.5	5.0	68.97
15	25	1.5	1.50	0.4	3.0	50.37
16	20	0.5	1.50	0.2	7.0	34.70
17	15	1.5	0.25	0.2	7.0	35.59
18	20	1.5	1.50	0.4	7.0	60.94
19	20	0.5	0.25	0.4	7.0	42.98
20	20	1.0	0.87	0.3	5.0	46.71
21	20	1.5	1.50	0.2	7.0	53.47
22	25	0.5	1.50	0.2	3.0	53.01
23	31	1.0	0.87	0.3	9.8	43.79
24	25	1.5	1.50	0.4	7.0	51.43
25	25	1.0	0.87	0.3	5.0	50.51
26	15	1.0	0.87	0.3	5.0	53.93
27	20	1.0	0.87	0.3	5.0	50.00
28	15	1.0	0.87	0.3	5.0	50.23
29	20	0.5	1.50	0.4	3.0	51.59
30	20	1.0	0.87	0.3	5.0	58.74
31	15	0.5	0.25	0.4	7.0	60.24
32	20	1.5	0.25	0.2	3.0	44.71
33	20	1.5	0.25	0.4	3.0	38.84
34	25	1.0	0.87	0.3	5.0	44.93
35	15	1.5	0.25	0.4	7.0	65.27
36	25	0.0	0.87	0.3	5.0	77.70
37	25	1.0	0.87	0.3	5.0	43.60
38	15	1.5	0.25	0.2	3.0	47.39
39	20	1.0	2.36	0.3	5.0	34.25
40	25	1.0	0.87	0.3	5.0	50.94
41	20	1.5	1.50	0.2	3.0	30.44
42	25	1.5	1.50	0.2	3.0	37.08
43	15	0.5	1.50	0.4	7.0	66.44
44	15	0.5	0.25	0.4	3.0	60.87
45	25	0.5	0.25	0.4	3.0	64.89
46	15	2.1	0.87	0.3	5.0	44.55
47	25	1.5	0.25	0.4	7.0	52.68
48	15	1.0	0.87	0.1	5.0	46.72
49	15	1.5	0.25	0.4	3.0	54.25
50	20	0.5	1.50	0.2	7.0	52.90

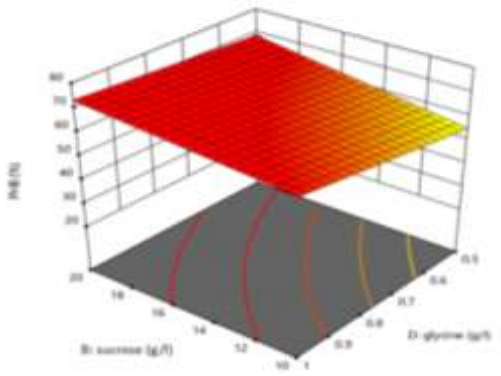

Fig 4. Three-dimensional response surface showing the effect of sucrose concentration and glycine concentration along with their mutual effect on the PHB production

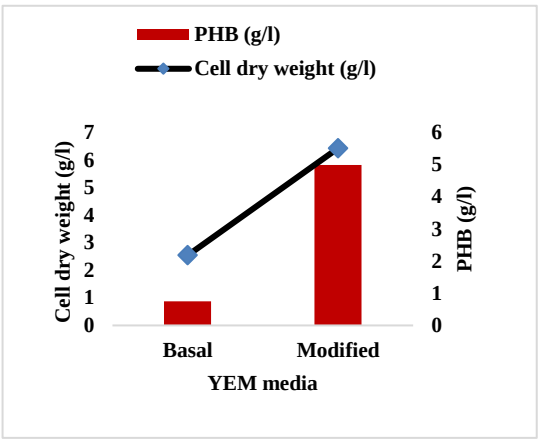

Fig 5. PHB production in the basal YEM medium against the modified YEM medium using *Rhizobium phaseoli* incubated at 28˚C for 48h

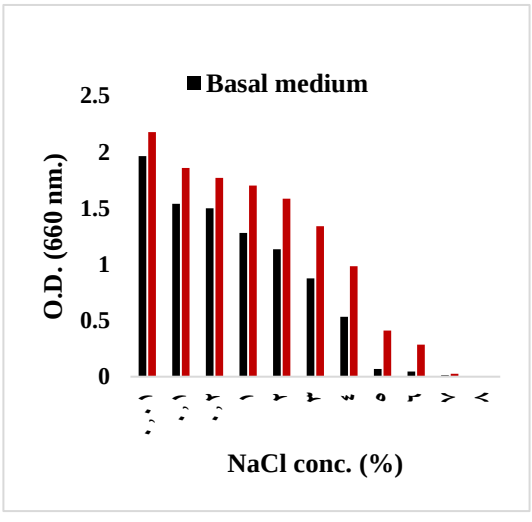

Fig 6A. Effect of salinity stress (NaCl: 4%) on *Rhizobium phaseoli* growth in the basal and modified YEM media at 28°C for 48 h

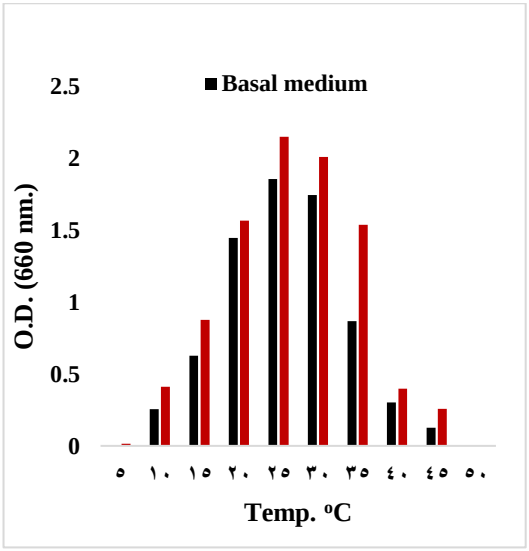

Fig 6B. Effect of temperature (43°C) on *Rhizobium phaseoli* growth in basal and modified YEM media for 48 h

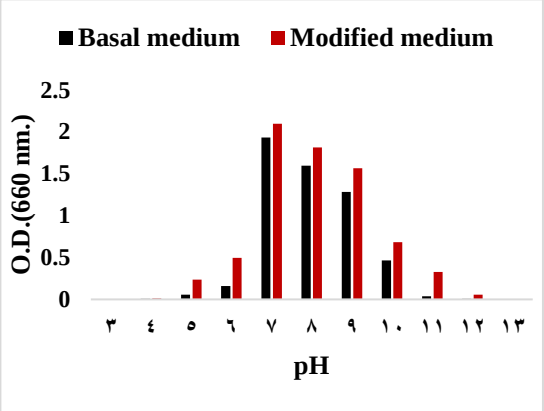

Fig 6C. Effect of alkalinity pH (10) on *Rhizobium phaseoli* growth in the basal and modified YEM media at 28°C for 48h

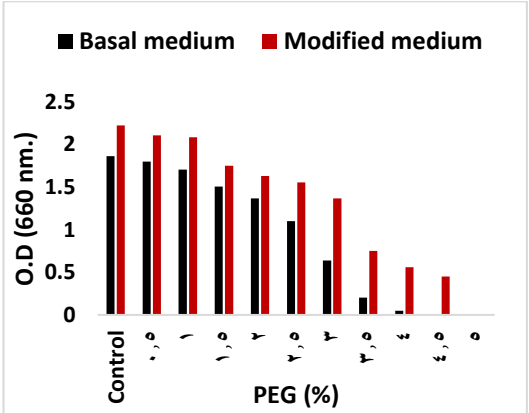

Fig 6D. Effect of drought stress using PEG (3.5%) on *Rhizobium phaseoli* growth in the basal and modified YEM medium at 28°C for 48 h

Fig 6A–5D. Effect of different abiotic stress conditions on *Rhizobium phaseoli* growth in basal and modified YEM media

Additionally, PHB synthesized by many species of bacteria has been shown to improve survival during starvation, as well as improve tolerance to osmotic stress (Kıvanç and Dombaycı 2016). Observed data of *Rhizobium leguminosarum bv. phaseoli* showed the ability of some strains to nodulate *Phaseolus vulgaris* at high temperatures (35°C and 38°C) but that the nodules formed at high temperatures were ineffective and plants did not accumulate N_2 in shoots. *Rhizobium* spp. can grow in the alkali medium (pH: 6–11). Therefore, tolerance to high salt, pH, and temperature stresses may be important for the survival, multiplication, and spread of *Rhizobium* spp. (Leonel et al 2019). Moreover, drought conditions negatively affect growth and persistence of rhizobia and bradyrhizobia in soils**.**

For example, salt-tolerant rhizobia such as *Sinorhizobium* sp. were isolated from the halophytic herb *C. rosea* and grown at 3.5% of NaCl. They were also isolated from *M. sativa*, grown at 4.5% NaCl (Bertrand et al 2016). Other rhizobial species such as *Mesorhizobium* strain CCNWGX035 showed a higher tolerance to NaCl, pH, and temperature; in addition, the halotolerant rhizobia that were recovered from the seedlings of *A. gummifera* and *A. raddiana* grew at about 6% NaCl **(Santos et al 2017).** Similarly, *Bradyrhizobium* sp. isolated from lupine grew at 5% NaCl and survived under acidic (pH: 4–5) and alkaline (pH 9–10) conditions (Kosmachevskaya et al 2020). In a follow-up study, about 25% of lentil rhizobia showed a higher tolerance to salinity while growing at electrical conductivity equaling to 10 ds/m (Tewari and Sharma 2020). Osmotolerant rhizobia strains, nodulating *P. vulgaris*, have been isolated from the saline soil of Morocco and identified by analyzing core genes (*rrs*, *atpD*, *recA*) and symbiotic (*nodC*) genes (Poole et al 2018). The most abundant strains were closely related to *R. etli* and *R. phaseoli* followed by those related to *R. gallicum* and *R. tropici*.

3.7 *In vivo* drought stress endurance efficiency in common bean plants

The data in **Fig 7** showed that *Rhizobium phaseoli* could resist the drought stress at 20% loss of WR to reach N_2-ase quantities of 0.756 and 0.601 m/mol of when grown on the modified and basal media, respectively. Plants inoculated with *Rhizobium phaseoli* grown in modified medium tolerate drought stress with 20% water loss to produce 75% of the crops. **Table 3** describes the effect of drought on plant growth parameters. Results show that inoculation with *R. phaseoli* grown on modified medium increased nodule number (49/plant),

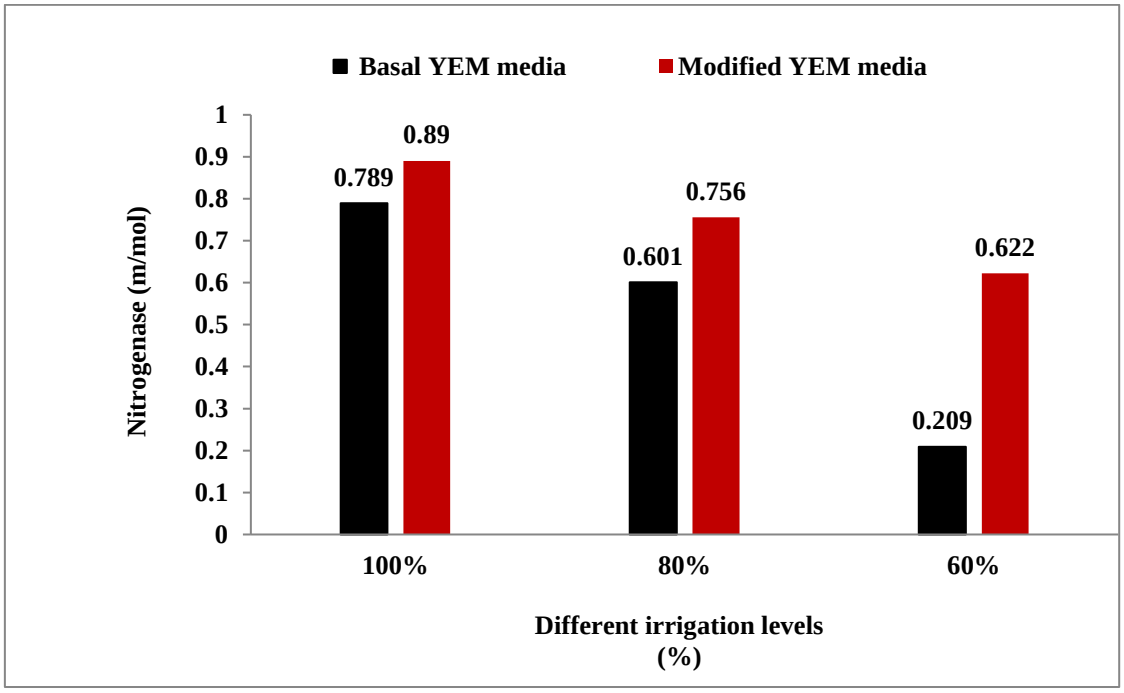

Fig 7. Effect of drought levels on the nitrogenase activity of common bean inoculated by *Rhizobium phaseoli* growing on the basal or modified media after 45 days

Table 3. Effect of drought levels on the plant growth parameters of common bean

Treatment		Number of nodules/plant	Nodules dry wt. (mg/plant)	Leghemoglobin content (mg/100 g nodules)	Root dry weight (g/plant)	Shoot dry weight (g/plant)	Nitrogen %
Rhizobium phaseoli in basal medium	100%	34^{c}	88^{c}	0.753^{c}	0.955^{c}	1.658^{d}	2.140^{c}
	80%	35^{c}	79^{d}	0.682^{d}	0.968^{c}	1.587^{e}	1.870^{d}
	60%	10^{e}	19^{f}	0.310^{f}	0.674^{f}	1.341^{f}	1.430^{f}
Rhizobium phaseoli in modified medium	100%	57^{a}	147^{a}	0.817^{a}	1.098^{a}	1.982^{a}	2.380^{a}
	80%	49^{b}	95^{b}	0.769^{b}	0.997^{b}	1.798^{b}	2.180^{b}
	60%	25^{d}	59^{e}	0.557^{e}	0.853^{e}	1.685^{c}	1.750^{e}

1- The small letters show the highly significant factors.
2-Means that within the same column followed by the same letters are not considerably totally different ($P < 0.05$), according to Duncan's test

nodule dry weight (95mg/plant) under drought conditions of 20% water loss when compared to plants inoculated with R. phaseoli grown on basal medium under both control and 20% water loss treatments. Yanni et al (2016) revealed the effect of *Rhizobium leguminosarum bv. viciae* inoculation on the growth of faba bean under water-deficit stress condition. They reported an increase in most of the growth parameters such as root dry weight, nodulation, number of nodules, total N content, day germination speed, mean day germination, and relative water content due to rhizobial inoculation. Finally, they recommended the inoculation of *Rhizobium leguminosarum bv. viciae* under water-deficit conditions to improve growth and yield of faba bean. While assessing the drought stress response of different bean genotypes inoculated with rhizobia, Hussain et al (2018) reported that rhizobial inoculation improved the production of nodule number (350/plant), nodule dry weight (0.55 mg/nodule), plant leaf area (96 cm^2), total biomass (1.4 mg/plant), and drought tolerance (in terms of trehalose contents of nodules up to 90 mg/g) under water-deficit conditions as compared to their well-watered counterparts.

The impact of inoculation of rhizobial isolates on nodulation, total N contents, and dry matter production of *Vigna mungo* grown under arid conditions was inconsistent (López et al 2016). Inoculation with rhizobial isolates had a maximum effect on nodulation, total nitrogen, and dry matter production in *Vigna mungo* grown in arid conditions (López et al 2016). When compared un-inoculated and inculatedv *V. mungo* plants, rhizobial inoculation substantially increased the number of nodules (23/plant), total N (3.3 mg/plant), and dry weight contents (298.6 mg/plant).

Salinity and drought have a negative impact on nodulation and nitrogen fixation in legume *Rhizobium* associations, which can prevent legume establishment and growth or reduce crop yield. Salinity has been shown to minimize nitrogen accumulation by the symbiotic system of soybean, alfalfa, and *Glycin javanica*. *Rhizobium* commercial strains are normally unable to tolerate or be active work in an uncontrolled environment (Staudinger et al 2016).

In addition, Wang et al (2010) asserted that the ability to synthesize PHB is critical for rhizobial nitrogen fixation. The stored energy in the lipid PHB, on the other hand, can help rhizobial survival when carbon (salinity) is reduced, either in nodules or in the soil. Furthermore, under growth-restricted conditions, bacteria from carbon storage compounds such as PHB and glycogen.

Plants, like legumes, suffer from drought stress when they are exposed to inadequate water for an extended period of time (Bukhari et al 2015). As a result, determining the effect of drought on the efficiency of the legume-rhizobial symbiosis under abiotic stress conditions is important. Under drought stress, uredines are nitrogenous compounds that contribute to nitrogen recycling, which accumulates in the shoots and nodules of legumes, causing SNF to rapidly decline. Furthermore, a lower transpiration rate decreases the N_2 demand of the shoot, which slows xylem translocation and reduces enzymatic activity, resulting in a lower nitrogen fixation rate. Moreover, drought affects the initiation of nodules, nodule growth, development, and function. Drought situation also decreases photosynthetic activity, which in turn adversely affects the SNF (Valentine et al 2011).

4 Conclusion

The main factors affecting PHB production, which were determined using selected *Rhizobium phaseoli* strain, were sucrose, yeast extract, glycine, and $MgSO_4$. PHB production reached 5.2-fold of maximum production in the basal medium. *In vitro* stress experiments showed that *Rhizobium phaseoli* strain could withstand high NaCl salt concentrations of 5%, the growth at high temperatures of 45°C, tolerance of alkalinity at pH 8-10, and responded to drought conditions at 3.5% PEG with growth

loss 50% when grown on modified medium and 75% growth loss when grown on the basal one. *In vivo* drought stress in the common bean plants showed that *Rhizobium phaseoli* could resist the drought stress at 20% loss of WR when grown in the modified medium Additionally, plants inoculated with *Rhizobium phaseoli* grown in the modified medium tolerate drought stress with 80% WR. Therefore, further studies are needed to produce abiotic-resistant rhizobial isolates as biofertilizers in different stress conditions.

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تعزيز المقاومة المستحثة لنبات الفاصوليا باستخدام بولي بيتا هيدروكسي بيوتيرات ب *Rhizobium phaseolei*

[17]

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الموجـــــــز

تم عزل 50 عزلة *Rhizobium* من العقد الجذرية لنبات الفاصوليا المزروعة في 9 محافظات مختلفة في مصر. وقد استخدمت التجربة العاملية لتحسين انتاج الـ PHB بواسطة السلالة المختارة المعرفة. تم دراسة تأثير 11 من العوامل البيئية مثل درجة الحموضة، ومدة التحضين، وحجم اللقاح، ودرجة الحرارة، وسرعة الرج والعوامل الغذائية من مصادر الكربون مثل المنيتول، السكروز، مستخلص الخميرة، الجلايسين، K_2HPO_4، $MgSO_4$على إنتاج PHB باستخدام تصميم بلكيت بورمان. وكانت أهم العوامل الرئيسية المؤثرة على إنتاج PHB تتمثل في السكروز، مستخلص الخميرة، الجليسين، و $MgSO_4$. تم اجراء تصميم CCD لاختيار المستويات المثلى للعوامل الهامة االناتجة من تصميم PBD. وصلت *Rhizobium phaseoli* إلى الإنتاج الأقصى (4.997 جرام / لتر) في وجود 25 جرام / لتر من السكروز، 0.0 جرام / لتر من مستخلص الخميرة، 0.87 جرام/لتر من الجليسين، 0.3 جرام / لتر من MgSO4، و 5٪ من حجم اللقاح. تم دراسة تأثير عوامل الإجهاد المختلفة من (درجة الحموضة: 6–11، درجة الحرارة:50 – 5 م° ، الملوحة: 0.01٪–7٪، والجفاف: 0–5% –3–5%PEG) على نمو *Rhizobium phaseoli* وأظهرت النتائج أن البيئة المعدلة ادت الي تحسين تحمل الرايزوبيم لظروف الاجهاد حيث تحملت الريزوبيوم 5٪–3 من كلوريد الصوديوم، ارتفاع درجة الحرارة من 30م° إلى 45م° ، درجة الحموضة عند درجة PH من 8–10، و تحمل 3.5% من البولي ايثيلين جلايكول مع فقدان النمو من 50٪ عندما نمت على البيئة المعدلة و 75٪ عندما نمت على البيئة الأساسية. تم اختبار تأثير مستويات الإجهاد الجفاف على مقاييس نمو نباتات الفاصوليا. بشكل عام، كانت جميع مقاييس النمو النباتي عند التلقيح *Rhizobium phaseoli* النامية علي البيئة المعدلة متفوقة على مقاييس النمو النباتي للنباتات الملقحة *Rhizobium phaseoli* النامية علي البيئة الاساسية. لم تلاحظ فروق كبيرة بين النباتات الملقحة *Rhizobium phaseoli* النامية علي البيئات المعدلة عند مستوي مائي 100٪ من الاحتياج المائي بالمقارنة مع النباتات المروية ب 80% من الاحتياج المائي.