Romanian Journal for Plant Protection, Vol. VIII, 2015
ISSN 2248 – 129X; ISSN-L 2248 – 129X

THE INFLUENCE OF SEVERAL ABIOTIC FACTORS ON SOME BACTERIAL STRAINS OF PHYTOSANITARY USE

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Abstract: The aim of this work was to evaluate the main abiotic factors that interact with bacterial strains of phytosanitary use, for their application as biological control agent against pests in order to obtain microorganisms with high tolerance to abiotic environmental factors. Two bacterial strains *Bacillus subtilis* Bce2 and *Bacillus* sp. 83.2s were tested. *In vitro* experiments have shown that, the multiplication rate of bacterial strains into temperature range analyzed decreased direct proportion to temperature and senescence of bacterial cells was more emphasized at the higher temperature than optimum value of 27°C. Also, the culture medium with acidic pH (4.5) was not conducive to development of the *Bacillus* strains. Thus, Bce2 strain of *B. subtilis* was multiplied much better under alkalinity conditions up to pH 8.5, while 83.2s strain of *Bacillus* sp. grew better in slightly acidic pH of 5.5, the multiplication rate being lower with increasing of pH value.

Key words: abiotic factors, bacterial strains, pH

INTRODUCTION

The environmental conditions may influence developing of microorganisms, either positively, favoring growth or slowing their rate of multiplication or synthesis of different metabolites. In extreme conditions, parameters such as temperature, aeration, nutrients, pH or tolerance of NaCl can become limiting factors for the growth of microorganisms. Thus, it was found that a low level of aeration coupled with a temperature of 15°C significantly decrease the multiplication of bacteria like *Bacillus*, cultivated in the bioreactor (De Sarrau et al., 2012). However, *Bacillus subtilis* and its affined species can grow in variable pH conditions, maintaining the cytoplasmically pH in a relatively close range, stable to the synthesis of proteins and nucleic acids. The cytoplasmic homeostasis to *B. subtilis* was been intensively studied, finding that when bacteria are grown under varying pH conditions, from 6 to 9, the values of cytoplasmic pH were maintained between 7.3 and 7.6 (Padan et al., 2005). An interesting feature of the *Bacillus* genus is to adjust catabolism and transport of protons such that bacteria to increase its acids production at low values of pH (Wilks et al., 2009). Under low pH conditions, it is triggered spores germination and vegetative growth (Illades-Aguiar et al., 1994).

The aim of this work was to evaluate the main abiotic factors that interact with bacterial strains of phytosanitary use, for their application as biological control agent against pests in order to obtain microorganisms with high tolerance to abiotic environmental factors.

MATERIALS AND METHODS

Biological material. Two bacterial strains *Bacillus subtilis* Bce2 and *Bacillus* sp. 83.2s, both isolated from soil, were used.

Culture media. Bacterial strains were cultivated on Luria-Bertani agarized culture medium (LBA: bactotryptone 10g; yeast extract 5g; NaCl 5g; agar-agar 18g; distilled water up to 1000ml; pH 7.2), incubated at 27°C for 48 hours.

Evaluation of temperature influence on bacterial strains growth

The bacterial strains were cultivated on LB agar culture medium and incubated at different temperatures, in order to assess the growth features on microorganisms multiplication under temperature influence. The bacterial increases were analysed at temperatures of 15, 25 and 35°C, compared to control of 27°C, optimum for the growth of *Bacillus.* For this experiment, bacterial suspensions and serial dilutions in phosphate saline buffer (TFS: 8g NaCl; 0.2g KCl; 1.44g Na_2HPO_4; 0.24g KH_2PO_4; 1000ml distilled water; pH 7.4; autoclaved) were prepared. Subsequently, 100µl of 5 and 7 dilutions were plated and uniformly distributed on agar medium with Drigalski spatula. Each experimental variant had three replicates. The evaluation of temperature influence on bacterial growth was carried out by notifications at 24, 48, 72 hours, and 5 days of incubation at the temperatures mentioned above. These observations consisted in counting of colonies formed to determine the concentration and analysis of the morphological traits such as aspect and size of colonies.

Evaluation of pH influence on bacterial strains growth

The bacterial growths were analyzed in Luria Bertani liquid medium at different pH values: 4.5; 5.5; 7.5 and 8.5, compared to reference control (neutral pH of 6.8 ± 2). To adjust the pH of culture medium, it was used a solution of 1N HCl to acidification and respectively, a solution of 1N NaOH for alkalizing. For achieve the experiment, the strains of bacteria have been conditioned as bacterial suspension in phosphate buffer saline, at an optical density of 0.85. The homogenous suspensions with a final concentration of 10% were used as starting inoculum for the test cultures. Serial dilutions were prepared, and then 100µl of 5 and 7 dilutions were plated and uniformly distributed on culture medium surface. Each variant had three replicates. Assessing of pH influence on bacterial growth was performed by observations at 24, 48, 72 hours, and 5 days of incubation at optimum temperature of 27°C. At each time of incubation, optical density was spectrophotometrically determined at 600 nm.

Evaluation of NaCl influence on bacterial strains growth

The bacterial growths developed in LB liquid medium at different concentrations of sodium chloride as compared to optimal requirement of 1% NaCl (control) were analyzed. The concentrations of 0.5, 4, 8 and 10% NaCl were tested. To perform the experiment, both strains of *Bacillus* have been formulated as bacterial concentrated suspension (10^8cfu/ml) in phosphate saline buffer. The homogenates with a final concentration of 10% were used as starting inoculum for test culture media. Each experimental variant had three replicates. Evaluating of salinity influence on bacterial growth was carried out by observations at 24, 48 and 72 hours, under optimum conditions of pH (7.0 ± 2), temperature (28°C) and aeration (150rpm orbital shaking). At each time of incubation, the optical density was spectrophotometrically determined at 600nm.

RESULTS AND DISCUSSIONS

Evaluation of temperature influence on bacterial strains growth

The cultivation of *Bacillus* strains in LB liquid medium incubated at different temperatures resulted in an optimum growth at temperature of 27°C with a concentration up 1.3 to 5.6 × 10^9cfu/ml and active growth after five days of incubation (table 1).

Table 1. The growth of bacterial strains at various temperatures

Temperature variant	Bacterial strain	Culture medium	The number of colonies at different times of incubation			
			24h	48h	72h	5 days
15°C	*Bacillus subtilis* Bce2	LBA	-	-	N.D.	$4,5\times10^{8}$ufc/ml
	Bacillus sp. 83.2s	LBA	-	-	N.D.	$1,97\times10^{9}$ufc/ml
25°C	*Bacillus subtilis* Bce2	LBA	3.4×10^{9}ufc/ml	3.8×10^{9}ufc/ml	3.8×10^{9}ufc/ml	3.8×10^{9}ufc/ml
	Bacillus sp. 83.2s	LBA	8.3×10^{9}ufc/ml	9.3×10^{9}ufc/ml	9.3×10^{9}ufc/ml	9.3×10^{9}ufc/ml
27°C (Control)	*Bacillus subtilis* Bce2	LBA	1.25×10^{9}ufc/ml	1.3×10^{9}ufc/ml	1.3×10^{9}ufc/ml	1.3×10^{9}ufc/ml
	Bacillus sp. 83.2s	LBA	5.27×10^{9}ufc/ml	5.59×10^{9}ufc/ml	5.59×10^{9}ufc/ml	5.59×10^{9}ufc/ml
35°C	*Bacillus subtilis* Bce2	LBA	1.5×10^{9}ufc/ml	1.5×10^{9}ufc/ml	1.5×10^{9}ufc/ml	1.5×10^{9}ufc/ml
	Bacillus sp. 83.2s	LBA	5.7×10^{9}ufc/ml	5.8×10^{9}ufc/ml	5.8×10^{9}ufc/ml	5.8×10^{9}ufc/ml

It was noted that, the number of germinated spores was higher at temperature of 25°C but the colonies size was considerably smaller compared to the control (27°C) and stagnated after the first three days of incubation. Also, the bacterial colonies had a good growth at 35°C in the first 72 hours of incubation, but they stopped to increase after the third day of incubation, suggesting an early senescence of cultures (figure 1).

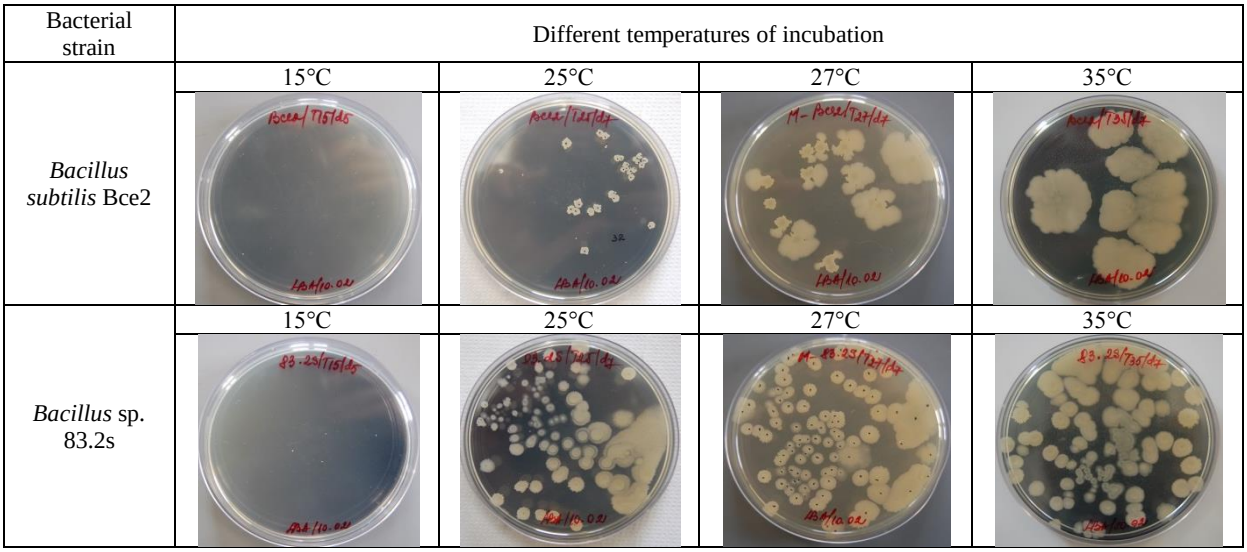

Figure. 1 The growth of *Bacillus* strains after three days of incubation at different temperatures

The lowest bacterial growths, both in terms of microbial load and size of colony were recorded at temperature of 15°C (tables 1 and 2). In the thermal range analyzed, it was found that the multiplication rate of bacterial strains increased with temperature and senescence of the cultures was more pronounced at values of higher temperature than optimum of 27°C for these bacteria.

Table 2. The growth of bacterial colonies at various temperatures

Temperature variant	Bacterial strain	The aspect of colonies at different times of incubation			
		24h	48h	72h	5 days
5°C	*Bacillus subtilis* Bce2	-	-	very small colonies, translucent, hardly visible	Ø = 3-5 mm, rough and flat colonies, slightly translucent, with fine crimped edge
	Bacillus sp. 83.2s	-	-	very small colonies, translucent, hardly visible	Ø = 3 mm, convex colonies, matte center but translucent edge, crimped
25°C	*Bacillus subtilis* Bce2	Ø ≤ 0,5 mm, flat and translucent colonies	Ø = 1-3.5 mm, rough and flat colonies with matte surface and	Ø = 0.1 -0.5 cm, rough and flat colonies with matte surface and	Ø = 0.1 – 0.5 cm, rough and flat colonies with matte surface and

			crimped edge	crimped edge	crimped edge
	Bacillus sp. 83.2s	Ø = 2 - 8 mm, rough and umbonate colonies with matte surface and fine crimped edge	Ø= 2 - 8 mm, rough and umbonate colonies with matte surface and fine crimped edge	Ø = 2 - 8 mm, rough and convex colonies, slightly umbonate, with matte surface and fine crimped edge	Ø = 2 - 8 mm, rough and convex colonies, slightly umbonate with matte surface and fine crimped edge
27°C (Control)	*Bacillus subtilis* Bce2	Ø = 1.5 - 3 mm, rough and flat colonies with serrated edge	Ø = 0.6 – 1.2 cm, rough and flat colonies with serrated edge	Ø = 0.6 – 2.1cm, rough and flat colonies with serrated edge	Ø = 0.7 – 3.2 cm, rough and flat colonies with serrated edge
	Bacillus sp. 83.2s	Ø = 2 - 4 mm, convex colonies with matte surface, EPS production	Ø = 0.3 – 0.8 cm, rough and umbonate colonies with matte surface and crimped edge	Ø=0.35 - 1,23cm, rough and umbonate colonies with matte surface and crimped edge	Ø = 0.4 – 2.1cm, rough and umbonate colonies with matte surface and crimped edge
35°C	*Bacillus subtilis* Bce2	Ø = 4 - 9 mm, rough and flat colonies with crimped edge	Ø = 1.05 – 2.15 cm, rough and flat colonies with crimped edge	Ø = 1.15 - 2,66 cm, rough and flat colonies with crimped edge	Ø = 1.8 – 3.3 cm, rough and flat colonies with crimped edge
	Bacillus sp. 83.2s	Ø = 5 mm, rough and flat colonies with regular outline	Ø = 0.3 – 0.93 cm, rough and flat colonies with regular outline	Ø = 0.33 – 1.36 cm, rough and flat colonies with regular outline	Ø = 0.33 – 1.36 cm, rough colonies, slightly umbonate with fine crimped edge

Evaluation of pH influence on bacterial growth

Bacterial cultures of both *Bacillus* strains grown in LB liquid medium with different values of pH were been spectrophotometrically analyzed at 600 nm (table 3). The cultivation of Bce2 strain of *B.subtilis* in different pH conditions reflected a good multiplication rate in alkaline LB medium, directly proportional to the increase of pH value up to pH 8.5 (figure 2). Regarding the *Bacillus* sp. 83.2s strain, the best multiplication rate was recorded when it was rown in slightly acidic culture medium, with pH 5.5 (figure 3).

Table 3. The optical density of bacterial cultures at different values of pH (LB culture medium)

pH variant	Bacterial strain	Absorbance at 600nm			
		24h	48h	72h	5 zile
pH 4,5	*Bacillus subtilis* Bce2	0.082	0.097	0.112	0.105
	Bacillus sp. 83.2s	0.095	0.094	0.097	0.012
pH 5,5	*Bacillus subtilis* Bce2	1.153	1.05	1.06*	0.237
	Bacillus sp. 83.2s	1.35	1.283	1.317	0.897
pH 6,8±2	*Bacillus subtilis* Bce2	1.17	1.23	1.093	0.407
	Bacillus sp. 83.2s	1.23	1.28	1.107	0.56
pH 7,5	*Bacillus subtilis* Bce2	1.146	1.173	1.133**	0.577
	Bacillus sp. 83.2s	1.333	1.253	1.01	0.433
pH 8,5	*Bacillus subtilis* Bce2	1.063	1.267	1.227**	0.733
	Bacillus sp. 83.2s	1.293	1.24	0.89	0.373

Note: *= floccules in suspension; ** = many floccules in suspension and insular pellicle of biofilm, formed on the flask.

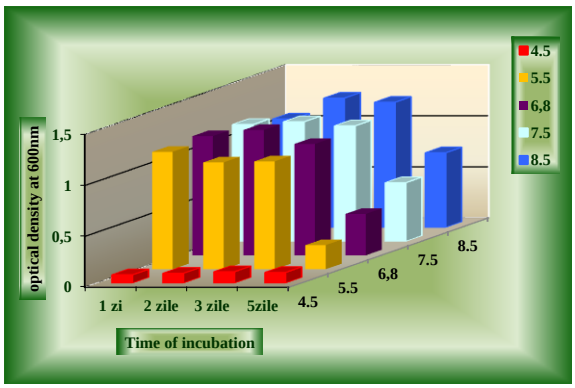

Figure 2. The multiplication rate of *Bacillus subtilis* Bce2 strain in different pH conditions

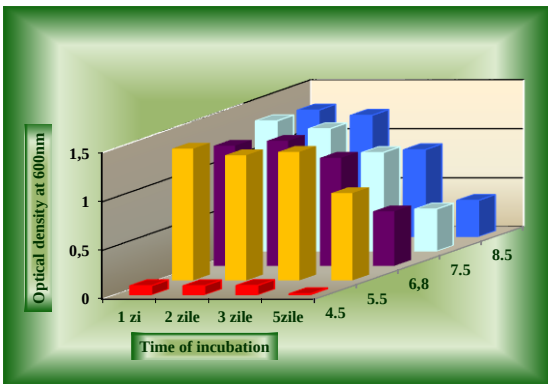

Figure 3. The multiplication rate of *Bacillus* sp. 83.2s strain in different pH conditions

Both strains of *Bacillus* went into decline after three days of incubation and their growth was more diminished under acidic conditions of pH 4.5, compared to the other analyzed variants.

Evaluation of NaCl influence on bacterial growth

The results that highlight the influence of salinity on the bacterial growths spectrophotometrically analyzed at 600nm, are presented in table 4.

Table 4. The optical density of bacterial cultures into different salinity conditions

Experimental variant	Bacterial strain	The absorbance at 600nm			
		24h	48h	72h	6 days
0,5% NaCl	*Bacillus subtilis* Bce2	0.92	0.84	0.79	0.28
	Bacillus sp. 83.2s	1.10	1.01	0.90	0.56
1% NaCl (Control)	*Bacillus subtilis* Bce2	1.20	1.11	0.99	0.56
	Bacillus sp. 83.2s	1.26	1.20	1.12	0.89
4% NaCl	*Bacillus subtilis* Bce2	1.12	1.09	0.89	0.31
	Bacillus sp. 83.2s	1.17	1.15	1.00	0.42
8% NaCl	*Bacillus subtilis* Bce2	1.04	0.96	0.69	0.32
	Bacillus sp. 83.2s	1.11	0.99	0.92	0.62
10% NaCl	*Bacillus subtilis* Bce2	0.99	0.90	0.68	0.23
	Bacillus sp. 83.2s	1.08	1.00	0.97	0.79

As a result of the *Bacillus* strains growing on nutrient substrate with different concentrations of sodium chloride, it was found a good tolerance of both strains at a concentration of 10% NaCI (figures 4 and 5).

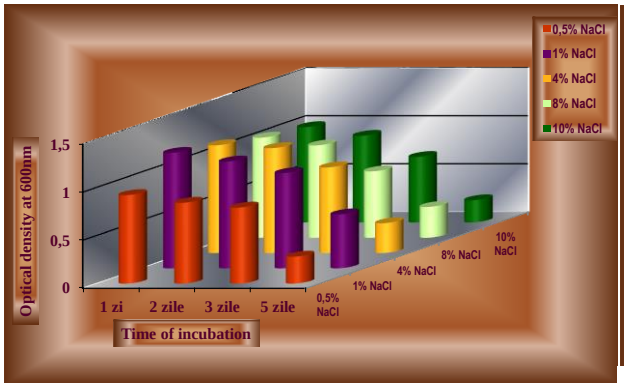

Figure 4. The multiplication rate of *Bacillus subtilis* Bce2 strain in different salinity conditions

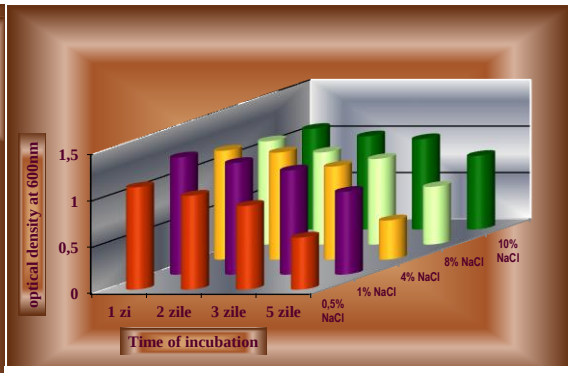

Figure 5. The multiplication rate of *Bacillus* sp. 83.2s strain in different salinity conditions

Since the start inoculum had a high concentration, both strains of *Bacillus* have been declining after 24h of growth. Thus, for Bce2 strain of *Bacillus subtilis*, the regression of growth was more sharply at concentrations of 0.5% and respectively, 10% NaCl. Regarding the *Bacillus* sp. 83.2s strain, regression was more intense at concentration of 4% NaCl, compared to the other analyzed variants.

CONCLUSIONS

- *In vitro* experiments have shown that, the multiplication rate of bacterial strains into temperature range of 25-35°C, decreased direct proportion to temperature and senescence of

bacterial cells was more emphasized at the higher temperature than optimum value of 27°C for these bacteria.

- Testing of the *Bacillus* strains at relatively low temperature of 15°C, reflected much slower growth, reaching optimal concentration to ensure the quality of biological control agent after five days of incubation in optimal conditions of nutrition and pH.
- The strains of *Bacillus* tested in temperature range examined, have reached concentration of 10^9cfu/ml after one day of incubation in optimal conditions of nutrition and pH.
- The multiplication rate of bacterial strains under various pH conditions was lower with increasing of pH value. Thus, the strain of *Bacillus subtilis* Bce2 was better multiplied under alkalinity conditions up to pH 8.5, while the strain of *Bacillus* sp. 83.2s grew better in slightly acidic pH of 5.5.
- The culture medium with acidic pH (4.5) was not conducive to the development of *Bacillus* strains tested.
- The bacterial strains showed a good tolerance to salinity, multiplication rate under 10% NaCl being slightly lower compared to the control (1% NaCl), only 14.3% for *Bacillus* sp. 83.2s, and 17.5% for *B. subtilis* Bce2 respectively, after 24 hours of cultivation in optimal conditions of temperature, pH and aeration.

AKNOWLEDGEMENTS

This study was financed by the NUCLEU Program 2009 and is part of the project no 40N/2009 with the title:'Microbiological means of plant protection, sustainable alternative to chemicals'.

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