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(RESEARCH ARTICLE)

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Effect of combined abiotic factors on the growth of PGPR associated with *Medicago Sativa*

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Abstract

Ten Plant growth promoting rhizobacteria (PGPR) were isolated from *Medicago Sativa* plants grown in two arid sites in south Morocco. Strains were recovered from functional root nodules, purified and checked for growth: they were all slow growing. They were evaluated for nodulation efficiency under controlled conditions: they were all infective and efficient with the host plant. Strains exhibited a wide tolerance to the main abiotic factors: half of the strains presented a good growth at 510 mM NaCl and 10% of strains tolerated levels up to 850 mM NaCl. All strains were able to grow at 32 °C and 10% of strains showed to be tolerant at 40 and 45 °C. Strains showed to be tolerant to alkaline pH: 50% of strains were all able to grow at pH 8.5 and 30% at pH 9. In vitro experiments of combined effects of NaCl x pH, NaCl x temperature and NaCl x pH x temperature indicated that pH and NaCl affected more growth than temperature. The combined effect of the three factors resulted in the decrease of growth; however, some strains showing a good performance were selected for subsequent inoculation tests under adverse environments.

Keywords: PGPR; *Medicago Sativa*; Abiotic Factors; Growth Rate

1. Introduction

Symbiosis is a key biological phenomenon through which biological nitrogen fixation takes place. The process, however, may be influenced by many factors. Soil properties, environmental conditions and the chemical interaction between the host plant and the bacterium are listed among the main factors. Soil water holding capacity, aeration, cropping system, organic matter and mineral content affect directly the plant root system development as well as the number of rhizobia in the soil [1]. The environmental conditions, affected by the global climate change, can disrupt all the symbiotic processes through various effects including rising temperatures and even more under arid climate by increasing drought. Under such conditions, the development and the performance of both partners of the symbiosis declines and the production as well [2]. All these facts make the use of synthetic or artificial inoculation a crucial necessity. However, there are possible actions to maintain an optimized N2-fixation; for instance, increasing the population density of specific and efficient indigenous strains of rhizobacteria [3] and which are naturally present in the soil but in low proportion. Exploring the performance and the persistence of these strains under extreme conditions is cost-effective and time-efficient [4]. This work aims to explore some PGPR strains associated with *Medicago Sativa* for their adaptation to salinity, pH, and extreme temperatures and to evaluate the combined effect of these factors on their survival and growth in order to select the most efficient for inoculation program.

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2. Materials and methods

2.1. Isolation of strains

Root nodules of alfalfa plants grown in fields in two arid regions Temsia and Laqliaa situated in south Morocco were harvested. They were rinsed, sterilized in 0.1 % sodium hypochlorite (NaOCl) for 1 min., re-rinsed several times, and then crashed in pure water under sterile conditions for bacterial extraction. The homogenate suspension was streaked on YEMA Petri dishes and incubated for 5 days at 28°C. Pure cultures of the different well-formed colonies were obtained by repeated streaking on the same media.

2.2. Determination of growth rate

The growth rate was evaluated form ln N curves inferred from the increase of the optical density (OD at 610 nm) of the strains cultured in YEM broth media (250ml) at 28°C under continuous shaking (200rpm). The medium was supplemented with the pH indicator bromthymol blue (BTB) at the end of growth to determine acid or alkali reaction.

2.3. Confirmatory nodulation test

Alfalfa seeds were surface sterilized and sown in pots filled with sterile soil (four seeds per pot). After seedling emergence, inoculation with 1 ml YEM broth culture from the late log-phase of the different strains was performed. After 15 days of growth under controlled conditions and adequate irrigation, plants were checked for the presence of nodules. Controls were not inoculated but placed under the same conditions.

2.3.1. Effects of abiotic stresses

Effect of salinity (NaCl), temperature and pH were first evaluated as a single factor in 100 ml YEM broth inoculated with 50 µl of late log-phase cultures of the different strains and incubated at 28 °C for 3 days in a rotary shaker as described above. For salinity test, the media broth was supplemented with 170, 340, 510, 680 and 850mM NaCl. For pH test, the media was adjusted to the following: 7, 7.5, 8, 8.5 and 9 units. For temperature test, the media was incubated after inoculation at the following degrees: 28, 30, 32, 35, 40, 45 °C.

The best performing strains were selected for evaluating the combined effect of temperature: 28 and 35°C with NaCl: 340 and 510 mM; NaCl: 170 and 340 mM with pH: 7, 8 and 8.5 units. The triple combination of the three factors was also studied NaCl: 170 and 340 mM with temperature: 28 and 35°C with pH: 7, 7.5 and 8 units.

The final selection of strains was based upon their tolerance to the combined abiotic factors.

2.3.2. Statistical analysis

All tests were evaluated in triplicate. SPSS Version 11.0 was used as a statistical analysis tool. Data were presented as means ± standard deviation (SD), $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Strain isolation

14 different strains were obtained from the root nodules. They were labeled and numbered according to their original site: TEM for Temsia and LQ for Laqliaa.

Morphological characteristics of the colonies: shape, color and size were uniform for all the strains. Creamy and white colonies with circular edges with 2 to 3 mm in diameter were obtained within 3 to 4 days.

3.2. Growth rate

Evaluation of the growth rate revealed that strains were all slow growing. BTB test showed that strains were alkali producing. However, the variation of pH at the end of growth should not be taken as a certain indication of the fast or slow growth.

Rhizobial strains associalted with alfalfa were reported as *Sinozhizobium meliloti* [5, 6] and exhibit a slow growth as we have found in this analysis.

3.3. Nodulation test

Strains were checked for their ability to induce nodulation with the plant host. 10 strains showed positive result with well-formed and functional nodule (figure 1).

Figure 1 Result of the confirmatory nodulation test with strain LQ 5.

3.4. Effect of NaCl

All the strains presented a good growth at 170 mM NaCl, half of the strains could tolerate 510 mM and 10 % of strains tolerated levels up to 850 mM NaCl (figure 2).

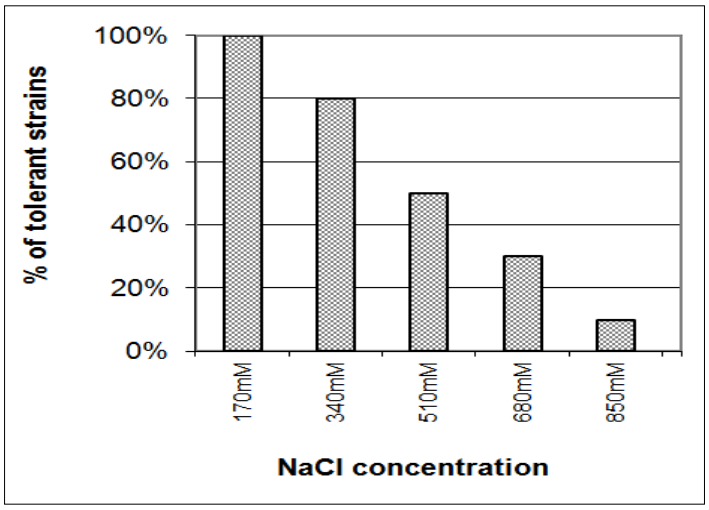


Figure 2 Tolerance of strains to different NaCl concentrations

Strains showed a large tolerance to salinity (NaCl). It was demonstrated that, at the molecular level, *Medicago* symbionts are tolerant to salinity [7]. Besides, the strains were isolated from arid sites in which both drought and salinity were recorded. In fact, aridity is another factor that causes increasing salinity as reported by Eswar et al. (2019) [8] and Gamalero et al. (2020) [9].

3.5. Effect of temperature

All strains were able to grow at 28, 30 and 32 °C. 40 % tolerated 35 °C, while only 10 % tolerated 40 and 45 °C (figure 3).

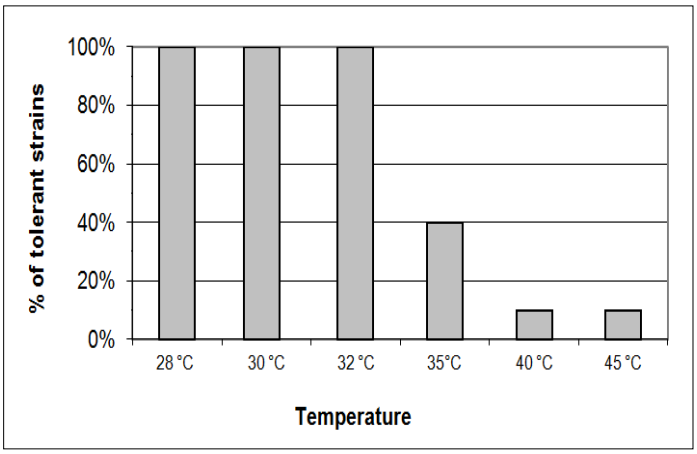


Figure 3 Effect of different temperatures on the growth of strains

Root zone temperature is known to have high impacts on the legume–Rhizobia symbiosis and the nitrogen fixation [10, 11, 12]. In this study, strains showed to be affected by high temperatures. In fact, rhizobia are described as mesophiles that can generally grow at temperatures ranging between 10 °C or above 37 °C with the optimum at 28°C [13]. However, many reports showed that strains isolated from hot and dry sites were more tolerant to high temperatures [14]. This was not observed in our analyses and no correlation with site is proved.

3.6. Effect of pH

Variable response was shown for pH. However, strains showed to be more tolerant to alkalinity. 100% of growth was recorded at pH 7 and 7.5, 80% at pH 8, 50% of strains were all able to grow at pH 8.5 and 30% at pH 9 (figure 4).

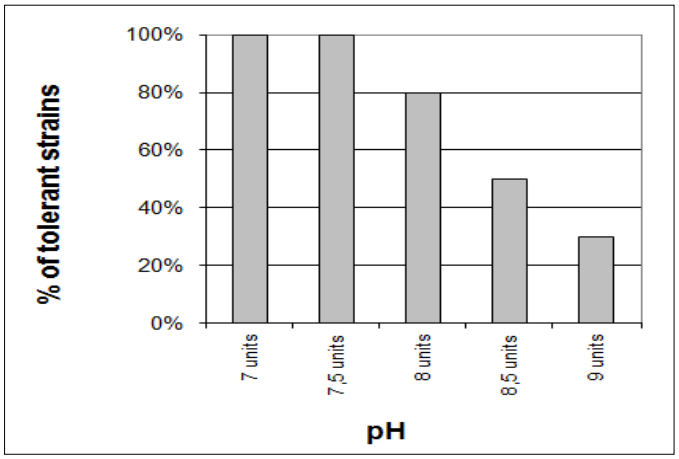


Figure 4 Effect of various pH values on the growth of strains

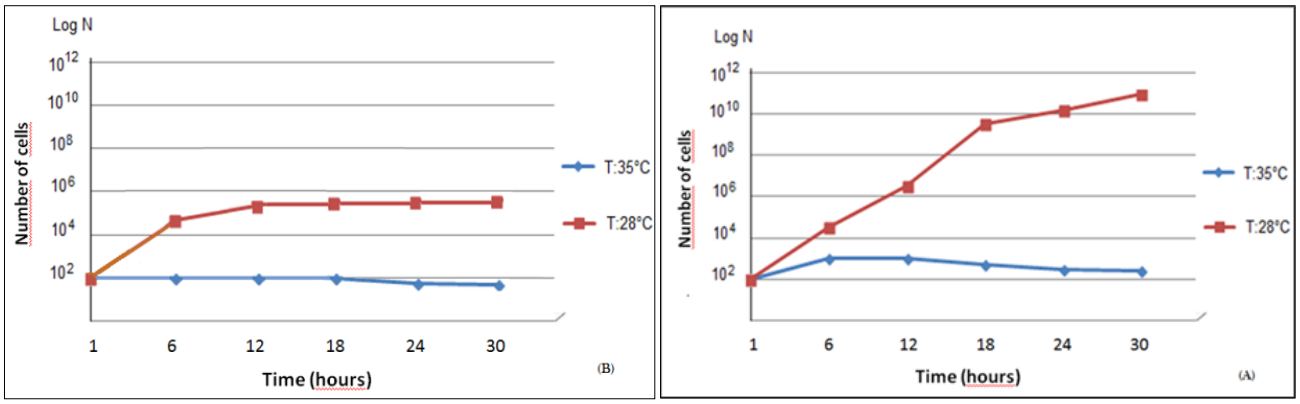
It is essential to evaluate the pH tolerance of the rhizobia for evaluating their performance in soil [15]. Soil pH plays a crucial role in the strain colonization of the rhizosphere, the attachment to the root hairs, the nodulation as well as many other effects [16]. Strains analyzed in this study showed slight tolerance to alkalinity. However, there is a large variability within rhizobia regarding their tolerance of acidity or alkalinity and even within the same species [17].

Results of the previous tested factors NaCl, temperature and pH were used to select the best performing strains: TEM 15, TEM 19, LQ 13 and LQ 5 for evaluating their tolerance and survival under the action of two combined factors.

3.7. Combined effect of NaCl and temperature

This test was performed for the four selected strains at two different NaCl concentrations: 340 mM and 510 mM combined each with two different temperatures: 28 and 35°C.

The four strains showed relatively the same results. Figure 5 illustrate results obtained for strain TEM 15. At the same salt concentration, results demonstrate the highly negative effect of the temperature on the strain's growth (Figure 5A). A slight growth was observed when salt concentration was increased (T = 28°C and NaCl = 510 mM) (Figure 5B). However, no growth was registered when both factors were at high levels (T = 35°C and NaCl = 510 mM).



(A: NaCl = 340 mM; B = 510 mM).

Figure 5 Effect of NaCl and temperature on the growth of strain TEM 15

3.7.1. Combined effect of NaCl and pH:

The test was performed for the four strains with 170mM and 340mM NaCl at 28°C with three pH values: 7, 8 and 8.5 units.

Figure 6 showed the results obtained with strain TEM 15 with 170mM NaCl. pH 8 induced a slight decrease of growth, while pH 8.5 inhibited totally the growth. The other strains showed relatively the same responses.

At 340mM NaCl, growth remained stationary at pH 7, decreased at pH 8 but was severely affected at pH 8.5 (data not shown).

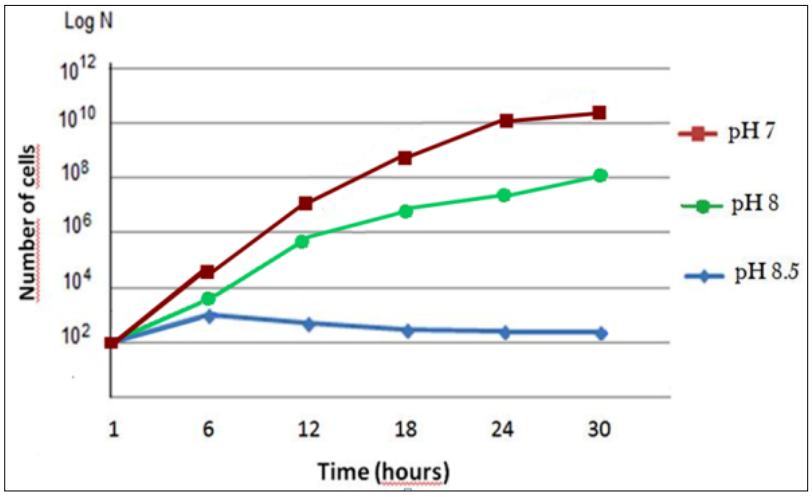


Figure 6 Effect of NaCl and pH on the growth of strain TEM 15

3.8. Effect of the combined three factors

From the test of combined effect NaCl x Temperature, it was showed that 510mM NaCl had a negative impact on growth, it was then omitted in the following test. The same for pH 8.5, as it showed also a negative impact in the test NaCl x pH. The other values of pH, NaCl and temperature degrees were considered.

The triple combination of the three factors tested showed that they have different impacts on the growth of strains. Figure 7 show the different effects for strain TEM 15. The same results were obtained for the other strains tested. The temperature showed to have a low impact than the other factors. At pH 7.5 and 340 mM NaCl, no difference in the number of cells was recorded with 28°C and 35°C; besides, at the same temperatures with pH 8 and 170 mM NaCl, the growth was not affected. Salt affected negatively the growth for all the combinations; however, it showed a synergic effect with pH. The pH showed a great impact: at the same temperature of 28°C, a noticed increase of growth was recorded for both 170 and 340mM NaCl when pH decreased from 8 to 7 units. On the other side, at the same temperature of 35°C, growth decreased when pH increased from 7.5 to 8 units for both salt concentrations.

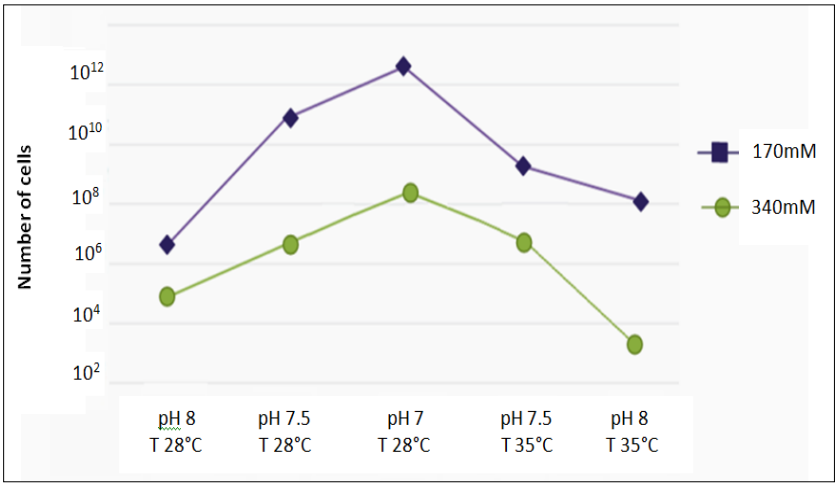


Figure 7 Effect of NaCl, pH and temperature combination on the growth of strain TEM 15.

4. Conclusion

Alfalfa (*Medicago Sativa*) is legume plant that establishes symbiosis with *Rhizobium* spp. to fix nitrogen for its nutrition and growth. Effective PGPR can increase the plant growth and the productivity. In soil, and especially in the rhizosphere, where bacterial populations are very diverse, the PGPR should compete to colonize the roots and to induce positive nitrogen fixation. However, the effectiveness depends on many factors, such as heat stress, salinity, drought, acidity or alkalinity. These factors occur in different ways and often simultaneously according to the environmental conditions, which may expose the PGPR to a severe decrease.

In this study, we have evaluated the impacts of three environmental factors: salinity (NaCl), high pH and high temperature - separately and in combination - on the growth of PGPR that were isolated from functional nodules of *Medicago Sativa* cultivated in arid areas. Five of ten strains gave encouraging results: they were infective and efficient under different combinations of the factors mentioned. These strains will be further tested in multiple inoculation assays with the host plant.

Compliance with ethical standards

Acknowledgement

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Conflict of interest

The authors declare that the research was conducted without any financial relationship that could be construed as a potential conflict of interest.

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