Study on plant growth promotion effects of legume symbionts

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Introduction:

Legume crops represent a relevant nutrient source in human and animal diets. The pea (*Pisum sativum* L.), soybean (*Glycine max* L.) and chickpea (*Cicer arietinum* L.) worldwide tend to conquer larger and larger sawing surface every year. For efficient growth rate and crop building these plants need symbiont rhizobacteria. The relevance of *Rhizobiales* in nitrogen fixation is well known, however it may be less obvious, that they perform other plant growth promoting (PGP) effects as well. These bacteria can also control the hormone system of plants, help them in nutrient uptake, etc. that makes the plants healthier and more resistant for climate extremities. For the efficient usage of bacterial strains it is crucial to know their optimums and tolerances.

Materials and methods:

In this study we described several PGP features and abiotic stress tolerance levels of selected legume symbiont strains: *Bradyrhizobium japonicum* Bj, *B. shewense* Bs, three strains of *B. diazoefficiens* Bd1, Bd2 and Bd3, *Ensifer sesbaniae* Es – symbionts of soybean, *Mesorhizobium ciceri* Mc – symbiont of chickpea and *Rhizobium leguminosarum bv viciae* Rl – symbiont of pea.

We screened the potassium (Hu et al., 2006), phosphorus (Lizama and Suzuki, 1988), zinc (Fasim et al., 2002) mobilization and siderophore production (Schwyn and Neilands, 1987) of our strains with agar diffusion methods.

To survey the pH optimum and abiotic stress tolerance of the strains we inoculated them into 96-well microplates. We used Nutrient broth and modified YEM liquid media. We modified these media to measure the pH tolerance – pH was set 3 to 10, without puffer – to test the salt stress tolerance – NaCl was added in 1-8% concentration – and to reveal the polyethylene glycol (PEG6000) induced osmotic stress tolerance – PEG6000 was added in 5-40% concentration. We measured the OD_{600} value of the wells at the end of the cultivation (30°C, 1050 rpm, inoculation rate: 2%, incubation times: Table 1.) to evaluate viability of the cells.

We measured levels of gibberellic acid, (GA_3) indole-3-acetic acid (IAA) (Tien et al., 1979), trans-zeatin (t-ZEA) and trans-zeatin riboside (t-ZEA-RIB) (Pooja et al., 2002) with thin layer chromatography (TLC) and quantified the results with CP-Atlas software. We applied liquid chromatography with tandem mass spectrometry (LC-MS/MS) equipment of ELTE Institute of Biology to supervise our results. Moreover we measured the amount of two metabolites of IAA synthesis: indole acetonitrile (IAN) and indole acetamide (IAM).

Strain	Incubation times (days)
Es	2
Bj	3
Mc	7
Bd1	6
Bd2	4
Bd3	3
Rl	2
Bs	4

Table 1.: Incubation times of microplate method.

Strain	P-mobilization	K-mobilization	Siderophore production
Es	-	-	++
Bj	-	-	+
Mc	++	-	++
Bd1	-	-	+
Bd2	-	-	-
Bd3	-	-	-
Rl	-	+	++
Bs	-	-	-

Table 2.: Results of agar diffusion methods.

Figure 1.: pH optimum

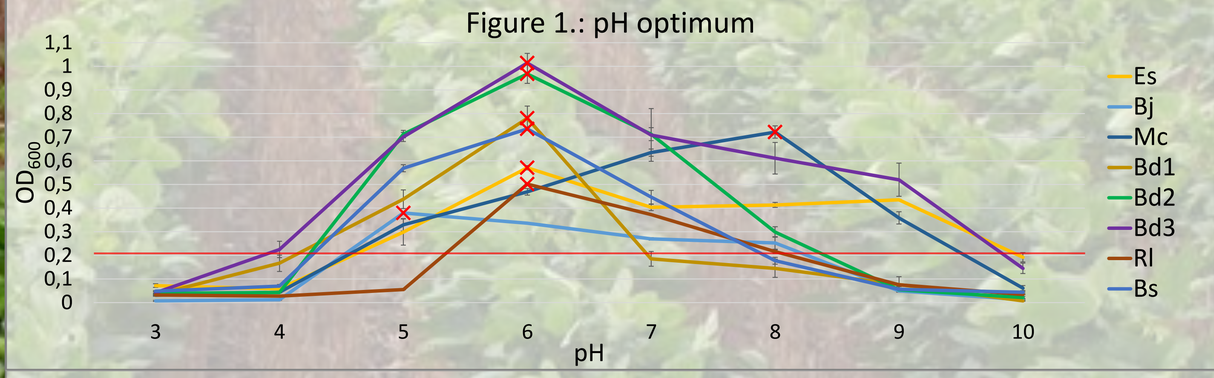


Figure 2.: Salt stress tolerance

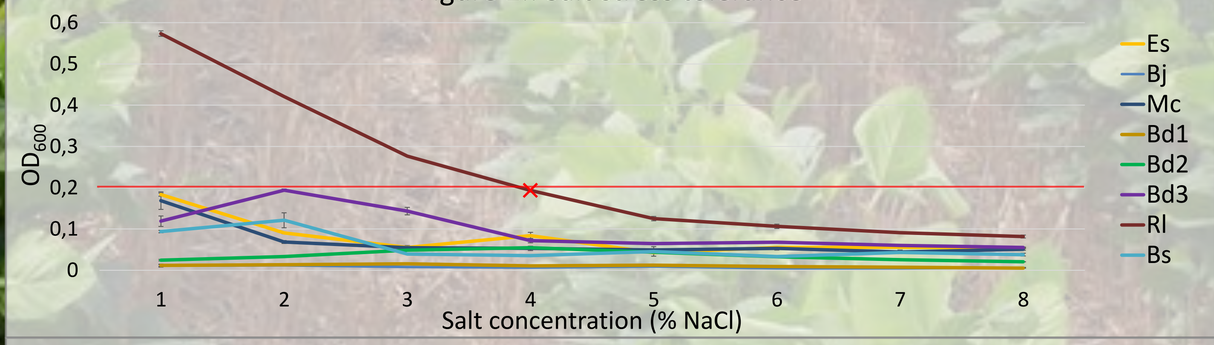
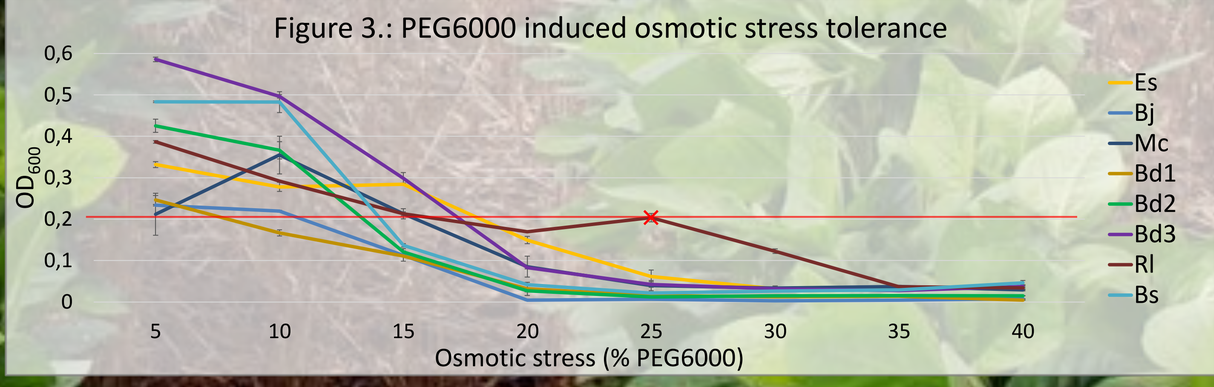


Figure 3.: PEG6000 induced osmotic stress tolerance



Results:

As presented in Table 2. P and K mobilization ability was scarce. Strain Mc showed moderate P-, and strain Rl showed weak K-solubilization. We could not detect Zn-mobilization for any of the examined strains. On the other hand weak to moderate siderophore production was observed for five strains, indicating Fe accumulation ability (Es, Bj, Mc, Bd1 and Rl).

pH tests (Figure 1.) revealed an average optimum value between 5.5-6.5 except for Bj (4.5-5.5) and Mc (7.5-8.5). With the exception of Rl all strains were able to grow between pH 4.5-5.5 and three of them were viable between pH 8.5-9.5 (Bd3, Es, Mc). Considerable salt stress tolerance (Figure 2.) has been shown only by Rl. This strain remained viable at 4% NaCl concentration. All species has proven to be moderately tolerant up to 15% PEG induced osmotic stress (Figure 3.). Higher PEG concentration reduced viability of the bacteria with the exception of Rl, performing outstanding osmotic stress tolerance.

TLC tests demonstrated IAA synthesis for Es (5.95±0.25 µg/ml), Mc (2.14±0.74 µg/ml) and Rl (2.80±0.34 µg/ml) (Figure 4.), however production of other hormones could not be detected in the examined strains. These results were further supported by LC-MS/MS, although this method showed one (Es) or two (Mc, Rl) orders of magnitude difference in the hormone levels (Figure 4.). LC-MS/MS results (Table 3. and Figure 5.) presented at least one positive strain for the production of each examined compound. GA_3 levels were detected only for Bj, and IAN only for Bd2. IAM production was recorded for all strains. We can highlight Es (270 ng/ml), Mc (42 ng/ml) and Rl (69 ng/ml) as the strains producing the highest levels of IAA. Moreover we have to mention Rl where most of the examined compounds were detected (IAN, IAA, t-ZEA, t-ZEA-RIB).

Hormone	Es	Bj	Mc	Bd1	Bd2	Bd3	Rl	Bs
GA_3	n. d.	$1.00*10^{0}$	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
IAN	n. d.	n. d.	n. d.	n. d.	$8.80*10^{-1}$	n. d.	n. d.	n. d.
IAM	$7.00*10^{0}$	$3.00*10^{-2}$	$1.30*10^{0}$	$4.00*10^{-1}$	$5.26*10^{-2}$	$2.10*10^{-2}$	$1.90*10^{0}$	$2,60*10^{-2}$
IAA	$2.70*10^{2}$	$1.00*10^{0}$	$4.20*10^{1}$	n. d.	n. d.	n. d.	$6.90*10^{1}$	$1.70*10^{-1}$
t-ZEA	$1.30*10^{-2}$	n. d.	$1.42*10^{-3}$	$1.09*10^{-3}$	$2.00*10^{-4}$	$1.00*10^{-4}$	$1.20*10^{-3}$	n. d.
t-ZEA-RIB	n. d.	n. d.	n. d.	n. d.	n. d.	$3.90*10^{-2}$	$6.90*10^{-2}$	$3.99*10^{-2}$

Table 3.: LC-MS/MS results of the strains in ng/ml magnitude. n. d. = not detectable.

Figure 4.: TLC and LC-MS/MS comparison

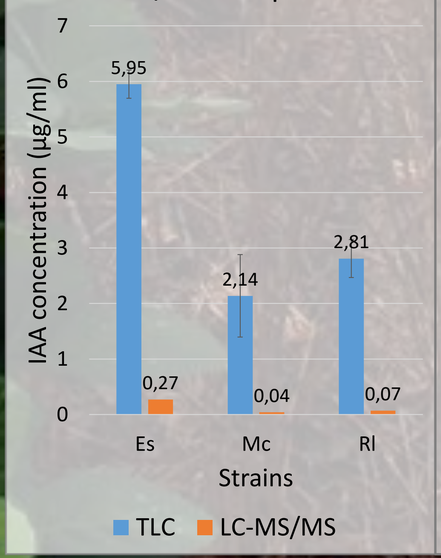
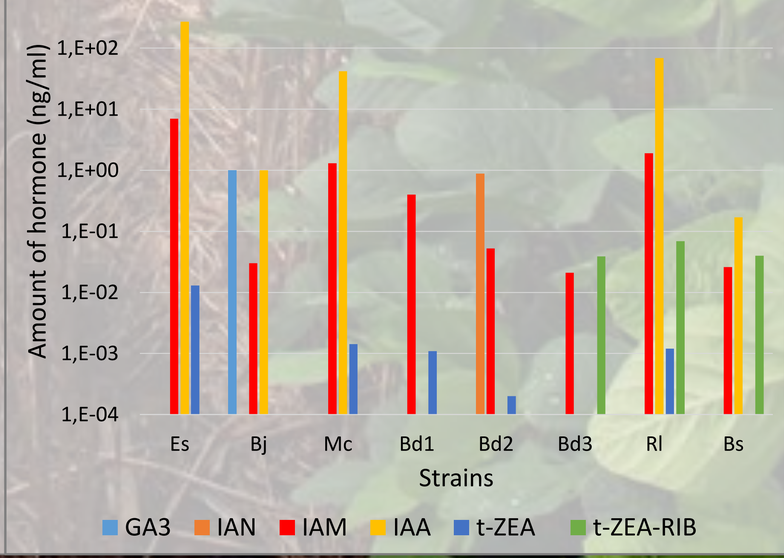


Figure 4.: LC-MS/MS results of the strains



Summary:

Agar tests revealed abundant siderophore production of legume symbionts. Average pH optimum is slightly acidic (5.5-6.5) with a few exceptions (Bj 4.5-5.5, Ms 7.5-8.5). Three strains had wide pH tolerance (Es, Mc, Bd3, 4.5-9.5). All strains demonstrated moderate osmotic and salt stress tolerance , with Rl strain shows significant salt stress tolerance.

Comparison of TLC and LC-MS/MS resulted in the conclusion that hormone concentrations up to 10 ng/ml are below the detection sensitivity of TLC. Another unreliability feature of TLC is that signal level reeds may bias one or two orders of magnitude. The LC-MS/MS results revealed that all strains produce IAM and/or IAN that are precursors of IAA. This finding may suggest that all examined strains are potential IAA producers, however this conclusion needs further experimental verification.

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