IMPACT OF SOYBEAN SEED INOCULATION WITH THE LEVAN-PRODUCING BACTERIA *PSEUDOMONAS AUREOFACIENS* ON SOIL INVERTASE AND LEVANSUCRASE ACTIVITIES UNDER SOIL WATER STRESS AND ELEVATED COPPER LEVEL

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The primary objective of this study was to examine the effect of seed inoculation with levan-producing bacteria Pseudomonas aureofaciens on soybean biomass production and invertase and levansucrase activities in the rhizosphere under water and high copper stress. Levansucrase activity increased in the rhizosphere of inoculated plants at reduced water content in agreement with our hypothesis. Bacterial inoculation increased soybean green mass at low water availability (35% WHC). The soybean cultivar Zodiac was not sensitive to high copper levels (300 ppm), but enzyme activities were significantly ($P<0.05$) reduced by copper in both the non-inoculated and inoculated rhizosphere soils. Further research is needed to elucidate the biochemical function of bacterial levansucrase, its expression under ecological stresses, and its utility in improving soil physical properties.

***Key words**: rhizosphere soil, invertase, levansucrase, Pseudomonas*

Soil invertase and levansucrase activities are of great agricultural importance because of their role in the carbon cycle and soil aggregation, [18]. Invertase (β-D-fructofuranosid – fructohydrolase, EC 3.2.1.26) is an extracellular enzyme that catalyzes the hydrolysis of sucrose liberating glucose and fructose. Levansucrase (sucrose-6-fructosyltransferase, EC 2.4.1.10) catalyzes levan (polyfructan) synthesis from sucrose by transfructosylation [12, 14-15]. According to Kiss and Dragan-Bularda [15-16] the synthesis of levan in toluene- and sucrose-treated soil was due to extracellular levansucrase in soil, although proliferating microorganisms could have participated as well. First, soil invertase activity was shown to be related to microbial numbers and metabolic activity. Later, the rate of invertase activity was shown to be the same in the presence and absence of toluene and proliferation of microorganisms during experimental determinations

contributed very little to the total invertase activity [16-17]. Toluene is widely used to inhibit enzyme synthesis during assessment of soil enzymatic activity. The only enzymatic activity which was reported to be seriously affected by toluene was dehydrogenase (EC1.1.1;1.2.1.), a strictly intracellular enzyme [23] commonly used to estimate soil microbial activity [4, 10].

Adsorption to clay minerals make invertase and levansucrase very stable against oxidative destruction [6]. The relationship between levansucrase and invertase activities in rhizosphere soil has not been determined, even though both enzymes require sucrose as substrate, which is commonly secreted into the rhizosphere by roots. In contrast to the majority of bacterial exopolysaccharides, which are synthesized from monosaccharide-nucleotide precursors and are little influenced by the nature of accessible nutrients, levan is formed directly from exogenous substrate – the disaccharide sucrose, only. Since levan synthesis does not require ATP and only requires a single enzyme – levansucrase, it is expected that this exopolysaccharide could be important in bacterial protection under different stress conditions (e.g., by complexing metals thereby reducing their toxicity or by imbibing water under water limiting conditions) as well as in soil structure improvement and water retention.

A *Pseudomonas* sp. strain has been demonstrated to increase its exopolysaccaride (EPS) production under low water conditions [22]. We isolated the levan-producing strain *Pseudomonas aureofaciens* from soybean (cultivar Zenit) roots growing in a Moldovan chernozem soil and demonstrated its beneficial effect on soybean plant wet and dry biomass accumulation (cultivars Zenit, Bucuria, Aura, Alina) under water and metal stress condition [7-8].

The objective of this research was to study the effect of seed inoculation with a levan-producing bacteria *Pseudomonas aureofaciens* on the biomass production of a new soybean cultivar Zodiac and on soil properties, including assessment of invertase and levansucrase activities in soybean rhizosphere soil.

MATERIAL AND METHOD

Soil properties and planting procedures . A carbonate-rich chernozem soil from the Moldovan Academy of Sciences Experimental Station (MASES,Chisinau, Moldova) was used in a greenhouse experiment. Selected soil properties are: pH 7.4; humus content 2.47 %; particle density 2.56 g cm^{-3}; total cation exchange capacity (CEC) 14.1 meq per 100 g of dry soil; and water holding capacity (WHC) 47.6 % of dry soil. Plastic pots with a volume of 7,5 kg were filled with 5.2 kg of dry soil. Soybean seeds (*Glycine max L.*) of the new cultivar "Zodiac" [2], grown on a field of MASES, were used without surface sterilization. The new cultivar is characterized by more than mean drought resistance.

Experimental design, bacterial inoculation, and plant growth. A total of 48 pots prepared as described above were incubated under several moisture and copper amendment conditions to mimic commonly encountered field cultivation stresses. Three specific soil treatments were used (16 pots each):

- **NS1** – nutrition status 1 - soil fertilized with 50 mg N kg^{-1} of dry soil, starting dose of nitrogen and 90 mg P kg^{-1} of dry soil, optimal for cultivation of legumes in the chernozem soil [25];

- **NS2** – nutrition status 2 - non-fertilized soil, resulting in N and P deficiencies;
- **NS2+Cu300 ppm** - non-fertilized soil + a toxic level of copper amendment, 300 ppm.

Two sets of pots - 24 pots for optimal soil water content (70% WHC) and 24 pots for reduced water (35% WHC) – were established in a greenhouse. For each group of 24 pots, 12 of them were planted with inoculated seeds, and 12 with non-inoculated seeds.

The *Pseudomonas* strain was isolated from soybean root-adhering soil (RAS) in 2003 [7], and identified as *Pseudomonas aureofaciens*. The strain was cultivated in liquid AS media at 28°C for 48-72 h. A bacterial suspension (10^9 CFU ml^{-1}) was used for seed treatment. The soybean seeds of the variety Zodiac were sprinkled with dystilled water (control) or bacterial suspension at a rate of $5 \cdot 10^7$ cells g^{-1} of dry seeds. Seeds were planted at a 2 cm depth (resulting in 4 plant per pot). Initial soil water content was 50% of WHC. At the blossom phase, plants were subjected to 14 days of water stress. In one set of 24 pots, soil water content was adjusted to 70% of WHC, while in the second 24 pots soil water content was 35% of WHC. After 14 days of water stress, the plants were harvested and root-adhering soil samples were collected and analyzed.

Soil sampling. Prior to soil sampling, the aboveground portions of the four plants per pot were cut for wet and dry mass determination. The whole root-systems of the four plants with soil were removed from the pot. Non-adhering soil was not retained for analysis. Soil adhering to the root system was gently shaken into a collection container and transported to the laboratory for analysis. Prior to analysis soil samples were passed through a 1 mm sieve and kept air-dried at 10-12°C.

Enzyme analysis: Soil invertase (INV) activity was determined by a method adapted from Chiunderova [5, 19] and Galstean [11]. Five grams of soil were treated with 6 drops of toluene, amended with 2,5 ml of acetic buffer (pH 5,6), and 2,5 ml of 20% (w/w) sucrose solution. Samples were incubated for 24 h at 37°C. The mixture was then filtered and the filtrate was used for analysis of reducing sugar by Bertran's method [21]. Copper content in the filtrate was measured by titrometric analysis with 0.01 N $KMnO_4$. Glucose content corresponding to reduced copper content was determined from specific table [21]. Invertase activity was expressed as mg glucose kg^{-1} soil h^{-1} at 37°C.

Soil levansucrase (LS) activity was measured by a method adapted from Kiss [13]. Briefly, 3g of soil was treated with 6 drops of toluene and incubated with 10 ml of 10% sucrose water solution in a closed container for 72 h at 37°C. Control tube with soil and water instead of sucrose solution was incubated under the same conditions. The soil was separated by filtration, and levan was precipitated from filtrate aliquots of 1 ml by 3 ml of cool acetone. The precipitation step was repeated twice. The precipitates were dried at 37 °C and dissolved in boiled distilled water. The quantity of fructose formed from levan after acid hydrolysis was determined as described by Filippovitch et al. [9]. Levansucrase activity was expressed as mg fructose kg^{-1} soil h^{-1} at 37°C.

Soil dehydrogenase (DH) activity was determined by the modified method of Galstean [11]. For determination of potential DH activity, 1 g of soil was treated with 1 ml of 0.1 M glucose and 1 ml of 1% (w/w) solution of 2,3,5-triphenyltetrazolium chloride (TTC). The tubes containing the soil plus reaction mixture were placed into an anaerobic jar and incubated at 30°C for 24 h [24]. Reaction mixture without soil and with previously autoclaved soil served as controls. After incubation, each mixture was treated with 12.5 ml of ethanol and shaken for 5 min before filtering. The red color density of triphenylformazan (TPF) was measured at 485 nm. Dehydrogenase activity was recorded as mg TPF kg^{-1} soil per 1 h at 30°C.

Statistical analysis. Microsoft Excel for Windows XP (Microsoft Office) was used for data analysis. An equal size matrix was analyzed. Means were evaluated by t-test

(with two-tail P-values, type 3 with unequal variances). The correlation analysis was performed with Pearson's coefficient (r), and determination coefficient (r^2). The limit of significance is given by $r \geq 0.45$, $r^2 \geq 0.20$ and $P \leq 0.05$ [1].

RESULTS AND DISCUSSIONS

Comparison of the all studied soil enzyme activities under soybean plants with that ones measured in initial soil before soybean planting has shown that all resulting activities were higher in fertilized soil, at the initial level in non-fertilized, and significantly lower in Cu-polluted soil (*table 1*).

Generally, DH was higher in NP-fertilized soil (NS1). Additionally, DH activity in the fertilized soil was not affected by water deficiency, whereas DH in the non-fertilized soil (NS2) was significantly lower at soil water content 35%WHC. Introduction of the levan-producing bacterial population into the soil with inoculated soybean seeds did not enhance soil DH activity, actual activity levels could be significantly ($P<0,05$) 11-18 % less than with the non-inoculated soil. Interestingly in the non-fertilized (NS2) but inoculated soil (+Inoc) under water deficiency (35%WHC), the DH activity was not reduced but was equal to that observed in fertilized soil. DH activity was strongly reduced in soil amended with 300 ppm Cu. Interpretation of these data is limited by the fact that copper can prevent formazan formation in the DH assay [3].

The soil INV activity was always statistically greater in fertilized soil. Additionally, this increased activity was not dependent on soil water content being maintained in soybean root zone with or without N and P fertilizer addition (NS1 and NS2), with only a single exception. INV activity was reduced significantly by 26% at 35% WHC in fertilized and inoculated soil. Inoculation of the rhizosphere soil in most cases did not change the level of soil INV activity, although in 1 case out of 6, a significant reduction of soil INV activity at 70% WHC (by 25%) after inoculation of non-fertilized soil was observed. Cu-pollution reduced INV activity in both non-inoculated and inoculated soils, although the differences were not always statistically significant (*tab. 1*).

Soil LS activity was stable even in the presence of water stress and a toxic level of 300 ppm of Cu; that is, the differences were not statistically significant. NP fertilizers were the strongest influencing factor at 70% WHC, resulting in an enhancement of LS activity by 8-35%. Contrary to expectations, inoculation of soil with levan-producing bacteria did not significantly alter the level of soil LS activity. The only exception occurred with inoculation of non-fertilized soil and subsequently subjected to reduced soil water. In this case, the soil LS activity reached the level observed in fertilized soil. Some contribution of levan-producing bacteria at more severe constraints could be assumed for water stress but not for chemical stress.

Table 1

The Student's t-test of enzymatic activities measured at different treatments

Treatment (for symbols see Material and Method)	Soil water, % of WHC	Mean enz. activity	SD	CV, %	P values, t-test, 2-tails, type 3 Compared treatments			
					WHC, 35% vs. 70%	Inoc. vs. non-Inoc.	NS1 vs. NS2	Cu300 vs. non-polluted
Dehydrogenase, mg TPF kg^{-1} h^{-1}								
NS1	70	47.1	7.0	15				
	35	41.8	2.6	6	0.23			
NS2	70	39.8	1.3	3			0.13	
	35	33.8	4.2	12	0.06		0.02*	
NS2 + Cu300 ppm	70	7.0	0.6	9				0.00***
	35	7.0	0.6	9				0.00***
NS1 + Inoc	70	43.4	3.2	7		0.39		
	35	37.1	2.6	7	0.02*	0.04*		
NS2 + Inoc	70	32.6	2.6	8		0.00**	0.00**	
	35	36.1	2.0	5	0.08	0.18	0.57	
NS2+Cu300+Inoc	70	6.8	0.8	11				0.00***
	35	6.6	0.5	7				0.00***
Initial soil		27.3	0.9	3				
Invertase, mg glucose kg-1 h-1								
NS1	70	409	86	21				
	35	409	112	27	1.00			
NS2	70	264	19	7			0.04*	
	35	221	47	21	0.17		0.04*	
NS2 + Cu300 ppm	70	159	6	4				0.00***
	35	171	14	8	0.19			0.12
NS1 + Inoc	70	517	78	15		0.11		
	35	385	60	16	0.04*	0.73		
NS2 + Inoc	70	198	37	19		0.03*	0.00**	
	35	208	25	12	0.69	0.65	0.00**	
NS2+Cu300+ Inoc	70	151	7	5		0.13		0.08
	35	153	10	6	0.74	0.08		0.02*
Initial soil		201	24	12				
Levansucrase, mg fructose kg^{-1} h^{-1}								
NS1	70	11.3	0.6	6				
	35	11.2	1.6	14	0.87			
NS2	70	7.3	2.4	33			0.04*	
	35	9.0	1.1	12	0.26		0.07	
NS2 + Cu300	70	5.8	2.7	48				0.45
	35	7.3	1.2	16	0.35			0.09
NS1 + Inoc	70	12.9	3.4	27		0.44		
	35	11.6	2.4	20	0.56	0.79		
NS2 + Inoc	70	7.5	1.5	20		0.86	0.04*	
	35	12.8	4.1	32	0.07	0.15	0.63	
NS2+Cu300+Inoc	70	6.3	5.3	84		0.88		0.67
	35	3.8	2.4	64	0.44	0.06		0.01*
Initial soil		8.2	3.4	42				

*, ** and *** significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

The coefficients of variation (CV) were in the range of 3-15% for DH activity, 4-27% for INV activity, and 6-33% for LS. For the copper amended soil, the data were highly variable. Thus, the LS activity revealed a higher range of deviation, compared with DH and INV activities (Table 1). This fact accentuates the adaptive function of LS activity in the changeable environment.

The Pearson's coefficient r-matrix (Table 2) shows that the investigated enzymes activities exhibited very high and significantly positive relationships with each other independently of nutrition status, water level and presence or absence of the inoculated levan-producing bacteria. A main observation is that soil INV and LS activities were always positively correlated despite the fact that the same substrate was necessary for expression of both invertase and levansucrase,. Thus, in the majority of the cases the competition for common substrate was not observed. A meaningful observation is the apparent reduction of the relationship between the two indicated enzymes in inoculated soil incubated under reduced water, as noted by the lower correlation coefficient for this situation (r = 0.586). The tendency for the increase of soil LS activity accompanied with a reduction of soil INV activity as a result of levan-producing bacteria activity in soil at water stress condition is convincing. Especially when it is considered together with the fact that soil DH activity in all circumstances had very high correlation to both enzymes involved in carbohydrate metabolism. It is worthy of mentioning that the enhanced soil LS activity in inoculated non-fertilized soil was concomitant with enhanced DH activity at the same treatment.

Table 2

Correlation matrix r (r^2)[a] between soil enzyme activities[b]

	Invertase	Levansucrase	Dehydrogenase
Non-inoculated			
70% WHC			
Invertase	1	0.985 (0.97)	0.901 (0.81)
Levansucrase		1	0.814 (0.66)
Dehydrogenase			1
35% WHC			
Invertase	1	0.972 (0.95)	0.810 (0.66)
Levansucrase		1	0.925 (0.86)
Dehydrogenase			1
Inoculated (+Inoc)			
70% WHC			
Invertase	1	0.998 (1.00)	0.803 (0.65)
Levansucrase		1	0.837 (0.70)
Dehydrogenase			1
35% WHC			
Invertase	1	**0.586 (0.34)**	0.702 (0.49)
Levansucrase		1	0.988 (0.98)
Dehydrogenase			1

[a] Values in parenthesis are the determination coefficient, r^2 , which measures the proportion of variance for a variable, that is explained by the variation of another estimated variable. The lower limit of significance is $r \geq 0.45$, $r^2 \geq 0.20$, and $P \leq 0.05$ (Aon et al., 2001); [b] conditions see Table 1 and Material and Method.

The new soybean cultivar, Zodiac, was significantly sensitive to water and N, P nutrient deficiencies. The plant green mass (PGM) was reduced by water stress by 25-29% in the fertilized soil, and by 33-41% - in the non-fertilized treatment. It is interesting to note that the cultivar was not sensitive to high copper levels (300 ppm) at either soil water contents. Inoculation of the soybean seeds with the levan-producing *P. aureofaciens* strain before planting was not accompanied by a significantly positive effect on biomass production, although there was a tendency for an increase in PGM production under the water stress condition. Probably, more replicates in the experiment with cultivar Zodiac could confirm a positive effect of seed inoculation for plant development under reduced water supply. Additionally, there was a high number of indigenous EPS-producing bacteria in the calcareous chernozem soil used in this study, thereby negating any potential impact of additional seed inoculation.

For all treatments, plant green mass (PGM) had a significant positive correlation with all three soil enzymatic activities. Although the invertase activity correlated more with plant characteristics and levansucrase activity correlated more with soil properties. Apparently, the inoculation of soil with levan-producing bacteria resulted in a dissociation of the positive correlation between INV and LS in favor of an increase in LS activity (Table 2), thus changing their relationship.

CONCLUSION

Inoculation of soybean Zodiac's seeds before planting by levan-producing *P. aureofaciens* strain revealed a steady tendency of increased plant green mass (PGM) under water stress. PGM had a significant positive correlation with all three soil enzymatic activities examined. At reduced soil water content (35% WHC), the inoculation of soil with levan-producing bacteria resulted in a lower value of the Pearson correlation coefficient ($r = 0.586$) between soil INV and LS activities than for the non-inoculated soil ($r = 0.972$). The soybean cultivar Zodiac was not sensitive to copper concentration (300 ppm) at either level of soil water content. All of studied soil enzyme activities were higher in fertilized soil than in non-fertilized soil, and significantly lower in Cu-polluted soil.

Acknowledgements. *We thank the NATO Science Program Board for supporting this research by grant EST, CLG 982852.*

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