BACTERIAL ENDOPHYTES AS POTENTIAL BIOSTIMULANTS TO INCREASE PLANTS GROWTH ON HEAVY METAL POLLUTED AREAS

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Abstract. Several endophytes were collected from root nodules of red and white clover, grown in unpolluted soils, tailing dumps, and heavy metal contaminated lands. Some of these strains revealed cadmium (Cd), copper (Cu), nickel (Ni), plumb (Pb) and zinc (Zn) tolerance to various concentrations. Among the 32 isolates, 22 strains revealed to be tolerant to at least one of the heavy metals tested. Best results were obtained for zinc tolerance, where 11 strains revealed bacterial growth in the presence of 5 mM of zinc sulphate. Eight strains were able to grow at 5 mM copper sulphate, while only 4 strains were tolerant to the same concentration of nickel chloride. Pb and Cd were more toxic to the studied bacteria. Eleven strains were able to grow at 1 mM plumb acetate, while only 4 strains at 1 mM cadmium sulphate. Most of the root nodule endophytes were *Rhizobium* species, although there were also *Stenotrophomonas maltophilia*, *Pseudomonas putida*, and *Rahnella aquatilis* bacteria. Most of these strains (28 bacterial endophytes) were able to solubilize dicalcium phosphate, while tricalcium phosphate solubilization was much slower, and seen only to 11 strains. Most of the tested strains increased clover germination and enhanced plant growth, compared to the untreated control, and improved root nodulation, compared with the reference strain *Rhizobium leguminosarum* biovar *trifolii* LMG 8820. Plant growth parameters were also improved in heavy metals contamination, when red clover was seed treated with selected bacterial mixture.

Keywords: biostimulants, clover, endophytes, heavy metals.

Rezumat. Bacterii endofite cu potențial biostimulator pentru promovarea creșterii plantelor în zonele poluate cu metale grele. Mai multe bacterii endofite au fost izolate din nodozități prezente pe rădăcinile plantelor de trifoi roșu și alb, colectate din soluri nepoluate, halde de steril și terenuri contaminate cu metale grele. Unele dintre aceste tulpini bacteriene au arătat toleranță la diferite concentrații de cadmiu (Cd), cupru (Cu), nichel (Ni), plumb (Pb) și zinc (Zn). Din cele 32 de izolate bacteriene, 22 de tulpini s-au dovedit a fi tolerante la cel puțin unul dintre metalele grele testate. Cea mai bună toleranță a fost la zinc, unde în prezența a 5 mM sulfat de zinc au fost capabile să se dezvolte 11 tulpini bacteriene. Dintre noile izolate, 8 tulpini au putut să se dezvolte la 5 mM sulfat de cupru, în timp ce la aceeași concentrație de clorură de nichel au crescut doar 4 tulpini. Plumbul și cadmiul au fost mai toxice metale grele, pentru izolatele bacteriene studiate. Doar 11 tulpini au putut să crească la 1 mM acetat de plumb și numai 4 tulpini la 1 mM sulfat de cadmiu. Majoritatea bacteriilor endofite izolate din nodozități au fost specii *Rhizobium*, deși au fost identificate și alte specii precum *Stenotrophomonas maltophilia*, *Pseudomonas putida* și *Rahnella aquatilis*. Majoritatea acestor bacterii endofite (28 de tulpini) au fost capabile să solubilizeze fosfatul dicalcic, în timp ce solubilizarea fosfatului tricalcic a fost mult mai lentă și observată la numai 11 tulpini. Majoritatea tulpinilor testate au stimulat germinația și creșterea plantelor, în comparație cu martorul netratat și au îmbunătățit nodularea rădăcinilor de trifoi, în comparație cu tulpina de referință *Rhizobium leguminosarum* biovar *trifolii* LMG 8820. Parametrii de creștere au fost, de asemenea, îmbunătățiți în urma tratamentului la sămânță cu un consorțiu de tulpini selecționate, atunci când plantele de trifoi roșu au fost expuse la metale grele.

Cuvinte cheie: bacterii endofite, biostimulatori, metale grele, trifoi.

INTRODUCTION

Heavy metal contamination is widespread today (NRIAGU, 1990). Soils can be contaminated with heavy metals as a result of a wide variety of anthropogenic sources, such as metallurgy, mining, energy industry, use of pesticides, chemical fertilizers, or application of sewage sludge to agricultural soils, and others (MC GRATH et al., 1995; ROBINSON et al., 2001).

Soil contamination with heavy metals can affect or inhibit plant development, or introduce toxic elements into the nutrient chain by plants absorption and accumulation in the edible parts, with undesirable consequences for herbivores and humans. At the same time, it can also affect soil microorganisms. However, there are natural soil microorganisms that can tolerate heavy metals, and can help soil remediation (AGUILAR et al., 2020; NURFITRIANI et al., 2020). Isolating such microorganisms and using them to prepare microbial inoculants for the treatment of heavy metal contaminated soils can increase the remediation efficacy (ATIGH et al., 2020). Bioaugmentation and microbial-based bioremediation are therefore promising approaches for increasing heavy metal removal efficiency (SAHA et al., 2021). To improve soil quality, phytoremediation is another promising approach for revegetation of tailing dumps and heavy metal polluted lands (YAN et al., 2020). Combining these methods, by using plant-microbial associations, is believed more efficient for soil remediation (RAKLAMI et al., 2022).

The present study is focused on selecting bacterial root nodule endophytes that can have biofertilizing and biostimulant activity, as well as heavy metal tolerance. The aim for such selection is to obtain a proper microbial inoculant that could help plants to grow in heavy metal contaminated areas. Such microbial inoculant could be of great interest in the revegetation process of tailing dumps.

MATERIALS AND METHODS

Bacterial isolation. Red and white clover, *Trifolium pretense* L. and *Trifolium repens L.* respectively, were collected from tailing dumps, heavy metal contaminated lands, and unpolluted soils from Romania. Their roots were gentry washed, and the root nodules were harvested. The best-looking nodules, pink coloured, were selected. The nodules were surface sterilised in two steps, with 70 % ethanol for 30 seconds, and with 4 % NaClO for 15 minutes, followed by five rinsed with sterile distilled water. The disinfected nodules were crushed in 1 ml of phosphate saline buffer (PBS), and let to infuse for 25 minutes. An amount of 100 µl suspension was plated on Yeast-Mannitol-Agar (YMA, containing 10 g mannitol, 1 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1 g $CaCO_3$, 18 g agar to one litre of distilled water, final pH 7.0) supplemented with Congo red 0.025 g/L. Bacteria were purified on YMA, maintained on agar plates or slants at 4 °C, and stored in 25 % glycerol at -20°C. Most probable number of rhizobia was also quantified in the studied soils.

***In vitro* heavy metal resistance assay.** The evaluation of isolated strains, for heavy metal resistance, was performed on YMA, in which four concentrations of heavy metals were added, 0.1 mM, 1 mM, 5 mM and 10 mM. Bacterial selection was performed on YMA containing $CdSO_4 \cdot 8H_2O$, $CuSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $Pb(CH_3COO)_2 \cdot 3H_2O$, and $ZnSO_4 \cdot 7H_2O$, respectively. Each time YMA was used as a control medium, without heavy metals. The strains were inoculated in spots of 5 µL, from freshly grown liquid cultures.

***In vitro* biostimulation tests.** Bacterial ability to solubilize inorganic phosphates was performed on two media, NBRIP and Pikovskaya (PVK). The NBRIP is based on dicalcium phosphate as insoluble phosphate source, while the Pikovskaya contained tricalcium phosphate (Table 1).

Table 1. Growth media and solutions.

Media / Solutions	Composition
YMA	10 g mannitol; 1 g yeast extract; 0.5 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7H2O$; 0.1 g NaCl; 1 g CaCO3; 18 g agar to one litre of distilled water, final pH 7.0
PBS	8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4; to one litre of distilled water, final pH 7.4
NBRIP	20 g glucose; 0.5 g yeast extract; 0.1 g $(NH_4)_2SO_4$; 0.25 g $MgSO_4 \cdot 7H_2O$; 0.1 g $MgCl_2 \cdot 6H_2O$; 0.2 g KCl; 5 g $CaHPO_4$; 18 g agar to one litre of distilled water, final pH 7.0
PVK	10 g glucose; 0.5 g yeast extract; 0.5 g $(NH_4)_2SO_4$; 0.01 g $MgSO_4 \cdot 7H_2O$; 0.2 g KCl; 0.0001 g $FeSO_4 \cdot 7H_2O$; 0.0001 g $MnSO_4 \cdot H_2O$; 5 g $Ca_3(PO_4)_2$; 18 g agar to one litre of distilled water, final pH 7.0
Hoagland solution	With nitrogen source (for 1 L): 5 ml of 1 M KNO_3; 5 ml of 1 M $Ca(NO_3)_2 \cdot 4H_2O$; 2 ml of 1 M $MgSO_4 \cdot 7H_2O$; 1 ml of 1 M KH_2PO_4, 1-5 ml of 1 g/L Fe-EDTA; 1 ml microelements stock solution. Without nitrogen source (for 1 L): 10 ml of 0.05 M $Ca(H_2PO_4)_2 \cdot H_2O$, 200 ml of 0.01 M $CaSO_4 \cdot 2H_2O$; 5 ml of 0.5 M K_2SO_4; 2 ml of 1 M $MgSO_4 \cdot 7H_2O$; 1-5 ml of 1 g/L Fe-EDTA; 1 ml microelements stock solution. Microelements stock solution 1000 X (for 1 L): 2.86 g H_3BO_3; 1.81 g $MnCl_2 \cdot 4H_2O$; 0.22 g $ZnSO_4 \cdot 7H_2O$; 0.08 g $CuSO_4 \cdot 5H_2O$; 0.02 g $MoO_3 \cdot H_2O$.

Microbial identification. Isolated bacteria were identified by Biolog GEN III technique, with two modifications from the manufacturers' protocol. Therefore, bacteria were grown on YMA instead of Biolog Universal Growth (BUG), and incubated for 3 days instead of one, as the isolated bacteria are slow-growers.

Pot trials. Selected bacteria, as well as the reference strain *Rhizobium leguminosarum* biovar *trifolii* LMG 8820, were analysed for nodule formation and plant growth promotion of red clover, in heavy metal free conditions (Experiment 1) as well as in the presence of 250 ppm Pd, 100 ppm Ni, 50 ppm Cd, 300 ppm Zn, 150 ppm Cu, and 1:5 v/v mixture (Experiment 2).

Pots were filed with 15 g sterile perlite, 0.5 to 2mm granulation, autoclaved at 121°C, for 1 h, three times at 24 h interval. Pots were watered with nitrogen-free Hoagland solution, in the experimental variants where seeds were bacterial inoculated. For the uninoculated controls, with no bacterial treatment, Hoagland solution with nitrogen source was used.

Red clover seeds were surface disinfected prior to use, as previously described for nodules. Disinfected seeds were inoculated by immersion in 10^9 cfu/ml bacterial suspension, supplemented with 1% carboxymethyl cellulose. Five seeds per pot were placed, and 3 replicates for each experimental variant were prepared. An extra inoculum was applied to the watered pots, injecting 100 µl of bacterial suspension near each seed. In the first experiment, single strain treatments were used, while in the second experiment, a bacterial consortium was tested. The bacterial consortium was composed of a mixture of 5 selected bacterial strains, combined in equal parts.

Plants were grown in controlled conditions of 16h light, with gradually increased light intensity from 15000 to 22000lx, up to 25°C, and 8h of complete darkness, at 20°C, with 70% constant relative humidity. Automated Sanyo MLR-351H growth chamber was used. Plants were kept under observation 3 weeks after germination. Germination, nodulation and plants hight were analysed.

RESULTS AND DISCUSSIONS

The microbial load of rhizobia-like bacteria was almost 3 times higher in the unpolluted soils (8.65×10^5 cfu/g) compared to the tailing dumps (9.93×10^5 cfu/g). 11 bacterial strains were isolated from red clover nodules, among which 2 were from tailing dumps; while 21 bacterial strains were isolated from the white clover, among which 14 were from tailing dumps and heavy metal contaminated lands.

Heavy metal resistance, or tolerance, to at least a single compound, was seen in 68.75 % of the isolated strains (Table 2). No strain was tolerant to 10 mM heavy metal, while strains were both tolerant and resistant to 5 mM of Cu, Ni and Zn.

Table 2. Bacterial growth in the presence of heavy metals (after 5 days of cultivation at 28°C).

Strain	Cd 10/5			Cu 15			Ni 17			Pb 18/11			Zn 19/11		
	0,5mM	1mM	5mM	1mM	5mM	10mM	1mM	5mM	10mM	0,5mM	1mM	5mM	1mM	5mM	10mM
1	**T**	S	S	**R**	**R**	S	S	S	S	S	S	S	**R**	**T**	S
2	**R**	**T**	S	**R**	**R**	S	**T**	S	S	**R**	**T**	S	**R**	**R**	S
3	S	S	S	**R**	S	S	**T**	S	S	S	S	S	**R**	**R**	S
4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6	**R**	**R**	S	**T**	**T**	S	**R**	S	S	**R**	**T**	S	**R**	**R**	S
7	**T**	S	S	S	S	S	**T**	S	S	S	S	S	**R**	**R**	S
8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S	**R**	**T**	S
11	S	S	S	**T**	S	S	S	S	S	**R**	**R**	S	**T**	S	S
12	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
13	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14	S	S	S	**T**	S	S	**T**	S	S	**R**	**T**	S	**T**	S	S
15	S	S	S	**T**	S	S	**T**	S	S	**R**	**T**	S	**T**	S	S
16	S	S	S	S	S	S	**T**	S	S	**T**	S	S	S	S	S
17	S	S	S	**R**	**T**	S	**R**	**T**	S	**T**	S	S	**R**	**R**	S
18	**R**	S	S	**R**	**R**	S	**T**	S	S	**R**	**T**	S	**R**	**R**	S
19	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
20	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
21	S	S	S	**T**	S	S	**T**	S	S	**T**	S	S	**T**	S	S
22	**T**	S	S	**T**	S	S	S	S	S	**T**	S	S	S	S	S
23	S	S	S	**T**	S	S	S	S	S	**T**	S	S	S	S	S
24	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S
1N	S	S	S	S	S	S	**R**	S	S	S	S	S	**R**	**R**	S
2N	S	S	S	S	S	S	**R**	**T**	S	**R**	**T**	S	**T**	S	S
3N	S	S	S	**R**	**R**	S	**T**	S	S	**T**	S	S	**R**	S	S
4N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
6N	**T**	S	S	S	S	S	**T**	S	S	**R**	**T**	S	**R**	S	S
7N	**R**	**R**	S	S	S	S	S	S	S	**R**	**R**	S	**R**	**R**	S
8N	S	S	S	S	S	S	**T**	S	S	**R**	S	S	**T**	S	S
LMG	S	S	S	**R**	S	S	**T**	S	S	**R**	**R**	S	**R**	**R**	S

Note: S = sensitive bacteria, no growth; T = tolerant bacteria, weak growth; R = resistant bacteria, normal to abundant growth,
LMG = reference strain *Rhizobium leguminosarum* biovar *trifolii* LMG 8820

There were 4 bacterial strains resistant to Cd, and one tolerant to 1 mM $CdSO_4 \cdot 8H_2O$. If the concentration of cadmium sulphate was reduced to 0.5 mM, the number of resistant bacteria increased to 6 strains, while the tolerant to 4 strains. To copper, there were 6 strains resistant to 5 mM $CuSO_4 \cdot 7H_2O$, and 2 strains were tolerant. To 1 mM copper sulphate the number of resistant bacteria was of 8 strains, and 7 strains were tolerant. Heavy metal resistance to Ni and Zn was similar, however there were more strains able to grow at 1 mM and 5 mM of zinc sulphate, than at copper sulphate or nickel chloride. To lead, there were 4 resistant strains and other 7 strains tolerant to 1 mM $Pb(CH_3COO)_2 \cdot 3H_2O$, while to 0.5 mM lead acetate there were 12 resistant strains and 6 tolerant strains.

The obtained results highlight strains no. 2, 6, 10, 18 and 24 as resistant, or at least tolerant, to all the five tested heavy metals. Various other isolated bacterial strains showed interesting tolerance to at least one of the heavy metals tested. Therefore, all strains were furtherly studied for other properties, useful from the practical point of view, such as phosphate solubilization.

Dicalcium phosphate was much more easily solubilized compared the tricalcium phosphate. This was shown by the larger clear halo surrounding the phosphate solubilizing bacteria, as well as by the higher number of strains that revealed a clear halo (Fig. 1).

On PVK medium, containing tricalcium phosphate, the strains no. 1, 6, 7, 11, 18, and 7N showed a semi-translucent halo, of 1mm, surrounding the bacterial colony, while strains no. 10, 14, 19, 21 and 2N showed a 2 to 3 mm halo. However, the halo was also incompletely cleared.

In comparison, on NBRIP medium, containing dicalcium phosphate, all tested strains revealed a clear halo surrounding the bacterial colonies, except for the strains no. 17, 18, 23 and 4N. Moreover, the halos were much wider, most strains revealing 3, 5, or 7 mm clear zone surrounding the colonies. Best results were obtained with strain no. 10, that revealed a clear zone of 18mm.

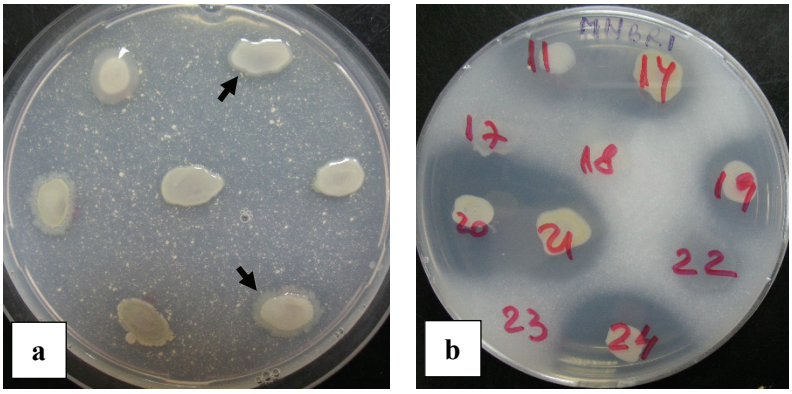

Figure 1. Phosphate solubilizing bacteria surrounded by halo
a. PVK medium containing tricalcium phosphate, **b**. NBRIP medium containing dicalcium phosphate.

Biolog identification revealed that strains no. 1, 2, 3, 5N, 7, 10, 11, 17, 18, and 24 most probably belong to the *Rhizobium* genus. However, no identification was available at species or biovar level, due to the limitation of this method. The strains no. 1N and 2N were identified as *Stenotrophomonas maltofila*, strains no. 6 and 7N as *Rahnella aquatilis*, while strains no. 14 and 21 were attributed to *Pseudomonas putida*. For the other strains, no identification was possible using the protocols A and C1 of the Gen III Biolog system.

Identifying endophytic bacteria from root nodules, also from other genera than *Rhizobium*, was not surprising. ABHINAV et al. (2020) isolated a plant growth promoting strain of *S. maltofila* from nodules of the medicinal legume *Mucuna utilis* var. *capitata* L. Although this bacterium is mentioned as a non-nodulating endophyte, it is confirmed as a nitrogen-fixing bacterium (REINHARDT et al., 2008; YU et al., 2011). Moreover, it can grow as endophyte, either individually, or as a co-inhabitant in the root nodules of leguminous plants (ABHINAV et al., 2020). *Rahnella aquatilis* was also found as co-inhabitant in root nodules (NOVELLO et al., 2022). Moreover, GEETHA et al. (2008), mentioned several other endophytes, such as *Pseudomonas*, *Burkholderia*, and *Enterobacteria*, isolated from root nodules of various leguminous plants, including clover. *Pseudmonas* sp. colonisation of leguminous root nodules was also confirmed by ZHAO et al. (2013).

Strains no. 1, 2, 3, 6, 7, 10, 11, 17, 18, 23, and 24, as well as strains 1N to 8N were tested for their nodulation ability compared to the reference strain *Rhizobium leguminosarum* biovar *trifolii* LMG 8820, on red clover. Seed treatment with selected bacteria positively influenced red clover germination. Results showed that seeds treated with strains no. 2, 17 and 24 had full germination, while plants developed larger size nodules (Table 3).

Table 3. Red clover germination and nodulation after bacterial seeds inoculation (4 weeks after germination).

Seed treatment	Germination (%)	Nodules/plant	Nodule size	Seed treatment	Germination (%)	Nodules/plant	Nodule size
Strain no. 1	83.3	28	Medium to large	**Strain no. 1N**	75	15	Medium and small
Strain no. 2	100	24	Large	**Strain no. 2N**	66.7	5	Small
Strain no. 3	83.3	31	Large and very large	**Strain no. 3N**	33.3	4	Small and very small
Strain no. 6	66.7	7	Small and very small	**Strain no. 4N**	50	4	Small and very small
Strain no. 7	66.7	37	Medium	**Strain no. 5N**	58.3	26	Medium and small
Strain no. 10	66.7	49	Small to medium	**Strain no. 6N**	33.3	6	Small and very small
Strain no. 11	66.7	48	Medium	**Strain no. 7N**	50.0	5	Small
Strain no. 17	100	45	Medium to large	**Strain no. 8N**	41.7	4	Very small
Strain no. 18	83.3	29	Large	**Reference strain**	83.3	30	Medium to large
Strain no. 23	83.3	53	Medium				
Strain no. 24	100	44	Large	**Untreated control**	33.3	0	-

Seed treatment with the bacterial strains no. 2, 17 and 24 envigored all clover plantlets to emerge.

Compared to the LMG 8820 reference strain, the rhizobia strains that significantly increased red clover nodulation were strains no. 23, 10, 11, 17 and 24. Seed treatments have influenced nodulation also in terms of nodule

size and colour (Fig. 2). Large and actively pink nodules were seen in plants treated with strains no. 1, 2, 3, 17, 18, 24, and reference strain, while medium side nodules of pink colour were seen in plants treated with strains no. 7, 10, 11, 23, 1N and 5N. Differences in nodules dimensions are possible due to the strain specificity as well as non-specific symbiosis among the bacterial strain and clover plants (YATES, 2008).

Surprisingly, a small number of nodules/ plants were also observed when clover seeds were inoculated with non-rhizobia strains. These nodules were of small size, with no visible pink colour. We consider this a consequence of plant cell proliferation, due to the phytohormone synthesis produced locally by the bacterial endophytes that colonised that root part.

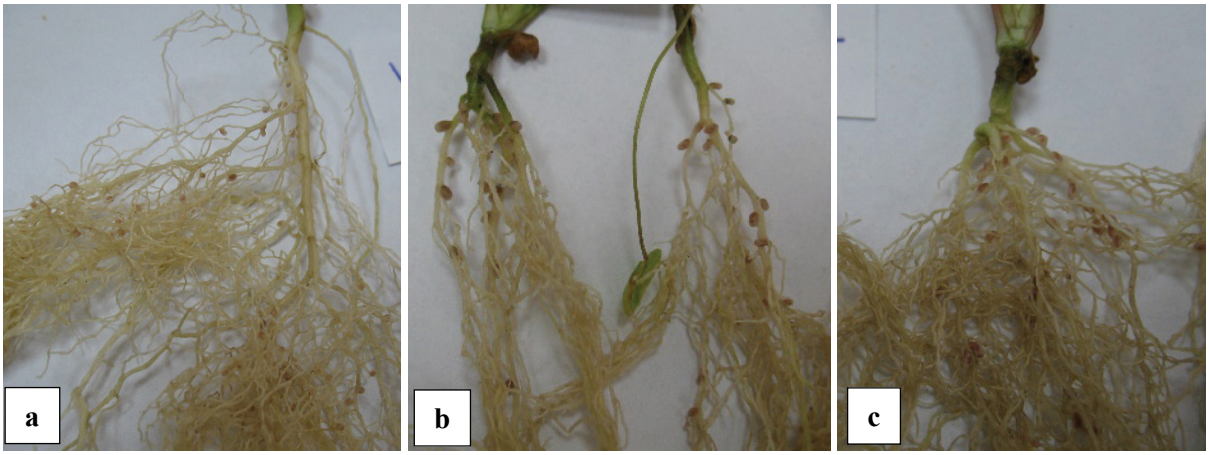

Figure 2. Clover root nodulation
a. small to medium size nodules formed by strain no. 10, b. large nodules formed by strain no. 18,
c. medium to large nodules formed by the reference strain.

Clover inoculation with strain no. 10, although it slightly improved seed germination (up to 66.7 %) and root nodulation,significantly enhanced plant growth compared to the reference strain (Fig. 3).

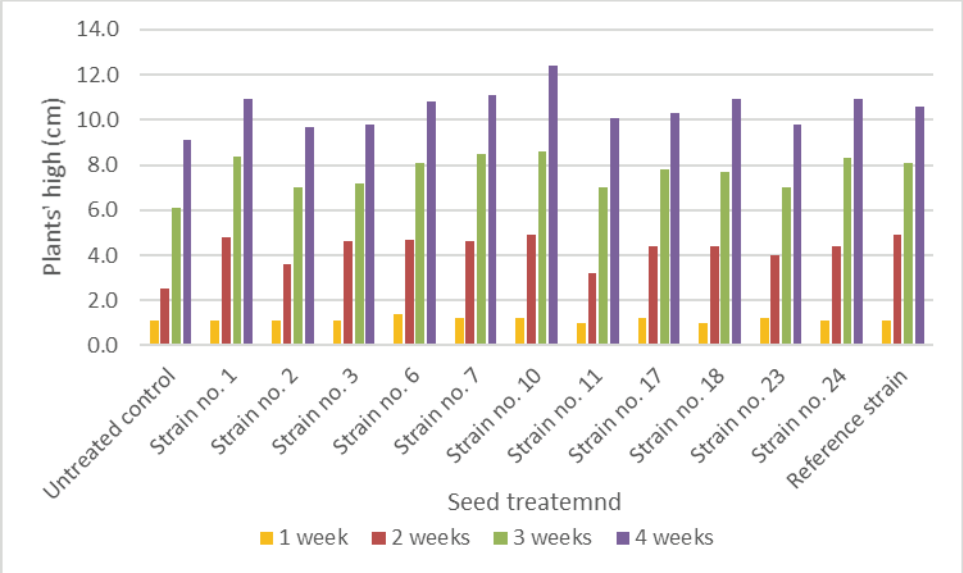

Figure 3. Red clover growth, when seed were inoculated with bacteria.

The experiment performed on heavy metal induced conditions confirmed their negative effects on seed germination and plant growth, and revealed the influence of plant beneficial inoculants applied as seed treatment to red clover.

In the V1 experimental variant, seeds were inoculated with bacterial consortium (strains no. 2, 10, 18, 24 and 2N) and plants were grown in 150 ppm Cu. Although clover emergence was 87%, plants survival decreased to 8% after three weeks of incubation. Most probably, this was due to the toxic effect of copper concentration within the substrate. Plants were weakly developed, and remained in the cotyledon stage, showing necrotic spots and yellowing. The root system was under-developed, showing brown areas, and lack of nodulation.

In the V2 experimental variant, were seeds were inoculated with bacterial consortium and plants were grown in 300 ppm Zn, the germination rate was 80%. Emerged plants survived during the incubation, although they were weakly developed, yellowed, having only the cotyledons and one mono-foliate leaf, with a short petiole and under-developed

leaf blade. Necrotic spots were present on the cotyledons. The root system was also poorly developed, with brown spots at the root branching points. Nodulation was not detected.

In the V3 experimental variant, where seeds were inoculated with bacterial consortium and plants were grown in 50 ppm Cd, the germination was 73%. After three weeks of incubation, 27% of the emerged plants died, due to the toxic effect of cadmium. The plants developed cotyledons, one mono-foliate and one tri-foliate leaf, with short petioles and reduced leaf blades. Plants were weakly developed, without nodules on the rooting system. No necrotic spots were seen on the plant.

In the V4 experimental variant, seeds were inoculated with bacterial consortium and plants were grown in 100 ppm Ni. The germination was 67%, but the survival rate decreased to 20% after three weeks of incubation. Plants remained at the cotyledonal stage, and showed necrotic spots at cotyledons' insertion points. The rooting system was also poorly developed, without nodules.

In the V5 experimental variant, seeds were inoculated with bacterial consortium and plants were grown in 250 ppm Pb. The germination rate was 87%. Plants presented normal appearance and root system. Small size nodules were formed, of an intense pink colour, proof of a nitrogen fixing activity. These confirmed the reduced toxic effect of the Pb concentration used, not only on the bacterial strains but also on the bacterial colonised plants.

In the V6 experimental variant, seeds were inoculated with bacterial consortium and plants were grown in a 1/5 (v/v) mixture of heavy metals (having 30 ppm Cu, 60 ppm Zn, 10 ppm Cd, 20 ppm Ni and 50 ppm Pb). In this experimental variant, the germination rate was 33%, but the survival rate decreased to 7% after three weeks of incubation. Plant remained in the cotyledonal stage, with necrotic spots of yellow appearance. The plant had a poorly developed root system, with several brown areas, and without nodules.

In the V7 experimental variant, seeds were treated with bacterial consortium, while plants were grown with no heavy metal application. The germination percentage in V7 was 87%. Plants were well developed, and formed up to 2 tri-foliate leaves, of an intense green colour. The root system was well-developed. The number of nodules/ plants varied between 2-7 nodules, of whitish pink to dark pink in colour.

In the V8 control variant, plants were uninoculated and grown without heavy metal application. However, plants were watered with nitrogen supplemented Hoagland solution. The germination percentage was 80%. Plants were normally grown, presented one or two tri-foliate leaves, with well-developed root system, without nodules.

Plants from the V9 experimental variant had normal growth conditions, without heavy metal application, and clover seeds were inoculated with the reference strain LMG 8820. The germination percentage for this variant was 80%, similar to the untreated control group, and slightly lower compared to the consortium treated seeds. Plants were well developed, intense green, with 1-2 tri-foliate leaves, and well-developed roots. The nodules were pink in colour, with an average of 2-3 nodules/ plant.

In the last two experimental variants, V10 and V11, plants were grown in a mixture of heavy metals, as in V6. In V10, seeds were inoculated with the reference strain LMG 8820, while in V11 seeds were untreated. However, in both of these experimental variants the germination rate was low, 45% in V10, while 33% in V11). Plants were improperly developed, with a reduced root system, without nodules. Various necroses were seen in both aerial and radicular parts of the plantlets. These confirm the extremely toxic effect of the heavy metals present into the growth substrate.

CONCLUSIONS

Several endophytic bacteria, 32 strains, were isolated from clover root nodules grown in tailing dumps, heavy metal contaminated lands and unpolluted soils. Only 31.25 % were identified as rhizobia (strains no. 1, 2, 3, 5N, 7, 10, 11, 17, 18, and 24), while 6.25 % were *Stenotrophomonas maltophilia* (strains no. 1N and 2N), 6.25 % were *Rahnella aquatilis* (strains no. 6 and 7N), 6.25 % were *Pseudomonas putida* (strains no 14 and 21), and the rest were unidentified. Rhizobia and *Stenotrophomonas* are nitrogen fixers, therefore they are able to help plant growth in less fertile environments. Most of these strains are able to solubilize dicalcium phosphate (87.5%), but only 34.4% were able to solubilize the tricalcium phosphate. Such traits are recommending the selected strains as biostimulants.

Among the studied strains, 68.75 % (22 strains) were tolerant to at least one of the five heavy metals Cd, Cu, Ni, Pb and Zn. The strains no. 2, 6, 10, 18 and 24 were found resistant, or at least tolerant, to all of these heavy metals, in concentrations less than 5mM.

The rhizobia strains as well as *Stenotrophomonas maltophilia* improved seed germination. Plant growth was also stimulated, if the red clover seeds were inoculated with rhizobia. Best results were obtained with strain no. 10, wich significantly increased the plant growth compared to the reference strain *Rhizobium leguminosarum* biovar *trifolii* LMG 8820.

The bacterial consortium made of five strains, no. 2, 10, 18, 24 and 2N, was able to promote red clover germination and growth in uncontaminated heavy metal conditions, as well as in the presence of 250 ppm Pb.

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