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Influence of bioremediation stimulators in soil on development of oat seedlings (*Avena sativa*) and their aminopeptidase activity

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Abstract: The selection of bioremediation techniques is important for purification of contaminated soil for agricultural use. Studies on soil contaminated with petroleum substances have indicated that the applied method of remediation has a bigger impact on the development of oat seedlings than the level of contamination. A yeast inoculum appeared to be a technique which was the friendliest to vegetation of oat.

Introduction

Development of plants, especially the young ones, is dominated by the processes of synthesis of new proteins necessary for their growth and full activity (eg. seed germination) (Bartling et al. 1992, Desimone et al. 2000, Matsui et al. 2006, Thayer et al. 1988). In the synthesis, degradation and conversion of plant proteins aminopeptidases play an important role. They are the enzymes being capable to cleave the amino acids from the N-terminus of the polypeptides and proteins (Bartling et al. 1992, Casano et al.1989, Chi et al. 2008, Desimone et al. 2000, Kowalczyk et al. 2009, Ogiwara et al. 2005). High concentration of aminopeptidases also appears in areas of plant tissue damage, for example as a result of pathogen attack, as a result of leaf damage. This is most probably due to the fact that the organism tries to defend itself by building or restoration of damaged tissues, which requires synthesis of new proteins and remodeling the old ones (Bartling et al. 1992, Chien et al. 2002, Matsui et al. 2006). The increase in aminopeptidase level in plants is also caused by other stress conditions, such as lack of water, salinity and pollution of the environment (Matsui et al. 2006). This causes that the level of aminopeptidases may serve as a marker of stressful conditions of plant growth and development.

Contamination of soil with petroleum derivatives leads to changes in its physical-chemical properties that in turn affect the plant development. The presence of hydrocarbons (aliphatic, monoaromatic and polycyclic aromatic hydrocarbons) in soil interferes with the biological balance in the result of increasing the amount of organic carbon and the deficit of assimilated forms of nitrogen, phosphorus and potassium. As an effect, there is the observed metabolic disorder and slow plant vegetation and, in extreme cases, plants languishment as the result of inhibition of the intake of: water, minerals, nutrients and oxygen. Certain amount of polycyclic aromatic compounds exists both in almost all plants and for instance in cereal crops they are present in concentrations of 46–66 µg/kg (Ellwardt 1977). Plants can take up hydrocarbons from contaminated soil through the root system and transport them to leaves. The problem of purifying the soil of petroleum derivative substances by a method of bioagumentation *Y. lipolytica* and the influence of the techniques on development of oat was also described by others (Kowalczyk et al. 2009).

On the other hand, there is no information about the effects of various soil bioremediation techniques from petroleum residues on the development of oat and aminopeptidase activity in plants.

Thus the purpose of the study was to determine the response of oat (*Avena sativa*) on used bioremediation stimulants in soil.

Material and methods

Materials

Plant material: Bohum oat seedlings were grown in pots filled with soil purified by various methods of bioremediation of petroleum substances.

Soil: Clay soil contaminated with petroleum derivatives taken from the site of pollution and stored for 2 years in natural conditions with initial content of petroleum derivative substances of 17 000 mg/kg d. m. The soil (S) was purified in laboratory conditions in the vases for 60 days using

the enzyme preparation Fyre-Zyme (FZ), yeast inoculum *Yarrrowia lipolytica* (Y), hydrogen peroxide (H) in following configurations:

1. enzyme preparation Fyre-Zyme (S + FZ)
2. yeast inoculum of *Y. lipolytica* (S + Y)
3. hydrogen peroxide (S + H)
4. enzyme preparation Fyre-Zyme and hydrogen peroxide (S + FZ + H)
5. yeast inoculum of *Y. lipolytica* and hydrogen peroxide (S +Y + H)

After discontinuation of bioremediation process (after 60 days) residues of separate groups of hydrocarbons were marked in averaged specimens: n-aliphatic C8 – C40), monoaromatic (BTEX) and polycyclic aromatic hydrocarbons (WWA) by method GC/MS. The conditions of conducting the analysis:

- BTEX – capillary column VF5 – ms with the length of 30 m; ID 0,25 mm and if 0,25 µm, carrier gas flow rate (He) – 1cm^3 per minute; injector temperature – 250°C, detector – 280°C; temperature program: 30-5/5/170-6; the current of detector MS from 1,2 to 1,4 kV.
- WWA – capillary column VF5 – ms with the length of 30 m; ID 0,25 mm and if 0,25 µm, carrier gas flow rate (He) – 1 cm^3 per minute; injector temperature – 300°C, detector – 310°C; temperature program: 80-8/10/270-12/2/300-12; the current of detector MS from 1,2 to 1,4 kV.
- n-aliphatic hydrocarbons – capillary column VF1 – ms with the length of 30m; ID 0,53 mm and if 1,50 µm, carrier gas flow rate (He) – 3 cm^3 per minute; injector temperature – 300°C, detector – 325°C; temperature program: 100-3/12/320-12; the current of detector MS from 1,2 to 1,4 kV.

Bioremedation stimulators:

- The enzyme preparation Fyre-Zyme (FZ) of the firm International Enzymes (as for the information given by the producer is a restricted formula of concentrated enzymes with addition of biosurfactants. For bioremediation of petroleum derivative contamination in soil and water using of 6% solvent of specimen is recommended.
- Inoculum *Y. lipolytica* A101 was originated from Biotechnology and Microbiology of Wroclaw University of Environmental and Life Sciences.
- Hydrogen peroxide pure for analysis POCH Gliwice in quantity of 1 mg O_2/g d. m.

Chemicals

L-leucine *p*-nitroanilide (Leu-pNA), 2-mercaptoethanol, triethanolamine, hydrochloric acid, dimethyl sulfoxide, PVP 40 (polyvinylpyrrolidone) and sodium chloride were purchased from Sigma (St Louis, MO, USA). The reagents were of analytical grade.

Apparatuses

Spectrophotometer UV-VIS, Varian Carry 100; centrifuge MPW 380R, Med. Instruments; Warszawa, homogenizer Kinematica, PT-2100, Switzerland, Moisture balance, Radwag.

Methods for evaluation of plant morphology

Oat seedlings were grown in soil in laboratory conditions in pots containing 1 kg of soil (S). Following bioremediation stimulators were used: (S + FZ), (S + Y), (S + FZ + H), (S + Y + H). The concentration of separate groups of hydrocarbons (residues after purification) were shown in Table 1.

After 60 days of bioremediation process 25 seedlings of oat were sown in each pot to a depth of 1cm (vertically – beard down) and incubated at room temperature of 22°C (± 2°C) in daylight. Humidity was maintained at 60% and every 2 days weight loss of water was replenished.

Plant grown without additives served as control (C), and absolute control was garden soil (C_0). Pot experiments have been done in 3 replications.

After 5 days, the percentage of germinated oat seeds was determined [%]. After 10, 30 and 90 days of vegetation the degree of development of plants was evaluated by measure of the length of roots and the hypocoty of oat seedlings [mm]. Finally, after 30 and 90 days the level of aminopeptidase activity of oat was determined and at 90 day a relative change in weight of plants by comparing with controls was determined (C_0) [%].

Isolation of aminopeptidase

For aminopeptidase activity measure 1 g of the plant tissue was homogenized in 2 ml of 50 mM Tris-HCl buffer (pH 8.0, 50 mM NaCl, 1% insoluble PVP). The suspension was stirred for 10 minutes at room temperature and centrifuged (6000 × g, 5 minutes, 4°C). The precipitate was discarded. The

Table1. Soil hydrocarbons concentration after 60-days bioremediation

Hydrocarbons	Concentration of hydrocarbons in soil after bioremediation stimulations [mg/kgdm]					
	C	S+FZ	S+Y	S+H	S+FZ+H	S+Y+H
n-aliphatic (C8 - C40)	10 550	8 136	13 233	6 990	9 397	16 002
BTEX monoaromatic	6	16	14	5	12	24
polycyclic aromatic hydrocarbons PAHs	63	55	55	31	61	76
Total	10 619	8 207	13 302	7 026	9 470	16 102

supernatant was tested for aminopeptidase activity and protein concentration.

Enzyme assays

Aminopeptidase activity was assayed at 37°C in 50 mM TEA-HCl buffer (pH 8.0). The substrate L-leucine p-nitroanilide (1.5 mM, dissolved in DMSO) was added to the assay buffer followed by addition of enzyme. The hydrolysis of leucine-p-nitroanilide (Leu-pNA) was followed by monitoring the appearance of p-nitroaniline versus time at 405 nm with a Cary 100 spectrophotometer equipped with a thermostated chamber. [$\Delta E_{405} = 9620\ M^{-1}\ cm^{-1}$] (Wachsmuth et al. 1966).

Protein determination

Protein concentration was determined by the method of Bradford (Bradford 1976) following the absorbance at 595 nm with bovine serum albumin used as standard.

Results and discussion

The development of oat seedlings in laboratory conditions was variable and depended on stimulants used in bioremediation process. The addition of stimulator influenced indigenous desorption and diffusion of petroleum derivative substances from different layers of soil (Table1). Supposed desorption of adsorbed particles of petroleum derivative compounds was also presumed by others (Robak et al. 2011).

Percentage of oat germination in the garden soil (C_0) was about 90%, while the use of residual hydrocarbons and introduced promoters of soil bioremediation process resulted in variable degrees of inhibition of oat germination. The lowest reduction (40%) in germination was observed in samples after bioaugmentation with inoculum of *Y. lipolytica* (S + Y), although the concentration of hydrocarbons was here higher than in other samples. In samples where Fyre-Zyme preparation was used or those, which were treated with yeast inoculum and oxygenated (S +Y + H) and (S + FZ + H) germination was about 40% (60% of reduction) (Figure 1).

The development of roots and hypocotyls of oat in tested soil samples during whole period of experiment was weaker than in the control garden soil (C_0), but periodically better in the cases when soil was not exposed to bioremediation (C). Stimulation of the development of the roots (as compared to control C) was observed in S + FZ after 90 days. We can assume that the concentration of hydrocarbons in the soil has little influence on the development of oat seedlings in comparison with those growing on soils where techniques of stimulation with ecological oxidant had been used (Figure 2).

At the beginning of aboveground development of oat seedlings in the soil contaminated with hydrocarbons it was similar and almost 50% lower than in the garden soil C_0, while in the following periods the highest plant growth was observed in yeast inoculum stimulated sites (S + Y) (Figure 3).

In the final effect, the best crop of oat biomass similar to that obtained from the garden soil (C_0) was also observed in samples treated with yeast inoculum yeast and oxygenated used for bioremediation (S + Y + H) (Figure 4).

The obtained results indicate that the condition of growth of oat seedlings is determined by the kind of bioremediation promoters used than by the concentration of hydrocarbons in the soil. They also showed that application of yeast inoculum is a method of choice for bioremediation.

Aminopeptidase activity determined in oat seedlings grown in soil purified by means of various techniques is in accordance with physiologic studies on aboveground parts of younger seedlings (30 days) and less in older ones (90 days).

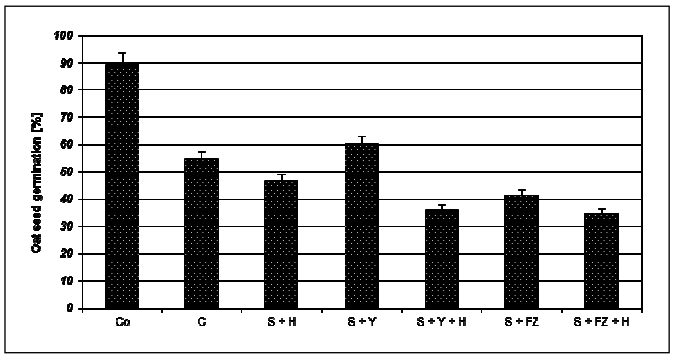

Fig. 1. Oat seeds germination in the soil after bioremediation by different techniques [%]

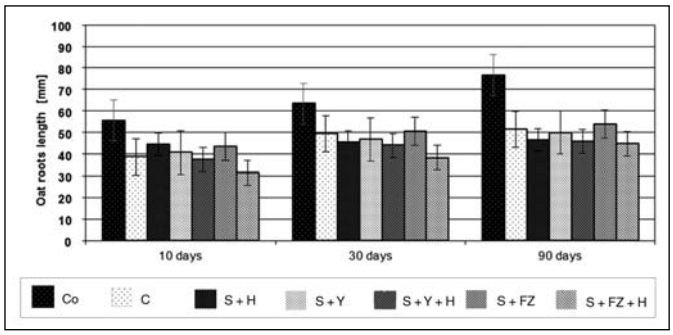

Fig. 2. Oat roots length [mm]

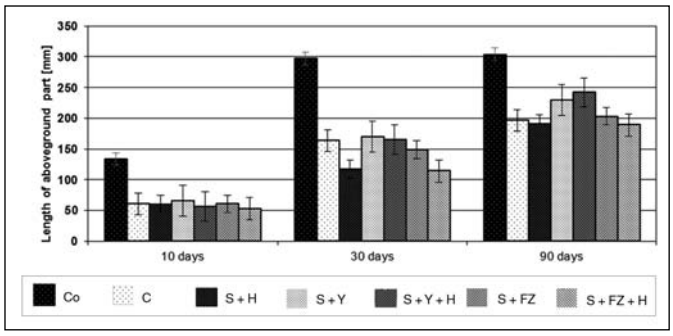

Fig. 3. Length of hypocotyls of oat seedlings [mm]

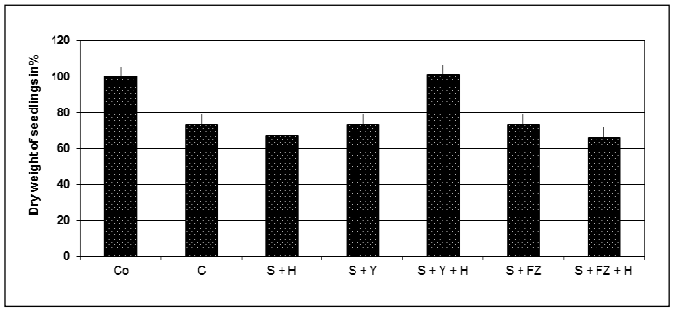

Fig. 4. Percent changes oat seedlings weight in relation to control

The highest activity of aminopeptidases has been determined is in seedlings grown in soil purified with yeast preparation (Table 2).

The lowest activity was determined in tissues of plants grown in the soil samples treated with Fyre-Zyme preparation (S + FZ). These results rather show intensification of the synthesis processes than the reaction of the plants to the stress condition; in older seedlings (90 day) regardless the bioremediation technique used. Aminopeptidase activity in seedlings of samples grown in soils bioremediated by S + Y, S + H, S + FZ and S + FZ + H compared to those grown in C_0 is reduced only to about 5%, while in the sample S + Y + H to 20%. On the other hand, in the seedlings from the soil without additives this activity disappears. The aminopeptidase activity in roots of seedlings grown in soil with yeast inoculum and S + Y, S + FZ + H and S + Y + H was similar to the activity in the roots of seedlings grown in garden soil. The highest aminopeptidase activity was observed in seedlings grown in S + H remediated soil during whole experimental period. This suggests that the oxygenation promotes aminopeptidase activity (Table 2).

Presented studies showed that the technique of bioremediation has greater impact on the development of oat seedlings than on the level of hydrocarbon contaminations of the soil. The friendliest technique of remediation seems to be the use of yeast inoculum since it resulted in lowest inhibition of oat vegetation. The yeast species – *Y. lipolytica,* in a form of cells suspension or immobilized liquid biomass was used for bioremediation of the soil contaminated with hydrocarbons (Kowalczyk et al. 2009, Robak 2006, Robak et al. 2011, Żogała 2005). It not only metabolizes harmful substances, but also secretes metabolites permitting the development of plants (Vassilev et al. 2001). Despite high concentration of hydrocarbons in the soil further introduction of the oxidant together with yeast inoculum accelerated the growth of seedlings. Probably use of oxidants results in stimulation of plant protein which indicates an increase in activity of aminopeptidase.

Table 2. Specific activity aminopeptidase in homogenates of radicle and hypocotyl of oat seedlings. One unit of enzyme activity was defined as the release of 1 μmol of free p-nitroaniline per minute per mg of protein

Soil after 60 days	Specific activity [U/mg] ± SD			
	Radicle		Hypocotyl	
	30-days	90-days	30-days	90-days
C_o	$0.00251 \pm 2.01 \cdot 10^{-4}$	$0.00154 \pm 1.23 \cdot 10^{-4}$	$0.00029 \pm 2.35 \cdot 10^{-5}$	$0.01211 \pm 9.69 \cdot 10^{-4}$
C	$0.00893 \pm 5.36 \cdot 10^{-4}$	$0.00158 \pm 9.48 \cdot 10^{-5}$	$0.00010 \pm 5.73 \cdot 10^{-6}$	$0.00010 \pm 6.08 \cdot 10^{-6}$
S+H	$0.07125 \pm 6.41 \cdot 10^{-3}$	$0.15675 \pm 1.41 \cdot 10^{-2}$	$0.00060 \pm 5.42 \cdot 10^{-5}$	$0.00039 \pm 3.50 \cdot 10^{-5}$
S+Y	$0.00370 \pm 4.44 \cdot 10^{-4}$	$0.00311 \pm 3.73 \cdot 10^{-4}$	$0.00075 \pm 9.00 \cdot 10^{-5}$	$0.00053 \pm 6.40 \cdot 10^{-5}$
S+Y+H	$0.00940 \pm 6.58 \cdot 10^{-4}$	$0.00231 \pm 1.61 \cdot 10^{-4}$	$0.00037 \pm 2.56 \cdot 10^{-5}$	$0.00217 \pm 1.52 \cdot 10^{-4}$
S+FZ	$0.01577 \pm 7.88 \cdot 10^{-4}$	$0.00349 \pm 1.75 \cdot 10^{-4}$	$0.00003 \pm 1.47 \cdot 10^{-6}$	$0.00088 \pm 4.42 \cdot 10^{-5}$
S+FZ+H	$0.00355 \pm 3.55 \cdot 10^{-4}$	$0.00095 \pm 9.51 \cdot 10^{-5}$	$0.00046 \pm 4.57 \cdot 10^{-5}$	$0.00053 \pm 5.28 \cdot 10^{-5}$

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References

[1] Bartling, D. & Weiler, E.W. (1992). Leucine aminopeptidase from Arabidopsis thaliana, *European Journal of Biochemistry*, 205, pp. 425–431.
[2] Bradford, M. (1976). A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principle of protein dye-binding, *Analytical Biochemistry*, 72, pp. 248–254.
[3] Casano, L., Desimone, M. & Trippi, V. (1989). Proteolytic activity at alkaline pH in oat leaves. Isolation of an aminopeptidase, *Plant Physiology*, 91, pp. 1414–1418.
[4] Chi, M.Ch., Liu, R.C. & Lin, L.L. (2008). Characterization of Bacillus kaustophilus, leucine aminopeptidase mobilized in Ca-alginate/k-carrageenan beads, *Biochemical Engineering Journal*, 39, pp. 376–382.
[5] Chien, H.Ch.R., Lin, L.L. & Chao, S.H. (2002). Purification, characterization, and genetic analysis of a leucine aminopeptidase from Aspergillus sojae, *BBA*, 1576, pp. 119–126.
[6] Desimone, M., Krüger, M., Wassel, T. & Hoffmann, R. (2000). Purification and characterization of an aminopeptidase from the chloroplast stroma of barley leaves by chromatographic and electrophoretic methods, *Journal of Chromatography B*, 737, pp. 285–293.

[7] Ellwardt, P. (1977). Variation in content of polycyclic aromatic hydrocarbons in soil and plants by using municipal waste composts in agriculture (In: Proceedings on a symposium on soil organic matter studies, Braunschweig, 1, pp. 291–298, Vienna 1976.
[8] Kowalczyk, A., Michniewicz, M. & Robak, M., (2009). Yarrowia lipolytica in bioremediation: cells entrapped in alginate and their influence on autochthonic mikroflora and oats germination, *Acta Scientiarum Polonorum, Biotechnologia*, 8 (3), pp. 15–24. (in Polish)
[9] Liu, X.Q. & Jagendorf, A.T. (1986). Neutral peptidase in the stroma of pea chloroplasts, *Plant Physiology*, 81, pp. 603–608.
[10] Matsui, M., Fowler, J.H. & Walling, L.L. (2006). Leucine aminopeptidases: diversity in structure and function, *Biological Chemistry*, 387, pp. 1535–1544.
[11] Ogiwara, N., Amano, T., Satoh, M. & Shioi, Y. (2005). Leucine aminopeptidase from etiolated barley seedlings: characterization and partial purification of isoforms, *Plant Science*, 168, pp. 575– 581.
[12] Robak, M. (2006). Is the environmental bioremediation possible with the participation of yeast Yarrowia lipolytica?, *Ekonatura,* 3, pp. 22–23. (in Polish)
[13] Robak, M., Boruczkowski, T., Drożdż, W., Lazar, Z., Baronowska, M., Prządo, D. & Steininger, M. (2011). Application of the yeasts Yarrowia lipolytica for *in-situ* bioremediation of soil contaminated with creosote oil – a case study, *Ochrona Środowiska*, 33 (2), pp. 27–33. (in Polish)
[14] Thayer, S.S., Choe, H.T., Rausser, S. & Huffaker, R.C. (1988). Characterizaton and subcellular localization of aminopeptidases in senescing barley leaves, *Plant Physiology*, 87, pp. 894–897.
[15] Wachsmuth, E.D., Fritze, I. & Pfeiderer, G. (1966). An aminopeptidase occurring in pig kidney. II A study on the mechanism of the hydrolysis, *Biochemistry*, 5, pp. 175–182.
[16] Vassilev, N., Vassileva, M., Azcon, R. & Medina, A. (2001). Interactions of an arbuscular mycorrhizal fungus with free or co-encapsulated cells of Rhizobium trifolii and Yarrowia lipolytica inoculated into soil-plant system, *Biotechnology Letters,* 23, pp. 149–151.
[17] Żogała, B., Robak, M., Rymowicz, W., Zientek, K., Rusin, M. & Maruszczak, J. (2005). Geoelectrical observation of Yarrowia lipolytica bioremediation of petrol-contaminated soil. *Polish Journal of Environmental Studies*, 14 (5), pp. 665–669.

Wpływ pozostałości substancji ropopochodnych w glebie na rozwój owsa i aktywność aminopeptydazową

Dobór technik bioremediacji jest bardzo ważny w oczyszczaniu gleby skażonej substancjami ropopochodnymi, która ma być wykorzystywanej rolniczo. Prezentowane wyniki wskazują, że na rozwój siewek owsa większy wpływ ma stosowana metoda bioremediacji niż poziom zanieczyszczenia węglowodorami. Z zastosowanych technik najbardziej przyjazne dla wegetacji owsa były te, w których wykorzystywano szczepionkę drożdżową.