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MICROALGAE EFFECTS ON THE BIOCHEMICAL PARAMETERS OF BARLEY GROWN ON SOIL CONTAMINATED WITH PETROLEUM PRODUCTS

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Abstract

A strategy to alleviate soil toxicity, which is gaining popularity in the last years, is bioremediation by inoculation with specific microorganisms. In the present study, we tested the influence of a mixture of four microalgae strains (Scenedesmus incrassatulus, Trachydiscus minutus, Chlorella sp. and Phormidium sp.) on some biochemical parameters of barley plants cultivated on soil contaminated with petroleum products. The aim was to evaluate the effect of microalgae suspension treatment on soil health and on the potential for phytoremediation. For the purpose, the nitrogen assimilation capacity, the levels of oxidative stress as well as the state of both enzymatic and non-enzymatic antioxidant systems in plants were measured. The results clearly show that petroleum-contaminated soil adversely affects the growth and development of the model culture, while treating the soil with the microalgae suspension significantly mitigates the negative impact. This is supported by the lower levels of stress markers and the increasing of some antioxidants in the plants grown on microalgae-treated soil. Therefore, the application of microalgae is an environmentally friendly strategy for improving soil health in areas affected by petroleum pollution.

Key words*: microalgae, oxidative stress, soil petroleum contamination, soil remediation, stress response.*

INTRODUCTION

Petroleum contamination, resulting from activities related to the petrochemical industry, is among the major environmental problems today. One of the reasons is the fact that petroleum-related components were shown to act as carcinogens and neurotoxic organic pollutants (Das & Chadran, 2011; Hatami et al., 2018). Moreover, the processes of natural purification of the affected soils are slow and take thousands of years to accomplish. Therefore, soils contaminated with petroleum products pose a permanent risk for the health of the human population as well as for the sustainable functioning of numerous ecosystems (Park & Park, 2011; Tang, 2019). In addition, some soil microorganisms, which are of particular importance for the proper biogeochemical cycles and maintenance of soil fertility, are sensitive to the presence of petroleum-based contaminants. The latter influence significantly the species distribution in the microbial community and generally have a negative impact on its biodiversity (Sutton et al., 2013). All these problems provoke serious scientific interest on the topic in the last years with the aim to develop technologies for safe and rational usage of such contaminated soils and their remediation (Chen et al., 2015; Tang, 2019). Classic methods to treat petroleum-contaminated soil are expensive and disturb the ecological balance at the treated sites (Juck et al., 2000; Liste and Alexander, 2000; Liu et al., 2012). A sound alternative is offered by the bioremediation approach, which utilizes living organisms to remove the toxic agents in the polluted soil or to alleviate their negative effects (Das & Chandran, 2011). The main advantages of bioremediation are its non-invasiveness and the relatively low cost (April et al., 2000). Though microalgae are important members of the microbial community in soil ecosystems, the information in the literature about their involvement in hydrocarbon biodegradation is sparse. The presence of microalgae, however, may synergize with the activity of oil-degrading bacterial strains. These consortia of microalgae/bacteria may scavenge various pollutants more effectively than individual microorganisms (Subashchandrabose et al., 2011; Chen et al., 2015). For instance, inoculation with blue-green algae such as *Calothrix elenkinii* stimulated the phyllosphere and rhizosphere microbiomes of okra (Manjunath et al., 2016). One of the main mechanisms responsible for the improvement of soil microbial communities in

response to inoculation with blue-green algae relates to the production of exopolysaccharides. Exopolysaccharides secreted by many micro algal species provide organic carbon for the growth and development of beneficial microbes (Xiao & Zheng, 2016; Xia et al., 2020). Their association with soil elements helps in the solubilization, mineralization, and bioavailability of macro and micronutrients, thus improving crop performance (Chiaiese et al., 2018). Soil contamination with petroleum is unfavorable for plant growth as well, due to the significant decrease in the available nutrients (Adam & Duncan, 1999) and the rise in the concentrations of certain elements such as iron and zinc to toxic levels (John et al., 2011). The usual symptoms observed in plants cultivated on petroleum contaminated soils include degradation of chlorophyll, general reduction of the photosynthetic activity and respiration, accumulation of toxic substances, size and biomass decrease, which in the case of crops leads to the consecutive loss of yield (Vange et al., 2004; Bona et al., 2011). The evaluation of the phytotoxicity is most often based on indirect methods like the assessment of the total yield loss in comparison to neighboring non polluted regions. Other readily accessible indicators of phytotoxicity are the data for seed germination, dry weight or similar biometric characteristics (Baud-Grasset et al., 1993; Lu et al., 2010). However, more accurate approaches utilize functional physiological and biochemical indicators as well, since germination efficiency and growth parameters by themselves do not provide sufficiently objective information. Such functional parameters include activities of the main antioxidant enzymes like peroxidases, catalase and superoxide dismutase, membrane integrity, changes in the photosynthetic parameters etc. (Cartmill et al., 2014; Wyszkowska et al., 2015; Hatami et al., 2018).

In the light of these actual problems, the major goal of this study was to perform a plant test, which combines growth and functional parameters, in order to assess the possibility for potential soil recovery by treatment with nonsterile microalgae cultures.

MATERIALS AND METHODS

Plant cultivation

The experiments were carried out with barley (*Hordeum vulgare* L.) as a model culture, variety Veslets. Plants were cultivated on soil in four different variants: Soil 4.5% + MS – polluted with 4.5% content of petroleum products, supplemented with microalgae suspension; Soil 4.5% - polluted with 4.5% content of petroleum products, no supplementation with microalgae suspension; Control + MS - non-polluted, supplemented with microalgae suspension; Control - non-polluted, no supplementation with microalgae suspension. Each of these variants was grown in three repetitions five plants per repetition in the following controlled conditions: photoperiod 16/8 h (light/dark), 250 μmol/m/s photosynthetic photon flux density (PPFD), 26/22ºC day/night temperature and 60-65% relative air humidity. After 21 days of cultivation, the plants were subjected to analyses for determination of various physiological and biochemical parameters.

For the variants with microalgae supplementation, before sowing the soil was irrigated daily with an inoculation mixture of 4 microalgae strains (*Scenedesmus incrassatulus*, *Trachydiscus minutus*, *Chlorella* sp. and *Phormidium* sp.) with a final concentration of 0.5 mg/ml for each of the species for a period of 5 days. Cultures of these four strains were previously isolated from an oil-spill contaminated site near Sofia, Bulgaria, and were therefore considered a suitable candidate for the evaluation of microalgae-assisted bioremediation of oil-contaminated soil. These strains were kindly provided by colleagues from the Bulgarian Academy of Sciences, the Institute of Algology. For the non-supplemented varieties the irrigation was carried out with water.

Within the period of their cultivation the plants were subjected to the following watering regimes: the variants supplemented with microalgae suspension were given 50 ml daily per pot, with alternation of microalgae suspension and water. For the other variants was used only water with the same quantity. Both groups were additionally supplemented twice with 50 ml ½ strength of modified nutrient solution: 0.505 mM KNO_3, 0.15 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.1 mM $MgSO_4 \cdot 7H_2O$, 4.63 mM H_3BO_3, 0.91 mM $MnCl_2 \cdot 4H_2O$, 0.03 mM $CuSO_4 \cdot 5H_2O$, 0.06 mM $H_2MoO_4 \cdot H_2O$, 0.16 mM $ZnSO_4 \cdot 7H_2O$, 1.64 mM $FeSO_4 \cdot 7H_2O$, and 0.81 mM Na_2-EDTA.

Lipid peroxidation

To determine the peroxidation state of membrane lipids the thiobarbituric acid (TBA) method (Heath & Packer, 1968) was used. It quantifies the malondialdehyde (MDA), considered to be one of the final products of lipid peroxidation. The spectrophotometric measurement is carried out at 532 nm wavelength, from the value of which is extracted the value of the non-specific absorption at 600 nm. The results are expressed as nmol MDA/g Fw. The quantity of the final MDA-TBA complex with red color is calculated with the specific molar extinction coefficient 155 mM^{-1} cm^{-1}.

Enzymatic analyses

In these experiments the following antioxidant enzymes were included: peroxidases (EC 1.11.1.7) - guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD), determined by the methods of Bermeyer (Bergmeyer, 1974) and Imberty (Imberty et al., 1984), respectively. Frozen material (0.5 g FW) was homogenized in 5 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM DTT and 1 mM EDTA, and centrifuged at 13 500 g (at 4 ºC for 10 min). The supernatant was collected and utilized for further experiments. Both methods for the antioxidant enzymes are spectrophotometric, with GPOD being measured at 436 nm and SPOD at 550 nm. In addition, the activity of the enzyme nitrate reductase (NR), which is crucial for nitrogen metabolism (NR; EC 1.7.99.4) was also assessed. NR activity was determined using *in vivo* assays following the methods of Al Gharbi & Hipkin (1984) and Downs et al. (1993). To determine the concentrations of nitrite produced during the assays, 1 ml samples were mixed with 1 ml sulphanilamide (1% weight/volume in 10 times diluted concentrated HCl) and 1 ml NEDA (0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water). The solution was left to stand at room temperature for 10 min, until the colour development was complete, and the absorbance was measured at 543 nm using a spectrophotometer.

Measurement of free proline

Free proline content was determined by the methodology of Bates et al. (1973).

Measurement of polyphenols

Determination of polyphenols was carried out by measuring the absorption at 765 nm by a modified method of Singleton and Rossi (Singleton & Rossi, 1965). In brief, 10 g of each sample were mixed with 20 mL of 60% acidic methanol in an extraction tube and subjected to homogenization. The extraction continued 24 hours at room temperature. The supernatant was used for determination of total polyphenol content (TPC) by spectrophotometry, using gallic acid (99% purity, Sigma Argentina) as standard. The reagent mixture contained 40 µl extract, 3160 µl distilled water, 200 µl Folin - Ciocalteu's phenol reagent (Merck Chemicals) and after 15 minutes 600 µl 20% $NaCO_3$ were added. The tubes were then left at room temperature for 60 min and thereafter the absorbance at 765 nm was measured. The TPC was finally expressed as gallic acid equivalents (GAE) in g/100 g material.

Statistical analyses

One-way ANOVA (for $P < 0.05$) was used for all experiments. Based on the ANOVA results, a Tukey's test for main comparison at a 95% confidential level was applied.

RESULTS AND DISCUSSIONS

To assess the overall influence of petroleum contamination on barley plants, initially experiments to measure biochemical parameters were conducted. In order to determine whether petroleum contamination and microalgae supplementation interfere with the assimilation of nitrogen and general nitrogen metabolism the activity of the enzyme nitrate reductase was measured. As shown in Table 1, when grown in petrol contaminated soil the barley plants manifested the highest nitrate reductase activity (100 µg NO_2/g/h). The plants grown on control soil showed lower enzyme activity (82 µg NO_2/g/h) compared to the contaminated variant. In turn, the presence of microalgae seems to affect negatively the activity of nitrate reductase both in the plants cultivated on normal soil and those subjected to petroleum contamination. The observed differences are all statistically significant with $P < 0.05$. This is in concordance with numerous previous studies from authors worked with petrol contaminated soil (Anoliefo and Edegbai, 2000; Akaniwor et al., 2007).

Different stress factors (abiotic or biotic) often triggers development of oxidative stress as a second stress. Oxidative stress is associated also with large-scale peroxidation of polyunsaturated fatty-acids, which ultimately leads to the accumulation of malondialdehyde (MDA). Therefore, MDA levels are used as a direct marker for this kind of stress (Shulaev & Oliver, 2006; Xu et al., 2019). The measurement of MDA concentration revealed as expected, that the lipid peroxidation was more expressed in the variants cultivated on contaminated soil (Table 1), confirming the stress on which these plants are subjected. This observation confirms the findings of other researchers worked with barley and petroleum contamination (Rajaei et al., 2016). On the other hand, supplementation with microalgae reduces lipid peroxidation as shown by the decreasing MDA quantities both in the controls and the contaminated variants.

Table 1. Measurements of the nitrate reductase activity (NR), lipid peroxidation state, expressed as malondialdehyde (MDA) content and free proline in young *Hordeum vulgare* L. plants, grown either on control non-polluted or polluted soil with petroleum products and treated or not with microalgae suspension (MS). Variants: polluted soil with 4.5% petroleum products + MS; polluted soil with 4.5% petroleum products; control soil + MS; control soil. The parameters were measured in leaves

Variants	NR activity (μg NO_2/g/h)	MDA (nmol/g FW)	Proline (mg/g FW)
Soil 4.5% + MS	75±11 b	18.3±1.2 b	0.19±0.08 a
Soil 4.5%	100±20 a	23±0.5 a	0.025±0.003 d
Control + MS	58±7 c	10±0.2 c	0.125±0.06 b
Control	82±4 b	12.4±0.5 c	0.073±0.002 c

The data in the columns followed by the same letter (a, b, c) are not statistically significant for $P < 0.05$.

A powerful biomarker for plant stress and the antioxidant status, which can be relatively easily determined, is the concentration of free proline. This amino acid is accumulated in plant cells in various stress conditions, mostly during drought, cold or salt stress factors (Miller et al., 2010; Hayat et al., 2012), and acts both as a compatible solute to facilitate water uptake and as a direct scavenger of reactive oxygen species (ROS) (Matysik et al., 2002). The analysis of free proline in our case demonstrates that when no microalgae are added barley plants contain almost three times as much free proline in normal conditions as when exposed to petroleum contamination (Table 1). This most probably reflects the state of stress of petroleum-treated plants which exhausts both carbon and nitrogen sources for the biosynthesis of this amino acid. The supplementation with microalgae significantly increased the amounts of proline in both variants. However, the microalgae-induced proline accumulation was much more pronounced in the contaminated samples than in the control ones reflecting the possible induction of defense mechanisms by the MS suspension.

Another non-enzymatic biomarker for stress is the accumulation of polyphenols (Krishnaiah et al, 2011). Their quantity expressed as gallic acid equivalents was assessed separately in roots and leaves (Table 2). In leaves the amounts of gallic acid equivalents (GAE) did not vary a lot between the different variants. In roots when the plants were cultivated on clean soil, the addition of MS-induced only a slight increase in GAE. Much higher amounts of total polyphenols in the roots were detected in the polluted variant without microalgae. Similar results were reported by other authors as well (Noori et al., 2012). However, in the presence of petroleum contamination, the microalgae supplementation led to a noticeable reduction of gallic acid equivalents. This observation is in contrast with the effect of microalgae on the other non-enzymatic antioxidant compound - the proline. The status of the antioxidant system in the studied plants was investigated not only by means of non-enzymatic antioxidants like proline and polyphenols but also with analyses of the activities of the antioxidant enzymes guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD). These two enzymes perform similar functions in protecting cells against the oxidative damage caused by H_2O_2, but can use different substrates – guaiacol and syringaldazine, respectively.

Table 2. Measurement of total polyphenols content, expressed as gallic acid equivalents (GAE), syringaldazine peroxidase (SPOD) activity and guaiacol peroxidase (GPOD) activity in young *Hordeum vulgare* L. plants, grown either on control non-polluted or polluted soil with petroleum products and treated or not with microalgae suspension (MS). Variants: polluted soil with 4.5% petroleum products + MS; polluted soil with 4.5% petroleum products; control soil + MS; control soil. The parameters were measured in leaves and in roots

Variants	GAE mg/g FW		SPOD U/g FW		GPOD U/g FW	
	roots	leaves	roots	leaves	roots	Leaves
Soil 4.5% + MS	0.62±0.03 b	0.52±0.02 b	92±15 b	10±1.1 b	52±1.2 b	4.5±1.2 b
Soil 4.5%	0.87±0.05 a	0.51±0.02 b	124±7 a	11±1.3 b	67±8 a	5.7±1.1 b
Control + MS	0.26±0.05 c	0.59±0.05 a	72±3 c	11±1.2 b	36±2 d	5.1±1 b
Control	0.18±0.03 d	0.41±0.03 c	84±5 b	20±1 a	44±2 c	10±1 a

The data in the columns followed by the same letter (a, b, c) are not statistically significant for $P < 0.05$.

In this case, the comparison between the four analyzed variants was also carried out separately for roots and leaves. The results for both enzymes were relatively similar (Table 2). The activities of both enzymes in the leaves were lower than those in the roots. The measured values representing the activity of SPOD in the leaves for all four variants were without significant differences. Only in the controls grown on clean soil without microalgae supplementation was observed a slightly higher activity (20 U/g FW). In the roots, contamination with petroleum products considerably induced the activity of SPOD. This is not surprising since roots are the organs that have direct contact with the petroleum derivatives and are thus subjected to stronger stress. On the other hand, the addition of microalgae suspension provokes a reduction of SPOD activities which suggests that treatment with microalgae mixtures may help mitigate the stress symptoms. A similar pattern of enzyme activity was observed for guaiacol peroxidase (GPOD) (Table 2) which is referred to as general peroxidase activity, while SPOD is associated with the specific activity in the apoplast and is related to the lignification of the cell walls. The first reaction of plants to the presence of the petroleum hydrocarbons in their growth environment and after the penetration of the contaminants into their tissues is the induction of internal defense mechanisms like enhancing antioxidant enzyme activity, which varies among plant species. The results presented here are in accordance with obtained from other authors who worked with soil contamination with gasoline (degree of contamination 0, 1, 2, 3, and 4%) and corn as a plant object. They also observed deviation of the activities of superoxide dismutase, peroxidase, and malondialdehyde concentration (Ghalamboran et al., 2019).

According to the newest classification, the methods used for remediation of soil contaminated by petroleum hydrocarbons, are divided into three groups i.e. physical methods, chemical methods, and biological methods. Biological methods can be further classified into bioremediation and phytoremediation. Bioremediation applies microorganisms, especially bacteria and fungi to remove soil contaminants or break them down into harmless compounds via, for instance, mineralization during which contaminants are used to produce carbon and energy. Phytoremediation removes contaminants from the environment by using plants and their micro-symbionts (Tang, 2019). The rationale behind the current research is investigating the possibility of the establishment of productive plants/microorganisms symbiotic interactions in problematic areas contaminated with petroleum products. This would, in turn, stimulate and accelerate the soil detoxification by biological means and lead to future enhancement of the crop yield. The plant species chosen as a model for the study was barley because of its good growth in controlled conditions and because of a report that barley could be a good marker for phytoremediation of contaminated areas (Asiabadi et al., 2014). Microalgae pose numerous advantages as remediation agents since they have relatively low nutrient requirements, grow fast and produce a lot of biomass due to their autotrophic metabolism, and rarely produce toxic byproducts (Kumar & Oommen, 2012). Moreover, these kinds of microorganisms have been already shown to be effective for other

kinds of soil pollution, for example with heavy metals (Suresh & Ravishankar, 2004). One of the interesting observations in the present study was the fact that the microalgae actually seemed to provoke reduction of nitrate reductase activity (Table 1). A plausible explanation is a competition between the microalgae and the plants for inorganic nitrogen sources from the soil. The decrease in the available inorganic nitrogen for the plants, however, does not affect their growth parameters. Moreover, it should be noted that it has been already documented that low-molecular-weight organic nitrogenous compounds can be exuded by the algae (Adam, 1999) and in this case, they may compensate for the reduction of inorganic nitrogen. As far as the influence of oil-contamination on nitrate reductase activity is concerned, in barley, the oil products appear to slightly induce the enzyme activity. In other studies, the activity of nitrate reductase has been shown to be increased in some cases or inhibited in others. For example, in the herbaceous plant, *Melilotus albus* L. after treatment with diesel, nitrate reductase seems to react similarly to the enzyme in barley (Hernández-Ortega et al., 2012). On the other hand, the response in *Amaranthus hybridus* L. grown in soil soaked in engine oil is the opposite, i.e. a reduced activity of nitrate reductase, for all concentrations of the pollutant (Odjegba & Atebe, 2007). Therefore, the influence of the presence of microalgae seems to be at the functional, not the structural level, most probably due to the alleviation of stress symptoms (Chen & Wang, 2020). In soils polluted with oil-derived chemicals, plants are subjected to combined stress from nutritional deficiency and chemical toxicity (Martí et al., 2009). The result of long-acting stress factors is an accumulation of reactive oxygen species (ROS) like the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•) which cause oxidative damage in numerous cellular organelles (Petrov et al., 2015). On the other hand, plants use ROS, especially hydrogen peroxide, as a second messenger in numerous signaling cascades. Thus, regulation of ROS levels and proper maintenance of the redox- status is crucial for plant development and to the proper functioning of all defense systems (Gechev and Hille, 2005; Eluehike et al., 2019). The level of oxidative stress in our barley plants was determined by measuring the concentrations of the markers MDA, free proline and phenolic substances while the status of the antioxidant systems was tested by quantification of the activities of two antioxidant enzymes GPOD and SPOD (Table 2). The results from all these experiments complement well each other and demonstrate that supplementation with microalgae suspension provides beneficial effects on the contaminated plants. This is illustrated by the reduced levels of lipid peroxidation, as determined by the decrease of MDA concentrations; the highly increased levels of the compatible solute with antioxidant properties proline; the lower activities of both antioxidant enzymes GPOD and SPOD in roots, which are directly exposed to the stress. The observed higher amounts of phenols in the roots of oil-treated plants, especially in the variant without microalgae supplementation, show that, in the absence of algae the root cells, which are in direct contact with the toxic substances in the soil, upregulate the synthesis of phenolic substances. The expected result is a thickening of the cell walls and limitation of the access of contaminants. This phenomenon correlates well with the data obtained for the activities of both antioxidant peroxidases, GPOD and SPOD because these enzymes are also involved in the synthesis of polyphenols and cell lignification. Similar results were obtained from other authors worked with soil contamination and remediation (Cuypers et al., 2002; Lu et al., 2010; Eluehike et al., 2019).

CONCLUSIONS

In conclusion, the supplementation of microalgae suspension had a positive effect on the growth and development of the barley plants cultivated on polluted soil. This is shown by the increase in the growth parameters, the overall boost of photosynthesis (data in press), the reduction of the levels of the stress marker MDA and the moderate activity of some of the main antioxidant enzymes (GPOD and SPOD). Therefore, inoculation with nonsterile microalgae cultures appears to be a promising approach to complement and accelerate phytoremediation in areas affected by oil spills. Follow-up studies on the topic would focus on

the development of suitable approaches to inoculate bacterial/microalgae cultures in affected soils, either on their own or by specific vectors, as well as comparison of the performance of barley and other plant species in order to select the most appropriate candidates to overcome the negative effect of petroleum contamination and to quicken soil recovery.

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