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# MICROALGAE EFFECTS ON THE PHOTOSYNTHETIC PERFORMANCE AND GROWTH PARAMETERS OF BARLEY GROWN ON SOIL CONTAMINATED WITH PETROLEUM PRODUCTS

**Lyubka KOLEVA-VALKOVA, Veselin PETROV, Adelina HARIZANOVA, Kiril MARINKOV, Atanas SEVOV, Andon VASSILEV**

Agricultural University of Plovdiv, 12 Mendeleev Blvd, 4000, Plovdiv, Bulgaria

Corresponding author email: l_koleva2001@yahoo.com

***Abstract***

*The aim of the present study was to evaluate the effect of microalgae suspension (MS) treatment on soil health and on the potential for phytoremediation. One of the strategies to alleviate soil toxicity, which has become increasingly popular in recent years, is bioremediation by inoculation with specific microorganisms. We tested the effect of a mixture of four microalgae strains (Scenedesmus incrassatulus, Trachydiscus minutus, Chlorella sp. and Phormidium sp.) on the specific physiological parameters of a model barley crop cultivated on petroleum-contaminated soil. Non-destructive methods of analysis were used to evaluate the state of the photosynthetic apparatus and the ability to carbon assimilation from the plants. 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 better growth and photosynthesis observed in plants grown on microalgae-treated soil. Therefore, the application of microalgae is an environmentally friendly and environmentally oriented strategy for improving soil health in areas affected by petroleum pollution.*

***Key words***: *microalgae, photosynthesis, soil contamination, soil remediation.*

## INTRODUCTION

Environmental pollution has been gaining ground in recent times. The daily use of products from the chemical and petrochemical industries is one of the major environmental problems today. One reason is the fact that the oil-related components have been shown to act as carcinogens and neurotoxic organic pollutants (Das & Chadran, 2011; Hatami et al., 2018). The processes of natural purification of the affected soils are slow and take thousands of years. Soils contaminated with petroleum products pose a constant risk to human health as well as to the sustainable functioning of ecosystems. (Park & Park, 2011; Tang, 2019). Some soil microorganisms, which are of particular importance for the course of biogeochemical cycles and for the maintenance of soil fertility, are sensitive to the presence of oil-based contaminants. The latest have a significant impact on the distribution of the species in the microbial community and generally have a negative impact on its biodiversity (Sutton et al., 2013). All these problems provoke a 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 proper remediation (Chen et al., 2015). Classic methods to treat petroleum contaminated soil include excavating its upper layers and their removal for treatment by physical or chemical methods (Hans-Holgar & Alexander, 2000; Juck et al., 2000). However, these procedures, despite being relatively effective, are expensive and disturb the ecological balance at the treated sites (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). It is well known that microalgae are important members of the microbial community in soil ecosystems, but there is little or no information available in the literature about their involvement in biodegradation of hydrocarbons. It is suggested that the presence of microalgae may favor the activity of oil-degrading bacterial strains. These microalgae/

bacteria consortia can clean different pollutants more efficiently 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). A possible mechanism explaining the favorability of soil microbial communities in response to inoculation with blue-green algae is related to the production and excretion of exopolysaccharides from the latter. Exopolysaccharides secreted by many microalgae species provide organic carbon for the growth and development of beneficial microorganisms, leading to the formation of beneficial biofilms in the rhizosphere (Xiao and Zheng, 2016; Chiaiese et al., 2018; Xia et al., 2020). Their association with soil elements helps in the solubilization, mineralization, and bioavailability of macro and micronutrients, thus improving crop performance (Manjunath et al., 2016; 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). Germination and seedling establishment are especially vulnerable stages in the plant life cycle (Vange et al., 2004). The usual symptoms observed in plants cultivated on petroleum contaminated soils include erosion of the epicuticular wax, 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 (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). 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). Photosynthetic performance and chlorophyll fluorescence are integral processes characterized the ability of the plant to cope with the stress factors (Gao et al., 2019; Tomar & Jajoo, 2019). By measuring these indicators, the degree of soil pollution and the effectiveness of the remediation techniques used can be estimated.

In the light of these actual problems the major goal of this study was to perform a plant assay for barley (*Hordeum vulgare* L.), which combines growth and functional parameters, in order to better evaluate the toxicity of petroleum product polluted soils as well as 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 replicates five plants per replicate 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 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 $Ca(NO_3)_2 \times 4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.1 mM $MgSO_4 \times 7H_2O$, 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.

**Determination of growth parameters**

The assessed growth characteristics include fresh and dry weight of the plants, leaf area, shoot height and root length. The absolute dry weight of the entire plants and their individual organs was measured after fixation of the material at 105°C for 30 min and consecutive drying at 80°C for 48 h. Leaf area was quantified with an electric digital leaf area meter NEO-2 (TU-Bulgaria).

**Assessment of photosynthetic parameters**

Net photosynthetic rate (A) was measured on the second developed leaf of the plants with an open photosynthetic system LCA-4 (Analytical Development Company Ltd., Hoddesdon, England), equipped with a narrow chamber. Chlorophyll fluorescence analysis was performed using a Handy PEA fluorimeter (Handy Plant Efficiency Analyzer, Hansatech Instruments Ltd., King's Lynn, UK).

**Measurement of photosynthetic pigments**

Photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) were extracted in 80% acetone, determined spectrophotometrically and calculated according to the formulae of Lichtenthaler (1987). Data are presented as mg pigments/g fresh weight (mg/g FW).

**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 biometric experiments to measure fresh weight, dry weight, root and leaf length, leaf area and total number of leaves were conducted. The results are presented in Table 1. It is clearly seen that soil pollution with oil products considerably reduced all growth parameters. The observed differences are all statistically significant with $P < 0.05$. This is in concordance with numerous previous studies from authors that report the negative effect of oil on crops (Anoliefo & Edegbai, 2000; Akaniwor et al., 2007). Interestingly, supplementation with microalgae suspension seems to improve the growth parameters both in normal and in stress conditions. In certain cases, like the root fresh weight and leaf area in clean soil (Table 1) the differences between the means of the samples are almost twice between the supplemented and non-supplemented variant. The variants subjected to petroleum contamination all parameters showed the lowest values compared to the control plants. The microalgae application alleviates this phenomenon as slightly improves the growth parameters. The results obtained from the growth analysis showed the inhibitory effect of petroleum contamination and are in accordance with the results of other authors (Odjegba & Sadiq, 2002).

Table 1. Biometric parameters of 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). FW - fresh weight; DW - dry weight; LA - leaf area

| Variants | Roots | | | Leaves | | | | |
|---|---|---|---|---|---|---|---|---|
| | cm | FW | DW | cm | FW | DW | LA $cm^2$ | Leaf count |
| Soil 4.5% + MS | 17.5c | 0.52c | 0.055b | 29b | 0.57bc | 0.063c | 30.45b | 3 |
| Soil 4.5% | 14c | 0.36c | 0.041b | 22.2c | 0.26c | 0.049c | 29c | 2.5 |
| Control + MS | 32.3a | 1.35a | 0.108a | 41.5a | 1.70a | 0.179a | 78.1a | 4.5 |
| Control | 23.5b | 0.72b | 0.088a | 32b | 0.99b | 0.129b | 39.2b | 3 |

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

To complement the data from the biometric experiments parameters indicative of the physiological status of the photosynthetic apparatus as well as the intensity of transpire-tion were determined (Table 2). These include photosynthetic activity (A), transpiration rate (E) and stomatal conductance (gs). The results unequivocally demonstrate that petroleum contamination significantly disturbs the photo-synthetic processes in those variants since all three parameters were reduced (photosynthesis - 7.66, transpiration - 3.27 and stomatal conductance - 0.127). In the control plants, differences between both supplemented and non-supplemented with microalgae variants were barely detectable. On the other hand, when grown in polluted soil the plants watered with microalgae suspension partially restored their photosynthetic activity (10.135), while the transpiration rate (4.38) and the stomatal conductance (0.205) reached the levels of the controls.

Table 2. Measurement of the photosynthetic activity (A), transpiration rate (E) and stomatal conductance (gs) 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

| Variants | A (µmol $m^2$/s) | E (mmol $m^2$/s) | Gs |
|---|---|---|---|
| Soil 4.5% + MS | 10.135b | 4.38a | 0.205a |
| Soil 4.5% | 7.66c | 3.27b | 0.127b |
| Control + MS | 12.35a | 4.61a | 0.209a |
| Control | 12.3a | 4.59a | 0.208a |

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

Not surprisingly the subsequent quantification of the main photosynthetic pigments also demonstrated that their levels are much lower in the variants grown on contaminated soil (Table 3). However, the ratio of chlorophylls relative to carotenoids was slightly augmented in the plants cultivated on contaminated soil (4.52) in comparison to the controls (4.38). This means that in barley carotenoids are more sensitive to the presence of petroleum products than chlorophylls. Since the carotenoids are supported by the findings for the inhibited photosynthesis in contaminated variants (Table 2). Photosynthesis is a process dependent on the photosynthetic pigments' quantity and the ability of all involved molecules to absorb, transform and transport the energy. This can be specific molecules which acts as antioxidants and protect against oxidative damage, their low concentration in the contaminated variants (0.48; 0.46) suggest for oxidative stress due to a photo-oxidation or other reason related to the petroleum-contaminated soil. The supplementation with microalgae didn't show any considerable effects neither in normal nor in stressful conditions, with the exception of a small increase of chlorophyll *a* and chl *a*/chl *b* ratio. These results and speculations are assessed by measuring chlorophyll fluorescence, one of the main markers for photosynthetic integrity and function (Paunov et al., 2018; Gao et al., 2019).

Table 3. Measurement of the photosynthetic pigment quantity 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

| Variants | Chl *a* (mg/gFW) | Chl *b* (mg/gFW) | Car (mg/gFW) | Chl*a*/Chl*b* | Chl/Car |
|---|---|---|---|---|---|
| Soil 4.5% + MS | 1.50b | 0.67b | 0.46b | 2.24b | 4.76a |
| Soil 4.5% | 1.43b | 0.74b | 0.48b | 1.92c | 4.52a |
| Control + MS | 2.30a | 0.84a | 0.69a | 2.74a | 4.55a |
| Control | 2.20a | 0.82a | 0.69a | 2.68a | 4.38b |

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

The most common and widely used chlorophyll fluorescence analyses were performed on dark- and light-adapted leaf samples and subsequently different parameters character-rizing the steady-state status of the photosynthetic apparatus were calculated.
As presented in Table 4, petroleum contamination disturbs the energy migration from the antenna complexes to the chlorophyll of the reaction centers which leads to an increased minimal chlorophyll fluorescence emission in dark-adapted objects (Fo = 760). The lowest fluorescence (Fo = 595), as well as the highest quantum yield (Y = 0.776), were detected in the control plants treated with microalgae suspension. Similarly, the electron transport rate (ETR) calculated in light-adapted plants had the biggest value in the control + MS sample. This demonstrates that supplementation with MS has a positive impact on PSII photochemistry even in normal conditions. On the other hand, the addition of MS to contaminated soil apparently induces a rescue effect on the Y parameter, which was restored to the levels of the controls (a transition from 0.698 to 0.764). An interesting result is that MS treatment actually leads to a decrease of ETR in barley plants on contaminated soil (ETR = 18.7). A possible explanation could be that the MS suspension stimulates the plants' protective mechanisms, including lowering of the ETR, in order to prevent additional stress.

Table 4. Measurement of chlorophyll fluorescence parameters in young *Hordeum vulgare* L. plants, grown either on control non-polluted or polluted with petroleum products soil and treated or not with microalgae suspension. Fo - minimal fluorescence in dark adapted plants, Fm - maximal fluorescence in dark adapted plants, Y - quantum yield, F' - minimal fluorescence in light adapted plants, Fm'- maximum fluorescence in the light adapted plants, ETR - electron transport rate

| Variants | Dark adapted | | | Light adapted | | | |
|---|---|---|---|---|---|---|---|
| | Fo | Fm | Y | F' | Fm' | Y | ETR |
| Soil 4.5% + MS | 611b | 2577b | 0.763a | 296d | 817c | 0.64a | 18.7c |
| Soil 4.5% | 760a | 2525c | 0.698b | 337c | 835b | 0.60b | 27.1b |
| Control + MS | 595c | 2664a | 0.776a | 342b | 841a | 0.59b | 31.6a |
| Control | 635b | 2514d | 0.747a | 372a | 660d | 0.44c | 27.2b |

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

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 for 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). Growth and physiological parameters were evaluated both when plants were cultivated by themselves and when a mixture of 4 microalgae species was added. 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 organisms 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 that the improvement of the photosynthetic parameters in the contaminated samples supplemented with microalgae was not due to higher quantities of photosynthetic pigments since the contents of the latter remained unaffected (Tables 2 and 3). Therefore the influence of the presence of microalgae seems to be at the functional, not the structural level, indirectly leading to increased efficiency of photosynthesis, most probably due to alleviation of stress symptoms.

In conclusion, the supplementation with 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 and the overall boost of photosynthesis. 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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