Photosynthetic and ultrastructure parameters of maize plants are affected during the phyto-rhizormediation process of degraded metal working fluids

Journal:	*International Journal of Phytoremediation*
Manuscript ID:	BIJP-2014-0312.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Grijalbo, Lucia; Universidad CEU-San Pablo, Gutierrez-Mañero, Francisco Javier; Universidad CEU-San Pablo, Fernandez-Pascual, Mercedes; Institute of Agricultural Sciences,, Department of Crop Protection, CSIC Lucas, Jose Antonio; Universidad San Pablo CEU,
Keywords:	Phytoremediation, Rhizoremediation, Metal-Working fluids

Photosynthetic and ultrastructure parameters of maize plants are affected during the phyto-rhizormediation process of degraded metal working fluids

Lucía Grijalbo[1], Francisco Javier Gutierrez Mañero[1], Mercedes Fernandez-Pascual[2], Jose Antonio Lucas[1]*

1. Departamento de CC. Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad San Pablo CEU, Urb. Monteprincipe, 28668, Boadilla del Monte, Madrid, Spain.
2. Institute of Agricultural Sciences, Department of Crop Protection, CSIC, Serrano, 115-bis, 28006 Madrid, Spain

* Corresponding author: Dr. Jose Antonio Lucas García, Dpto. Departamento de CC. Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad San Pablo CEU, Urb. Monteprincipe, 28668, Boadilla del Monte, Madrid, Spain. Phone number:+34913724733. Fax number: +34913510496. alucgar@ceu.es.

Running head: Phyto-rhizormediation of metal working fluids

Abstract

A phyto-rhizoremediation system using corn and esparto fibre as rooting support to remediate degraded metal working fluids (dMWFs) has been developed in the present study. In order to improve the process, plants were inoculated at the root level with bacteria either individually, and with a consortium of strains. All strains used were able to grow with MWFs. The results show that this system significantly lowers the Chemical Oxygen Demand below legal limits within 5 days. However, results were only improved with the bacterial consortium. Despite the effectiveness of the phyto-rhizoremediation process, plants are damaged at the photosynthetic level according to the photosynthetical parameters measured, as well as at the ultrastructure of the vascular cylinder and the Bundle Sheath Cells. Interestingly, the bacterial inoculation protects against this damage. Therefore, it seems that that the inoculation with bacteria can protect the plants against these harmful effects.

Keywords

Phyto-rhizoremediation, chlorophyll fluorescence, Metal working fluids, maize.

1. Introduction

The generation, distribution and accidental spills of different organic pollutants (weedkillers, insecticides, acaricides, hydrocarbons) have resulted in environmental deterioration, with a direct or indirect accumulation in soils, water and air. Its accumulation rate is higher than the planet capacity to remove these xenobiotics (Kvesitadze and Gordeziani 2001). In the next decades, billions of dollars will be spent to clean up all sites polluted with polycyclic aromatic hydrocarbons (PAHs) (Rosenberg

1993). In the United States alone, restoration of all contaminated sites will cost approximately $1.7 trillion (Kuiper et al., 2003).

The Metal Working Fluids (MWFs) are oils of different nature. They serve for cooling of work pieces and tools, lubricating the process, and flushing away chips, fines, swarf, and residues (Moscoso et al., 2012). **MWF used in this work is a common product used by companies that use metal pieces that rubbed together, and that in their manufacturing processes have to turning, milling, drilling, grinding, boring, etc. annual worldwide production in 2000, reached 22.4 x 10^9 litres (Great Britain, 2000), and this amount will have increased year after year.**

The exact composition of the oils cannot be determined because substances of 85-95% purity are used (Rabenstein et al., 2009). In addition, it is known that MWFs can contain more than 60 different components, including biocides, foaming inhibitors, emulsifiers, corrosion inhibitors, extreme pressure additives, etc, and the percentages and recipe of each compound are secrets of the MWF manufacturers. When these MWF lose their properties, they are known as degraded MWFs and become a problem for industry.

In the area where this work was made (Madrid, Spain), degraded MWFs handling is regulated by the regional law 10/1993 on industrial waste discharges into urban sanitary sewer system. **This law details about the physic-chemical characteristics that degraded MWFs should have to be released into the environment.**

Among the many parameters regulated by the law, chemical oxygen demand (COD) and pH values are usually above the maximum permitted values, and therefore must be treated prior to release to the environment. However, the decrease in COD and pH does not necessarily rule out biotoxic effects of the degraded MWFs (Lucas García et al., 2013).

Plants are exposed to the degraded MWFs released to the environment during all the development stages, from germination to reproduction (Wild et al., 1992). **Plants can absorb these compounds by roots, through waxy leaf cuticle or by their uptake through stomata (Wild et al., 2006; Meudec et al., 2006). After absorption, plants can accumulate, translocate and transform these compounds, and during these processes can affect to biochemical and/or physiological mechanisms and negative affect to grow and** biomass production (Kummerová et al., 2006).

Some bacteria of the soil are able to promote plant growth by colonizing the plant root (Kloepper et al., 1989; Dutta and Podile 2010). These free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) (Hayat et al., 2010). Some of them, can improve the plant's defensive metabolism, provoking a physiological status of the plant named priming (Conrath 2011). Primed pants can respond faster and/or stronger when subsequently challenged by biotic or abiotic stress, because these plants have activated their defensive responses (Conrath et al., 2006).

The use of plants in combination with microbes (phyto-rhizoremediation) in remediation processes has the advantage of causing an increase in microbial population numbers and metabolic activity in the rhizosphere. The exudation of phothosyntates by plants roots creates a nutrient rich environment in which microbial activity is stimulated (Kuiper et al., 2003). The type of root exudates is one of the most important factors in successful rhizoremediation (Yoshitomi and Shann 2001). Application of the interaction between plant roots and rhizobacteria for rhizoremediation has been investigated because it is an environmentally friendly and cost effective technology for the

remediation of contaminated soils (Gerhardt et al., 2009). The key mechanism for a succesful rhizoremediation is the rhizosphere effect that results in enhanced biodegradation (Gerhardt et al., 2009; Newman and Reynolds, 2004).

The aim of this work was to develop a phyto-rhizoremediation system able to reduce the chemical oxygen demand (COD) and pH below that required by law for degraded MWFs, and to study the impact of these pollutants on the physiology of maize.

2. Materials and methods

2.1. Degraded metal-working fluids (dMWFs)

John Deere Ibérica S. A. kindly provided the degraded MWFs for this study from its headquarters in Getafe (Madrid). dMWF were previously remediated in the company with physicochemical procedures and showed a chemical oxygen demand (COD) and pH values over the regulatory values, according to the regional Law 10/1993.

~~The main chemical constituents include a formaldehyde-based biocide; alkyl benzotriazole (metal passivator); C16/C18 fatty alcohol polyglycol ether (corrosion inhibitor); isopropanolamine (lubrication agent), and 3-iodo-2-propynylbutylcarbamate. Fresh MWFs is typically supplied as a concentrate, which is diluted with water to form a 2% v/v working fluid prior to use in machining operations.~~

2.2. Strains used

Microorganisms used in the experiments were selected for their capacity to grow in degraded MWFs (data not shown). All microorganisms belong to the Universidad San Pablo CEU collection. The strains used in the experiments were isolated from different sources: i) two strains were isolated from the esparto fibre and ii) consortium

microorganisms from a membrane bioreactor. Strains from esparto: A bacterial strain identified as *Enterobacter* sp. (denoted as Esp. 1) and a yeast strain identified as *Rhodotorula dairenensis* (denoted as Esp. 21). These strains were selected from those present in esparto; the *Enterobacter* sp. was the more abundant genus (66.7%) and the yeast was the only microorganism able to grow in agar with degraded MWFs supplied with nutrientsor not (data not shown). The microorganisms for the consortium were selected from a membrane bioreactor (MBR) fed with a 6,000 ppm COD metal working-fluid pretreated solution. Four bacteria formed the consotium: MBR-A11 (*Pseudomonas sp)*; MBR-A12 (*Acinetobacter johnsonii)*; MBR-A16 (*Acinetobacter johnsonii)* and MBR-A23 (*Sphingobium xenophagum*). These strains were selected for being the most abundant genera among over 100 colonies in the MBR (data not shown). Taxonic affliliation of these bacteria was performed through the partial sequencing of the gene 16S rRNA, and its later comparison by the BLASTN 2.2.26 (Zhang et al., 2000) algorithm in the GenBank database. The sequences of the esparto bacteria were deposited in the GenBank with the numbers: JF690924 and, AF444501 respectively, and those of the consortium bacteria were deposited in the GenBank with the numbers: JF937328, JF937329, JF937331 and JF937337, respectively.

2.3. Maize plants

After surface-sterilized with ethanol (70%) during 30 s, and sodium hypochlorite (5%) for 6 min, maize seeds were washed with sterile deionized water for five times. Seeds were placed in petri dishes on agar-agar. On 1 L-containers filled with 11.5 g of esparto fibre and a metal grille, germinated seeds were deposited. These containers were filled with 1 L of tap water supplemented with 1 g of Hoagland (Sigma-Aldrich) medium~~Seeds of maize were surface-sterilized with ethanol (70%) during 30 s, followed~~

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

~~by sodium hypochlorite (5%) for 6 min. Then, seeds were rinsed five times with sterile deionized water and then deposited in petri dishes with agar-agar. After two days, germinated seeds were transferred to 1 L containers filled with 11.5 g of esparto fibre and a metal grille where seeds were deposited; the device was filled with with 1 L of tap water supplemented with 1 g of Hoagland (Sigma-Aldrich) medium~~. Twelve days after, 50% of the pots were inoculated with microorganisms to achieve 10^7 cfu mL^{-1}. Three days later, 50% of the containers (3 with microorganisms and 3 without) were filled with degraded MWFs. Each container had 20 seedlings. The experiment consisted of 12 containers: three non-inoculated without degraded MWFs (control containers); three inoculated without degraded MWFs; three non-inoculated with degraded MWFs (final COD between 1,678 ppm to 1,821 ppm) and three inoculated with degraded MWFs (final COD between 1,678 ppm to 1,821 ppm). Five days later, COD and pH of the bioremediated degraded MWFs were measured in order to assess the success of the phytoremediation process; physiological determinations were made in maize plants.

The assays were made in a controlled environment chamber with a 25/18ºC day/night temperature, a PPFD of 600 mmol/(s*m^2) and a 60-70% relative humidity.

2.4. COD and pH determinations

COD and pH were measured following the specifications of the EPA method 410.4 (the determination of chemical oxygen demand by semi-automated colorimetry) , and EPA method 150.1 (pH, Electrometric Method) respectively. To COD colorimetric analysis using a Merck Photometer SQ 118 with COD cuvette test kits (range 500-10,000 mg/L) was used. To pH a CRISON micro pH 2100 pHmeter used directly on degraded MWFs was used.

~~COD was determined by colorimetric analysis using a Merck Photometer SQ 118 with COD cuvette test kits (range 500-10,000 mg/L). Analyses were performed according to the manufacturer`s instructions. This method follows the specifications of the EPA method 410.4 (the determination of chemical oxygen demand by semi automated colorimetry). pH was measured directly on degraded MWFs with a CRISON micro pH 2100 pHmeter, following the EPA method 150.1 (pH, Electrometric Method).~~

2.5. Chlorophyll fluorescence, Hill reaction and chlorophyll analysis determinations

To measure the fluorescence emitted by chlorophyll a pulse amplitude modulated (PAM) fluorometer (Hansatech FM2, Hansatech, Inc, UK) was used. One h after leaves were adapted to dark conditions Fo (dark adapted minimum fluorescence) was measured with weak modulated irradiation (1 µmol m^{-2} s^{-1}). After a 700 ms saturating flash (9000 µmol m^{-2} s^{-1}) maximum fluorescence (Fm) was determined. Fv (variable fluorescence) was calculated as the difference between Fm and Fo. With these data, Fv/Fm (maximal PSII quantum yield) was calculated. ~~Chlorophyll fluorescence was measured with a pulse amplitude modulated (PAM) fluorometer (Hansatech FM2, Hansatech, Inc, UK). After dark adaptation of leaves for 1 h, the minimal fluorescence (Fo; dark adapted minimum fluorescence) was measured with weak modulated irradiation (1 µmol m^{-2} s^{-1}). Maximum fluorescence (Fm) was determined for the dark adapted state by applying a 700 ms saturating flash (9000 µmol m^{-2} s^{-1}). The variable fluorescence (Fv) was calculated as the difference between the maximum fluorescence (Fm) and the minimum fluorescence (Fo). The maximum photosynthetic efficiency of photosystem II (maximal PSII quantum yield) was calculated as Fv/Fm.~~

Immediately, the leaf was continuously irradiated with red-blue actinic beams (80 µmol m^{-2} s^{-1}) and equilibrated for 15 s to record Fs (steady-state fluorescence signal). Following this, another saturation flash (9000 µmol m^{-2} s^{-1}) was applied and then Fm` (maximum fluorescence under light adapted conditions) was determined. Other fluorescent parameters were calculated as follows: the effective PSII quantum yield ϕPSII=(Fm`-Fs)/Fm` (Genty et al., 1989); the photochemical quenching coefficient qP=(Fm´-Fs)/(Fm´-Fo); the non-photochemical quenching coefficient NPQ=(Fm–Fm´)/Fm´ and the electron transport rate ETR=ϕPSIIxPARx0.5x0.85. All measurements were carried out in 10 plants of each container.

In order to measure the Hill reaction and to determine the chlorophyll quantity, 1 g maize leaves were taken from different plants from each container. Leaves were homogenised with 10 mL phosphate buffer 75 mM pH 6.9. Chloroplast lamellae used in the Hill reaction were obtained after filtering the macerate through 6 gauze layers. The Hill reaction was carried out mixing 4.5 ml of dichlorophenolindophenol (DCPIP) solution and 0.5 ml of chloroplasts suspension. This mix was exposed for 3 min to irradiance of 174 µmol m^{-2} s^{-1} PAR at 22 °C. Afterwards, absorbance at 600 nm (UV–VIS Spectrophotometer Thermo Electron Corporation (Biomate 5) was measured, and Hill reaction activity was expressed as the rate of DCPIP reduction by the chloroplast suspension during irradiation, and the activity of chloroplasts was evaluated as a rate of DCIP photoreduction in µg reduced DCPIP µg $^{-1}$ (chl) min^{-1}. The measurements were repeated three times and the data are presented as arithmetic means. ~~These fragments of leaves were macerated with 10 mL phosphate buffer 75 mM pH 6.9. This macerate was filtered through 6 gauze layers, obtaining a liquid containing chloroplast lamellae.~~

~~The Hill reaction mixture contained 4.5 ml of dichlorophenolindophenol (DCPIP) solution and 0.5 ml of chloroplasts suspension. This reaction mixture was exposed for 3~~

Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight

~~min to irradiance of 174 µmol m^{-2} s^{-1} PAR at 22 °C. Hill reaction activity was measured spectrophotometrically at 600 nm and expressed as the rate of DCPIP reduction by the chloroplast suspension during irradiation, using UV VIS Spectrophotometer Thermo Electron Corporation (Biomate 5) in a cuvette of 1 cm light pass. The activity of chloroplasts was evaluated as a rate of DCIP photoreduction in µg reduced DCPIP µg^{-1} (chl) min^{-1}. The measurements were repeated three times and the data are presented as arithmetic means.~~

Chlorophyll quantity was measured mixing 1 mL of chloroplast lamellae and 4 mL of acetone (Strain et al., 1971). After centifigate 5 min at 4,000 r.p.m, absorbance of the supernatant at 649 and 665 nm was measured. Total chlorophyll, chlorophyll a and chlorophyll be was calculed following the math expressions proposed by Arnon, 1949.

~~To measure the chlorophyll quantity, 1 mL of chloroplast lamellae and 4 mL acetone were used (Strain et al., 1971). This mixture was centrifugated 5 min at 4,000 r.p.m. and absorbance of the supernatant was measured at 649 nm and at 665 nm. Total chlorophyll and chlorophyll a and b contents were calculated as previously described by (Arnon 1949) and expressed as nmol g^{-1} leaf fresh weight.~~

2.6. Low Temperature Scanning Electron Microscopy (LTSEM)

Leaves and roots were sampled 5 days after MWFs application. The third youngest leaf was detached from non-inoculated controls and MWFs treated plants seeking same physiological status to obtain a reliable comparation.

Ultraestructure of fresh leaves and roots were studied by low temperaure scanning electron microscopy (LTSEM). For this, organs were fixed to a sampler with an adhesive (Gurr®, OCT, BDH), were cryofixed into slush nitrogen (-196 °C), and vacuum-cryo-transferred to a -180 °C camera to be fractured and then gold coated (de

Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight
Formatted: Not Highlight

María et al, 2005). Ultraestructures of leaves and roots were observed with a Zeiss Digital Scanning Microscope DSM 960 at low temperature.

~~Low-temperature scanning electron microscopy (LTSEM) was performed on fresh leaves and roots just detached from the plant. These structures were fixed to a sampler with an adhesive (Gurr®, OCT, BDH) and were cryofixed into slush nitrogen (-196 °C), vacuum cryo-transferred to a -180 °C camera to be fractured and then gold coated (de María et al, 2005). The structures were observed at low temperature with a Zeiss Digital Scanning Microscope DSM 960.~~

Formatted: Indent: First line: 0.49"

2.7. Statistics

Simple ANOVA analyses were performed to compare data obtained in each experiment. Significant differences between the analysed variables were considered when $P< 0.05$, and in these cases, average values were compared by means of LSD statistic (Sokal and Rholf 1979). For these analyses Statgraphics software was used.

3. Results

3.1. COD and pH determinations

The reduction in the chemical oxygen demand (COD) from the degraded MWFs achieved by the phyto-rhizoremediation system developed, ranged between 48.79 % and 64.42 % (Table 1). This was further improved only by inoculation with the consortium. Similarly, reduction in pH values of the degraded MWFs ranged between 8.58 % to 11.84 % (Table 2). Only Esp. 1 improved the reduction of pH. In both cases, values obtained after phyto-rhizoremediation processes were below local legislation limits.

3.2. Chlorophyll fluorescence, Hill reaction and chlorophyll analysis determinations

Tables 3, 4 and 5 show values of the main chlorophyll fluorescence parameters, Hill reaction and chlorophyll analyses in inoculated and non-inoculated plants with and without degraded MWFs.

Data from the experiment in which the Esp. 1 was used appears in Table 3. Fo, ΦPSII and Hill reaction values decreased significantly in plants treated with degraded MWFs with regard to the control (without degraded MWFs and non-inoculated). Total chrophyll significantly increased. Plants treated with degraded MWFs and incoculated had the same behaviour described above, except that ΦPSII did not significantly decreased. In addition, Chlorophyll a/b ratio significantly increased, and total chlorophyll decreased.

Data from the experiment in which the Esp. 21 was used appears in Table 4. In this case, Fo and ΦPSII values significantly decreased in plants treated with degraded MWFs with regard to the control (without degraded MWFs and non-inoculated). In plants treated with degraded MWFs and incoculated all chlorophy fluorescences parameters measured except Fv/Fm significantly decreased with regard to control (without degraded MWFs and inoculated). Chlorophyll a/b ratio value significanrly increased and total chlorophyll decreased.

Data from the experiment in which the consortium was used appears in Table 5. ΦPSII and Hill reaction values significantly decreased in plants treated with degraded MWFs with regard to the control (without degraded MWFs and non-inoculated). In addition, Chlorophyll a/b ratio and total chrorophyl values increased. In plants treated with degraded MWFs and incoculated all chlorophy fluorescences parameters measured except Fv/Fm significantly decreased with regard to control (without degraded MWFs and inoculated). Total chlorophyll value significantly decreased.

LTSEM micrographs show the ultrastructure of the vascular cylinder and the Bundle Sheath Cells (BSC; Puertas Mejías et al, 2010) of maize cells after cryofracture. In control plants (figure 1A) vigorous chloroplasts were observed. However, degraded MWFs changed the leaf structure less vacuoles were observed and boundaries were not as sharp (Figure 1B). Apparently, chloroplast number is reduced in the MWFs treatments (figure 1B) with respect of the number observed in the tap water treatments (figure 1A) and these organelles seem to be crushed between tonoplast and cell wall (figure 1B). Micrographs of plants treated with degraded MWFs and inoculated with bacteria or consortium do not reveal differences with plants growing on degraded MWFs but non-inoculated (data not shown).

4. Discussion

Phytoremediation of organic compounds has been widely reported (Susarla et al., 2002; Arthur et al., 2005; Vangronsveld et al., 2009; Batty and Dolan 2013). Most studies have focused on chlorinated solvents, explosives and petroleum hydrocarbons. However, in recent years, researchers have started to address the potential of phytoremediation to treat other organic pollutants including polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or MWFs (Ferro et al., 1994; Nwoko 2010; Ndimele 2010; Batty and Dolan 2013; Lucas García et al., 2013).

The phyto-rhizoremediation system developed in this research was composed by maize, esparto fibre and different microorganisms. Root exudation by the plant is crucial in the rhizoremediation process, since it provides nutrients to soil and stimulates

microbial activity. Root exudation contributes to xenobiotic degradation because by addition of organic compounds, microbial metabolic necessities are supplied (Kim et al., 2002; Gerhardson 2002; Hadacek 2002; Barrutia et al., 2011) or by release of enzymes that complement the microbial cathabolic ways (Pilon-Smits 2004; Wenzel 2008). The microorganisms used were isolated from different systems, and were checked for its capacity to grow in degraded MWFs (data not shown). The bioaugmentation improved the biodegradative capacities from the polluted sites by the introduction of individual microorganisms or consortium with the deserable cathalytic capacities (Mrozik and Piotrowska-Seget 2010). Within the phytoremediation field, rhizoremediation has been suggested as the primary mechanism responsible for hydrocarbon degradation in soil (Yateem et al., 2007; Barrutia et al., 2011).

The process designed in this work was performed to reduce in a short period of time the COD and pH values below the maximum indicated by the local law. The results of this study indicate the effectiveness of this procedure, because these parameters were significantly reduced (Table 1 and 2), consistent with the reported effects of corn as a plant that has already demonstrated its ability to remediate different types of organic and inorganic contaminants (Ogbo et al., 2010; Hong et al., 2010; Han et al., 2011). In addition, we have demonstrated this procedure in a previuos work with other bacteria (Lucas García et al., 2013).

The pollutants degradation could be attributed to the plant, the microorganisms or the plant-microorganisms interaction. However, the pollutants could be adsorbed to the esparto fibre as reported for many organic pollutants, such as the oil derivatives, which are hydrofobic and tend to adsorb to soil (Martin and Gallego, 2003).

In our work, the consortium was the only one able to reduce COD over the non-inoculated treatments (Table 1) suggesting that the mixed inoculum could be better for

phyto-rhizoremediation treatments of degraded MWFs. The bioaugmentation with pure inoculums in diffent habitats (polluted water, underground water, soil or clay) is usually less effective than the use of consortia, as it is very difficult for a single microorganism to perform a complete mineralization (Bouchez et al., 2000; Arthur et al., 2005; Mrozik and Piotrowska-Seget 2010), although the opposite has also been reported (Moscoso et al., 2012).

This work is mainly focused on the study of the damage produced by MWFs on plant fitness, especially on photosynthesis. Photosynthesis is one of the metabolic process more investigated, since it is a central route in plants responsible for synthesis of carbon scaffoldings for growth and metabolism (Kummerová et al., 2008).

The fluorescence emission from dark-adapted leaves (Fo) decreased in all degraded MWFs treatments, being the lowest decreases detected in Esp. 1 and Esp. 21 inoculated treatments (Tables 3 and 4). Increased Fo values are considered as indicator of damaged photosynthetic systems (Bradbury and Baker 1986; Osmond 1994; Baker 2008); unexpectedly, pollutants from degraded MWFs decreased this parameter.

The electronic microscopy images showed most organelles crushed between the tonoplast and the cell wall (Fig. 1) and evidenced a decrease in the chloroplast number due to degraded MWFs, consistent with the sensitivity of chloroplasts to abiotic stress (Alscher et al., 1998). The irreversible inactivation of photosystem II in the thylakoid membrane produced by the organic compounds (Huang et al., 1997; Mallakin et al., 2002) underlies the increase in Fo values generally reported. However, the unexpected decreases detected here seem to be associated to a decrease in the number of chloroplasts, not to a better photosynthetic system. Considering this fact, it seems that inoculation with strains Esp. 1 and Esp. 21 could be protecting plants from the pollutants, especially Esp. 21 that achieves significant differences with respect to non-

inoculated plants (Table 4) although micrographs do not reveal differences with all other treatments (data not shown).

Hill reaction determination (Tables 3, 4 and 5) revealed a significant reduction on degraded MWFs grown plants, either inoculated or non-inoculated. Photosystem II is inactivated by differents stresses, such as air pollution, high metals or organic compounds (Felipe et al., 1988; Hernandez-Terrones et al, 2003; Kumerova et al 2008) The damage is first localyzed in D1 protein, where electron transfer between the first donor (Tyr Z) and the second acceptor plastoquinone (Qb) takes place (Barber and Andersson 1992). The chlorophyll a/b ratio (Tables 3, 4 and 5) increases when plants are grown in degraded MWFs consistent with the reported increase of this ratio upon stressful situations (Garcia-Plazaola et al., 2008). Chlorophyll a/b ratio is inversely proportional to the antenna size (Boardman, 1977).

5. Conclusion

The phyto-rhizoremediation system developed (maize – esparto) was able to reduce the COD below the limits stablished regional Law 10/1993. Only the inoculation with the consortium further improved this reduction. Degraded MWFs strongly affects plant physiology, lowering chloroplast number, probably associated to damage in the differents acceptors from the electronic transport chain. Inoculation with microbial strains, particulary the consortium, prevent damage of some of the physiological parameters measured. Therefore, it seems that that the inoculation with bacteria can protect the plants against harmful effects of degraded MWF.

Acknowledge

This work was funded by Comunidad de Madrid grants S-0505/AMB/0321 and S-2009/AMB/1511 (Microambiente-CM), by the Spanish Ministry of Science and Innovation [grant CGL2010-15675, sub-programme BOS; grant FPU (Personal University Formation)] and by John Deere S.A Company. Gaiker Foundation (Vizcaya – España) is aknowldeged for the use of the MBR.

References

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. Plant Physiol 24: 1–15.

Arthur E, Rice P, Rice P, Anderson T, Baladi S, Henderson K, Coats J. 2005. Phytoremediation—An Overview. Crit Rev Plant Sci 24: 109–122.

Baker NR. 2008. Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. Annu Rev Plant Biol 59: 89–113.

Barber J, Andersson B. 1992. Too much of a good thing: light can be bad for photosynthesis. Trends Biochem Sci 17: 61–66.

Barrutia O, Garbisu C, Epelde L, Sampedro MC, Goicolea MA, Becerril JM. 2011. Plant tolerance to diesel minimizes its impact on soil microbial characteristics during rhizoremediation of diesel-contaminated soils. Sci Total Environ 409: 4087-4093.

Batty LC, Dolan C. 2013. The Potential Use of Phytoremediation for Sites With Mixed Organic and Inorganic Contamination. Crit Rev Environ Sci Technol 43: 217–259.

Bouchez T, Patureau D, Dabert P, Juretschko S, Doré J, Delgenès P, Moletta R, Wagner M. 2000. Ecological study of a bioaugmentation failure. Environ Microbiol 2: 179–190.

Bradbury M, Baker NR. 1986. The kinetics of photoinhibition of the photosynthetic apparatus in pea chloroplasts. Plant Cell Environ 9: 289–297.

Conrath U. 2011. Molecular aspects of defence priming. Trends Plant Sci 16: 524–531.

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L, Mauch-Mani B. 2006. Priming: Getting Ready for Battle. MPMI 19: 1062–1071.

Dutta S, Podile AR. 2010. Plant Growth Promoting Rhizobacteria (PGPR): the bugs to debug the root zone. Crit Rev Microbiol 36: 232–244.

Felipe MR, Golvano MP, Lucas MM, Lang P, Pozuelo JM. 1988. Differential effects of isoproturon on the photosynthetic apparatus and yield of two varieties of wheat and L. rigidum. Weed Res 28: 85–92.

Ferro AMA, Sims RCR, Bugbee BB. 1994. Hycrest crested wheatgrass accelerates the degradation of pentachlorophenol in soil. J Environ Qual 23: 272–279.

Garcia-Plazaola JI, Esteban R, Hormaetxe K, Becerril JM. 2008. Seasonal reversibility of acclimation to irradiance in leaves of common box (*Buxus sempervirens* L.) in a deciduous forest. Flora 203: 254–260.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. BBA - General Subjects 990: 87–92.

Gerhardson B. 2002. Biological substitutes for pesticides. Trends Biotechnol 20: 338–343.

Gerhardt KE, Huang XD, Glick BR, Greenberg BM. 2009. Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. Plant Sci 176: 20–30.

Bio-Wise. 2000. A guide to biological treatment for metalworking fluids disposal. Oxon: DTI.

Hadacek F. 2002. Secondary Metabolites as Plant Traits: Current Assessment and

Future Perspectives. Crit Rev Plant Sci 21: 273–322.

Han M, Li G, Sang N, Dong Y. 2011. Investigating the bio-toxicity of coking wastewater using Zea mays L. assay. Ecotoxicol Environ Saf 74: 1050–1056.

Hayat R, Ali S, Amara U, Khalid R, Ahmed I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60: 579–598.

Hernandez-Terrones MG, Aguilar MI, King-Diaz B, Lotina-Hennsen B. 2003. Inhibition of photosystem II in spinach chloroplasts by trachyloban-19-oic acid. Pestic Biochem Physiol 77: 6–6.

Hong SH, Ryu H, Kim J, Cho KS. 2010. Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp. S2RP-17. Biodegrad 22: 593–601.

Huang XD, McConkey BJ, Babu TS, Greenberg BM. 1997. Mechanisms of photoinduced toxicity of photomodified anthracene to plants: Inhibition of photosynthesis in the aquatic higher plant *Lemna gibba* (duckweed). Environ. Toxicol Chem 16: 1707–1715.

Kim D, Park MJ, Koh SC, So JS, Kim E. 2002. Three separate pathways for the initial oxidation of limonene, biphenyl, and phenol by *Rhodococcus* sp. strain T104. J Microbiol 40: 86–89.

Kloepper JW, Lifshitz R, Zablotowicz RM. 1989. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol 7: 39–44.

Kuiper II, Lagendijk ELE, Bloemberg GVG, Lugtenberg B. 2003. Rhizoremediation: a beneficial plant-microbe interaction. MPMI 17: 6–15.

Kummerová M, Krulová J, Zezulka S, Tríska J. 2006. Evaluation of fluoranthene phytotoxicity in pea plants by Hill reaction and chlorophyll fluorescence. Chemosphere 65: 489–496.

Kummerová M, Vánová L, Krulová J, Zezulka S. 2008. The use of physiological characteristics for comparison of organic compounds phytotoxicity. Chemosphere 71: 2050–2059.

Kvesitadze G, Gordeziani M. 2001. Some aspects of the enzymatic basis of phytoremediation. J Biol Phys Chem 1: 49-57.

Lucas García JA, Grijalbo L, Ramos B, Fernández-Piñas F, Rodea-Palomares I, Gutierrez-Mañero FJ. 2013. Combined phytoremediation of metal-working fluids with maize plants inoculated with different microorganisms and toxicity assessment of the phytoremediated waste. Chemosphere 90: 2654–2661.

Mallakin AA, Babu TST, Dixon DGD, Greenberg BMB. 2002. Sites of toxicity of specific photooxidation products of anthracene to higher plants: inhibition of photosynthetic activity and electron transport in *Lemna gibba* L. G-3 (duckweed). Environ Toxicol 17: 462–471.

Martín JS, Gallego JR. 2003. Biorremediación. Fundamentos y aspectos microbiológicos, Industria y Minería 351: 12-16.

Meudec AA, Dussauze JJ, Deslandes EE, Poupart NN. 2006. Evidence for bioaccumulation of PAHs within internal shoot tissues by a halophytic plant artificially exposed to petroleum-polluted sediments. Chemosphere 65: 8–8.

Moscoso F, Deive FJ, Villar P, Pena R, Herrero L, Longo MA, Sanromán MA. 2012. Assessment of a process to degrade metal working fluids using Pseudomonas stutzeri CECT 930 and indigenous microbial consortia. Chemosphere 86: 420–426.

Mrozik A, Piotrowska-Seget Z. 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol Res 165: 13–13.

Ndimele PEP. 2010. A review on the phytoremediation of petroleum hydrocarbon. Pak J Biol Sci 13: 715–722.

Newman LA, Reynolds CM. 2004. Phytodegradation of organic compounds. Curr Opin Biotechnol 15: 225–230.

Nwoko CO. 2010. Trends in phytoremediation of toxic elemental and organic pollutants. Afr J Biotechnol 9: 6010–6016.

Ogbo EM, Tabuanu A, Ubebe R. 2010. Phytotoxicity assay of diesel fuel-spiked substrates remediated with Pleurotus tuberregium using Zea mays. Int J Appl Res Nat Prod 3: 12–16.

Osmond, C.B. 1994. What is photoinhibition? Some insights from comparisons of shade and sun plants. In: Baker NR, & Bowyer JR editors. Photoinhibition of Photosynthesis, from Molecular Mechanisms to the Eield. Oxford: Bios Scientific Publishers,. p 1-24.

Pilon-Smits E. 2004. Phytoremediation. Annu Rev Plant Biol 56: 15–39.

Rabenstein A, Koch T, Remesch M, Brinksmeier E, Kuever J. 2009. Microbial degradation of water miscible metal working fluids. Int. Biodeter Biodegrad 63: 1023–1029.

Rosenberg E. 1993. Exploiting microbial growth on hydrocarbons ? new markets. Trends Biotechnol 11: 419–424.

Sokal, R. 1979. Biometria. Barcelona: H. Blume.

Strain HH, Cope BT, Svec WA. 1971. Analytical procedures for the isolation, identification, estimation, and investigation of the chlorophylls. Methods Enzymol 23: 452–476.

Susarla S, Medina V, McCutcheon S. 2002. Phytoremediation: an ecological solution to organic chemical contamination. Ecol Eng 18: 647–658.

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, Lelie D, Mench M. 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ Sci Pollut Res 16:

765–794.

Wenzel WW. 2008. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321: 385–408.

Wild EE, Dent JJ, Thomas GOG, Jones KCK. 2006. Visualizing the air-to-leaf transfer and within-leaf movement and distribution of phenanthrene: further studies utilizing two-photon excitation microscopy. Environ Sci Technol 40: 907–916.

Wild SR, Jones KC, Johnston AE. 1992. The polynuclear aromatic hydrocarbon (PAH) content of herbage from a long-term grassland experiment. Atmos Environ Part A 26: 1299–1307.

Yateem A, Al-Sharrah T, Bin-Haji A. 2007. Investigation of Microbes in the Rhizosphere of Selected Grasses for Rhizoremediation of Hydrocarbon-Contaminated Soils. J Soil Contam 16: 269–280.

Yoshitomi K, Shann J. 2001. Corn (*Zea mays* L.) root exudates and their impact on 14C-pyrene mineralization. Soil Biol Biochem 33: 1769–1776.

Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A Greedy Algorithm for Aligning DNA Sequences. J Comput Biol 7: 203–214.

Table 1. Chemical oxygen demand (COD) parameter values in the bioremediated systems, with and without inoculation. Different letters indicate significant differences ($p<0,05$). The percentage of reduction appears in brackets.

	Initial COD	Final COD
Without Esp. 1	1779 a	632.89±22.81 b (64.42 %)
Esp. 1	1779 a	683.78±28.18 b (61.56 %)
Without Esp. 21	1800 a	902±40.01 b (49.88 %)
Esp. 21	1800 a	921.67±15.97 b (48.79 %)
Without consortium	1740 a	836.33±46.54 b (51.93 %)
Consortium	1740 a	754.67±40.29 b (56.62 %)

Table 2. pH values in the bioremediated system, with and without inoculation. Different letters indicate significant differences (p<0,05). The percentage of reduction appears in brackets.

	Initial pH	Final pH
Without Esp. 1	9.71a	8.65±0.007b (10.91 %)
Esp. 1	9.71a	8.56±0.06b (11.84 %)
Without Esp. 21	9.43a	8.54±0.01b (9.43 %)
Esp. 21	9.43a	8.62±0.008b (8.58 %)
Without consortium	9.01a	7.99±0.05b (11.32 %)
Consortium	9.01a	8.03±0.02b (10.87 %)

Table 3. Fluorescence emission from dark adapted leaves (Fo), maximum photosynthetic efficiency of photosystem II (Fv/Fm), the effective PSII quantum yield (ϕPSII), Hill reaction, Chlorophyll a/b and total chlorophyll content in maize samples, without and with inoculation with the strain Esp. 1 (*Enterobacter* sp.), after the phytoremediation process (5 days). Different letters indicate significant differences ($p<0.05$).

Esp. 1	Fo	Fv/Fm	ϕPSII	Hill reaction[1]	Chlorophyll a/b	Total Chlorophyll content[2]
Control plants (non-treated with dMWF and non –inoculated	162.80±7 a	0.77±0.004 b	0.33±0.01 c	53.59±2.16 a	1.72±0.023 a	1082±30 a
Plants non-treated with dMWF and inoculated	180.30±7 c	0.76±0.006 ab	0.33±0.012 bc	81.70±1.85 b	1.93±0.027 b	1327±55 b
Plants treated with dMWF and non-inoculated	144.60±5 b	0.76±0.008 b	0.29±0.013 a	28.70±1.35 c	1.83±0.042 ab	1290±22 b
Plants treated with dMWF and inoculated	154.60±3 ab	0.74±0.009 a	0.29±0.014 ab	18.82±1.54 d	2.27±0.054 c	953±18 c

[1] µg DCPIP reduced * min^{-1} * µg Clorophyll^{-1}

[2]nmol * g^{-1} leave fresh weight

Table 4. Fluorescence emission from dark adapted leaves (Fo), maximum photosynthetic efficiency of photosystem II (Fv/Fm), the effective PSII quantum yield (ϕPSII), Hill reaction, Chlorophyll a/b and total chlorophyll content in maize samples, without and with inoculation with the strain Esp. 21 (*Rhodotorula dairenensis* sp.), after the phytoremediation process (5 days). Different letters indicate significant differences ($p<0.05$).

Esp. 21	Fo	Fv/Fm	ϕPSII	Hill reaction[1]	Chlorophyll a/b	Total Chlorophyll content[2]
Control plants (non-treated with dMWF and non – inoculated	180±5 c	0.75±0.007 a	0.49±0.02 c	17.30±0.34 b	1.28±0.08 a	962±12 b
Plants non-treated with dMWF and inoculated	182±5 c	0.77±0.006 a	0.43±0.01 b	36.50±3.53 c	2.93±0.16 b	1128±13 c
Plants treated with dMWF and non-inoculated	120±6 a	0.76±0.010 a	0.30±0.02 a	18.70±1.18 b	1.45±0.11 a	940±8 b
Plants treated with dMWF and inoculated	158±6 b	0.74±0.087 a	0.32±0.02 a	13.07±0.58 a	8.31±0.72 c	893±24 a

[1] µg DCPIP reduced * min^{-1} * µg $Clorophyll^{-1}$

[2]nmol * g^{-1} leave fresh weight

Table 5. Fluorescence emission from dark adapted leaves (Fo), maximum photosynthetic efficiency of photosystem II (Fv/Fm), the effective PSII quantum yield (ϕPSII), Hill reaction, Chlorophyll a/b and total chlorophyll content in maize samples, without and with inoculation with the consortium (see Material and Methods), after the phytoremediation process (5 days). Different letters indicate significant differences ($p<0.05$).

Consortium	Fo	Fv/Fm	ϕPSII	Hill reaction[1]	Chlorophyll a/b	Total Chlorophyll content[2]
Control plants (non-treated with dMWF and non – inoculated	199±4 b	0.76±0.006 a	0.32±0.01 b	90.05±4 a	2.99±0.01 a	1531±21 a
Plants non-treated with dMWF and inoculated	214±5 c	0.77±0.006 a	0.34±0.01 b	51.92±1.4 b	2.94±0.03 a	1565±13 a
Plants treated with dMWF and non-inoculated	186±6 ab	0.75±0.009 a	0.26±0.01 a	26.87±0.73 c	3.90±0.12 b	1191±17 b
Plants treated with dMWF and inoculated	182±6 a	0.75±0.007 a	0.24±0.01 a	28.91±1.34 c	2.92±0.04 a	1286±19 c

[1] µg DCPIP reduced * min^{-1} * µg $Clorophyll^{-1}$

[2]nmol * g^{-1} leave fresh weight

Figure captions

Figure 1. LTSEM micrographs of leaf mesophyll cells of 20-days old maize plants non-inoculated and grown for 5 days with and without degraded metal working fluids (MWF). Treatments tap water without bacteria (A); non inoculated and growing in dMWFs (B). Bundle sheath cells (BSCs), Chloroplast (C), vascular bundles (VB), Cell wall (CW), Tonoplast (T) and Vacuole (V).