Changes in total phenol and flavonoid contents in *Chrysanthemum leucanthemum* under crude oil contamination

Azam Sadat Noori, Hassan Zare Maivan, Ebrahim Alaie

1Department of Plant science, College of Biology Science, Tarbiat Modares University  
2Research Institute of Petroleum Industry (RIPI)

Azam Sadat Noori, Hassan Zare Maivan, Ebrahim Alaie: Changes in total phenol and flavonoid contents in *Chrysanthemum leucanthemum* under crude oil contamination

ABSTRACT

Oil contamination of soil serves as damaging oxidative stress and impairs plant cell membrane. Tolerant plants modify their secondary metabolism under crude oil to lessen oxidative damage accordingly. Mycorrhizal inoculation helps plants to manage their primary and secondary metabolism more efficiently and access nutrient under abiotic stress. *Chrysanthemum leucanthemum* has proven to adapt well under environmental stress; as such, one year old mycorrhizal *C. leucanthemum* plants were planted in pots containing soil mixed with petroleum at 2.5%, 5%, 7.5% and 10% w/w. Plants were grown under ambient conditions in greenhouse 16/8 h light/dark and average 30/5°C day/night for 6 months. Mycorrhizal inoculation, flavonoid and phenolic content of plants were determined three times once every 2 months. Results showed increased flavonoid and phenol production in mycorrhizal *C. leucanthemum* plants under different concentrations of crude oil. After 6 months, the highest rate of flavonoids and phenol occurred in leaves and roots at 7.5 and 10% (w/w) of crude oil, respectively. Mycorrhizal inoculation percentages increased in all treatments over time except in soil treated with 10% (w/w) crude oil, in which mycorrhizal percentage decreased after 4th month. Results of this investigation showed mycorrhizal *C. leucanthemum* could survive in soil under crude oil contamination through altering of its metabolic processing.

Key words: *Chrysanthemum leucanthemum*, Crude oil, flavonoids, Mycorrhizae, Phenol

Introduction

Petroleum and its products are the main economic source in many countries which bring along both economical profits and high environment pollution drawbacks in many ways [9]. Petroleum industry pollutions have raised the need for environmental awareness in populations and taking of affirmative action to remedy its effects.

Oil has different fractions, part of which will evaporate immediately, but some parts may remain in soil for years [12]. In recent decades, although environmental scientists have focused on improving the ecophysiological and phytotechnological knowledge on plants growing in polluted sites, still more is needed to be known about functional and physiological responses of plants growing in oil contaminated sites.

Phenolic compounds and flavonoids are important groups of secondary metabolites and bioactive products, are produced as a response to environmental pollution through improving defense mechanism of injured plants, are antioxidants capable of scavenging free superoxide radicals and as such, are crucial for plant growth and reproduction [18,6,11,1].

Early works of authors [13,14] have shown the effects of petroleum on seed germination of *C. leucanthemum* and sunflower as well as on their mycorrhizal development. These authors showed seed germination rate increased over time, regardless of percent petroleum in the medium upto 10% (w/w) and mycorrhizae developed in pots containing 7.5% (w/w) crude oil. It is well known that mycorrhizae improve production of plant secondary metabolites that assist in contaminant degradation. Mycorrhizae change soil characteristics and alter contaminant quantity and quality throughout phytoremediation process [7,16] and ameliorate soil for facilitated growth and development of symbiotic plants [17]. Since, mycorrhizal *C. leucanthemum* is able and displays improved growth under stress, this investigation was undertaken to investigate its mycorrhizal inoculation potentials and changes in flavonoids and total phenols content under petroleum contamination in pot cultures in greenhouse.

Materials and methods

Corresponding Author

Hassan Zare Maivan, Department of Plant Biology, College of Biological Sciences, Tarbiat Modares University, 14115-175, Tehran, Iran.  
E-mail: zare897@yahoo.com; Tel: (+98) 9123831060, Fax: (+9821) 82884717
Soil properties:

Soil samples, collected from depth of 0-30 cm Tarbiat Modares University campus, were sieved through 2mm mesh screen, soil samples were divided in two series and labeled Non-Sterilized soil (NS) and mycorrhizal soil (M) which steam-sterilized at 121°C for 2 hours, cooled and mixed with sterilized perlite and vermiculite 5:4:1 (w:w), proportionally. M soil contained inoculum propagules (spores) of *Glomus mossae* and *G. intraradices* which were added 4:1 (w:w).

Crude oil was obtained from Tehran Refinery. Crude oil was added and mixed with M and NS soils at concentration of 2.5, 5, 7.5, 10 percent (w:w). Non-contaminated soil served as control. Plastic pots were filled with 1 kg of M and NS contaminated soils.

- C. leucanthemum planting:

Roots of one year old *C. leucanthemum*, grown under natural ambient conditions in greenhouse, were washed carefully in distilled water, then transferred to M and NS soils. All experiments were carried out under ambient condition 16/8 h and 30/5°C day/night for 6 months, respectively. Plants roots and leaves were collected every two months for analytical determination.

- Total flavonoids measurement:

Total flavonoid content was determined according to the method of [8], briefly 0.2 g of frozen tissue was homogenized with acidic EtOH (contain EtOH and HOAc with ratio of 99:1), and centrifuged at 12000×g for 15 min. The supernatant was placed in 80°C water bath for 10 min. After cooling, the absorbance of flavonoids was read at 270, 300 and 330 nm by double beam UV visible spectrophotometer Analytikjena Spekol 2000, Germany. Flavonoids content was determined by using extinction coefficient of 33000 Cm⁻¹M⁻¹.

- Total phenolics measurement:

Total phenolics of plant extracts of all treatments were determined using Folin-Ciocalteu reagent as described by Maizura et al., 2011. Samples were inserted into different test tube and mixed thoroughly with 5 ml of 10 % Folin-Ciocalteu reagent. After 5 mins, 4 ml of 7.5% sodium carbonate (Na₂CO₃) was added and allowed to react for 2 hrs at room temperature. The absorbance was measured at 765 nm using a double beam UV visible spectrophotometer Analytikjena Spekol 2000, Germany. Triplicate samples were analyzed for each treatment. Standard curve of Gallic acid (GA) solution (10, 20, 40, 60, 80 and 100 ppm) was prepared using the similar procedure. The results were expressed as mg GA/100 g extract sample.

- Measuring mycorrhizal extension:

To measure mycorrhizal colonization, root samples were cleared in KOH 90%, acidified in HCl 10% and stained with acidic fuscin [15]. Percentage of root colonization was determined by the line intersect method [4].

2.6 Analytical methods:

Experiments were conducted with three replicates per treatment. Data were subjected to one way ANOVA. When, statistical difference between the means of the treatments existed, LSD test at the 5% level was applied using SPSS version 19.

Results

- Flavonoid content:

The highest flavonoid content in leaves of *C. leucanthemum* was observed in M soils containing 2.5% (w/w) crude oil six months after planting. Flavonoids increased steadily over time (Figure 1-A,B) in M soil, but not in NS soil. The least flavonoid content was observed in NS soil after 4 months.

- Total phenol:

Total phenol measurement in leaves of *C. leucanthemum* increased gradually over time in all treatments except in treatments with 10% crude oil which showed significant decline after 4 months (Figure 3 A, B). Total phenol content in root of *C. leucanthemum* increased gradually over time in all treatments of both M and NS soils, particularly in plants treated with 7.5 and 10% (w/w) crude oil increasing significantly over time (Figure 4 A, B).

- Mycorrhizal inoculation:

*C. leucanthemum* developed AM mycorrhizae. Mycorrhizal development in roots progressed steadily over time in all treatments throughout the study period except in plants treated with 10% (w/w) crude oil which showed significant decrease following 4 months of mycorrhizal development. Furthermore, plants grown in M soil showed greater mycorrhizal percentage than plants grown in NS soil (Table 1).
Discussion:

In current study, effects of crude oil contamination and mycorrhizae on the contents of flavonoids and total phenol compounds were examined. Crude oil is an abiotic stress factor and causes oxidative stress in plants. Many plant species are able to tolerate crude oil contamination in soil and even many other species are used remediating oil contamination. Tolerant plant species modify their metabolic processes to alter production of phenolics and flavonoids and reduce oxidative damage to their cells. Flavonoids serve as signaling molecules during mycorrhizal inoculation as well as responding to abiotic stresses.

Results of this study showed that *C. leucanthemum* increased its total phenol and flavonoids contents gradually throughout the exposure period, more so in greater concentrations of oil in the soil. Altering of flavonoids and phenolic compounds production under crude oil stress is in agreement with findings of other investigators [21]. Zhou et al. in their investigation showed that alfalfa and fescue plants increased their phenolic compound contents after exposure to PAH. This seems to be as a result of changes in gene expression of enzymes such as, PAL and CHS and explains that plant secondary metabolism improves under petroleum contamination, especially under high concentrations to help plants resist pollution.

Early works of authors [13,14] have showed seed germination rate increased over time regardless of percent petroleum in the medium upto 10% (w/w) and mycorrhizae developed in pots containing 7.5% (w/w) crude oil. This adaptation might be because mycorrhizal inoculation increases plants primary and secondary metabolites, especially under oxidative stress and could start as early as rootlet development following seed germination. Phenol and flavonoids compounds, products of secondary metabolism, primarily increase in mycorrhizal plants because of promotion in plant root cell gene expression. Zhang et al [20] reported AM colonization promoted phenolic compounds through altering gene expression of PAL and CHS enzymes. Results of this investigation also showed that mycorrhizal inoculation had significant effect on increasing total phenol and flavonoids content of *C. leucanthemum*.

Role of mycorrhiza on flavonoids content in root cells has been reported in other investigations. For example, Amalesh et al. [2] reported that mycorrhizal inoculation increased flavonoid content, and vice versa flavonoids content enhance mycorrhizal inoculation. However, whether the response and development of *C. leucanthemum* root cells to mycorrhizal inoculation and to crude oil stress is similar or follows a different pathway needs to be investigated in more detail for specific flavonoids and phenolic compounds.

Results of this research showed that mycorrhizal development and secondary metabolite production of *C. leucanthemum* cells occurred to a certain concentration of crude oil contamination in soil and beyond that phenol and flavonoid content decreased depending on the plant organ. For example, flavonoids content in *C. leucanthemum* leaves increased up to 7.5% (w/w) crude oil and decreased afterwards under 10% w/w crude oil treatment. However, root responded differently and showed gradual increase in flavonoids and phenolic contents over time in all treatments (Table 1, Figures 1-4). Different response of leaves and roots to crude oil contamination might be explained as follows: (1) Roots produce total flavonoids and phenolic compounds in greater variety and quantity than leaves, (2) Roots are exposed early and there is a lag time for leaves to respond accordingly (3) mycorrhizal inoculation might have reinforced effects of crude oil contamination in roots, but not in leaves and (4) petroleum hydrocarbons affect soil physical properties, more so on oxygen and water diffusion in soil [5]. Since, petroleum hydrocarbons display hydrophobic and lipophylic properties, thus, could reduce water availability and root exchange capacity. Therefore, high concentration of oil in soil could cause low water uptake and nutrient availability and subsequently might insert osmotic, drought and oxidative stress leading to low plant growth.

As far as application of mycorrhizal *C. leucanthemum* under crude oil treatment is concerned, it could be concluded that *C. leucanthemum* produced greater quantities of total phenol and flavonoids compounds under oil contamination, which could be exploited commercially, and also its cultivation could be extended to remedy oil contaminated sites.
Fig. 1A: Flavonoids content in leaf of MS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation has been shown. The mean difference is significant at $p < 0.05$. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: content before transferring of plants, T1, T2 and T3: contents in 2, 4 and 6 months after transferring of plants, respectively.

Fig. 1B: Flavonoids content in leaf of NAS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation has been shown. The mean difference is significant at $p < 0.05$. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: content before transferring of plants, T1, T2 and T3: contents in 2, 4 and 6 months after transferring of plants, respectively.
Fig. 2A: Flavonoids content in root of MS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation has been shown. The mean difference is significant at p < 0.05. Means followed by the same letter are not significantly different based on the Duncan test (p < 0.05). T0: content before transferring of plants, T1, T2 and T3: contents in 2, 4 and 6 months after transferring of plants, respectively.

Fig. 2B: Flavonoids content in root of NAS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation has been shown. The mean difference is significant at p < 0.05. Means followed by the same letter are not significantly different based on the Duncan test (p < 0.05). T0: content before transferring of plants, T1, T2 and T3: contents in 2, 4 and 6 months after transferring of plants, respectively.
Fig. 3A: Total phenol content in leaf of MS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 level. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: First day of examination before transferring of plants, T1: Two months after plants transferring, T2: Four months after plants transferring, T3: Six months after plants transferring.

Fig. 3B: Total phenol content in leaf of NAS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 level. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: First day of examination before transferring of plants, T1: Two months after plants transferring, T2: Four months after plants transferring, T3: Six months after plants transferring.
Fig. 4A: Total phenol content in root of MS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 level. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: First day of examination before transferring of plants, T1: Two months after plants transferring, T2: Four months after plants transferring, T3: Six months after plants transferring.

Fig. 4B: Total phenol content in root of NAS under crude oil contamination. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 level. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T0: First day of examination before transferring of plants, T1: Two months after plants transferring, T2: Four months after plants transferring, T3: Six months after plants transferring.

Table 1: Mycorrhizal percentage under crude oil contamination

<table>
<thead>
<tr>
<th>Treatment % (w/w)</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time course</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1M</td>
<td>50.58±2.52</td>
<td>58.57±2.9</td>
<td>30.55±1.52</td>
<td>34.21±1.71</td>
<td>11.66±0.58</td>
</tr>
<tr>
<td>T1N</td>
<td>85.00±5.00</td>
<td>59.66±4.5</td>
<td>74.7±7.9</td>
<td>55.83±5.20</td>
<td>69.13±3.77</td>
</tr>
<tr>
<td>T2M</td>
<td>93.33±2.88</td>
<td>90.5±5</td>
<td>72.16±2.56</td>
<td>59.33±4.04</td>
<td></td>
</tr>
<tr>
<td>T2N</td>
<td>54.11±2.70</td>
<td>27.47±1.34</td>
<td>25.7±1.28</td>
<td>15.01±0.56</td>
<td></td>
</tr>
<tr>
<td>T3M</td>
<td>88.33±2.88</td>
<td>80.33±4.72</td>
<td>72.5±2.5</td>
<td>59.16±3.81</td>
<td></td>
</tr>
<tr>
<td>T3N</td>
<td>75.00±5.00</td>
<td>52.66±6.8</td>
<td>45.83±6.29</td>
<td>62.5±2.5</td>
<td></td>
</tr>
</tbody>
</table>

* = Average of three repetitions; ± standard deviation. The mean difference is significant at $p < 0.05$; Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$); T0: content before transferring of plants, T1, T2 and T3: contents in 2, 4 and 6 months after transferring of plants, respectively.
Reference