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# Tolerance of *Impatiens balsamina* L., and *Crotalaria retusa* L. to grow on soil contaminated by used lubricating oil: A comparative study

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ABSTRACT

Screening of plant species with an ability to grow on contaminated soil is the most critical step in the planning of a phytoremediation program. While flourishing growth of *Impatiens balsamina* L. and *Crotalaria retusa* L. has been observed in areas adjacent to automobile service stations in Sri Lanka, no systematic study of their tolerance to used lubricating oil (ULO) contaminated soil has been carried out. Therefore, the aim of the present study was to investigate the comparative responses of *I. balsamina* L. and *C. retusa* L. to soil contaminated with ULO. Both species exhibited 100% seed germination in soils treated with 1%–5% w/w ULO. After 120 h exposure, root lengths and biomass of germinated seedlings of both species were significantly ($p < 0.05$) reduced in all treatments above 3% w/w ULO. The measured growth parameters of plants following 90 d exposure to 0.5–3% w/w ULO, indicated significant ($p < 0.05$) negative effects on *I. balsamina* and *C. retusa* at > 1% w/w and > 2% w/w ULO, respectively. There were no significant effects on chlorophyll content or root anatomy of either species under any treatments. Therefore, we concluded that *I. balsamina* can tolerate up to 1% of ULO and *C. retusa* up to 2% w/w ULO without displaying any negative effects. Comparatively higher biodegradation of ULO in the rhizosphere, root nodule formation, increases in root length and root hair density are all possible strategies for the exhibited higher tolerance of *C. retusa*. Therefore, the overall results indicate that *C. retusa* has the greater potential to be used in phytoremediation of ULO contaminated soils. The findings of the present study will be beneficial in planning phytoremediation program for ULO contaminated soil.

## 1. Introduction

Soil is a dynamic and complex ecosystem that plays a major role in the continuation of life on earth. Contamination of soil with used lubricating oil (ULO), as a result of anthropogenic activities such as indiscriminate disposal of ULO into gutters, water drains and vacant plots by roadside motor mechanics and allied workers has become a serious global issue (Odjegba and Sadiq, 2002; Glibovytska et al., 2019). Used lubricating oil is a mixture of hydrocarbons consisting of 73–80% aliphatic compounds, 11–15% monoaromatic, 2–5% diaromatic and 4–8% polyaromatic hydrocarbons, polar fractions and heavy metals (Vazquez-Duhalt, 1989). Further, ULO may persist for a long time in ecosystems (Ramadass et al., 2018). Therefore, soil contaminated with ULO may negatively affect soil physicochemical and biological properties (Okonokhua et al., 2007; Lum and Chikoye, 2018) and consequently may pose threats on terrestrial ecosystems (Ramadass et al., 2015). Therefore, an effective method is required to remediate the ULO contaminated soil.

Phytoremediation is a novel, cost effective and environmental friendly method (Malik et al., 2017; Ugwu et al., 2019) that has potential application in remediation of ULO contaminated soil (Sharifi et al., 2007). The approach has a number of technical advantages over conventional ex-situ treatment methods such as excavation, off-site storage, incineration and soil washing using surfactants, emulsifiers and additives (Chaudhry et al., 2005; Hussain et al., 2018). The most critical step in the planning of a phytoremediation program is the screening of plant species for their ability to grow on ULO contaminated soil and its success will mainly depends on the degree of resistance to hydrocarbons of the selected species (Gaskin, 2008). Previous studies have reported that the effect of ULO on the growth and tolerance of crop and ornamental plants is species-specific e.g. *Capsicum annuum* L. (Anoliefo and Vwioko, 1995), *Abelmoschus esculentus* (Agbogidi and Nweke, 2005); *Medicago sativa* (Liu et al., 2012) and *Echinacea purpurea* (Heidari et al., 2018). However, there is a scarcity of data relevant to plant species with tolerance to sites contaminated with multiple pollutants (Batty and Dolan, 2013). Hence, it is vitally important to identify different

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plant species with higher tolerance to contaminated soil with a mixture of contaminants such as ULO.

*Impatiens balsamina* L. is an ornamental species of the family Balsaminaceae. It is native to India and Myanmar but has been introduced to other countries in Europe, Asia, the Americas, Australia and some parts of tropical Africa as an ornamental species. *Crotalaria retusa* L. is a leguminous species within the family Fabaceae. It has a worldwide distribution and widely spread in tropical Asia, Africa, America and Australia (Cabi.org, 2019). Both species are annuals that produce attractive flowers. In our preliminary studies, we observed flourishing growth and widespread dispersion of *I. balsamina* L and *C. retusa* as single stands on wastelands adjacent to automobile service stations in semi-urban areas of Sri Lanka. If abundant, ornamental plants could be used to remediate contaminated soils, with the additional advantage of enhancing the aesthetic value of the contaminated site providing therapeutic benefits to local residents (Ikeura et al., 2016). However, little attention has been paid to the suitability of ornamental plants to remediate ULO contaminated soils (Wang and Zhou, 2005), and thus further studies are required. Moreover, there are additional benefits to using leguminous plants in phytoremediation because of their ability to fix nitrogen. Thus, leguminous plants not only grow well in petroleum-contaminated soils with low availability of nitrogen (Gudin and Syratt, 1975, Wenzel 2009), they also stimulate microorganisms in the rhizosphere by releasing exudates of carbon, energy, nutrients, enzymes and sometimes oxygen from their roots (Cunningham et al., 1996; Yan-de et al., 2007; Pérez-Montaño et al., 2014). The suitability of using leguminous plants to recover petroleum-contaminated soils has been reported previously (Gudin and Syratt, 1975; Muratova et al., 2008; Inckot et al., 2011; Akhter et al., 2018).

Seed germination and seedling growth of a particular plant species in contaminated soil is an indication of the tolerance of that species (Peng et al., 2009). Chen et al. (2013) reported the importance of plant tolerance as an indication of the phytoremediation potential of a plant. Different stages in the plant life cycle might show different responses to contaminants and therefore, it is vitally important to measure a range of endpoints at different stages of development and levels of biological organization (physiological and cellular). However, comprehensive and comparative studies on seed germination and growth performance of *I. balsamina* and *C. retusa* in ULO contaminated soil have not been carried out to date. Therefore, the aim of the present study was to investigate the effects of ULO contaminated soil on seed germination, growth performance, root anatomy and chlorophyll contents of *I. balsamina* and *C. retusa*.

## 2. Materials and methods

### 2.1. Plant material

Five hundred mature seeds from each of *I. balsamina* and *C. retusa* were collected from plants growing in the plant-house of the University of Ruhuna, Matara, Sri Lanka. Mature seeds of the two species identifiable by the pod color; pods of *C. retusa* change from greenish to dark brown to black when mature and those of *I. balsamina* change from greenish to yellow when mature. Collected seeds were carefully observed for physical damages and fungal infections and apparently healthy seeds with approximately the same size were selected for experiments. A sub-sample of seeds was tested for viability using Tetrazolium/TTC test (Souza et al., 2010). According to the results both seed samples were suitable for experimentations.

### 2.2. Soil sampling and preparation

Soil was collected from the surface layer (5–10 cm) of an undisturbed site of the University of Ruhuna, Matara, Sri Lanka in July 2017. Following air drying, soil was passed through a 2 mm × 2 mm size sieve and homogenized. This soil was used as an uncontaminated control in all experiments in this study. Prior to experimentation a physiochemical analysis of soil samples was carried out. Soil texture was determined by using a standard sieve set (Retsch AS 200 basic, RETSCH, Germany) and analyzed by using soil texture triangle (USDA-NRCS, 1999). The pH was determined with a digital pH meter (Eutech pH 150, Thermo Fisher Scientific India Pvt. Ltd, India) using a 1:5 w/v soil/water suspension. Gravimetric water potential (GWP) was determined by loss of weight after drying soil at 105 °C for 24 h, according to Black (1965). The organic matter content (OM) of the soil was determined according the loss on ignition method (LOI) following the protocol of the American Society for Testing and Materials (ASTM) standards (2014). Soil nitrate-nitrogen was determined by the KCl extractable method (Robertson et al., 1999) and extractable P was determined according to the method described by Murphy and Riley (1962).

Used lubricating oil (ULO) was collected from an automobile service station at Kekanadura, Matara, Sri Lanka. In order to obtain different ULO contamination levels in the soil, uncontaminated soil was spiked with ULO and homogenized to obtain 10,000 mg/kg (1% w/w), 20,000 mg/kg (2% w/w), 30,000 mg/kg (3% w/w), 40,000 mg/kg (4% w/w) and 50,000 mg/kg (5%w/w).

### 2.3. Determination of seed germination and seedling growth performance

A preliminary experiment was conducted according to the ISO method (1993) to investigate seed germination and seedling growth performance of *I. balsamina* and *C. retusa* in ULO contaminated soil containing 10,000 mg/kg, 20,000 mg/kg, 30,000 mg/kg, 40,000 mg/kg and 50,000 mg/kg w/w ULO. Briefly, samples of 150 g control and contaminated soils were placed in 150 mm diameter Petri dishes and soil water holding capacity was adjusted to 60%. Ten seeds per Petri plate and five replicates per treatment and control were used. Following 96 h incubation at 25 °C in the dark, the percentage seed germination, defined as radicle and plumule was calculated using the formula:

$$Percentage\ seed\ germination\ (GP) = (Number\ of\ germinated\ seeds) /(Total\ number\ of\ seeds) \times 100$$

After a 120 h incubation period, five seedlings from each replicate were randomly selected and seedling shoot length, main root length and dry biomass of shoots and roots were measured. Shoot and root length was were measured prior to drying using a millimeter ruler and the dry weight was measured by oven drying samples to constant weight at 60 °C. Percentage inhibition of seed germination, seedling height, root length and dry weight were calculated using following the formula:

$$Percentage\ inhibition = ((A - B)/A) \times 100$$

where A is the mean of the measured parameter in the control soil and B is the mean of the measured parameter in the tested contaminated soil.

### 2.4. Plant performance

A pot experiment was carried out in the plant house at about 30 °C to study early growth performance of *I. balsamina* and *C. retusa* in ULO contaminated soil. Four kilograms of prepared contaminated (5000 mg/kg (0.5% w/w), 10,000 mg/kg (1% w/w), 15,000 mg/kg (1.5% w/w), 20,000 mg/kg (2% w/w), 25,000 mg/kg (2.5% w/w), 30,000 mg/kg (3% w/w)) and uncontaminated (control) soils were placed in individual plastic pots (17 cm × 22 cm) and the soil water holding capacity was adjusted to 60% and maintained during the 90 d experimental time period. The experiment used a randomized block design (RBD) with 4 replicates per treatment and control. In each pot two seeds of either *I. balsamina* or *C. retusa* were planted and watered regularly.

### 2.5. Measurement of plant height, root length, biomass and morphological characterization of root systems

Shoot height (cm) was measured from soil level to the tip of the shoot using a meter ruler (Omosun et al., 2008) after 30 d and thereafter at 30 d intervals. Length of the main root (cm), shoot fresh and dry biomass (mg) and root fresh and dry biomass (mg) were measured and morphological characters of the root system, including root color, lateral branching, presence of root hairs in both tested species and presence of root nodules in *C. retusa*, were carefully observed in all treatments and control at the end of 90 d experimental period. Root length was measured using a meter rule by taking measurement from the base of plant to the tip of the longest root. Fresh and dry biomass were measured by using an electronic balance (EK - 410i 400 g × 0.01 g). Root fresh biomass was measured after washing off soil adhering to the root surface followed by blotting and air-drying for 1 h. Dry biomass of shoots and roots were determined after oven-drying samples to constant weight at 60 °C.

### 2.6. Tolerance index (Ti)

To determine the degree of growth inhibition in *C. retusa* and *I. balsamina* grown in soil contaminated with ULO, an index of tolerance was calculated according to Szulc et al. (2010).

$$Ti = dry\ weight\ of\ plant\ grown\ in\ ULO\ contaminated\ soil\ (Tp) / dry\ weight\ of\ plant\ grown\ in\ control\ soil\ (Tn)$$

### 2.7. Determination of chlorophyll content

Chlorophyll *a*, *b* and total chlorophyll concentrations were determined after 90 d following the method described by Maclachalam and Zalik (1963). Chlorophyll was extracted from the fourth leaf from the tip of the main stem. Briefly, 0.5 g o f leaf tissue were placed in 100 mL flasks containing 25 mL of acetone. Flasks were kept in the dark for 24 h and were shaken twice during incubation. The chlorophyll suspensions were centrifuged at 67,080 × g for 20 min. Absorbance of the supernatant was measured at 645 nm and 665 nm using UV/Visible spectrophotometer (Evolution 260 Bio, Thermo Fisher Scientific Inc. Germany). Chlorophyll *a*, *b* and total chlorophyll contents were calculated using following formulae:

$$Ca = (12.3OD663 - 0.86OD645) / (d \times 1000 \times W) \times V$$

$$Cb = (19.3OD645 - 3.60OD663) / (d \times 1000 \times W) \times V$$

where $C_a$ is the chlorophyll a concentration (mg/kg FW), $C_b$ is the chlorophyll b concentration (mg/kg FW), OD is the optical density, V is the final volume (mL) of extract, W is the fresh weight of the leaf sample (g), d is the path-length of light (cm).

### 2.8. Root anatomy

Anatomical studies were carried out to determine whether there were any structural differences in roots of *C. retusa* and *I. balsamina* grown in soil contaminated with ULO compared to plants grown in control soil. Fresh root sample was collected from *C. retusa* and *I. balsamina* and washed thoroughly using distilled water. Cross sections from the middle of roots (10 cm back from the root tip) were cut using a razor blade and mounted in water and observed under the medium power (10x) of a digital photo microscope (Olympus DP 20, Olympus Cooperation, Japan).

### 2.9. Percentage biodegradation of ULO

The percentage oil biodegradation in the soil taken from the rhizosphere of tested species was determined at intervals of 30 d using the gravimetric method described by Agamuthu et al. (2010). Total petroleum hydrocarbon (TPH) concentrations were measured from soil with contamination levels of 1%, 2% and 3% w/w ULO. Ten grams of soil were suspended in 20 mL of dichloromethane in a 250 mL Erlenmeyer flask. After shaking for 1 h on an orbital shaker (Lab Companion SK 300 Benchtop shaker, GMI Inc. USA) at 200 rpm, the oil suspension was filtered through a Whatman No. 4 filter paper, the filtrate centrifuged for 20 min at 966 × g and the supernatant decanted into a clean dry beaker of known weight. This procedure was repeated 5 times for each sample in order to recover the maximum amount of oil extracted into the solvent. The solvent was completely evaporated on a water bath at 40 °C. The beaker (containing residual oil) was re-weighed and the TPH content was calculated using the following formula:

$$TPH\ content = (final\ weight\ of\ the\ beaker\ containing\ residual\ oil - weight\ of\ the\ empty\ beaker) / (weight\ of\ the\ soil\ sample)$$

Percentage biodegradation was calculated according to the following formula.

$$Percentage\ biodegradation = [weight\ of\ oil\ (control) - Weight\ of\ oil\ (degraded)] / [weight\ of\ oil\ (control)] \times 100$$

### 2.10. Statistical analysis

Statistical analyses were performed using the standard statistical software MINITAB version 17. All the measured parameters were analyzed using one way ANOVA followed by Turkey's post hoc test to determine significant difference among individual means. Two way ANOVA was performed to determine the interactive effect of ULO concentration and species on plant growth performances. The statistical significant was defined at $p < 0.05$ and $p < 0.001$.

## 3. Results

### 3.1. Physicochemical characterization of control soil

According to the measured physico-chemical parameters, soil texture of the control soil was determined as sandy loam and pH was slightly acidic (Table 1).

### 3.2. Effect of ULO on seed germination and seedling growth

Since results showed 100% seed germination of both *C. retusa* and *I. balsamina* in all ULO contamination levels, the percentage inhibition of seed germination was zero.

After 120 h exposure, there was a downward trend in root length, seedling height and biomass of both species with the increasing ULO

**Table 1**
Physicochemical properties of uncontaminated soil (control).

| Soil property | Control soil |
|---|---|
| TPH (mg kg $^{-1}$) | 0 |
| Soil texture | Sandy loamy |
| Soil pH | 5.36 ± 0.03 |
| GWP (%) | 5.37 ± 0.04 |
| OM (%) | 14.64 ± 0.46 |
| N–$NO_3^-$ (mg kg $^{-1}$) | 3.06 ± 0.02 |
| P (mg kg $^{-1}$) | 0.12 ± 0.01 |

GWP gravimetric water potential, OM organic matter content, N–$NO_3^-$ nitrogen nitrate concentration, P extractable phosphorous.

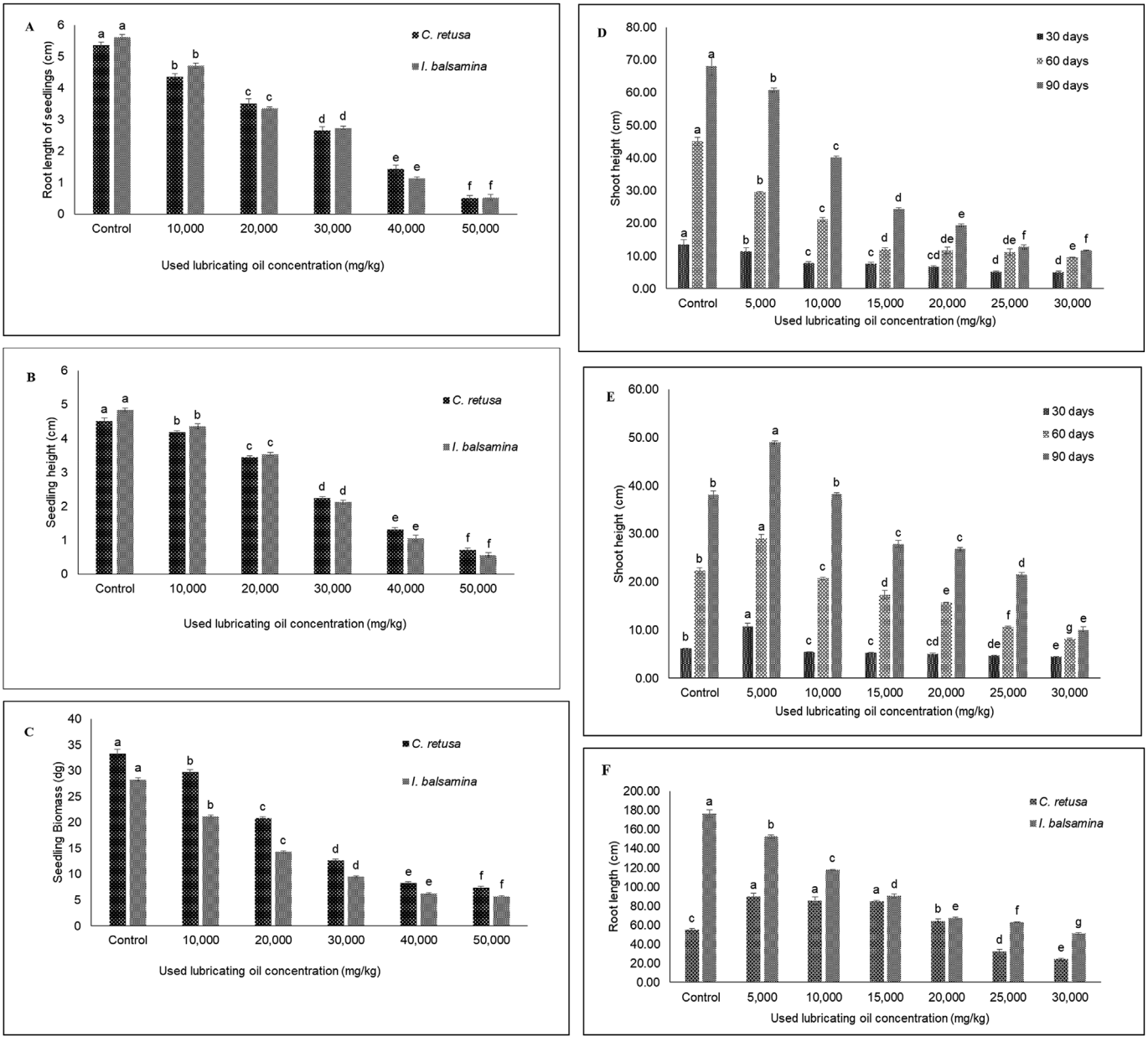


**Fig. 1.** Effects of used lubricating oil on (A) root length, (B) seedling length and (C) biomass of *Crotalaria retusa* L. and *Impatiens balsamina* L. after 120 h incubation period. (D) Shoot height of *Impatiens balsamina* L and (E) shoot height of. *Crotalaria retusa* L. after 30 days, 60 days and 90 days of exposure. (F) Root length of *Crotalaria retusa* L. and *Impatiens balsamina* L. after 90 days of exposure. Means that do not share a letter are significantly different ($p < 0.001$). Error bars represent the standard deviation of three independent measurements.

concentration (Fig. 1-A, 1-B and 1-C). The root lengths of *C. retusa* and *I. balsamina* seedlings in uncontaminated control were 5.36 cm and 5.62 cm, respectively compared with significantly smaller roots in the ULO contaminated soils. Mean seedling height and biomass of *I. balsamina* grown in control soil were 4.84 cm and 28.3 dg respectively and the same parameters measured in *C. retusa* were 4.52 cm and 33.3 dg respectively. At 3% w/w ULO, root length inhibitions was 48.88% and 49.47% of controls for *C. retusa* and *I. balsamina* respectively. For seedling height, values were 48.23% and 47.93% for *C. retusa* and *I. balsamina* respectively and for biomass inhibition were 49.43% and 48.83% for *C. retusa* and *I. balsamina* respectively. More than 50% inhibition in root length, seedling height and biomass were recorded in seedlings of both species that grew in soils contaminated with ≥3% w/w ULO.

### 3.3. Effect of used lubricating oil on plant growth performance and morphological characteristics of root system

For *I. balsamina,* after 90 d there was a significant contamination level dependent decrease in all the measured growth parameters, shoot height, (Fig. 1-D); root length, (Fig. 1-F); shoot and root fresh and dry biomass, (Table 2). At 1% w/w ULO, percentage inhibition values for shoot fresh biomass (46.77%), shoot dry biomass (44.45%), root fresh biomass (43.10%) and root dry biomass (38.20%) of *I. balsamina* were all less than 50%, whereas at higher contamination levels all these parameters were inhibited by more than 50%. In contrast, *C. retusa* grown in soil containing 0.5% w/w ULO showed growth enhancement compared to controls. The recorded percentage increases in shoot height, shoot fresh biomass, shoot dry biomass, root length, root fresh biomass and root dry biomass were 28.61%, 8.42%, 23.62%, 63.72%, 13.39% and 6.36% (Fig. 1-E and 1-F and Table 3), respectively.

**Table 2**
Effects of Used Lubricating Oil (ULO) on shoot system biomass (fresh and dry) and root system biomass (fresh and dry) of *Impatiens balsamina* L. after 90 days exposure.

| Treatment (mg/kg) | Shoot fresh biomass (g) | Shoot dry biomass (g) | Root fresh biomass (g) | Root dry biomass (g) |
|---|---|---|---|---|
| Control | 53.97 ± 0.52$^{a}$ | 15.14 ± 0.26$^{a}$ | 26.54 ± 0.94$^{a}$ | 5.47 ± 0.05$^{a}$ |
| 5000 | 42.56 ± 0.41$^{b}$ | 12.31 ± 0.29$^{b}$ | 22.10 ± 1.08$^{b}$ | 4.86 ± 0.04$^{b}$ |
| 10,000 | 28.73 ± 0.39$^{c}$ | 8.41 ± 0.07$^{c}$ | 15.10 ± 0.65$^{c}$ | 3.38 ± 0.01$^{c}$ |
| 15,000 | 12.29 ± 0.68$^{d}$ | 2.25 ± 0.04$^{d}$ | 5.34 ± 0.30$^{d}$ | 1.17 ± 0.02$^{d}$ |
| 20,000 | 7.95 ± 0.33$^{e}$ | 1.60 ± 0.01$^{e}$ | 4.21 ± 0.11$^{de}$ | 0.98 ± 0.01$^{e}$ |
| 25,000 | 5.17 ± 0.04$^{f}$ | 1.09 ± 0.004$^{f}$ | 3.06 ± 0.11$^{ef}$ | 0.77 ± 0.02$^{f}$ |
| 30,000 | 3.67 ± 0.03$^{g}$ | 0.06 ± 0.002$^{g}$ | 2.34 ± 0.22$^{f}$ | 0.67 ± 0.01$^{g}$ |

*Means that do not share a letter are significantly different. ($P < 0.001$).

However, at ULO levels above 0.5% w/w there was a significant contamination-level dependent decrease in all growth parameters, except for root length. For example, at 1% w/w ULO, values for shoot fresh biomass, shoot dry biomass, root fresh biomass and root dry biomass were 23.10%, 31.16%, 20.35% and 18.18%, respectively (Table 3). These values are lower than those recorded for I. *balsamina* at the same contamination level. In *C. retusa*, > 50% inhibition in the measured growth parameters was observed in plants grown in soils above 2% w/w ULO contamination.

When considering the root length of *C. retusa,* there was a significance increment up to 2% w/w ULO levels compared to control at the end of 90 d experimental time. At higher contamination levels of 2.5% and 3% w/w ULO, inhibition of root length was observed compared to the control. The shoot height of both *C. retusa* and *I. balsamina* (Fig. 1-D and 1-E), measured every 30 d time interval up to 90 d showed a time dependent increase at ULO treatment levels. Despite a contamination-level dependent inhibition in plant performance of both species over the course of the experiment there was no evidence of plant death. All plants of both species reached maturity 90 days after sowing seeds, even at the most extreme level of ULO contamination.

There was no significant change in root color of either tested species in any of the treatment at the end of the experiment. However, increases in lateral branching, presence of root hairs and root nodule formation were all apparent in the root systems of *C. retusa* plants grown in contaminated soil up to 2% ULO w/w compared with root systems of controls ((Fig. 2-A and 2-B).

Two way ANOVA revealed a significant effect ($p < 0.001$) of ULO contamination level, species and an interaction of species and contamination level on plant growth performance of both species. Further results clearly showed that the marked increase in growth of *I. balsamina* in low level of ULO contamination level up to 1% w/w ULO compared to the growth performance of *C. retusa*. But higher growth performance was recorded from *C. retusa* grown in contaminated soils with contamination levels higher than 1% ULO w/w compared to the growth performance of *I. balsamina*.

### 3.4. Tolerance index (Ti)

The calculated tolerance index of both *C. retusa* and *I. balsamina* (Fig. 2-C) showed contamination level dependent decrease. However, Ti of *C. retusa* at each tested contamination level was higher compared to that of *I. balsamina.* However, higher Ti was recorded at low contamination level for both species compared to that at high contamination levels.

### 3.5. Effect of ULO on chlorophyll content

Statistical analyses (one way ANOVA) revealed no significant differences ($p < 0.05$) in the chlorophyll *a*, *b* or total chlorophyll contents in plants of either C. *retusa* or *I. balsamina* between treatments. Mean concentrations of total chlorophyll of *C. retusa* plants (1.63 mg/g) were significantly higher than those of *I. balsamina* plants (0.88 mg/g). The results obtained from two way ANOVA showed the absence of significant ($p < 0.05$) interactive effect of species and contamination level on chlorophyll content (Fig. 3-A, 3-B and 3-C).

### 3.6. Root anatomical study

No differences in root anatomical features, including cell size, shape, cellular arrangement and the thickness of different types of cell layers of tissues, were observed in plants of either *I. balsamina* or *C. retusa* amongst treatments.

### 3.7. Percentage oil biodegradation

Percentage oil biodegradation of soil taken from the rhizosphere of the tested species showed contamination-level dependent decreases (Fig. 4). In soil with a 1% w/w ULO contamination level percentage biodegradation was 51.7% and 46.3% for C. *retusa* and *I. balsamina*, respectively. At3% ULO, percentage biodegradation has dropped to 22.8% and 18.5% from soils taken from the rhizosphere of *C. retusa* and *I. balsamina*, respectively.

## 4. Discussion

Measuring seed germination and seedling growth can provide valuable information on the acute toxicity of contaminants to plants. The process of seed germination is a critical step in a plant's life cycle and its sensitivity to soil contaminants has been demonstrated in various studies (Banks and Schultz, 2005). Thus, the ability of seeds to germinate in contaminated soil is often used as a first step in screening the tolerance of a species to chemical pollutants (Gaskin, 2008). Therefore, the ability of *C. retusa* and *I. balsamina* seeds to germinate in soils contaminated with different levels of ULO was assessed and used as a preliminary screening for tolerance of the tested species. However, the resultant 100% germination of both species in all tested ULO-contaminated soils, including the highest contamination level of 5% w/w ULO, infers that ULO does not negatively impact on this process. These

**Table 3**
Effects of Used Lubricating Oil (ULO) on shoot system biomass (fresh and dry), root system biomass (fresh and dry) and root nodule weight (fresh) of *Crotalaria retusa* L. after 90 days exposure.

| Treatment (mg/kg) | Shoot fresh biomass (g) | Shoot dry biomass (g) | Root fresh weight biomass (g) | Root dry biomass (g) | Nodule fresh weight (g) |
|---|---|---|---|---|---|
| Control | 24.46 ± 0.48$^{b}$ | 3.98 ± 0.50$^{b}$ | 14.94 ± 0.67$^{b}$ | 2.20 ± 0.13$^{a}$ | 0.36 ± 0.04$^{a}$ |
| 5000 | 26.52 ± 0.77$^{a}$ | 5.42 ± 0.43a | 16.94 ± 0.24$^{a}$ | 2.33 ± 0.06$^{a}$ | 1.10 ± 0.02$^{b}$ |
| 10,000 | 18.81 ± 0.15$^{c}$ | 2.74 ± 0.12$^{c}$ | 11.90 ± 0.39$^{c}$ | 1.80 ± 0.13$^{b}$ | 1.01 ± 0.02$^{c}$ |
| 15,000 | 16.17 ± 0.73$^{d}$ | 1.87 ± 0.04$^{d}$ | 8.69 ± 0.20$^{d}$ | 1.55 ± 0.06$^{c}$ | 0.78 ± 0.02$^{d}$ |
| 20,000 | 15.02 ± 0.23$^{d}$ | 1.61 ± 0.01$^{d}$ | 7.83 ± 0.31$^{e}$ | 1.39 ± 0.04$^{c}$ | 0.50 ± 0.02$^{e}$ |
| 25,000 | 5.56 ± 0.67$^{e}$ | 0.81 ± 0.05$^{e}$ | 3.72 ± 0.07$^{f}$ | 0.89 ± 0.02$^{d}$ | 0.41 ± 0.02$^{f}$ |
| 30,000 | 3.9 ± 0.86$^{f}$ | 0.39 ± 0.01$^{e}$ | 1.51 ± 0.08$^{g}$ | 0.40 ± 0.02$^{e}$ | 0.34 ± 0.02$^{a}$ |

*Means that do not share a letter are significantly different.

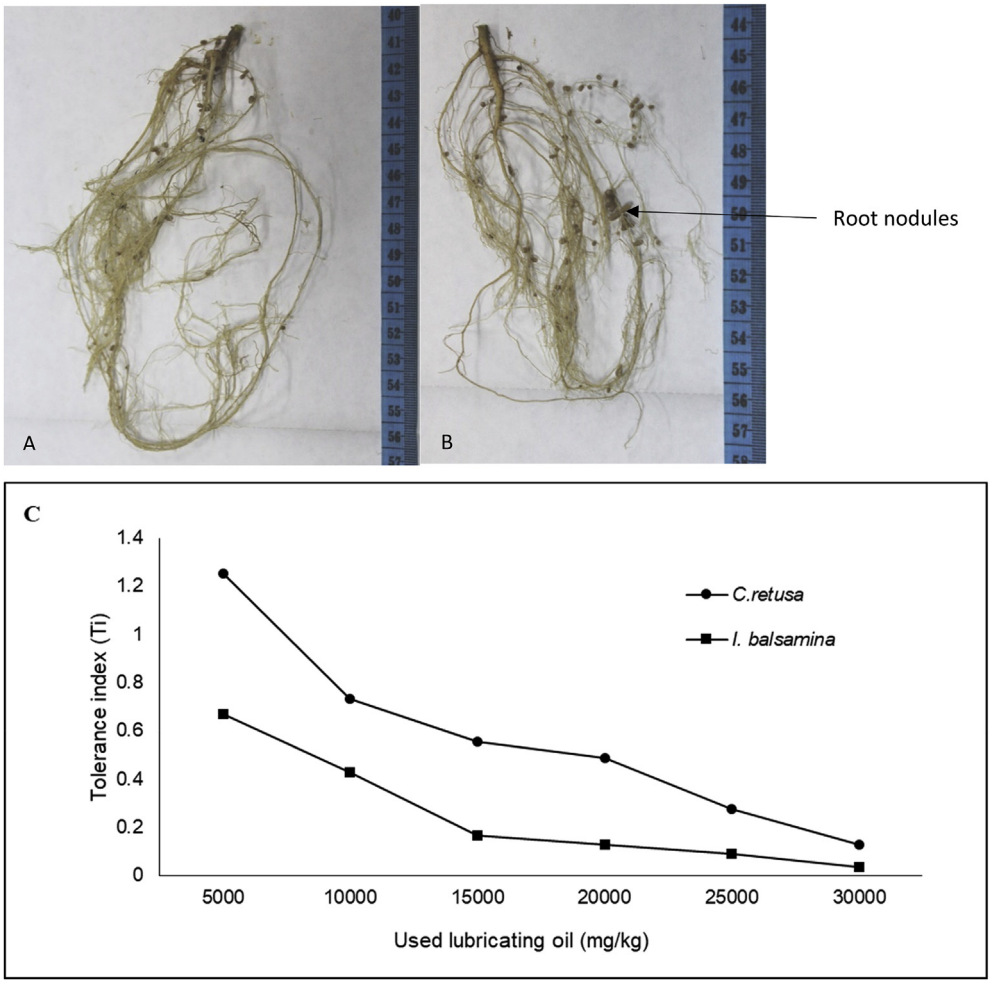


**Fig. 2.** Root morphology of *Crotalaria retusa* L. plants grown in (A) control soil (B) 0.5% w/w ULO. (C) Tolerance index of *Crotalaria retusa* L. and *Impatiens balsamina* L. in used lubricating oil contaminated soil.

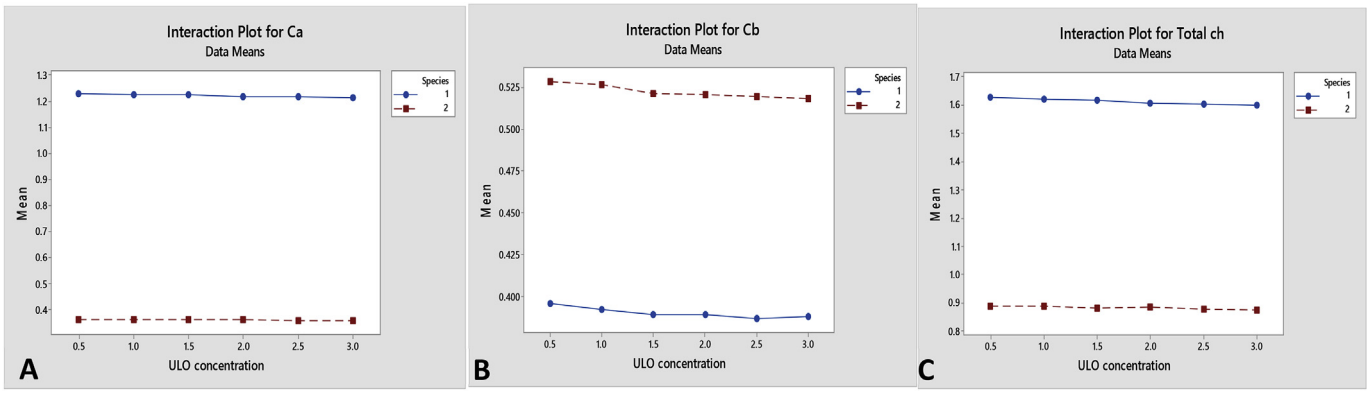


**Fig. 3.** Interaction plot for (A) Chlorophyll a; (B) Chlorophyll b; (C) Total chlorophyll content vs. used lubricating oil concentration (species 1 = *Crotalaria retusa*; species 2 = *Impatiens balsamina*, $p < 0.001$).

results are in agreement with those from several previous studies using soils contaminated with different types of hydrocarbons. For example, Ogbo et al. (2009) reported 100% seed germination of *Paspalum scrobiculatum* L. in soils containing 0–15% w/w crude oil. Similarly, Odokuma and Ubogu (2014) found no reduction in germination of *Phragmitis australies* seeds exposed to soil contaminated with 1–6% crude oil. In contrast, Bona et al. (2011) reported a significant reduction in seed germination of *Schinus terebinthifolius* in soil contaminated with diesel oil.

During seed germination, water is absorbed into the seed through the imbibition, where stored carbohydrates are hydrolyzed resulting in rapid growth of radical cells marking germination (Bewley and Black, 1994). In ULO-contaminated soils, oil may negatively influence the rate of seed germination by acting as a physical barrier that block or reduce access of water (Odokuma and Ubogu, 2014). Also, seed germination could be inhibited by hydrocarbon compounds or their metabolites that entered into the seed through imbibition due to their toxic influence or negative effect on hydrolysis (Masakorala et al., 2013a). Therefore, the

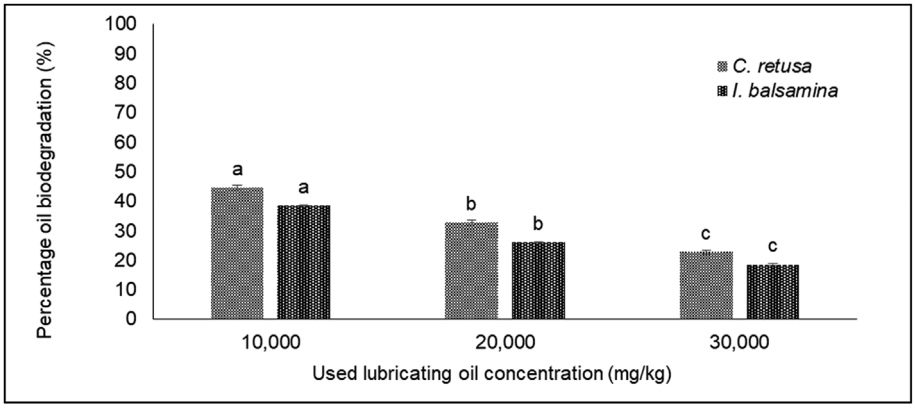


**Fig. 4.** Percentage oil biodegradation at the end the 90 d experimental period. Error bars represent the standard deviation of three independent measurements. Means that do not share a letter are significantly different ($p < 0.001$). Error bars represent the standard deviation of three independent measurements.

exhibited tolerance of *I. balsamina* and *C. retusa* seeds to ULO contaminated soil might be associated with species specific factors such as thickness and the chemistry of the seed coat. As highlighted by Adam and Duncan (2002), seed germination in soil contaminated with hydrocarbons is likely to be dependent on factors such as the type of hydrocarbon, the level of contamination and plant species. Thus, seed germination may not always be a reliable indicator of a species tolerance to soil contaminants.

Root growth parameters have relatively higher sensitivity to toxicants than percentage seed germination (Dorn et al., 1998). The root is the first organ to be fully exposed to the contaminants in soil and therefore the effects on roots are is important endpoints in evaluating plant susceptibility to soil contaminants. Root growth is a complex process involving number of biochemical and physiological processes (Masakorala et al., 2013a). The results of the present study indicated not only an inhibitory effect of ULO on early root elongation, but also on seedling height and biomass at contamination level above 3% w/w ULO. These findings are in agreement with previous studies on *Glycine max* L., *Capsicum annuum*, three pepper species, *Vigna radiata* L., *Vigna unguiculata* L. and *Senna alata* L. grown in hydrocarbon contaminated soils (Olayinka and Arinde, 2012; Njoku et al., 2008; Anoliefo and Vwioko, 1995; Obazuaye and Obueh, 2014; Akhter et al., 2018; Ugwu et al., 2019). The recorded inhibition in seedling growth might be due to combined effect of toxicity of hydrocarbon contaminants and unfavorable soil conditions developed in soil with ULO contamination.

Several studies have reported contamination-level dependent decreases in shoot length, root length, and shoot and root system biomass of plant species grown in hydrocarbon polluted soil (Anoliefo and Vwioko, 1995; Bona et al., 2011; Chupakhina and Maslennikov, 2004; Kuhn et al., 1998; Lum and Chikoye, 2018; Merkl et al., 2004; Odokuma and Ubogu, 2014; Ogbo et al., 2009; Omosun et al., 2008; Oyedeji et al., 2012; Proffitt et al., 1995; Sharifi et al. 2007). Therefore previous findings are in agreement with the results of the present study. However, typically effects of chemical are considered as negative to a test organism when a 50% reduction in the measured parameter results (Hodgson, 2004). Therefore, the recorded < 50% reduction in all measured growth parameters of *I. balsamina* and *C. retusa* grown in soils above 1% w/w ULO and 2% w/w ULO respectively compared to the plant growth in control soils infer that *I. balsamina* and *C. retusa* are able to tolerate up to 1% and 2% w/w ULO respectively.

As ULO forms a hydrophobic layer over the root, absorption of water and nutrients might be interrupted (Omosun et al., 2008). In addition, ULO contamination can result in anaerobic conditions in the surrounding environment and as a consequence phytotoxic compounds such as $H_2S$ are produced by microorganisms inhabiting the soil (DeJong, 1980). Also, as oil penetrates into soil pores this can result in poor aeration, immobilization of nutrients, changes in soil pH and soil fertility (Shukry et al., 2013). As Shirdam et al. (2008) highlighted, plant growth can be inhibited by toxic compounds especially hydrocarbons with low molecular weight. Therefore, the reduction in growth performance of *I. balsamina* and *C. retusa* in ULO contaminated soils. is likely due to the combined effects of the direct toxicity of the hydrocarbon together with the unfavorable soil conditions that have developed.

Merkl et al. (2005) has reported an enhancement of growth of some plant species in soil with low level of petroleum contamination. Some plant species tend to synthesize plant growth-regulating substances as a response to the stress condition (Baker, 1970). Since *C. retusa* is a leguminous plant belongs to the family Fabaceae, plants are able to fix atmospheric nitrogen and restore the nitrogen pool in soil. Thus, these plants have a competitive advantage over other plants and possess a wide range of adaptations to tolerate harsh conditions. Also at low levels of contamination, diverse groups of hydrocarbon degrading microorganisms may enhance the plant growth by providing nutrients through the degradation of contaminants (Chaudhry et al., 2005; Macek et al., 2000; Martin et al., 2014). Microorganisms inhabited in the rhizosphere may decrease the production of stress hormones (Dams et al., 2007) and may increase the production of growth promoting substances at the low contamination levels. Thus the recorded growth enhancement in *C. retusa* in soil contaminated with 0.5% w/w ULO might be a result of the combined effect of the above mentioned reasons. Further investigations are necessary to establish the mechanisms involved.

The time-dependent increase in shoot growth of both *C. retusa* and *I. balsamina* grown in ULO contaminated soils reflects the overall growth enhancement. The colonization of microorganisms in the rhizospheres of *C. retusa* and *I. balsamina* with the potential to degrade ULO and produce growth promoting substances could in part be responsible for this. Further, the influence of the rhizospheric effect (RE) is strengthened by the synergistic effects of microorganisms and plant roots (Nie et al., 2010). Therefore, the observed time dependent increase in growth might be due to the time dependent reduction in toxicity levels as a result of enhancement of biodegradation of ULO with the increase in ULO degrading microbial population size in time dependent manner. Also time dependent improvement in soil physico-chemical properties parallel to the toxicity reduction might be contributing to the recorded time dependent growth enhancement.

Exposure to hydrocarbon contaminated soil can also result in changes to morphological characteristics of roots systems (Reynoso-Cuevas et al. 2008). Therefore, careful observations were made on root systems of both species grown in ULO contaminated soils after 90 d of exposure. Plants with a broader root systems are able to take up larger quantities of water and nutrients (Hutchinson et al., 2001). The population density of microorganisms in the rhizosphere is dependent on

roots which offer substrate for growth and metabolism of microbes (Hinsinger et al., 2005). Hydrocarbon biodegradation can also be stimulated by the physical effects of plant roots such as soil aeration and the provision of sites for microbial attachment (Martin et al., 2014). Therefore, the observed increases in lateral branching of roots and the presence of root hairs in *C. retusa* be considered as an adaptive strategy for growth in soil containing up to 2% w/w ULO and can help explain the relative differences in tolerance between *C. retusa* and *I. balsamina*.

In a previous study, Agamuthu et al. (2010) observed death of *Jatropha curcas* plants grown in 2.5% w/w ULO contaminated soil. However, in the present study, plants of both species survived in all treatments. Thus, we can infer from the growth performances of *I. balsamina* and *C. retusa* that both species have a greater tolerance to grow in ULO contaminated soil. *C. retusa* belongs to family fabaceae. Hall et al. (2011) has reported that members of family fabaceae as a well-adapted group of plant to grow on petroleum contaminated soil. Therefore, the apparently higher tolerance of *C. retusa* compared to *I. balsamina* might be due to nitrogen fixation and other intrinsic properties of *C. retusa*.

According to Bellout et al. (2016), there was a difference in cellular arrangement of roots of *Pisum sativum* L. grown in hydrocarbon contaminated soil. Plant may tend to produce more intercellular spaces as a response to stress posed by contaminated soil (Pezeshki et al., 2000). Also some plant species may response to water stress by increasing root diameter. When considering the root anatomical structure of both species at the age of 90 days, there were no visible differences in cell size, shape, cellular arrangement, thickness of different types of cell layers of tissues in cross section of both *I. balsamina* and *C. retusa* grown in soil contaminated with ULO compared to control plants grown in uncontaminated soil. Thus results infer that there is no stressful condition in ULO contaminated soil by the age of 90 days of both species.

Chlorophyll act as a stress biomarker in plants (More and Chaubal, 2017). Thus, chlorophyll content is considered as an important parameter to determine whether plants are in physiological stress. Previous studies have shown inhibition in chlorophyll content with increasing total petroleum hydrocarbon contamination levels (Masakorala et al., 2013b). However, as significant reductions ($P < 0.05$) in the measured chlorophyll contents at the age of 90 d of tested plants grown even at the highest test ULO contamination level (3% w/w) were not observed. Thus the results infer that both *I. balsamina* and *C. retusa* were not under stress condition at the age of 90 d or at the time plant enter into full mature stage in their life cycle. This might be due to combined effect of time dependent reduction in ULO through the biodegradation in the rhizosphere, time dependent improvement in soil physico-chemical parameters with reduction of ULO. Therefore, the measured chlorophyll contents further highlight the tolerance of *I. balsamina* and *C. retusa* to grow on ULO contaminated soil.

Previous studies have reported that host plants have an ability to maintain a rhizospheric microbial community with selective reactions towards contaminants by secreting specific root exudates (Berendsen et al., 2012). Bio-surfactants produced by plant roots and bacteria inhabiting in the rhizosphere may increase the bioavailability of organic pollutants (Truu et al., 2015). The expression and abundance of degrading genes in microorganisms are enhanced by the presence of particular organic compounds having structural analogy with different types of petroleum hydrocarbons in the rhizosphere (Hussain et al., 2018). Furthermore, hydrocarbon degradation in soil is increased by root exudates through making a favorable environment with good aeration and enhancing root growth and microbial activity (Hajabbasi, 2016). Thus, the higher percentage biodegradation of hydrocarbons in the rhizosphere of *C. retusa* than of *I. balsamina* might be due to species specific differences in rhizospheric effect which may have contributed higher tolerance of *C. retusa* compared to *I. balsamina* to grow in ULO contaminated soils. The measured time dependent increases in percentage biodegradation of ULO might be due to the time depended increases in root growth in ULO contaminated soil. Further, results infer interrelation between time dependent enhancement in growth performance and degradation of hydrocarbons which results gradual elimination of stress condition posed by ULO contaminants.

## 5. Conclusion

Seeds of both *I. balsamina* and *C. retusa* species were able to germinate in ULO contaminated soil even at contamination levels of 5% w/w ULO due to species-specific tolerance of seeds. Growth performance of *C. retusa* enhanced by low ULO contamination level such as 0.5% w/w. Significant reductions in measured growth parameters of *I. balsamina* and *C. retusa* were evident only at contamination levels greater than 1% w/w ULO and 2% w/w ULO, respectively. Of the two species investigated, the calculated tolerance indices (based on dry biomass) indicate that *C. retusa* has a greater ability to grow in ULO contaminated soil than *I. balsamina*. Lateral branching and increased density of root hairs in both species and increases in root nodule formation in *C. retusa*, together with efficient rhizodegradation of ULO are all strategies for overcoming the toxic effects or unfavorable conditions of ULO contaminated soils. The overall results indicate the greater potential for using *C. retusa* rather than *I. balsamina* in any future phytoremediation programs to deal with ULO contaminated soil. Our findings provide valuable information for planning phytoremediation strategies for ULO contaminated soil.

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