VARIETAL DIFFERENCES IN *ZINNIA HYBRIDA* FOR REMEDIATION IN OIL-CONTAMINATED SOIL

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Abstract

In this study our goal was to select the variety of Z. hybrida that shows the best growth in oil-contaminated soils and the highest remediation of contaminated soil. We chose 'Profusion White', 'Profusion Cherry' and 'Profusion Orange' in Z. hybrida cultivars and cultivated the plants under 18,597 mg diesel/Kg soil condition. The dry weights of shoot and root of 'Profusion White' were more than 'Profusion Cherry' and 'Profusion Orange'. Soil total petroleum hydrocarbons (TPH) concentration decreased in all three varieties throughout the study period and no significant differences could be observed among the varieties. Dead cells of plant roots and rotten roots after plant death are nutrient sources for microorganism in soil. We propose that 'Profusion White', whose root growth was the greatest among the three varieties, is the best suitable variety for the remediation of oil-contaminated soil under the initial soil TPH concentration of 20,000 mg/kg.

Key words: *oil-contaminated soil, phytoremediation, varietal differences, Zinnia hybrid*

1. INTRODUCTION

Petroleum is an essential material for daily life and is used for various purposes, including lubrication of machines and as a fuel for cars and heaters. However, its wide use has led to contamination of soils by oil in many places; however, this issue has been researched to a very limited extent until now (Huang et al. 2005). Soil and groundwater pollution by oil have a negative effect on human health and the environment (Peña-Castro et al. 2006; Phillips et al. 2006; Dowling and Doty 2009), and may also cause decreases in land values (Hall et al. 2003). Therefore, in 2006, the Ministry of the Environment in Japan issued specific guidelines against oil pollution (The Geo-Environmental Protection Center) and established methods to counter soil contamination by oil.

Soil contamination by oil is often found in vacated gas stations and factories in Japan. It is treated by various processes, including excavation removal, thermal treatment, and liquid matter extraction. While such processes effectively remove the oil in a short period of time, they are problematic because they are expensive (requiring huge energy inputs, manpower, and large-scale equipment). In addition, these processes place major stress on the environment and alter the soil quality (Zhou and Song 2004; Kaimi et al. 2007). Low-cost and environmentally friendly removal processes, such as those using plants (phytoremediation), have been assessed in recent years (Anderson et al. 1993). Phytoremediation is a method for reducing or inhibiting the flow of contaminants in soils, bottom mud, or groundwater, using plants.

Until now, most studies on phytoremediation of oil-contaminated soils have used herbaceous plants including grasses (Lin and Mendelssohn 1998; Nedunuri et al. 2000; Merkl et al. 2005; Kaimi et al. 2006, 2007; Gaskin et al. 2010; Afzal et al. 2012) and legumes (Merkl et al. 2005; Schwab et al. 2006). Grasses such as Italian ryegrass (*Lolium multiflorum* L.) and legumes such as alfalfa (*Medicago sativa*) are capable of increasing the hydrocarbon decomposition activity of soil microorganisms, thereby decreasing the concentrations of TPH in the soil. The root system of grasses can grow remarkably well in contaminated soils, promoting the development of a rhizosphere with organisms that can break down the oil (Tang et al. 2010). Legumes can grow without adverse effects in contaminated soil due to their symbiosis with nitrogen fixing bacteria (Kelner et al 1997; Tang et al 2010). Other advantages in using

herbaceous plants for phytoremediation are that they can be used as pasture, they can be sown at low cost, and their management is easy.

However, unlike in other oil-producing countries, the contaminated soils in Japan are often found and treated at locations adjacent to urban areas. In these areas, the use of ornamental flowering plants is desirable. The advantages of using ornamental flowering plants include beautification of the environment with the addition of some possible therapeutic effect, as well as the prevention of pollutants from entering the food chain. Zhang et al. (2010) grew *Impatiens balsamina* in oil-contaminated soil for 120 days and found that it promoted the decomposition of persistent organic petroleum hydrocarbons such as resin and asphaltene. In another study, Peng et al. (2009) grew *Mirabilis jalapa* for 127 days in soil contaminated by less than 10,000 mg/kg TPH and found that the TPH concentration decreased significantly faster in the test plot with plants than in the plot without plants, with the growth rate similar to that of plants grown in non-contaminated soil. Liu et al. (2012) studied phytoremediation of oil-contaminated soil using 9 ornamental plant species, 4 grass species, and one legume. They reported high TPH degradation rates by purple coneflower (*Echinacea purpurea* in the family Asteraceae) and tall fescue (*Festuca arundinacea* in the family Gramineae). In our previous study we compared 33 ornamental plant species grown under Japanese environmental conditions and demonstrated that species belonging to the genus *Zinnia* were the most effective for the phytoremediation of oil-contaminated soils. Among the four zinnia species that we tested (*Z. elegans, Z. angustifolia*, *Z. haageana*, and *Z. hybrida*), we found that *Z. hybrida* is best suited for high remediation of contaminated soil (Ozawa et al. 2014). Therefore, although studies of the use of ornamental flowering plants in phytoremediation are rare, the results are encouraging. Our objective in this study was to select the variety of *Z. hybrida* that shows the best growth in oil-contaminated soils and the highest remediation of contaminated soil.

2. MATERIALS AND METHODS

2.1. Plant Materials

Seeds of *Z. hybrida* cultivars ‘Profusion White’, ‘Profusion Cherry’ and ‘Profusion Orange’ were purchased from Sakata Seed Corporation (Yokohama, Japan) since other varieties of *Z. hybrid* were raised based on these three varieties. *Z. hybrid* is an intraspecific hybrid between *Z. angustifolia* and *Z. elegans*.

2.2. Preparation of Oil-contaminated Soil

Oil-contaminated soil was prepared by mixing 4% diesel oil by weight with soil that had been air-dried in a greenhouse to less than 1% water content for 2 weeks according to the method of Kaimi et al. (2007). Commercial Kanto-loamy soil was used. The oil was mixed evenly into the soil by gradually spraying it with a pump sprayer while the soil was stirring in a soil mixer. The contaminated soil was stirred once every 2 days in a greenhouse for 10 days, and then the remaining oil particles were volatilized to prevent effects on early plant growth by low molecular weight hydrocarbons (Kaimi et al. 2006). The initial TPH concentration of the contaminated soil after volatilization was 18,597 ± 409 mg diesel/kg soil.

2.3. Treatments

The experiment was conducted in the greenhouse at Meiji University. Two hundred g of diesel oil-contaminated soil was provided in the pots (internal diameter 10 cm, height 8.5 cm). Eight seeds were planted in each pot and three individuals were thinned out after emergence. The pots were separated into 3 groups for treatment: ‘Profusion White’ (White), ‘Profusion Cherry’ (Cherry), and ‘Profusion Orange’ (Orange). Every 2 days the pots were irrigated with 20 mL of water. The quantity was just enough and no water ran out of the pots. Once a week, 20 mL of HYPONeX diluted 500-fold in tap water ($N:P_2O_5:K_2O$=6:10:5; HYPONeX Japan Corp., Ltd., Osaka, Japan) was provided instead of water.

At 20, 40 and 60 days after sowing (DAS), four pots of each treatment were selected randomly and the plants and soil were used for the following measurements: plant height, longest root length, shoot and root dry weights, and soil TPH concentration.

2.4. Analysis

2.4.1. Plant Height and Dry Matter Weights of Plants

Plant height was measured three plants per each pot. Roots were taken from each pot and washed carefully. The dry weights of shoot and root of the plants were measured after oven drying for 3 days at 80°C.

2.4.2. TPH Concentration in Soil

The TPH concentration in the soil was measured using a method developed by the Ministry of the Environment in Japan and issued in guidelines for preventing oil pollution in 2006 (The Geo-Environmental Protection Center 2006). After removing roots from each pot, the soil was mixed and about 30 g of soil was dried at 30ºC for 4 days. Then, 5 g of soil were placed in a 50 mL conical flask, 15 mL carbon disulphide was added, and the mixture was shaken for 30 min. The supernatant was separated from the residue 2 h after shaking. Again, 15 mL carbon disulphide was added to the residue, the mixture was shaken for 30 min, and the supernatant was separated 1 h after shaking. This process was repeated one more time and the three supernatants were combined. The recovered supernatants were diluted to 50 mL and filtered through a membrane filter (pore diameter, 0.45 µm). Then, 1 µL of the filtrate was injected into a gas chromatograph hydrogen flame ionization detector (GC-FID, GC-2010, Shimadzu Corp., Ltd., Kyoto, Japan) for analysis. The analysis was conducted using a GC equipped with an Intercap 1MS capillary column (liquid phase, 5% phenylmethyl silicon; i.d., 30 m × 0.32 mm; and film thickness, 0.25µm; GL Science Inc., Japan) and FID. For the analysis, the injection temperature and detector temperature were set at 320°C. The heating program was set to maintain 35°C for 5 min, and was then increased to 320°C at a rate of 10°C min^{-1}. Helium was used as the carrier gas in a splitless mode. The analysis was repeated three times for each sample.

2.5. Statistical Analysis

Data were collected as the means of 4 pots. Data analysis for plant growth and TPH concentration was carried out using statistical software (Excel Statistics 2012 for Windows, Social Survey Research Information Co., Ltd., Tokyo, Japan). Significant differences in plant height, longest root length, shoot and root dry weights, and TPH concentrations among three varieties of *Z. hybrida* were compared using one-way analysis of variance followed by Fisher's LSD Test for mean comparison at the 5% level of probability.

3. RESULTS

3.1. Growth of the three Z. hybrida varieties in comtaminated soil

Changes in plant growth of the three *Z. hybrida* varieties are shown in Tables 1-4. No differences were observed in plant height among different Zinnia varieties. The plant height increased in all three varieties throughout the study period and no differences were observed in plant height at the specified DAS. The shoot dry weight of the "White" variety was significantly higher than the others. No differences existed in shoot dry weight of the three varieties at 20 DAS and the 'White' shoot was the heaviest at 40 and 60 DASs.

The "White" variety had the longest root compared to other varieties. No differences existed in the longest root length of the three varieties at 20 and 40 DASs, and the root length of 'White' was the most at 60 DAS. The root dry weight was significantly higher in the "White" variety compared to others. The root dry weight of 'White' was more than that of 'Cherry' and 'Orange' throughout the study period. This result shows that the root growth of 'White' is the most superior among the three varieties in oil-contaminated soil.

Table 1 Changes in plant height of the three *Z. hybrida* varieties.

Plant height (cm±SE)			
Treatment	20 DAS	40 DAS	60 DAS
White	2.84 ± 0.09a[z]	6.32 ± 0.06a	12.42 ± 1.10a
Cherry	2.71 ± 0.11a	6.48 ± 0.16a	13.12 ± 1.18a
Orange	3.13 ± 0.08a	6.53 ± 0.11a	12.82 ± 1.16a

[z] Different letters denote significantly different values at the 5% level of probability based on Fisher's LSD among varieties for the same sampling day (n=12).

Table 2 Changes in shoot dry weight of the three *Z. hybrida* varieties.

Shoot dry weight (mg/pot±SE)			
Treatment	20 DAS	40 DAS	60 DAS
White	16.35 ± 0.44a[z]	29.83 ± 1.93a	47.25 ± 3.34a
Cherry	13.33 ± 0.74a	21.18 ± 2.04b	37.33 ± 3.38b
Orange	15.30 ± 0.96a	24.90 ± 2.11b	41.18 ± 1.73b

[z] Different letters denote significantly different values at the 5% level of probability based on Fisher's LSD among varieties for the same sampling day (n=4).

Table 3 Changes in the longest root length of the three *Z. hybrida* varieties.

Longest root length (cm±SE)			
Treatment	20 DAS	40 DAS	60 DAS
White	6.22 ± 0.94a[z]	6.44 ± 0.29a	8.68 ± 0.67a
Cherry	5.07 ± 0.61a	5.11 ± 0.23a	5.40 ± 0.31b
Orange	4.83 ± 0.83a	6.03 ± 0.50a	7.08 ± 0.60ab

[z] Different letters denote significantly different values at the 5% level of probability based on Fisher's LSD among varieties for the same sampling day (n=12).

Table 4 Changes in root dry weight of the three *Z. hybrida* varieties.

Root dry weight (mg/pot±SE)			
Treatment	20 DAS	40 DAS	60 DAS
White	11.05 ± 1.72a[z]	21.23 ± 3.52a	42.58 ± 3.89a
Cherry	7.08 ± 0.84b	15.43 ± 1.19b	26.25 ± 1.57b
Orange	7.30 ± 0.90b	14.80 ± 1.27b	31.45 ± 3.03b

[z] Different letters denote significantly different values at the 5% level of probability based on Fisher's LSD among varieties for the same sampling day (n=4).

3.2. TPH concentration of the three Z. hybrida varieties in contaminated soil

Changes in soil TPH concentration of the three *Z. hybrida* varieties are shown in Table 5. Soil TPH concentration decreased in all three varieties with increasing DAS and no differences could be observed in soil TPH concentration among the three varieties at each DAS. The reduction rates of soil TPH concentration at 60 DAS from the initial concentration were about 35% in all three cultivars.

Table 5 Changes in soil TPH concentration of the three *Z. hybrida* varieties.

Soil TPH concentration (mg diesel/kg soil±SE)				
Treatment	0 DAS	20 DAS	40DAS	60 DAS
White	18597 ± 409	15961 ± 432a[z]	14741 ± 295a	11769 ± 497a
Cherry	18597 ± 409	16046 ± 504a	14922 ± 204a	11638 ± 814a
Orange	18597 ± 409	15737 ± 181a	14125 ± 180a	11977 ± 670a

[z] Different letters denote significantly different values at the 5% level of probability based on Fisher's LSD among varieties for the same sampling day (n=12).

4. DISCUSSION

'Profusion White' used in this study is reared by crossing between two different Zinnia lines (*Z. angustifolia*×amphidiploid of *Z. elegans*). 'Profusion Orange' and 'Profusion Cherry' are screened by crossing of 'Purity' (*Z. linearis*×*Z. elegans*) onto 'Thumbelia mini-salmon' (*Z. linearis*×*Z. elegans*). The dry weights of shoot and root of 'Profusion White' were more than 'Profusion Cherry' and 'Profusion Orange' in contaminated-soil indicating that this variety thrived better than the other two. Soil TPH concentration decreased in all three varieties throughout the study period and no varietal differences existed at each sampling day.

Few studies have investigated the varietal differences of plants on phytoremediation. Remediation differences among plant species and plant genotypes are known to influence the degradation of petroleum oil by microbes in the rhizosphere (Chiapusio et al. 2007). Banks et al. (2003) indicated that the growth rate of four *Sorghum* (*S. bicolor* L.) genotypes differed when cultivated in oil-contaminated soil, despite there being no significant difference in TPH degradation ability among the varieties. In addition, metal concentrations in plants vary with plant species and genotypes (Grant et al. 2008; Liu et al. 2011). Thus, the correlation does not often exist between the growth of plants and the remediation in oil-contaminated soil among varieties of the same plant genotypes. In our previous study we did not observe any correlation between the root dry weight and the reduction rate of soil TPH concentration among the four zinnia species that we tested (*Z. elegans*, *Z. angustifolia*, *Z. haageana*, and *Z. hybrida*) (Ozawa et al. 2014). On the other hand, deep growth-rooted plants that have plenty of rootlets were useful for phytoremediation (Basumatary et al. 2012). Roots play the following roles in the phytostimulation of oil-contaminated soil: (1) dying cells of the roots activate microorganisms by becoming the source of their nutrients; (2) substances excreted from plant roots (organic acid, amino acids, sugars, etc.) become a source of nutrients and growth-stimulating substances for microorganisms; (3) roots create pore space in soil and improve permeability and aeration; (4) roots destroy soil particles and make the contact between microorganisms and non-water-soluble pollutants adsorbed onto the soil pores easier; and (5) rotten plant roots become a source of nutrients for microorganisms (Cunningham et al. 1996; Banks et al. 2003; Singer et al. 2003). Root surfaces also become the location of growth for microorganisms because of the abundance of nutrients of the roots exude, such as carboxylic acid, amino acid, protein, and sugars (Kaimi et al. 2006). Exudates and enzymes from roots have greater effects on a large part of microbial community and rhizosphere (Karthikeyan and Kulakow 2003). In fact, exudates are known to increase microbial activity and plant dry weight when high quality organic compounds are added to the soil (Walton et al. 1994).

Although this study was conducted only for 60 days after sowing, it is expected to remediate for longer than 60 days after sowing. Dead cells of plant roots and rotten roots after plant death are nutrient sources for microorganisms. Moreover, it is reported that rhizosphere microbiota change by soil, plant cultivation, and different cultivars of plants (Hirano et al. 1979). Thus, it is suggested that different varieties on the same species were different from exudates from plant root and then have effect on rhizosphere microbiota.

No differences existed in the reduction rate of the soil TPH concentration among the three *Z. hybrida* varieties in the oil-contaminated soil. However, the growth of both the shoot and root was the most superior in 'Profusion White'. Since oil-contaminated soil is expected to be continued to remediate after plant death, we propose that 'Profusion White', with its prolific root growth was the greatest among the three varieties and is the best suited variety for the remediation of oil-contaminated soil under the initial soil TPH concentration of 20,000 mg diesel/kg soil.

5. CONCLUSION

In this study the variety of *Z. hybrida* that shows the best growth in oil-contaminated soils and the highest remediation of contaminated soil was selected. The dry weights of shoot and root of 'Profusion White' was the maximum among the three varieties of Zinnia tested. No varietal differences existed in soil TPH concentration. Dead cells of plant roots and rotten roots after plant death are nutrient sources for microorganisms in soil. Because of the superior growth of the roots, we propose that 'Profusion White'is the best suitable variety for the remediation of oil- contaminated soil under the initial soil TPH concentration of 20,000 mg diesel/kg soil.

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