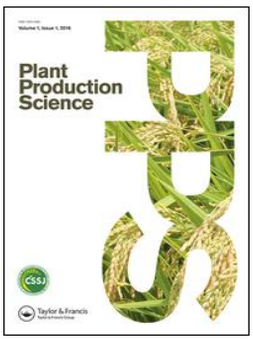

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Screening of Twelve Plant Species for Phytoremediation of Petroleum Hydrocarbon-Contaminated Soil

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Abstract : Twelve plant species were screened for their phytoremediation ability for the cleanup of hydrocarbon-contaminated soil in Japanese environmental conditions. The plants were cultivated in a greenhouse for 141 days in 1/5000 a Wagner pots containing the experimental diesel-contaminated soil. During plant cultivation, the changes in the total petroleum hydrocarbon (TPH) concentration, soil dehydrogenase activity (DHA) and the number of aerobic bacteria were evaluated. The results of the screening experiment indicated that eight plant species (Italian ryegrass, sorghum, maize, alfalfa, Bermuda grass, rice, kudzu and beggar ticks) caused a more significant decrease in the TPH concentration in the planted diesel-contaminated soil than in the unplanted soil, and would be effective in the phytoremediation of petroleum hydrocarbon-contaminated soil in Japan. The TPH concentration was more closely related to the soil DHA than to the aerobic bacterial number. In this study we discussed the characteristics of the plants which are suitable for phytoremediation.

Key words : Diesel oil, Microbial activity, Phytoremediation, Root, Soil DHA.

With the development of human economic activity, petroleum products have widely contaminated soil. Petroleum hydrocarbon-contaminated soil can be treated by various physical and chemical engineering-based technologies such as thermal treatment, soil washing, gaseous or liquid matter extraction, solidification and stabilization or by using combinations of the above (Langbehn and Steinhart, 1995). However, these techniques are not only expensive but also involve high energy consumption. Therefore, there is a growing interest in the development of phytoremediation, which is environment-friendly, less expensive and uses less energy.

Therefore, many researches have studied the phytoremediation of organic contaminants such as polyaromatic hydrocarbons (PAHs) (Aprill and Sims, 1990; Siciliano et al., 2003), polychlorinated biphenyls (PCBs) (Donnelly et al., 1994) and hydrocarbons (Günther et al., 1996; Banks et al., 2003a; Kirk et al., 2005) by using different plant species in Europe and North America. Italian ryegrass, sorghum, maize and alfalfa are recognized as phytoremediators (Radwan et al., 1995; Wiltse et al., 1998; Pradhan et al., 1999; Châineau et al., 2000; Banks et al., 2003b; Parrish et al., 2004). Bermuda grass, sunflower, southern crabgrass and red clover are recognized as hydrocarbon-tolerant plants (Adam and Duncan, 1999; Olson and Fletcher, 2000). These studies suggested that grass species and Leguminosae could be suitable for phytoremediation of petroleum hydrocarbon-contaminated soil. Grasses have fibrous root systems with long roots that have a large surface area per unit volume of soil. Fibrous roots provide a larger surface than taproots for colonization by soil microorganisms (Anderson et al., 1993); they also allow a close interaction between the rhizosphere microbial community and the contaminant (Schwab and Banks, 1994). Leguminosae have a symbiotic relationship with nitrogen-fixing bacteria. This symbiotic relationship suggests that Leguminosae could grow well in petroleum-contaminated soil in which the C/N ratio tends to be high (Adam and Duncan, 2003).

However, few studies have been conducted in Japan, where soil contamination has become a civic problem. In order to develop a practical use for phytoremediation in Japan, it is important to select plant species that are suitable for use under Japanese environmental conditions.

In this study, we screened twelve plant species that grow under Japanese environmental conditions for their possible use in the phytoremediation of petroleum hydrocarbon-contaminated soil. Four species were selected as phytoremediators, and four species as hydrocarbon-tolerant plants. The remaining four species were rice and spinach, which are popular Japanese crops, and kudzu and beggar ticks, which are typical Japanese weeds. Kudzu occurs in the mountains and fields of Japan, and beggar ticks occur naturally beside water bodies in wetlands and by the road.

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Table 1. Twelve plant species used in this study.

Common name / habitat	Genus and Species	Notes[a]		Notes[b]
Italian ryegrass	*Lolium multiflorum* L.	F	P	(Parrish et al., 2004)
Sorghum	*Sorghum vulgae* Pers.	F	P	(Banks et al., 2003b)
Maize	*Zea mays* L.	F	P	(Radwan et al., 1995; Châineau et al., 2000)
Alfalfa	*Medicago sativa* L.	L	P	(Wiltse et al., 1998; Pradhan et al., 1999)
Bermuda grass	*Cynodon dactylon* (L.) Pers.	F	T	(Olson and Fletcher, 2000)
Sunflower	*Helianthus annu us* L.		T	(Olson and Fletcher, 2000)
Southern crabgrass	*Digitaria ciliaris* (Retz.) Koeler	F	T	(Olson and Fletcher, 2000)
Red clover	*Trifolium pratense* L.	L	T	(Adam and Duncan, 1999)
Rice	*Oryza sativa* L. cv. Natate-shinsenbon	F		
Spinach	*Spinacia oleracea* L. cv. Ohrai			
Kudzu / The mountains and fields throughout Japan.	*Pueraria lobata* (Willd.) Ohwi	L		
Beggar ticks / The water's edge, waste land and the roadside.	*Bidens frondosa* L.			

a: indicates potential for phytoremediation.
F: plants with a fibrous root system, L: plants belonging to Leguminosae.
b: indicates suitability for phytoremediation.
P: recognized phytoremediator, T: hydrocarbon tolerant.

Materials and Methods

1. Plant material and growth conditions

Table 1 shows the 12 plant species analyzed. Wild plant seeds of southern crabgrass and kudzu and wild seedlings of beggar ticks were sampled from the campus of Hiroshima Prefectural University, Japan (34°49´S and 132°58´W; altitude, 320 m). Seeds of the other nine species were obtained commercially.

The seedlings of the beggar ticks were transplanted, and the seeds of the other eleven species were sown in 1/5000 a Wagner pots. The plants were grown in a greenhouse at Hiroshima Prefectural University, Japan, from April 30, 2003 to September 17, 2003; no supplemental light was provided. The average temperature of the greenhouse was between 8°C and 28°C. The growing periods of plants were 126 to 141 days. A compound fertilizer ($N : P_2O_5 : K_2O$ = 10% : 8% : 9%) was mixed as basal manure into the soil. In addition, at an early growth stage, a 1/1000-strength commercial liquid fertilizer ($N : P_2O_5 : K_2O$ = 5% : 10% : 5%) was applied weekly. After germination, the seedlings of each plant species were thinned uniformly. Irrigation was adjusted daily to 80% of the pots' water-holding capacity to prevent run-out from the bottoms of the pots.

2. Experimental design

For each of the twelve plant species, three sets of pots were prepared with five replicates: planted pots, unplanted pots with irrigation (unplanted control) and unplanted pots without irrigation (no-irrigated control). The unplanted control pots received identical liquid fertilizer treatment as the planted pots. Soil samples were collected four times from the planted pots; however, the time of collection differed among the species because the plants were not at the same growth stage. Each plant was sampled at the seedling, flowering and mature stages. Soil was sampled at each growth stage at the initial stage (April 30) and at the end of the experiment (September 17).

The procedure for sampling the soil and the plants from the planted pots was as follows. After the measurement of plant height, the aboveground parts of the plants were cut off at the soil surface, dried at 60°C for 72 h and then weighed. The soil samples were gently crushed and shaken in a vat to carefully collect the roots. The soil that was firmly attached to the roots was then collected for the analysis of total petroleum hydrocarbon (TPH) concentration, soil dehydrogenase activity (DHA) and the number of aerobic bacteria. The total root length was measured after the roots were washed with water. For the unplanted and non-irrigated controls, the soils in each pot were mixed uniformly before analysis.

3. Measurements

(1) TPH concentration

The TPH concentration of the soil was measured according to the method published by the Ministry of the Environment of Japan in bulletin no. 64 (JIS K-0102-1993). The soil samples were first dried at room temperature. From a 1-g soil sample, petroleum hydrocarbons were extracted by an ultrasonic extraction method with CCl_4 as the solvent. The extractant was filtered through a Florisil column and then quantified using the Fourier transform infrared (FT-IR) method. The TPH concentration was revised to dry weight by water contents obtained from the loss of soil weight dried at 105°C.

(2) Soil DHA

The soil DHA was determined according to the method described by Hayano (1997). One gram of soil was exposed to 0.2 mL of 0.4% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in 1.0 ml of 0.25 M Tris buffer solution containing 50 μl of 1% glucose for 6 h at 30°C in a dark environment. The iodonitrotetrazolium formazan (INTF) that was formed by the enzyme reactions was extracted using 10 ml of methanol, shaken vigorously for 1 min and filtered. It was then measured spectrophotometrically at 485 nm.

(3) Number of aerobic bacteria

For reference to soil DHA, aerobic microbial numbers were counted at the initial and mature stages with five replications and at the other growth stages without replication.

In order to enumerate the aerobic bacteria, aqueous extracts of 1-g soil samples were serially diluted and plated on nutrient agar (DIFCO Co., catalog no. 213000). The plates were incubated at 30°C for 3 days prior to counting the colony-forming units (cfu) (Kato, 1997).

(4) Root length

The washed roots were stained by soaking in 0.1% methylene blue for 3 min. Then the roots were rinsed in water to remove any excess dyeing solution. They were spread on a transparent acryl case filled with water without any overlapping and then scanned using the Epson TWAIN PRO software. The resulting image was then analysed using specialized software (Kimura and Yamasaki, 2001) to measure the root length for each range of diameter.

4. Preparation of the experimental soil

In order to ensure the adsorption of diesel oil, the decomposed granite soil masado was sun dried in a greenhouse and mixed every 3 days until its water content was less than 1%. Diesel oil was gradually sprayed into the masado in a mechanical mixer in order to obtain a homogeneous soil/pollutant mixture. Diesel oil was added up to 2% (w/w), and the mixing was continued for an additional 5 min.

Table 2. Initial chemical properties of experimental soil.

Analysis	Value	Method
pH	7.6	1:2.5 soil/water slurry
TOC (%)	1.5	Flash combustion at 1000°C followed by thermic conductivity detection
Texture (%)	Sandy	
–Sand	86.2	Light dispersion
–Silt	9.7	Light dispersion
–Clay	4.1	Light dispersion
T-N (mg kg^{-1})	560	Kjeldahl method
T-P_2O_5 (mg kg^{-1})	630	Peroxide digest
Available P_2O_5 (mg kg^{-1})	340	Truog method
Water content (%)	6	Loss on ignition at 105°C
CEC (meq 100 g^{-1})	5.8	NH_4^+ saturation

The TPH concentration immediately after the addition of diesel was 20,700 ± 1,520 mg kg^{-1} (n = 5). The data showed that the recovery of diesel oil in the analysis process was almost 100%. In order to stabilize the diesel concentration, the masado-diesel mixture was further stirred at 4- to 5-day intervals for 3 weeks during solar drying. After 3 weeks, the TPH concentration was 9,800 ± 200 mg kg^{-1} (n = 5). In a preliminary experiment, we observed that after the TPH concentration had decreased by 50%, further TPH loss was negligible. Commercial leaf mold and perlite were subsequently added at a concentration of 20% by volume to improve the physical characteristics of the soil.

Table 2 summarizes the characteristics of the experimental soil. The soil texture was sandy, and it contained sufficient nitrogen and phosphorous for plant growth.

5. Statistical analysis

Data were tested for homogeneity of variance by using Bartlett's test. The mean values were compared using a one-way ANOVA followed by Bonferroni's multiple comparisons.

Results

Table 3 shows the mean values of plant height and aboveground-part dry weight at the mature stage. Since plant height of the spinach was 2-3 cm at one month after germination due to poor growth, the experiment was terminated at 1 month. As the sunflower and red clover plants died after the flowering stage, soil was not collected from the sunflower and red clover plants at the mature stage. The other plants grew well.

Table 3 lists the total root length in different plant

Table 3. Maximum plant height, aboveground dry weight per pot and total root length in different plant species grown in the contaminated soil.

Plant species	Plant height height (cm)	Dry weight (g pot^{-1})	Total root length (cm)		
			Seedling stage	Flowering stage	Mature stage
Italian ryegrass	52 d	18.6 c	1,083 b	2,648 e	18,525 c
Sorghum	132 a	56.9 b	488 b	9,006 d	12,684 c
Maize	137 a	83.2 a	4,232 a	20,992 b	27,657 c
Alfalfa	53 d	2.4 c	141 c	191 e	11,251 c
Bermuda grass	65 c	17.9 c	4,534 a	—	85,767 b
Sunflower*	43 —	1.3 —	913 b	1,093 e	—
Southern crabgrass	135 a	90.0 a	269 b	23,983 a	139,849 a
Red clover*	2 —	0.1 —	345 b	362 e	—
Rice	79 c	54.7 b	603 b	11,324 c	13,419 c
Spinach	—	—	—	—	—
Kudzu	116 b	3.7 c	314 b	—	16,377 c
Beggar ticks	63 c	5.5 c	—	883 e	14,010 c

Data are means (n = 5).
' __ ' indicates no data.
Maximum plant height and aboveground dry weight were measured at the mature stage in all plants, except for sunflower and red clover, which were examined at the flowering stage.
Means with different letters are significantly different at a 5% level of significance.

Table 4. TPH concentration in planted and unplanted soils at different growth stages.

Plant species	TPH (mg kg^{-1})			
	Initial value	Seedling stage	Flowering stage	Mature stage
Italian ryegrass	9,800	8,160 c	6,877 f	4,410 a
Sorghum	9,800	9,603 e	5,267 b	4,083 a
Maize	9,800	6,663 a	4,717 a	4,330 a
Alfalfa	9,800	7,463 b	8,230 h	3,730 a
Bermuda grass	9,800	8,733 d	—	3,640 a
Sunflower	9,800	7,860 c	6,150 d	—
Southern crabgrass	9,800	7,567 b	5,410 c	4,763 b
Red clover	9,800	7,743 c	7,410 g	—
Rice	9,800	8,343 c	5,810 c	3,367 a
Spinach	9,800	—	—	—
Kudzu	9,800	8,150 c	—	3,537 a
Beggar ticks	9,800	—	6,723 e	3,763 a
Unplanted	9,800	7,333 b	7,940 h	5,790 b
No-irrigation	9,800	—	—	10,800 c

Data are means (n = 5).
' __ ' indicates no data.
Means with different letters are significantly different at a 5% level of significance.

species. Italian ryegrass, sorghum, Bermuda grass and southern crabgrass have fibrous root systems. Southern crabgrass and Bermuda grass have longer roots than the other species ($P<0.05$). The root growth of the sunflower plants was very poor between the seedling and flowering stages. The root length of the beggar ticks and alfalfa demonstrated an increase of more than 10-fold between the flowering and mature stages.

At the mature stage, the TPH concentrations of the soils on which Italian ryegrass, sorghum, maize, alfalfa, Bermuda grass, rice, kudzu and beggar ticks were planted, had significantly lower than that of the unplanted control at a 5% level of significance (Table 4). On the other hand, the TPH concentration of the soil planted with southern crabgrass was not significantly different from that of the unplanted control.

The TPH concentration in non-irrigated control did not decrease during the experimental period, while TPH in irrigated unplanted control was decreased by the evaporation loss. However, the decrease in unplanted control was less than that in planted soil.

The value of the soil DHA increased from the initial stage to the mature stage in all the soil sets-the planted soil, unplanted control and non-irrigated control (Table 5). At the mature stage, the DHA in all the planted soils was significantly higher than that in the unplanted control at a 5% level of significance. The most significantly higher value was observed in the soil planted with beggar ticks, followed by those

Table 5. Soil DHA in planted and unplanted soils at different growth stages.

Plant species	DHA (mg formazan 100g soil dw^{-1} $6hr^{-1}$)			
	Initial value	Seedling stage	Flowering stage	Mature stage
Italian ryegrass	2.8	6.2 c	18.1 c	23.0 d
Sorghum	2.8	11.4 a	19.8 c	28.1 c
Maize	2.8	11.4 a	22.5 b	23.2 d
Alfalfa	2.8	6.9 c	11.4 e	36.9 b
Bermuda grass	2.8	7.1 c	—	36.3 b
Sunflower	2.8	12.1 a	17.2 d	—
Southern crabgrass	2.8	5.1 d	20.0 c	21.4 e
Red clover	2.8	7.9 b	8.2 e	—
Rice	2.8	7.5 c	21.4 c	23.7 d
Spinach	2.8	—	—	—
Kudzu	2.8	6.5 c	—	36.4 b
Beggar ticks	2.8	—	28.4 a	41.8 a
Unplanted	2.8	10.8 a	12.6 e	14.6 f
No-irrigation	2.8	—	—	5.8 g

Data are means (n = 5).
' __ ' indicates no data.
Means with different letters are significantly different at a 5% level of significance.

with alfalfa, Bermuda grass and kudzu. The third significantly higher value was observed in the soil with sorghum, and the fourth, with Italian ryegrass and rice. The DHA value in the soil with southern crabgrass was the lowest.

The number of aerobic bacteria in the soil planted with southern crabgrass, rice and beggar ticks decreased from the flowering stage to the mature stage (Table 6). At maturity, the value in the sorghum-planted soil was significantly higher than that in the soil with the other plants and that in the unplanted control at a 5% level of significance. There was no difference in the number of aerobic bacteria between planted soil and unplanted control, except for sorghum.

In this study, the soil DHA was measured based on the colour formed by INT (Trevors, 1984) by a reduction reaction that indicates the total activity of not only aerobic bacteria but also of various other microorganisms. On the other hand, the microbial number includes only the microorganisms that can be cultured on aerobic agar plates. It is considered that the soil DHA could be a general and simple index of the degradation activity of rhizosphere microorganisms in diesel-contaminated soil. Fig. 1 shows the correlation between the soil DHA and the TPH concentrations in the planted and unplanted soils. TPH concentration was strongly related to soil DHA. In addition, the degree of correlation between soil DHA and TPH concentration differ between plant species. On the other hand, the microbial count was not correlated with the TPH concentration (data not shown).

Discussion

1. Rhizosphere effect

TPH concentration in soil planted with sorghum, Italian ryegrass, Bermuda grass, rice, maize, beggar ticks, alfalfa and kudzu was significantly lower than in the unplanted soil. These plant species grew well in diesel-contaminated soil. However, the growths of spinach, sunflower and red clover were inhibited. There was a large variation in the plant growth among the twelve species. The growth of the spinach plant was inhibited immediately after germination, and that of the sunflower plant was inhibited after the flowering stage. The red clover showed poor growth throughout the experiment. Hydrocarbon toxicity is caused by volatile compounds and hydrophobicity. Volatile hydrocarbons, primarily small and lightweight hydrocarbons, can easily move through cell membranes, thus causing toxic effects (Adam and Duncan, 2002). On the other hand, the hydrophobicity in oil-contaminated soils prevents water infiltration and aeration that are required for the growth of plant roots (Kirk et al., 2005). Therefore, we assumed that the spinach was severely damaged due to the toxicity of the volatile compounds in the soil. On the other hand, the growth of the sunflower plant was inhibited probably by the hydrophobicity of the diesel oil rather than by the volatile compounds. The red clover was considered to be influenced by both the volatile compounds and the hydrophobicity. Leguminosae such as kudzu, alfalfa and red clover have a symbiotic relationship with nitrogen-fixing bacteria. This symbiotic relationship suggests that Leguminosae could grow well in petroleum-contaminated soil in which the C/N ratio tends to be high (Adam and Duncan, 2003) and could therefore be effective in phytoremediation. However, the red clover showed poor growth in this experiment. This result suggests that Leguminosae is not always advantageous to phytoremediation.

It is considered that the decrease of TPH concentration was caused by microbial activity. Fibrous roots provide a larger surface than taproots for colonization by soil microorganisms (Anderson et al., 1993); they also allow a close interaction between the rhizosphere microbial community and the contaminant (Schwab and Banks, 1994). Grasses have fibrous root systems with long roots that have a large surface area per unit volume of soil. Then, grass species have been suggested as plants that can improve microbial activity for cleanup petroleum hydrocarbon-contamination (Aprill and Sims, 1990). In a previous study, we evaluated the enhancement of biodegradation in diesel-contaminated soil by the

Table 6. Aerobic microbial numbers in planted and unplanted soils at different sampling stages.

Plant species	Microbial numbers (CFU g soil dw^{-1})				
	Initial value	Seedling stage	Flowering stage	Mature stage	
Italian ryegrass	3.6E+07	2.4E+07	3.5E+07	1.4E+08	b
Sorghum	3.6E+07	4.2E+07	3.4E+08	6.8E+08	a
Maize	3.6E+07	3.3E+07	3.0E+08	1.4E+08	b
Alfalfa	3.6E+07	2.0E+08	2.9E+07	5.6E+07	b
Bermuda grass	3.6E+07	5.1E+07	—	1.5E+08	b
Sunflower	3.6E+07	2.4E+07	4.1E+07	—	
Southern crabgrass	3.6E+07	1.5E+07	2.2E+08	1.6E+08	b
Red clover	3.6E+07	6.6E+07	4.5E+07	—	
Rice	3.6E+07	2.7E+07	2.7E+08	1.6E+08	b
Spinach	3.6E+07	—	—	—	
Kudzu	3.6E+07	3.6E+07	—	5.8E+07	b
Beggar ticks	3.6E+07	—	2.5E+08	1.4E+08	b
Unplanted	3.6E+07	3.0E+07	1.1E+07	1.2E+08	b
No-irrigation	3.6E+07	—	—	2.1E+07	b

Data are for initial value and mature stage.
' __ ' indicates no data.
N = 5 for the initial value and mature stage, and n = 1 for the seedling and flowering stages.
Means with different letters are significantly different at a 5% level of significance.

growth of Italian ryegrass (Kaimi et al., 2006). We observed that the growth of fine roots is apparently required for rhizodegradation. However, in this study, southern crabgrass did not show a significant decrease in the TPH concentration, although its fibrous root density is approximately one order of magnitude greater than that of the other species. The DHA of the southern crabgrass-planted soil was lower than that of the soil planted with the other species. The lower DHA of the southern crabgrass-planted soil appears to be related to the limited degradation ability. This indicates that physically high-density root systems do not always guarantee effective microbial activity for rhizodegradation. It is therefore assumed that the quality of exudates or other factors besides the physical characteristics of the roots contribute to the effectiveness of rhizodegradation. Plants exude soluble organic matter into soil. The rhizosphere microbial community is greatly influenced by root exudates that depending on the plant species (Rovira, 1959; Bowen, 1969). The strong correlation of TPH concentration with soil DHA, and the difference in the degree of the correlation among plant species support the above assumption.

The consortium of various microorganisms can metabolize various intermediates of the degradation pathway (Pelz et al., 1999). Therefore, they are more effective than aerobic bacteria alone, although aerobic bacteria are also important for the degradation of hydrocarbons (Obuekwe et al., 2003). Günther et al. (1996) reported that the soil DHA can reflect the rhizodegradation effect of perennial ryegrass in hydrocarbon-contaminated soil. In a previous study, we revealed that the rhizodegradation effect of Italian ryegrass was significantly correlated to soil DHA (Kaimi et al., 2006). The results of this study indicate that the soil DHA is correlated to the degradation ability of the nine plant species, and that this observation is not limited to perennial ryegrass and Italian ryegrass.

2. Validity of measurements

The soil TPH concentration decreased in the planted soil, probably due to both biodegradative and non-biodegradative processes. In general, non-biodegradative processes include leakage due to irrigation, evaporation, direct plant uptake and adsorption by soil or organic matter. However, the leakage was prevented in this study, and the loss by evaporation was less than biodegradation. The loss of TPH through direct plant uptake was assumed to be negligible because other studies have shown that mixtures of petroleum hydrocarbons similar to diesel oil was not taken up by the plant (Schwab and Banks, 1994; Reilley et al., 1996). Organic matter in soil adsorbs petroleum contaminants, and it decreases mobility, biocidal activity and bioavailability by the formation of non-extractable bound residues (Kästner et al., 1995). Plants exude soluble organic matter into soil, and this organic matter has the potential to increase the adsorption of the abovementioned contaminant (Banks et al., 2003b). In this study, we postulated that the formation of bound residues by plants contributed to the cleanup of soil contaminants. The effects of the rhizosphere were then evaluated based on the concentration of TPH extracted from the soil by using an organic solvent.

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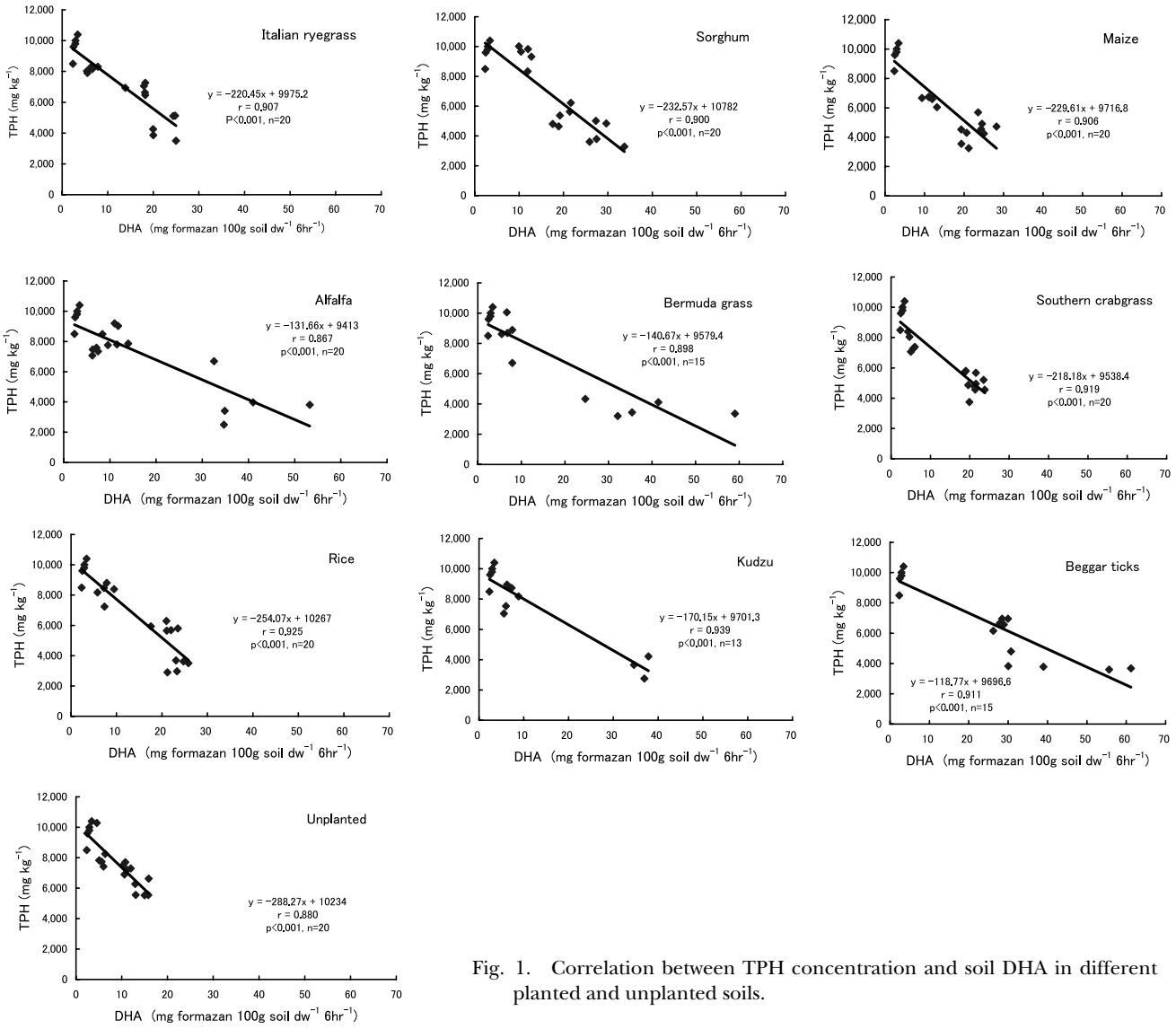

Fig. 1. Correlation between TPH concentration and soil DHA in different planted and unplanted soils.

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