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Protein Biomarkers of Phytotoxicity in Hazard Evaluation

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Protein biomarkers induced by stress can serve as diagnostic screening tool in environmental monitoring. Stress proteins are part of the primary cellular protective response from environmental stress, are induced by wide variety of stress factors, and are usually highly conserved in all organisms from bacteria to man (Schlesinger et al. 1982). Rice (*Oryza sativa*) plants which are relatively tolerant to environmental changes showed alteration of several polypeptides when exposed to NaCl, drought, and low and high temperature (Lim et al. 1992; Koga et al. 1991; Mathias and Walbot 1989), anoxia (Mujer et al. 1993), and heavy metals (Kaneta et al. 1986; Illangovan and Vivekanandan 1990). Apart from toxicity tests, induction of stress related proteins in plants could further identify and characterize hazardous waste sites. The major objectives of this study were to determine 1) alteration of proteins in rice plants that were exposed to contaminated soil samples collected from selected areas at an abandoned oil refinery site and 2) to evaluate if shoot and root growth inhibition observed in rice plants correlates with change in protein pattern in the plant tissues.

MATERIALS AND METHODS

The study site was an abandoned oil refinery located in Cyril, at Caddo county, Oklahoma. The refinery site covered approximately 63 ha which included the main processing plant facility in addition to an array of unlined earthen ponds, storage pits, and a 3.4 ha soil farm facility used to treat oily sludges. Crude oil had been refined into petroleum products for distribution since early 1920's. This practice had produced considerable quantities of waste materials requiring treatment, storage, and disposal. From 1920 to 1984 process wastes were placed in over 50 impoundments, many unlined. Also, some process waste was applied to the soil and treated in a land-farming operation. For this study soil samples were collected from predefined grids covering known areas of contamination (Fig. 1 and Table 1). The trap grids were used by other

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co-investigators for collecting small feral rodents from the site and the enclosures were used for confining populations of lab-raised white-footed deer mice on site for controlled mesocosm exposures (McMurry 1993). Random surface soil samples were collected during April 1992 from the grids, during May 1992 from the enclosures, and during January 1993 from both the grids and enclosures. A composite consisting of > 6 random soil samples was mixed on site and subsampled to obtain aliquots for lab analyses. Soil samples from each area were homogenised, air dried and ground in a blender for the rice shoot growth test. Aqueous extracts of soil samples were prepared in accordance with Dredged Material and Testing Manual USA-ACE method (USA-ACE 1991). Rice (*Oryza sativa* L. cultiver Gulmont) seeds were obtained from Rice Research of USDA in Beaumont, Texas.

Shoot growth test was performed acccording to the Modified Neubauer Seed Germination Phytoassay method (Thomas and Cline 1985). Rice seeds were soaked with 50% Chlorox solution for 20 min and rinsed with sterile water four

Table 1. Cyril Refinery, Oklahoma, sampling site locations, and site description.

Site	Grids	Enclosures
1	North side and upstream upslope of the Refinery. A reference site, unused land.	Placed in Grid 1. A reference site, unused land.
2	Within the Refinery, Asphalt Drum Storage Area with pits filled with asphalt wastes.	Within the Refinery. Placed in the area of leaded gasoline tank and the soil is soaked with gasoline.
3	Within the Refinery, Area surrounding the oil/sludge	Within the Refinery and placed near API separator traps.
4	Within the Refinery, Land Farming Area, used for processing sediments from oil/sludge traps.	Within the refinery, placed in Land Farming Area.
5	A reference site, 1 mile southwest of the Refinery A cultivated land.	Reference site, placed at the southern border of the Refinery, unused land.
6	A reference site, 4 miles east of the Refinery, unused land.	A reference site, adjacent to Enclosure 5, unused land.

times to prevent fungal growth. Soil (100 g) was placed in the bottom half of a plastic petri dish (100 mm wide x 20 mm depth). Twenty seeds were placed on the soil and pushed into the soil with a glass rod. Six replicates were used for each treatment. The soil was hydrated with deionized water to 80% water holding capacity. Each petri dish was enclosed in a Ziploc plastic bag (1-gal size) and placed in a controlled environment at 28 to 30°C and photoperiod (16:8, 1ight:dark) and light (4300 lux) for 10 d. After 10 d shoots were severed above the soil for shoot dry weight determination. For protein analysis rice plants were grown for 10 d in petri dishes in similar soils, leaves were then severed and stored under -80°C prior to protein analysis.

Rice root elongation tests were performed according to the seed rack method described by Myhill and Konzak (1967). Rice seeds were germinated in blotters placed in seed racks. Three-day-old seedlings were transferred to glass jars containing about 450 mL of aqueous soil extract. Seedlings were exposed to 100% of the aqueous soil extracts except for the more toxic 1992 Enclosure 3 soil extract which was diluted to 50 and 25% by mixing with reference Grid 1 soil extract. Seedlings were grown on screens placed on the neck of glass jars that were coated with silica beads. Approximately 200 seedlings per treatment and two replicates per treatment, were used. The glass jars were enclosed in plastic bags (1- gal size) and were incubated at 28 to 30°C, light 4300 lux and photoperiod (16:8, 1ight:dark) for 5 d. After 5 d 10 roots were selected randomly from each replicate/treatment and the primary or seminal root lenghts were measured. The remaining rice roots from each treatment were rinsed with distilled water and stored in plastic bags at -80°C prior to protein analysis.

The rice roots and leaves were homogenized with prechilled extraction buffer (0.05M Tris (pH 6.8), 1mM phenyl methyl sulfonyl fluoride). The homogenate was filtered through two layers of Miracloth. The filtrate was placed in eppendorf tubes and centrifuged at 14000 rpm for 10 min. The supernatant was then drawn into another eppendorf vial and stored at -20°C. About 20 µL of each sample was used for protein quantification using the dye binding assay (Biorad Laboratories, Richmond, CA). A standard curve was constructed with bovine serum albumin. Proteins in aliquots from the root and leaf samples were analyzed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) as described by Laemmli et al. (1970). The acrylamide matrix for separating the protein consisted of 12.0% acrylamide separating gel. The protein samples (75 µL) were dissolved in 4X sample buffer (25µL) (Hurkman and Tanaka 1986) and boiled for 2 min and centrifuged. Each sample was loaded into a well of the polymerized stacking gel. The power supply was connected to the gel was run at 100 volts for 2 hr. At completion of the run the gels were removed and stained by the silver staining procedure of Morrisey (1981).

Data analysis was performed using Dunnett's procedure consisting of an analysis of variance (Anova), and t-test to compare treatment means with the control means. If the data were not normally distributed Steele's Many-one Rank Test a multiple comparision method was used.

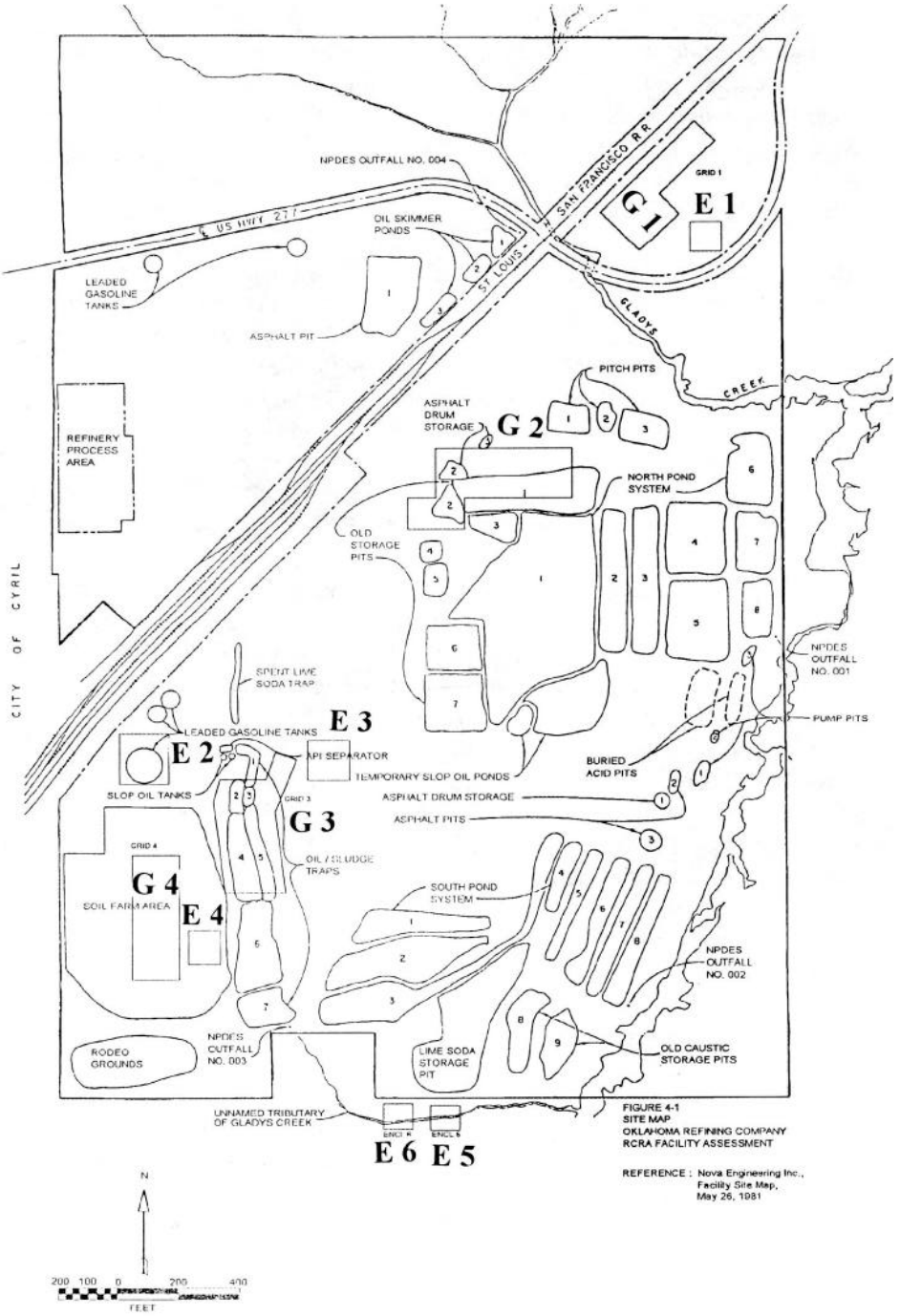
RESULTS AND DISCUSSION

The contaminated Grids 3 and 4 caused significant effect (Dunnett's procedure, Anova) on rice shoot growth when compared with the uncontaminated site, reference Grid, G1 (Fig. 2). Contaminated Grid 2 did not cause any significant toxic effect on shoot growth when compared with reference G1. Rice shoot growth was inhibited by 21% in Enclosure 2 soil when compared with reference G1. The most significant effect was caused by Enclosure 3 soil where shoot growth was suppressed to 71% when compared with reference G1.

Root length of the seedlings was significantly inhibited in Enclosure 3 soil collected in 1992 (I E3) when compared with reference G1(Fig. 3). Root growth did not increase in I E3 soil dilutions though the 100% soil extract was diluted with the reference G1 soil extract up to 25 % . The extract was very toxic so addition of nutrients by mixing reference soil extract still did not overcome growth inhibition. The roots also appeared thick and stubby. Although aquaeous extracts of Enclosure 3 soil collected in 1993 (II E3) promoted root growth, shoot growth was inhibited to 71 percent in II E3 soil (Fig. 2). This could be due to the seeds having direct contact with all contaminants present in the soil. The contaminated Enclosure 2 and Grid 2 soil did not cause any significant toxic effects on root growth when compared with the uncontaminated site, reference Grid 1.

Figure 4 shows the results of one dimensional SDS-polyacrylamide gel electrophoresis of rice root proteins. Marked changes in patterns of protein were observed in seedlings treated with I E3 soil extract. Proteins were suppressed in roots treated with I E3 soil extract. Polypeptides 74 , 70 and 56 kDa were suppressed in roots exposed to I E3 soil extract while they were present in roots exposed to reference G1 soil extract and the blank, deionized water control. Polypeptides were also induced in roots treated with contaminated soil extracts. Polypeptides 34 kDa was induced in roots exposed to contaminated soil extracts Grid 2, Enclosure 2, and II E3. The II E3 soil extract which had stimulatory effect on root growth had induced polypeptides 34, and 19 kDa in the roots. In this study proteins were induced when both shoot and root growth were inhibited. Induction of proteins also occurred independent of toxic effect. Both roots as well as leaves have changes in protein pattern in plants exposed to contaminated soil extracts. Polypeptide 98 kDa was suppressed in leaves of the plants exposed to I E3 soil extract (Fig. 5).

Changes in protein pattern were also observed in leaves of rice plants grown in



Cyril, Oklahoma - N 34° 53' 30"longitude, W 98° 12' 6" latitude

Figure 1. Map of Cyril Refinery, Oklahoma and sampling areas in the refinery - grids (G 2, 3, 4) and enclosures (E 2, 3, 4). Reference areas - grid G1 and enclosures E 1, 5, and 6.

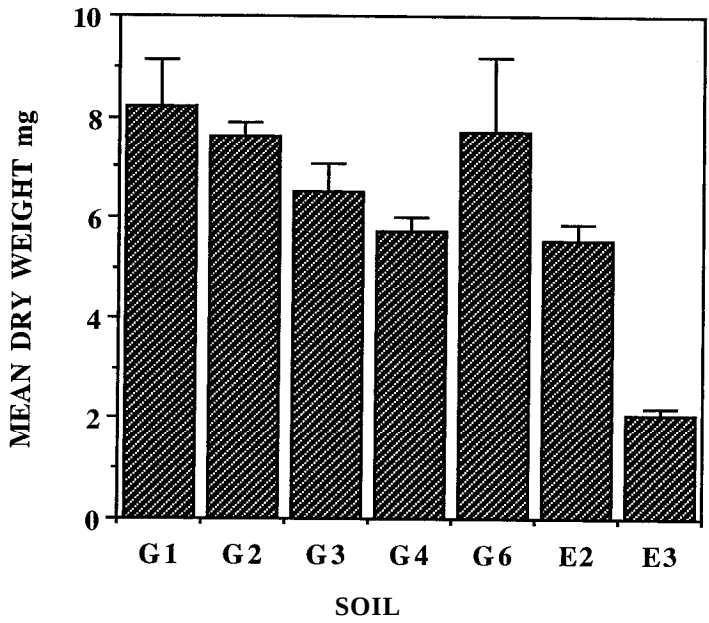


Figure 2. Rice shoot growth in soil G1, G6 (reference grids), G2, G3, and G4 (contaminated grids, Cyril Refinery, Oklahoma), and E2, and E3 (contaminated enclosures, Cyril Refinery, Oklahoma)

contaminated soil. Polypeptide 94 kDa was highly induced in leaves of plants grown in contaminated soil Grid 3 and Enclosure 3 (Fig. 6). Polypeptide 51 kDa was suppressed in leaves exposed Enclosure 2 and Enclosure 3 soil when compared with leaves exposed to reference Grids land 6. The leaves of plants exposed to contaminated soils namely Grid 2, Grid 3, Enclosure 2, and Enclosure 3 all had highly induced polypeptides 29, and 21 kDa when compared with proteins in leaves of plants that were exposed reference Grids 1 and 6. Interestingly the proteins in leaves of plants exposed to Grid 1 soil and soil elutriate were different due to the different media of exposure.

Response of rice plants to the environmental stress is evidenced by the induction and suppression of polypeptides in the roots and leaves after exposure to the refinery soil. The changes in the patterns of protein synthesis observed in rice plants in this study could be due to the effect of multiple contaminants present in the refinery soil. Polyteptides 94 kDa was highly induced in the rice plants grown in Grid 3 and Enclosure 3 soil which could be due to the presence of similar contaminants in both sites. The Grid 2 soil had no significant effect on shoot and root growth when compared with reference G1 but still had induced polypeptides in roots and leaves. Polypeptides were induced even after 10 d of plant growth in the contaminated soil which shows plant tolerance to environmental toxicity. Protein biomarkers will serve as additional tool to determine if adverse effects still exist in the ecosystem in cases where toxicity tests or field tests may fail to show any adverse effects. Protein biomarkers could be employed as biomonitoring tool and for site characterization of hazardous waste sites.

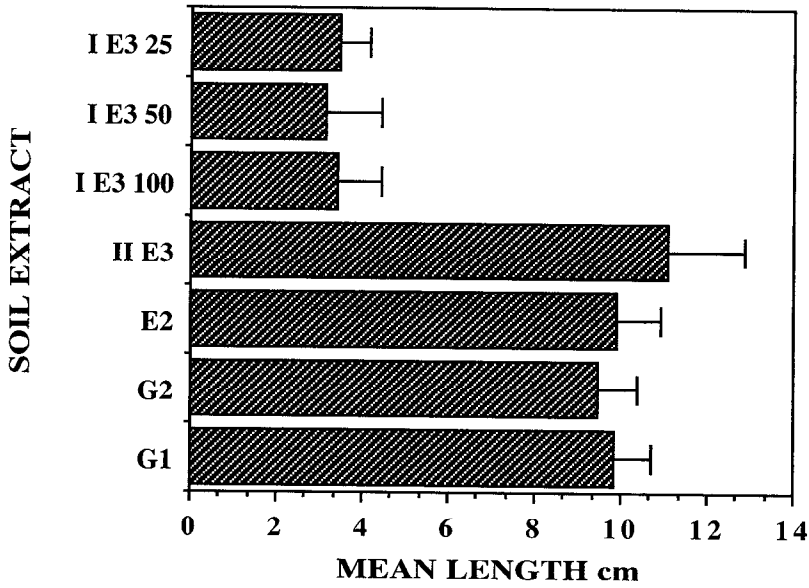


Figure 3. Root growth of 3-day rice seedling grown for five days in the aqueous soil extracts G1 (reference grid), G2 (contaminated grid), and contaminated enclosures E2, II E3 (1993), and I E3 (1992) 100, 50, 25%

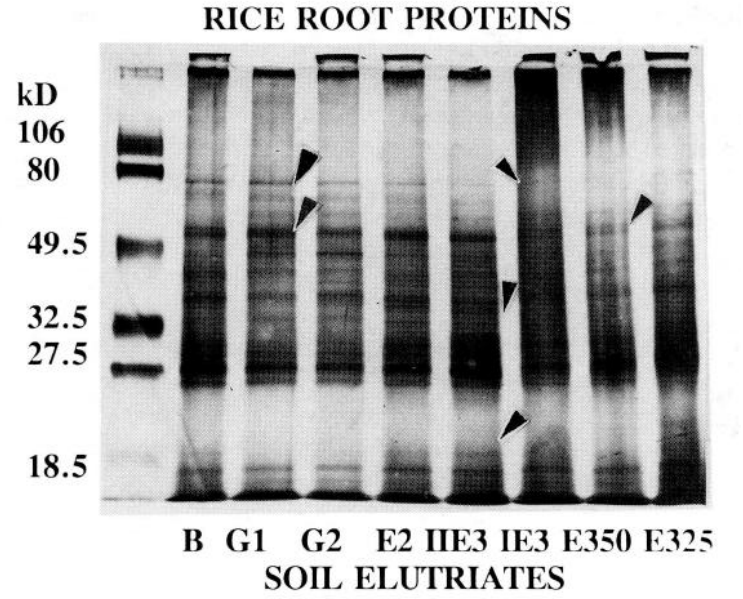


Figure 4. Root proteins of rice plants after exposure to aqueous soil extracts -B (blank, deionized water), G1 (reference grid), G2 (contaminated grid), and contaminated enclosures II E3 (1993), and I E3 (1992) 100, 50, 25%. Arrowheads indicate 74 kDa protein in reference Grid G1, suppression of 74, and 56 kDa protein in IE3, and induction of 34, and 19 kDa proteins in II E3

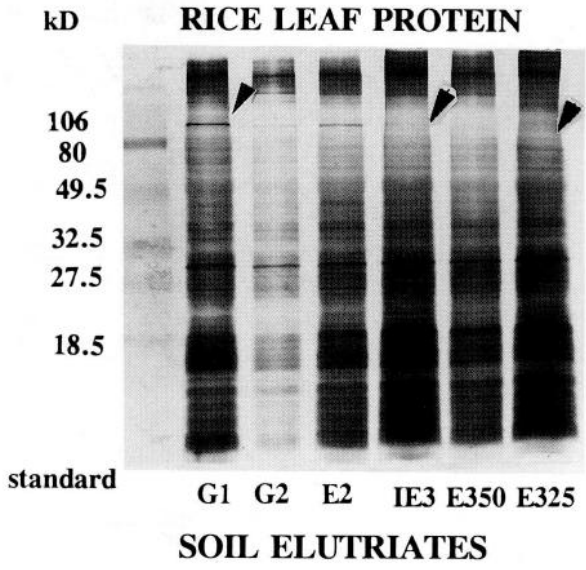


Figure 5. Leaf proteins of rice plants after exposure to aqueous soil extracts of G1 (reference grid), G2 (contaminated grid) and contaminated enclosures E2, and I E3 (1992) 100, 50, 25%. Arrowheads indicate 98 kDa protein in reference grid G1 and suppression of 98 kDa in I E3 100, 50, and 25%

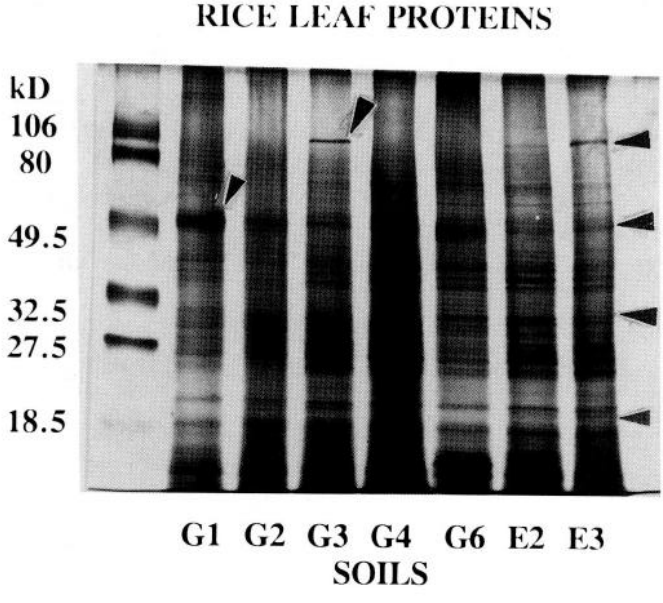


Figure 6. Leaf proteins of rice plants after exposure to soil G1, and G6 (Reference Grids), G2, G3, and G4 (contaminated grids), and E2, and E3 (contaminated enclosures). Arrowheads indicate induction of 94 kDa protein in G3 and E3, presence of 51 kDa protein in reference G1 and suppression of 51 kDa protein in E3, and induction of 29 and 21 kDa proteins in E3

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