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Phytoremediation of Crude Oil Contaminated Soil by *Acacia farnesiana* L. Willd. and Spraying Glutathione

Ahmed R. AL-Tameemi
Horticulture and Landscaping
College of Agriculture
University of Basrah
Basrah/Iraq
ahmraz94@gmail.com

Taha Y. Al-Edany
plant Protection
College of Agriculture
University of Basrah
Basrah/Iraq
tahaeledany@yahoo.com

Ali H. M. Attaha
Horticulture and Landscaping
College of Agriculture
University of Basrah
Basrah/Iraq
attaha1955@yahoo.com

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***Abstract*— This experiment was carried out in the field of medicinal plants of the College of Agriculture, Basra University to investigate the efficiency of sweet acacia *Acacia farnesiana* (L.) Willd. in the phytoremediation of contaminated soil with crude oil in four concentrations (control , 20000 , 40000 , 60000 mgkg^{-1}), and sprayed with glutathione (0 , 100 mgl^{-1}). Experimental measurements were taken after 9 months of planting in the polluted soil. The results of the experiment showed the plant's tolerance to crude oil. Vegetative growth measurements include plant height, stem diameter, number of leaves and leaf area, as well as chemical characters such as, total chlorophyll, carbohydrates and enzymes. The combination treatment of 20,000 mgkg^{-1} crude oil and 100 mg l^{-1} glutathione recorded the highest increase in plant height (83 cm) as well as the other vegetative growth characters. The plant showed the highest percentage of phytoremediation at the combination of 20,000 mg kg^{-1} crude oil and glutathione 100 mgl^{-1} it was (56.1 %).**

Keywords*— *Crude oil, Phytoremediation, Glutathione, Rhizodegradation

I. INTRODUCTION

Disposal huge amounts of pollutants into the environment is the negative side of the development of industry, agriculture, urbanization and other human activities and many of these pollutants accumulate in large quantities despite the increase in the growth of public awareness, but population growth is increasing and globalization is also increasing the complexity of this problem (Landrigan et al., 2018). Environmental pollution with crude oil may occur due to exploration, production, transportation, storage and refining operations, as well as oil marketing operations (Oberdorster and Cheek, 2000). Environmental pollution with crude oil is one of the most dangerous types of pollution that is in the oil producing countries due to drilling for it in many countries required for economic development, which caused great sabotage in the environment (Okotie et al., 2018). Production and export of crude oil is one of the most important resources of Iraq, on which it relies mostly in the annual budget, as 95% of the total financial budget depends on crude oil production in 2014 (Faucon et al., 2014). Crude oil consists of a complex mixture of aliphatic and aromatic hydrocarbons and heterocyclic hydrocarbons (Falkova et al., 2016). Pollution affects essential components of the biosphere such as water, air quality and soil fertility, as well as forests, biodiversity and climate changes, in many government are ignored for negative effect of pollution especially in low and middle-income countries (Landrigan et al., 2018). Petroleum hydrocarbons are a permanent hazardous pollutant and include compounds that can bioaccumulation in food chains (McElroy et al., 1989). Pollution can affect directly or indirectly on human health because of high toxicity may causes cancer, mutations, and birth defects (Chen et al., 2015; Rein et al., 2016). There are several techniques for cleaning the environment contaminated with crude oil and choosing the most appropriate technique is crucial to reduce the negative effects of this pollution (Moreira et al., 2011). All the process to clean site contaminated by crude oil implemented using chemical, physical and thermal are very expensive (Ezeji et al., 2007). It is established on the basis of chemical and physical treatments, such as, incineration (burning) and thermal adsorption (Kastanek et al., 2016). These methods are usually highly energy-consuming and harmful to the environment. The limited methods of traditional engineering treatments sparked the search for alternative biological technologies (Gomez et al., 2019). Phytoremediation is the use of green plants to clean up contaminated sites and is an environmentally friendly and simpler method as compared to other cleaning methods (Joner et al., 2002). It has an advantage over chemical and physical treatment techniques in decontamination because it provides a biodegradation of the parts of the crude oil to treat hazardous pollutants and has no environmental harmful effects (Zhang et al., 2011). Gerhardt et al. (2017) mentioned the main common plant techniques for cleaning the environment phytoextraction,

rhizofiltration, phytostabilization, phytovolatilization, phytodegradation and Rhizodegradation. Phytodegradation is the absorption of pollutants by plant roots and their degradation through metabolic processes in the plant or the degradation of external pollutants through the influence of compounds produced by enzymes in the plant (Mukhopadhysy and Maiti, 2010). Rhizodegradation is the process of degrading organic pollutants in the soil through the activity of microorganisms in the rhizosphere (Germida et al., 2002). Microorganisms provide vitamins and amino acids to increase plant growth while plant roots provide a favorable environment for the growth of microorganisms to degrade hydrocarbons (Dominguez-Rosado and Pichtel, 2004; Qixing et al., 2011). Many stage can accord to degradation of hydrocarbons, the primary stage is hydroxylation, adding a hydroxyl group to produce the transformation of non-polar hydrocarbon molecules into molecules containing a polar group (Hydroxyl) to make it easier to accept further oxidation degradation (Kvesitadze et al., 2001, 2006). Glutathione is a component of in plant and animal cells this is acts as an antioxidant, it is the most abundant form of organic sulfur in plants, glutathione has two forms, oxidized (GSSG) and Reduced form (GSH) (Tandogan and Ulusu, 2006).

II. MATERIALS AND METHODS

The experiment was carried out at the College of Agriculture / Basrah University at the period from September 2018 to June 2019. The *Acacia farnesiana* L. Willd. was used, a tree belonging to the Fabaceae family, which is a local tree and that suits the climatic conditions of Basrah Governorate.

A. Pot experiment

The plastic plates with 200 eyes with dimensions of 2 x 2 x 5 cm for one eye filled with organic fertilizer (pitmos) used for planted the seeds, as three seeds were in each eye and after the seedlings reached a height of 10 cm they were transferred to pots containing soil contaminated with crude oil. Coverage was used with an open plastic cover on both sides in the winter to keep the crude oil from washing with rain. Two concentrations of glutathione were used (control ,100)mgL^{-1}. Plants were sprayed with glutathione every month until the end of the experiment. The experimental measurements were taken 9 months after planting trees in the polluted soil.

TABLE I. physical and chemical properties of the soil

Parameters	***Value***
pH	8.02
Electrical conductivity (EC) dSm^{-1}	6.22
Total nitrogen $mgkg^{-1}$	1140
Total phosphorus $mgkg^{-1}$	20.3
Total potassium $mgkg^{-1}$	254
Sand %	14.9
Silt %	44.2
Clay %	40.9
Soil Textural	silty clay

B. Soil preparation

Soil brought from southwest of Basra was used, and some Physical and chemical properties of the soilwas shown in the Table 1. The soil spread under the sunlight until it was completely dried and sifted the soil using a sieve with a diameter of 2 mm and took the weight of 10 kg of soil and contaminated with four levels of pollution with crude oil (control , 20000, 40000, 60000) mgKg-1, the concentrations were prepared by the gravimetric method. The soil mixed well with the crude oil and put in pots with a diameter of 30 cm and a depth of 35 cm. Each pot contains 10 kg of soil contaminated with crude oil and prepared for planting.

TABLE II. Concentrations of crude oil spiked with soil

Treatments		***code***
Conc. Crude oil	***Conc. GSH***	
0 $mg\ kg^{-1}$	0 $mg\ l^{-1}$	C1
20 000 $mg\ kg^{-1}$	0 $mg\ l^{-1}$	C2
40 000 $mg\ kg^{-1}$	0 $mg\ l^{-1}$	C3
60 000 $mg\ kg^{-1}$	0 $mg\ l^{-1}$	C4
0 $mg\ kg^{-1}$	100 $mg\ l^{-1}$	Cg1
20 000 $mg\ kg^{-1}$	100 $mg\ l^{-1}$	Cg2
40 000 $mg\ kg^{-1}$	100 $mg\ l^{-1}$	Cg3
60 000 $mg\ kg^{-1}$	100 $mg\ l^{-1}$	Cg4

C.Statistical analysis

GenStat discovery edition 3 was used for the statistical evaluation. The experiment was designed as completely randomized with three replicates for each treatment. The result was analysed using the analysis of variance (ANOVA). The differences between means were compared by LSD range at a significance level at $p < 0.05$.

Plant measurement

Vegetative growth

Three randomly replicates were taken in duplicates for vegetative growth which were measured by measuring the height of the plant (cm) using the measurement metal tape, and the main stem diameter by feet Vernier caliper (mm), the number of leaves (leaf/plant) and leaf area (m^2 /plant) and the area was measured by Paper based Image software according to the method used by Darwish *et al*. (2014).

Chemical characteristics

1. Chlorophyll pigment

According Zaehringer *et al*. (1974) method, total chlorophyll (mgg^{-1}) was measured by a Spectrophotometer at 645 and 663 nm.

2. Carbohydrates

Estimated total soluble carbohydrates in the leaves were determined using the phenol-sulfuric acid, modified method according to the Duboies *et al*. (1956).

3. Glutathione

Determination of glutathione content in leaf was used according to Moron *et al*. (1979),glutathione interacts with (DTNB) 5, 5'-dithiobis-2-nitrobenzoic acid, measured by the spectrophotometer at 412 nm.

Enzyme activity determination

1. Extract

Extraction of plant leaf samples was used by Luhova *et al*. (2003), plant leaf crushed by a 0.1 M phosphate buffer, pH 7 , 1: 2 (W:V). The extract was filtered through nylon cloth and centrifuged at 12,000 Rpm for 30 minutes at 4 ° C.

2. Catalase (CAT)

The Goth (1991) modified method was used to estimate the enzymatic efficacy of CAT, 0.2 ml of plant sample extract and incubated with 1 ml of substrate (65 µmol per ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) for 4 minutes at about 25 C°, the reaction is stopped by adding 1 ml of ammonium molybdate (32.4 mm) then measured at 405 nm.

3. Peroxidase (POD)

Estimating the enzymatic activity of peroxidase (POD) was made using the method of Angelini *et al*. (1990) In guaiacol 1 ml of 5 mM guaiacol and 1 ml of 2 mM hydrogen peroxide and 1 ml of 0.1M, phosphate buffer, pH 7 add 0.1 ml of the sample extract and measured at 436 nm for 3 minutes, the reading was recorded every 30 seconds at a 30 C°.

4. Glutathione peroxidase (GPX)

The method of Flohe and Gunzler (1984) was applied, 0.3 ml of sample extract in test tubes and added 0.3 ml of 0.1 M phosphate buffer PH 7.4 and 0.2 ml of 0.002 M reduced glutathione and 0.1 ml of 0.010 M sodium azide With 0.1 ml of 0.03 M hydrogen peroxide. Test tubes were placed in a water bath at 37 C° for 15 minutes. Add 0.3 ml of 5% Trichloroacetic acid (TCA), then the tubes were cooled in an ice bath and then placed in a centrifuge at 1500 rpm for 5 minutes, measured at 420 nm.

Determination of total petroleum hydrocarbons in soil (TPHs)

Estimating the total petroleum hydrocarbons TPH remaining after remediation was made using the method of Miles *et al*. (1977), take 10 g of dry soil and put in a 250 ml beaker and add 50 ml of hexane and put it in the Rotator for two hours, Pass the extract through filter paper and through a separating column containing glass wool and anhydrous sodium sulfate then evaporate the extract and re-dissolve the residual by hexane and measured by Fluorescence spectrophotometer at 310 nm.

III. RESULTS

Results in Table 3 included the effect of different concentrations of crude oil on the height of the plant. Results showed a significant decrease in plant height by treatment C4 (53.7 cm) as compared to treatments C1, C2 and C3, (72.0 ,72.3 and 63.7 cm) respectively, while no significant difference was found between C4 (53.7cm) and Cg4 (57.7 cm). Also noted that the height of the plant Cg4 (57.7 cm) decreased significantly as compared to Cg1.Cg2 and Cg3 (73 , 83 , 66.3 cm) respectively.

Table 3 showed the results of stem diameter, in which the treatment Cg2 (9.2 mm), was significantly higher than the other treatments C1, C3, C4 (7.7 , 7.4 , 7.1 mm), while no significant difference was found between Cg1 and Cg2 treatments (8.3 , 9.2 mm) respectively. Also, the treatment Cg2 significantly increased stem diameter as compered Cg3 and Cg4 (7.8 , 7.2 mm) respectively.

TABLE III. Effect of crude oil on the vegetative growth

treatment	height (cm)	Diameter (mm)	Number of leaves	Leaf area (cm^2Plant^{-1})	Total Chlorophyll (mgg^{-1})	Total Carbohydrate (mgg^{-1})	Total Glutathione ($\mu molg^{-1}$)
C1	72.0	7.7	67.3	676.8	1.048	95.2	37.92
C2	72.3	8.2	75.7	794.5	1.165	127.3	37.05
C3	63.7	7.4	44.7	427.0	0.920	93.0	42.92
C4	53.7	7.1	43.0	333.4	0.932	82.2	43.88
Cg1	73.0	8.3	71.7	816.4	1.270	122.6	41.81
Cg2	83.0	9.2	85.3	991.5	1.260	127.4	39.51
Cg3	66.3	7.8	51.0	542.3	0.990	106.5	49.42
Cg4	57.7	7.2	53.7	453.3	1.034	105.7	59.78
L.S.D at p< 0.05	7.95	1.21	9.14	62.86	0.1878	8.86	6.303

Also showed a significantly increase in the number of leaves for the treatment Cg2 (85.3 leaves) as compared to the remaining treatments, while increased significantly C2(75.7 leaves) as compared to treatments C3 and C4(44.7 , 43.0 leaves) respectively, whereas no significant difference was found between C3, C4 and Cg3 treatments (44.7 , 43.0 , 51.0 leaves) respectively (table 3).

As for leaf area, the treatment, Cg2 increased significantly (991.5 cm^2Plant^{-1}) as compared to the other treatments, and the C2 increased significantly (794.5 cm^2Plant^{-1}) as compared to C1, C3 and C4 treatments (676.8 , 427.0 , 333.4 cm^2Plant^{-1}) respectively.

As noted in Table 3, results of total chlorophyll increased significantly for treatment Cg1 (1.270 mgg^{-1}) as compared to the remaining treatments, and also increased significantly C2 (1.165 mgg^{-1}) as compared to treatments C3, C4 (0.920 , 0.932 mgg^{-1}) respectively. In general, total chlorophyll was observed to decrease with increasing the concentration of crude oil in the soil.

Table 3 included the results of total soluble carbohydrates content in the leaves. Treatments C2, Cg1 and Cg2 were significantly increased (127.3 , 122.6 , 127.4 mgg^{-1}) respectively as compared to the other treatments, while no significant difference was found between them.

The effect of soil contaminated with crude oil on the total content of glutathione GSH was shown in Table 3, results showed a significantly increase in treatment Cg4 (59.78 $\mu molg^{-1}$) as compared to the other treatments. As for the treatment, Cg3, (49.42 $\mu molg^{-1}$) significantly increased as compared to the treatments Cg1 and Cg2, (41.81 , 39.51 $\mu molg^{-1}$) respectively. Treatments C1, C2, C3 and C4 (37.92 , 37.05 , 42.92 , 43.88 $\mu molg^{-1}$) while no significant difference was found among them regarding glutathione content af leaf.

Fig. 1 showed the results of the effect of contaminated soil with crude oil on the enzymatic activity of catalase CAT, a significant increase was observe in the enzymatic activity of CAT for Cg2 treatment (23.91 Ug^{-1}) as compared to treatments Cg3 , Cg4 (13.31 , 12.36 Ug^{-1}) respectively . The treatment C2 (21.31 Ug^{-1}) significantly increased CAT activity as compared to the treatments C3 and C4(8.04 , 5.61 Ug^{-1}) respectively.

Fig. 2 showed the effect of soil contaminated with crude oil on the enzymatic activity of peroxidase POD. It was observed that treatment Cg2 (79.6 Ug^{-1}) decreased significantly POD activity as compared to the other treatments, except treatment C2 (82.6 Ug^{-1}). The treatment, C2 decreased POD activity significantly as compared to all treatments.

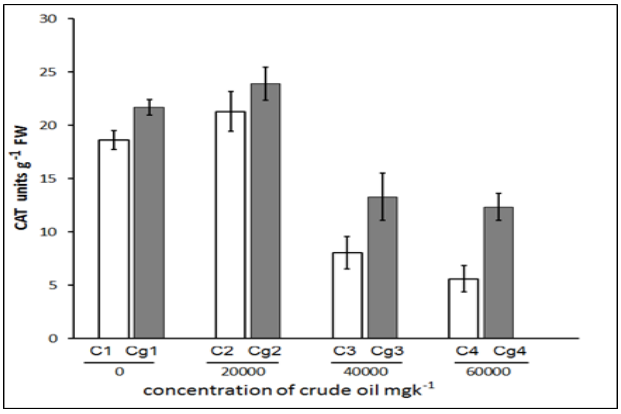

Fig. I Effect of crude oil on the CAT enzymatic activity

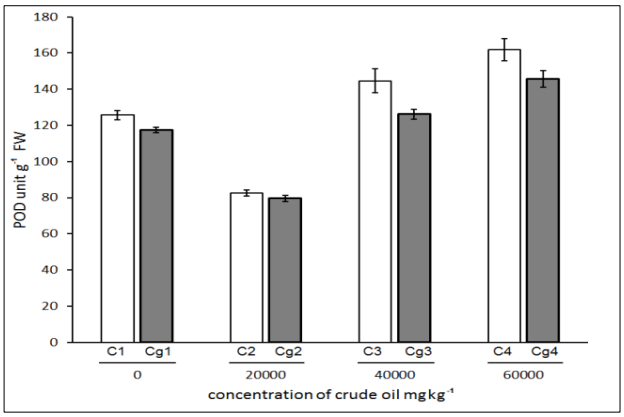

Fig. II Effect of crude oil on the POD enzymatic activity

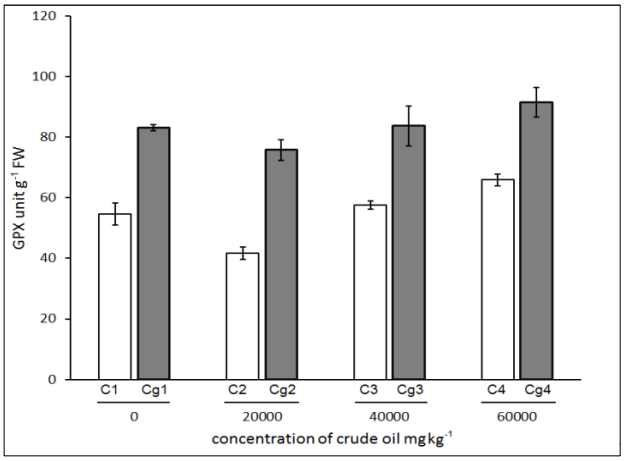

Fig. III Effect of crude oil on the GPX enzymatic activity

Fig. 3 showed the effect of soil contaminated with crude oil on the enzymatic activity of the glutathione peroxidase GPX enzyme. Results showed a significantly decrease in GPX activity treatment C2 (41.7 Ug^{-1}) as compared to the other treatments. The treatment Cg2 (75.7 Ug^{-1}) decreased GPX activity significantly as compared to the Cg4 treatment (91.4 Ug^{-1}).

Fig. 4 showed the percentage of crude oil removal from contaminated soil and the results showed significantly increase in treatment Cg2 (56.1 %) as compared to the control treatment (31.6%). Also Cg2 treatment increased percentage as compared to treatment C2 (54%). The treatment Cg3 (46%) increased the % of crude oil removal significantly as compared to the Cg4 treatment (38%). The C2 treatment (54%) increased the percentage of crude oil removal significantly as compared to the C3 and C4 treatments (43.2 and 34.8%) respectively.

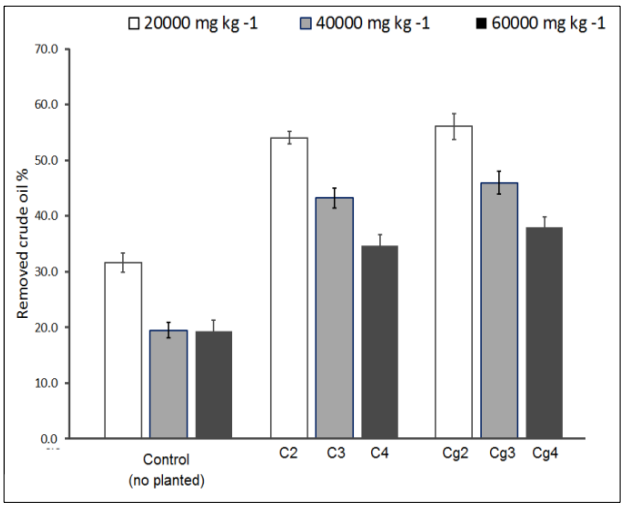

Fig. IV Percentage of removal crude oil in contaminated- soil

IV. DISCUSSION

Results showed the effect of contaminated soil with crude oil on the vegetative growth of *Acacia farnesiana* L. Willd. Soil contamination decreased the height of the plant and stem diameter may be due to the nutrients decrease with increasing oil concentration (Odjegba and Atebe 2007). The height plant decrease when the availability of nutrients decreased, because plant depends

on the presence and availability of nutrients in the soil (Clarkson and Hanson 1980). Some previous study observed plant growth reduce with increased soil pH interacted with the increase in the concentration of crude oil in the soil (Odiyi *et al*., 2020), pH is an important factor in availability nutrients for plant absorption (Okonokhua *et al*., 2007).

These results are in according with Ramos *et al*.,(2009), Who studied to phytoremediation of contaminated soil with crude oil using *Sebastiania commersoniana*. And also Merkl *et al*.(2004) for phytoremediation of contaminated soil with crude oil using tropical plants, six types of which are from the legume family, The results showed a significantly decrease in the height of the plant.

The superiority of the treatments C2 and Cg2 was noted, which is the concentration of crude oil 20,000 mg. Kg^{-1} and the reason may be due to asymmetric effect of petroleum hydrocarbons on plant growth (Malallah *et al.*,1996). The results showed in Table 3 a decrease in the number of leaves and the growth of plants may be inhibited because the plant absorbs toxic hydrocarbons of small molecular weight that cause damage to the integrity and permeability of cellular membranes (Reis, 1996),also, may be attributed to a change in the chemical properties of oil contaminated soil (Balasubramaniyam and Harvey, 2014). Soil contamination by crude oil led to a decrease in leaf area, as in Table 3. Contamination of soil reduced leaf growth because reduces soil aeration by blocking air spaces and thus creates a state of air causes stress on plant roots (Shukry *et al*., 2013). Decreases in the leaves content of the total chlorophyll as in Table 3 due to the availability of nutrients and environmental stress, such as, soil pollution with crude oil (Onwurah *et al*., 2007). Crude oil is a mixture of aliphatic and aromatic compounds with high molecular weights that inhibit the enzymes needed for the synthesis of chlorophyll (Anthony, 2001). This was in accordance with Arellano *et al*. (2017) in his study of tropical trees in Amazon regions, have less chlorophyll content in polluted areas as compared to non-polluted areas. Al-Hawas *et al*. (2012) reported that increasing the concentration of crude oil reduced the leaf content of chlorophyll A, chlorophyll B, carotene and all pigments in the plant. Al-Hawas *et al*. (2012) registered increasing the concentration of crude oil reduced the leaf content of chlorophyll A, chlorophyll B, carotene and all pigments in the plant.

Table 3 also showed a decrease in leaf content of total carbohydrates. Furthermore the photosynthetic rates in plants decrease due to contamination by petroleum hydrocarbons (Caudle and Maricle 2014). Reduced contacting stomatal, which is a mechanism used by the plant to reduce lossing of water in drought-tight conditions, to avoid losing more water (Li, 1991). This leads to decrease CO_2, which results in a decrease in the rate of photosynthesis, which is attributed to a decrease for two reasons, either a decrease in the stomta contact or a decrease in the effectiveness of mesophyll cells (Farquhar and Sharkey, 1982). And decreased chlorophyll, protein and carotenoids (Agrawal, 1992).

Increase in leaf content of glutathione which has an important role in resisting biotic stress and abiotic stress, and it has a role in the glutathione - ascorbate cycle that reduces toxic hydrogen peroxide by scavenges toxic hydrogen peroxide (Noctor and Foyer, 1998). Because the glutathione is an antioxidant, it protects cells from breakdown by free radicals and also helps the cells to remain active (Mamdouh, 1995).

Decrease in the CAT enzyme, due to changes in chemical structures and enzymes. It is one of the hypotheses observed in plants growing in soils contaminated with crude oil (Eriyamremu *et al*., 1999). The treatments that sprayed with GSH have an increase in the enzyme activity of the CAT and POD. The reason is that GSH is an antioxidant that protects cells and keeps cells active, so it leads to increased activity of enzymes (Mamdouh, 1995).

Increase in the POD enzyme, in accordance with Adeyemi (2020), who observed that oil pollution caused an increase in POD enzymes for *Phaseolus vulgaris* and the reason that soil contaminated with crude oil impeded the availability of water, air and nutrients to plant roots, creating drought conditions that led to oxidative stress on plant.

Enzymic activity GPX in leaves of *Acacia farnesiana* L. Willd.increased which was due to abiotic stress conditions (water deficit, metal stresses, and photooxidation) also induce modifications of the Gpx levels in plant (Navrot *et al*., 2006). Glutathione peroxidase GPX is an antioxidant enzyme that is ubiquitously in plant cells in cytosol, chloroplasts, mitochondria, peroxisome and apoplast and catalyzes the reduction of H_2O_2 , organic and lipid hydroperoxides directly employ GSH, and thus protects cells from oxidative damage (Anjum *et al*., 2010).GPX functions provide protection as an antioxidant and salicylic regulator, and target free radicals ROS (Chang *et al*., 2009).

The removal of crude oil from the soil by *Acacia farnesiana* L. Willd. showed in figure 4., this may be attributed to the compounds released by plant roots which play a catalytic role for microorganisms in the soil to break down hydrocarbon pollutants and plant roots that help these organisms tolerate petroleum hydrocarbons in the soil (Liu *et al.*, 2011). Plants have the ability to absorb organic pollutants and their metabolism or modify them to less toxic metabolites once the organic chemicals are absorbed and transported under three types of transformations: I) Chemical modification (oxidation, reduction, hydrolysis, etc.), II),conjugation with (carbohydrates, glutathione, amino acids, etc.), III),compartmentalization in cell wall and vacuoles (Burken,2003; Kvesitadze *et al*., 2006). This is agreement with the study of Hultgren *et al*. (2009) to treat PAH in contaminated soil, and with Rezek *et al*. (2009) who studied phytoremediation of contaminated soil by PAHs which used two types of trees *Betula pendula* and *Morus rubra*, the results showed that after one year of treatment, the PAH concentration was able to remove 50% of contamination. It also agree with Ramos *et al*.,(2009). Figure 4. showed an increase in the removal of crude oil when sprayed with glutathione, due to glutathione which binds to molecules and then the enzymes bind to glutathione, or perhaps to some enzymes which use glutathione as a catalyst in the glutaredxion process (Rouhier *et al*., 2008).

V. CONCLUSION

This study demonstrated an ability of the *Acacia farnesiana* L. Willd. plant to tolerate different levels of crude oil concentration in soil. This result was demonstrated by measures of vegetative growth, such as, the height of plant, stem diameter, number of leaves and leaf area, as well as biochemical characteristics, such as, total chlorophyll and total carbohydrates, This ability came from the antioxidant enzymes that enabled the plant to tolerate abiotic stress, The result also showed that the plant had the ability to phytoremediation of crude oil, and it was also observed that plants sprayed with glutathione increased the tolerance of crude oil and caused greatly increase in the removal of crude oil from the soil.

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