Abstract

In an attempt to enhance crude oil tolerance in tissue cultures and intact plants of *Triticum aestivum* L., several experiments were carried out. Callus was induced using embryos isolated from the seeds of the two wheat varieties (Abu Ghraib-3 and Dijila) which were used as explants. Callus was induced and maintained on Murashige and Skoog, 1962 medium (MS) supplemented with (0.5) mg/l kinetin and (2) mg/l 2,4-D.

Crude oil was added to the culture medium at different concentrations as a contaminating agent. Results showed that callus fresh weight for both varieties decreased with increasing crude oil concentrations to (590.4, 442.8, 485.7, 288.0, 200.2 and 102.8)mg for Abu Ghraib-3 and (614.7, 4590.0, 434.7, 300.6, 230.2 and 110)mg for Dijila at crude oil concentrations (0, 2, 4, 6, 8 or 10)% in the culture medium respectively.

Cells that survived on different concentrations of crude oil for the two wheat varieties were subcultured on oil free MS medium for maintenance. Then they were recultured on MS medium containing different concentrations of crude oil.

Results showed that callus fresh weight of the two wheat varieties decreased with increasing crude oil concentrations in culture medium reaching (599.4, 468.9, 427.5, 370.6, 85.5 and 60.0)mg for Abu Ghraib-3 and (632.7, 486.9, 455.4, 393.3, 95.9 and 70.4)mg for Dijila at (0, 2, 4, 6, 8 and 10)% respectively.

From the results above it was shown that there was an increase tolerance ability to the low concentrations of crude oil in both of the two wheat varieties (Dijila and Abu Ghraib-3). Results showed that Dijila variety gave better tolerance to crude oil than Abu Ghraib-3 variety.

In order to asses the effect of crude oil on seed germination and plant height, seeds were sown in soil contaminated with different concentrations of crude oil. Results showed that germination percentage and plant height increased overtime. However a reduction occurred in these parameters with increasing crude oil levels.

الملخص

في محاولة لدراسة تحمل نبات الحنطة *Triticum aestivum* L. للنفط الخام على مستوى النبات الكامل و مزارعه النسيجية، تم تنفيذ عدد من التجارب لهذا الغرض. أستحث الكالس و أديم على وسط MS مجهز ب (0.5) ملغم\ لتر من Kin و (2) مغم\ لتر من 2,4- D باستعمال الأجنة الناضجة المستأصلة من بذور الحنطة لصنفي أبو غريب-٣ و دجلة كمصدر لنشوء الكالس. أضيف النفط الخام بتراكيز مختلفة (0، 2، 4، 6، 8 أو10)% الى الوسط الغذائي Murashige and Skoog، 1962 (MS) كعامل ملوث.

أوضحت النتائج نقصان في وزن الكالس الطري لكلا صنفي الحنطة مع زيادة تراكيز النفط الخام في الوسط حيث وصلت الى (590.4، 442.8، 425.7، 288.0، 200.0 أو 102.2) ملغم لصنف أبو غريب- ٣ و لصنف دجلة(614.7، 459.0، 434.7، 300.6، 230.2 أو 110.8) ملغم عند التراكيز (0، 2، 4، 6، 8 أو 10)% على التوالي.

أخذت الخلايا الناجية من مختلف تراكيز النفط الخام و كثرت و أديمت على وسط MS الغذائي خال من النفط الخام و بعدها أعيدت زراعتها على وسط MS الغذائي الحاوي على تراكيز مختلفة من النفط الخام.

أظهرت النتائج نقصان في وزن الكالس الطري لكلا صنفي الحنطة مع زيادة تراكيز النفط الخام حيث وصل الى (599.4، 468.9، 427.5، 370.6، 85.5 أو 60.0) ملغم لصنف أبو غريب- ٣ و (632.7، 486.9، 455.4، 393.3، 95.9 أو 70.4) ملغم لصنف دجلة عند التراكيز (0، 2، 4، 6، 8 أو10) % من النفط الخام عى التوالي.

من النتائج الموضحة أعلاه وجدت زيادة في قابلية تحمل صنفي الحنطة للتراكيز المنخفضة من النفط الخام. حيث أظهرت النتائج ان صنف دجلة اعطى نسبة تحمل افضل من صنف أبو غريب-٣ .

و لغرض دراسة أثر هذه الملوثات من النفط الخام على إنبات البذور و نمو الباذرات، زرعت بذور نبات الحنطة (أبو غريب- ٣ و دجلة) في تراكيز مختلفة من النفط الخام. أوضحت النتائج زيادة في نسبة الإنبات و أرتفاع النبات مع زيادة الوقت لكنها انخفضت بزيادة تراكيز النفط الخام.

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Preliminary study on the tolerance of two wheat varieties of *Triticum aestivum* L. to the crude oil at the whole and cellular level

A thesis
Submitted to the College of Science, AL- Nahrain University in Partial Fulfillment of Requirements for the Degree of Master of Science in Biotechnology

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وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم

دراسة أولية عن تحمل صنفين من نبات الحنطة *Triticum aestivum* L. للنفط الخام على مستوى الخلية و النبات الكامل

رسالة
مقدمة الى كلية العلوم في جامعة النهرين
و هي جزء من متطلبات نيل درجة الماجستير
في علوم التقانة الإحيائية

من قبل
زينب فرقد الأشقر
بكالوريوس تقانة إحيائية- كلية العلوم- ٢٠٠٤
جامعة النهرين

بإشراف
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1.1- Introduction

After the increased need of crude oil and its derivatives all over the world, great hazards resulted from oil leaks due to bad storage or transportation. Oil pollution may occur in water, public lands and agricultural lands due to leaks from shipment processes and oil pipes (Farrel and Mccurdy, 2002).

Spills on lands can be removed by long exposure to air and weathering conditions but this cleaning process is one of the slowest methods used that needs long periods of time. Spills could also be removed using chemical and mechanical methods (Hutchison *et al.*, 2002).

Rock (1993) of Georgia State showed that plants have the ability to remove oil spills from soil. He called this process phytoremediation. Phytoremediation is a technology that aims to provide a cheap, soft and safe treatment applicable to contaminated sites based on the consideration: short-term effectiveness, reduction in the toxicity, mobility, or volume of crude oil.

Tissue culture is a powerful tool that gives the possibility to grow millions of cells under controlled conditions. The technique provides a preliminary physiological information about the behavior of the plant cells under stress conditions like salt, heat, cold, drought and crude oil (Stefano and Edoardo, 2003).

The genus *Triticum* belongs to the family Poaceae (Gramineae previously), which include important plants that are used for treatment of contaminated soils. It's known for its good growth rates and it has several uses. It is the main source of flour for the world bread making; its grains are the main source of alcoholic beverages, beer and industrial alcohol. (Watson and Dallwitz, 2004).

Due to the importance of enhancing crude oil tolerance for phytoremidation purposes especially in northern regions of Iraq where wheat is grown in arid and semi arid areas. Oil pipes transporting crude oil or oil derivatives are exposed to sabotage very commonly. This has led to soil contamination. Therefore, this project is aimed to investigate crude oil tolerance in locally grown *T. aestivum* varieties, as potential candidates for phytoremediation application in crude oil contaminated lands at the whole plant and cellular levels.

1.2- Literature review

1.2.1- Plant Taxonomy

Triticum aestivum L. poir, common names are wheat, bread wheat, winter wheat and flour wheat. The genus *Triticum* is important all over the world especially in Iraq, Australia and United Kingdom and many other countries. It has a large number of species like *T. crithodium*, *T. spelta* which are both found mostly in the United States (Watson and Dallwitz, 2004).

Kingdom: plantae
Division: Manoliophyta
Class: Liliopsida
Order: Poales
Family: Poaceae
Subfamily: Pooideae
Tribe: Triticeae
Genus: *Triticum* L.
Species: *Triticum aestivum* L. (Kartesz, 2000).

1.2.2- Plant Origin and Distribution

T. aestivum L. is an annual grass, it is widely spread in the United Kingdom, Europe, Westren and Middle Asia, North Africa, United State of America. The place of origin is the area known in early history as Fertile Crescent in the upper region of the Tigris and Euphrates rivers (Briggle and Curtis, 1987). *Triticum* is tolerant to a wide range of conditions starting from moist to rain and dry zones, annual temperature 4.9˚C to 27.8˚C. *T. aestivum* could tolerate soils having pH 4.5 to 8.3, so it is well adapted to a wide range of soils (Watson and Dallwitz, 2004).

1.2.3- Plant Uses

Triticum species have different uses, they are the main world source of flour used mainly in bakeries. It is used as livestock feed, both as fodder and as hay. *Triticum* seed embryo is a rich source of vitamin E which is an essential nutrient for healthy skin, hair, gland, kidneys and muscles. Wheat embryo is considered one of the best remedies for miscarriage and birthing prematurely. It is also used in beer manufacturing, biodegradable plastics from wheat starch. It is also used as a row material for cosmetics, ethanol production and turkey feed. *Triticum* byproducts are now used like ethanol from wheat straw (Richman, 2002).

1.2.4- Crude oil

Crude oil is an organic compound formed millions of years ago. It was formed from the remains of tiny aquatic plants and animals that lived in ancient seas. Petroleum owes its existence largely to marine organisms, as these organisms died they sank to the sea bed then buried with sand and mud forming an organic rich layer. Increased pressure and heat from the weight of the layers above caused partial distillation of the organic remains, transforming them slowly into crude oil and natural gas.

Crude oil is one of the environmental pollutants and its toxicity is a problem of increasing significance for ecological, evolutionary and nutritional reasons. Crude oil is a smelly yellow to black liquid and is usually found under ground areas called reservoirs. It contains hydrocarbonic compounds having different characteristics in their chemical structure and they are alkanes like ethan, propane and methan. Aromatic hydrocarbons like benzene, toluene, ethyl benzen and xylene.

Polycyclic aromatic hydrocarbons like nephthallene, phenathrene and anthracene. Although various types of hydrocarbon molecules made of hydrogen and carbon atoms that form the basis of all petroleum, they differ in their configurations. The carbon atom may be linked in a ring or a chain, each with a full partial complement of hydrogen atoms. Some hydrocarbons combine easily with other materials and some resist such binding. The petroleum industry often characterizes crude oils according to their geographical source. Oils from different geographical areas have unique properties; they can vary in their constancy from light volatile fluid to semi solid oils (Macaky, 1991; Committee on *in situ* Bioremediation, 1993; Lyons, 1996).

Crude oils are generally classified by Romano (2007) into the following types:

1. Class A: light, volatile oils. These oils are highly fluid often clear, spread rapidly on water or solid surfaces, have strong odor, a high evaporation rate and are usually flammable. They penetrate porous surfaces such as dirt and sand, they do not adhere to surfaces; flushing with water generally removes them. This class of oils may be highly toxic to humans and animals. Most refined products and many of the highest quality light crude's can be included in this class.
2. Class B: non sticky oils. These oils have a waxy or oily feel, they are less toxic and adhere more firmly to surfaces, as temperature increase their tendency to penetrate porous increases. Medium to heavy paraffin based oils all into this class.
3. Class C: heavy, sticky oils. They are viscose, sticky or tarry, they are brown to black in colure flushing with water will not readily remove this material from surfaces, but the oils do not penetrate

porous surfaces. Their density is like water and they often sink. They have low toxicity. This class includes residual fuel oils and medium to heavy crude's.

4. Class D: non fluid oils. They are relatively non toxic, do not penetrate porous surfaces and are usually black or dark brown in color. When heated they melt and coat other surfaces making cleanup very difficult. Residual oils, heavy crude oils and some high paraffin oils fall into this class.

Crude oil contains non hydrocarbonic compounds like sulfur, nitrogen and oxygen in addition to small amounts of trace metals like cadmium (cd), mercury (Hg), nickel (Ni), sodium (Na) and potassium (K) (Schnoor *et al.*, 1995).

1.2.5- Sources of soil contamination

Soil is a complex system with many interacting components. The soil is part of the earth's crust, which is composed of minerals and dead organic matter supporting the biological population of its own and varying wildly in its ability to produce crops in favorable climatic conditions.

The soil is exposed to different kinds of contaminants such as chemicals, oil spills, food waste and metal elements. Most of these contaminants could be removed through physical and chemical treatments (Desord *et al.*, 2002). Another factor of soil contamination is alteration of the natural soil environment. This type of contamination typically arises from the rapture of underground storage tanks, application of pesticides, percolation of contaminated surface water to sub surface strata, leashing of wastes from landfills or direct discharge of industrial wastes to the soil.

The most common chemicals involved are petroleum hydrocarbons, solvents, pesticides, and heavy metals (Bauman, 1991).

Heavy metals are a source of soil contamination. It represents a hazardous waste, including materials from chemical products, dyeing, electroplating and heat treatment, the production of batteries, metal treatment, mining, scrap yards, service station and tanning (Chen, 2002).

Okolo and Ode (2005) stated that crude oil pollution adversely affects the soil ecosystem through the adsorption to soil particles, provision of an access carbon that might be unavailable for microbial use and induction of a limitation in soil nitrogen and phosphorus. Bauman (1991) stated crude oil and its derivatives have paused a serious problem of environmental pollution because of their toxicity to most of the living organisms. Crude oil is a source of soil contamination; it comes from leaks in the oil transporting pipes due to sabotage operations. Car leaks from car accident and leaks from refinery factories are considered another source of soil contamination.

1.2.6- Phytoremediation

The term Phytoremediation ("phyto" meaning plant, and the Latin suffix remedium" meaning to clean or restore) actually refers to a divers collection of plant based technology that use either naturally occurring or genetically engineered plants for cleaning contaminated environment (Cunningham *et al.*, 1997; Flathman and Lanza, 1998).

Elevated concentrations of crude oil results in growth inhibition and toxicity symptoms in plants. Plants possess a range of potential cellular mechanisms that may be involved in the detoxification of crude oil and thus tolerance to oil stress (Hall, 2002).

The use of plants to clean up contaminated environments is not new, about 300 years ago plants were proposed for use in the treatment of waste-water (Hartman, 1975). Studies before the nineties of the last century presented the crude oil effects negatively on plants not knowing the plant advantages in the removal of oil from soil. (Cunningham *et al*., 1996).

Several studies illustrated that plants absorb crude oil via their roots and accumulate it at a small degree in their roots and shoots. Durmisheidze (1977) reported that rice seedlings take up [^{14}C] methane through their roots and that bean and corn seedlings take up ethane, propane, and butane through their roots and leaves.

Desord *et al.,* (2002) and Lasat *et al.,* (2000) reported that phytoremidation is an emerging technology that employs the use of higher plants for the clean up of contaminated environments. Soil is exposed to different kinds of pollutants like heavy metals and oil compounds that could be removed by chemical and physical treatments but the use of plants is better due to its low expenses and also improve the landscape in addition this type of cleaning preserve the basic structural compounds of the soil.

Phytoremediation works best with low to medium amount of pollution. Plants remove harmful chemicals from the ground when their roots take in water and nutrients from polluted soil, streams and ground water. Plants can clean up chemicals as deep as their roots can grow. Tree roots grow deeper than small plants, so they are used to reach pollution deeper in the ground. Once inside the plant, chemicals can be either stored in the roots, stem or leaves, changed into less harmful chemicals within the plant or change into gases that are released into the air as the plant transpires (Boyajian and Devedjian, 1997).

The time that phytoremediation takes to clean up sites depends on several factors:

- Type and number of plants being used.
- Time and amount of harmful chemicals present.
- Size and depth of the polluted area.
- Type of soil and conditions present.

These factors vary from site to site. Plants may have to be replaced if they are destroyed by bad weather or animals. This adds time to the clean up; often it takes many years to clean up a site with phytoremediation (Schnoor *et al*., 1995). Phytoremediation may be applicable for the remediation of metals, pesticides, solvents, explosives, crude oil, for example some plant species have the ability to store metals in their roots. They can be transplanted to sites to filter metals from wastewater. As the roots become saturated with metal contaminants, they can be harvested. Hyper-accumulator plants may be able to remove and store significant amount of metallic contaminants (Doe, 1995).

Phytoremediation consists of plant based remediation of oil polluted soil, this includes:

1. Phytotransformation, which involves the root absorbance to the oil contaminants from soil then decomposing them to a less toxic compounds accumulating in plant tissues which are used later in the plant metabolism. This is an important ecological application due to its effect in decreasing contamination in soil (Schnoor *et al.*, 1995). Plant absorbance efficiency depends on the chemical and physical properties of the contaminants.

Transpiration is the key that specify the absorbance of the chemicals. Increasing transpiration increases contaminants absorption and vise versa. This depends on plants type, leaf area, type of nutrition, soil moisture and temperature (Eddan, 2005).

2. Phytostimulation, which it is also known as rhizosphere bioremediation via encouraging the microorganisms found in the root area through the secretion of exudates (Hudge and Flecher, 1996). Bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Depending on the site and its contaminants Bioremediation may be safer and less expensive than alternative solutions such as incineration or landfilling of the contaminated materials. It also has the advantage of treating the contamination in situe so that large quantities of soil, sediments, or water do not have to be dug up or pumped out of the ground for treatment. Microorganisms population multiplication depends on the roots secretions (Foth, 1990).

They benefit either directly or indirectly from these secretions like the secretion of root enzymes which is considered a source of carbon, nitrogen and phosphorus to these organisms, secretion of the mucigel which is an oily substance that decreases the fractionation between the root and the contaminated soil (Horvath, 1972).

3. Phytostabilization: includes plants which are used to stabilize oil contaminants in the soil and prevent their spreading. This type of remedy is mostly used with plants grown in contaminated swamps with metals especially organic contaminants. It occurs through the evaporation of large quantities of water by the plant pores which prevent the translocation of contaminants to other places.

Using fertilizers like phosphate in contaminated lands with heavy metals like zinc and lead in the presence of plants contribute in the stabilization of these metals by binding with them (Schnoor, 1997).

4. Rhizosphere filtration: It involves the use of plants to clean various aquatic environments. This type of remediation is used with weapon powder and explosives like TNT (2,4,2-Trinitrotoluene) that could be easily absorbed by plant roots like Canary Grasses (Young, 1996). Using this type of remedy in aquatic swamps contaminated with toxic metals and crude oil gives high successes ratio when it's used for long period of time leading to the reduction of sulfur, increase in the pH and decrease in the contaminants concentration (Wielder, 1993 and Walski, 1993).

5. Phytoextraction: it is the use of plants to remove contaminants from soils, sediments or water into harvestable plants biomass. It is a process used for the extraction of heavy metals more than organics in the contaminated soils. It is emerged as s cost-effective, environment-friendly cleanup alternative. To restore soil excavation by using soil washing followed by physical or chemical separation of the contaminants (Salt *et al.*, 1995).

The cleaning process for the crude oil polluted sites is more successful when the contaminants concentration is not too high otherwise it will inhibit plants growth in these areas (Lee and Banks, 1993). As shown in Figure (1) plants use one of the following mechanisms to remove oil spills and contaminants of petroleum hydrocarbons:

1. Degradation:

Plants and microorganisms are involved in the degradation of oil contaminants (petroleum hydrocarbons) to different products such as alcohol, water, carbon dioxide that are considered products less toxic and less dangerous to the environment when compared to the untreated

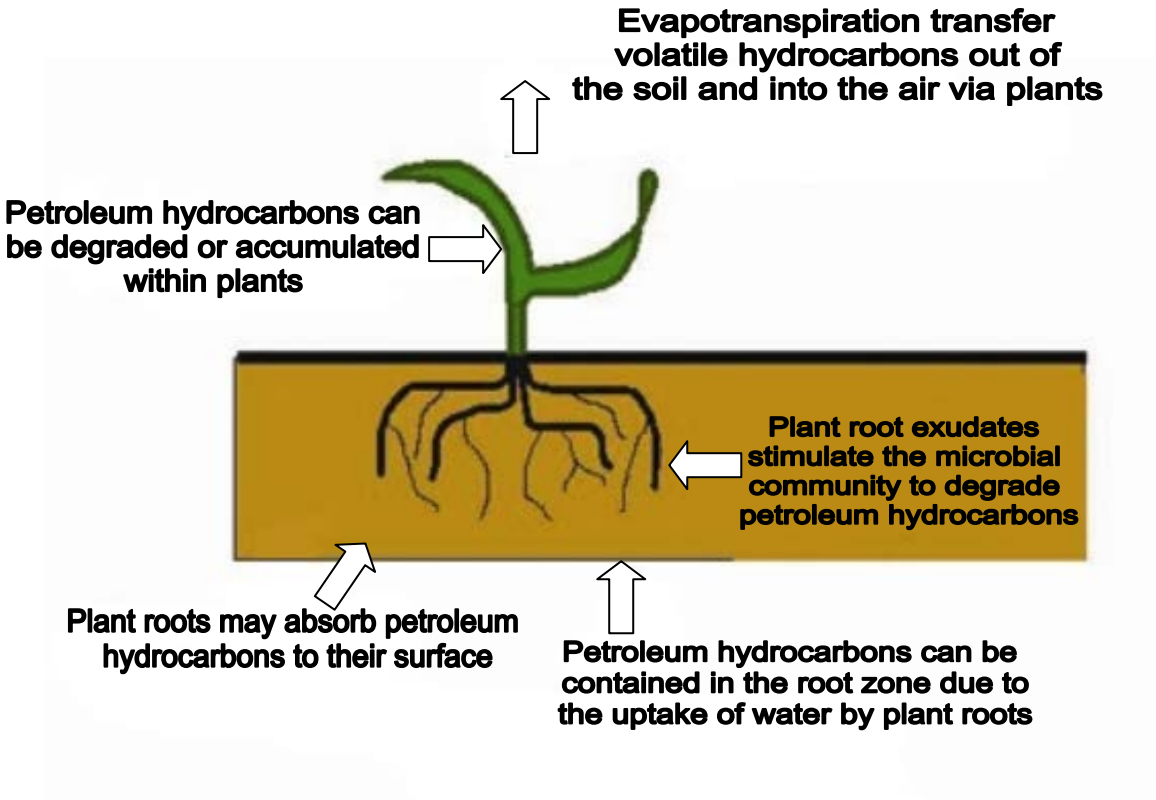


Figure (1): Phytoremediation mechanisms, degradation, contaminants, or transfer of petroleum hydrocarbons in soil via interactions with plants and microorganisms (Frick *et al.*, 1999).

crude oil components (Eweis *et al.*, 1998). Another indirect role that plants play in the degradation of petroleum hydrocarbons involves the release of enzymes from the roots; these enzymes are capable of transforming organic contaminants by catalyzing chemicals in soil. Schnoor *et al.*, (1995) identified plant enzymes as the causative agents in the transformation of contaminants mixed with sediments and soil. These findings suggested that plant enzymes might have an effect extends beyond the plant itself and temporal effects continuing after plant death (Cunningham *et al.*, 1996).

2. The Rhizosphere Effect

The Rhizosphere also called the root area is the area were the soil is connected to the plant roots and its under direct control of the root system. It supplies the microorganisms with exudates that contain

important nutrients for the microorganism's growth (Campbell, 1985). In turn, these microorganisms work on cleaning the soil from contaminants (Paul and Clark, 1989; Anderson *et al.,* 1993; Gunther *et al.,* 1996; Atlas and Bartha, 1998).

The roots exudates can influence the type of interaction between plants and soil microorganisms. For instance, the interaction can be specific or non-specific depending on the exudates. Specific interaction occurs when the plant exudes a specific compound or compounds in response to the presence of contaminants. Non specific interaction occurs when typical or normal plant exudates are chemically similar to organic contaminants, resulting in increased microbial activity and increased degradation of contaminants (Silica and Germid, 1998).

The rhizosphere soils of all plants containing greater numbers of these hydrocarbons utilizing bacteria than bulk soils and this rhizosphere effect was more pronounced for plants growing in oil contaminated soil compared to clean soil.

3. Decrease oil contaminants from spreading

Oil contaminants are redacted or deactivated by plants. Plants have different methods to decrease contaminants spreading through its roots that works as an organic pump which work on pulling the contaminants to the rhizosphere through water absorbance leading to the isolation of contaminants from the soil. Plant roots also have indirect effect on decreasing contaminants spreading through its enzyme secretion that binds with the contaminants in the soil forming the humus (Cunningham *et al.,* 1996).

4. Phytovolatization

It is a process where plants have the ability to transfer oil volatile compounds from the soil to the atmosphere (Flathman and Lanza, 1998).

Plants deal with crude oil contaminants on the basis of they are either volatile organic compounds which are easy to evaporate making them hard to be detected in soil or plants. Or non-volatile organic compounds, which have hydrophobic nature, making them hard to be absorbed by the plant roots to be transferred to the atmosphere through the plants pores.

Plants absorb compounds with low molecular weight but do not do so with high molecular weighted compounds. In addition to environmental conditions, plant features like plant size, plant age and root type have an effect on the absorbance of oil contaminants from the soil by the roots (Anderson *et al.*, 1993).

The benefits and limitations of using phytoremediation techniques phytoremediation are:

1. Inexpensive.
2. Improves the landscape.
3. More effective in cleaning surface polluted areas near the root.
4. Used as a final cleaning step to the polluted sites after exposing the contaminated area to the sunray for specific period of time (Gatlife, 1994).

Schnoor (1996) summarized the limitations of phytoremediation as follows**:**

1. The difficulty of growing plants in the contaminated soil with depths more than 3 meters.
2. The contaminants could be absorbed and stored in the plant leaves, and then these leaves will decompose and the contaminants will return to the soil.

3. Lacking the ability to ensure the lowest levels of actual cleaning in a certain period of time.
4. The difficulty of creating a wide range of plant cover due to the contaminants toxicity.

1.2.7- Plant tissue culture

Plant tissue culture techniques have many applications in life. It can be defined as the production of plants from very small parts (such as shoot tip, embryos, nodes and axially buds) in sterile controlled environments. It also includes cell culture and the initiation of callus and cell suspension cultures which are used for different purposes (Stiff, 2006).

Croughan *et al.,* (1981) and Gibbs *et al.,* (1989), outlined the advantages of tissue culture techniques over traditional breeding methods as follows:

1. Large variation in genotypes may be obtained.
2. Large numbers of individuals can be evaluated and selected using relatively little space.
3. Time between generations can be reduced.
4. The environment is closely controlled.
5. Physiology can be studied at the cellular level.
6. Cultured cells are relatively undifferentiated, which reduce the complications caused by differences in the morphology and stage of growth.
7. Culture of plant cells on rigidly defined media permits uniform and precise treatments

Ramawat (2004) and Anderson (1980) reported four sequential stages in plant tissue culture system, namely, establishment, multiplication, rooting and acclimating. Explants (starting point for all tissue culture) from any plant

structure or part, can be excised, disinfested, and placed on the surface of culture medium to produce callus that is as a result of wounding and in response to plant growth regulators, either endogenous or supplied into the medium. A number of different culture media have been used to grow callus, but the most common is Murashige and Skoog (MS) medium. This medium is rich in macroelements, microelements, sucrose and certain vitamins (Purohit, 2003).

One major application of plant tissue culture is *in vitro* screening and selection for resistant plants, which provide the variation, required for the crop improvement program. An assumption is made during the selection at the cell level that the resistant to a particular set of a cultural or environmental conditions is expressed in the same way in the cells as in the intact plant (Collin and Dix, 1990). Several screening and selection methods have been used to select tolerant variants. The efficiency of certain method depends on the effectiveness of selection-desired variants out of a large number of individuals. Most have used callus culture for selection to environmental stresses tolerant cell lines (Naik and Babu, 1988).

Callus cultures have been used routinely in selection for resistance or tolerance to a selective agent in the medium. The risk that not all the callus aggregate may be uniformly exposed to the agent. Small pieces of the callus may be used to overcome such a problem. Cells tolerant to the agent grow out as a small mass on the side of an otherwise dead callus piece. The new growth can be easily identified and subcultured (Collin and Dix, 1990).

One of the important steps in the application of biotechnology for crop improvement is the successful plant regeneration from cells, organs or tissues. Many investigations on tissue culture and plant regeneration in a variety of plants including wheat have been well documented.

Plant regeneration from wheat tissue culture has mainly been obtained from callus derived from immature embryos (Sears and Deckard, 1982; Ozgen *et al*., 1996). Callus induction from immature wheat embryos are commonly used for genetic transformation (Simmonds and Grainger, 1993; McCormac *et al.*, 1998). Thus, first of all, for biotechnological research on wheat requires reliable callus induction and then plant regeneration. Especially, for an efficient genetic transformation, establishment of a successful wheat tissue culture system using immature or mature embryos is essential. Immature embryos in wheat have been reported as a good source of callus induction (Redway *et al*., 1990).

However, immature embryo production is time dependent procedure and difficult to obtain all seasons of the year. Varshney *et al.*, (1996) stated that the maturity stage of explants is also important for embryogenic callus formation. Therefore, this problem led researchers to develop mature embryo derived callus.

A few attempts have been made to use cell culture systems to screen for variants to increase callus tissues resistant to toxins secreted into media (Toyoda *et al.,* 1989*).* Fungal growth in coculture with callus cultures was correlated with tolerance to a particular stress like heat, cold, drought and crude oil.

Regeneration of shoots from poplar leaf explants exposed to glyphosate (Herbicide) gave rise to glyphosate tolerant plants (Michler and Haissing, 1988). In pea, *in vitro* sensitivity of some commercial cultivars showed some correlation with field sensitivity to glyphosate (Yenne *et al.,* 1987). Cell lines tolerant to elevated levels of salt in the medium have been selected in *Coleus blumei* (Ibrahim *et al.*, 1992). Watad *et al*., (1991) reported tobacco plants tolerant to elevated levels of salt in the medium.

Conventional plant breeding has improved yield partly by increasing crude oil tolerance of crop plants. Genetic improvement in grain yield has been demonstrated under both favorable and stressed conditions over a period of several decades. Therefore, breeders are continuing to look for genetic variability and more effective selection criteria, especially under unfavorable conditions. Recent developments in plant tissue culture techniques in combination with genetic engineering offer a much wider scope for improvement in crude oil tolerance.

Plant cell tissue culture techniques offer several advantages for wheat improvement, particularly in respect of drought tolerance and some crude oil derivatives. Immature embryos of wheat may be used as an explant source to study embryogenesis, plant regeneration and somaclonal variations (Maddock *et al.*, 1983; Cooper *et al.*, 1986).

2.1- Materials

2.1.1- Apparatus and equipments

The following equipments and apparatus were used through out the experimental work:

Apparatus	Company
Autoclave	Karl /Germany
Distillator	GFL / Germany
Electric balance	Mettler (Switzerland)
Incubator	Sanyo / Japan ESCO
Laminar air flow cabinet	Sanyo / Japan ESCO
Micropippettes	Brand / Germany
pH-meter	Metter Gmbh-Teledo / England
Refrigerator	Ishtar
Sensitive balance	Delta Range / Switzerland
Oven	Gallenkamp / England

2.1.2- Chemicals

Chemicals	Company
Ethanol	BDH
Ammonium nitrate	Mall
Potassium nitrate	BDH
Calcium chloride anhydrate	Fluka
Magnesium sulphate anhydrate	Fluka
Potassium phosphate monobasic	Fluka
Boric acid	Merk
Potassium iodide	Tetanal
Manganese sulphate.$4H_2O$	BDH
Zinc sulphate.$7H_2O$	BDH
Molybdic acid (sodium salt).$2H_2O$	BDH
Cupric sulphate.$5H_2O$	BDH
Cobalt chloride.$6H_2O$	BDH
Sodium ethylene diamine tetraacetate	Fluka
Ferrous sulphate.$7H_2O$	BDH
Thiamine.HCl	BDH
Nicotinic acid (free acid)	Kochligh
Pyrodoxine.HCl	BDH

Continued

Sodium hypochlorite	Local market
Diethyl ether	BDH
2,4-diclorophenpxyacetic acid	BDH
Glyine	BDH
Kinetin	BDH
Agar- Agar	Sleeze
Crude oil	Kirkuk Government/ Iraq

2.2- Methods

2.2.1- The place of study

This study was carried out in the plant tissue culture laboratory, Biotechnology Department, College of Science, Al–Nahrain University. The field experiment was conducted in pots in the gardens of the college of science during the period 1/11/2005 to 1/4/2007.

2.2.2- Plant material

The two varieties of *T. aestivum* seeds (Abu Ghraib-3 and Dijila) were kindly supplied by Dr. Ibrahim AL- Shamari. Ministry of Science and Technology.

2.2.3- Preparation of culture medium

Half power (Murashige and Skoog, 1962) medium was prepared and used (Table 1). Sucrose 30 g/l, Myoinositol 100 mg/l and the plant growth regulators 2,4-D and Kin at different concentrations were added.

Table (1). Components of Murashige and Skoog (1962) culture medium.

Macronutrients		
Components	Chemical formula	Weight (mg/l)
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride anhydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate anhydrate	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate.$4H_2O$	$MnSO_4.4H_2O$	22.30
Zinc sulphate.$7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate.$5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt chloride.$6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetraacetate	Na_2-EDTA	37.3
Ferrous sulfate.$7H_2O$	$FeSO_4.7H_2O$	27.8
Vitamins and Organics		
Thiamine.HCl	$Cl_2H_{17}C1N_4OS.$ HCl	0.1
Nicotinic acid(free acid)	$C_8H_{11}NO_3.HCl$	0.5
Pyrodoxine.HCl	$C_6H_5NO_2$	0.5
Glycine(free base)	$C_2H_5NO_2$	2.0
Myoinstol		100

The pH was adjusted to 5.8 using NaOH or HCl (1N), then 7 g/l agar type (Agar – Agar) was added to the medium, placed on a hot plate magnetic stirrer till boiling. Aliquots of 20 ml were dispensed into (10×5.5) cm culture vessels. Culture media were sterilized by autoclaving at 121 °C under pressure (1.04 Kg/cm^2), for 15 min. Glassware and other instruments were either autoclaved or placed in electric oven (180-200) °C for 2 hrs (Cappuecino and Sherman, 1987). The medium was left at room temperature to cool and became ready to culture the explants.

2.2.4- Plant growth regulators

Different concentrations of the auxin 2,4-D (0.0, 1.0, 2.0, 3.0 or 4.0)mg/l and the cytokinin kinetin (0.0, 0.25, 0.50, 0.75 or 1.0)mg/l were prepared and added to the culture media before autoclaving.

2.2.5- Seeds surface sterilization

T. aestivum L. seeds were surface sterilized. Briefly, they were rinsed with continuous shaking in 3% NaOCl for 15 min., and then rinsed three times with sterilized DDH_2O. Seeds were then submerged in DDH_2O for 24 hrs, to imbibit seeds. Embryos were dissected and then cultured on half power MS medium (George and Sherrington, 1984).

2.2.6- Initiation of callus cultures

Combinations of plant growth regulators were prepared and added to the cultural medium to determine the most effective one on callus induction. Embryos excised from the seeds were placed onto half power MS medium containing different concentrations of 2,4-D and Kin as in 2.2.4. Each concentration was containing 9 replicates.

The response of the embryos to callus induction was evaluated after 6 weeks in culture to determine the proper combination for callus induction.

2.2.7- Maintenance of callus cultures

The initiated callus was removed using forceps and scalpel, then pieces weighting 40mg were subcultured onto fresh medium supplemented with the same combinations of 2,4-D and Kin as in 2.2.4. Callus fresh weight was determined using sensitive balance.

2.2.8- Incubation of cultures

Surface sterilized explants (embryos) were inoculated into the culture vessels under aseptic conditions, placed in the incubator at 25±2 °C in total darkness.

2.2.9- Exposure of callus cultures to crude oil

Stock solution of Kirkuk crude oil whose chemical and physical properties are displayed in table (2) was prepared by mixing 100ml of the crude oil with 900ml of DDH_2O to achieve a concentration of 10%. To prepare the rest of the stock solutions, aliquots of 20, 40, 60 and 80ml of the stock (10%) crude oil solution were taken and diluted with 80, 60, 40 and 20ml of DDH_2O respectively, giving the following concentrations

Table (2). The physical and chemical properties of Kirkuk crude oil (Iraq Institute of Oil Research) cited from Al-Gelawi (1985).

Characteristics	Results
Specific gravity 15/4	0.844
Specific gravity 20/4	0.841
API gravity	36.1
Kinematics viscosity (CS) 20 ºC	7.05
Kinematics viscosity (CS) 37 ºC	4.55
Pour point ºC	-21
Flash point ºC	20
Reid vapor pressure (bar) 37.8 ºC	0.386
Reid vapor pressure (Psi) 37.8 ºC	5.6
Water by distillation (Vol. %)	Nil
Total sulfur (wt. %)	2.10
Hydrogen sulfide (ppm)	20
Salt content NaCl (wt. %)	0.002
Total salinity (wt. %)	0.004
Wax content (wt.)	2.40
Melting point of waxes (ºC)	2.50
Ash content (wt. %)	0.007
Lower heating value (mth/kg)	10.205
Gross heating value (mth/Kg)	10.885
Nitrogen (wt. %)	0.12
Vanadium (ppm)	8.0
Nickel (ppm)	5.0
Iron (ppm)	4.0
Aluminum (ppm)	1.5
Sodium (ppm)	1.0
Calcium (ppm)	1.0
Copper (ppm)	0.4
Lead (ppm)	0.1
Manganese (ppm)	0.1

(2, 4, 6 and 8%). Those were added to the culture medium before autoclaving.

Callus pieces (40mg each) were placed on the surface of the culture medium supplemented with the different concentrations of the crude oil. Each concentration included 9 replicates. Results of this experiment were evaluated after 6 weeks.

2.2.10- Callus subculture on oil free medium

The survival cells of the callus pieces for the two wheat varieties exposed to different concentrations of crude oil were subcultured onto oil free MS callus maintenance medium. This process was repeated every 6 weeks for 3 subcultures until sufficient amount of callus fresh weight was obtained.

2.2.11- Second exposure of callus cultures to crude oil

After a sufficient amount of callus fresh weight was collected, callus pieces weighting 40mg each were subcultured for the second time on maintenance medium containing different concentrations of crude oil as prepared in 2.2.9. The results were evaluated after 6 weeks in culture.

2.2.12- Field Experiment

T. astivum seeds were germinated on different concentrations of crude oil at six concentrations with three replicates. Each replicate included 20 seeds for each concentration of crude oil. These were (0.0, 40, 80, 120 160 or 200)ml of crude oil each diluted with (120)ml of Diethyl ether (BDH) to give the following concentrations of crude oil (0, 2, 4, 6, 8 and 10%) respectively (Al- Gelawi, 1985).

This experiment was accomplished at (December- February), in pots with diameter of 16 cm using clay silt soil. Mechanical analysis of soil samples were kindly carried out in soil laboratories of the Technical College, Musaib. Each pot was containing 2Kg of soil (sand (280)g, clay (1280)g and silt (440)g) contaminated with crude oil. These pots were placed under field conditions. Seeds started germinating after 7 days. Germination percentage and seedling height were recorded weekly for 6 weeks.

2.2.13- Statistical analysis

Mean and standard errors were calculated as follows:

Mean $= X = \frac{\Sigma x.}{n}$

Variance $= \sigma^2 = \frac{\Sigma(x - X)^2}{n-1}$

Standard deviation $= \sqrt{\sigma^2} = \sigma$

Standard error = $\frac{\sigma.}{\sqrt{n}}$ (Finney and Thomas, 1989).

3.1- Initiation of callus cultures

The effect of different concentrations of 2,4-D and Kin on the response (%) of callus induction on mature embryos excised from the seeds of the two wheat varieties is shown in tables 3 and 4. Response occurred at the concentrations (1.0, 2.0, 3.0 and 4.0)mg/l of 2,4-D in the presence of (0.5)mg/l Kin. They achieved 97, 100, 99 and 95 for Abu Ghraib-3 and 100% response for Dijila at the above mentioned four concentrations.

Table (3): Percentages of embryos initiated callus on MS medium supplemented with different concentrations of 2,4-D and Kin for Abu Ghraib-3 variety after 6 weeks in culture (n=9).

Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	97	100	99	95
0.75	*	*	*	*	*
1.0	*	*	*	*	*

(*) No callus induction occurred.

Table (4): Percentages of embryos initiated callus on MS medium supplemented with different concentrations of 2,4-D and Kin for Dijila variety after 6 weeks in culture (n=9).

Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	100	100	100	100
0.75	*	*	*	*	*
1.0	*	*	*	*	*

(*) No callus induction occurred.

As shown in tables 5 and 6, the highest callus fresh weight initiated on mature embryos excised from the seeds of the two wheat varieties occurred at the combination of (2)mg/l 2,4-D and (0.5)mg/l Kin giving (120.6)mg for Abu Ghraib-3 and (131.2)mg for Dijila (figure 2). Callus fresh weight decreased reaching (0.0, 100.0, 117.2 and 79.4)mg for Abu Ghraib-3 and (0.0, 120.0, 129.5 and 90.4)mg for Dijila at (0.5)mg/l Kin and (0.0, 1.0, 3.0 and 4.0)mg/l 2,4-D respectively. In addition no callus was reported on the rest of the interactions between the other combinations of the two growth regulators. Al-Shammari, (2007) reported that mature embryos excised from some wheat varieties require relatively high concentrations of auxins for callus initiation. Generally, callus induction requires a balance between auxin(s) and cytokinin(s) as reported by Skoog and Miller (1957). In a number of plant species callus induction favors higher auxins than cytokinins (Ramawat, 2004).

Table (5): Callus fresh weight (mg) initiated from mature embryos on MS medium supplemented with different concentrations of 2,4-D and Kin for Abu Ghraib-3 variety after 6 weeks (n=9).

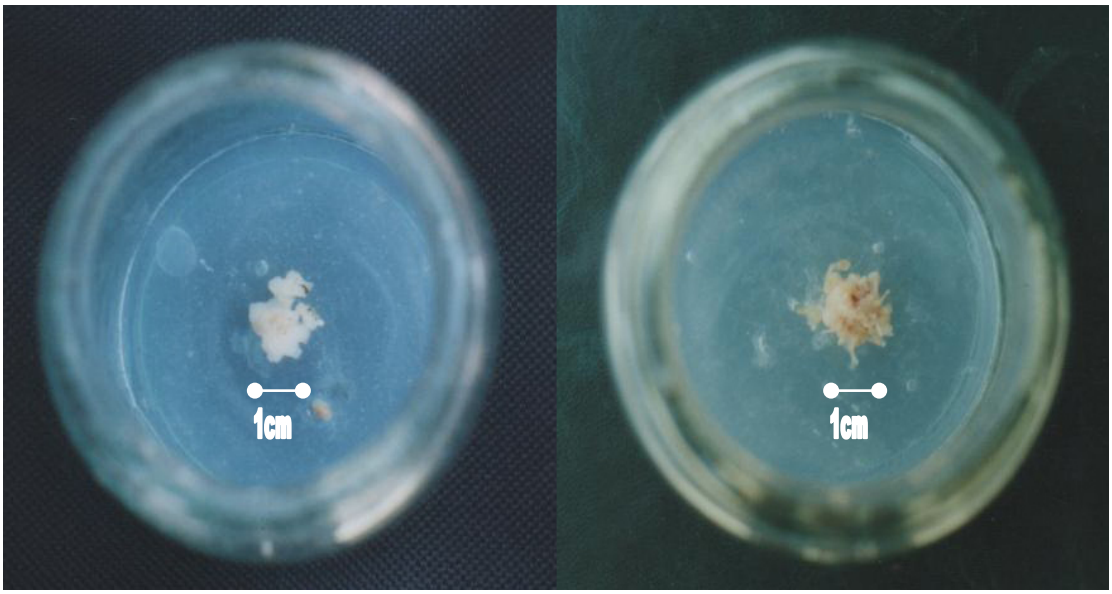
Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	100.0	120.6	117.2	79.4
0.75	*	*	*	*	*
1.0	*	*	*	*	*

(*) No callus initiation occurred.

Table (6): Callus fresh weight (mg) initiated from mature embryos on MS medium supplemented with different concentrations of 2,4-D and Kin for Dijila variety after 6 weeks (n=9).

Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	120.0	131.2	129.5	90.4
0.75	*	*	*	*	*
1.0	*	*	*	*	*

(*) No callus initiation occurred.



Abu Ghraib-3 **Dijila**

Figure (2): Callus induction from mature embryos for the two wheat varieties cultured on MS medium containing a combination of 0.5 mg/l Kin and 2 mg/l 2,4-D after 6 weeks.

The results are in agreement with Mohammad and Hassan (1998), who reported that the increase in callus fresh weight that induced on sunflower explants grown on MS medium supplemented with 2,4-D and Kin is due to the effect of these growth regulators in encouraging cells to divide and expand. But, the decrease in callus fresh weight at higher concentrations of 2,4-D was due to the depression effect of this auxin leading to less cell division and expansion in different plants like corn and barley calli (Devlin and Wethman, 1998; Al-Romi, 2001).

Auxins are organic acids that have the ability to affect plant biological processes at low concentrations. Auxins induce cell elongation, root initiation, cell division and callus formation. Additionaly, they encourage apical dominance and inhibits lateral buds and leaf abscission. Auxins are important due to their effect on the cell wall, since they increase it's plasticity leading to an increase in the cell wall extensibility.

The turgor pressure caused by osmotic forces in the cell sap causes water to inter the cell resulting in cell enlargement. Plasticity is not reversible as a result of breaking the cross links between the cellulose microfibrils of the cell wall. The increase in cell size occurs in two stages, first, loosing of the cell wall and the second, uptake of water and an expansion of the cell wall (Taiz and Zeiger, 2002). This conclusion agrees with Varshney *et al.*, (1999) for the induction of callus from mature embryos excised from wheat seeds.

3.2- Maintenance of callus cultures

Callus cultures induced on mature embryos for the two wheat varieties obtained from the best combination of 2,4-D and Kin (2 mg/l and 0.5 mg/l) respectively, were inoculated into the same combination of plant growth regulators used for callus induction to determine the appropriate concentration for callus maintenance (Tables 7 and 8).

Table (7): Callus fresh weight (mg) grown on MS medium supplemented with different concentrations of 2,4-D and Kin for Abu Ghraib-3 variety, after 6 weeks. The initial callus fresh weight is 40 mg (n=9).

Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	560.1	601.2	594.4	518.8
0.75	*	*	*	*	*
1.0	*	*	*	*	*

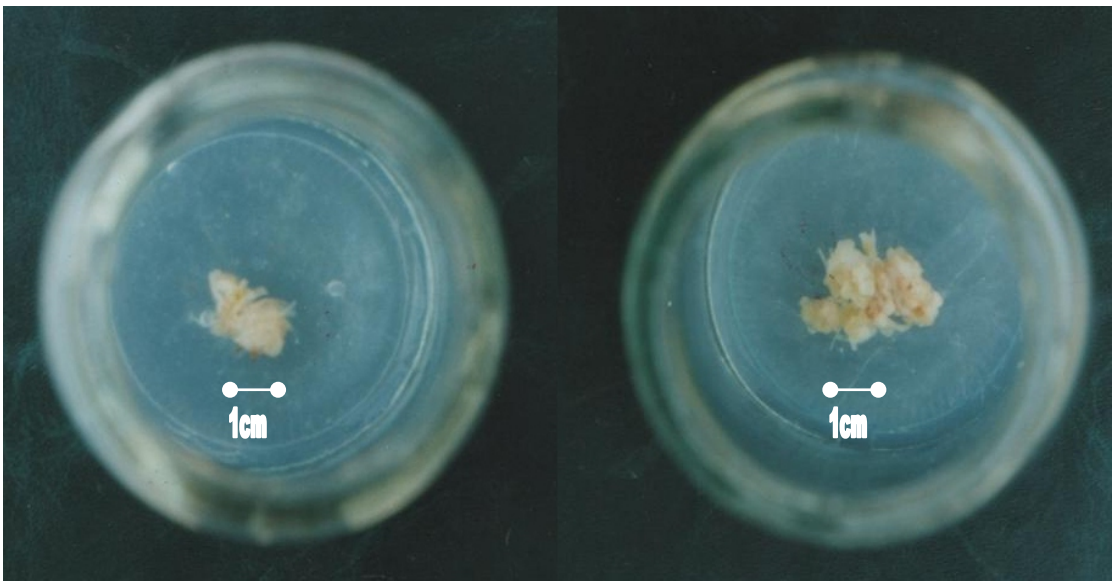
(*) No callus initiated originally.

Table (8): Callus fresh weight (mg) grown on MS medium supplemented with different concentrations of 2,4-D and Kin for Dijila variety, after 6 weeks. The initial callus fresh weight is 40 mg (n=9).

Kinetin (mg/l)	**2,4-D (mg/l)**				
	0.0	1.0	2.0	3.0	4.0
0.0	*	*	*	*	*
0.25	*	*	*	*	*
0.5	0.0	600.0	622.5	619.1	540.8
0.75	*	*	*	*	*
1.0	*	*	*	*	*

(*) No callus initiated originally.

A combination of 2 mg/l 2,4-D and 0.5 mg/l Kin produced more callus fresh weight reached (601.2 mg) for Abu Ghraib-3 and (622.5 mg) for Dijila variety. Inclusion of 2,4-D in the culture medium gave (0.0, 560.1, 594.4 and 518.8)mg for Abu Ghraib-3 and (0.0, 600.0, 619.1 and 540.8)mg for Dijila at the concentrations (0.0, 1.0, 3.0 and 4.0)mg/l of 2,4-D with (0.5)mg/l Kin respectively. However, increasing or decreasing levels of Kin prohibited callus formation at concentrations above or lower than 0.5 mg/l. Thus the combination of 2 mg/l 2,4-D and 0.5 mg/l Kin was chosen to maintain callus cultures in all subsequent experiments (Figure 3).



Abu Ghraib-3 **Dijila**

Figure (3): Callus of the two wheat varieties grown on maintenance medium supplemented with 0.5 mg/l of kin znd 2 mg/l of 2,4-D after 6 weeks in culture.

According to the results stated above callus induced from mature embryos and maintained for three subcultures on MS medium supplemented with 0.5 mg/l Kin and 2 mg/l 2,4-D. Induction and maintenance of callus cultures of *T. aestivum* L. seems to favor high levels of 2,4-D and low levels of Kin.

Auxin is generally included in a culture medium to stimulate callus production, cell growth, induction of somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures (Stiff, 2006). Cytokinin is very important for cell division in the presence of auxin, so the interaction between auxin and cytokinin led to cell division and callus induction (Abdol, 1987). Callus induction and differentiation is influenced by many factors: medium components, type and concentration of plant growth regulator, plant physiology, source of explant and environmental conditions (Torbert *et al*., 1998).

3.3- Callus cultures with crude oil

The effect of different concentrations of crude oil on callus fresh weight was determined (Figures 4 and 5). There was a progressive decline in the callus fresh weight with the increasing crude oil concentrations. Maximum callus fresh weight occurred at (0.0)% of crude oil for the two wheat varieties reaching (590.4)mg for Abu Ghraib-3 and (614.7)mg for Dijila after 6 weeks in culture. Reduction in callus fresh weight occurred at (2%) and higher concentrations recording (442.8, 425.7, 288.0, 200.2 and 102.8)mg for Abu Ghraib-3 and (459, 434.7, 300.6, 230.2 and 110.8)mg for Dijila at the concentrations (2, 4, 6, 8 and 10)% of crude oil respectively.

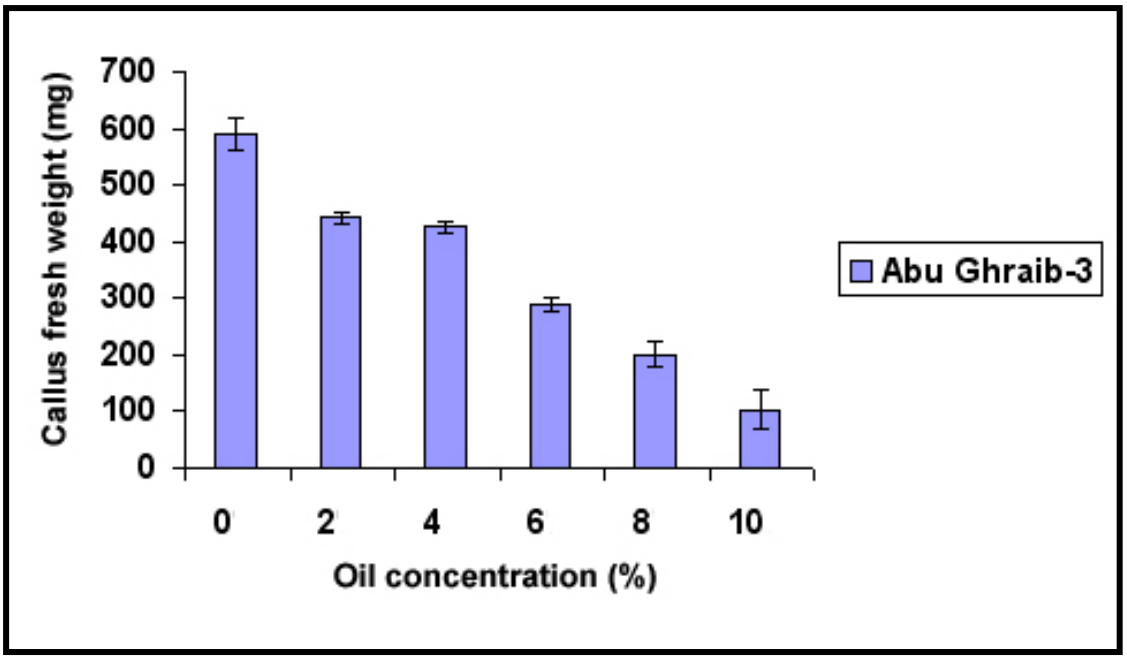


Figure (4): Effect of different concentrations of crude oil on Abu Ghraib-3 callus fresh weight (mg) grown on MS medium supplemented with 0.5 mg/l Kin and 2 mg/l 2,4-D, after 6 weeks. The initial callus fresh weight is 40 mg. Bars represents SE. (n=9)

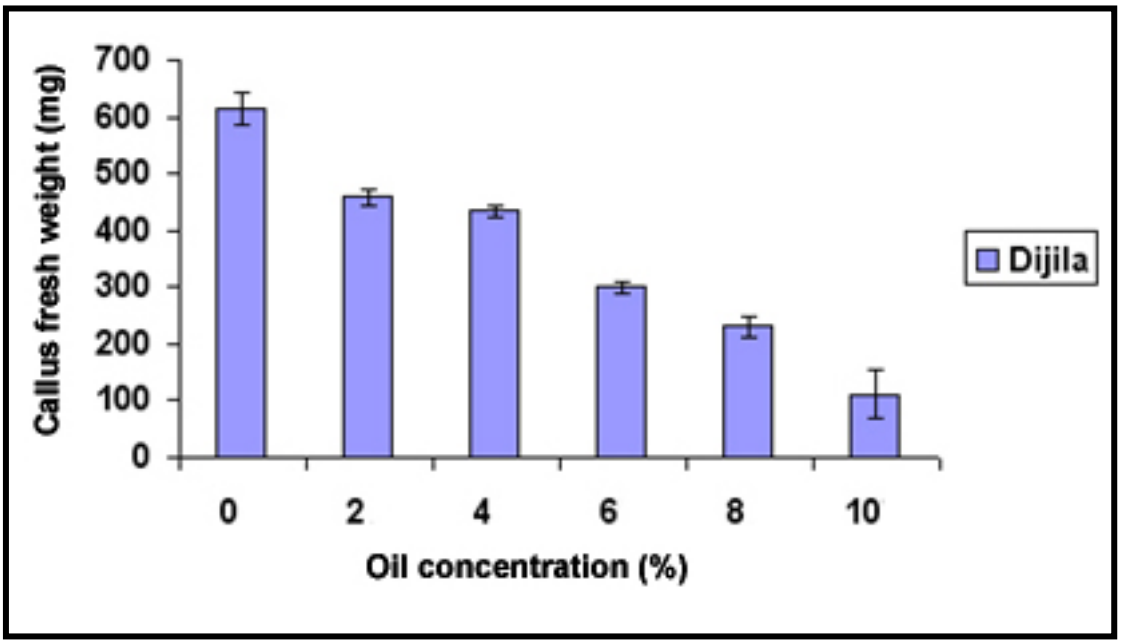


Figure (5): Effect of different concentrations of crude oil on Dijila callus fresh weight (mg) grown on MS medium supplemented with 0.5 mg/l Kin and 2 mg/l 2,4-D, after 6 weeks. The initial callus fresh weight is 40 mg. Bars represents SE. (n=9)

Most of the callus pieces at higher concentrations of crude oil showed browning and shrinkage in callus size (Figures 6 and 7) that may due to the coverage of callus by a thin layer of crude oil which prevented the entry of nutrients from the media to the callus.

Tables (9 and 10) show cells of the two wheat varieties were subcultured on crude oil free maintenance medium. This process was repeated every 6 weeks for 3 subcultures. Results showed that callus fresh weight increased with each subculture giving (3970.2, 2542.4, 2003.1, 1319.3, 890.6 and 309.8)mg for Abu Ghraib-3 and (4517.6, 3015.2, 2477.8, 1704.8, 981.4 and 488.9)mg for Dijila after 18 weeks. These calli recultured on different concentrations of crude oil which mixed with maintenance medium.

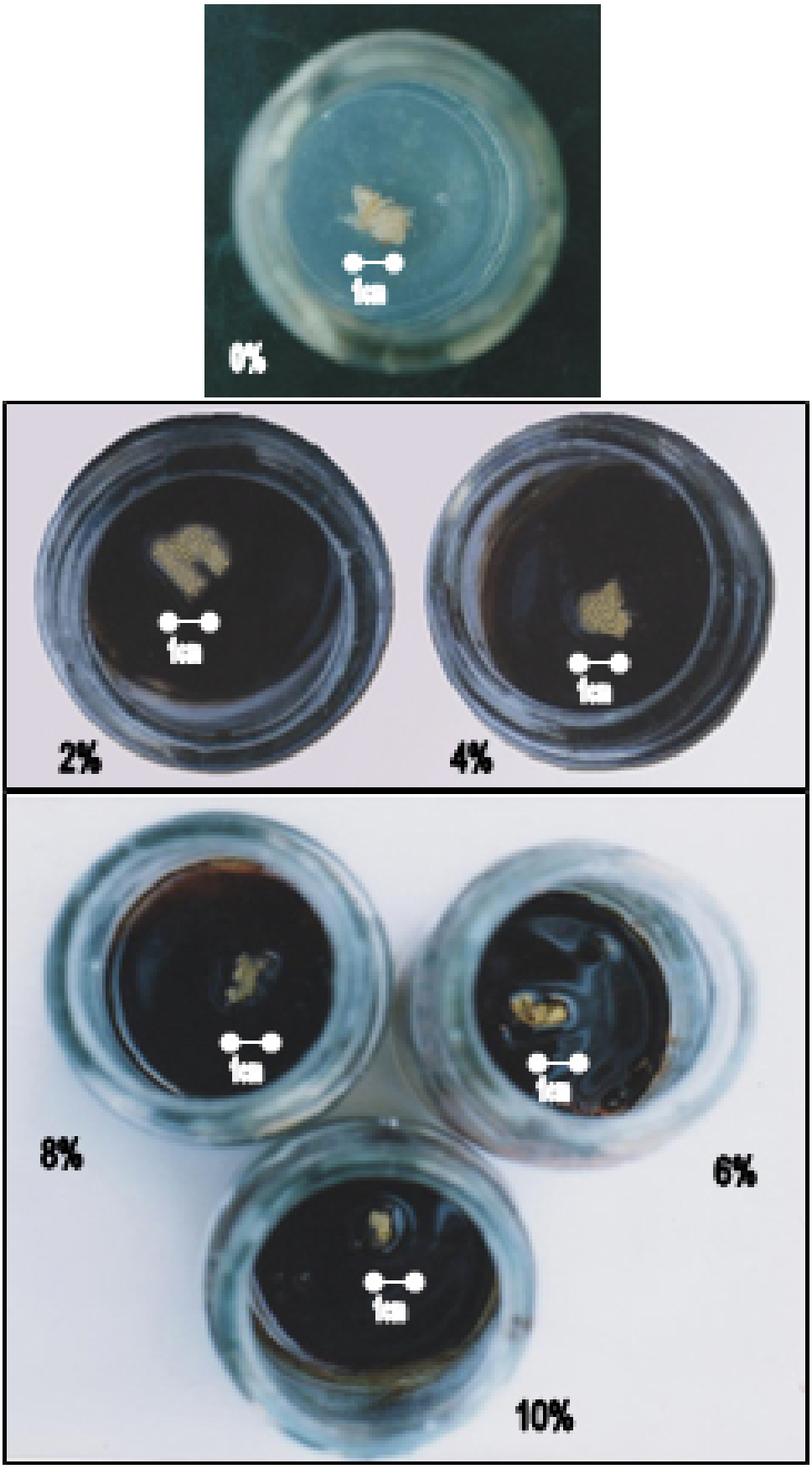


Figure (6): Callus of Abu Ghraib-3 variety grown on MS medium containing different concentrations of crude oil after 6 weeks.

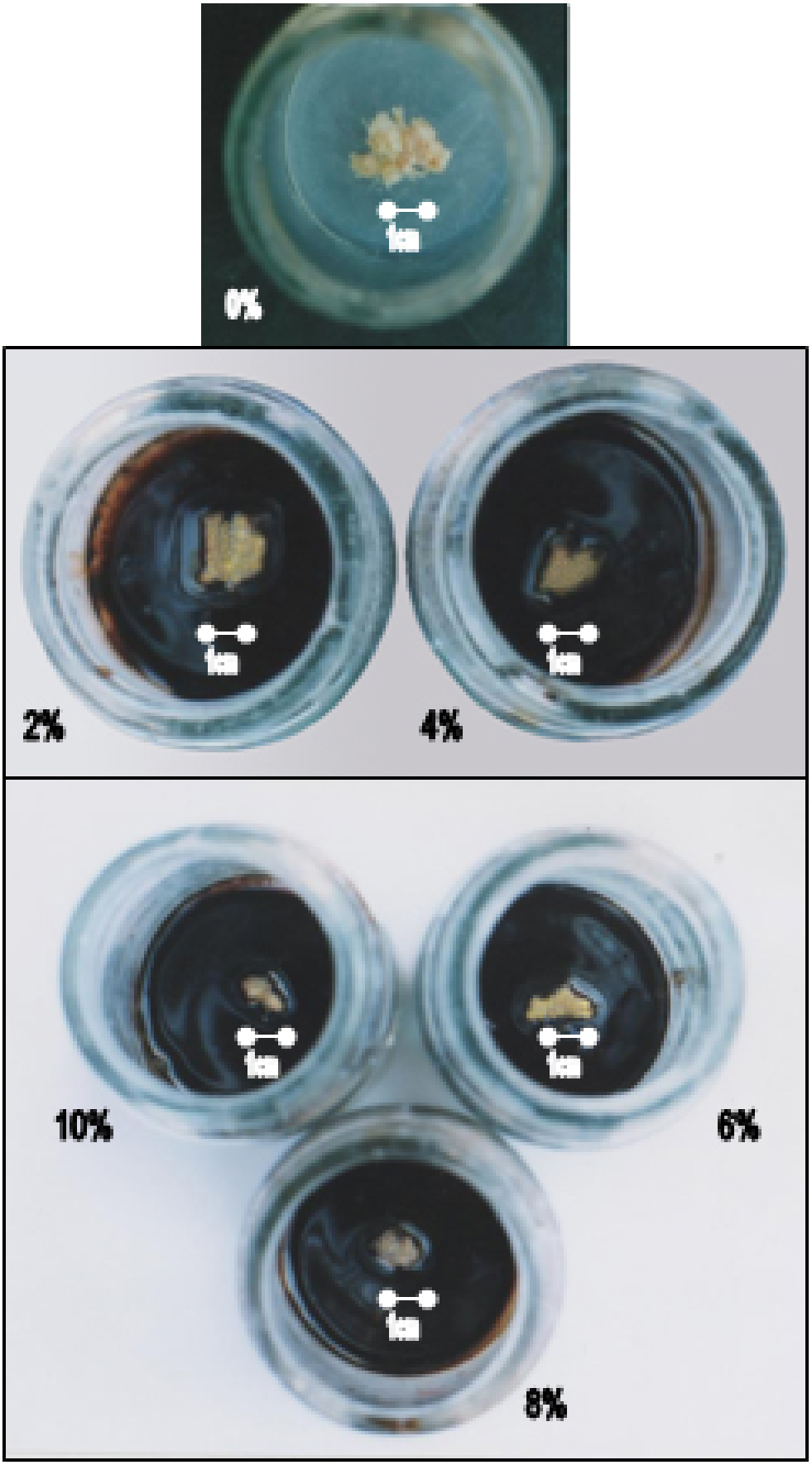


Figure (7): Callus of Dijila variety grown on MS medium containing different concentrations of crude oil after 6 weeks.

Table (9): Callus fresh weight (mg) of Abu Ghraib-3 variety at the end of three subsequent subcultures on oil free maintenance medium previously cultured at different concentrations of crude oil recultured at six weeks intervals.

%Oil conc.	Callus fresh weight (mg)		
	First subculture (after 6 weeks)	Second subculture (after 12 week)	Third subculture (after 18 week)
0	1150.8	2200.6	3970.2
2	785.6	1600.2	2542.4
4	650.8	1201.3	2003.1
6	476.2	790.4	1319.3
8	310.3	525.7	890.6
10	120.7	243.1	309.8

Table (10): Callus fresh weight (mg) of Dijila variety at the end of three subsequent subcultures on oil free maintenance medium previously cultured at different concentrations of crude oil recultured at six weeks intervals.

%Oil conc.	Callus fresh weight (mg)		
	First subculture (after 6 weeks)	Second subculture (after 12 week)	Third subculture (after 18 week)
0	1209.4	2358.8	4517.6
2	818.1	1736.3	3015.2
4	769.4	1538.4	2477.8
6	590.2	1202.2	1704.8
8	430.7	613.9	981.4
10	200.8	353.7	488.9

Figures 8 and 9 display the examination of callus tissues of the two wheat varieties grown previously on different concentrations of crude oil. Results showed that the highest callus fresh weight was obtained at (0.0)% crude oil reaching (599.4)mg for Abu Ghraib-3 and (632.7)mg for Dijila. Callus fresh weight decreased with the increase of crude oil concentration reaching (468.9, 427.5, 370.6, 85.5 and 60.0)mg for Abu Ghraib-3 and (486.9, 455.4, 393.3, 95.9 and 70.4)mg for Dijila at crude oil concentrations (2, 4, 6, 8 and 10)% respectively. The lowest callus growth was on 8% and 10% of crude oil concentrations, due to the high toxicity of oil on the callus cultures.

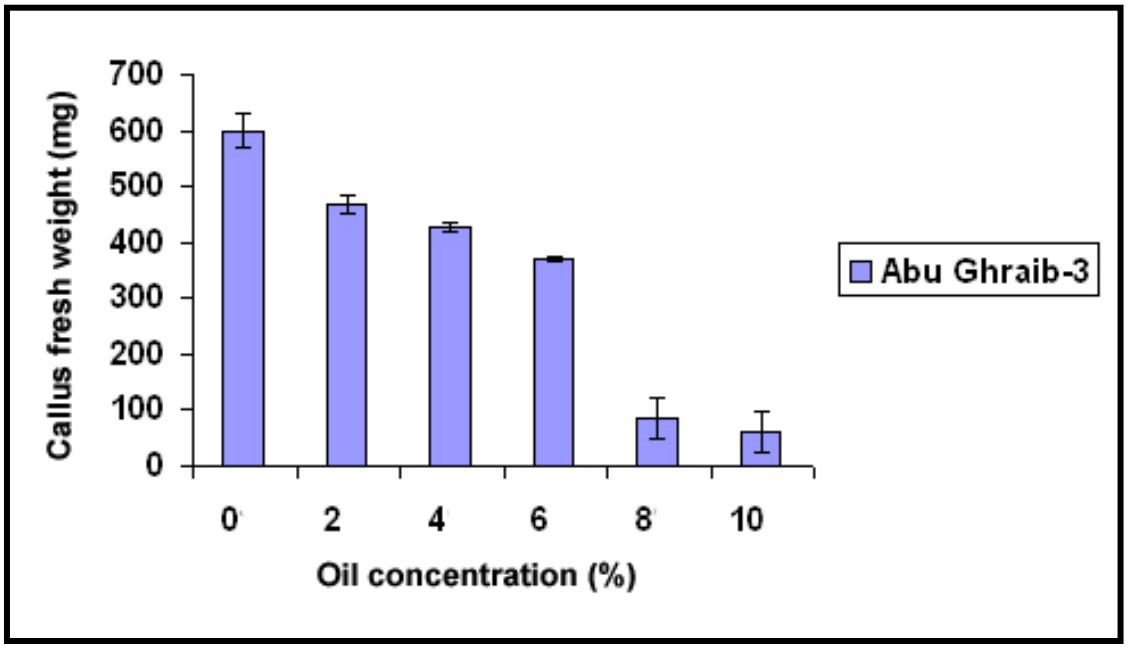


Figure (8): Examination of Abu Ghraib-3 callus tissues grown previously on different concentrations of crude oil after 6 weeks. The initial callus fresh weight is 40 mg. Bars represents SE. (n=9).

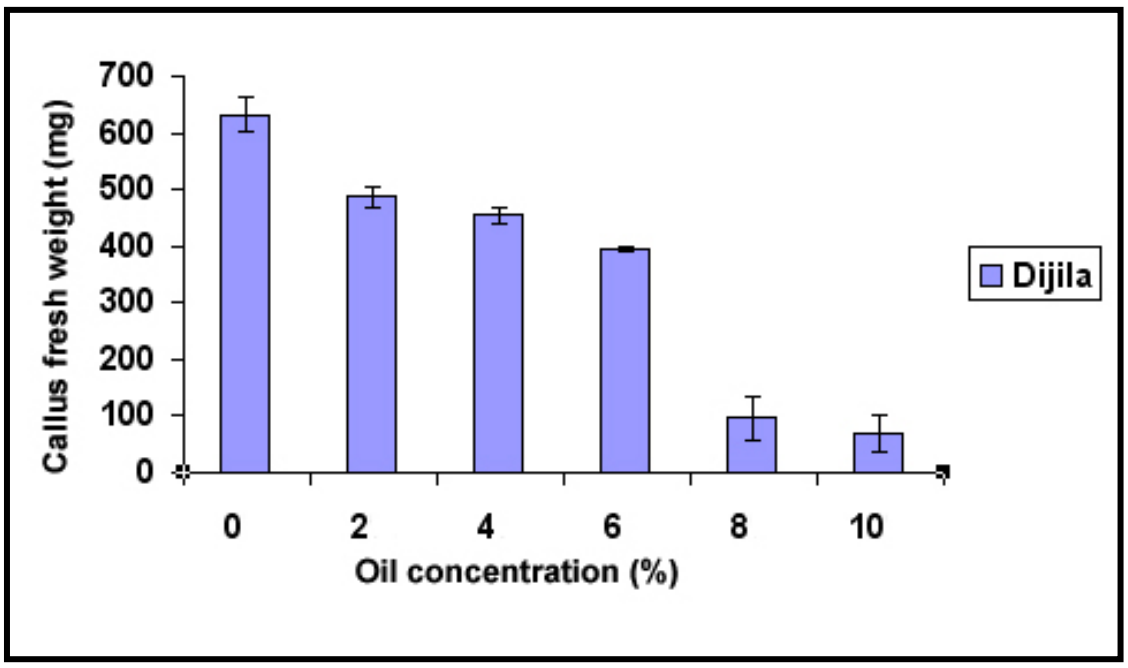


Figure (9): Examination of Dijila callus tissues grown previously on different concentrations of crude oil after 6 weeks. The initial callus fresh weight is 40 mg. Bars represents SE. (n=9).

There was increasing tolerance in callus tissues of Abu Ghraib-3 and Dijila varieties (Figures 10 and 11). Results showed that Abu Ghraib-3 callus fresh weights subcultured on maintenance medium containing crude oil in the second exposure reaching (599.4, 468.9, 370.6, 85.5 and 60.0)mg. While in the first exposure to crude oil, Abu Ghraib-3 callus fresh weight was (590.4, 442.8, 425.7, 288.0, 200.2 and 102.8)mg knowing that the (r) value was (-0.94) for the second exposure and (-0.99) for the first exposure. Dijila callus fresh weight in oil subculture (second exposure) also showed tolerance to different crude oil concentrations reaching (632.7, 486.9, 455.4, 393.3, 95.5 and 70.4)mg and the (r) value was (-0.94) but it reached (614.7, 459.0, 434.7, 300.6, 230.2 and 110.8)mg and the (r) value was (-0.99) in the first exposure to crude oil.

From this study it was shown that Abu Ghraib-3 and Dijila callus have the ability to tolerate low concentrations of crude oil for limited period (Duncan *et al.*, 1996).

It was shown that Dijila produced higher callus fresh weight and better tolerance to crude oil stress than Abu Ghraib-3. This could be due to of the genetic differences between the two wheat varieties (Dornelles *et al.*, 1997).

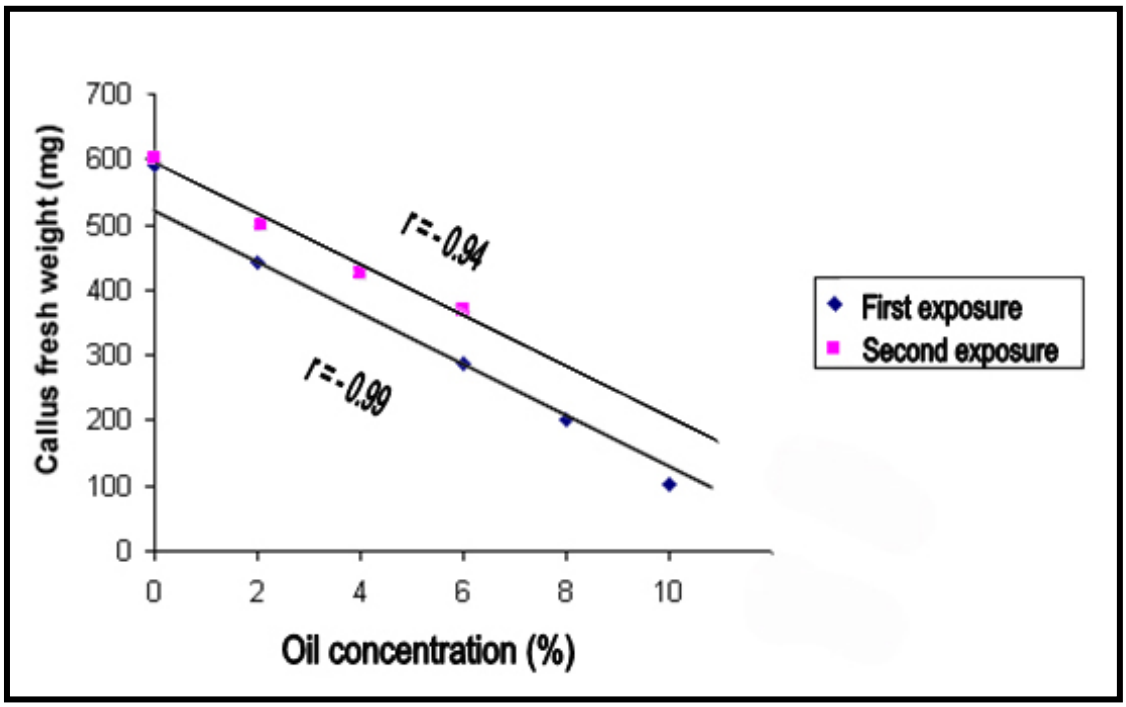


Figure (10): Enhancement in crude oil tolerance for Abu Ghraib-3 callus between the first and the second exposure to different concentrations of oil after 6 weeks in culture.

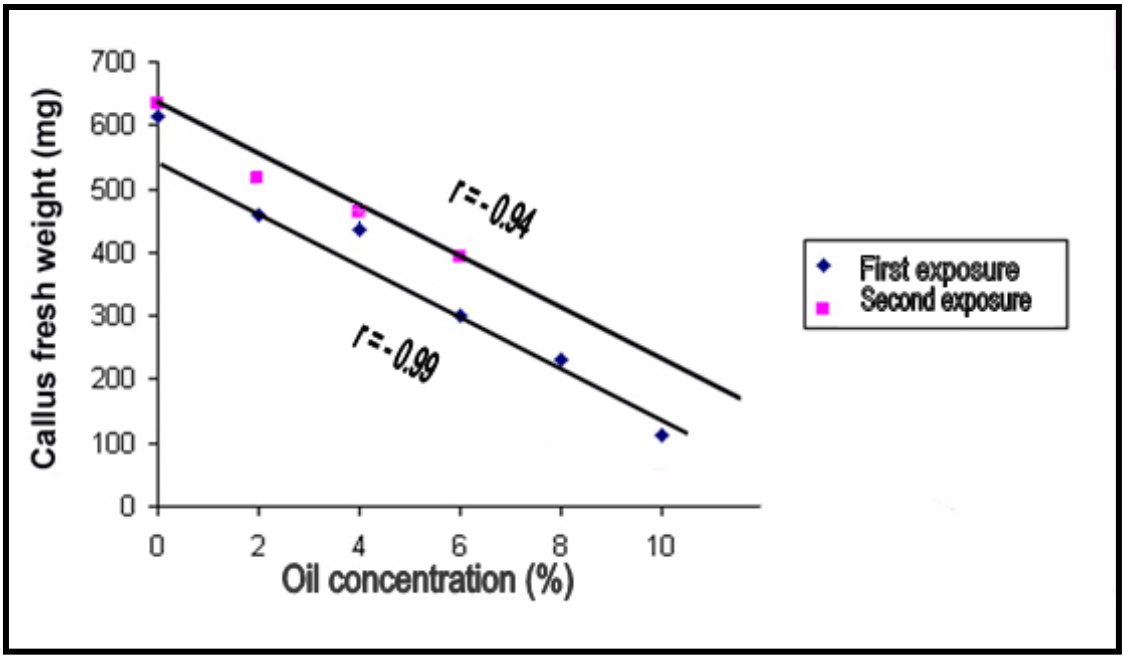


Figure (11): Enhancement in Dijila callus between the first and the second exposure to different concentrations of oil after 6 weeks in culture.

3.4- Field experiment

In order to estimate the germination percentage of the two wheat varieties subjected to crude oil concentrations under field conditions a field experiment was conducted.

Germination started for both Abu Ghraib-3 and Dijila at day (7) in most crude oil treatments reaching (16.6%) for Abu Ghraib-3 and (28.3%) for Dijila at the control treatment (Figures 12 and 13). Germination increased steadily even at high crude oil levels over the time course. At the end of the sixth week, germination percentage reached (96.6, 75.6, 60.0, 41.6, 25.0 and 18.3)% for Abu Ghraib-3 and (100, 88.3, 80.0, 88.3, 66.6 and 36.6)% for Dijila in crude oil concentrations (0, 2, 4, 6, 8 and 10)% respectively. The fluctuation in seed germination may be due to the variation exists among seeds in their ability to tolerate oil concentrations.

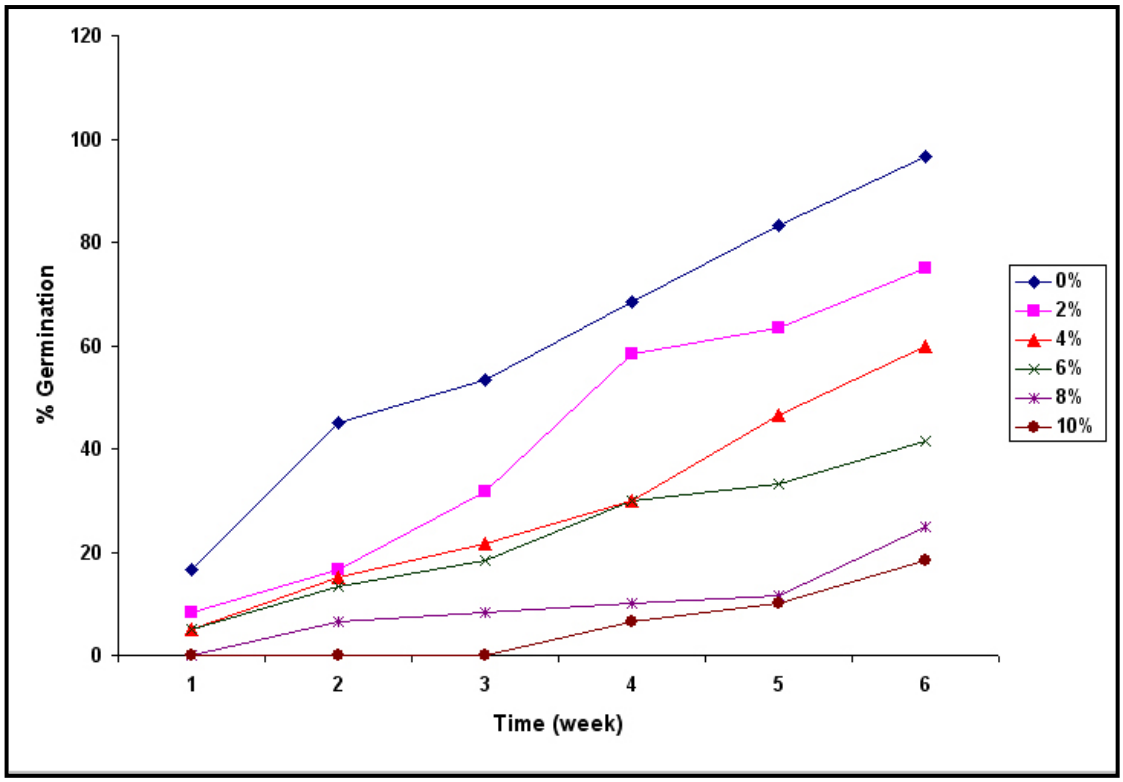


Figure (12): Germination percentages of Abu Ghraib-3 seeds germinated on different concentrations of crude oil over 6 weeks of time course.

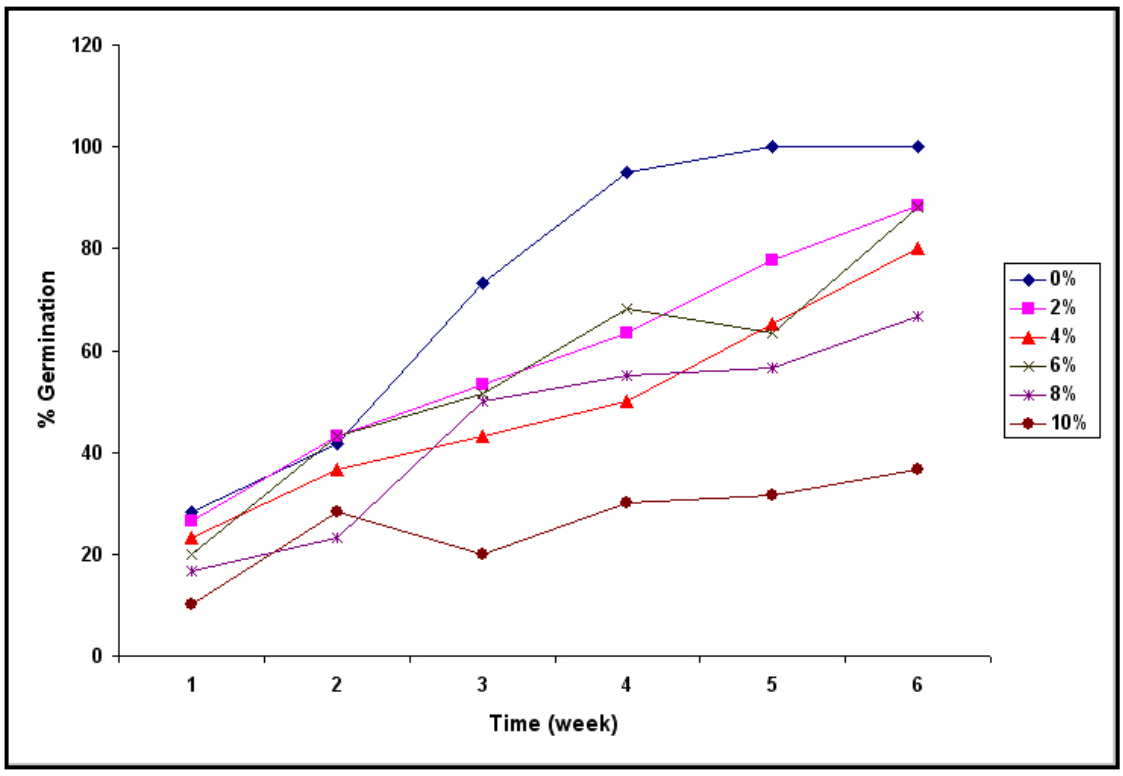


Figure (13): Germination percentages of Dijila seeds germinated on different concentrations of crude oil over 6 weeks of time course.

Plants height for the two wheat varieties started from (1.35)cm for Abu Ghraib-3 and (0.25)cm for Dijila in control treatment after 7 days of seeds sowing (Figure 14 and 15). Plant heights increased steadily with the increase of crude oil levels reaching (9.5, 5.2, 6.0, 4.5, 4.7 and 3.3)cm for Abu Ghraib-3 and (12.7, 6.6, 6.7, 5.8, 5.5 and 4.4)cm for Dijila at the crude oil levels (0, 2, 4, 6, 8 and 10%) respectively after 6 weeks of seeds sowing. It's clear that seeds of Abu Ghraib-3 variety started germinated after the third week at the highest crude oil concentration (10%), while the seeds of Dijila variety started germination at the first week even in the highest crude oil concentration (10%).

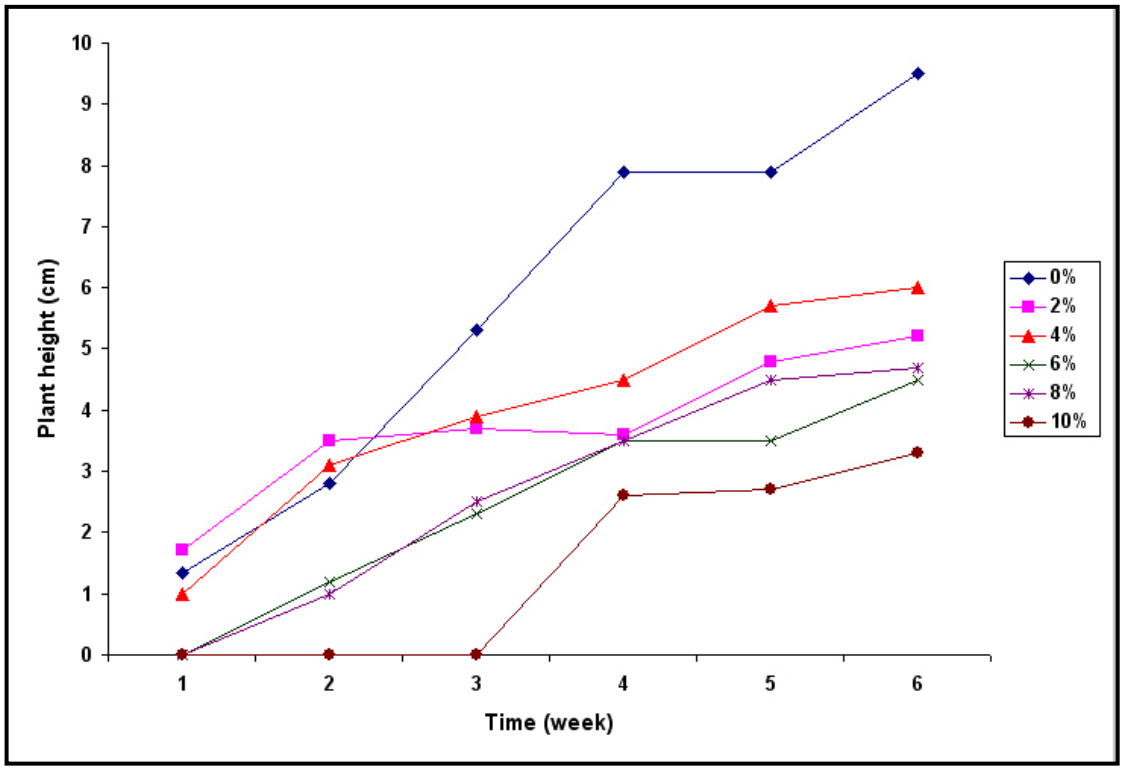


Figure (14): Effect of different concentrations of crude oil on Abu Ghraib-3 variety plant height during 6 weeks of time course.

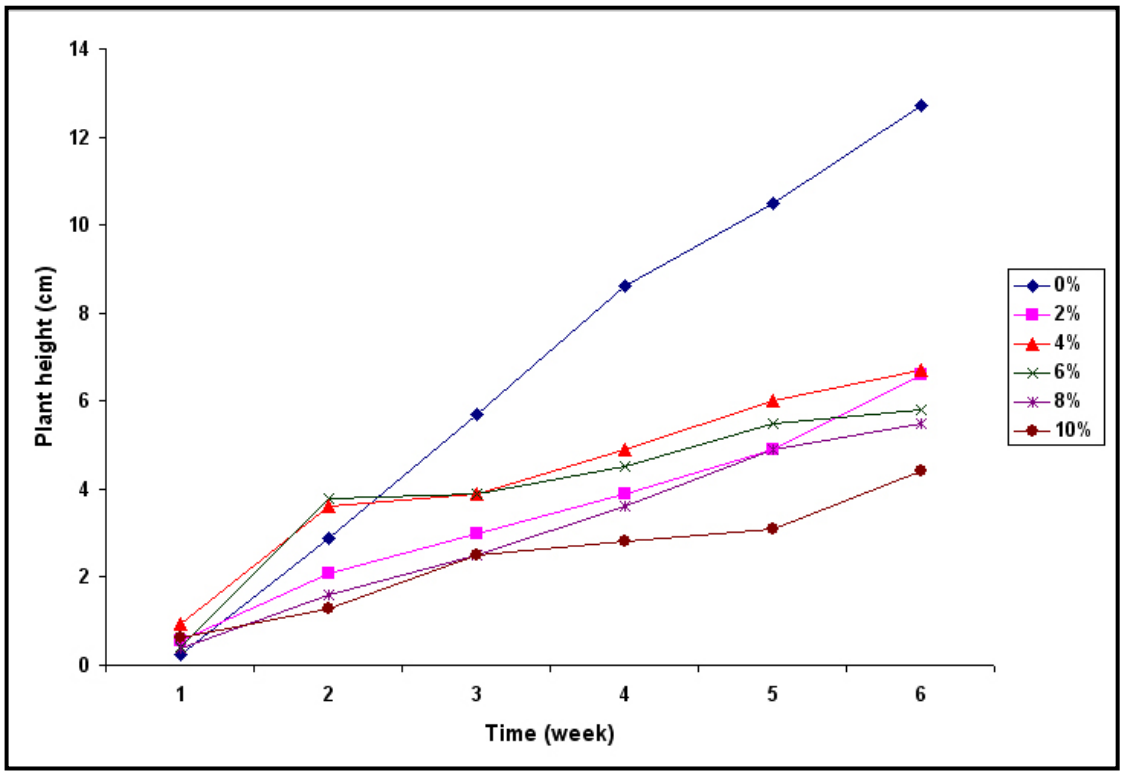


Figure (15): Effect of different concentrations of crude oil on Dijila variety plant height during 6 weeks of time course.

Crude oil is a toxic compound at high concentrations having a negative effect on seed germination at different ratios. Some plants could resist certain crude oil levels like *Hordeum vulgare* L. , *Zea mayz* L. and *Triticum aestivum* L. (Farrell *et al.*, 2002). Crude oil at high concentrations may prevent water and dissolved nutrients to enter root hairs (Schnoor, 1996; Landau *et al*., 1999). Results also showed that plant height decreased in treated plants compared with control ones.

This may be due to oil compounds, which prevent or reduce water absorption by the roots. Crude oil has a hydrophobic nature and the root is hydrophilic in its nature. It also decreases the reach of oxygen to the plant roots (Landau *et al.*, 1999; Vavrek and Campbell, 2002).

Seeds require longer periods of time to germinate in oil contaminated soils compared with the control due to the negative effect of crude oil on the germination velocity and germination stimulators (Figures 16 and 17).

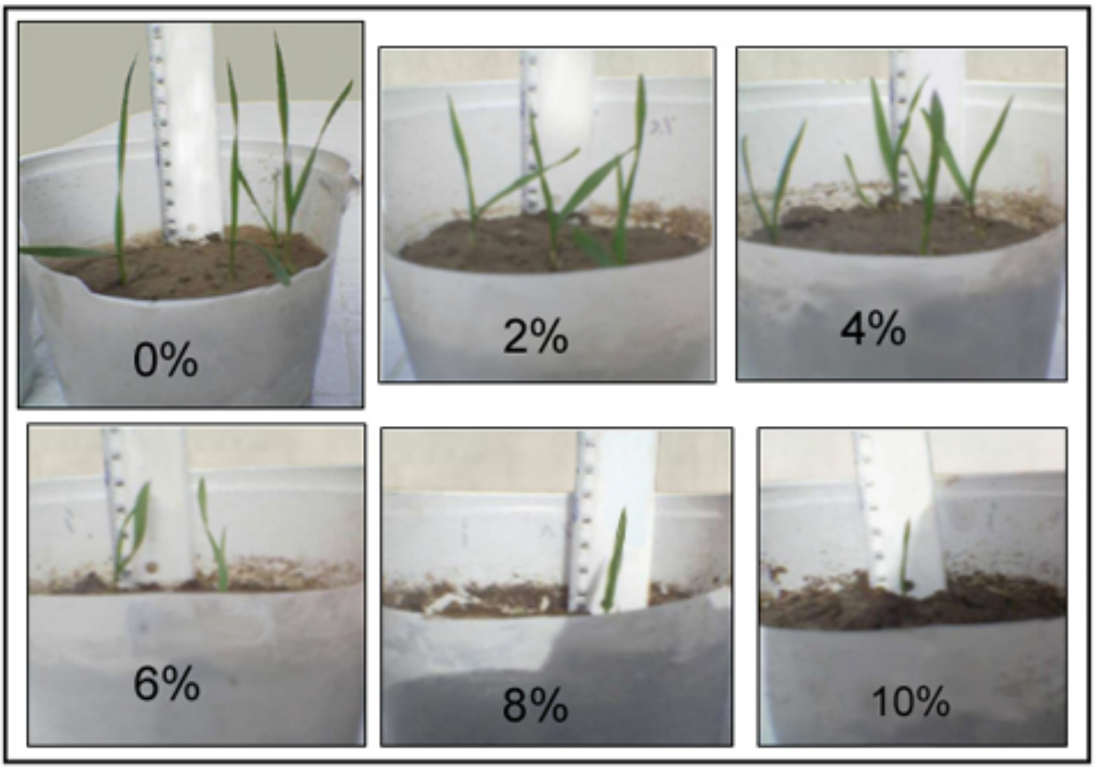


Figure (16): Abu Ghraib-3 plants grown in soil contaminated with different concentrations of crude oil after 6 weeks from sowing

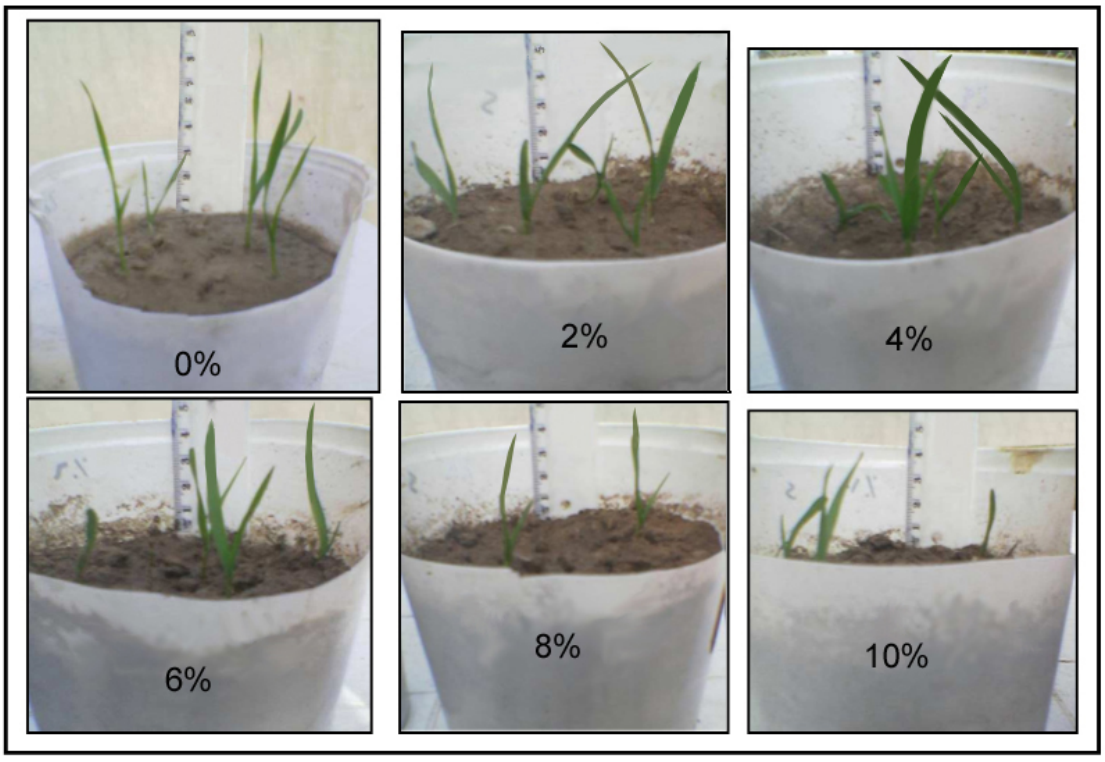


Figure (17): Dijila plants grown in soil contaminated with different concentrations of crude oil after 6 weeks from sowing.

The presence of aromatic compounds in crude oil has the ability to bind with some organic nutrients resulting in plant nutrient deficiency (Al- Gelawi, 1991). Additionally, crude oil may act as isolating layer between soil and water which may lead to less water absorption (Tagood, 1977). The conclusion is in agreement with Vedo *et al.*,(1975) who stated that oil makes oily coatings around seeds which prevent water and gas exchange.

Conclusions

1. Callus cultures of *T. aestivum* L. can be induced and maintained on MS medium supplemented with 0.5 mg/l Kin and 2 mg/l 2,4-D using mature embryos excised from seeds.
2. Growth of *T. aestivum* L. callus reduced with increasing of crude oil concentration in culture medium.
3. Callus tissues of the two wheat varieties showed tolerance to certain concentrations of crude oil.
4. It is possible to select cell lines tolerate to some extent to crude oil concentration.
5. Seeds germination and plant height of the two wheat varieties are severely affected with increasing crude oil concentrations.

Recommendations

1. Investigation of other plants as a source for phytoremediation at the cellular level using tissue culture techniques.
2. Regeneration of *T. aestivum* L. plants resulted from screening and selection of callus cultures then examination of the resulted regenerates for their tolerance to crude oil.
3. Examination of the inheritance of crude oil tolerance in seeds progeny in plants derived from *in vitro* selection.
4. Assessments of crude oil residues in the soil and plants using chemical analysis.

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List of Abbreviations

Abbreviation	Full name
DDH_2O	Double distilled water
^oC	Degree Celsius
HCL	Hydrochloric acid
PAH	Poly aromatic hydrocarbons
MS	Murashige and Skoog (1962) medium
n	Number of replicates
hrs	Hours
NaOCL	Sodium Hypochlorite
ppm	Part per million

wt.	Weight
2,4-D	2,4-Diclorophenoxy acetic acid
Kin	Kinetin
API	American Petroleum Institute
PSi	Pascal
Bar	Barometer
Vol.	Volume
SE	Standard Error
min	Minute
r	Correlation between two variants
N	Normality
NaOH	Sodium hydroxide
$\bar{X}$	Means of the variable
Σ_X	Means of the added values
n	Number of the individuals in the sample
σ^2	Variance
σ	Standard deviation
x	Variables