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INDUCTION OF CALLUS FROM LEAF AND STEM TISSUES OBTAINED FROM *CAPSICUM ANNUUM* EXPLANT GROWN ON WASTE ENGINE OIL-POLLUTED SOILS

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ABSTRACT

The present study investigated callusing frequency of explants from leaf and stem tissues obtained from *Capsicum annuum* explant exposed to waste engine oil contamination. The explants obtained from 21-31 days old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of BAP (0, 1, 2, 3, 4, 5 and 6 mg/l) and NAA, IAA, 2, 4-D and IBA (0, 5, 10, 15 and 20 mg/l). Results showed that efficient callusing in leaves and stems explants (21 days old) was achieved after explants were cultured on MS semi-solid media containing 1-5 mg/l BAP and 0.5-1.5 mg/l IBA for 4 weeks; while callus formation was hindered in older tissues. An effective protocol for callogenesis of 21 day-old explants (stems) of *C. annuum* obtained from oil-polluted soil in a semi-solid state MS medium using BAP and IBA was thus developed. The study also documented the ability of IBA to induce callus.

Keywords: Callus, Callogenesis, morphogenesis, explant, tissue culture, oil-polluted soil

INTRODUCTION

In order to survive poor economic conditions, there is usually the option of diversification of income by poor locals. One of such is the proliferation of alternative employment like artisans including auto mechanics. In many poverty-impoverished countries, particularly in the third world, there seem to have been an increase in the siting of mechanic workshops as well workshops of other artisans which use and dispose of engine oils and other waste petroleum products (**Ikhajiagbe, 2010; Ikhajiagbe and Anoliefo, 2012a)**. These workshops range from repairers of automobiles, industrial machines and engines, as well as generating sets. These mechanics replace engine oil from motor vehicle and generator engines, and other machines. The engine oil wastes are in most cases disposed inappropriately into open vacant plots and fields as well as drainages. This action directly or indirectly leads to soil pollution with oil and its constituents; thus leading to phytotoxicity. The adverse impact oil and water-soluble factions of oil have on plant growth and development, as well as oversell crop yield has been reported (**Vwioko and Fashemi, 2005; Ikhajiagbe, 2010**).
The impact of oil on plants starts from germination. **Ikhajiagbe *et al.* (2014)** reported effects of oil on germination parameters of *Vigna unguiculata*. **Anoliefo and Vwioko (1995)** also reported reduced germination rate of *Capsicum frutescens* and *Zea mays* due to oil pollution. The inhibitory effects of oil on seed germination may be due to a number of reasons including physical limitations and biological impairment on the seeds (**Amakiri and Onofeghara, 1984**). When this occurs, crop sustainability can no longer be guaranteed, particularly during the time when the clamour for food security seems loudest. The idea of possible regeneration and proliferation of seedlings to guarantee the sustenance of species and species lines is thus imperative. This informs the basis of the study.
Recent works by **Ikhajiagbe (2010)** and **Ikhajiagbe and Anoliefo (2012a, b)** showed that soil polluted by waste engine oil resulted in chlorosis and the death of the crop plants sown within five (5) months of pollution. This finding was for current oil-in-soil concentrations of above 5% w/w. This begs the question whether it was possible for any crop plant that was sown in oil-polluted soil within 5 months of pollution to be regenerated from explants of such plants. If this were achieved, then it would be possible to save the oil-threatened plants. In the present study, pepper (*Capsicum annuum L.*) has been selected. *Capsicum annuum L.* plays a vital part in many cuisines. Apart from their use as spices, pepper contains capsaicin which has use in many pharmaceutical preparations. This plant species is usually propagated by seed; however the use of tissue culture has become an important tool in clonal propagation and breeding programs.
Although there are not yet documented evidences that oil pollution currently threatens the *in vitro* regeneration of peppers, the possibility is not far-fetched, especially in a world that now revolves round the petroleum sector. The need to address this possible threat is not the only rationale for the study; there is also the need to satisfy the ever-increasing demand for the crops. This therefore calls for more dependable breeding methods for mass proliferation of the crop in the face of the present challenges.
Most plants in oil-contaminated soils accumulate heavy metals and toxic poly aromatic hydrocarbons leading to chlorosis and eventually, necrosis (**Ikhajiagbe, 2010**). When this happens, harvesting explants for the purpose of regeneration or other in vitro studies can become very challenging. The present study intends to find out at what time in the pepper's developmental phase where successful explants exposed to waste engine oil polluted soil can be regenerated using tissue culture techniques. The objective is to induce callus from leaf and stem tissues obtained from *C. annuum* explant grown on polluted soils, employing standard *In vitro* techniques and using defined medium, supplemented with various combinations of plant growth hormones and to determine the growth rate of callus formation.

MATERIAL AND METHODS

This study was carried out in the Tissue Culture Laboratory, Physiology and Tissue Culture Division, Nigerian Institute for Oil Palm Research (NIFOR), located at 1735 land area, 29km off the City centre, off Benin - Akure Road, Benin, Edo State, Nigeria.

Collection and preparation of materials for the experiment

Top soil (0 – 10 cm) was collected from a cleared field, beside Tissue Culture Laboratory (NIFOR) and sun-dried to constant weight. Thereafter, 12.5kg of the sun-dried soil was collected using shovel and hand trowel, weighed (using measuring scale) and poured into ten (10) perforated nursery bags with three replicates labeled as control. Waste engine oil obtained as pooled from an automechanic workshop was added to the soil; this was thoroughly mixed to obtain a constant concentration of 5%w/w WEO-in-soil. The bags of soil were left for one month to attenuate, after which physiochemical parameters of polluted and control soils were determined (Table 1). Thereafter, planting of

viable pepper seeds (which were pre-soaked in water for ten hours) were carried out in the morning (7am) and left to grow for a month.

Collection of Explants

Stem and leaf explants were randomly collected at three different ages; 21, 28 and 31 days old. These three (3) age groups were preferred because previous reports by **Ikhajiagbe (2010)** revealed that plants sown in 2.5 – 10.0% w/w oil-polluted soil began to show signs of physiological stress within 2 weeks (or 14 days) of exposure to polluted soils. These explants were cultured *In vitro*.

Development of MS protocol for explants

The callusing efficiencies of *Capsicum annuum* leaf and stem explants were examined by using Murashige and Skoog (MS) basal medium (**Murashige and Skoog, 1962**) with different concentrations of plant growth regulators (PGRs). However, modified MS protocols were developed for the purpose of the study for explant regeneration. In developing the MS protocols, stock solutions were first prepared using the facilities of Tissue Culture Laboratory of the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Nigeria. All the apparatus used were thoroughly washed, rinsed twice and dried using the oven at 65^0C for 1 hour. After preparation, the solutions were stored in a refrigerator at 60^0C.

Stock Solutions

MS Macro x10 (500ml): Two hundred (200) ml of distilled water was poured in a 400ml Pyrex bottle and placed on a magnetic stirrer with the stirring bar and allowed to stir for about 5mins. A measured 9.5g of potassium nitrate (KNO_3), 8.25g of ammonium nitrate ($NH_4 NO_3$), and 0.85g of potassium dihydrogen orthophosphate ($KH_2 PO_4$), 2.2g of calcium chloride dehydrate ($Cl.2H_2O$) and 1.85g of Magnesium sulphate ($MgSO_4.7H_2O$) were weighed and poured into the bottle. After pouring the first compound, it was allowed to stir for 5 - 7mins before the other compounds were added. The solution was poured into a 500ml measuring cylinder and rinsed with distilled water. It was then made to 500ml and stored for use in a 1-litre flask.

MS Micro A x10 (500ml): A measured 250ml of distilled water was poured into a 400ml Pyrex bottle, which was placed on a magnetic stirrer (with the stirring bar) and allowed to stir. Thereafter, 1.11g of Maganous sulphate ($MnSO_4.4H_2O$), 0.315g of Boric acid (H_3BO_3), 0.43g of zinc sulphate ($ZnSO_4.7H_2O$) and 0.415g of potassium iodide were weighed and added into the stirring bottle. After 10mins, it was turned into a 500ml measuring cylinder and then made up to the mark. The solution was stored for use.

MS Micro B (500ml): Distilled water (300ml) was measured into a 400ml Pyrex bottle which was placed on a magnetic stirrer (with the stirring rod). Measured qualities (0.125g) of sodium molybdate ($Na_2MOD_4.2H_2O$), 0.0125g of cupric acid ($CuSO_4.5H_2O$) and 0.0125g of cobalt chloride ($Cl_2.6H_2O$) were poured into the bottle. After 10mins, it was turned into a measuring cylinder and then made up to the 500 ml mark.

MS Iron x100 (500ml): Fifty (50) ml of distilled water was poured into a conical flask (containing stirring bar) and placed on a hot plate. Measured 1.865g of ethylene diamine tetraacetic acid (EDTA) was weighed into a conical flask and allowed to heat at 260^0C for 10mins. Thereafter, 1.39g of ferrous sulphate ($FeSO_4.7H_2O$) was weighed into a beaker containing 30ml of water which was placed on a stirrer machine and allowed to dissolve. This was poured into the EDTA solution and allowed to heat for 10mins, and thereafter made up to the 500ml mark.

MS Vitamins x100 (500ml): 200ml of distilled water was poured into a 400ml Pyrex bottle which was placed on a magnetic stirrer and allowed to stir. Then 0.25g of thiamine-HCL, 0.125g of pyridoxine-HCL, 0.25g of Nicotinic acid, 0.1g of Glycine acid and 0.125g of calcium parthotenate were weighed and poured into the Pyrex bottle. After 10mins the entire solution mixture was turned into a measuring cylinder and made up to the 500ml mark.

Establishment of MS protocol (1000ml) for 21 and 28 day-old explants

Macro x10 ---------- 100ml
Micro (A and B) x100 ---------- 10ml
Iron x100 ---------- 10ml
Vit. x100 ---------- 10ml
Inositol ---------- 0.1g
Ascorbic acid ---------- 0.28g
Sucrose ---------- 30g

The media were divided into 28 bottles of 36ml each

MS Medium for pepper (28days old) callogenesis

Full strength MS medium supplemented with 30g sucrose, macro x10, micro x10 A and B, iron x100,vitamins x100, plant growth hormones (PGH). 36ml of the solution was dispensed into 28 bottles, each containing 0.35g of agar pH of 5.8. An aliquot of 12ml was dispensed into 84 labeled (control and polluted) screw-cap McCartney bottles, three replicates of each concentrations of PGH, autoclaved and allowed to cool at ambient temperature (28^0C). The explants (stems and leaves) were removed from the plants, rinsed with tap water; surface sterilized using 5% hypochlorite solution (containing a drop of $Tween_{20}$) for 5mins. Sterile water was used to remove traces of sodium hypochlorite solution five times to get rid of the sterilizing solution which is toxic to the stems and leaves on delayed exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 6 weeks.

MS Medium for pepper (21days old) callogenesis

The medium was in semi-solid state. The amount of agar used was reduced to 0.28g for 36ml of the solution. The explants (stems) were removed from the plants, rinsed with tap water; surface sterilized using 0.4% mercuric chloride ($HgCl_2$) solution containing a drop of $Tween_{20}$ for 5mins. Traces of the $HgCl_2$ were removed by rinsing with sterile distilled water five times to get rid of the sterilizing solution which is toxic to the stems and leaves on prolonged exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 8weeks.

Establishment of MS protocol (1350ml) for 31day-old explants

Macro x10 ---------- 135ml
Micro (A and B) x100 ---------- 13.5ml
Iron x100 ---------- 13.5ml
Vit. x100 ---------- 13.5ml
$NaH_2PO_4.2H_20$ ---------- 0.229g
Inositol ---------- 0.135g
(ASP, ARG, GLU) ---------- 0.135g
Ascorbic acid ---------- 0.338g
Sucrose ---------- 40.5g

Then, the media were divided into 45 bottles of 30ml each

MS Medium for pepper (31 days old) callogenesis

Standard MS medium is supplemented with 40.5g sucrose, macro x10, micro x10 A and B, iron x100,vitamins x100, plant growth hormones (PGH). Measured quantity (30ml) of the solution was dispensed into 45 bottles, each containing 0.28g of agar pH of 5.8. An aliquot of 10ml was dispensed into 135 labeled (control and polluted) test tubes (50ml), three replicates of each concentrations of PGH, autoclaved and allowed to cool at ambient temperature (28^0C). The explants (stems and leaves) were removed from the plants, rinsed with tap water; surface sterilized using 5% hypochlorite solution (containing a drop of $Tween_{20}$) for 5mins. Sterile water was used to remove traces of sodium hypochlorite solution five times to get rid of the sterilizing solution which is toxic to the stems and leaves on delayed exposure. The explants were cut into thin sections (using sterilized forceps and surgical blades on sterilized Petri dishes) and transferred aseptically into the test tubes containing the culture medium using sterilized forceps (i.e. inoculation) and covering them up immediately with aluminium foil passed over flame. They were then incubated in the dark growth room at 27^0C for 8weeks. The media were prepared in the main laboratory and the initiation was carried out in the Laminar flow cabinet.

Plant growth hormones (PGH) modification and supplementation for 31days old pepper

Three types of auxins and one cytokinin were used. The auxins used were Indole acetic acid (IAA), Naphthalene acetic acid (NAA) and 2,4 dichlorophenoxyl acetic acid (2,4-D), each at concentrations of 0, 1.5, 3, 4.5 and 6 mg/L. The cytokinin used was Benzyl amino purine (BAP) at concentrations of 0, 0.1 and 1 mg/L (Table 2).

Testing the effects of IAA and BAP

IAA at concentrations of 1.5, 3, 4.5 and 6 mg/L as well as BAP at concentrations of 0.1 and 1 mg/L was added. Control experiment without IAA and BAP was

also included. The possible combinations for various concentrations of the two PGH (IAA and BAP) led to an experiment with 15 treatments.

Testing the effects of NAA and BAP

NAA at concentrations of 1.5, 3, 4.5 and 6 mg/L as well as 0.1 and 1 mg/L concentrations of BAP were added. Control experiment without NAA and BAP was also included. The possible combinations for various concentrations of the two PGH (NAA and BAP) led to an experiment of 15 treatments.

Testing the effects of 2, 4-D and BAP

2, 4-D at concentrations of 1.5, 3, 4.5 and 6 mg/L and BAP at concentrations of 0.1 and 1 mg/L were added. Control experiment without 2, 4-D and BAP was also included. The possible combinations for various concentrations of the two PGH (2, 4-D and BAP) led to an experiment with 15 treatments.

Plant growth hormones (PGH) modification and supplementation for 28 and 21days old pepper

One type of auxin and one cytokinin were used. The auxin used was Indole butyric acid (IBA) at concentrations of 0, 0.5, 1 and 1.5 ppm. The cytokinin used was Benzyl amino purine (BAP) at concentrations of 0, 1, 2, 3, 4, 5 and 6 ppm (Table 3).

Testing the effects of IBA and BAP

IBA at concentrations of 0.18, 0.36 and 0.54 mg/L as well as 0.36, 0.72, 1.08, 1.44, 1.8 AND 2.16 MG/L concentrations of BAP were added. Control experiment without IBA and BAP was also included. The possible combinations for various concentrations of the two PGH (IBA and BAP) led to an experiment of 28 treatments.

Determination of source of contamination in inoculum

In other to ascertain contamination rate for explants obtained from control and polluted soils, swaps from the contaminated media were taken and cultured on PDA and nutrient agar media, using the spread plate method (Cheesebrough, 2001).

Experimental Design

The experimental design chosen was the completely randomized design (CRD). As a result, treatments were randomized over the media. Each treatment consisted of 3 replicates. The treatments were the various levels of Indole acetic acid (IAA), Naphthalene acetic acid (NAA), 2,4 dichlorophenoxyl acetic acid (2,4-D), Indole butyric acid (IBA) and Benzyl amino purine (BAP) in the media. In order to avoid bias and misidentification, treatment bottles were properly labeled according to a given treatment name and replicate number. Results were presented as mean ± standard error.

RESULTS

The aim of this experiment was to study the growth of callus formation from pepper (*Capsicum annuum* L.) leaf and stem explants, obtained from polluted soils (see Table 1), employing standard *in vitro* techniques and using defined medium, supplemented with various combinations of cytokinin and auxins. Results showed that naphthalene acetic acid (NAA) and Benzyl amino purine (BAP) supplemented on solid media resulted in the callogenesis of the control 31 day-old stem explants. Callus initiation was obtained at the concentrations of 1.5 and 4.5 (mg/l) after 8weeks. This treatment showed a low percentage of callogenesis. Callogenesis did not occur in the Indole acetic acid (IAA) and Benzyl amino purine (BAP)-amended media, as well as in the 2, 4 dichlorophenoxyl acetic acid (2,4-D) and Benzyl amino purine (BAP) media. However, 33.3% callogenesis was obtained in the control explants in the 0.1 mgl^{-1} BAP/1.5 mgl^{-1} IAA combination (Table 4). No callus was obtained in the polluted treatments. Similarly, 66.7% callogenesis was obtained in 1.0 mgl^{-1} BAP/4.5 mgl^{-1} NAA combination. Appropriate combinations of 0.1 mgl^{-1} BAP/1.5 mgl^{-1} IAA as well as 1.0 mgl^{-1} BAP/4.5 mgl^{-1} IAA showed 33.3% and 66.7% callogenesis respectively in control explants (Table 5). No callus was obtained in the polluted experiments. Similar results were obtained with the BAP/2, 4 - D combinations (Table 6). The addition of IBA (0 and 1.0 mg/l) to the culture medium containing different BAP levels greatly stimulated further callus proliferation beyond the levels obtained for IAA and 2, 4-D (see Table 7). However, there was no callus formation when the concentration of IBA was increased to 1.5mg/l at any given BAP concentration.

Callus induction with IBA/BAP-supplemented semi-solid medium at various levels of concentration of both the control and polluted explants source (21days old) after 2weeks of initiation was reported (Table 8). The age of the explants and medium state greatly stimulated further callus proliferation beyond the levels obtained for other treatments. Fresh weight of callus was obtained at increased concentrations (3, 4, 5 and 6) of BAP in polluted plant source, and control source (1, 2, 4 and 5). Calli formations were effective when the plant's (pepper) age was 21days old, medium state (which was in semi-solid state) and the type of sterilant ($HgCl_2$) used. Callus was induced with IBA in combination with the cytokinin, BAP at various levels of concentration. The use of semi-solid medium containing different levels of concentrations stimulated further callus proliferation beyond the levels obtained from other treatment.

Results also showed that the contamination rate for leaf and stem explants (31, 28 and 21days) obtained from control and polluted soils (Table 9). The rate shows seven days interval after inoculation of the explants. For the 31day-old leaf explants; the contaminated media after 8 weeks indicates fungal infection. This observation is similar to the 31day-old stem explants. The contaminated media for both 28 day-old leaf and stem explants indicates bacterial infection after 8 weeks. Results for the 21 day-old leaf and stem explants indicate bacterial and fungal infections respectively. Young plant tissues possess higher capability for response to *in vitro* culture, compared to older plant materials. This may explain the favourable responses recorded for the young stem explants. The standard medium for callogenesis of *Capsicum annuum* has been presented to be the semi-solid state MS medium that is enhanced with BAP 1, 2, 3, 4 and 5 and IBA 0.5 and 1.5, 0 and 1, 0, 0.5, 0.5 and 1.5 mg L^{-1}. Survival rate of the established calli ranged from 60 – 80%, and were kept in a dark growth room. The study confirms attainment of maximum callogenesis as long as proper concentrations of plant growth hormones were applied.

Table 1 Physicochemical properties of soil at one month after pollution, prior to sowing of pepper

Parameters	Unpolluted soil	Oil-polluted soil
pH	5.59	5.47
Electric conductivity (µs/cm)	289	339
Total organic carbon (%)	0.59	2.32
Total nitrogen (%)	0.15	0.29
Na `(meq/100 g soil)	11.10	11.10
K (meq/100 g soil)	1.16	1.16
Ca (meq/100 g soil)	22.30	22.30
Mg (meq/100 g soil)	15.40	15.40
Fe (mg/kg)	720.34	997.64
Mn (mg/kg)	13.64	11.70
Zn (mg/kg)	10.36	21.80
Cu (mg/kg)	<0.01	2.60
Cr (mg/kg)	<0.01	1.60
Cd (mg/kg)	<0.01	0.12
Pb (mg/kg)	<0.01	1.26
Ni (mg/kg)	<0.01	0.70
V (mg/kg)	<0.01	0.64
Total hydrocarbon content (mg/kg)	100.78	1268.00

Table 2 A schematic arrangements of treatments used for explants obtained at 31 days. pH - 5.8, Agar - 0.28g

	0ppm	5ppm	10ppm	15ppm	20ppm
NAA_{100ppm}/BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml
IAA_{100ppm}/ BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml
$2,4\text{-}D_{100ppm}$/BAP_{100ppm}					
0ppm	0ml / 0ml	1.5ml / 0ml	3ml / 0ml	4.5ml / 0ml	6ml / 0ml
0.1ppm	0ml / 0.03ml	1.5ml/ 0.03ml	3ml/ 0.03ml	4.5ml/0.03ml	6ml/0.03ml
1ppm	0ml/0.3ml	1.5ml/0.3ml	3ml/0.3ml	4.5ml/0.3ml	6ml/0.3ml

Table 3 A schematic arrangements of treatments applied to explants obtained from 21 and 28 days old plants. pH - 5.8; Agar - 0.35g (for 28day-old explants) and 0.28g (for 21day-old explants).

IBA_{100ppm}/ BAP_{100ppm}	0ppm	0.5ppm	1ppm	1.5ppm
0ppm	0ml/0ml	0.18ml/0ml	0.36ml/0ml	0.54ml/0ml
1ppm	0ml/0.36ml	0.18ml/0.36ml	0.36ml/0.36ml	0.54ml/0.36ml
2ppm	0ml/0.72ml	0.18ml/0.72ml	0.36ml/0.72ml	0.54ml/0.72ml
3ppm	0ml/1.08ml	0.18ml/1.08ml	0.36ml/1.08ml	0.54ml/1.08ml
4ppm	0ml/1.44ml	0.18ml/1.44ml	0.36ml/1.44ml	0.54ml/1.44ml
5ppm	0ml/1.8ml	0.18ml/1.8ml	0.36ml/1.8ml	0.54ml/1.8ml
6ppm	0ml/2.16ml	0.18ml/2.16ml	0.36ml/2.16ml	0.54ml/2.16ml

Table 4 Effects of BAP and NAA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	NAA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0	0.429±0.012 (B)	0.424±0.0.098(C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0.0	0	0.350±0.0.035(LB)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	66.7±0.0	0	0.082±0.016 (DB)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 5 Combination of different concentrations of BAP and IAA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	IAA mg	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0	0.510±0.035 (B)	0.452±0.083(C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0	0	0.054±0.009 (B)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	66.7±0	0	0.499±0.031 (DB)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 6 Effects of different concentrations of BAP and 2, 4-D in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	2,4-D mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0	100±0.0	0.481±0.078 (B)	0.462±0.0.046 (C)
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
0.1	0	0	0	-	
	1.5	33.3±0.0	0	0.114±0.027 (B)	-
	3.0	0	0	-	-
	4.5	0	0	-	-
	6.0	0	0	-	-
1.0	0	0	0	-	-
	1.5	0	0	-	-
	3.0	0	0	-	-
	4.5	63.2±1.8	0	0.231±0.019 (C)	
	6.0	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 7 Shows the effects of appropriate combinations of BAP and IBA in solid medium, callogenesis (%) and weight (colour) of callus of *C. annuum* L.

BAP mgL^{-1}	IBA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0.0	100±0	0.444±0.102 (B)	0.520±0.114 (C)
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
1.0	0	0	0	-	
	0.5	0	0	-	-
	1.0	66.7±0.0	0	0.343±0.083 (C)	-
	1.5	0	0	-	-
2.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
3.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
4.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

Table 8 Effects of different concentrations of Benzyl amino purine (BAP) and Indole butyric acid (IBA) in a semi-solid state, callogenesis (%) and weight (colour) of callus of *C. annuum L.*

BAP mgL^{-1}	IBA mg^{-1}	Callogenesis (%)		Wt. g (colour) of callus	
		Control	Polluted	Control	Polluted
0	0	100±0.0	100±0.0	0.544±0.102 (B)	0.613±0.11 4 (C)
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-
1.0	0	0	0	-	
	0.5	30.9±1.3	0	0.213±0.092 (B)	-
	1.0	33.3±0.0	62.9±4.2	0.280±0.045 (C)	0.333±0.10 0 (B)
	1.5	0	0	-	-
2.0	0	0	100±0.0	-	0.563±0.13 2 (C)
	0.5	33.3±0.0	0	0.034±0.008 (B)	-
	1.0	97.7±2.1	0	0.251±0.074 (B)	-
	1.5	33.3±0.0	0	0.232±0.062 (B)	-
3.0	0	0	66.7±0.0	-	0.182±0.07 5 (LB)
	0.5	0	0	-	-
	1.0	0	33.3±0.0	-	0.222±0.07 3 (B)
	1.5	0	0	-	-
4.0	0	0	0	-	-

	0.5	33.3±0.0	0	0.138±0.088 (C)	-
	1.0	33.3±0.0	0	0.031±0.011 (B)	-
	1.5	0	0	-	-
5.0	0	0	0	-	-
	0.5	33.3±0.0	0	0.227±0.052 (B)	-
	1.0	31.7±1.9	0	0.125±0.037 (DB)	-
	1.5	33.3±0.0	66.7±0.0	0.097±0.024 (C)	0.572±0.102 (C)
6.0	0	0	0	-	-
	0.5	0	0	-	-
	1.0	0	0	-	-
	1.5	0	0	-	-

Legend: B- Brown; C- Cream; DB- Dark brown; LB- Light brown. Results have been presented as mean ± standard error

DISCUSSION

The most significant characteristic in plant tissue culture is the explant; and until the appropriate choice of explant effective regeneration may not be achieved. **Bhojwani and Dantu (2013)** reported that the most commonly used explant for most micropropagation work is either apical bud or nodal segment. In the present study, regeneration potential of leaf and stem explants was investigated. Auxins like Indoleacetic acid (IAA) and napthaleneacetic acid (NAA) generally stimulate cell expansion, particularly cell elongation; thus promoting adventitious root development. The capacity for NAA to initiate rapid tissue response even in very small amounts has been reported (**Mineo, 1990)**. This auxin is relatively stable in plant tissues, and their hormonal influences last for extensive periods.

The functionality of most phytohormones, whether synthetic or natural, is not to the extent of acting in isolation within the plant body; the hormones rather function in relation to each other (**Mineo, 1990**). This may account for improved weights of calluses, as reported in the study, than when the auxins were used in isolation. **Sharma and Shahzad (2008)** also earlier reported no callus formation or seed germination in MS medium alone, during the induced regeneration from cotyledonary node explant of *Abelmoschus moschatus.* The capacity for NAA and BAP-supplemented MS medium to initiate callogenesis was reported. Although the popularity of use of both indolebutyric acid and naphthaleneacetic acid for propagation of plants from stem and leaf cuttings have been well documented (**Hopkins & Hüner, 2009; Taiz & Zeiger, 2010**); the supplementation of the culture media with the hormones did not initiate callogenesis in polluted explants, except in the unpolluted. The presentation of differential callus colour as reported in the study is most likely due to differences in type of explant used, the solid state of the medium, as well as possible presence of contamination of explant or medium. **Jin et al. (2014)** reported significant changes in callus colour with increase in age of *Pogostemon cablin* callus, from white to green. Initial contamination of explant is a significant factor that determines the success of callus formation. Such contamination may be of biological origin, for example microbial contamination, or chemical. In the study, plants from which explants were obtained were originally exposed to waste oil pollution, and as such the possibility for contamination from phyto-accumulated oil constituents like heavy metals and polyaromatics suffice as like possible factors for poor callus initiation. This was generally reported in the study. However, in the BAP/IBA-supplement semi-solid medium, significant callogenesis was reported. Generally, the use of semi-solid medium containing different levels of concentrations stimulated further callus proliferation beyond the levels obtained from other treatment. Hormone stability is ostensibly more significant than the total concentration of any single hormone. It is also an important factor in considering the overall effects of hormones on plant growth and morphological changes. This therefore means that the hormone variances in the experimental media states (solid or semi-solid state) ought to generate rather dissimilar effects on the growth and development of excised explants. This may be responsible for the differences in percentage callogenesis and callus weight gain in the BAP/IBA-supplemented media in solid and semi-solid states (**Mineo, 1990**). **Aghaei et al. (2013)** earlier reported significant callus induction of stem explants in *Pistacia atlantica* subsp. Kurdica inoculated in IBA-supplemented medium. Similar reported were provided by **Jin et al. (2014)** in the *in vitro* propagation of *Pogostemon cablin*, and **Caraballo et al. (2010)** *in Agave fourcroydes*. **Agrawal and Chandra (1983); Phillips and Hustenberger (1985); Agrawal *et al.* (1989); Harini and Sita (1993); Christopher and Rajam (1994, 1996); Hyde and Phillips (1996); Hassan *et al.* (1999)** reported that at elevated concentrations of BAP and low levels of auxins, *Capsicum annuum* could be regenerated from explants including hypocotyls and cotyledons. The study also reported significant callogenesis of the 21 day-old stem explants. Contamination rate for this treatment also gave a low result. The effectiveness of use of IBA in culture media has been further buttressed in this study. According to **Sharma and Shahzad (2008)**, however, the inclusion of either (0.05 mg/L) IBA or (0.05 mg/L) NAA in BA-containing media did not improve the regeneration efficiency of *Abelmoschus moschatus.* In many other species similar combinations were advantageous for shoot multiplication as reported in *Macuna pruriens* (**Bretagne et al., 1994**). The magnitude of broad differentiation in tissue culture is subject to the hormonal balance of the support medium as well as the physiological state of the tissue, which usually swings in the direction of the young tissues than older ones **(Mineo, 1990).** Explants from numerous intact plant parts can be used to form callus. However, the most successful explants are often young tissues of one or a few cell types. More so, given the fact that some nutrients like nitrogen, phosphorus and potassium can readily relocate from older to younger leaf, leaving the possibility for deficiency symptoms in older leaves. Young plant tissues possess higher capability for response to *in vitro* culture, compared to older plant materials. This may explain the favourable responses recorded for the young stem explants in this study.

Results of **Debauza and Pena (2001); Peddabonia *et al.* (2006)** on the organogenesis of *C. annuum* confirmed the claim in this study that the standard medium for callogenesis of *Capsicum annuum* is a semi-said state MS medium that is enhanced with BAP and IBA. **Hassan *et al.* (1999); Dabauza and Peña (2001)** however reported MS medium supplemented with silver nitrate to be suitable as an elongation medium, whereas NAA- and IBA – supplemented MS media enhances rooting in sequential experiments. Survival rate of the established calli ranged from 60 – 80%.

Table 9 Contamination rate for explants obtained from control and polluted soils

No. of days after initiation	Leaf explants		Stem explants		Contamination source			
					Leaf explant		Stem explant	
	Control	Polluted	Control	Polluted	Control	Polluted	Control	Polluted
31 days-old explants								
1 – 7	-	+	-	-	-	B,F	-	-
8 – 14	-	++	-	++	-	B,F	-	B,F
15-21	+	+	-	+	B,F	B,F	-	B,F
22-28	+	++	-	+	B,F	B,F	-	B,F
29-35	+	++	++	+++	B,F	F	B,F	F
36-42	+	+++	++	+++	F	F	B,F	F
43-49	++	++++	+++	+++++	F	F	F	F
50-56	+++	++++	++++	+++++	F	F	F	F
28 days-old explants								
1 – 7	-	-	-	++	-	-	-	F
8 – 14	-	++	-	+++	-	B,F	-	F
15-21	-	+	+	++	-	B,F	B,F	F
22-28	-	+	++	+	-	B,F	B,F	F
29-35	-	+	+	++	-	B,F	B,F	B,F
36-42	+	+++	++	++	+	B	B,F	B
43-49	++	+++	++	+++	++	B	B	B
50-56	+++	+++++	++++	+++++	+++	B	B	B
21days-old explants								
1 – 7	-	-	-	-	-	-	-	-
8 – 14	-	++	-	+	-	B	-	+
15-21	+	++	-	+++	+	B	B	B,F

Legend: - No contamination; + not profuse; ++ slightly profuse; +++ profuse; ++++ very profuse; +++++ highly profuse.
F majorly fungal infection, B majorly bacterial infection.

CONCLUSION

In this study, an effective procedure for callogenesis of *Capsicum annuum* explants obtained from pepper plants sown in oil-polluted soil had been developed, using BAP - and IBA – supplemented MS media in a semi-solid state. This protocol could be very useful for callus production which could be used in large scale production of this cultivar of pepper from polluted soils over a short period of time; and offers a potential method for the genetic enhancement of the crop. Similarly, the ability of IBA to induce callus was documented. However, a lot of difficulties were observed during the induction of callus, over all the experiments, therefore a further investigation is needed to overcome these problems.

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