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EFFECT OF HEAVY METALS ON SOME GROWTH PARAMETERS IN CALLUS OF IRAQI *CYNODON DACTYLON* L. PLANT CULTIVATED *IN VITRO*.

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Abstract

Callus initiated from internodes of Iraqi *Cynodon dactylon*.L were used as a source for explants. Internodes were sterilized under aseptic conditions and cultured in media MS supplemented with different combinations of 2, 4-D at concentrations of (0, 1.0, 2.0, 3.0, 4.0 mg/l) and Kinetin (0, 0.5, 0.75 , 1.0 mg/l). The maximum % of callus induction occurred when 2 mg/l of 2, 4-D was interacted with 1.0 mg/l of Kin achieving 93.33%. The interaction between hormones treatments showed that the highest callus fresh weight was recorded at 2 mg/l 2, 4- D and 0.5 mg/l kin reached 673.00 mg. While callus fresh weight decreased and reached 316.13 at 3 mg/l 2, 4-D and 1.5 mg/l kin when both 2, 4-D and kin concentrations were increased. For screening and selection for heavy metal tolerance, Pb, Cd and Ni were used at different concentrations (75,100,150 ppm), (100, 150, 200 ppm) and (600, 800, 1000 ppm) respectively. The results revealed that, the interaction between Pb concentration, Pb uptake and fresh weight were negative that mean when the lead concentration increase the fresh weight is decrease, while the Pb uptake is increase. Regarding to the effect of Nickel on callus relative fresh weight (RFW), Results displayed highest value of relative fresh weight was recorded at 600 ppm Ni concentration, reached (573 mg/Fw), then FW values were decreased with increasing Nickel concentrations and reached (482 and 320 mg/Fw) at the concentration (800 and 1000 ppm) respectively. Concerning to the effect of Cadmium Calli RFW values were very low at the high Cd concentration Cd (200 ppm) reached (352mg/FW), while RFW at (100 ppm) was high, reached (809mg/FW).

***Key words** : In Vitro*, Cynodon, Pb, Cd, Ni, Co, Callus.

Introduction

Soils normally contain low levels of heavy metals. Excessive levels can be hazardous to man, animals and plants. The term heavy metal, however, is often broadly applied to include other potentially hazardous elements. The most important heavy metals involved in soil contamination are arsenic (As), cadmium (Cd), chromium (Cr), nickel (Ni), and selenium (Se) (Tucker *et al.,* 2005). Phytoremediation is one of the promising methods for reclamation of soils contaminated with toxic metals by using hyper accumulator plants (Ghosh and Singh, 2005; Lazaro *et al.,* 2006). More than 400 plant species belonging to 45 plant families have been identified and reported from temperate to tropical regions with the ability to tolerate and hyper accumulate trace elements (Baker and Brooks, 1989). Plant tissue culture and molecular genetics have opened new avenues in plant improvement. Screening and selection at the plant cell level has established plant clones with increased tolerance or resistance in plants to various environmental stresses like salt, heat, cold, drought, disease, insects, heavy metals and herbicides (Tal, 1983). Cell lines tolerant to elevated levels of salt in the medium have been selected in *Brassica juncea* (Jain., 1991). Cell lines resistant to elevated concentrations of aluminum have been selected in *Nicotiana plumbaginifolia* (Meredith., 1988). The *Cynodon* genus comprises of nine species and 10 varieties and contains grasses of economic importance for livestock herbage, turf and soil stabilization (Assefa *et al., 1999)*. *Cynodon dactylon* (L.) Pers. is the most conspicuous and successful member of the genus and is ubiquitous through the tropics and subtropics (Harlan and de Wet, 1969; Assefa *et al.,* 1999). Recent studies have shown that *Cynodon dactylon* has the ability to pull heavy metals from the soil and reclamation it (Madejó *et al.,*2010; Soleimani *et al.,*2009).The aim of this study was to

evaluated the effect of heavy metals on some growth parameters in Cynodon dactylon plant cultivated *In Vitro*.

Materials and Methods

The work was carried out in the plant tissue culture Lab., Institute of Genetic engineering and Biotechnology., Baghdad University, during the period 15/11/2017 - 15\1\2019.

Plant materials

Cynodon dactylon plantlets were obtained from the gardens of Institute of Genetic engineering and Biotechnology, Baghdad University, Iraq.

Sterilization of explants and media preparation

Iraqi *C. dactylons* Stolons containing a node with axillary buds and 10mm of the internodes above and below the node, (about 1.0 cm in length) were used as a source for explants, sterilized with 1 % (v/v) sodium hypochlorite containing a few drops of Tween 20, followed by washing with sterilized distilled water (D. D. Water) three times. All the steps of sterilization were carried out under aseptic conditions using laminar Air flow cabinet. Explants were transferred into sterilized Petri dishes having sterile filter papers to remove excess of water. Murashige and Skoog (MS) basal salts medium (Murashige and Skoog, 1962) fortified with Inositol, Glycine, Pyrodoxine- HCl, Nicotinc acid, Thiamine- HCl and sucrose at concentration of 100, 2,0.5, 0.5, 0.1,3000 mg/l respectively and growth regulators at different concentrations.

Callus induction

The surface sterilized explants were cut into small segments, about 1cm, and Planting into callus induction medium, supplemented with different combinations of various concentrations of 2, 4-D (0, 1.0, 2.0, 3.0, 4.0 mg/l) and Kinetin (0, 0.5, 0.75 , 1.0 mg/l). Cultures were incubated at 25 C in the dark. After six weeks of incubation, callus induction frequency (%) was calculated using the following formula. Callus induction frequency (%) = No. of explants produced callus/total No. of cultured explants × 100.

Maintenance of callus cultures

Small pieces of calli weighting 200 mg each were transferred into MS medium supplemented with (1.0, 2.0, 3.0 mg/l) 2, 4-D and combined with (0.0, 0.5, 1.0, 1.5) mg/l Kin. Vigorous growing portions of calli were transferred while necrotic or brown calli were discarded before subculture on a fresh medium. Callus fresh weight was measured after six weeks of subculture under aseptic conditions.

Screening and selection for heavy metal tolerance

Stock solutions (1000 ppm) of the metals Pb, Cd, Ni were used to prepare different concentrations of these metals as follows (75,100,150 ppm), (100, 150, 200 ppm) and (600, 800, 1000 ppm) respectively, then add to the maintenance medium (MS supplemented with 2mg/l 2,4-D and 1.0 mg/l kin. Fresh weights were calculated after six weeks and the selected concentration of the heavy metals were determined. Relative fresh weight of the embryogenic calli was calculated at different stress levels according to the following formula (Daud *et al.,* 2014).

Statistical Analysis

All experiments were performed in Completely Randomized Design (C.R.D) and each treatment was replicated trice time. Analysis of variance were compared using GenStat software (12^{ed}).Means were compared at Least Significant Differences (L.S.D) at F Probability 0.05.

Results and Discussion

Callus induction

Effect of 2,4-D and Kin on callus induction

An experiment was carried out to induce callus on nodal segment explants. After two weeks of culturing on MS medium, callus induction was observed at the nodal then covering the entire explants surface after four weeks. The resulted callus appeared watery texture. The percentage of callus induction was also assessed (table1). Addition of Kin increased the % of callus induction at the concentration 1.0 and 0.75 mg/l significantly recording 48.67 and 45.33% compared with 0.0 mg/l Kin (20%). However, All 2,4-D concentrations (1, 2, 3 mg/l) led to a significant increase in the % of callus induction which gave 40.83, 70 and 43.33 % while the control treatment (0.0 mg/l) recorded 16.67 %, and the high concentration of 2,4-D (4 mg/l) led to reduce in the % of callus induction which gave 17.5 %. The maximum % of callus induction occurred when 2 mg/l of 2, 4-D was interacted with 1.0 mg/l of Kin achieving 93.33%. Our results are agree with Ramgareeb (2004) who referred to the importance of 2, 4-D for callus induction in *C. dactylon* and showed that a further increase in 2, 4-D concentration resulted in decreased callus proliferation. Also our results are agree with Rashid *et al.* (2009), who stated that the addition of 2 mg/L of 2, 4-D was the most effective for callus induction where 97.18% callus induction was recorded in *Triticum aestivum* cv. tatara. Furthermore, The maximum % of callus induction occurred when 2 mg/l of 2, 4-D was interacted with 0.75 mg/l of Kin achieving 93.33%. Similar results were found by Bano *et al.,* (2005) who reported that good callus of rice (*Oryza sativa* cv. Swat-II) resulted on MS medium supplemented with 2,4-

D and kin. Parco (2007) reported that maximum % of callus induction in *C. dactylon* (Brazos cultivar) was achieved with 3 mg/L 2, 4-D, 1 mg/l Kin and 3mg/l ABA which yielded compact yellowish callus.

Maintenance of callus cultures

For maintenance, increasing and improving callus Quantity, calli obtained from induction stage were transferred to other MS medium supplemented with different concentrations of plant growth regulators (Table 2). Mean of callus fresh weight recorded 234.47 mg at 0.0 mg/l kin and 507.86 at 0.5 mg/l then reduced at 1 mg/l and 1.5 mg/l 438.73 and 408.93 mg respectively.

Table 1: Effect of 2,4-D and Kin and their interaction on the % callus induction, after inoculating explants on MS medium for six weeks.

2,4 D (mg/l)	Kin (mg/l)				Mean
	0.0	0.5	0.75	1.0	
0.0	0	23.33	16.67	26.67	16.67
1	26.67	36.67	46.67	53.33	40.83
2	33.33	73.33	80	93.33	70
3	23.33	36.67	60	53.33	43.33
4	16.67	13.33	23.33	16.67	17.5
Mean	20	36.67	45.33	48.67	
LSD: 2,4-D= 6.026 Kin= 5.390 , 2,4-D*Kin= 12.051 (P<0.05)					

Highest callus fresh weight at 2 mg/l 2, 4-D reached 494.53 mg compared to concentrations 1 mg/l and 3 mg/l recorded 390.45 and 307.51 mg respectively. The interaction between hormones treatments showed that the highest callus fresh weight was recorded at 2 mg/l 2, 4- D and 0.5 mg/l kin reached 673.00 mg. While callus fresh weight decreased and reached 316.13 at 3 mg/l 2, 4-D and 1.5 mg/l kin when both 2,4-D and kin concentrations were increased.

Effect of Lead, Cadmium, Nickel on callus relative fresh weight (RFW)

It's clear from Fig. (1), there is an increase in accumulations of lead in callus of *C. dactylon* reached 60% (150 ppm) as a compared with (75 ppm) in all three replicates, the figure also show a decrease in fresh weight with increase lead media concentration (75, 100 and 150 ppm) reached (796, 605 and 426 mg/Fw) respectively. Also the interaction between Pb concentration , Pb uptake and fresh weight were negative that mean when the lead concentration increase the fresh weight is decrease, while the Pb uptake is increase. The results are in line with Taghizadeh (2015) who reported that 100 mg/L of Pb treatment gave acceptable growth for the callus of *C. dactylon*. Krishania and Agarwal (2012). show that lead concentration above 100 µm were toxic for callus induction in *Eleusine coracana*. While Nawrot (2017) reported that lead at concentration (20 mg/L) has the most toxic effects on the tissue of *Abies nordmanniana*.

Table 2: Effect of 2,4-D and kinetin and their interaction on mean callus fresh weight (mg) after inoculating callus pieces on solid MS medium for 6 weeks. Initial weight was 200mg.

2,4 D (mg/l)	Kin (mg/l)				Mean
0	0.5	1	1.5		
1	234.47	493.57	449.33	384.43	390.45
2	234.47	673.00	544.43	526.23	494.53
3	234.47	357.00	322.43	316.13	307.51
Mean	234.47	507.86	438.73	408.93	.....
LSD: 2,4-D= 35.944, Kin= 41.505 , 2,4-D*Kin= 71.889 (P<0.05)					

Also the results are in line with Taghizadeh (2015). Who reported that 100 mg/L of Pb treatment gave acceptable growth for the callus of *C. dactylon* and Krishania and Agarwal (2012) who Showed that lead

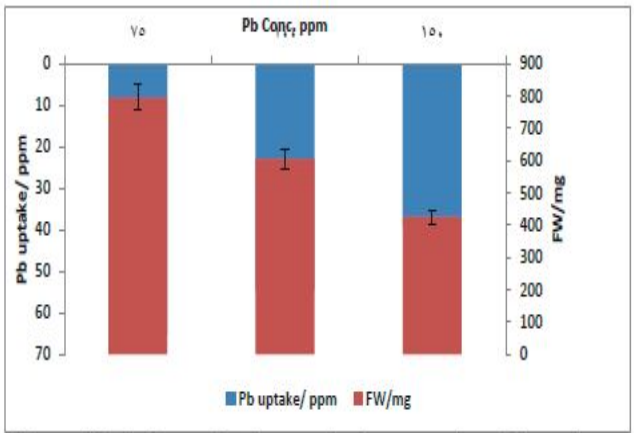

Fig. 1: The interaction between lead concentration , Pb uptake and callus fresh weight under Pb stress after four weeks.

concentration above 100 µm were toxic for callus induction in *Eleusine coracana*. Pb deposits occupy a significant part of vacuoles and, in extreme cases, fill it almost entirely. Lead causes disturbances in cellular metabolism, which is manifested in the form of intensive vacuolization

Concentric endoplasmic reticulum, lobed nucleus and numerous multivesicular bodies present in the cell (Wozny and Przybyl, 2004).

Concerning to the effect of Cadmium on callus relative fresh weight (RFW) in the current study, Calli RFW values were very low at the high Cd concentration Cd (200 ppm) reached (352mg/FW), while RFW at Cd concentration of (100 ppm) was high, reached (809mg/FW). As shown in fig. 2, increasing cadmium

concentration resulted in decreasing relative fresh weight. Moreover, data analysis showed that there are significant differences in callus fresh weight. The interaction between three properties (fresh weight, Cd concentration, Cd uptake) were negative that mean when the Cadmium concentration is an increase the fresh weight is decrease, while the Cd uptake is increase.

Results is in line with Israr *et al*., (2006) who reported that callus grew well up at (50 µM) Cd concentration while the growth was significantly inhibited at a

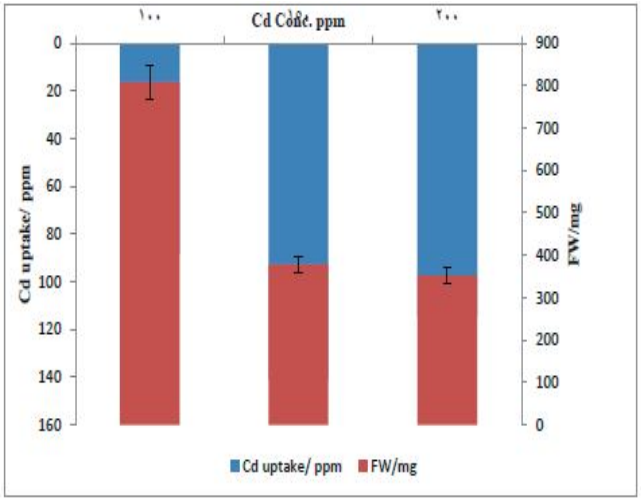

Fig. 2: The interaction between Cadmium concentration, Cd uptake and callus fresh weight under Cd stress after four weeks.

concentration of (100µM) Cd. While Fornazier *et al.* (2002) reported that low concentration of CdCl2 (0.1 mM) caused a significant increase in callus growth, whereas concentrations (0.5 and 1.0 mM) inhibited growth of callus culture in *Saccharum officinarum* callus cultures. Moreover, Namjooyan *et al*. (2012) showed that the growth of calli was comparable to that of the control at lower concentrations of Cd (25 and 50 µM Cd), while the higher concentrations (75 and 100 µM Cd) reduced callus growth in *Carthamus tinctorius*. However, Shekhawat *et al.,* (2010) showed a reduction in the fresh growth with the increasing concentration of Cd in calli of Brassica. Similar results were reported by Sobkowiak and Deckert (2003) in soybean (*Glycine max*) cultures. The increasing in callus fresh weight at low cadmium concentrations could be explained by competition between Zn or Fe and Cd for the same cellular binding sites since there is similarity in structural, geochemical, and environmental properties and can functionally substitute for Zn in plant cells. High concentrations of Cd result in toxicity represented by various functional-based alterations in plants such as growth retardation, changes in root morphology, root and leaf anatomy, and damages to cell structures as well as disturbance in water balance, mineral nutrition, photosynthesis, and plant development (Prasad, 1995).

Regarding to the effect of Nickel on callus relative fresh weight (RFW), Results displayed in figures 2 and 3.8 indicate that the highest value of relative fresh weight was recorded at 600 ppm Ni concentration, reached (573 mg/Fw), then FW values were decreased with increasing Nickel concentrations and reached (482 and 320 mg/Fw) at the concentration (800 and 1000 ppm) respectively with increasing in Ni uptake**.** The interaction between three properties (fresh weight, Nickel concentration, Ni uptake) were negative that mean when the Ni concentration is an increase the fresh weight is decrease, while the Nickel uptake is increase. That was similar of Pb and Cd behavior in *C. dactylon* plant tissue. Rout *et al*., (1998) reported that callus fresh weight was increase at the lower concentrations of Ni (0.1301-5 mg.dm^{-3}), but the few callus was grew on at higher concentrations of nickel (2.0-2.5 mg.dm^{-3}) and other calli did not exhibit any growth. Krishania and Agarwal (2012). Show that Ni concentration above 100 µm were toxic for callus induction in *Eleusine coracana*. Seregin and Kozhevnikowa (2006). reported that low Ni concentrations have promotional effect on plant growth and development. High concentrations of Ni may contribute to the deficiency of nutrients or the divalent cations that can compete with Ni. Nickel stress has been shown to cause a substantial decrease in all macro- and micronutrients. higher Ni levels decreased the concentrations of Ca, Mn, and Fe in achenes and N, K, Zn, Mn, and Cu decreased consistently with increasing level of Ni (Ahmad *et al.,* 2011).

Conclusions

In general, the results in our research show first steps

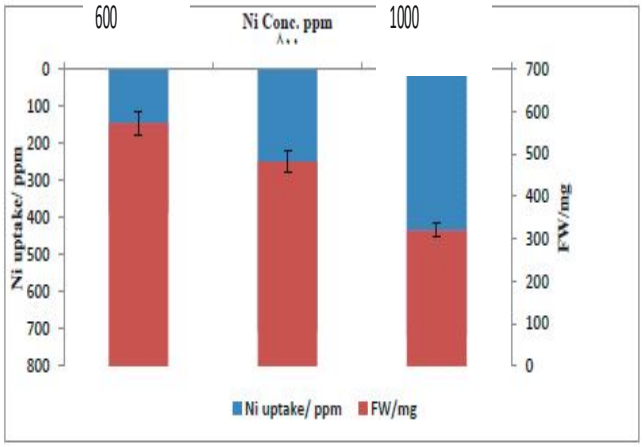

Fig. 3: The interaction between Nickel concentration, Ni uptake and callus fresh weight under Ni stress after four weeks.

for micropropagation of *Cynodon* grass and there adopted for heavy metals stresses. Recently, Phytoremedators are new strategy for arid and semiarid area are needed and therefore, further studies for plant regenerations from stressed callus of *Cynodon dactylon* grasses and improving soils problems by field performance for phytoremediation.

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