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Investigating Zinc Tolerance in Saline and Drought Tolerant *Ruta graveolens* Tissue Cultures

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Abstract

Tolerance of *Rutagraveolens* to Zn was investigated *in vitro*. Heavy metal stress was induced by subculturing salt and drought tolerant callus pieces weighting 100 mg on Murashige and Skoog (MS) medium supplemented with 1 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/l kinetin (kin). Zn was added at concentrations 0.1, 0.5, 1.0, 2.0 or 3.0 ppm. Results showed that callus relative fresh weight (RFW) decreased with increasing metal concentrations. Tolerance index (TI) was assessed at different metal concentrations and stress durations. Results also indicated that callus cultures were more tolerant to heavy metals at the concentration 1 ppm. Spectrophotometric estimation of metal concentrations inside plant tissue samples showed that callus cultures stand for moderately high concentrations of Zn reached 7100 µg/g dry weight. Heavy metal tolerant calli were selected and shoots were regenerated on ½ MS medium supplemented with 2.5 mg/l Benzyl adenine (BA).

Keywords: *Ruta graveolens*, callus cultures, Zn tolerance, abiotic stresses.

Introduction

Ruta graveolens commonly known as rue or herb of grace is a species of *Ruta* grown as a herb. Survival of plants under adverse environmental conditions relies on the integration of stress adaptive metabolic and structural changes in target plant. Abiotic environmental factors such as drought and salinity are significant plant stressors with major impact on plant development and productivity thus causing serious agricultural yield losses. The complex regulatory processes of plant drought and salt adaptation involve control of water flux and cellular osmotic adjustment by osmoprotectants biosynthesis. Since salinity induced imbalance of cellular ion homeostasis, it is coped with regulated ion influx and efflux at the plasma membrane and vacuolar ion sequestration. Drought and salinity have additionally major detrimental impacts on the cellular energy supply and redox homeostasis that are balanced by global re-programming of plant primary metabolism and altered cellular architecture [1].

Soil contamination with heavy metals represents abiotic stress results from human activities. Some manmade activities like sewage sludge, fertilizers, and the direct discharge of domestic and industrial wastes are the main source of higher concentrations of heavy metals. Toxic heavy metals are biomagnified through the food chain. They contaminate the environment by altering soil properties biomass, fertility, and crop yields and thus the human health [2]. Heavy metals toxicity reflects its impact on plant growth including leaf chlorosis, a decrease in the rate of seed germination, and a crippled photosynthetic apparatus, often correlated with plant death. Contaminated soils pose a major environmental and human health concern; phytoremediation technology provides solution to this critical problem [3].

Plants have developed their own specific responses against abiotic stresses. Actually, investigating these responses is difficult under field conditions. Plant tissue culture techniques are performed under aseptic and controlled environmental conditions for the purpose of both commercial, like mass production, and scientific studies like germplasm preservation, plant breeding, physiological, and genetic. These advantages of plant tissue culture allow various opportunities for researcher to study the unique and complex responses of plants against environmental stresses [4]. Development of abiotic stress tolerant plants using *in vitro* selection has been reported in a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species [5].

There is an urgent need for hyperaccumulating plants to be grown widely on soil that is stressed by salt, drought and

sites contaminated with heavy metals to enhance the vegetation of such sites. Thus, this study was carried out to investigate the ability of *R. graveolens*to tolerate Zn*in vitro* and examination of regenerated shoots for their tolerance to Zn.

Materials and Methods

Plant material and surface sterilization

Ruta graveolens plantlets were obtained from the Biotechnology Dept. College of Science, Al-Nahrain University, Iraq. Stem segments (about 1.0cm in length) were used as a source for explants. Stems were placed under tap water for 30 minutes, and then sterilized by continuous shaking with 2.0% v/v sodium hypochlorite (Clorox) for 20 minutes, followed by washing with sterilized distilled 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 water [6].

MS medium [7] was used and supplemented with 30 g/l sucrose and growth regulators. The pH of the medium was adjusted to 5.8, and then 7 g/l agar was added to the medium. The culture medium was autoclaved at a pressure of 1.04 kg/cm^2, 121°C, for 15 minutes, and then left at room temperature until use. The medium was dispensed into 10x1cm tubes (3 ml/tube).

The surface sterilized explants were cut into small segments, about 1 cm, and inoculated into callus induction medium, different combinations of 2,4-D (0.75, 1.0 or 1.25) mg/l, and various concentrations of kinetin (0.75, 1.0 or 1.25) mg/l were added in addition to control treatment. Cultures were incubated at 25±2°C in dark. Stem explants were cultured in each Petri dish (5 explants per dish) with 10 replicate for each treatment. 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

Small pieces of calli weighting 50 mg each were transferred into MS medium supplemented with factorial combinations of 0.0, 1.0, 1.5 or 2.0 mg/l 2,4-D and 0.0, 0.5, 1.0 or 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 tissues were incubated as in callus induction experiment. Each treatment included 10 replicates. Callus fresh weight was measured after four weeks of subculture under aseptic conditions. Callus pieces formed on stems explants weighting about 100 mg/tube were subcultured into MS medium supplemented with 1mg/l 2,4-D and 0.5 mg/l Kin and manitol at the concentration 300 mg/l to induce drought stress or with saline water (drainage water) with an electrical conductivity (EC) 30ds.m^{-1} for inducing salt stress [8].

Stock solution (1000 ppm) of Zn was used to prepare different concentrations of Zn (0.0, 0.1, 0.5, 1.0, 2.0, 3.0 ppm) to be added to the maintenance medium (MS supplemented with 1mg/l 2,4-D and 0.5 mg/l Kin). About 100 mg of salinity and drought tolerant calli were subcultured directly into previously prepared medium. Fresh weights were determined after three weeks and the selected concentrations of the heavy metal were determined. Callus cultures were re-cultured three times on the same medium. Each treatment was carried out with 10 replicates.

Relative fresh weight of the embryogenic calli was calculated at different stress levels according to the following formula [9]

$$RFW=\frac{FWF-FWI}{FWI} \quad \text{..... (1)}$$

Where, FWI = initial fresh weight and FWF = final fresh weight.

Fresh biomass-based tolerance index (TI) of *R. graveolens* callus culture was calculated according to the following formula:

$$TI\ (\%) =\frac{\text{mean biomass in stressed medium}}{\text{mean biomass in control medium}} \times 100 \quad \text{..... (2)}$$

Since TI = Tolerance Index

Data were taken for four different stress periods at three week intervals, that is, 21, 42, and 63 days.

Shoot regeneration

Tolerant calli were selected and transferred into regeneration medium under aseptic conditions. The regeneration medium

consisted of half strength MS medium supplemented with BA (2.5 or 3.0 mg/l), NAA (0.5 mg/l). All cultures were maintained at 25±2 °C for 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. All multiple shoots obtained were transferred to elongation medium that was the same regeneration medium butsupplemented with 0.5 mg/l of GA_3. Shoots were transferred onto half strength MS medium supplemented with 0.1 mg/l IBA for rooting [10, 11].

Results and Discussion

Table (1) shows that addition of kin increased significantly the percentage of callus induction at the concentration 1.0 and 0.75 mg/l recording 75.24 and 55.02% compared with 0.0 mg/l kin (32.72%). However, the percentage of callus induction reduced significantly (16.14%) when the kin concentration was increased to 1.25 mg/l. All 2,4-D concentrations (0.75, 1.00, 1.25 mg/l) led to a significant increase in the percentage of callus induction which gave 41.47, 59.12 and 56.14% respectively while the control treatment (0.0 mg/l) recorded 22.39%. The maximum percentage of callus induction occurred when 1.0 mg/l of 2,4-D was interacted with 1.0 mg/l of kin achieving 100%. Data presented in table (1) was similar to those obtained by Ahmad *et al.* (2010) who reported light yellow friable callus induction in *R. graveolens* using MS medium supplemented with different concentrations of 2,4-D, and showed that the frequency of callus production increased with increased concentration of 2,4-D [11]. Callus induced on stem explants was transferred to a maintenance medium. The highest callus fresh weight was recorded at 1.0 mg/l 2,4-D and 0.5 mg/l kin (data not shown).

Considering the stimulatory effect of 2,4-D on plant growth and its basic role in cell elongation, a significant effect of 2,4-D was confirmed in increasing callus formation. Kin also increased cell division and thus callus production, but increasing its concentration caused a considerable decline in callus growth. This shows the negative effect of increasing the concentration of cytokininon callus formation. Low concentrations of 2,4-D and kinetin enhance mitosis, but high concentrations have inhibitory effect. Auxin has aneugenic potential by acting as spindle poison and disturbing the correct separation of the chromosomes at cell poles [12].

Table (1)
Effect of 2,4-D, kin and their interaction on the % callus induction, after inoculating explants on MS medium for six weeks, n=10.

Kin (mg/l)	*2,4-D (mg/l)*				*mean*
	0.00	*0.75*	*1.0*	*1.25*	
0.00	0.00	32.58	44.00	54.33	32.72
0.75	33.45	55.67	70.33	60.63	55.02
1.00	43.64	64.33	100.0	93.00	75.24
1.25	12.47	13.32	22.18	16.62	16.14
mean	22.39	41.47	59.12	56.14	
LSD 0.05	2,4-D= 7.2; Kin= 6.8 Interaction= 13.4				

Effect of zinc on callus relative fresh weight (RFW)

It's clear from the results shown in Fig.(1.1) that salinity tolerant calli, after three weeks of culturing, there is a linear increase in callus RFW with the increasing of Zn at the concentrations 0.1, 0.5, and 1.0 ppm reached 1.82, 2.41, 2.81 g respectively. A decline in callus RFW occurred at the concentration 2.0 ppm recorded 1.26 g and further at 3.0 ppm, as the RFW reached minimum value (0.93).

While in case of drought tolerant calli Fig.(1.2), RFW under Zn stress recorded the highest value at the concentration 0.5 ppm, reached 2.00, compared with the concentrations 0.0 and 0.1 ppm, which reached 1.62 and 33 respectively. Means of RFW decreased at high concentrations 1.0, 2.0 and 3.0 ppm, recorded 1.58, 0.94 and 0.16 respectively. Similar findings were obtained by Long *et al.* (2013) and Yahya (2014) who reported a correlation between callus fresh weight and Zn concentration in callus cultures [13, 14]. Zinc is involved in many cellular functions such as protein metabolism, photosynthetic carbon metabolism and indole acetic acid metabolism, and the only metal presented in all six enzyme classes, oxidoreductases, transferases, hydrolases, lyases, and isomerases, yet its higher concentrations cause toxicity [15]. Toxicity

leads to an inhibition of cell activity or disruption of structures. High levels of Zn may stimulate the formation of free radicals; witch may lead to oxidative stress [16].

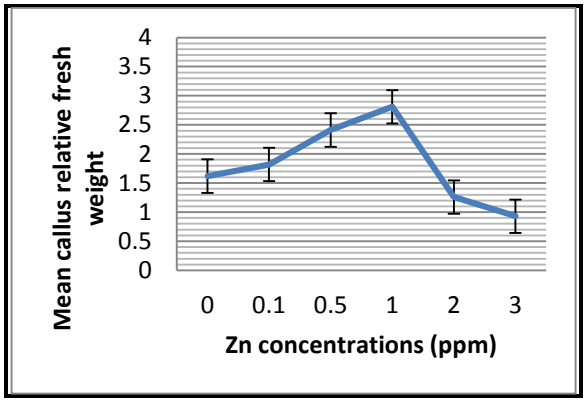

Fig.(1): Callus relative growth curve for salt tolerant calli under Zn stress after three weeks. Bars represent standard errors.

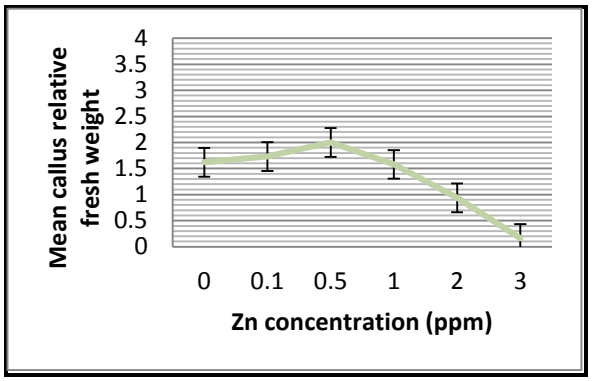

Fig.(2): Callus relative growth curve for drought tolerant calli under Zn stress after three weeks. Bars represent standard errors.

Tolerance index percentage

In order to study the dose and time-dependent effect of heavy metals on *R. graveolens* callus cultures, fresh biomass-based tolerance capability under various heavy metals stress levels for different durationwas also investigated Table (2).

Table (2)
Tolerance index percentages (TI %) for R. graveolenscallus cultures tolerate salinity and drought stresses then subjected to different concentrations of Zn after different time periods.

Callus	*Zn Conc. (ppm)*	*Tolerance index (%) after*		
		21 days	*42 days*	*63 days*
Non-stressed	Normal MS medium	0.00	00.00	0.00
Salinity tolerant	1.0	145.40	217.7	339.70
	2.0	86.20	75.00	74.00
	3.0	73.00	34.00	32.64
Drought tolerant	1.0	98.47	107.03	172.76
	2.0	74.04	62.76	54.67
	3.0	44.27	27.08	19.33

Shoots regeneration

Results shown in Table (3) exhibited that half strength MS medium supplemented with BA at 2.5 mg/l gave the highest rate of shoot formation percentage reached 72% compared with 40% and 0% when the same medium supplemented with 2.5 mg/l BA plus 0.5 mg/l NAA and with 3.0 mg/l BA plus 0.5 mg/l NAA respectively.

Table (3)
Effect of BA and NAA on % shoot formation after 8 weeks of culturing callus pieces on half strength MS medium. n=3.

BA(mg/l)	*NAA(mg/l)*	*Shoot formation %*
0.0	0.0	*
2.5	0.0	72
2.5	0.5	40
3.0	0.5	0
Mean		56
LSD 0.05		5.40

However, BA at 2.5 mg/l induced the development of greenish nodular calluswich gave rise to minishoots Fig.(3A) which later developed to shoots. The earliest sign of shoot formation and subsequent elongation was noticeable in un-stressed callus after 6 and 8 weeks of incubation, respectively, while tolerant callus took two weeks more Fig.(3B). Although the combination of 2.5 mg/l BA plus 0.5 mg/l NAA gave rise for high morphogenic

capacity, a few numbers were developed to shoots later and the resulted shoots exhibited glassines appearance Fig.(3C). Increasing BA to 3 mg/l resulted in inhibiting shoot induction but callusing at the shoot bases was noticed Fig.(3D).These results come in line with those of Ahmad *et al.* (2010) who indicated that BA at 2.5 mg/l induced greenish nodular callus in *R. graveolens* cultures forming microshoots which later developed to shoots[11]. Also in line with Tejavathi and Manjula (2010) who stated that the rate of shoots multiplication reduced with increasing cytokinin concentrations [17]. On the other hand, these results disagree with Ahmad *et al.* (2010) who studied the effect of adding low concentrations of NAA to the regeneration medium of the same taxon. Results also disagree with Tejavathi and Gayathramma, (2005) who stated that low auxin concentration in combination with a high cytokinin is the most suitable combination for the proliferation of shoots in *Agave vera-cruz* Mill [18].

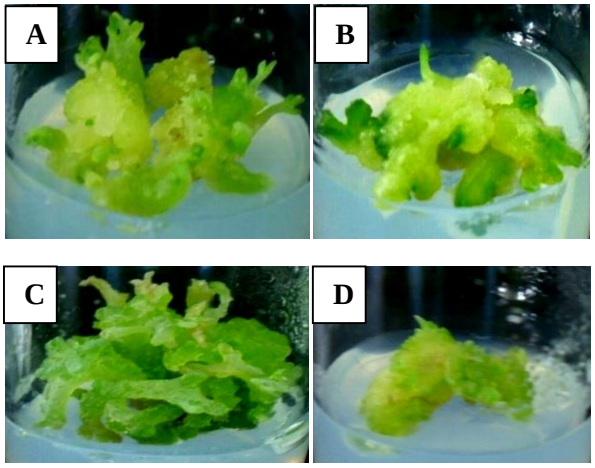

Fig.(3): Regeneration of microshoots.
A: un-stressed callus regenerated on half MS medium supplemented with 2.5 mg/l BA.
B: shoots regenerated from salt, drought and heavy metals tolerant callus on half MS medium supplemented with 2.5 mg/l BA.
C: shoots intiated on half MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l NAA.
D: shoots regenerated on half MS medium supplemented with 3.0 mg/l BA and 0.5 mg/l NAA.

Shoots elongation and rooting

Single shoots (50-150 mm length) were transferred to the same regeneration medium supplemented with 0.5 mg/l GA_3. More shoots and longer were observed after two subculture cycles at two week intervals Fig.(4). Similar results were obtained by Gonbad*et al.* (2014) who supplemented different cytokinin concentrations combined with GA_3[19]. They found that highest shoots number and longest shoots were obtained at combination of 3 mg/l BAP and 0.5 mg/l GA_3. The outcome of the previous investigation was that the combination of BA and GA3 could have two products simultaneously. The first product was the appropriate shoots for shoot multiplication by applying BA and the second one was shoots multiplication due to the use of GA_3, which is convenient for rooting. Well developed shoots were re-inoculated on MS medium supplemented with Zn at a concentration 1ppm to examine their tolerance capacity and found to be tolerant to this metal.

Elongated and healthy shoots were transferred into a rooting medium consisted of half strength MS medium supplemented with 0.1 mg/l IBA Fig.(5). It is interesting that, un-stressed callus-derived plantlets showed early signs of root initiation than stressed callus-derived plantlets which took too long for root induction (about eight weeks).Batty and Younger (2003) stated aweak root growth, lack of branching of roots and root flaccidity in reed cultured under high Fe concentrations [20]. Decreased roots number and length, and sharp depression in the mitotic activity of roots from sugarcane were reported by Jain (2010) under heavy metals stress [21].

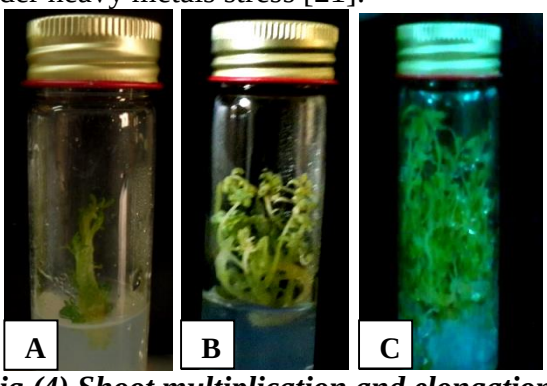

Fig.(4) Shoot multiplication and elongation.
A: shoots (50-150 mm length) were transferred to half strength MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l

GA_3 after two weeks. B: after four weeks. C: after six weeks.

Fig.(5) : Healthy elongated shoots cultured on half strength MS medium supplemented with 0.1 mg/l IBA for rooting.

Characterization of regenerated shoots

Noticeable morphological difference between shoots derived from non-stressed calli and others derived from stressed calli after eight weeks. Longer shoots, a wide leaves surface area, typical leaf edges and more leaf number were recorded in non stressed shoots. While short shoots, small leaf surface area, and a sharp leaves edges were noticed in shoots derived from stressed callus Fig.(6).

Changes in leaf characteristics have been widely reported as a mechanism to cope with adverse environmental conditions. The phytotoxic effect of heavy metals in plants through visual symptoms such as reduced growth and biomass accumulation was also reported by Gomes *et al.* (2011)[22]. Gomes *et al.* (2013) stated that metals induce the appearance of xeromorphic characteristics in leaves as result of decreased water potential, increased numbers and decreased stomata size in *Pfaffia glomerata* cultures.

This may be beyond the production of ethylene at high concentrations as a result of salt, drought and heavy metal stresses applied on plant *in vivo* which have adverse effect on plant in terms of growth and appearance. This was confirmed in the acs2-1acs6-1 double knockout mutants, which showed a decreased ethylene production, positively affecting leaf biomass [23].

Fig.(6) : Morphological description of callus cultures differentiated to shoot. A: shoots derived from non stressed callus. B: shoots derived from stressed callus.

Quantitation of heavy metals

Heavy metal concentrations inside the dried plant tissues were determined spectrophotometrically according to procedure described by Yousir, (2007) [24]. As shown in Table (4), *R. graveolens* accumulated high concentrations of Zn in stressed callus reached 7100µg/g compared with leaves grown in a typical soil reached 202µg/g and non-stressed callus reached 213µg/g respectively. Many enzymes require Zn^{+2} to enhance their activity since it may be required for chlorophyll biosynthesis in some plant species. However, Zn^{+2}deficiency may lose the capacity to produce sufficient quantities of IAA, which required for cell division [25].

Table (4)

A comparison between heavy metals content in leaves dissected from plants grown in a typical soil, non-stressed callus and stressed callus exposed to Zn at a concentration of 1 ppm.

Tissue type	*Zn (µg/g)*
leaves	202
Non-stressed callus	213
Stressed callus	7100

As a conclusion it is possible to select plants tolerant to Zn *in vitro.*

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5000 لتقدير تراكيز المعدن داخل عينات الأنسجة التي شملت أوراق النبات النامي في تربة اعتيادية ونوعي الكالس المعرض وغير المعرض للإجهاد. بينت النتائج بان المزارع النسيجية لنبات السذاب تتحمل تراكيز عالية لحد ما من المعادن الثقيلة (7100 مايكروغرام/ غرام من الزنك). أُنتخب الكالس المتحمل للمعادن الثقيلة وسجلت اخلافا للافرع بعد تعزيز وسط MS بنصف القوة بالسايتوكاينينبينزلادينين BA بتركيز 2.5 ملغم/ لتر.

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الخلاصة

تم التحري عن تحمل نبات السذاب *Ruta graveolens* للزنك خارج الجسم الحي. استحث إجهاد الزنك بنقل قطع من الكالس المتحمل للملوحة والجفاف تزن ١٠٠ ملغم الى وسط موراشجوسكوك (Murashige and Skoog, 1962) مجهز بمقدار ١ ملغم/ لتر من دايكلوروفينوكسي استك اسد (2,4-D) و 0.5 ملغم/ لتر من الكاينتين (kin).أُضيف الزنك بتراكيز 1.,٠,٥, ١,٠, ٢,٠ او ٣,٠ جزء بالمليون إلى الوسط. بينت النتائج حصول نقصان في وزن الكالس الطري مع زيادة تراكيز المعدن. تم حساب مقياس التحمل بعد تعريض الكالس لتراكيز وفترات زمنية مختلفة من الإجهاد. وكانت المزارع النسيجية اكثر تحملا لوجود تركيز ١ جزء بالمليون من الزنك. أُستعمل مطياف الإمتصاص الذري