World Journal of
Experimental Biosciences

Volume 5, Number 1: 14 -18
ISSN: 2313-3937
2017

Research article

Evaluation of *Helianthus annuus* L. tolerance for Zinc in vitro post combination with Naphthalene acetic acid and Benzyl adenine

Zainab F. Mahmood*[1], Kadhim M. Ibrahim[2]

ABSTRACT

The tolerance of zinc (Zn) in tissue culture and whole plant of *Helianthus annuus* was studied. Callus was induced and maintained on Murashage and Skoog (1962) medium (MS) supplemented with 0.2 mg/L Kinetin (KIN), 0.4 mg/L Naphthalene acetic acid (NAA) and 0.5 mg/ L 2,4- dichlorophenoxy acetic acid (2,4-D) used hypocotyl as the source explant for callus induction. Different concentrations of Zn were added directly to the culture medium as contaminant. Selected tolerant cell lines were subjected to regeneration. Concentration of Zn in callus tissues was 29 ppm when 3.0 mg/L of Zn used. The study focused on effect of the cytokinin Benzyl adenine (BA) and the auxin, NAA on number of regenerated shoots percentage from tolerant callus to Zn. Results exhibited that 82% of callus tolerant Zn, occurred when the medium supplemented with combination of 1.0 mg/L BA and 0.5 mg/L NAA. Effect of NAA on rooting of shoots showed that 2.0 mg/L of NAA gave a rooting percentage mounted to 75%. The study also included measurement of plant height and fresh weight. Maximum Zn accumulation reached to 17.5 ppm in the shoots.

Keywords: Benzyl adenine, callus, Helianthus annuus L., Naphthalene acetic acid, Zinc.

Citation: **Mahmood ZF, Ibrahim KM. (2017)** Evaluation of Helianthus annuus L. tolerance for Zinc in vitro post combination with Naphthalene acetic acid and Benzyl adenine. World J Exp Biosci 5: 14 – 18.

Received December 17, 2016; Accepted January 6, 2017; Published January 29, 2017.

INTRODUCTION

Sunflower (Helianthus annuus L.) is an annual plant of the genus Helianthus grown as a crop for its edible oil and the endosperm of seeds. Sunflower is also used as bird food, as livestock forage (as a meal or a silage plant), and used in some industrial applications. It is also used in soil cleaning due to its moderate tolerance to different types of heavy metals [1, 2].

Plant survival under adverse environmental conditions relies on integration of stress adaptive metabolic and structural changes in plants including abiotic environmental factors such as heavy metals. Some manmade activeties like irrigation with sewage sludge, adding fertilizers, and the discharge of domestic wastes in land were the main source of soil and water contamination. Pollution of heavy metals is common in industrial cities, in the air, soil and water and poses many problems causing adverse effects on ecosystems and a risk to human health. Additionally, contaminants enter the food chain through contaminated drinking water or agricultural products. Additionally, contaminants alter soil properties biomass, fertility, crop yield and thus the human health [3]. Toxicity with heavy metals 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. Phytoremediation technology provides solution to this critical problem which is considered a safe and cheap method for the

* Correspondence: Mahmood, ZF.zainab.farqad@gmail.com
Department of Biotechnology, College Science, University of Baghdad, Baghdad, Iraq.
Full list of author information is available at the end of the article.

removal of these pollutants [4, 5].

Tissue culture is a powerful tool that provides the possibility to grow millions of cells under controlled conditions, and to get physiological information about the behavior of the plant cells under stress conditions. Screening and selection at the plant cell level has established plants with increased tolerance to various environmental stresses like salt, drought, diseases, herbicides and heavy metals [6].

Due to the widespread contamination with heavy metals in soils as a result of industrial activates, thus this project was designed to investigate the ability of *H. annuus* to tolerate Zn, screening and selection of tolerant cell lines and develop a regeneration protocol to whole plants and estimation of Zn in the vegetative and root systems of the plants raised from tissue culture.

MATERIALS and METHODS

Seed sterilization

Seeds of *H. annuus* were washed with tap water and then surface sterilized with 70% ethanol for 10 min followed by washing with sterile distilled water three times, then they were sterilized with 50% chlorex (6.25% sodium hypochlorite) with a drop of tween-20 for 30 min. Finally they were washed with sterile distilled water for three times. All steps of sterilization were carried out under aseptic conditions using laminar air flow cabinet. Seeds were transferred to petri dishes containing sterile filter paper to remove excess water [7].

Ready-made Murashage and Skoog **(MS) medium**

Murashage and Skoog, 1962 (MS) medium components were used according to the manufacture instructions [8]. A quantity of 4.99 g of the powder medium was dissolved in one liter of distilled water supplemented with 30 g/L sucrose and growth regulators at different concentrations. Medium pH was adjusted to 5.8, and then 7 g/L agar was added to the medium. The medium was dispensed in universal tubes (8×2.5) cm 10 ml/tube. The culture medium was autoclaved at a pressure of 1.04 Kg/cm^2, 121ºC for 15 min and then left at room temperature till use.

Raising of seedlings

Surface sterilized seeds were germinated in universal tubes (8×2.5) cm/tube on hormone free MS medium, and then incubated in a growth chamber under total darkness at 25 ºC for 5-7 days.

Callus induction and maintenance

The seedlings were transferred to sterilized petri dishes under sterile conditions, the hypocotyls were cut into 0.5 cm and inoculated into callus induction medium containing 0.5 mg/L 2,4-D, 0.4 mg/L NAA and 0.2 mg/L KIN. Explants were cultured with 10 replicates for each treatment; cultures were incubated at 25±2ºC at 1000 lux light intensity for 16/8 h light/darkness. Results were recorded after four weeks of incubation [9].

Small pieces of calli weighting 50 mg were transferred into fresh MS medium supplemented with the same hormonal combination used for callus induction after four weeks. The experiment was carried out with 10 replicates.

Inoculation of callus cultures with medium containing Zn

Different concentrations (0.0, 1, 2, 3, 4 and 5 ppm) of Zn were added to the maintenance medium. About 100 mg of fresh weight calli were subcultured directly onto the prepared medium. Callus fresh weights were recorded after four weeks and re-cultured three times on the same medium. Each treatment was carried out with 10 replicates.

Determination of Zn in callus cultures

Before digestion, samples were dried at 65 ºC for 48 h. One gram of the sample was placed into 250 ml digestion tube and aliquot of 10 ml of concentrated HNO_3 was added to the mixture and then gently boiled for 30-40 min to oxidize all easily oxidizable materials. After cooling, 5 ml of (70%) $HClO_4$ was added and the mixture was boiled gently until dense white fumes appeared. After cooling, 20 ml of distilled water were added and the mixture was boiled further to release any fumes. The solution was cooled, further filtered through 0.4 µM Millipore filter, then transferred to a 25 ml volumetric flask. The Zn was estimated by flame atomic absorption spectrophotometer [10].

Selection of Zn tolerant cell lines

The concentration of Zn at 3.0 ppm was considered under the lethal dose hence tolerant cell lines can be selected. These Zn tolerant cell lines were re-cultured on MS free medium then transferred to MS medium containing the same Zn concentration for further confirmation.

Determination of callus relative fresh weight

Relative fresh weight of the callus initiated from hypocotyl was recorded at different of Zn levels and calculated according to the formula, RFW= $\frac{FWF-FWI}{FWI}$, **(**FWI, initial fresh weight; FWF, final fresh weight) [11].

Regeneration

Shoot regeneration on Zn tolerant shoots

Tolerant calli were selected and transferred into the regeneration medium under aseptic conditions. The regeneration medium consisted of full strength MS medium supplemented with BA (0.0, 0.5, 1.0, 1.5 or 2.0) mg/L and (0.0, 0.3, 0.5, 0.7 or 1.0) mg/L NAA. All cultures were maintained at 25±2ºC for 16/8 h (light/dark) photoperiod with a light intensity of 1000 lux.

Root regeneration on Zn tolerant shoots

Shoots were transferred into the same regeneration medium except the replacement of plant growth regulators with 2 mg/L NAA only [12].

Determination of Zn in plantlets (ppm)

Zn was determined in the vegetative and root tissues separately after drying in an oven depending on previous method [13].

RESULTS and DISCUSSION

Callus induction, maintenance and dry weights

Callus was initiated and maintained on MS medium supplemented with 0.5 mg/L 2,4-D, 0.4 mg/L NAA**,** 0.2 mg/L KIN [9]. This combination was re-examined to confirm that the medium is convenient for callus initiation and maintenance under the experimental conditions in our laboratories (**Tables 1** and **2**).

Table 1. Percentage of callus induction on *H. annuus* hypocotyl explants cultured on MS medium supplemented with 0.5 mg/L 2,4-D, 0.4 mg/L NAA and 0.2 mg/L KIN for four weeks; n, 10.

Treatment	Callus induction (%)
MS free hormones	0.0
MS + plant hormones	100

The addition of auxins and cytokinins are important for callus induction, since cytokinins work as a key for cellular division in the presences of auxins. Inclusive of 2,4-D, NAA and KIN at certain combinations to MS medium gave better callusing compared to other basal media for initiating callus and inducing somatic embryogenesis in H. annuus [14]. In order to maintain and increase callus mass, portions of calli obtained from previous experiment were transferred to MS medium supplemented with the same combination of plant growth regulators giving 650 mg of callus fresh weight and 122 mg of callus dry weight (**Table 2**).

Table 2. Callus fresh and dry weights (mg) grown on MS medium supplemented with 0.5 mg/L 2,4-D, 0.4 mg/L NAA and 0.2 mg/L KIN after four weeks. Initial callus fresh weight was 35 mg. n, 10.

Treatment	Fresh weight (mg)	Dry weight (mg)
MS free hormones	0.0	0.0
MS + plant hormones	650	122

Effect of Zn on callus RFW

Callus cultures were inoculated into MS medium containing different concentrations (0.0, 1.0, 2.0, 3.0, 4.0 or 5.0) ppm of Zn to investigate their expected adverse effect on callus RFW. It is clear from the results shown in **table 3**, a stepwise increase in callus RFW at Zn concentrations 1.0, 2.0 and 3.0 ppm was observed at 2.02, 2.51 and 2.91 gm, respectively. none of these increments were significantly higher than 0.0 ppm except at 3.0 ppm of Zn. Then a decline occurred at the concentrations 4.0 and 0.5 ppm reached 1.36 gm and 1.0 gm, respectively. The reduction in RFW was significantly low at the highest treatment 5 ppm (1.0 gm) compared with the control (1.9) gm indicating a serious deterioration in callus mass. Zn is involved in many cellular functions such as protein metabolism, photosynthetic carbon metabolism and indole acetic acid metabolism, it is the only metal present in all six enzyme classes, oxidoreductases, transferases, hydrolases, lyases, and isomerases. Zn is an important mineral which causes callus RFW to increase at low concentrations but at higher concentrations represents toxic concentration [15]. Toxicity leads to an inhibition of cell activity or disruption of cell organelles and as a result, a decrease in callus RFW occurred since high levels of Zn may stimulate the formation of free radicals; which may lead to oxidative stress.

Table 3. Effect of Zn on callus RFW after culture on maintenances medium. Initial callus fresh wt. 100 mg. n=10**.** ± represents standard error for mean values**.**

Zn Conc. (ppm)	Callus RFW (gm)
0.0	1.90 ±0.08
1.0	2.02 ±0.22
2.0	2.51 ±0.16
3.0	2.91 ±0.25
4.0	1.36 ±0.05
5.0	1.00 ±0.02
L.S.D: 0.05	0.772

Results in **table 3** are in line with those reported by previous study [16], it studied effects of different Zn concentrations on the growth and biomass of *Jatropha curcas* seedlings and found, biomass of cotyledons and hypocotyls, increased gradually at 0.5, 1.0 and 1.5 ppm Zn followed by a decline, when Zn concentration was higher than 3.0 ppm toxic symptoms were observed on the cotyledons, hypocotyls and radicles in Jatropha plant. *In vitro* tolerance to Zn mainly depends on metal exclusion, where Zn uptake and transport is restricted, this restriction is modified by plant cells through the conversion of Zn to a detoxified form by binding it to the cell wall, active pumping of Zn ions into vacuoles or specific metal binding proteins.

Quantitation of Zn in callus tissues

Accumulated Zn in hypocotyl derived callus was determined spectrophotometry. **Table 4** showed Zn accumulation increased in callus tissues with the increase of Zn concentration reaching 15, 18 and 29 ppm at 1.0, 2.0 and 3.0 ppm, respectively. At 4.0 and 5.0 ppm of Zn, the accumulation levels declined recording 12 and 10 ppm due to the toxic effect of these high concentrations on callus cells.

Table 4. Zn accumulation (ppm) in *H. annuus* callus tissues after treatment with different concentrations of Zn after four weeks of culture.

Zn conc. (ppm)	Accumulated Zn (ppm)
0.0	0.0
1.0	15
2.0	18
3.0	29
4.0	12
5.0	10
Mean	14.0
L.S.D 0.05	2.61

Shoot regeneration

Results exhibited that MS medium supplemented with BA at 1.0 and 0.5 mg/l NAA gave the highest shoot formation percentage reached 90% for control, while 82% in calli tolerated 3.0 ppm of Zn (**Table 5**). No shoots formed neither in the absence of BA and NAA nor at high concentrations of BA (2.0) and 1.0 mg/l NAA. This indicates that a synergistic relation between auxins and cytokinins promots tissues differentiation with the preference of higher cytokinin level over the auxin [4]. Similar results were also reported in previous study [17], it showed that good shoot regeneration from sunflower callus cultures when 1 mg/l BA and 0.5 mg/l NAA were added to the medium.

Table 5. Shoot formation percentage from Zn tolerant calli grown on MS medium supplemented with different concentrations of BA and NAA. After four weeks. n=10.

BA (mg/l)	NAA (mg/l)	Non-treated	Shoot formation % from Zn tolerant callus
0.0	0.0	0.0	0.0
0.5	0.3	81	60
1.0	0.5	90	82
1.5	0.7	68	25
2.0	1.0	0.0	0.0
mean		48.0	33.4

Presence of cytokinins at relatively high level accompanied with low level of auxins promotes shoots initiation and

multiplication. Results also showed no shoot formation at high levels of BA and NAA. This may due to the negative effect of NAA on callus response to regenerate shoots [18].

Rooting of regenerated shoots

Elongated and healthy shoots were transferred to a rooting medium consisted of MS medium supplemented with 2.0 mg/l NAA (**Table 6**) [12]. Root formation varied depending on the origin of callus derived shoots, when 2.0 mg/l NAA was added to the medium. The highest percentage of rooting was 90% in the Zn free callus medium, 80% in shoots derived from Zn tolerant calli which is considered to be very high.

Table 6. Root formation percentage of Zn tolerated callus and untreated callus grown on MS medium supplemented with 2 mg.l^{-1} NAA after six weeks.

Treatment	Root formation %
Untreated callus	90
Zn tolerated	80

Effect of Zn on H. annuus plantlets shoot and root fresh and dry weights before acclimatization

A negative effect of Zn on the fresh and dry weight of shoots and roots was recorded. The highest shoots fresh weight was 9.3 gm at the control and 6.1 gm at 3.0 ppm for plantlets derived from Zn tolerant callus (**Table 7**). Root fresh weights were 3.6 and 4.2 gm for the control and 3.0 ppm Zn concentration. Shoot dry weights recorded 4.1 and 2.9 gm for the control and plantlets derived from Zn tolerant callus respectively. However, root dry weights reached 3.1 and 2.3 gm for control and plantlets derived from Zn tolerant callus. Results also showed a significant reduction in plantlets heights, the highest was 13.9 cm in control and 10.3 cm in plantlets derived from Zn tolerant callus. Zn is one of the important cellular minerals used by many enzymatic activities; elevated levels of this mineral cause cells toxicity which in turn leads to decrease plants growth and biomass [19].

Table 7. Effect of Zn on the fresh, dry weight and height of *H. annuus* plantlets derived from Zn tolerated callus and untreated callus after 6 weeks of rooting.

Treatment	Conc. (ppm)	fresh wt. (g)		Dry wt. (g)		Height (cm)
		shoot	Root	shoot	Root	
Non- treated callus	0.0	9.3	3.6	4.1	3.1	13.9
Zn treated	3.0	6.1	4.2	2.9	2.3	10.3
L.S.D: 0.05		1.083	0.503	0.785	0.563	1.628

Accumulation of Zn in H. annuus plantlets

Table 8 shows significant increase in Zn accumulation at 3.0 ppm in plantlets tissue when compared to the control treatment; the highest accumulation level was in the shoots recording 17.5 ppm while it recorded 14.5 ppm in the roots. Zn is involved in many cellular functions such as protein metabolism, photosynthetic carbon metabolism and indole acetic acid (IAA) metabolism, yet at higher concentrations it causes cells toxicity [15]. Other investigators [20] suggested that the production of transgenic plants to enhance pollutants removal from contaminated sites, a technology may negatively affects the ecosystem when plants are genetically modified.

Table 8. Accumulation of Zn in shoots and roots of *H. annuus* plantlets derived from callus treated or non-treated with Zn after 6 weeks of rooting.

Treatment	Conc. (ppm)	Content (ppm) in	
		Shoot	Root
Non- treated callus	0.0	0.0	0.0
Zn treated	3.0	17.5	14.5
L.S.D: 0.05		3.094	2.772

In conclusion, it was found that *H. annuus* callus can be manipulated *in vitro* to increase tolerance to Zn in plantlets derived from selected cell lines. This gain in Zn tolerance can be expressed at the whole plants regenerated from callus cultures and thus, it is possible to enhance Zn tolerance in *H. annuus* plants grown in the field. It seems that sunflower plants accumulate Zn in their vegetative parts and can be manipulated to be hyperaccumulator. Examination of progeny would be the key for evaluating the heritability of Zn in next regenerants.

Conflict of interest

The authors declare that they have no conflict of interests.

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Author affiliation
1. *Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.*
2. *College of Biotechnology, Al-Nahrain University. Baghdad, Iraq.*