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Toxicity of Chloride Strongly Hinders Regeneration of Basil (*Ocimum basilicum* L.) Plants *via* Somatic Embryogenesis

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Abstract: Little information is available on the toxic effects of chloride on *in vitro* culture of plants, therefore, the aim of the present study was to investigate the influences of chloride concentrations on regeneration of *Ocimum basilicum* L. plants *via* somatic embryogenesis. Thus, MS medium, salts of MS medium and the improved revised medium (modified MS medium) containing 2.99 mM of calcium as calcium nitrate and different concentrations of chloride as $CaCl_2.2H_2O$; control; 0 % (0.1 µM Cl^-), 1 % (0.0599 mM), 10 % (0.599 mM) and 100 % (5.99 mM) was examined. The obtained results revealed that, the deleterious effects caused by chloride toxicity on leaf cells of *O. basilicum* were confirmed, also the reduction of chloride concentration in the improved revised MS medium through the reduction of the amount of calcium chloride; 0.0 % (control; 0.1 µM Cl^-) significantly led to increase the percentage of callus induction (from 77 % to 99 %), the percentage of explants induced somatic embryos (from 6 % to 15%) and the average number of plantlets/leaf explant of *O. basilicum* (from 2.10 to 4.32) in comparison with modified MS medium containing different levels of calcium chloride; 1 % (0.0599 mM), 10 % (0.599 mM) and 100 % (5.99 mM). Therefore, these results proved that the presence of chloride in culture medium even at low level 1% (59.9 µM Cl^-) has negative effects on leaf cells of *O. basilicum* in comparison with the control treatment (0.1 µM Cl^-). Also, these results confirmed that the presence of chloride in MS medium with this high concentration (5.99 mM Cl^-) which exceeded the level of some major essential elements such as calcium, sulphur, magnesium and phosphorus which about 3 mM, 1.63 mM, 1.5 mM and 1.25 mM, respectively, is inappropriate for *O. basilicum* cell growth and development and the cells suffer from this toxic concentration of chloride in MS medium which strongly hindered callus induction, the development of somatic embryos and consequently the regeneration capacity of *O. basilicum* was significantly reduced. In the present study, it could be concluded that, although obvious toxic effects of chloride on leaf explants of *O. basilicum* were detected on many leaf explants which developed brown necrotic areas and extended over the entire tissue, then led to explant death, but the most important influences of chloride toxicity on *O. basilicum* cells were the hidden toxic effects which could be appear or conclude from the pronounced inhibition of callus induction, callus quality, development of somatic embryos and led to severe reduction of regeneration capacity *via* embryogenesis. Therefore, the obtained results in this study proved that, enhancement of regeneration *via* somatic embryogenesis from leaf explants of *O. basilicum* was achieved using the improved revised medium (mMS) containing low level of chloride (0.1 µM Cl^-) and this work could be considered as an essential step and an efficient protocol of regeneration *via* somatic embryogenesis for *O. basilicum*, as well as for developing efficient regeneration protocols for different plants in particular the recalcitrant species.

Key words: Basil · *Ocimum basilicum* · Chloride toxicity · Regeneration · Embryogenesis

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) belongs to the family *Lamiaceae* and considers one of the most important aromatic herbs and is widely cultivated worldwide [1]. Basil leaves are largely employed as a flavoring agent for food. Sweet basil is used for pharmaceutical and cosmetic preparations due to the high

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content of essential oils, [1, 2]. The leaves of *O. basilicum* are rich in rosmarinic acid, a predominant phenolic acid detected in the methanolic extract of the leaves and stems [3-5]. Rosmarinic acid (RA) is a caffeic acid ester with several important biological properties, including antioxidant, antibacterial, antiviral and anti-inflammatory activities [6]. Overall, the medicinal properties of *O. basilicum* are that the plant is considered to be stomachic, anthelmintic, antipyretic, diaphoretic, expectorant, carminative, stimulant and pectoral as well as having antiseptic, antiallergic and anticancer effects [7, 3]. It is also used to treat purulent discharge of the ear, bronchitis, hiccup and diseases of the heart and brain [8].

Plant tissue culture is an important tool in plant biotechnology which including micropropagation, production of natural compounds, plant conservation and plant genetic engineering for improving plant productivity and quality in particular under biotic or abiotic stress conditions. In fact it is still difficult to establish an efficient protocol for somatic embryogenesis in *O. basilicum* plant, hence, until now there is only one report on regeneration *via* somatic embryogenesis is available [9]. Recent literature used organogenesis and induction of multiple shoots from node segments as the common protocol of regeneration in this plant [10-17]. Also there is only one report on genetic transformation of *O. basilicum* using *Agrobacterium tumefaciens*-mediated transformation system and the transgenic plants were regenerated from leaf explants [18]. Consequently, there is an urgent need to develop an efficient and reliable embryogenesis protocol as a basic step to improve this important plant through genetic engineering.

But unexpected results were obtained from the preliminary experiments which exhibited low regeneration capacity of *O. basilicum*, hence, these results strongly drove us to investigate the potentialities of reasons related to this problem. Regeneration *via* somatic embryogenesis in plants is the most complicated pathway, thus, the whole genome of the cell is sequentially expressed to develop the entire plant in the form of an embryo and hence, optimal growth conditions including the medium composition and the environmental conditions are indispensable to sustain the progress of cell division and development of embryo formation. Therefore, it is important to optimize the growth conditions to be as possible as optimal requirements to complete the pathway of embryogenesis. The composition of culture medium effectively affects the regeneration capacity of the explants. Therefore, optimization of culture medium is essential to overcome limitation of embryogenesis occurrence in plants. The essential mineral nutrients added to the culture media enhances cell proliferation and regeneration either *via* organogenesis or somatic embryogenesis in plants and according to several intensive studies there are different formula are established for the most of plants, although, there are some recalcitrant plant species still need specific media to regenerate plants in particular through somatic embryogenesis.

It is well known that micronutrients can cause toxic effects when added at high concentrations in culture medium. These toxic effects occur when plant cells absorb and accumulate these micronutrients to the level of toxicity which including competition between ions and interference with some ions involving in the activity of some enzymes which known as ion toxicity to enzymes. Although, chloride is an essential micronutrient it has only a relatively small nutritional significance and if present at high concentration in culture medium toxic influences are occur which including leaf chlorosis with brown necrosis and the necrotic areas could extend over the entire tissue and led to explant death and could cause leaves death and abscission, in addition to growth retardation of the plants which have weak stems and roots and most of these plants fail to survive during adaptation and with high potentiality these plants could collapse and die. In this context, some species are sensitive to chloride ions. Sensitivity to high Cl^- concentrations varies widely between plant species and cultivars. Generally, most nonwoody crops tolerate excessive levels of Cl^-, whereas many woody plant species and beans are susceptible to Cl^- toxicity [19]. Therefore, steps are sometimes taken to reduce the concentration of chloride ion in culture media. The concentration of chloride 5.99 mM in MS medium [20] is very high since this element is micronutrient not macronutrient, although this concentration exceeded the level of some major essential elements (macronutrients) in this medium such as calcium, sulphur, magnesium and phosphorus which about 3 mM, 1.63 mM, 1.5 mM and 1.25 mM, respectively.

Little information is available on the toxic effects of chloride on *in vitro* culture of plants. *In vitro* effect of chloride toxicity can result if too much calcium chloride is added to the culture medium. The most common concentration of chloride in culture media is ranged between 3 and 6 mM for instance; MS medium [20] contains 6 mM Cl^-. McCown and Sellmer [21] reported that too high Cl^- concentration seemed to cause woody species to have yellow leaves and weak stems: sometimes tissues collapsed and died. To solve this problem many

researchers used low amount of calcium chloride or different source of calcium such as calcium nitrate. For example, the medium of Quoirin and Lepoivre [22] contains only 0.123 µM of chloride and calcium nitrate was used as a source of calcium. An excess of Cl^- has been thought to be one cause of the induction of hyperhydricity and omission of the ion does seem to prevent the development of these symptoms in *Prunus*. Thus, Pevalek-Kozlina and Jelaska [23] deliberately omitted chloride ions from WPM medium for the shoot culture of *Prunus avium* and obtained infrequent hyperhydricity in only one genotype. The presence of 7 mM Cl^- can be toxic to pine suspension cultures [24]. Some researchers added 6 mM calcium gluconate to Lloyd and McCown [25] WPM medium to correct Ca^{2+} deficiency, without altering the concentrations of the customary anions [26, 27].

Therefore, the aim of the present study was to investigate the influences of chloride concentrations on regeneration of *O. basilicum* L. plants *via* somatic embryogenesis. To achieve this goal, different media; (a) original MS medium [20], (b) salts of MS medium and (c) the improved revised medium (modified MS medium) containing 2.99 mM of calcium as calcium nitrate and different concentrations of chloride as $CaCl_2.2H_2O$; control; 0 % (0.1 µM Cl^-), 1 % (0.0599 mM), 10 % (0.599 mM) and 100 % (5.99 mM) were examined.

MATERIAL AND METHODS

Media Preparation: In this work, (a) original MS medium [20], (b) salts of MS medium and (c) the improved revised medium (modified MS medium) which containing 14.61 mM (1170 mg/l) or 2 mM (165 mg/l) of NH_4NO_3 instead of 20.61 mM (1650 mg/l) in original MS were used, as shown in (Table 1) and 2.99 mM calcium as calcium nitrate (706.8 mg/l) was used instead of 440 mg/l of $CaCl_2.2H_2O$ to reduce the concentration of chloride, thus, the amount used of this salt to provide 2.99 mM of Ca^{+2} also provides 5.99 mM of Cl^- which is known as trace element, therefore, the $CaCl_2.2H_2O$ was completely omitted to reduce chloride concentration in original MS medium to be only 0.1 µM Cl^- due to the presence of 0.025 mg/l $CoCl.6H_2O$ and to determine the effect of chloride concentrations on callus induction and formation of somatic embryos, different concentrations of $CaCl_2.2H_2O$; 0.0 % (0.1 µM Cl^-) 0 mg/l, 1 % (0.0599 mM Cl^-) 4.4 mg/l, 10 % (0.599 mM Cl^-) 44 mg/l and 100 % (5.99 mM Cl^-) 440 mg/l were added to culture media. All culture media was prepared using individual stocks of MS salts; macronutrients (20x) of each stock and micronutrients (100 x) of each stock, in addition to vitamins of B5 medium [28], additional vitamins (Pantothenate, Biotin, Riboflavin and Folic acid) myo-inositol and casein enzymatic hydrolysate (Sigma- C0626) in case of (b) salts of MS medium and (c) the improved revised medium (modified MS medium), these media formula were used for callus induction medium (CIM), somatic embryogenesis medium (SEM), plantlets development medium (PDM) and root development medium (RDM) as shown in Table 1. All media used in this work were filter-sterilized using Durapore PVDF 0.22 µm, WHPL 47 mm (Millipore Cat. No. GVWP04700), i.e. double concentrated media solution (2x) 500 ml was filter-sterilized then mixed with 500 ml of 2x Gelrite® 70°C (6g/l was autoclaved for 20 min at 121°C and 15 psi) and poured 25 ml aliquots into glass jars (volume 200 ml) or 50 ml into magenta boxes.

Plant Material and Preparation of *in vitro* basil plants: Seeds of sweet basil (*O. basilicum* L.) were provided by the Medicinal and Aromatic Plants Division, Horticultural Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. Basil seeds were treated with 0.3% fungicide (Rizolex-T50% WP) and sown in germination cubs filled with peat moss, then germinated seedling of basil plants were transferred to plastic pots 30 cm in diameter filled with peat moss under greenhouse conditions. Young shoots were excised from basil plants at the time of flowering and were carefully washed 3 times with tap water and liquid detergent, then rinsed under running tap water for 30 min. These shoots were surface sterilized under aseptic condition in laminar flow hood (NUAIRE™, USA) with 0.2 % fungicide (Rizolex-T50% WP) for 5 min followed by soaking for 1 min in 70 % (v/v) ethanol, then washed once with sterile distilled water (D. H_2O), sterile (D. H_2O) and soaking in 0.1% (w/v) $HgCl_2$ (4 min) and thoroughly rinsed 5 times with sterile (D. H_2O) to remove any traces of $HgCl_2$.

Nodal segments of 5 cm long with leaves were cut from sterilized shoots and cultured in glass jars (volume 400 ml) containing 50 ml of culture medium which contains full strength of MS medium [20], supplemented with 2 mg/l BAP, 30 g/l sucrose and 2 g/l gelrite as a solidifying agent and were incubated at 24 °C under cool white fluorescent light with 5000 lux under 16 hr /8 hr light/dark cycle. Then these shoots were subcultured each 2 weeks on the same fresh medium and used as a stock culture for providing leaf explants for regeneration experiments.

Table 1: Composition of different media used for induction and development of somatic embryogenesis in *O. basilicum* L.; (a) original MS medium, (b) salts of MS medium and (c) the improved revised medium (modified MS medium), these media formula were used for (CIM), (SEM), (PDM) and (RDM).

			(c) modified MS			
Substances	(a) MS mg/l	(b) MS salts mg/l	CIM mg/l	SEM mg/l	PDM mg/l	RDM mg/l
Salts						
MS salts *	2,543.33	2,543.33	2,543.33	2,543.33	2,543.33	1,271.67
$CaCl_2.2H_2O$	440	440	-	-	-	-
NH_4NO_3	1650	1650	1170	165	165	585
$Ca(NO3)_2.4H_2O$	-	-	706.8	706.8	706.8	353.4
Amino acids						
Casein hydrolysate	-	300	300	300	300	-
Glycine	2	-	-	-	-	-
Carbohydrates						
Sucrose	30,000	30,000	30,000	30,000	30,000	20,000
Myo-inositol	100	250	250	250	250	-
Vitamins						
Thiamine.HCl	0.1	10	10	10	10	-
Pyridoxine.HCl	0.5	1	1	1	1	-
Nicotinic acid	0.5	1	1	1	1	-
Pantothenate	-	0.5	0.5	0.5	0.5	-
Biotin	-	0.01	0.01	0.01	0.01	-
Riboflavin	-	0.01	0.01	0.01	0.01	-
Folic acid	-	0.01	0.01	0.01	0.01	-
Plant growth regulators						
6-Benzylaminopurine (BAP)			1	1	1	0.5
Kinetin			-	0.5	0.5	
2,4-Dichlorophenoxyacetic acid			0.5		-	-
Naphthalene acetic acid (NAA)			-	1	0.1	-
Indole butyric acid (IBA)			-	-	-	0.1
Gelrite	3,000	3,000	3,000	3,000	2,000	2,000
pH	5.8	5.8	5.8	5.8	5.8	5.8

MS salts *; MS salts without (NH4NO3 and CaCl2.2H2O), (CIM); callus induction medium, (SEM); somatic embryogenesis medium, (PDM); plantlets development medium and (RDM); root development medium.

Callus Initiation and Regeneration *via* somatic embryogenesis: Leaves were excised from *in vitro* basil plants then leaf explants were cut about 1 cm^2 and cultured on different media as shown in Table 1 callus induction medium (CIM). Cultures were incubated at 24 °C and kept under cool white fluorescent light with 5000 lux under 16 hr /8 hr light/dark cycle and subcultured after 2 weeks on the same fresh medium for two more weeks. After 4 week of culture on CIM the produced callus of each explant was transferred to somatic embryogenesis medium (SEM) for 2 weeks, then subcultured on fresh medium for another two weeks. Afterwards, Embyogenic callus containing somatic embryos was transferred to plantlets development medium (PDM) for 4 weeks (subcultured each two weeks). The developed plantlets were transferred to root development medium (RDM) in magenta boxes for 4 weeks. The developed plantlets with strong root system were then transferred to soil mixture peat moss : sand (3:1) respectively, in small pots and covered with plastic pages and kept in a controlled growth chamber at 20 °C with high humidity 80% for 3 weeks, then successfully transferred to big pots and placed in the greenhouse until maturity.

Statistical Analysis: Data were statistically analyzed according to Snedecor and Cochran [29], all treatments were replicated thrice and each replicate with 10 jars (10 explants/jar) and mean values were compared using the least significant difference test (L.S.D.) at 5% level.

RESULTS AND DISCUSSION

At the beginning of this work *in vitro* stock culture of *O. basilicum* plants for providing leaf explants for regeneration experiments was established using shoot explants form cultivated basil plants grown in the greenhouse, thus nodal segments of 5 cm long with leaves were cut from sterilized shoots and cultured on MS

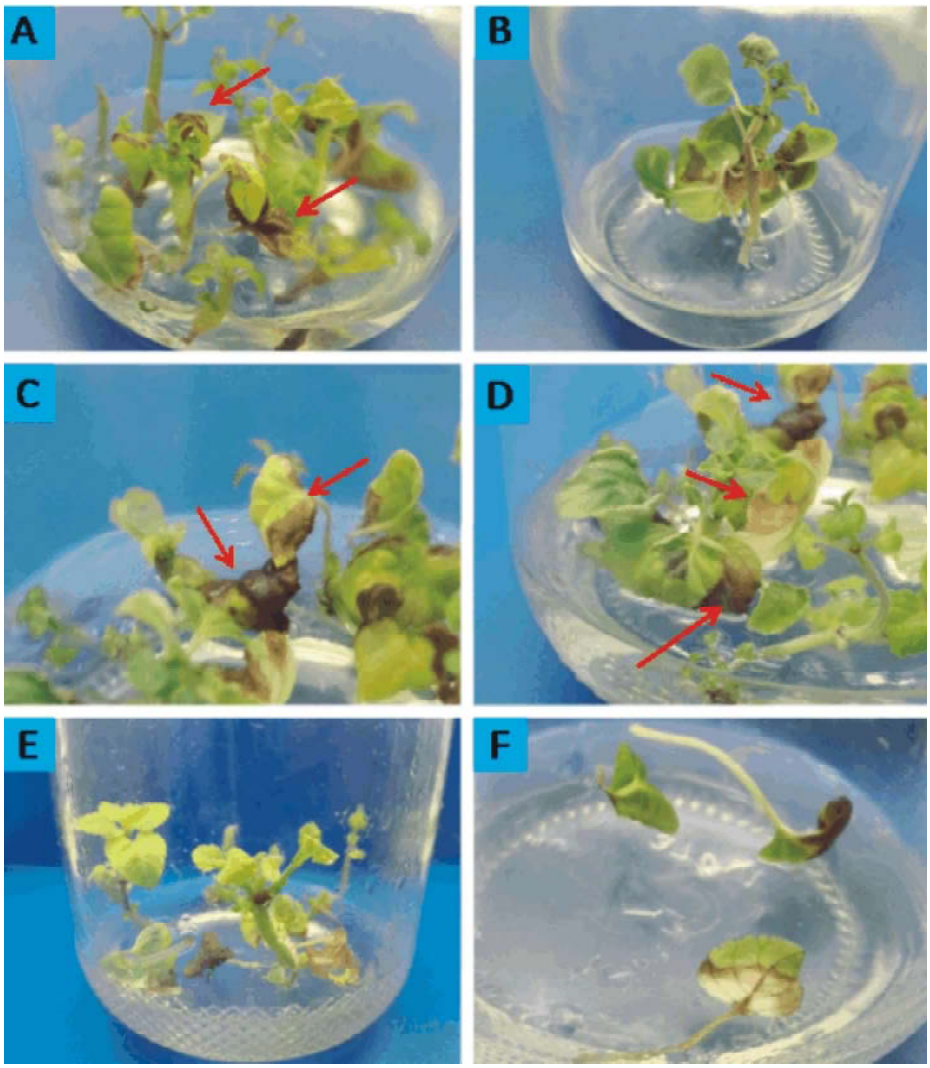

Fig. 1: Symptoms of chloride toxicity on *in vitro O. basilicum* L. cultured on MS medium.
(A) Leaves of plants exhibited light green color then turned to yellow; arrows indicate to the developed brown necrotic tissues on leaves. (B) Weak regenerated shoot with leaves contain brown necrotic tissues. (C and D) in focus the arrows indicate to the developed brown necrotic tissues on leaves. (E) Shoots with light green leaves and abscission of dead leaves. (F) Abscission of leaves with brown necrotic tissues from regenerated shoots.

medium [20], supplemented with 2 mg/l BAP. In this stage, it was observed that, the color of some leaves of the regenerated shoots of *in vitro* plants turned to light green then to yellow. Afterwards, most of these leaves exhibited brown necrotic tissues which were developed and extended over the entire leaves, hence abscission of these leaves were occurred as shown in Fig. 1. In addition, in this study preliminary experiments were conducted to establish an efficient regeneration system of *O. basilicum*, *via* somatic embryogenesis from leaf callus using MS medium [20], supplemented with plant growth regulators according to the protocol of Gopi and Ponmurugan [9]. Also, it was observed from these preliminary experiments that, about 56% of leaf explants produced callus and about 44% of leaf explants developed brown necrotic areas which extended over the entire tissue and led to explant death as shown in Fig. 2A. Overall, inhibition of callus induction and production of non-embryogenic callus was occurred and consequently a strong retardation of somatic embryogenesis was detected.

Table 2: Effect of different levels of calcium chloride in modified MS medium (c) on percentage of callus induction, percentage of explants induced somatic embryos and average number of plantlets/explant using leaf explants of *O. basilicum* compared to (a) original MS medium or (b) salts of MS medium

Concentration of CaCl2.2H2O	Percentage of explants induced callus	Percentage of explants induced somatic embryos	Average No. of plantlets /explant
(a) MS (100%)	56± 2.36	1.79± 0.06	1.0± 0.00
(b) MS salts (100%)	65± 2.92	3.07±0.11	1.50± 0.04
(c) mMS (0.0 %)	99± 4.83	15.15±0.73	4.33±0.31
(c) mMS (1%)	81± 3.96	7.41±0.35	2.33±0.10
(c) mMS (10%)	78± 3.79	6.41±0.32	2.20±0.09
(c) mMS (100%)	77± 3.74	5.19±0.28	2.00±0.12
L.S.D.at 0.05	6.11	2.93	0.64

The mean (±SE) of three replicates.

The obtained results of these experiments revealed that a very low percentage of calli produced somatic embryos (1.79 %) and also the number of regenerated plantlet was about (1.0 plantlets/explants) as the same obtained results with the treatment of original MS medium as shown in Table 2. Therefore these results drove us to conduct a set of experiments to examine the effect of chloride concentrations in culture medium on callus induction from leaf explants of *O. basilicum*, callus quality and regeneration *via* somatic embryogenesis.

The Influence of Chloride Concentrations in Culture Medium on Regeneration of *O. Basilicum Via* Somatic Embryogenesis: The use of the original MS medium (including salts and vitamins) for callus induction medium (CIM), somatic embryogenesis medium (SEM), plantlets development medium (PDM) as a control treatment in all experiments resulted in the lowest level of the percentage of callus induction (56%), the percentage of explants induced somatic embryos (1.79 %) and the average number of plantlets/leaf explant of *O. basilicum* (1.0) as shown in Table 2. While, the use of MS salts only in addition to the vitamins and other supplements as in the improved revised medium (modified MS medium; mMS) as shown in Table 1, resulted in significant increase in the percentage of callus induction (from 56% to 65%) and relative enhancement in the percentage of explants induced somatic embryos (1.72-fold) and the average number of plantlets/leaf explant of *O. basilicum* (1.50-fold) in comparison with the original MS medium.

On the other hand, the use of the improved revised medium (modified MS medium) containing 100% (5.99 mM Cl^-) as the same concentration in original MS medium, for callus induction medium (CIM), somatic embryogenesis medium (SEM), plantlets development medium (PDM) and root development medium (RDM), was significantly led to increase the percentage of callus induction (from 56% to 77%), the percentage of explants induced somatic embryos (from 1.79 % to 5.19 %) and the average number of plantlets/leaf explant of *O. basilicum* (from 1.0 to 2.0) in comparison with the original MS medium. Moreover, the obtained results in Table 2 revealed that, the reduction of chloride concentration in the improved revised medium (mMS) through the reduction of the amount of calcium chloride; 0.0 % (control; 0.1 µM Cl^- due to the presence of 0.025 mg/l $CoCl.6H_2O$) was significantly led to increase the percentage of callus induction (from 77% to 99%), the percentage of explants induced somatic embryos (from 5.19 % to 15.15 %) and the average number of plantlets/leaf explant of *O. basilicum* (from 2.0 to 4.33) which about 2-fold increase in comparison with 100% (5.99 mM Cl^-) treatment as shown in Table 2 and Fig. 2. On the other hand, unforeseen results showed that, no significant differences on percentage of callus induction, percentage of explants induced somatic embryos and average number of plantlets/explant were detected between these levels of calcium chloride (1%, 10% and 100%) added to mMS medium. Therefore, these results proved that the presence of chloride in MS medium even at the low level 1% (59.9 µM Cl^-) has negative effects on the above mentioned parameters in comparison with the control treatment 0.0 % (0.1 µM Cl^-).

Little information is available on the toxic effects of chloride on *in vitro* culture of plants, therefore in this study, the deleterious effects caused by chloride toxicity on leaf cells of *O. basilicum* were confirmed, which hindered callus induction and development of somatic embryos in addition, many leaf explants developed brown necrotic areas which extended over the entire tissue and led to explant death.

The obtained results revealed that, the effects of Cl^- toxicity did not disappeared with low levels of chloride and that may be because these concentrations 1% (59.9 µM Cl^-), 10% (599 µM Cl^-) and 100% (5990 µM Cl^-) represent about 599 times, 5990 times and 59900 times, respectively, of the concentration in the control treatment 0.0 % (0.1 µM Cl^-); MS medium without $CaCl_2.2H_2O$. Therefore, these concentrations of chloride proved to be

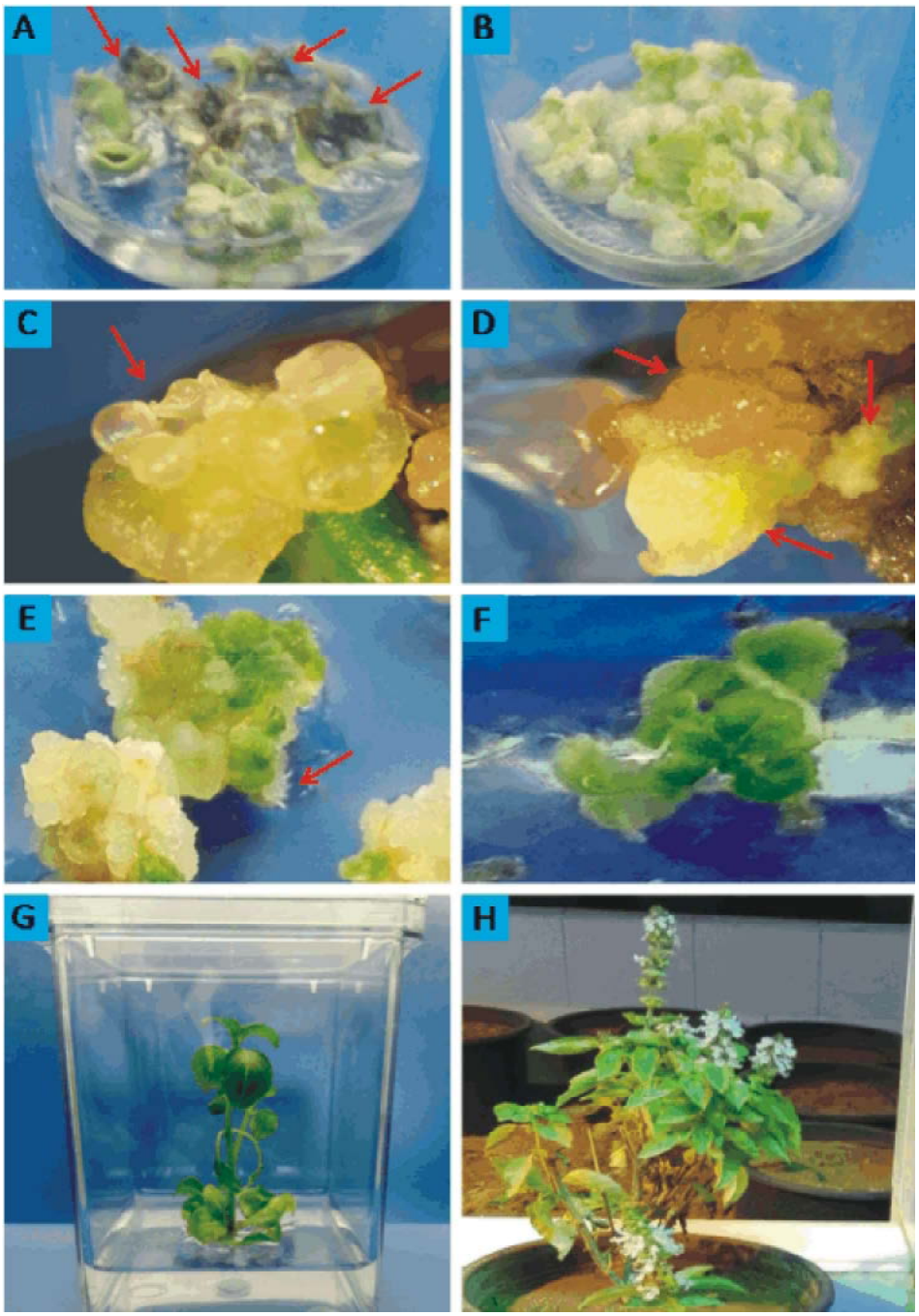

Fig. 2: The improved revised medium (modified MS medium; mMS) enhanced regeneration of *O. basilicum* L. *via* somatic embryogenesis. (A) Leaf explants after 10 days of culture on original MS medium for callus induction; arrows indicate to the developed brown necrotic tissues on leaf explants. (B) Leaf explants with embryogenic calli after 4 weeks of culture on mMS medium (CIM) for callus induction. (C) In focus; the arrows indicate to the developed somatic embryos after 2 weeks and (D) after 4 weeks on SEM; somatic embryogenesis medium. (E) Development of plantlets from somatic embryos after 2 weeks; arrow indicate to the developed roots of embryos and (F) after 4 weeks on PDM; Plantlets development medium. (G) Elongation of plantlets with full developed roots in magenta box after 4 weeks on RDM; Root development medium. (H) Successful growth and development of fertile regenerated plant in the greenhouse.

very high and toxic to the cells of *O. basilicum* and this may explain why the effects of toxicity did not disappeared even with the lowest level 1% (59.9 µM Cl^-) of $CaCl_2.2H_2O$ in comparison with the control treatment (0.1 µM Cl^-). These results proved that the presence of chloride in MS medium with this high concentration (5.99 mM Cl^-) which exceeded the level of some major essential elements such as calcium, sulphur, magnesium and phosphorus which about 3 mM, 1.63 mM, 1.5 mM and 1.25 mM, respectively, is inappropriate for *O. basilicum* cell growth and development and the cells suffer from this toxic concentration of chloride in MS medium which hindered callus induction, the development of somatic embryos and consequently the regeneration capacity of *O. basilicum* was significantly reduced. In this study it could be concluded that, the most important influences of chloride toxicity on *O. basilicum* are the hidden toxic effects of high concentrations of chloride in culture medium which could be appear or conclude from the inhibition of callus induction or low quality of callus such as watery callus or non-embryogenic callus and reduction of regeneration capacity in particular through embryogenesis.

In this context, some species are sensitive to chloride ions. Sensitivity to high Cl^- concentrations varies widely between plant species and cultivars. Generally, most nonwoody crops tolerate excessive levels of Cl^-, whereas many woody plant species and beans are susceptible to Cl^- toxicity [19]. *In vitro* effect of Chloride toxicity can result if too much calcium chloride is added to the culture medium. The most common concentration of chloride in culture media is ranged between 3 and 6 mM for instance; MS medium [20] contains 6 mM Cl^-. McCown and Sellmer [21] reported that too high Cl^- concentration seemed to cause woody species to have yellow leaves and weak stems: sometimes tissues collapsed and died. To solve this problem many researchers used low amount of calcium chloride or different source of calcium as calcium nitrate used in this study as shown in Table 1. In this respect, the medium of Quoirin and Lepoivre [22] contains only 0.123 µM of chloride and calcium nitrate was used as a source of calcium. An excess of Cl^- has been thought to be one cause of the induction of hyperhydricity and omission of the ion does seem to prevent the development of these symptoms in *Prunus*. Thus, Pevalek-Kozlina and Jelaska [23] deliberately omitted chloride ions from WPM medium for the shoot culture of *Prunus avium* and obtained infrequent hyperhydricity in only one genotype. The presence of 7 mM Cl^- can be toxic to pine suspension cultures [24]. As chloride has only a relatively small nutritional significance, steps are sometimes taken to reduce the concentration of chloride ion in culture media, but in order to adjust the concentration of other ions, it is then often necessary to make a marked increase in (SO_4) Zeldin and McCown [26], Russell and McCown [27] added 6 mM calcium gluconate to Lloyd and McCown [25] WPM medium to correct Ca^{2+} deficiency, without altering the concentrations of the customary anions.

Here it is worthy to mention that, the improved revised medium used in this study was developed based on a wide survey and intensive investigation on the impact of media composition on the development of somatic embryos in different plant groups such as barley and rice plants as monocotyledons and *O. basilicum* plant as dicotyledon. Thus, the concentration of ammonium in the media formula was reduced to 1/10 of the concentration in MS medium [20], therefore, 165 mg/l of NH_4NO_3 was added to obtain the modified MS medium containing 1/10 of NH_4NO_3 [30-34]. Also the use of the vitamins of B5 medium [28] and the additional vitamins (Pantothenate, Biotin, Riboflavin and Folic acid), myo-inositol and casein enzymatic hydrolysate proved to be essential in this modified medium which significantly enhanced the regeneration capacity of *O. basilicum via* somatic embryogenesis.

In conclusion, enhancement of regeneration *via* somatic embryogenesis from leaf explants of *O. basilicum* was achieved in this study using the improved revised medium (mMS) containing low level of chloride (0.1 µM Cl^-) and this work could be considered as an essential step and an efficient protocol of regeneration *via* somatic embryogenesis for *O. basilicum*, as well as, for developing efficient regeneration protocols for different plants in particular the recalcitrant species.

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