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Response of Shoot Multiplication and Characteristics of Chinese Mandarin as Affected by Modification of MS Medium

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ABSTRACT

Micro scions of Citrus which are formed *in vitro* are very important in virus-free plants production. Micro scion multiplication faces multi obstacles such as drop leaves and shoots tip necrosis. So, this investigation aims to develop the micro propagation medium through examine the effect of modification of the mineral's concentrations in medium of chinese mandarin. Explants were cultured on full, half and quarter strength of MS medium individual or in combination with different concentrations (25, 50 and 100% of recommended rate) of $MgSO_4$, $7H_2O$, $MnSO_4$, $4H_2O$, $CaCl_2$, $2H_2O$ and KH_2PO_4. All media supplemented with 2 mg/l BA. The highest axillary shoot number (13 shoots/ jar) was obtained from full MS strength medium supplemented with 25% of $MgSO_4$, $7H_2O$, $MnSO_4$. $4H_2O$, $CaCl_2$, $2H_2O$ and KH_2PO_4. While, full MS strength supplemented with 50% of $MgSO_4$. $7H_2O$ and KH_2PO_4 recorded the highest value (55shoots/jar) of axillary shoots. On contrary, the highest number of adventitious shoots (65shoots/jar) resulted from full MS strength supplemented with 50% of $MgSO_4$, $7H_2O$ and $CaCl_2$, $2H_2O$. Interestingly, full MS strength supplemented with 50% of $MgSO_4$, 7H2O, $MnSO_4$, $4H_2O$ and KH_2PO_4 or supplemented with 25% of $MgSO_4$, $7H_2O$, $MnSO_4$. $4H_2O$, $CaCl_2$, $2H_2O$ and KH_2PO_4 prevent drop leaves phenomenon and shoot tip necrosis as compared to control treatment.

Keywords: MS modification, micro scion, mandarin, citrus, Ca, Mg, Mn, K

1. Introduction

Citrus are the most important fruit crop as well as apples and bananas (FAO 2001)**.** Citrus mainly includes oranges (with 55% of the world production), mandarins (25%), lemons/limes (13%) and grapefruits (7%) (FAO 2017). It was thought to be originated in South East Asia and now growing in more than 30 countries around the world (Gottwald *et al.,* 2002). It is widely grown at latitude 35°N-35°S (Ruiz *et al.,* 2000). *Citrus aurantifolia* has important fleshy, juicy and edible fruit plant belonging to Family Rutaceae (Katz and Weaver, 2003; Rathore *et al.,* 2006). The sustainable development of the Citrus industry is mainly depending on a continuous supply of new and improved cultivars (Tornero *et al.,* 2010; Wu *et al.,* 2018) divided mandarins into 3 groups pure *C. reticulata*, unnamed Chinese mandarins, and the ancient Chinese cultivar.

Conventional methods for *Citrus* propagation are based on budwood selection and grafting for scion varieties (Carimi and Pasquale, 2003). Micropropagation through nucellar embryos eliminate many viral diseases (Onghia *et al.,* 2001; Hamza *et al.,* 2013), the plant material obtained by somatic embryos regenerated *in vitro* can be used to establish healthy citrus stocks. Shoot multiplication is described for *Citrus latifoliaTan*. (persian lime) using nodal segment explants of young one year old trees by two different pathways with and without callus phase. The best result for multiple shoot formation and regenerated shoot formation was 3.2 and 2.6 shoots per explants with 4.44 µM BA plus 0.053 µM NAA and 4.44 µM BA plus 0.049 µM IBA respectively. A like shoot regeneration, shoot elongation was occurred in medium supplemented with 4.44 µM BA and 0.049 µM IBA. Micropropagated and regenerated plants are under other experiments (Chamandoosti, 2017). However in vitro micropropagation technology can overcome some constraints to Citrus improvement and

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cultivation, also can increase fruit quality and resistance to diseases and environmental stresses (Gresser, 1994). Also, micropropagation system with high multiplication rates is an important asexual method that can be used for the production of clonal plants (Cardoso *et al.,* 2010; He *et al.,* 2011; Ali *et al.,* 2012).

In all cases, it is necessary to be able to regenerate viable shoots, which can be propagated by either organogenesis or somatic embryogenesis (Tornero *et al.,* 2010). Compared to MS medium, best results were obtained on the modified ME medium (supplemented with 0.2 mg l^{-1} Topolin, 10 mg l^{-1} glutamine, 100 mg l^{-1} sequestrene and double concentration of $MgSO_4$ and KH_2PO_4) significantly increased elongation and improved vigor of in vitro shoots and decrease the rate of shoot tip necrosis (Mirabbasi and Hosseinpour, 2014). Calcium, in the form of calcium pectate, is responsible for holding together the cell walls of plants. When calcium is deficient, new tissue such as root tips, young leaves, and shoot tips often exhibit distorted growth from improper cell wall formation. Calcium is also used in activating certain enzymes and to send signals that coordinate certain cellular activities (Hepler, 2005).

Magnesium (Mg) has diverse physiological roles in biological systems. Particularly, Mg is important to plants, 15–20% of total Mg associated with chlorophyll pigments, with its tendency to form octahedral complexes, acts mainly as a cofactor of a series of enzymes involved in photosynthetic carbon fixation and metabolism, and the remaining fraction stored in the vacuole (Wanli, 2019).

Manganese (Mn) plays an important role in oxidation and reduction processes in plants, such as the electron transport in photosynthesis. Manganese also has played a role in chlorophyll production, and its presence is essential in Photosystem II. Manganese acts as an activating factor which is causes the activation more than 35 different enzymes. Due to the metabolic role of manganese in the nitrate-reducing enzyme activity and activation of enzymes which play roles on carbohydrate metabolism (Mousavi *et al.,* 2011).

Mesos salts ($CaCl_2$, $2H_2O$, $MgSO_4$, $7H_2O$, and KH_2PO_4) are currently some of the most studied factors influencing growth of the plants in vitro. Rarely, K_2SO_4 is also included in the mesos group (Kovalchuk *et al.,* 2017). The majority of studies showed that increased mesos salts have a positive effect on growth parameters such as shoot length or shoot number, theraby contributing to a better quality of treated shoots (Poothong and Reed, 2015). The results indicate that mesos manipulation significantly influences the number and length of shoots in most of the studied cultivars. The greatest multiplication rate for A. alnifolia was achieved with tripled mesos, whereas 'Black Satin' and 'Loch Ness' reacted positively to a lower (1–2x) concentration of mesos. Decreasing the concentration of mesos to half led to worse quality in both blackberry and Saskatoon shoots. 'Brigitta Blue' was more sensitive to greater mesos concentrations compared to 'Toro'. Optimizing the mineral nutrition of plants cultivated in vitro enhances their multiplication rate and contributes to a higher production of good quality plantlets (Hunková *et al.,* 2020).

This investigation aimed to optimize medium composition to determine the most efficient medium for citrus shoot multiplication induction and overcome some citrus micropropagation disorders such as, leaves drop and shoot tip necrosis.

2. Materials and Methods

This study was carried out in Plant Tissue Culture Laboratories, Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt, during the period from 2014 to 2018.

2.1. Plant Material

Mother plant stock was planted in the Ali Moubark research farm, El-Bostan, Behera Governorate. Explants used in tissue culture experiments were dissected from mother plant stock in 1st November.

2.2. Explant sterilization

Seeds separated from fruit were used as in vitro initiation malterials (explants). Which were soaked in 5, 25 % (v/v) NaOCl solution for 15 min. subsequently, washed three times with sterile distilled water. Then fruit were just dipped in 70% Ethanol and taken out ethanol and were flamed. Fruits were opened in order to collect the seeds. Then seeds were washed with sterile water.

2.2.1. *In vitro* seed germination

Seeds were cultivated in jars (210 ml) contained 50 ml Murashige and Skoog (MS) free hormones (Murashige and skoog, 1962). The cultures were incubated at temperature of 26±2°C and total darkness until seeds germination, and then transferred to photoperiod 16 to 8h (light and dark) and light intensity 2000 lux to maintain growth.

2.3. Effect of MS strengths individual or in combination with different concentrations of Ca, K, Mg, and Mn on growth parameters of Chinese mandarin

After *in vitro* germination of seeds, shoot tips of nucellar embryos of china mandarin were planted on full, half and quarter strength of MS medium individual or in combination with different concentrations (25, 50 and 100% of recommended concentrations of Ca, K, Mg and Mn).The sources and concentrations of the salts in the experiments were $CaCl_2.2H_2O$ (110, 220 and 440 mg/l) KH_2PO_4 (42.5, 85 and 170 mg/l) $MgSO_4.7H_2O$ (92.5, 185 and 370 mg/l) and $MnSO_4.4H_2O$ (5.5, 11.15 and 22.3 mg/l) as sources of Ca, K, Mg and Mn. All media supplemented with 2mg/l BA each treatment consists of three replicates each replicate three jars. The cultures were incubated at a temperature of 26±2°C and photoperiod 16 to 8h (light and dark) and light intensity 2000 lux. The following parameters were observed and recorded after 30 days: direct shoots number, leaves number, number of dropping leaves, shoot necrosis, growth vigor and shoot length (cm).

2.4. Effect of MS medium supplemented with different concentrations of individual or combinations of Mg, Mn, Ca and K on Chinese mandarin multiplication

In this experiment, shoot tips of nucellar embryos of Chinese mandarin were planted on MS medium individual or MS supplemented with Ca, K, Mg and Mn (from these sources: $CaCl_2.2H2O$, KH_2PO_4, $MgSO_4.7H_2O$ and $MnSO_4.4H_2O$,) at 25% and 50% of its concentrations in MS medium, with different combinations as shown in Table 2. All media supplemented with 2mg/l BA each treatment consists of three replicates each replicate three jars. The cultures were incubated at the same conditions of the previous experiment. Axillary and Adventitious shoots number, leaves number, dropping leaves number, shoot necrosis, growth vigor and shoot length (cm), were observed and recorded after three months.

2.5. Data analysis

Experiments design and statistical analysis: Experiments designed was complete randomized. Data were analyzed using analysis of variance (ANOVA). Comparisons between average values from the different treatments were made by L.S.D. test at a 0.05 probability significance level using SAS (1988) package using computer software MSTAT-C (MSTAT Development Team, 1988). The least significant difference among levels of each treatment was compared using LSD. Test at 5% level according to Steel *et al.* (1997).

3. Results and Discussion

3.1. Effect of MS strengths individual or in combination with different concentrations of Mg, Mn, Ca and K on growth parameters of Chinese mandarin presented in Table 1 and Figure 1.

3.1.1. Shoots Number

As shown in Table 1 and Figure 1, the strength of MS medium affected the shoots number. The highest value of shoots number was obtained from full MS strength while the lowest value was obtained from quarter MS strength. Concerning the salt concentrations, the highest shoots number was observed from 25%Ca+25%Mg+25%Mn+25%K treatment while no significant difference was detected among the other treatments. Regarding the interaction between the MS strength and salt concentrations, the full MS strength combined with the lowest salt concentrations (25%Ca+25%Mg+25%Mn+25%K or 50% Ca+50% Mg+50% Mn+50% k) gave the highest values of shoot number (13.00 and 12.67shoot/ replicate respectively). While, the lowest value of shoot number resulted from half strength of MS without any addition of salts (4.667 shoot/ replicate).

3.1.2. Number of the leaves.

Data in Table 1 revealed that the full MS strength gave the highest number of leaves (43.17 leaf/ replicate), while no significant difference was observed between the other MS strength. The high

concentration of salts (100%Ca+100%Mg+100%Mn+100%K) gave the highest value of number of the leaves (32.56 leaves/ replicate), while, using full MS strength without any salt addition gave the lowest number of leaves (24.11 leave/ replicate). In relation to interaction between the previous two factors, using full MS strength with all salt's concentrations (100, 50 and 25% of the recommended salts doses) gave the highest values of number of the leaves (46.67, 44.00 and 48.00 leaves/jar, respectively). while using half strength of MS with addition of 50% Ca+50% Mg+50% Mn+50% k gave the lowest Number of the leaves (17.33 leave/ replicate) with no significant difference between the later treatment and MS without salt additions.

3.1.3. Number of the drop leaves.

Drop leaves was affected by MS strength but the differences were not significant. Addition of salt concentrations positively affected the decreasing of number of drop leaves compared with MS without salt additions. The interaction data cleared that full MS with all addition of salt concentrations inhibited the number of drop leaves compared with MS without addition of salts. Also, half MS strength in combined with100% addition of salt concentration resulted in decreasing the drop leaves number.

Table 1: Effect of MS strengths individual or in combination with different concentrations of Mg, Mn, Ca and K on growth parameters of Chinese mandarin

Concentration of additive salts (As % of MS)	MS strength			
	Full MS	**Half MS**	**Quarter MS**	**Means**
		Shoots Number		
Without Salt additive	8.67	6.33	4.67	6.56
25%Ca+25%Mg+25%Mn+25%K	13.00	5.67	7.67	8.78
50% Ca+50% Mg+50% Mn+50% k	12.67	5.00	6.00	7.89
100%Ca+100%Mg+100%Mn+100%K	11.00	6.33	7.33	8.22
Means	11.33	5.83	6.42	
LSD at 5%	A 2.216	A&B 3.838	B 1.92	
		Number of the leaves		
Without Salt additive	34.00	19.33	19 .00	24.11
25%Ca+25%Mg+25%Mn+25%K	46.67	18.67	26.33	30.56
50% Ca+50% Mg+50% Mn+50% k	44.00	19.33	17.33	26.89
100%Ca+100%Mg+100%Mn+100%K	48.00	22.33	27.33	32.56
Means	43.17	19.92	22.50	
LSD at 5%	A 4.12	A&B 6.12	B 3.48	
		Number of the drop leaves.		
Without Salt additive	10.00	6.33	10.0	8.78
25%Ca+25%Mg+25%Mn+25%K	2.33	2.67	7.00	4.00
50% Ca+50% Mg+50% Mn+50% k	0.00	4.37	5.67	3.38
100%Ca+100%Mg+100%Mn+100%K	5.33	4.667	2.700	4.233
Means	4.44	4.51	6.342	
LSD at 5%	A 2.32	A&B 4.02	B 2.011	
		Number of the shoot necrosis		
Without Salt additive	2.00	0.40	3.00	1.80
25%Ca+25%Mg+25%Mn+25%K	0.40	3.67	1.67	1.91
50% Ca+50% Mg+50% Mn+50% k	0.00	2.37	0.70	1.06
100%Ca+100%Mg+100%Mn+100%K	1.41	2.00	0.40	1.27
Means	0.98	2.11	1.44	
LSD at 5%	A 0.6854	AXB 1.187	B 0.59	
		Shoot length		
Without Salt additive	1.10	2.00	0.93	1.34
25%Ca+25%Mg+25%Mn+25%K	0.93	0.27	0.13	0.44
50% Ca+50% Mg+50% Mn+50% k	0.33	0.70	0.97	0.67
100%Ca+100%Mg+100%Mn+100%K	0.47	0.27	0.13	0.29
Means	0.71	0.81	0.52	
LSD at 5%	A 0.31	AXB 0.53	B 0.27	

*Concentration was calculated as percentage of the salt concentration on MS medium

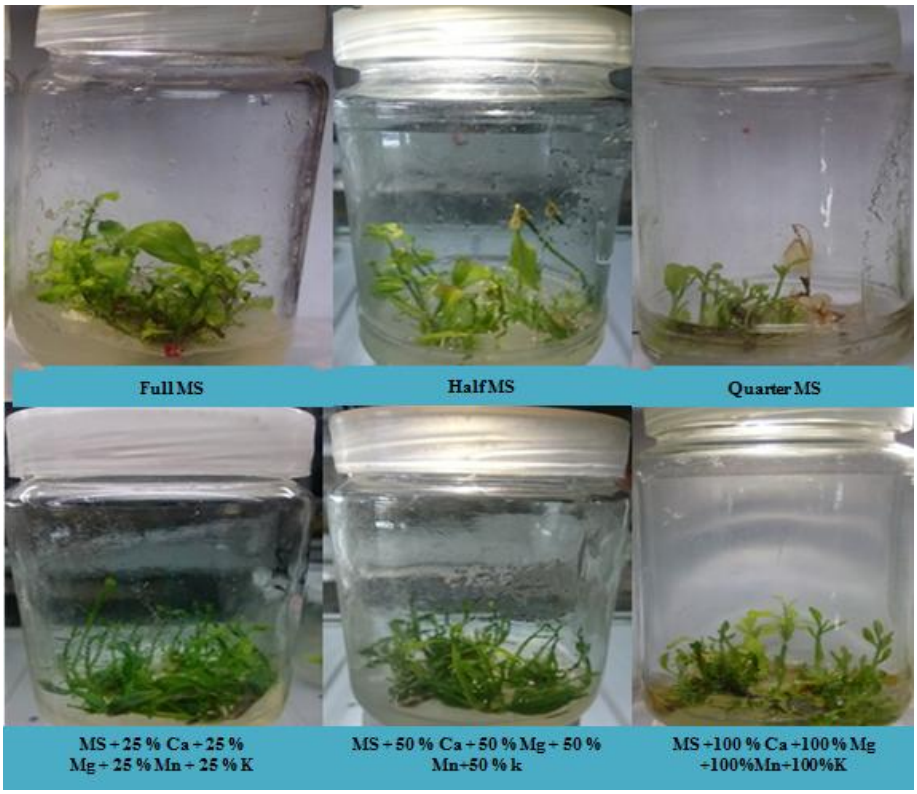

Fig. 1: Effect of different MS strength (full, half and quarter) individual or in combination with different concentrations of Mg, Mn, Ca and K on Chines mandarin multiplication

3.1.4. Shoot necrosis

Shoot necrosis is obstacles observed during citrus micropropagation. MS strength cleared slight differences without significant between various treatments. Addition of different concentrations of salts resulted in significant decreasing of number of shoot tip necrosis. Anyway, using full MS strength with addition of 50% Ca+50% Mg+50% Mn+50% k could overcome this obstacle and eliminated shoot necrosis.

3.1.5. Shoot length.

MS strength affected chines mandarin shoot length, half MS strength gave the highest shoot length (2.00 Cm) followed by full MS strength and quarter MS strength (1.10. and 0.93 cm, respectively). On the other hand, addition of different salts concentrations significantly minimized shoot length compared with MS without salt addition. Interaction data revealed that half MS strength without addition of salts significantly produced the highest shoot length (2.00cm) followed by full MS strength without addition of salts and with addition of 25%Ca+25%Mg+25%Mn+25% K.

3.2. Effect of MS medium supplemented with different concentrations of individual or combinations of Mg, Mn, Ca and K on Chinese mandarin multiplication:

3.2.1. Shoots Number

Shoot number was recorded as axillary and adventitious shoots and presented in Table 2.

3.2.2. Number of axillary shoots

As shown in Table 2 and Figure 2, the highest value of axillary shoot number (55 shoots/replicate) was obtained from MS supplemented with half recommended concentrations from Mg and K. The most treatments showed significant superior response and gave axillary shoots more than MS without additives.

Table 2: Effect of different concentrations of individual or combinations of Mg, Mn, Ca and K on Chinese mandarin multiplication

Salts concentrations* and combinations	Axillary Shoots NO.	Adventitious shoots NO.	Leaves NO.	Drubbing Leaves NO/shoot.	Shoot necrosis	Growth Figure	Shoot length
MS without salts addition	15.0	0.0	60.0	3.30	8.0	1.0	1. 0
25% Ca	15.0	10.0	60.0	1.80	3.0	1.0	0.8
25%Mg	22.0	8.0	125.0	1.40	0.0	2.0	1.2
25%Mn	15.3	40.0	130.	1.60	5.0	1.0	0.7
25%K	12.0	30.0	127.0	2.90	7.0	1.0	1. 0
50%Ca	25.0	15.0	120.0	0.80	3.0	2.0	0.8
50%Mg	4.0	5.0	45.0	0.40	0.0	1.0	1.5
50%Mn	10.0	45.0	140.0	1.50	3.0	3.0	1.1
50%K	20.0	1.0	80.0	2.00	4.0	1.0	0.9
025%Ca+25% Mg	10.0	15.0	95.0	1.30	2.0	1.0	1. 3
25%Ca +25% Mn	7.0	25.0	95.0	1.50	6.0	1.0	0.8
25%Ca +25% K	11.0	8.0	60.0	1.50	2.0	1.0	0.7
25%Mg +25% Mn	20.0	30.0	170.0	1.00	2.0	2.0	1.3
25%Mg+25%K	30.0	7.0	165.0	1.00	3.0	1.0	1.2
25%Mn+25%K	19.0	5.0	98.0	2.00	7.0	1.0	0.9
25%Ca+25%Mg+25%Mn	35.0	15.0	170.0	1.10	3.0	3.0	1.2
25% Ca+25%Mn+25%K	25.0	0.0	90.0	0.80	0.0	1.0	0.7
25%Mg+25%Mn+25%K	25.0	0.0	99.0	1.50	0.0	1.0	1. 0
25%Ca+25%Mg+25%Mn+25%K	25.0	0.0	95.0	0.0	2.0	2.0	1. 0
50%Ca+50% Mg	5.0	65.0	210.0	00	0.0	4.0	2.5
50% Ca +50% Mn	19.0	6.0JK	100.0	0.50	6.0	1.0	1.4
50%Ca +50% K	14.0	1.0	50.0	0.70	9.0	1.0	1.2
50% Mg +50% Mn	10.0	20.0	95.0	0.50	3.0	3.0	1.5
50%Mg+50%K	55.0	5.0	180.0	0.50	5.0	2.0	2.2
50% Mn+50% K	20.0	0.0	80.0	0.80	6.0	1.0	1.3
50%Ca+50%Mg+50%Mn	40.0	0.0	150.0	0.20	5.0	2.0	2.0
50%Ca+50%Mn+50%K	15.0	5.0	85.0	0.40	5.0	1.0	1.8
50%mg+50%Mn+50%K	10.0	55.0	200.0	0.0	0.0	5.0	3.0
50%Ca+50%Mg+50%Mn+50%K	25.0	1.0	110.0	0.30	5.0	1.0	2.0
LSD at 5%	2.2	2.7	3.1	0.13	0.7	0.1	0.1

*Concentration was calculated as percentage of the salt concentration on MS medium

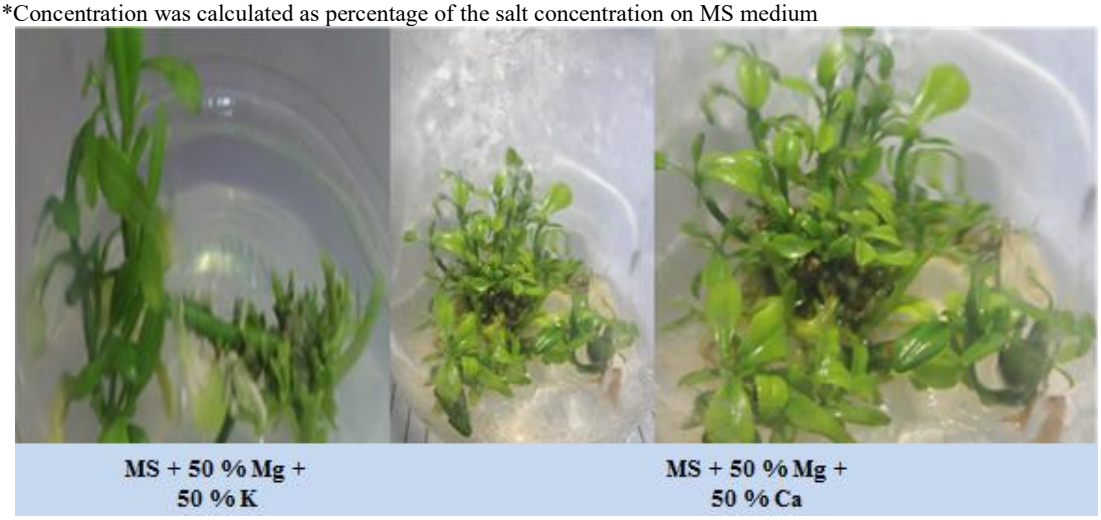

Fig. 2: Effect of MS medium supplemented with different combinations from Ca, Mn, Mg and K on Chinese mandarin multiplication

3.2.3. Adventitious shoots

The highest number of adventitious shoots (65 shoots / replicate) were obtained from full strength MS medium supplemented with half recommended concentrations from Mg and Ca, MS without additive did not produced adventitious shoots, the most treatments significantly maximized the number of adventitious shoots.

3.2.4. Number of the total leaves.

Using full strength MS medium supplemented with half recommended concentrations from Mg and Ca gave the highest number of leaves (210 leaves/ replicate). While using full strength MS medium supplemented with half recommended concentrations from Mg gave the lowest number of leaves (45 leaves/ replicate).

3.2.5. Number of the drop leaves.

Among all treatments used in this experiment, three of them (MS+ quarter concentrations of Ca, Mg, Mn and K, as well as MS+ half concentrations of Ca and Mg and MS+ half concentrations of Mg, and Mn) prevent dropping leaves phenomenon.

3.2.6. Number of shoot tip necrosis

Data in Table 2 revealed that, shoot Tip necrosis phenomenon can be avoided by using 6 treatments (MS quarter concentrations of Mg, MS+ quarter concentrations Ca, Mn, and K, MS+ quarter concentrations Mg, Mn and K, MS+ half concentrations of Mg, or MS+ half concentrations of Ca and Mg or MS+ half concentrations of Mg, Mn, K) Among all treatments used in this experiment.

3.2.7. Growth vigor and Shoot length.

The highest value of growth vigor and shoot length obtained from full strength MS medium supplemented with MS supplemented with half recommended concentrations of Mg, K and Mn.

These results were in agreement with those obtained by (Chevre *et al.,* 1983) who stated that shoot cultures grew and well proliferated on MS medium modified by doubling the usual levels of calcium and magnesium. Also, (Barghchi and Alderson, 1996; Debergh, 1988) showed that there is a negative correlation between Ca ions concentration and shoot tip necrosis. However, the average level of phosphate in ME medium was lower than MS because of the double concentration of phosphate in ME medium. Where the level of phosphate which introduced in plant culture medium was up to 19.8 mM. However, MS medium contains 1.25 mM and many reports note that such level may be too low for plant culture (Hall and klerk, 2008). Also, the same findings of (Perez *et al.,* 2009) reported that the

best results for productivity (number of shoots\9 the average shoot length) were obtained with 2 mg^{-1} BA and 2 mg^{1} GA, although explants with chlorosis and narrow leaves were observed.

4. Conclusion

The most striking results of this work were, using full strength MS supplemented with half recommended concentrations from $MgSO_4$. $7H_2O$ and KH_2PO_4 and full MS strength supplemented with half recommended concentrations from $MgSO_4$. $7H_2O$ and $CaCl_2$, $2H_2O$ gave the best Chinese mandarin multiplication. Finally, using MS+ quarter or half concentrations of Ca, Mg, Mn and K in micropropagation can solve Chinese mandarin dropping leaves problem and can prevent shoot tip necrosis phenomenon.

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