Journal of Horticultural Science & Ornamental Plants 3 (2): 115-129, 2011
ISSN 2079-2158
© IDOSI Publications, 2011

Growth and Cytogenetical Properties of Micro-propagated and Successfully Acclimatized Garlic (*Allium sativum* L.) Clones with a Modified Shoot Tip Culture Protocol

[1]*Seif El-Nasr H. GadEl-Hak,* [2]*Kasem Z. Ahmed,* [1]*Yasser M.M. Moustafa and* [1]*Asmaa S. Ezzat*

[1]Horticulture Department, Faculty of Agriculture, Minia University, Minia, Egypt
[2]Genetics Department, Faculty of Agriculture, Minia University, Minia, Egypt

Abstract: A shoot tip micro-propagation modified protocol was used in this study to propagate and obtain virus-free garlic clones from two Egyptian parental cultivars (Egaseed1 and Sids40). The study aimed to create and evaluate a micro-propagation protocol to obtain a big number of healthy virus-free garlic clones and succeed in acclimatizing the obtained plantlets and bulblets to the ex-vitro growing conditions. Also, study the cytological behavior of the original and micro-propagated obtained plants. The obtained laboratory, open field and cytogenetical results were interesting and could be valuable for garlic breeders and researchers. Moreover, the *in vitro* obtained clones of both parental cultivars were evaluated under the open field conditions and the acclimatization protocol was successful as a big number of economical normal bulbs were obtained from those micro-propagated plantlets and bulblets derived garlic clones, which could be one step forward to successful garlic micro-propagation.

Key words: Garlic · Micro-propagation · Acclimatization · Horticultural and cytogenetical properties · Chromosomal aberrations

Abbreviations: CMS: complete MS (Murashege and Skooge) medium · HMS: half MS medium · MI: mitotic index, Kin: kinetin · BAP: benzyl amino purine

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the most important vegetable crops belongs to the *Alliaceae* family and genus *Allium* which has more than 600 available species, this family includes onions, shallots and leeks. Mostly all *Allium* crops originated from the Mediterranean Basin to Central Asia [1, 2], moreover, an evidence has been found to show that garlic was grown and consumed at the age of building of pyramids in Egypt 2780 -2100 BC [3].

Cultivated garlic is a vegetative propagated plant due to its sexual sterility and about 10% of the harvested crop is used for next field crop production [4]. In the year 2009, garlic was grown in an area around 1.165.758.30 hectares world wide with an annual production of 15.183.600.00 metric tons [5]. In Egypt, the cultivated area of garlic reached 17.589.6 fed in 2009 yielding 174.659 ton with an average of 9.45 ton/fed [5].

The diploid number of common garlic is 16 (2n=16) with a karyotypic formula of 6 metacentric chromosomes, 4 submetacentric chromosomes and 6 acrocentric chromosomes as reported by Bozzini [6]. The karyotype of Egyptian garlic clones seems to be different when compared with other genotypes grown worldwide [7-9]. The mitotic Index (MI) and the mitotic parameters, reflect chromosomal aberrations in several organisms including garlic [10, 11]. The current variation in garlic probably occurred through natural mutation [12]. This characteristic, limits the breeding of garlic species to clonal selection protocols.

Tissue culture and genetic transformation techniques have been shown as efficient tools for breeding of many crops such as artichoke [13], strawberry [14], garlic [15] and could be useful in the future improvement of these genetically closed-plants [16]. Also, Tissue culture provides a useful technique for eliminating viruses from infected plantlets and for producing virus-free garlic plantlets. In Japan, these viruses caused about 70% yield reduction in garlic cultivations [17]. Meristem culture and shoot tips culture also, have been used for this purpose [18-20]. Various tissue culture techniques have been reported to improve the efficiency of plant micro-propagation [17, 21-23]. However, most of these

Corresponding Author: Yasser M. M. Moustafa, Department of Horticulture, Faculty of Agriculture, Minia University, El-Minia, Egypt. E-mail: yasser73@gmail.com.

protocols of propagation of virus-free plantlets are limited and time-consuming techniques. Hence, the need for long-term cultivation, still have relatively low propagation rates and still in need of more modifications in garlic production protocols.

This study was conducted to provide some modified protocols of *in vitro* colored garlic propagation and aims to get a suitable protocol for garlic micro-propagation by using shoot-tips culture, choose the best medium from different media tested and obtain high number of disease-free plantlets by using shoot-tips as ex-plants, form *in vitro* bulblets from garlic parental clones, get a good protocol of adaptation for the *in vitro* derived plantlets in the open field and study the cytological profiles of *in vitro* obtained plantlets of the parental and micro-propagated derived clones.

MATERIALS AND METHODS

Plant Materials: Two garlic (*Allium sativum* L.) selected clones from cultivars: Sids40 and Egaseed1 were used throughout these studies. Garlic bulbs of those two clones were kindly provided by the Department of Horticulture, Faculty of Agriculture, Minia University, Minia, Egypt. The experiments were carried out at the Laboratories and Experimental Farm of the Horticulture and Genetics Departments as well as Minia Center for Genetic Engineering and Biotechnology, Faculty of Agriculture, Minia University, Minia, Egypt during the period from 2007 to 2011.

Micro-Propagation Protocols: The cloves of the two garlic selected clones were maintained under refrigeration at 4°C for 2 weeks to stimulate the sprouting process [20], then the cloves were washed several times with tap water. The protective dry leaves were removed from each clove; the pealed cloves were consequently disinfected in the laminar flow hood by immersing in 70% ethanol for 40 sec. Then, the cloves were soaked for 20 min in 20% solution of commercial bleach (clorex^R) containing 5.25% sodium hypochlorite (NaOCl). After each disinfectant, cloves were washed three to five times with sterile distilled water. The disinfected garlic cloves were transversally half sectioned carefully, with the purpose of showing the bud. Subsequently, the shoot-tips of 2-5mm length approximately were separated from the buds. The excised shoot-tips were individually cultured in 100ml vessels containing 50ml of one of the following combinations of MS medium [24] as well as 30 g/L sucrose and solidified with 8 g/L agar:

Table 1: The main constituents of MS media used in the present study

	Constituents	Concentration (mg/L)
Macronutrients:		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride	$CaCl_2.2H_2O$	440
Magnesium chloride	$MgSO_4.7H_2O$	370
Potassium phosphate	KH_2PO_4	170
Micronutrients:		
Potassium iodide	KI	0.83
Phosphoric acid	H_3PO_3	6.20
Manganese sulphate	$MnSO_4.4H_2O$	22.30
Zinc sulphate	$ZnSO_4.7H_2O$	8.60
Sodium molibidate	$Na_2MoO_4.2H_2O$	0.25
Cupper chloride	$CuSO_4.7H_2O$	0.025
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Vitamins:		
Nicotinic acid		1.0
Pyridoxine.HCl		1.0
Thiamine		10.0
Myo Insitol		100.0
Sucrose		30.0 g/L
Agar		8.0 g/L

- Complete MS (CMS) hormone-free (control)
- Complete MS + IAA (0.1 mg/L) + Kin (1mg/L)
- Complete MS + IAA (0.2mg/L) + BAP (2mg/L)
- Half MS (HMS) hormone-free (control)
- Half MS + IAA (0.1mg/L) + Kin (1mg/L)
- Half MS + IAA (0.2mg/L) + BAP (2mg/L)

The MS components as shown in Table 1 and other growth regulators were mixed with the other constituents at room temperature and heated to 85°C with vigorous stirring until translucent [25]. The pH of the prepared media was adjusted to 5.7 before autoclaving.

Three sterilized shoot-tips were cultured in each vessel and were considered as one replicate; 5 replicates were used for each tested medium and each experiment was repeated three times. The healthy cultures were grown for 2-4 weeks in the incubation room at 28±2°C with 16:8 h photoperiod and (2000-4000 lux, light intensity).

Garlic Plantlets Multiplication and Sub-Culturing: In order to multiply the plantlets, the formed plantlets from the previous stage were vertically divided into two sections by cutting the plantlet at equidistance along the stem bases and each section was sub-cultured on fresh media for 4 sub-cultures. The sub-cultures were incubated for 18-23 days under the previously mentioned conditions to obtain complete *in vitro* plantlets and data were recorded at these different stages.

Garlic Bulblets Formation: Some *in vitro* derived obtained plantlets in previous phase were used to induce the *in vitro* bulb formation. Bulblets were produced by culturing the plantlets of the selected clones of the two garlic cultivars; Sids40 and Egaseed1 on free hormone MS medium [24] without growth regulators with sugar concentration, 60 g/L sucrose [20]. The pH was adjusted to 5.7 before autoclaving. The cultures were incubated for 50 days at 28±2°C with 16:8h photoperiod and (2000-4000 lux, light intensity), then, the weight of formed bulblets was measured. The obtained induced bulblets were stored for two weeks to break their dormancy then were cultured on media containing only 8.0g/L agar and sterilized water [26] to be ready for the acclimatization and data were recorded at this stage.

Garlic Plantlets Acclimatization: At least 100 plantlets obtained from previous stages; plantlets and bulblets were washed with tap water and transferred to sterilized pots (30 cm in diameter) containing previously prepared soil which consisted of (as stock enough for 10 trays): three blocks of pet moss (Kokopeet), three blocks vermiculite, 500g combined fertilizers (19N:19P:19K), 250g lime super phosphate, 500g potassium sulphate, 50mg magnesium sulphate, 50g Microme (Fe+Zn+Mn+B+Cu) and 50mg fungicide. The culture containers after plantation were incubated in a greenhouse and covered with plastic film for 7 to 9 days and then the plastic cover sheet was gradually removed. The containers were exposed to the normal indoor growing conditions after 5 days from plastic film removal. After 9 days, the containers were placed in the open field previously prepared soil (3m long and 50cm wide rows were used and garlic plantlets were spaced 10cm apart within each row and two lines were planted in each side of the row and the distance between the double rows in each side was 20cm). Plants were irrigated every week for three weeks and all other horticultural activities suitable for garlic cultivations and production were applied. At the three-week-old stage, the following data were recorded: plant height (cm), number of leaves/plant and number of roots/plant.

Field Data

Plant Height (cm): After 90 days from plantation, the height of 5-10 guarded plants was measured for each selected clones from the true stem to the terminal point of the tallest leaf and the average of these 10 plants was recorded.

Number of Leaves per Plant after 90 Days from Plantation: All leaves of 5-10 plants above the soil were counted and the average was recorded.

Bulb Characteristics

Bulb Diameter and Neck Diameter at Harvesting Time (mm): Five harvested plants from each replicate were taken and then, neck and bulb diameters were measured using a calipers and values were recorded.

Garlic Bulbing Ratio: The previously obtained neck and bulb diameters values were subjected to the estimation of bulbing ratio the following formula [27]:

$$\text{Bulbing ratio} = \frac{\text{Nick diameter(mm)}}{\text{Bulb diameter (mm)}} \times 100$$

Average Weight of Single Fresh Bulb (g): ten harvested fresh bulbs were weighed and the average was recorded.

Cytological Study

Mitotic Preparation from Root Tips: To study the mitotic features of garlic selected parental and micro-propagated obtained clones, the root tips (2-3 cm) of the formed plantlets from each medium were squashed in acetocarmen dye (1.5-2.0%) treated with colchicine 0.05% for 3 hours. Then, the root tips were fixed in Farmer's solution (3 parts of 96% ethanol to one part glacial acetic acid) overnight and stored in 70% ethanol at 4°C. Before the cytological examination the roots were hydrolyzed with 1N HCL at 60°C for 3 min then transferred to water and finally were examined under Olympus microscope.

Mitotic Index: The mitotic index was calculated as the percentage of the mitotically divided cells (i.e., cells with condensed chromosomes) among total observed cells as described by Yasuhara and Shibaoka [28].

Mitotic Abnormalities: The found chromosomal abnormalities in the divided cells (i.e., cells with condensed chromosomes) were recorded. The structural changes (i.e. multi-bridges and fragments) at mitotic ana-phase stage were examined. The mitotic spreads were photographed using the SIS computer program with OLYMPUS camera 4040.

Statistical Analyses: The CRD (Complete Randomized Design) was used in all laboratory studies with at least three replications and each experiment except the field

study was repeated 3 to 4 times, All recorded data were subjected to the analysis of variance procedures and treatment means were compared using the least significant difference (L.S.D.) at 95% of confidence as described by Gomez and Gomez [29] and Duncan's Multiple Range [30]. The statistical analysis was done by using the computer program MSTATC software version 4.

RESULTS AND DISCUSSION

Days to *in vitro* Shoots and Roots Formation: Data in Fig. 1 and Plate 1 showed that garlic ex-plants (shoot tips) of clone Sids40 propagated on the control medium (medium No1), which is hormone-free and medium No 2 took short time to form shoots (3 days) comparing to medium No 6 (13.67 days) with significant differences among them. Also, clone Egaseed1 showed the same behavior to take shorter time to form shoots with the control medium (8.67 days) while took the longest time (14.67 days) when its ex-plants were propagated on medium No 6. On the other hand, regarding to days to roots formation, Sids40 behaved different from clone Egaseed1 as ex-plants of the former one took the shortest time when propagated on medium No 6 and the longest time on Medium No 1 with highly significant differences between them. However, ex-plants of Egaseed1 took the shortest time (9.6 and 10.6 days) to form roots when were propagated on medium No 3 and No 1, respectively and took the longest rooting time when propagated on medium No 6 as shown in (Fig. 1 and Table 2).

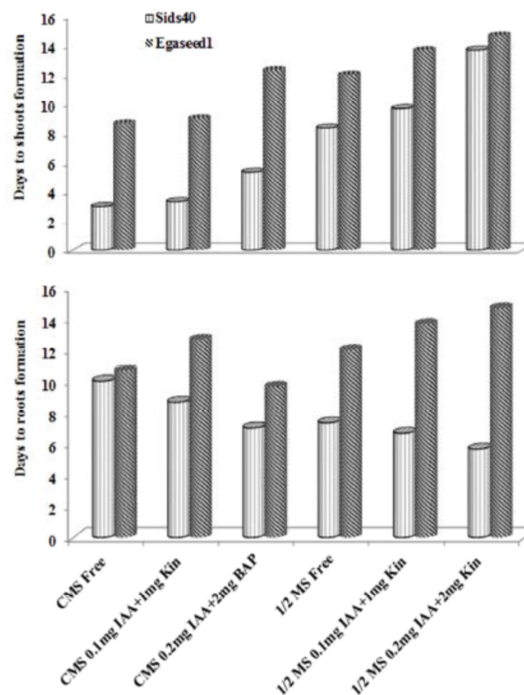


Fig. 1: Effect of different media on days to shoots and roots formation of *in vitro* micro-propagated two colored Egyptian garlic cultivars.

Number of Roots/ Plantlet: As shown in Fig. 2 and Table 3, the *in vitro* propagated Sids40 clones on different used media significantly differed in No of roots/plantlet characteristic Ex-plants of clone Sids40 grown on medium No 1 (the control medium) gave the highest No of roots/plantlet (5.0) and gave the lowest number when its ex-plants were grown on medium No 6 (2.6). However, clone Egaseed1 gave the highest No of roots/plantlet (6.0) when its ex-plants were grown on medium No 4 and the lowest one on medium No 6 (1.3) with highly significant differences between both clones. The other media used came in between of the aforementioned treatments with the two used garlic genotypes. In a similar study,

Table 2: Effect of different media on days to shoots and roots formation of *in vitro* micro propagated two colored Egyptian garlic cultivars

	Days to shoots formation		Days to roots formation	
Media*	Sids40	Egaseed1	Sids40	Egaseed1
1	3.0 F**	8.6 D**	10.0 EF	10.6 DE
2	3.3 F	9.0 D	8.6 FG	12.6 BC
3	5.3 E	12.3 BC	7.0 HI	9.6 EF
4	8.3 D	12.0 C	7.3 GH	12.0 CD
5	9.6 D	13.6 AB	6.6 HI	13.6 AB
6	13.6 AB	14.6 A	5.6 I	14.6 A
Means	7.2	12.2	7.5	12.2
L.S.D at 0.05	1.3		1.4	

* 1: Complete MS (CMS) hormone-free, 2: CMS+IAA+BAP, 3: CMS+IAA+Kin,4: Half MS (HMS) hormone-free, 5: HMS+IAA+BAP, 6:CMS +IAA +Kin.
**Values in each clone followed by the same letter are not significantly different

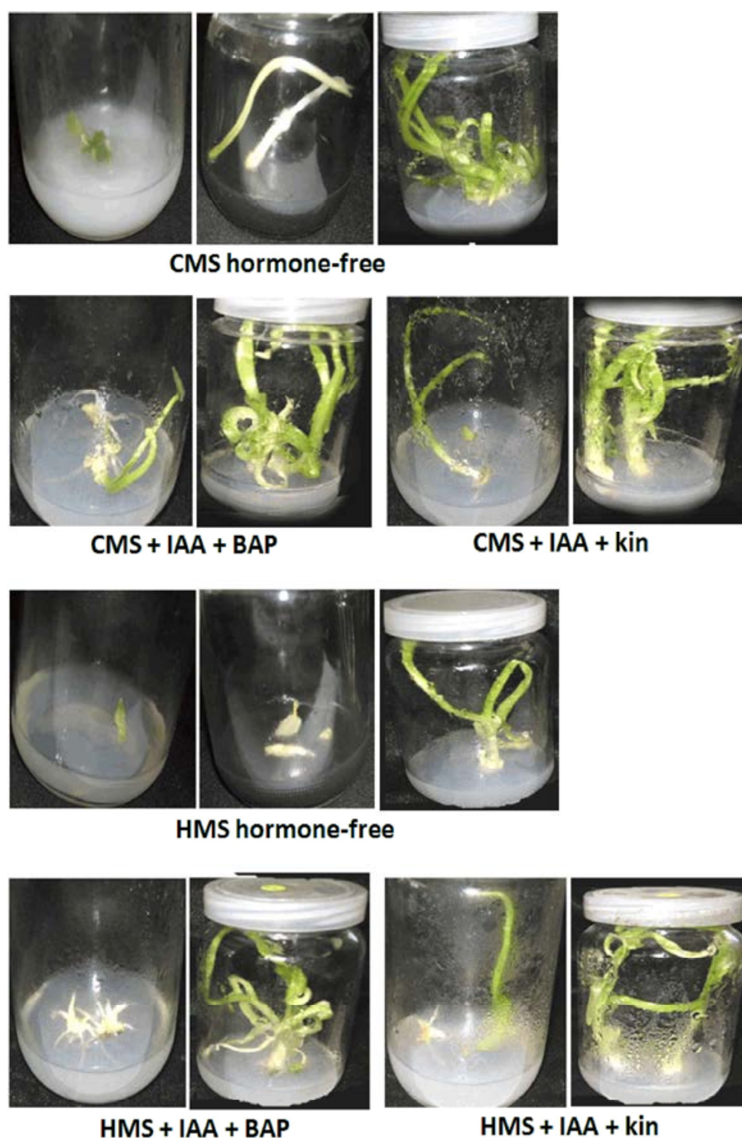


Plate 1: Photos of i*n vitro* obtained colored garlic shoots regenerated from the shoot tips grown on complete MS (CMS) and half MS (HMS) hormone-free or amended with IAA with BAP or IAA with kin at different growth stages.

Lee *et al*. [31] studied the effect of some growth regulators at different concentrations on growth of *in vitro* garlic in which explants were grown on MS medium and found that most of the obtained shoots were formed in medium supplemented with 2 mg/L kinetin+0.5 mg/l NAA.

No of Plantlets/Ex-Plant: The average number of plantlets/ex-plant differed between the two used garlic cultivars when they were *in vitro* propagated on the different six used media. In the mean time, the different used media did not differ for cultivar Sids40 in this characteristic but it differed significantly with Egaseed1. Moreover, ex-plants of Sids40 gave three plantlets/ex-plant when were grown on all used media but that of Egaseed1 gave only 2.0 plantlets/ex-plant with medium No 6, 2.33 plantlets/ex-plant with medium No1 and 3.0 plantlets/ex-plant with the other used media, respectively (Fig. 2 Table3).

No of Plantlets after the First and Fourth Sub-Culturing: The two used garlic clones which selected from Sids40 and Egaseed1 differed significantly in the mean number of regenerated plantlets/ex-plant after the first (5.94 and 5.28 plantlets/ex-plant), respectively and fourth sub-culturing (23.56 and 21.11 plantlets/ex-plant),

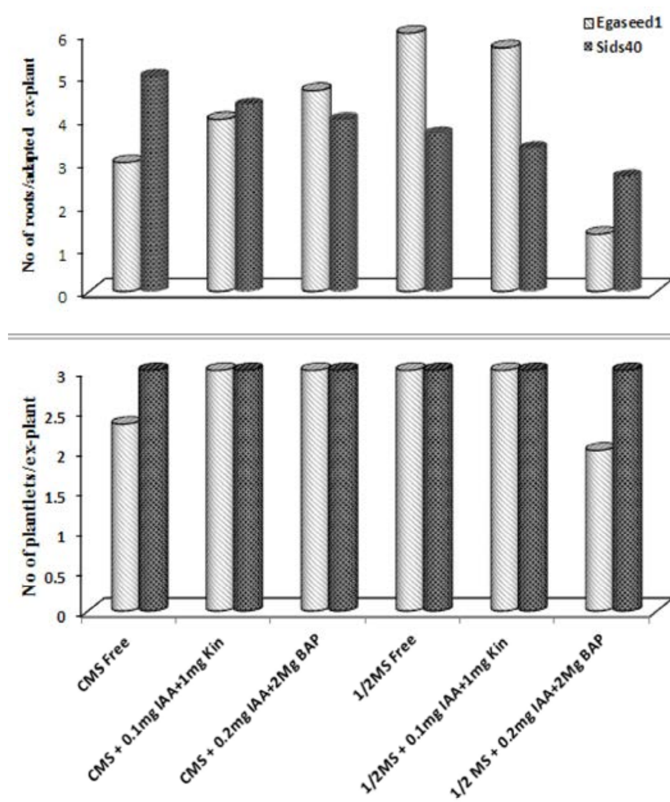


Fig. 2: Effect of different media on No of roots/plantlet and No of plantlets/ex-plant of *in vitro* micropropagated two colored Egyptian garlic clones.

Table 3: Effect of different media on No of roots/plantlet and No of plantlets/ex-plant of *in vitro* micro propagated two colored Egyptian garlic clones

	No. of roots/plantlet		No. of plantlets/ex-plant	
Media*	Sids40	Egaseed1	Sids40	Egaseed1
1	5.0 BC **	3.0 GH**	3.0 A	2.3 B
2	4.3 CDE	4.0 DEF	3.0 A	3.0 A
3	4.0 DEF	4.6 CD	3.0 A	3.0 A
4	3.6 EFG	6.0 A	3.0 A	3.0 A
5	3.3 FGH	5.6 AB	3.0 A	3.0 A
6	2.6 H	1.3 I	3.0 A	2.0 C
Means	3.8	4.1	3.0	2.7
L.S.D at 0.05		0.8		0.1

* 1: Complete MS (CMS) hormone-free, 2: CMS+IAA+BAP, 3: CMS+IAA+Kin,4: Half MS (HMS) hormone-free,
5: HMS+IAA+BAP, 6:CMS +IAA +Kin.
**Values in each clone followed by the same letter are not significantly different.

respectively when their ex-plants were micro-propagated on six different media (Fig. 3 and the included table). Clone sids40 gave 5.67 plantlets/ex-plant when its ex-plants were grown and sub-cultured for one time on medium No 3 and gave 6.0 plantlets with the other used five media with significant differences among them. On the other hand, Egaseed1 gave the lowest number of plantlets/ex-plant with media No 1 and 6 (4.0 plantlets) followed by medium No 3 (5.67 plantlets) and followed by the other three used media (6.0 plantlets). After four sub-culturing processes ex-plants of Sids40 gave higher number of plantlets/ex-plant when they were grown on media No 1, 2, 4 and 6 (24.0 plantlets/ex-plant) and gave lower number on media significant differences among them (Fig. 3 and the included table). On the other hand, ex-plants of clone Egassed1gave the lowest number of

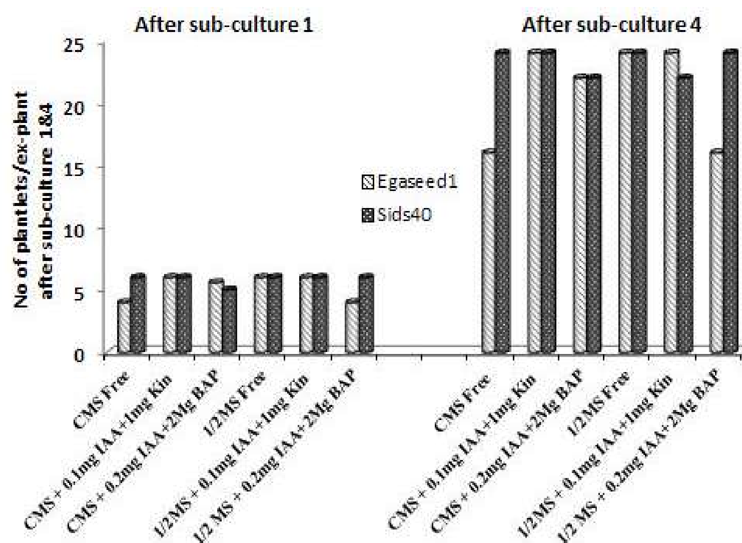


Fig. 3: Effect of different media on No of plantlet/ex-plant after subculture 1 and 4 of *in vitro* micropropagated two colored Egyptian garlic cultivars.

Table 4: Effect of different media on No of plantlet/ex-plant after subculture first and fourth of in vitro micro propagated two colored Egyptian garlic cultivars

	No. Plantlets/ex-plant after the first sub-culturing		No. Plantlets/ex-plant after the fourth sub-culturing	
Media	Sids40	Egaseed1	Sids40	Egaseed1
1	6.0 A	4.0 C	24.0 A	16.0 C
2	6.0 A	6.0 A	24.0 A	24.0 A
3	5.6 B	5.6 B	22.0 B	22.6 B
4	6.0 A	6.0 A	24.0 A	24.0 A
5	6.0 A	6.0 A	22.6 B	24.0 A
6	6.0 A	4.0 C	24.0 A	16.0 C
Means	5.9	5.2	23.5	21.1

* 1: Complete MS (CMS) hormone-free, 2: CMS+IAA+BAP, 3: CMS+IAA+Kin,4: Half MS (HMS) hormone-free, 5: HMS+IAA+BAP, 6:CMS +IAA +Kin.

plantlets/ex-plant when were grown on media No 1 and 6 (16.0 plantlets/ex-plant) for both of them, followed by medium No 3 (22.67 plantlets/ex-plant) and media No 2, 4 and 5 (24.0 plantlets/ex-plant) for all of them with significant differences among these used media as shown in (Fig. 3 and Table4).

In the present work, the results were found to be in agreement with those findings by Camara *et al*. [32] who indicated that garlic plantlets formation was the best at low concentration of growth regulators. Furthermore, complete plantlets (shoot and roots) were formed with MS medium (HMS and CMS) with or without growth regulators however, this finding did not agree with Maggioni *et al.* [33] who found that the rooting of *in vitro* garlic growth was induced on MS medium when 0.1 mg/L IBA was added to the medium. Suh and Park [34] found that the highest number of shoots was obtained from the medium supplemented with 2.0 mg/l NAA and 3.0 mg/l BA. Lu *et* al. [35] found that tissue culture of garlic on basal medium supplemented with various growth regulators resulted in the production of virus-free garlic. Moreover, number of shoots/ex-plant of garlic increased with increasing the BA concentration and root formation was inhibited by increasing the BA concentration [36-38] and these results did not agree with the present results. The genotype differences in regeneration from root-tips were observed by Khan *et al*. [39]. According to the previous results and the available complete MS medium (CMS) and half MS medium (HMS) facilities two media were chosen for further studies.

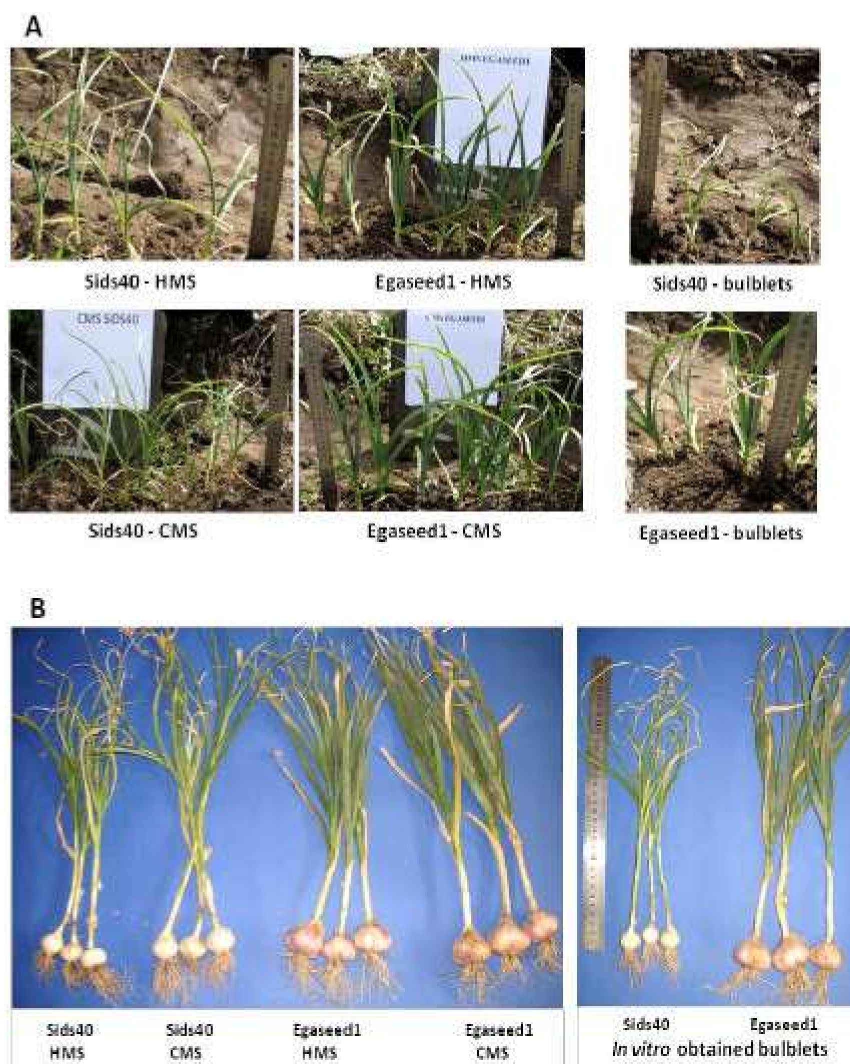
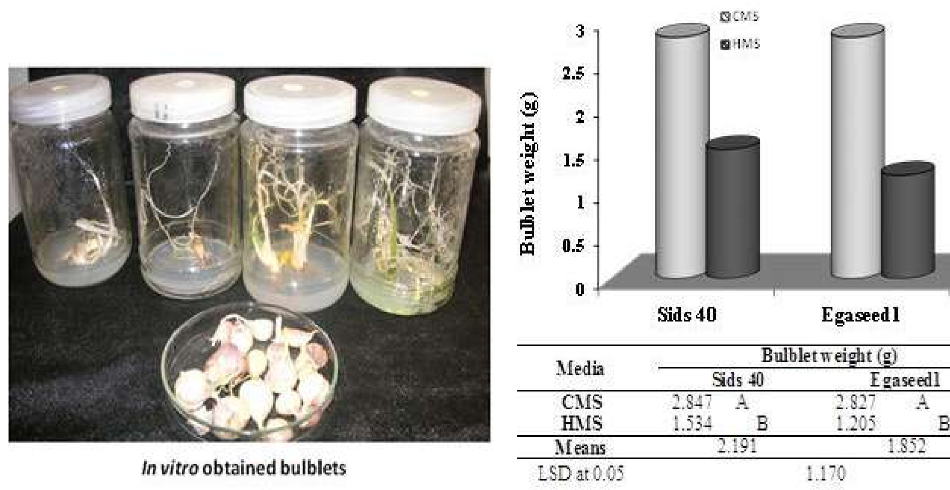


Plate 2: Photos of *In Vitro* obtained garlic bulblets regenerated from the shoot tips grown on hormone-free complete MS media (left) and bulblet weight of the obtained bulblets of both tested garlic clones (right).

Bulblet Weight and Performance of *in vitro* Micro-Propagated Garlic Clones: When two different micro-propagation media (CMS and HMS) were used to micro-propagate bulblets of two Egyptian colored garlic cultivars, the obtained results of used media and cultivars were significantly different. As the CMS medium gave higher mean values of bulblet weight (2.85 g) for Sids40 and (2.83 g) for Egaseed1 but the HMS medium gave (1.54 g) for Sids40 and (1.21 g) for Egaseed1 with averages of 2.19 and 1.85g of the two media for Sids40 and Egaseed1, respectively (Plate 2 and the included table). In similar studies, Bhojwani *et al*. [40] and Choi *et al*. [41] found that MS medium generally gave better results than B5 medium in producing bulblets from garlic shoot tips. Concerning the ability to *in vitro* bulblet formation, the regenerated shoots formed bulblets after 6–7 weeks and also similar results were also found by Robledo *et al*. [42]. Gad El-Hak *et al*. [43] studied callus formation, plantlet regeneration and cytological behavior of *in vitro* garlic cultures. They found that both Egyptian and Chinese garlic cultivars were able to *in vitro* culture but the Egyptian cultivar produced higher percentages of bulblets *in vitro* than that of the Chinese one on the MS medium. Moreover, Ayabe and Sumi [44] found that more than 90% of the shoots formed bulblets *in vitro*; however, Park *et al*. [45] observed that the rate of *in vitro* normal bulblet formation was about 82% on 0.1 mg/l NAA with 9% sucrose. Meanwhile, Seabrook [46] found that the mean number of regenerated bulblets/50 shoot explants ranged from 9 to 62 depending upon the genotype.



Media	Bulblet weight (g)	
	Sids 40	Egaseed1
CMS	2.847 A	2.827 A
HMS	1.534 B	1.205 B
Means	2.191	1.852
LSD at 0.05	1.170	

Plate 3: Photos of *ex-vitro* garlic plants (of Sids40 and Egaseed1 cultivars) derived from plantlets or bulblets regenerated from the shoot tips grown on hormone-free (CMS) or (HMS): A) two-month-old plants; B) mature plants after harvesting.

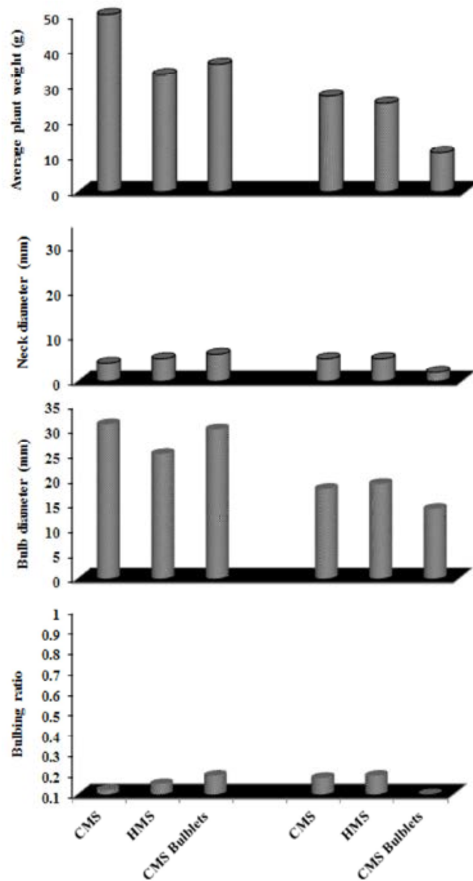


Fig. 4: Effect of different media on average plant weight (g), neck diameter (mm), bulb diameter (mm) and bulbing ratio of *in vitro* micro propagated Egyptian garlic clones after harvesting.

Yun *et al*. [47] also reported that bulblets were also obtained on MS medium either supplemented with 0.5 mg/L IAA, 0.1 mg/L 2ip and 70 g/L sucrose or contained 3.0 mg/L 2-isopentenyl adenine and 0.3 mg/L NAA.

Acclimatized Plants' Characteristics: As shown and described in Plate 3, Fig. 4 and Table 5, the total plant weight values of mature harvested garlic plants were higher with plants obtained from the hormone-free CMS media plantlets for both Sids40 and Egaseed1 clones However, Egaseed1 yielded 50g with this media comparing to that of Sids40 (27.0g). Furthermore, the three media gave higher values of plant weight with Egassed1 than those of Sids40. On the other hand, the bulbing ratio values were lower in plants of the hormone-free CMS media only with plants of clone Egaseed1 than that of bulb-formation CMS or hormone-free HMS media (Fig. 4).

Cytological Studies: In this study, plants of two selected clones from the garlic genotypes (Egaseed1 and Sids40) as well as their tissue culture derived micro-propagated plants were mitotically analyzed to check their cytological stability. Root tip cells of field grown plants were tested for percentage of different mitotic stages and mitotic index (MI) as well as percentage of divided cells with abnormal chromosomal behavior at mitotic anaphase stage respect to the total divided cells to assess the effects of tissue culture process on cytological stability of micro-propagated pants and its reflect on field growing stages.

Table 5: Effect of different media on Plant weight (g), Nick diameter Bulb diameter and Bulbing ratio of harvested plants in the open field of *in vitro* micro propagated two colored Egyptian garlic cultivars

Genotypes	Media*	Plant height (cm)	No. of leaves/plant	Bulb diameter (cm)	Nick diameter (cm)	Bulbing ratio
Egaseed1	CMS	49.4 C	9.4 A	3.1 A	0.9 A	28.6 BC
	CMS Bulblets	48.8 C	9.2 A	3.0 A	0.8 A	26.5 C
	HMS	43.0 D	9.0 A	2.5 B	0.6 B	27.0 BC
Means of media	46.0	9.2	2.9	0.8	27.4	
Sids40	CMS	54.0 B	5.8 B	1.8 C	0.5 CD	32.3 B
	CMS Bulblets	43.0 D	5.4 B	1.4 D	0.6 BC	32.3 B
	HMS	58.4 A	5.6 B	1.8 C	0.5 D	46.2 A
	Mean of media	51.8	5.6	1.71	0.57	36.9
LSD at 0.05		3.3	2.3	0.6	0.08	5.07

*CMS: Complete MS medium, HMS: Half MS medium, CMS Bulblets: bulblets obtained from the complete MS medium.

Table 6: Cytological study of mitotic root tip cells of two colored Egyptian garlic cultivars (Sids 40 and Egaseed1) and their tissue culture derived micro propagated plants (M. plants); Percentage of different mitotic stages and mitotic index (MI) were recorded respect to the total observed cells

Clones	Treatments [a]	Total No. of Observed Cells	Prophase %	Metaphase %	Ana & telo-phase %	MI %
Sids 40	Original Parental plants (Control)	1665	34.5	54.8	10.5	15.9
	M. plants* CMSf	1968	48.5	37.1	16.4	7.30
	M. plants CMSIAA+Kin	2250	50.3	32.2	19.3	12.7
	M. plants CMSIAA+BAP	2174	54.2	25.8	19.4	16.7
	M. plants HMSf	2593	43.0	32.1	24.8	10.1
	M. plants HMSIAA+Kin	2489	38.3	35.5	26.1	9.3
	M. plants HMSIAA+BAP	1969	61.6	17.8	20.5	11.8
Egaseed1	Original Parental plants (Control)	2007	61.8	20.2	17.8	14.4
	M. plants CMSf	2264	56.9	29.8	13.1	15.3
	M. plants CMSIAA+Kin	1959	41.6	38.6	17.5	11.1
	GM. plants CMSIAA+BAP	2334	52.8	29.6	18.3	11.6
	M. plants HMSf	1213	61.7	20.1	18.0	17.2
	M. plants HMSIAA+Kin	1784	61.9	24.0	13.9	16.1
	M. plants HMSIAA+BAP	1780	45.4	30.7	20.5	11.7
LSD at 0.05		5.55	5.14	3.23	5.55	

Micropropagated plants followed by the name of tissue culture medium used for *in vitro* micropropagation

Mitotic Index: Data in Table 6 and Plate 4 show the mean percentage of mitotic index (MI) of different garlic plants (i.e. original parental genotypes and their obtained micro-propagated plants). In respect to Sids40 clone, its original plants showed 15.9% of MI, while their derived micro-propagated plants exhibited MI ranged from 7.3% to 16.7% and this may be attributed to the tissue culture media used in the propagation process. The highest value of MI (16.7%) was recorded for micro-propagated plantlets from CMS medium supplemented with IAA+BAP. However, significant differences were observed only between these plants and those plants showing lower MI which regenerated on hormone-free CMS (7.3%), HMS+IAA+kin (9.3%) and hormone-free HMS media (10.1%). For the second used clone from cultivar Egaseed1 which showed 14.4% MI, there are insignificant differences between the original genotype and all their derived tissue cultured clones. It is interesting that the Egaseed1 plants which regenerated on HMS medium without growth regulators showed the highest value of MI (17.2%) comparing to all other studied treatments. These results explain the important role of tissue culture used media in the cytological stability of the *in vitro* regenerated plants.

Mitotic Stages Percentages: The mean percentages of prophase, metaphase and ana-telophase of control and micro-propagated plants of the studied garlic genotypes are given in Table 6, Fig.5 and Plate 4. Generally, the highest percentage value of observed divided cells were

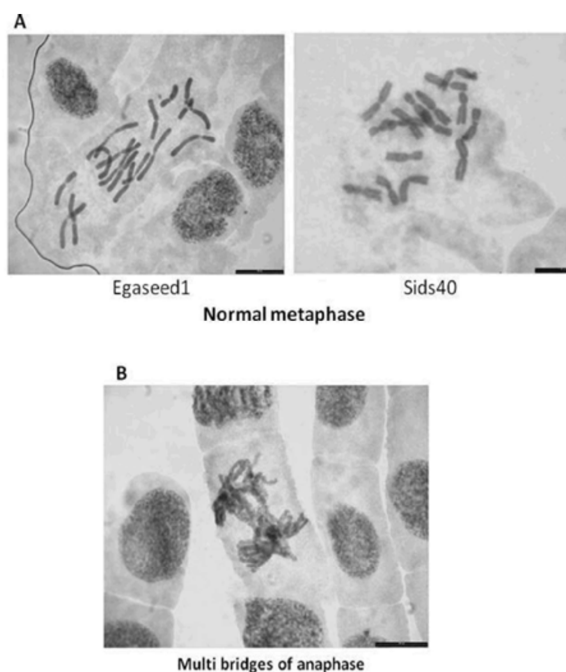


Plate 4: Mitotic chromosomes of Sids40 and Egaseed1 garlic cultivars showing a) normal metaphase (2n=16) plate, b) anaphase with multi-bridges. Scale bar= 20 µ.

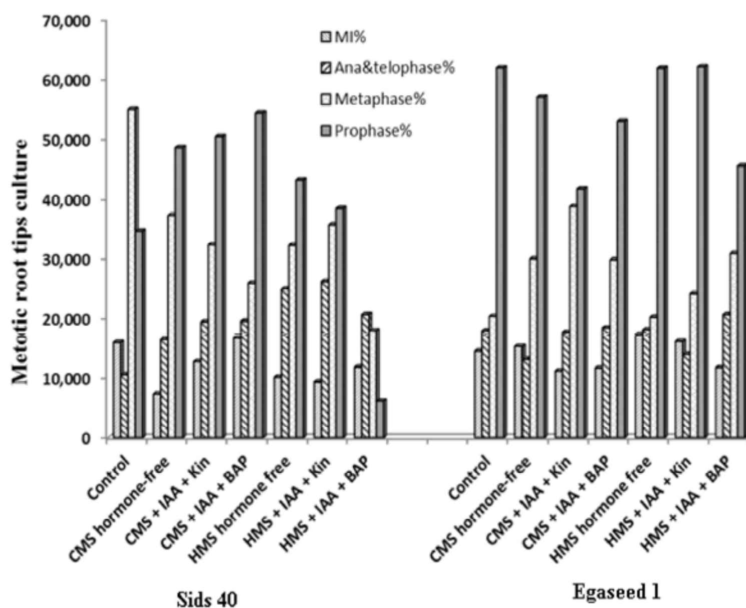


Fig. 5: Cytological study of mitotic root tip cells of two colored Egyptian garlic cultivars (Sids40 and Egaseed1) and their tissue culture derived micro propagated plants (M. plants); Percentage of different mitotic stages and mitotic index (MI) were recorded respect to the total observed cells.

in the prophase stage followed by metaphase and the least was in the ana-telophase stage, respectively, except in case of the original plants of clone Sids40 as the highest obtained value (54.8 %) belonged to the metaphase stage. However, the highest percentages of prophase (61.6%) and (61.9%) were detected in micro-propagated plants on HMS+IAA+BAP medium from Sids40 clone and on HMS+IAA+kin medium from Egaseed1 clone, respectively and the least value was 34.5% for Sids40 control. The range of percentage of

Table 7: Cytological study of mitotic root tip cells of two colored Egyptian garlic cultivars (Sids40 and Egaseed1) and their tissue culture derived micro propagated plants (M. plants); percentage of divided cells with abnormal chromosomal behavior at mitotic anaphase stage respect to the total observed divided cells

			Cells with		
Clones	Treatments [a]	Total No of divided cells	Multi bridges %	Fragments%	Total %
Sids 40	Original Parental plants (Control)	123	2.7	2.1	4.9
	M. plants CMS hormone-free	389	3.0	1.4	4.4
	M. plants CMS+IAA+Kin	181	6.6	5.2	11.8
	M. plants CMS+IAA+BAP	325	3.1	3.4	6.6
	M. plants HMS hormone-free	136	2.5	2.4	5.0
	M. plants HMS+IAA+Kin	280	3.5	2.3	5.9
	M. plants HMS+IAA+BAP	191	5.2	5.2	10.4
Egaseed1	Original Parental plants (Control)	121	5.2	4.0	9.2
	M. plants CMS hormone-free	273	11.7	6.8	18.5
	M. plants CMS+IAA+Kin	228	3.1	2.8	5.9
	M. plants CMS+IAA+BAP	247	1.4	1.4	2.8
	M. plants HMS hormone-free	319	5.6	3.4	9.1
	M. plants HMS+IAA+Kin	252	4.1	2.8	7.0
	M. plants HMS+IAA+BAP	268	5.3	5.7	11.1
LSD at 0.05		1.03	1.05	2.09	

[a] Micro-propagated plants followed by the name of tissue culture medium used for *in vitro* micro-propagation.

divided cells in the metaphase stage was 17.8-54.8% and for the ana-telophase one was 10.5- 26.1% and both belonged to Sids40 plants. These findings are strongly indicating a wide range of variation among the tested treatments under this study.

Abnormal Chromosomal Behavior: Percentages of divided cells with abnormal chromosomal behavior at mitotic anaphase stage (multi-bridges and fragments) in respect to the total observed divided cells are shown in Table 3 and Plate 4. Wide range of total abnormal cells percentages (4.4-11.8%) for clone Sids40 and (2.8-18.5%) for clone Egaseed1 were observed. Most of these values showed significant variation. Plants of Mitotic plants of CMS hormone-free clone showed 7 times higher of abnormal cells than in the Mitotic plants of CMS+IAA+BAP clone (Table 7 and plate 4). These results are in agreement with most published studies on garlic tissue cultured derived plants [7, 48] or even normal garlic clones; hence garlic as a vegetative propagated crop can keep all cytological abnormalities without any effects on yield for several years [49, 50]. This is in contrast with sexually propagated crops which can strongly been affected with any cytological abnormalities [8, 51].

Generally, tissue culture method could be a successful alternative avenue to produce large number of propagules in a short in several *Allium spp*. using root tips [52], shoot tips [53], true seeds [54], mature cloves [55] and stem domes [56] explants. The *in vitro* regenerated plants developed higher number of leaves to be able to intercept incoming radiation for photosynthesis earlier than conventionally propagated plants. This may explain the differences between the micro-propagated and clove-derived plants [57]. Moreover, the better vegetative growth in micro-propagated plants means the support in better establishment of plants against any biotic stress. The protected *in vitro* environment during micro-propagation favored the plantlets to express their complete potentiality towards morphological attributes *in vivo*, too. This study supported the earlier report of Gustavsson and Stanys [58] who observed the better performance of *in vitro* derived lingo berry plants than the conventional propagated plants. The ability to regenerate whole plants from individual cells and various tissues provides the application of plant cell and tissue culture to the improvement of plants. Extensive research has improved plant regeneration system in garlic and has applied to breeding program for crop improvement. However, much of the progress has been empirically based and plant regeneration is still devoted of a satisfactory theoretical foundation [59].

Micro-propagation results in uniform batches of plants which normally grow, flower and fruit [59]. Although, it can be expected that eventually plants multiplied *in vitro* would equivalent or superior to those

propagated by traditional techniques, it can not be assumed that their *in vivo* growth behavior in long term would necessarily be the same [60]. The outmost importance was assessed in the field performance of micro-propagated plantlets to ensure whether their fidelity or superiority to conventionally propagated plantlets or not. Hence, obtaining normal plants from micro-propagated garlic plantlets is not easy and many investigators in previous studies failed to get healthy survived plants [61]. Fortunately, we got such like normal garlic plants with good economic marketable bulbs. Hence, the protocols used and findings in this study may be beneficial for garlic micro-propagation and breeding interested researchers.

CONCLUSION

The protocol used in this study to micro-propagate two colored garlic clones which selected from the Egyptian parental cultivars (Sids40 and Egaseed1) using the shoot tips tissue culture techniques and study the cytological behavior of the original and micro-propagated obtained plants was successful and results were interesting and could be valuable for garlic breeders and researchers. Moreover, the *in vitro* obtained clones of both parental cultivars were evaluated under the open field conditions and the acclimatization protocol was successful along with economical and normal bulbs were obtained from those micro-propagated derived garlic clones.

REFERENCES

1. Hong, C.Y. and T. Etoh, 1996. Fertile clones of garlic (*Allium sativum* L) abundant around Tiennshan Mountains. Breed Sci., 46: 349-353.
2. Meer, Q.P., D.L. Botha and C.R. Galmerini, 1997. Old and new crops within edible *Allium*. Proceeding of the first international symposium on edible Alliaceae. Acta. Hortic., 433: 13-17.
3. Yamaguchi, M., 1983. World vegetables: Principles, Production and Nutritive Values.. AVI Publ. Company, Westport, USA and Ellis Horwood Limited Publishers, Chichester, England.
4. Ahmed, H.E.M., 1997. *In vitro* propagation of garlic (*Allium sativum* L.). M.Sc.Thesis, Faculty of Agriculture, Assuit University, Assuit, Egypt.
5. FAO, 2011. Food and Agricultural Organization Of United Nations: Economic and Social Department: The Statistical Division.
6. Bozzini, A., 1991. Discovery of Italian fertile tetraploid line of garlic. Econ. Bot., 45: 436-438.
7. El-Mamlouk, E.A.K., A.M. Ata, M.A.H. Mahmoud, H.M. Foly and H.Z. Allam, 2002. Cytological features and isozymes profile of some *Allium sativum* genotypes (garlic) cultivated in Egypt. Minia J. Agricultural Res. and Development, 22: 1420-1440.
8. Ata, A.M., 2005. Constitutive heterochromatin diversification of two *Allium* species cultivated in Egypt. Minia J. Agricultural Research and Development, 25: 663-676.
9. Osman, S.A.M. and Y.M.M. Moustafa, 2009. Horticultural and cytological characters of some Egyptian and foreign garlic cultivars. African Crop Science Conference Proceeding, 9: 459-465.
10. Swanson, C.P., T. Merz and W.J. Yoyng, 1990. Cytogenetics, The Chromosome In Division, Inheritance and Evaluation. Prentice-Hall Inc, 2nd Ed, USA.
11. Kaushik, G.C., 1996. Cytotoxicity of cement kiln dust on mitosis of root tip cells in *Vicia faba*. J. Ecotoxico Environ. Monit., 1: 53-57.
12. McLaurin, W.J., D. Adams and T. Eaker, 2002. Garlic production for the gardener. University of Georgia Cooperative Extension Service publication, circular, 854: 1-7.
13. Max-Wanger, G. and S. Schittenhelm, 1991. *In vitro* propagation methods of Jerusalem artichoke (*Helianithus tuberosus* L.) in medium trem storage.FAO IBPGE plant Genet. Resources NWSI, 86: 5-8.
14. Reed, B.M., 1991. Application of gas-permeable bags for in vitro cold storage of strawberry germplasm. Plant cell Rpt, 10: 431-434.
15. Moriconi, D.N., V.C. Conci and S.F. Nome, 1989. *In vitro* plantlet formation from callus in garlic (*Allium sativum* L.) Phyton. Argentina, 49: 97-103.
16. Martin, J., 1999. An efficient method for callus culture and shoot regeneration of garlic (*Allium sativum* L). Hort. Sci., 34: 348-349.
17. Nagakubo, T., A. Nagasawa and H. Ohkawa, 1993. Micro-propagation of garlic through *in vitro* bulblet formation. Plant Cell Tissue and Organ Culture, 32: 175-183.
18. Bhojwani, S.S., D. Cohen and P.R. Fry, 1982. Production of virus-Free garlic and field performance of Micropropagated plants. Scientia Hortic., 18: 39-43.

19. Walkey, D.G.A., M.J.W. Webb, C.J. Bolland and A. Miller, 1987. Production of virus-free garlic (*Allium sativum* L.) and shallot (*A. ascalonicum L.*) by Meristem-Tip culture. J. Hortic. Sci., 62: 211-219.
20. Haque, M., T. Wada and K. Hattori, 2003. Shoot regeneration and bulblet formation from shoot and root meristem of garlic cv. Bangladesh Local. Asian J. Plant Sci., 2: 23-27.
21. Heverek, L. and F. Novak, 1973. The bud formation in the callus cultures of (*Allium sativum* L). Flanzenphysiol, 68: 308-318.
22. Kehy, A.E. and G.W. Schaaeffer, 1976. Tissue culture and differentiation of garlic. Hortic. Sci., 11: 422-423.
23. Abo El-Nil, M.M., 1977. Organogenesis and embryogenesis in callus cultures of garlic (*Allium sativum* L.). Plant Sci. Lett., 9: 259-264.
24. Murashige, T. and F. Skooge, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol., 15: 473-497.
25. Zimmerman, R.H., 1995. Use of starch- gelled medium for tissue culture of some fruit crops. Plant Cell Tissue Organ Cult., 43: 207-213.
26. Ahmed, H.E., N.M. Shalaby, N.M. Kandel and M.N. Aboul-Nasr, 2000. *In vitro* Propagation of garlic (*Allium sativum* L). Proceedings of the 2nd Scientific Conference of Agricultural Science, Assuit, Egypt Oct., pp: 101-113.
27. Mann, L.K., 1952. Anatomy of the garlic bulb and factors affecting bulb development. Hilgardia, 2l: 195-251.
28. Yasuhara, H. and H. Shibaoka, 2000. Inhibition of cell-plate formation by brefeldin A inhibited the depolymerization of microtubules in the central region of the phragmoplast. Plant Cell Physiol., 41: 300-310.
29. Gomes, K.A. and A.A. Gomes, 1984. Statistical procedures of agricultural research. John Willy and Sons, New York.
30. Duncan, D.B., 1955. Multiple range and multiple F test. Biometrics, 11: 1-42.
31. Lee, E.M., S.W. Ra, J.Y. Lee, S.R. Min, H.N. Song and V.B. Lee, 1988. The effect of growth regulators and conditions of stalk bulb storage on callus growth of garlic *in vitro*. Research Reports of the Rural Development Administration Hort., 30: 90-95. (cf. Hort. Abstr., 60: 4261).
32. Camara, F.A.A., M. Pasqual and J.E. Pinto, 1989. Obtaining plants from garlic (*Allium sativum* L) cultivars Chinese and Chonan using *in vitro* cultured meristems. Ciencia-e-pratica., 13: 97-102.
33. Maggioni, L., C. Cordi and C. Fogher, 1989. Callus induction ploidy level and plant regeneration *in vitro* garlic (*Allium sativum* L.). Journal of Genetics and Breeding, 2: 251-254.
34. Suh, S.K. and H.G. Park, 1995. Plant regeneration from the culture of garlic root explants. J. Korean Society for Horticultural Sci., 36: 31-37.
35. Lu, Q.Y., W.Y. Zhou and Y.Y. Dong, 1982. Induction of callus from young leaves of garlic and plantlet regeneration. Acta Hort. Sci., 9: 67-96.
36. Matsubra, S. and D. Chen, 1989. *In vitro* production of garlic plants and field acclimatization. Hort Sci., 24: 677-679.
37. Choi, S.Y., J.Y. Oh, J.S. Kim, J.H. Lim, D.J. Choi, S.B. Lee and D.U. Choi, 1992. Meristem culture of garlic (*Allium sativum* L.). 1-Effects of growth regulators through in vitro multi-propagation of garlic (*Allium sativum* L.). Research Reports of the Rural Development Administration. Biotechnol., 34: 24-29.
38. Kudou, R., Y. Fujime, N. Fukada and K. Amimoto, 1995. Effects of sampling position, plant growth regulators and low temperature treatment on bulblet formation *in vitro* of garlic. Technical Bulletin of the Faculty of Agriculture, Kagesawa University, 47: 93-98.
39. Khan, N., M.S. Alam and U.K. Nat, 2004. *In vitro* regeneration of garlic through callus culture. J. Biological Sci., 4: 189-191.
40. Bhojwani, S.S., 1980. *In vitro* propagation of garlic by shoot proliferation. Scientia Hortic., 13: 47-52.
41. Choi, S.L., K.Y. Paek, K.C. Kwan, S.G. Son and J.T. Cho, 1985. Effect of explant source trimming on shoot multiplication and bulb formation of garlic. Sci., 26: 304-312.
42. Robledo, P.A., A.V.M. Villalobos and G.A.E. Jofre, 2000. Efficient plant regeneration of garlic (*Allium sativum* L.) by root tip culture. *In vitro* Cell. Dev. Biol. Plant., 36: 416-419.
43. Gad El-Hak, S.H., A.T. Abdel-Rahem, S.A. El-Shobaky and H.S. Tony, 1996. Callus formation, plantlet regeneration and cytological behavior of *in vitro* garlic cultures. Fourth Arabic Conf. Hort. Crops, March 26-30, El-Minia, Egypt, pp: 33-46.
44. Ayabe, M. and S. Sumi, 1998. Establishment of a novel tissue culture method, stem disc culture and its practical application to micro-propagation of garlic (*Allium sativum* L). Plant Cell Rep., 15: 17- 21.
45. Park, H.G., 1993. Rapid multiplication through immature bulbil cultures of garlic. J. Korean Soc. Hortic. Sci., 34: 173-178.

46. Seabrook, J.E.A., 1994. *In vitro* propagation and bulb formation of garlic. Can. J. Plant Sci., 74: 155-158.
47. Yun, W.D., Q. Su, Z.Y. Luo, H. Teng, Y.Q. Li, X.E. He, 1998. Effect of garlic and garlic-green tea mixture on serum lipids in MNNG-induced experimental gastric carcinoma and precancerous lesion. World J. Gastroenterol., 4: 29-34.
48. Al-Zahim, M.A., B.V. Ford-Lloyd and H.J. Newbury, 1999. Detection of soma-clonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. Plant Cell Rep., 18: 473-477.
49. Dolezel, J. and F.J. Novák, 1985. Caryological and cytophotometric study of callus induction in *Allium sativum* L. J. plant physiol., 118: 421-429.
50. Novak, F.J., L. Havel and J. Dolezol, 1986. Allium In:Evans, D.A., W.R. sharp and P.V. Animato (eds).Handbook of plant cell culture. Macmillan, New York, pp: 419-459.
51. Osman, S.A.M., A.M. Ata and S.H. Gad El-Hak, 2007. Morphological, germination, bolting and cytological characteristics of fourteen promising garlic genotypes. African Crop Science Conference Proceeding, 8: 2005-2012.
52. Haque, M.S., T. Wada and H. Kazumi, 1997. High frequency shoot regeneration and plantlet formation from root tip of garlic. Plant Cell Tissue Org. Cult., 50: 83-89.
53. Gantait, S., N. Mandal, S. Bhattacharyya and P.K. Das, 2009. *In vitro* mass multiplication with genetic clonality in elephant garlic (*Allium ampeloprasum* L.). J. Crop Weed, 5: 100-104.
54. Warosch, C., P.R. Malla and B. Kopp, 2001. Micro-propagation of Allium. A threatened medicinal plant of Nepal. In vitro Cell Dev. Biol. Plant., 37: 555-557.
55. Roksana, R., M.F. Alam, R. Islam and M.M. Hossain, 2002. *In vitro* bulblet formation from shoot apex in garlic (*Allium sativum* L.). Plant Tissue Cult., 12: 11-17.
56. Kamstaityte, D. and S. Stanys, 2004. Micro-propagation of onion (*Allium cepa* L.). Acta Univ. Latv. Biol., 676: 173-176.
57. Buah, J.N., Y. Kawamitsu, S. Yonemori and S. Murayama, 2000. Field performance of *in vitro*-propagated and sucker-derived plants of banana (*Musa spp*.). Plant Produc. Sci., 3: 124-128.
58. Gustavsson, B.A. and V. Stanys, 2000. Field Performance of “Sonna” lingonberry derived by Micro-propagation versus stem cuttings. Hort. Sci., 35: 742-744.
59. Gantait, S.I., N. Mandal and P.K. Das, 2010. Field evaluation of micro-propagated vs. conventionally propagated elephant garlic. J. Agric. Technol., 7: 97-103.
60. George, E.F., 1996. Plant propagation by tissue culture. Exegetics Ltd., Part 2, UK.
61. Gad El-Hak, S.H., A.Z. Osman, A.A. Galal, Y.T. Abd El Mageed and N.S. Yossef, 2001. Effect of gelling agents and antioxidant treatments on *in vitro* potato micro-propagation using virus indexed plantlets. The 5th Arab Horti. Conf, March, Ismailia, Egypt, pp: 24-28.