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ARTICLE

In vitro propagation of ginger (*Zingiber officinale* Rosco)

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Abstract An efficient and promising protocol for *in vitro* propagation of *Zingiber officinale* Rosco using sprouting buds was established. Sprouting buds were sterilized and cultured onto MS medium supplemented with different growth regulators. Augmentation of MS-medium with 4.5 mg/l BAP recorded the highest percentage of shootlets multiplication. Shootlets were highly rooted on half strength of B_5 medium supplemented with 1.0 mg/l NAA. The maximum percentage of acclimatization, hardening and rhizomes production of *in vitro* derived plants in greenhouse was 80–100%.

1. Introduction

Ginger plant (*Zingiber officinale* Rosco) is belonging to the family Zingiberaceae. It is one of the world's most important spices and produces a pungent, aromatic rhizome. Rhizomes of ginger are valuable all over the world not only as a spice but also as herbal medicine. As documented in several reports [11,33], ginger is constrained severely can summarized as following: (1) ginger normally propagates by rhizome with a low proliferation rate, and the reproducing part (rhizome) is also the economically used part of the ginger plant, which restricts the availability of ginger seeds needed for cultivation, (2) easily infected by soil-borne pathogens such as bacterial wilt (*Pseudomonas solanacearum*), soft rot (*Pythium aphanidermatum*) and nematodes (*Meloidogyne* spp.), which cause heavy losses in yield, (3) ginger rhizomes show variations and degeneration under long-term vegetative propagation and (4) normal breeding of ginger is a real problem due to poor flowering and seeds set. Indeed, most crop improvement programs for these species were confined to evaluation and selection of naturally occurring clonal variants. Clonal multiplication of ginger through multiple shoots induction has been reported by several workers [20,14,19]. The present study was carried out to highlight an effective, reliable and reproducible protocol for *in vitro* propagation of ginger.

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2. Materials and methods

2.1. Plant material

Healthy ginger rhizomes were secured from Institute of Medicinal and Aromatic Plants, Dokki, Cairo, Egypt. Then they carefully cleaned with soap and tap water and kept under darkness condition. After a few days, the induced bud sprouts were excised, and then surface sterilized by immersion in 70% ethanol for 2 min, followed by three washes using sterile distilled water. Further, they were immersed in 50% (v/v) of commercial Clorox solution (5.25% sodium hypochlorite) containing a drop of Twin 20 for 15 min, and subsequently rinsed several times with sterile distilled water. The obtained sterilized buds were cultured aseptically on selected MS-media [21] for achievement of multiplication process.

2.2. Culture media and incubation conditions

MS-culture media were solidified using 0.7% (w/v) agar and containing 3% sucrose. The pH media were adjusted to 5.8 before autoclaving at 1.2 kg/cm^2 for 20 min at 121 °C. Cultures of all treatments were maintained under light condition 16 h/day photoperiod at intensity of 3000 Lux from cool white light of fluorescent lamps for 30 days. All cultures were incubated at 26 ± 1 °C. Five replicates from each treatment were used.

2.3. Multiplication stage

MS medium was supplemented with different concentrations of BAP, Kin and NAA to investigate their effects on enhancement of shootlets multiplication. This experiment consisted of 13 treatments as shown in Table 1. The following parameters were recorded with intervals of three subcultures, after 4, 8 and 12 weeks of cultivation as follow:-

— Numbers of shootlets/bud sprout.
— Shoot length (cm).
— Number of leaves/shoot.

2.4. Rooting stage

The following parameters affecting on shootlets root formation were studied as follow:-

2.4.1. Effect of NAA and IAA

Shootlets produced from *in vitro* multiplication stage (4–5 cm long) were individually separated and cultured on solidified MS medium. MS nutrient media were augmented with different concentration of NAA or IAA as shown in Table 2.

The following parameters were considered:

- Number of roots per shoot.
- Root length (cm).

2.4.2. Effect of different types of nutrient media

In this experiment different types of nutrient media i.e., MS; Heller's [13]; B_5 [9] and NN media [22] at full or half of salt strength were used to study their effects on rootlets shootlets formation. All cultures were augmented with 1 mg/l NAA (the best growth regulator for rootlets formation). The compositions of the culture media are presented in Table 3.

The following parameters were considered as follow:-

- Number of roots per shoot.
- Root length (cm).

2.5. Hardening and ex vitro acclimatization

In vitro grown plantlets were thoroughly washed with tap water to remove residual agar from roots. Then they were immersed in 0.2% aqueous solution of Bavistin (a fungicide) for 15–20 min and washed with tap water. Thereafter, the treated plantlets were transplanted in pots filled with the following media:

(1) Peatmoss.
(2) Peatmoss:sand (1:1).
(3) Peatmoss:sand:perlit (1:1:1).
(4) Peatmoss:sand:vermiculite (1:1:1).

Table 1 Effect of various growth regulators added to MS medium on shootlets multiplication of *Zingiber officinale* after 4 weeks of cultivation.

Code	MS medium supplemented with	Number of shoots/explant	Number of leaves/shoots	Shoot length (cm)
MC	Free growth regulators	1.00	3.25	2.73
M1	1.5 mg/l BAP	4.25	10.50	3.18
M2	2.5 mg/l BAP	6.00	11.00	2.93
M3	3.5 mg/l BAP	6.25	12.00	3.85
M4	4.5 mg/l BAP	8.00	15.50	4.10
M5	0.4 mg/l BAP + 0.2 mg/l IAA	2.25	7.75	4.03
M6	0.6 mg/l BAP + 0.2 mg/l IAA	3.25	9.75	3.33
M7	0.8 mg/l BAP + 0.2 mg/l IAA	4.00	8.25	3.38
M8	1 mg/l BAP + 0.2 mg/l IAA	3.00	6.50	2.28
M9	1 mg/l Kin	4.19	11.25	4.10
M10	1 mg/l Kin + 1 mg/l NAA	3.75	10.25	3.63
M11	2 mg/l Kin + 1 mg/l NAA	4.00	11.00	4.18
M12	3 mg/l Kin + 1 mg/l NAA	4.75	12.75	4.58
M13	4 mg/l Kin + 1 mg/l NAA	5.00	13.75	4.03
$LSD_{0.05}$		2.08	5.23	1.10

Each value is the average of five replicates.

Table 2 Effect of MS medium supplemented with NAA and IAA at different concentrations on roots number/shoot and root length of *Zingiber officinale* after 4 weeks of cultivation and incubation under light conditions at 25 ± 1 °C.

Code	MS medium supplemented with	Roots number/shoot	Root length (cm)
RC	Free growth regulator	1.67	3.87
R1	0.2 mg/l NAA	5.00	6.10
R2	0.4 mg/l NAA	2.33	2.10
R3	0.6 mg/l NAA	3.67	4.40
R4	0.8 mg/l NAA	7.67	3.40
R5	1 mg/l NAA	13.67	6.37
R6	1.2 mg/l NAA	9.00	6.05
R7	0.2 mg/l IAA	2.67	4.60
R8	0.4 mg/l IAA	3.00	3.07
R9	0.6 mg/l IAA	3.33	5.50
R10	0.8 mg/l IAA	2.67	4.77
R11	1 mg/l IAA	3.67	1.97
R12	1.2 mg/l IAA	3.00	2.97
LSD $_{0.05}$		1.66	1.71

Light: 16 h/day.
Each value is the average of five replicates.

Table 3 Effect of different type of nutrient media (MS, NN, B_5 and Heller) at full and half salt strength and supplemented with 1 mg/l NAA on number of roots/shoot and root length of *Zingiber officinale* after 4 weeks of cultivation.

Code	Type of nutrient media	Roots number/shoot	Root length (cm)
NC	Sugar and agar	3.0	0.90
N1	Full MS + 1 mg/l NAA	12.7	5.27
N2	Half MS + 1 mg/l NAA	12.3	3.60
N3	Full NN + 1 mg/l NAA	12.3	6.13
N4	Half NN + 1 mg/l NAA	19.0	6.30
N5	Full B_5 + 1 mg/l NAA	11.0	3.70
N6	Half B_5 + 1 mg/l NAA	25.3	7.93
N7	Full Heller's + 1 mg/l NAA	9.7	4.20
N8	Half Heller's + 1 mg/l NAA	23.7	7.33
LSD at ($P \leqslant 0.05$)		3.78	1.04

Each value is the average of five replicates.

Cultured pots were covered with polyethylene bags to ensure high humidity around plantlets. Trays were kept in green house and sprayed with water 2–3 times during the first week and as needed during rest time. The top corners of bags were cut after 1 week and completely removed after 2 weeks. Each treatment consisted of five replicates. The obtained *ex vitro* plants were subjected to the following measurements:-

(1) Percentage of survival plants.
(2) Plant height (cm).
(3) Number of leaves/plant.
(4) Development figure.

2.6. Statistical analysis

The resulted data were subjected to statistical analysis, employing *F*-test for significance at $P \leqslant 0.05$ and computing of LSD values to separate means in different statistical groups according to described method by Gomez and Gomes [10].

3. Results and discussion

3.1. Multiplication stage

3.1.1. Effect of some growth regulators on shoots multiplication
3.1.1.1. Number of shoots/bud. Data tabulated in Table 1 and Fig. 1 clearly showed that, between different treatments, the number of produced multiple shoots/bud was significantly changed at the level of 5%. M4, M3 and M2 recorded the maximum numbers of shootlets multiplication (8, 6.25 and 6 shoots/bud, respectively) compared with other treatments. Conversely, the lowest number of shoots multiplication (1 shoot/bud) was recorded with MS free growth regulators (MC). The positive effect of MS medium augmented with BAP on increasing of shoots number was also found by Balachandran et al. [3] who reported that, a concentration of 3.0 mg/l BAP was found to be the optimum for *in vitro* multiplication of turmeric and ginger species. Furthermore and in agreement of our obtained results, Muda et al. [20] indicated that *in vitro* propagation procedures for three varieties of *Z. officinale* were successfully established on MS medium supplemented with a range of 1.0–3.0 mg/l BAP. Moreover, and in same respect, Panda et al. [23] reported that using BAP alone with *Curcuma amada gave* the maximum number of shoots per explant. Further as mentioned by Buah et al. [5] the efficiency of BAP as a marked growth regulator on shoot formation compared to kinetin and 2ip may be attributed to its high *in vitro* cultures stability. However in contrast, Kambaska and Santilata [16] indicated that, MS supplemented with high concentration of BAP combined with Kin or NAA gave the best multiplication rate of *Z. officinale*.

3.1.1.2. Number of leaves/shoot. Data in Table 1 showed significant differences concerning the number of leaves/shoot at the level of 5% among the applied different treatments. Moreover, the same data revealed that there is a positive relationship between increasing of Kin concentrations combined with NAA and number of leaves/shoot. The maximum number of leaves (15.5) was recorded with M4 followed by M13, M12 and M3 without significant differences between them. However, the minimum number of leaves/shoot appeared in MC followed by M8, respectively without significant differences between them. These results are in harmony with those obtained by Jagadev et al. [14] who stated that, MS medium supplemented with 3.0 mg/l BAP and 0.4 mg/l NAA was ideal for maximum number of leaves formation in *Z. officinale* Rosc. While, Shukla et al. [29] found that MS medium supplemented with 3.0 mg/l BAP produced the optimum number of leaves of *Curcuma angustifolia*.

3.1.1.3. Shoot length (cm). Data in Table 1 revealed that the longest shoot (4.58 cm) was recorded with M12, followed by M11 (4.18 cm) and M4 (4.10 cm), respectively, without significant differences between them. In contrast, lowest shoots (2.28, 2.73 and 2.93 cm) were recorded with M8, MC and M2, respectively, also without significant differences between them. These results are convergent with that obtained by Sharma and Singh [28] who found that the best medium for

Figure 1 Multiple shootlets formation/bud of *Zingiber officinale* after 4 weeks of cultivation on MS medium supplemented with, (A) 4.5 mg/l BAP, (B) 4 mg/l Kin + 1 mg/l NAA, (C) 3.5 mg/l BAP and (D) 1 mg/l BAP + 0.2 mg/l IAA.

shoot length (6.8-cm) of *Z. officinale* was obtained with MS medium containing 2.0 mg Kin + 2.0 mg NAA. Similarly, Kambaska and santilata [16] reported that, in *Z. officinale* cultures, the combination of MS with BAP (2.0 mg/l) and NAA (0.5 mg/l) elicited optimal response in shoot length of 6.2 ± 0.37 cm per shoot. However, Baskaran and Jayabalan [4] found that Kin was more effective than BAP and 2iP for enhancement of shoot length and higher concentrations of BA (8.8–22.2 μM) which reduced the shoot length of *Eclipta alba*.

3.2. Rooting stage

3.2.1. Effect of growth regulator on roots formation

A well developed of the elongated shoots about 4–5 cm in length were excised from shoot clump and cultured on MS medium containing different concentrations of NAA or IAA for enhancement of roots formation. Rooting responses includes number of roots/shoot and mean root length over a period of four weeks is presented in Table 2 and Fig. 2. Statistical differences between all tested concentrations of the two auxins on number of roots per shoot and root length at ($P \leqslant 0.05$) were recorded.

3.2.1.1. Number of roots per shoot. Presented data in Table 2 and Fig. 2 showed that MS medium supplemented with NAA at different concentrations was more efficiency on number of roots formation than those formed on MS medium supplemented with IAA at different concentrations with average 6.90 and 3.05 roots per shoot, respectively. The maximum number of roots 13.67, 9.00 and 7, 67 was recorded with R5, R6 and R4, respectively. On other hand poor rooting formation was observed in case of shoots cultured on RC medium. This obtained result goes in line with those findings by Salvi et al. [27] who cleared that, regenerated shoots of *Curcuma longa* were rooted and grown on MS medium supplemented with 0.1 mg/l NAA. Moreover, Jagadev et al. [14] found that for rooting of *Z. officinale* Rosc, MS supplemented with

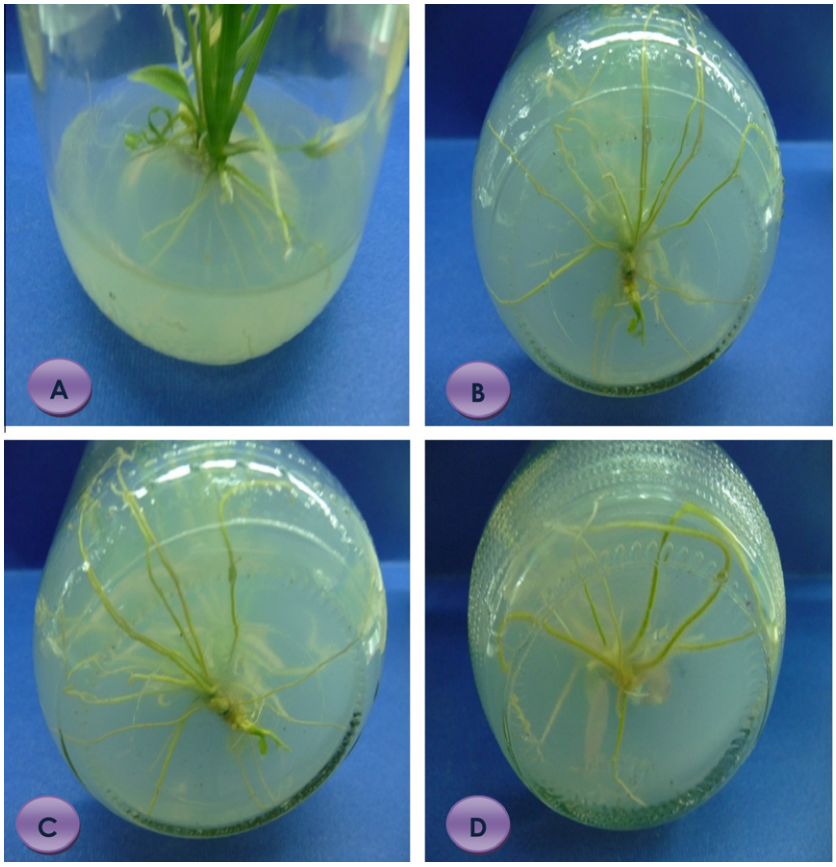

Figure 2 Rooting response of *Z. officinale* cultured on MS medium containing various concentrations of NAA after 4 weeks of cultivation. Where (A) represent roots formation per shoot, (B) 0.8 mg/l NAA, (C) 1 mg/l NAA and (D) 1.2 mg/l NAA.

NAA (0.5 mg/l) was found to be more effective and produced the maximum number of roots per shoot (13.3). On other hand, Rout et al. [26] indicated that the use of either IAA or IBA in the culture medium affect the root induction from shoots of *Acacia chundra*.

3.2.1.2. Root length (cm). The experimental results indicated that the type of auxins and their concentrations significantly influenced on root length. High concentration of NAA and low to moderate concentrations of IAA increased of root length but high concentration of IAA depressed of root length. The longest roots (6.37, 6.10 and 6.05 cm) were recorded with R5, R1 and R6, respectively (Table 2 and Fig. 2). However, the shortest roots (1.97, 2.10 and 2.97 cm) were observed with R11, R2 and R12, respectively, without significant difference between them (Table 2 and Fig. 2). These obtained results were in harmony with those obtained by [16] they concluded that *in vitro* shoots of *Z. officinale* Rosc were rooted on half strength MS basal medium supplemented with 2.0 mg/l NAA. However, Badoni et al. [2] cleared that in micropropagation of *Hedychium spicatum* (Zingiberaceae), rooting percentage (80%) was reported on MS medium with 5.0 μM/l Kin and 1.0 μM/l IAA.

3.2.2. Effect of different types of nutrient media on roots formation

The influence of different type of nutrient media and their concentrations have important roles for promoting well developed roots. To determine the optimum nutrient medium, four nutrient media in full- and half-of salt strengths supplemented with 1 mg/l NAA were tested on number of roots/shoot and root length.

3.2.2.1. Number of roots/shoot. Generally, half-strength of all different nutrient media improved the overall growth of roots compared to full strength salts of nutrient media for roots formation of *Z. officinale*. This result goes in line with those found by Abbasin et al. [1] who confirmed that, half strength basal salts media were more effective than corresponding full strength basal salts media for root formation of *Taxus* species. Results showed significant differences at the level of 5% among the applied different treatments. However, data in Table 3 cleared that, the maximum numbers of roots formed per shoot 25.3, 23.7 and 19 were recorded with N6, N8 and N4 media, respectively. Also, it was observed that the root system formed with these treatments, presented well developed and widely branched vigorous roots with insignificant differences between them. In other side, the minimum numbers of formed roots per shoot were appeared with NC (3) followed by N7 (9.7), N5 (11) and N2 (12.3) media with significant differences between them. Present results were in agreement with those reported by Ma and Gang [18] who demonstrated that, the best root growth was observed with B_5 medium supplemented with 2 mg/l Kin and 1 mg/l of IAA. Moreover, Hedayat et al. [12] concluded that, all regenerated shoots of *Tanacetum cinerariifolium* developed roots on B_5 medium containing 2 mg/l NAA.

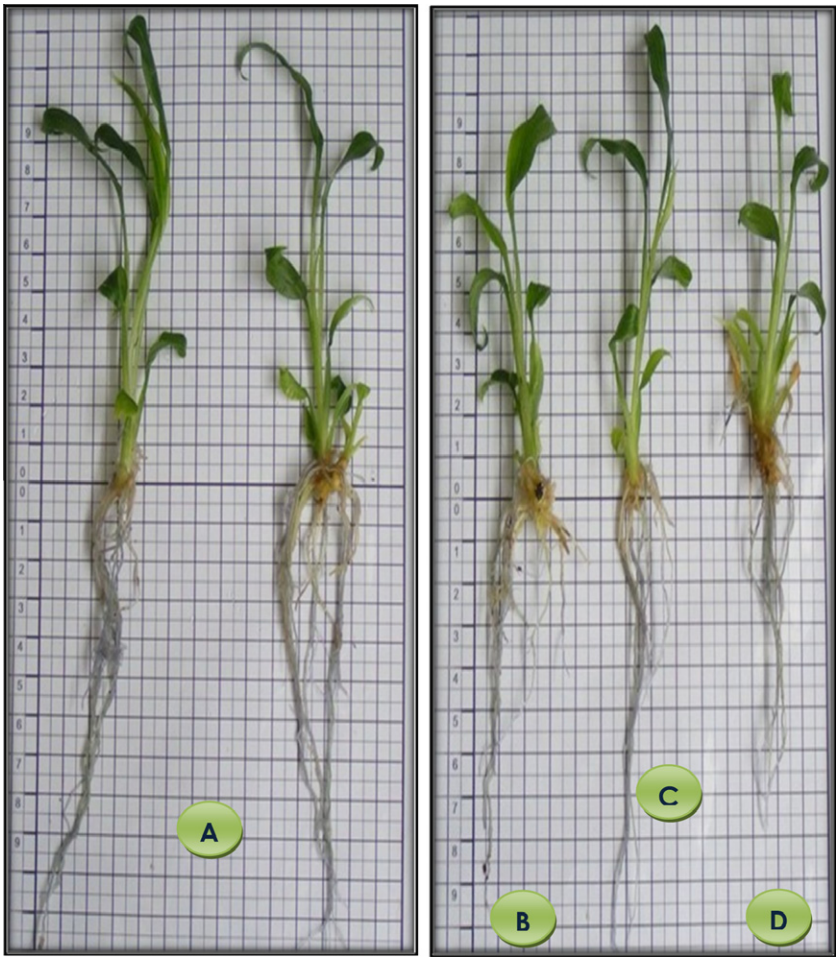

Figure 3 *In vitro* well developed roots of *Zingiber officinale* prior transfer to field, (A) half strength B_5 medium, (B) half strength Heller's medium, (C) half strength NN medium and (D) half strength MS medium.

3.2.2.2. Root length (cm). Data in Table 3 and Fig. 3 concerning the length of root formation/shoot, data clearly recorded insignificant differences between N6 (7.93) and N8 (7.33) media, respectively. In contrast, a significant difference between the shortest length of roots were appeared with NC (0.9 cm), N3 (3.60 cm), N5 (3.70 cm) and N7 (4.20 cm) media, respectively. Overall results show clear relationship between lowering of medium salt strength and root length. The promoting effect of reducing MS salt concentration on *in vitro* rooting has been described in several reports. Sivanesan and Jeong [30] demonstrated that regenerated shoots of *Sida cordifolia* were successfully rooted on half-strength of MS medium supplemented with 2.0 mg/l IBA. In addition, Kalidass and Mohan [15] indicated that, IBA was found to be most effective for root production of *Phyllanthus urinaria* with half salts of MS medium. Moreover, Dhabhai et al. [7] found that, half strength MS medium supplemented with 0.5 mg/l of IBA resulted 80% of root initiation of *Acacia nilotica* microshoots.

3.3. Hardening and ex vitro acclimatization

After transplanting, micro-propagated ginger plantlets experienced an acclimatization stage to adapt to the fluctuating environmental factors. During this stage, the *in vitro* derived plantlets showed special growth and photosynthetic characteristics. Percentage of shoots survival, increase in shoot length, number of leaves and development rate were measured on 30th day after transplanting in mixtures soil (Table 4). However, these parameters were changed insignificantly at the level of 5% after 30 days of acclimatization under greenhouse conditions.

3.3.1. Percentage of survival plantlets

The *in vitro* derived plants were acclimatized better under *ex vitro* condition when transferred on specially made plastic trays containing different soil mixture and moistened uniformly at periodic intervals taking special care not to damage the roots. In general, about 80% of the regenerated plantlets were tolerated and survive under *ex vitro* environment in greenhouse conditions. A number of plantlets were lost maybe due to these plantlets showed less adaptive abilities for photoautotrophic and/or lowing of relative humidity during acclimatization in *ex vitro* condition. Similar observations have been described by Preece and Sutter [25] who reported that, plantlets growing under *in vitro* conditions exhibit no or reduced photosynthetic capacity, and during acclimatization there is a need for rapid transition from the heterotrophic to the photoautotrophic state for survival. In addition, Pospisilova et al. [24] cleared that,

Table 4 Influence of soil mixtures on acclimatization of micropropagated plants of *Zingiber officinale* under green house conditions after 4 weeks of cultivation.

Treatments	Survival (%)	Plant height (cm)	Number of leaves/plant	Development rate
Peatmoss	100	8.02	5.40	[b]
Peatmoss:sand (1:1)	80	6.18	4.20	[a]
Peatmoss:sand:perlit (1:1:1)	80	6.58	6.00	[c]
Peatmoss:sand:vermiculite (1:1:1)	60	4.50	2.80	[a]
LSD 0.05		NS	NS	

NS: Non-significant.
[a] Low development rate.
[b] Moderate development rate.
[c] High development rate.

photosynthetic parameters seem very important for growth of *in vitro* plantlets have been grown. According to this, transfer of plantlets from *in vitro* to *in vivo* condition can be accompanied with a transient decrease in photosynthetic parameters. While, Cha-um et al. [6] found that *in vitro* acclimatization of ginger plantlets under high relative humidity with CO_2 enrichment produced plantlets with both vigorous shoot and root systems. Data in Table 4 show the effect of different growing media used in this study on the survival percentage. The growing medium of peatmoss gave highest survival percentage (100%) than those grown on peatmoss + sand and peatmoss + sand + perlit which produced 80% for each one.

3.3.2. Plant height (cm)

It is evident from the obtained data (Table 4 and Fig. 4) that, the different growing soil mixtures had insignificant effect on increasing of shoot length. The tallest plantlets 8.02 and 6.58 (cm) were measured with peatmoss medium followed by peatmoss + sand + perlit, respectively. While, shortest plantlet (4.50 cm) was measured with peatmoss + sand + vermiculite.

Figure 4 Acclimatization of micropropagated *Zingiber officinale* plantlets under greenhouse conditions, (A) after 2 weeks of transferring and (B) 2 month old plants, (C) after 7 months.

3.3.3. Number of leaves per plant

Results in Table 4 and Fig. 4 indicate that, the various treatments had insignificant affect on the number of leaves per plant. The maximum number of leaves (6) was produced by using peatmoss + sand + perlit as a growing medium. On the contrary, the lowest number of leaves was created by using peatmoss + sand + vermiculite growing medium.

3.3.4. Development rate

Development rate had been concluded from a comparative study between different treatments during acclimatization stage and performed on the morphological characteristics (percentage of shoots survival, increase in shoot length and number of leaves per plant) after 30 days of culturing in greenhouse. According to development rate, the acclimatized plants seem healthy vigorous and got domesticated with peatmoss:sand:perlit treatment, which has highest development rate (***) while, the treatments of peatmoss:sand and peatmoss:sand:vermiculite gave low development rate (*). However, moderate development rate (**) was recorded in acclimatized plants cultured in peatmoss alone. The rest of these procedures, plantlets were transferred to bigger pots. The potted plants were maintained under greenhouse conditions with regularly irrigated and *in vitro* propagated ginger plants completed growth under greenhouse conditions. After 7 months initial rhizomes were appeared and harvested as shown in Fig. 4. Similarly, the proposed effect of soil mixtures on *ex vitro* acclimatization has been described by Faria and Illg [8] they found that acclimatization of *Zingiber spectabile* was performed in plastic pots containing a mixture of 1:1 autoclaved soil and vermiculite. Vigorous plants with four to five visible roots started to produce new leaves with 95% survival rate. While, Tyagi et al. [32] found that, in *vitro* regenerated plantlets of *Curcuma* were planted in soil and acclimatized to the outside temperature in a net-house displayed 2–4 *in vitro* generated roots for every shoot had a survival rate of 96–100%. In this regard, Loc et al. [17] acclimatized *Curcuma zedoaria* plantlets on pots containing a mixture of soil and rice husk ashes (3:1 ratio), and healthy roots appeared after 2 weeks. The clone survival rate was about 95%. Also, Jagadev et al. [14] reported that, acclimatization of *in vitro* plantlets was done on media mixture of soil:sand:farm yard manure (1:1:1) which found to be best for survival of the plantlets in ginger. However, Stanly and Lai-Keng [31] found that *in vitro* rooted plantlets of *C. zedoaria* and *Zingiber zerwnbet* cultured in pots containing mixture of organic soil and sand (1:1) without direct sunlight were successfully acclimatized with 100% of the plantlet survived.

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