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ORIGINAL ARTICLE

# Micropropagation of carnation (*Dianthus caryophyllus* L.) in liquid medium by temporary immersion bioreactor in comparison with solid culture

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**Abstract** Developing scale-up system and automation of micropropagation in a bioreactor has been a possible way of cost reduction and intensive manual handling. We report a comparison between the results of experiments aimed at improving carnation micropropagation using new bioreactor according to Temporary Immersion Bioreactor (TIB) and solid culture. By applying different levels of BAP, at the concentration of 3 mg $L^{-1}$, we observed 14.3 new shoots in TIB, but the number of new shoots on solid medium reached to 5.7 at the same treatment. Our results also showed that with 3 mg $L^{-1}$ BAP in TIB, the initial fresh weight of plant material increased from 10 g to 450 g after 15 days. It is concluded that TIB showed more than 10 times shoot production of carnation. Shoot elongation and rooting induction was successfully stimulated in TIB by applying 1 mg $L^{-1}$ IBA. Rooting of proliferated plantlets from TIB and solid culture were successfully happened, and led to highest number of roots (4.6 cm) and highest length of roots (6.87 cm) in TIB. More than 90% of plantlet was acclimatized to *ex vitro*. Our results suggested that mass production of carnation shoots in our simple TIB, with effective result, can be considered as a critical first step toward large scale production of carnation.



## 1. Introduction

Carnation (*Dianthus caryophyllus* L.) from dicotyledonous Caryophyllaceae family is one of the most popular commercial flowers all over the world [9]. As an important floricultural crop, carnation is cultivated all-year-round in temperate areas

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[22]. Carnation can be propagated through different ways of propagations like, seed, cutting, layering and tissue culture. Among the cut flowers and pot plants commercialized in the Alsmeer market, which is located in Netherlands, nearly 60% of them are produced by micropropagations [4]. Micropropagation is a modern system for production of healthy and virus free stock plants and supports breeding activities in agriculture and forestry [2,4]. Producing of plants through *in vitro* technique is labor intensive and expensive [17]. Since solid medium does not make the full automation of plant propagation possible, scientists shift to moving liquid culture for automation of tissue culture system without using solidifier. These movements to liquid culture make all tissues to be connect with nutrient solution and resulting in growing faster [12]. However, there is still a great obstacle, which is called hyperhydricity. Under precise conditions, hyperhydricity can irreversibly lead to loss of the regenerative ability of the tissue [33]. Vitreous appearance and wrinkled leaves are secondary events resulting from waterlogging of the apoplast [19]. For decreasing unpleasant effects of this physiological disorder, instead of permanent contact of explants with liquid nutrient medium, the times and the frequency of contact should be controlled. In this regard, bioreactors which work according to temporary immersion have been used [20]. Bioreactor technology can be readily adapted for the growth of cells and tissues in liquid media, as demonstrated by the successful mass propagation of *Phalaenopsis* Orchid [28], pistachio [11], *Dendrobium* Orchid [5], pineapple [30], grape rootstock [25], *Oncidium* Orchid [14], *Gerbera jamesonii* [27], and many other plants. Mass production of plants and automation of culture through culture of cells, tissues, somatic embryos and tubers by bioreactors is one of the suitable methods to reduce the cost of industrial propagation in many of plants [17]. The main purpose of applying bioreactors is to provide suitable growth conditions through adjusting physical and chemical parameters in order to get maximum quality and quantity of desired plants [36]. In some cases, bioreactor cultures have many advantages compared with solid cultures, with a better control of the contact of the plant with the culture medium, and optimal nutrient and growth regulator supply, as well as aeration and medium circulation [23]. Nowadays, commercializing and mass propagating of many ornamental species according to somatic embryogenesis and shoot proliferation by using temporary immersion bioreactor is one of the promising ways of rapid and continues multiplication [20,12]. In order to introduce an efficient micropropagation method for carnation micro shoots, our newly designed bioreactor with the supplementation of plant growth regulators (PGRs) to the medium were assessed and compared to solid culture.

## 2. Material and methods

### 2.1. Preparation of explants

Carnation (*Dianthus caryophyllus*) "Tessino Cherry" cultivar (a cultivar with important market value in Iran) was transferred to tissue culture laboratory of Tarbiat Modares University from a commercial green house in Pakdasht, Iran. Axillary buds were selected as explants, and they were isolated and were washed under running tap water and surface sterilized by 70% ethanol for 70 s and 2.5% sodium hypochlorite solution for 2 min followed by immersing in Nano Silver solution for 10 min by the concentration of 200 ppm. Disinfected explants were placed in vessels containing 25 mL of MS [35] medium in 250 mL jar supplemented with 30% sucrose and 8 g $L^{-1}$ agar. The pH of medium was adjustment to 5.8 ± 0/2 and explant was culture in establishment medium without any plant grow regulators. Cultures was carried to growth room at light intensity of 30 μmol $m^{-2}$ $s^{-1}$ PPFD emitted by two cool white fluorescent lamps at 25/18 °C (day and night) with 16 h' photoperiod and 40% relative humidity. One month later, we followed two strategies: first, adventitious shoots from this solid medium were cut into 1.5 cm length single nodes with the average weight of 0.2 g for each shoots (10 g total weight of incoming shoots in TIB), and placed in 3 l translucent glass containers containing 1.5 L multiplication medium of carnation according to HIMEDIA™[1] laboratories protocol (control treatment), and other multiplication medium which is supplemented with different level of BAP 1, 2 and 3 mg $L^{-1}$ instead of Kinetin in HIMEDIA™ protocol, and at the second strategy, explants were entered in solid multiplication medium with the same composition of the medium of TIB.

### 2.2. Preparation and adjustment of TIB

This bioreactor has been invented in a joint project by Tarbiat Modares University and HPTCL. For construction of TIB, we used two translucent glass containers of 3 l capacity, two timers TEBAN®, two pumps HAILEA® ACO-5501, silicone hoses 1/4″ high temperature resistant which is equipped with micro pore filters of Poly tetrafluoroethylene 0.22 μm and two solenoid valves ASCO® which is shown in both Fig. 1A and B. The required power for performing the immersion is produced by the pumps. By pumps, air was distributed through silicone hoses. The culture system (TIB) was placed on aluminium shelves. Aluminium shelves were equipped with white fluorescent lamps to provide the systems with 30 μmol $m^{-2}$ $s^{-1}$ PPFD, and other environmental condition was same as solid culture. Our designed TIB was equipped with timers, which make the adjustment of the numbers of immersion in a day in each immersion. When one of the solenoid valve become opened, air pushes culture medium from its container to plant material container which was controlled via timer based on its program. Then, the second solenoid valve became opened to make the culture passing back to medium container.

In order to find the best frequency of immersion in a day, three treatments of 2, 4 and 6 times of immersion per day (with duration of 2 min) with three replications for each treatment were carried out. After four weeks, the rate of hyperhydricity and the numbers of new shoots were evaluated. Assessment of hyperhydricity was performed via visual observation presented in Fig. 2.

### 2.3. Multiplication phase

For assessment of the PGRs efficiency on multiplication, plantlets obtained from axillary buds in solid medium were inoculated in bioreactor containing different levels of BAP

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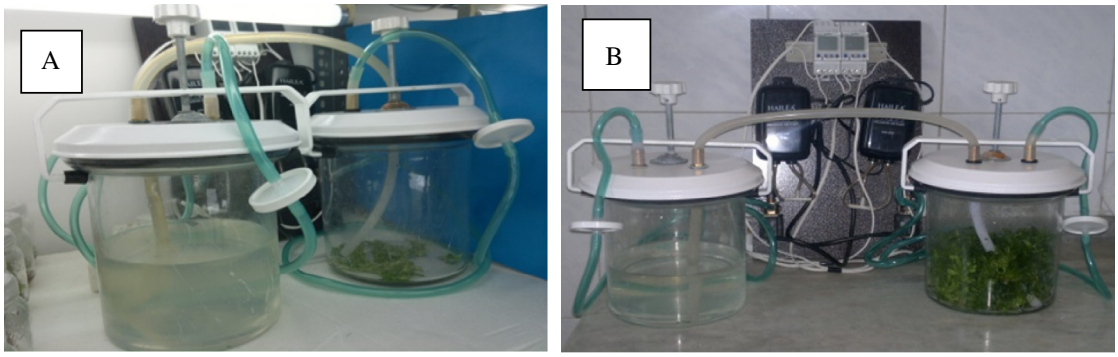


**Fig. 1** (A) Plant material at the first day in liquid medium by TIB; (B) plant material at 15th day in multiplication medium in TIB; in both (A, and B figures) the TIB system are shown.

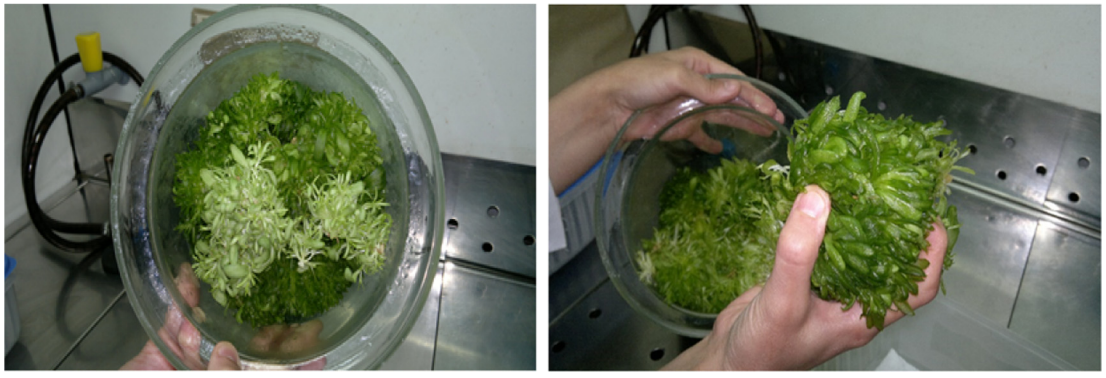

**Fig. 2** Hyperhydric planlets. Loss of color in leaves, and vigorous shoots was the criterion of assessing hyperhydricity (from 1 to 4).

(0, 1, 2, 3 mg $L^{-1}$) to ascertain the best concentration of BAP in the bioreactor. After two weeks in multiplication medium in TIB and 4 weeks on solid medium, the number, the length (cm) and fresh weight (g) of new shoots were measured.

### *2.4. Rooting phase, and acclimatization*

The multiplication medium changed to rooting induction medium, and we used 1 mg $L^{-1}$ IBA as rooting induction element in TIB, then plant materials were harvested and transferred to solid medium which was supplemented with 0, 0.5, 1 and 1.5 mg $L^{-1}$ IBA. In solid culture, proliferated plant materials transferred to rooting medium for producing roots. The medium was supplemented with 0, 0.5, 1 and 1.5 mg $L^{-1}$ IBA. After four weeks in both systems, the length of the roots and the numbers of healthy roots was measured. After carefully removing plantlets from the rooting medium using water, they were potted in a (1:1, v/v) of coco peat and perlite, and transferred directly to the greenhouse. At the first days, they were located under mist to provide 90% relative humidity. Then, they were irrigated once a day.

### *2.5. Statistical analysis*

Experiments were set up in a completely randomized design. Each treatment had ten replications, except different level of BAP which had three replications in TIB. Analysis of variance (ANOVA) and mean comparisons were performed by SAS software. When $F$ test was significant ($P = 0.05$), Duncan's test was used to separate treatment means.

## 3. Result

### *3.1. Immersion times test*

#### *3.1.1. Frequency of immersion, and rate of hyperhydricity*

The result showed that 6 times immersion per day (every 4 h) caused the highest number of new shoots (17.33), although this treatment led to the highest rate of hyperhydricity (4) (Table 1, Figs. 2 and 3). The results were showed significant and distinctive differences among all the treatments (Table 1). According to our records, 4 times immersion per day via interval of 6 h (produced 14.33 new shoots) was the most desirable treatment. It is completely true that the 6 times immersion caused the highest number of new shoots, but we observed the highest rate of hyperhydricity, too which is not a sign of efficient performance of TIB (Figs. 2 and 3).

### *3.2. Multiplication phase in solid medium and liquid medium by TIB*

In solid medium, the average number of emerged new shoots was 5.7 shoots with the average length of 2.74 cm (Table 2), however in TIB, 14.33 was the average number of new shoots

**Table 1** The comparison of the immersion frequency and their effects on numbers of new shoots and rate of hyperhydricity.

| Numbers of immersion | Numbers of new shoots | Rate of hyperhydricity |
|---|---|---|
| 2 | 7.33 c ± 0.12 | 1.66 c ± 0.12 |
| 4 | 14.33 b ± 0.14 | 2.20 b ± 0.11 |
| 6 | 17.83 a ± 0.14 | 4.00 a ± 0.10 |
| *F*-value | 1513.24** | 118.71** |

Values in each column represent means ± SE. Different letters within columns indicate significant differences.
** Means significant at 1% levels.

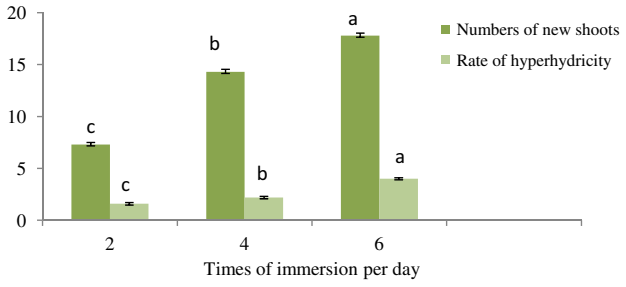


**Fig. 3** The effects of the times of immersion on numbers of new shoots and the rate of hyper hydricity.

by the length of 4.66 cm. (Table 2). BAP at the concentration of 3 mg $L^{-1}$ was the best treatment. From this experiment, it was found that TIB has shown great potential to be developed and established for enhancing the mass propagation of carnation plantlets (Fig. 1A and B).

### *3.3. Rooting phase in solid medium and liquid medium by TIB, and acclimatization*

Rooting plantlet which were proliferated on solid medium showed the length of 6.12 cm and the numbers of healthy roots were 2.9 both at the concentration 1 mg $L^{-1}$ IBA (Table 3), while proliferated plantlets in TIB, showing highest numbers of healthy roots (4.6 roots/explant) by the length of 6.87 cm, after four weeks on solid medium (Fig. 4A and Table 3).

More than 90% of our plant material which were rooted acclimatized in green house successful (Fig.4B). No pets and disease were not seen during acclimatization step.

## 4. Discussion

Propagation from axillary shoots has been proved to be a promising way for the micropropagation of a large number of species, although not the most efficient method [26]. In most of commercial micropropagation, micropropagated plants are derived from axillary shoot formations [1].

Proper regeneration of adventitious shoots of carnation rely on composition of nutrient medium, plant grow regulators and types of explant [10,15]. Cytokinin promotes cell division and cell expansion and growth is stimulated by adding a cytokinin to a proliferation medium [21]. Cytokinin induces the initiation and capability of axillary meristems which result in shoot formation. In this way, different concentrations of one of the common cytokinin type of PGR (BAP) were applied to compare the capability of two culture systems in mass propagation of carnation.

TIB, as a kind of bioreactor, has been known to be applicable in culturing of horticultural crops [17]. Times, duration and frequency of every immersion are the most critical features in order to reach the highest proliferation rate in TIB [3]. It is clear that the frequency of immersion is an important parameter to control nutrients and hyperhydricity. The interval between two immersions has a definite role in the multiplication rate of different species. Due to the contact of plant material with nutrient compounds in each immersion, growth and multiplication will occur in this interval. The significant interaction on multiple shoot formation of nodal buds obtained in temporary immersion system could be due to the elimination of apical dominance and the induction of lateral bud growth [23]. 4 times immersion per day via interval of 6 h was the most desirable treatment, in our experiment. According to different experiments, it is proven that the times of immersion is not the same for all the plant species [20]. It is clear that by adjusting the numbers of immersion the rate of hyperhydricity will be controlled in TIB. We observed that 6 times immersion per day ($6 \times 2 = 12$ min contact per day) increased hyperhydricity and made the adaption of these plants to ex vitro more and more difficult. Increasing the immersion frequency from every 6 h to 12 h decreased the grade of hyperhydricity in propagation of *Gerbera jamesonii* in a commercial TIB (BIT®) [27]. This result is in agreement via our obtained result.

**Table 2** Comparison of the different concentrations of BAP and their effects on numbers of new shoots, length of new shoots and fresh weight by explants growing in solid medium and liquid medium by TIB.

| Medium | Solid Medium | | | Liquid medium by TIB | | |
|---|---|---|---|---|---|---|
| Concentrations of BAP (mg $L^{-1}$) | Numbers of new shoots | Length of new shoots (cm) | Fresh weight (g) | Numbers of new shoots | Length of new shoots (cm) | Fresh weight (g) |
| 0 | 3.8 b ± 0.32 | 2.51 ± 0.53 | 0.17 ± 0.005 | 8.66 d ± 0.33 | 4.06 c ± 0.06 | 6.63 c ± 0.08 |
| 1 | 4.8 ab ± 0.24 | 2.9 ± 0.57 | 0.18 ± 0.005 | 10.33 c ± 0.33 | 5.06 a ± 0.12 | 7.00 c ± 0.11 |
| 2 | 5.3 a ± 0.33 | 2.79 ± 0.36 | 0.19 ± 0.006 | 12.00 b ± 0.57 | 4.83 a b ± 0.08 | 7.90 b ± 0.30 |
| 3 | 5.7 a ± 0.51 | 2.74 ± 0.22 | 0.20 ± 0.179 | 14.33 a ± 0.33 | 4.66 b ± 0.08 | 9.10 a ± 0.11 |
| *F*-value | 4.91** | 1.35 $n^s$ | 1.29 $n^s$ | 35.11** | 21.19** | 87.86** |

Values in each column represent means ± SE. Different letters within columns indicate significant differences.
** Means significant at 1% levels.

**Table 3** The comparison of the different concentrations of IBA and their effects on numbers of healthy roots and the length of new roots of proliferated plants from solid culture and liquid medium by TIB.

| Medium | Solid Medium | | Liquid medium by TIB | |
|---|---|---|---|---|
| Concentrations of IBA (mg $L^{-1}$) | Numbers of healthy roots | Length of new roots (cm) | Numbers of healthy roots | Length of new roots (cm) |
| 0 | 1.50 b ± 0.16 | 4.91 c ± 0.18 | 4.6 a ± 0.16 | 6.56 a ± 0.13 |
| 0.5 | 1.8 b ± 0.20 | 5.11 b c ± 0.21 | 3.4 b ± 0.16 | 5.9 b ± 0.18 |
| 1 | 2.9 a ± 0.17 | 6.12 a ± 0.22 | 4.6 a ± 0.22 | 6.87 a ± 0.10 |
| 1.5 | 2.6 a ± 0.16 | 5.59 a b ± 0.19 | 3.8 b ± 0.24 | 6.06 b ± 0.21 |
| *F*-value | 13.68** | 6.98** | 8.76** | 7.48** |

Values in each column represent means ± SE. Different letters within columns indicate significant differences.
** Means significant at 1% levels.

**Fig. 4** (A) Rooted plantlet; (B) acclimatized plantlets in greenhouse.

It is completely well known that one of the most important advantages of temporary immersion bioreactor lies in the higher proliferation rates when compared to solid medium. TIB has positive effects on the whole procedure of multiplication of shoots. Temporary immersion system enhanced the elongation of new shoots in St. John's wort (*Hypericum perforatum* L. cv. 'New Stem') [32]. In present study, obtained results indicated the efficient effects of TIB on carnation multiplication. Reaching to 4.66 cm shoot length at the best treatment in comparison to 2.74 cm in solid culture was another proof for the efficiency of our designed TIB.

Temporary immersions systems result in higher biomass to avoid intensive manual handling, utilizing liquid media, diminishing production costs regarding labor force, saving energy than conventional techniques [29]. In addition, they have been adapted to many propagation and breeding programs to decrease costs and increase the production of elite clones and release new cultivars [24]. Commercial production of cultivar Kaly of carnation through direct organogenesis in temporary immersion bioreactor has been done by applying of 0.05 mg $L^{-1}$ TDZ [13]. The preliminary study on multiplication of carnation using temporary immersion bioreactor has shown great potential to be used for mass propagation in comparison with solid media. In our study it was suggested that 3 mg $L^{-1}$ BAP is the optimal plant growth regulators for shoot multiplication of carnation plantlets, due to better performance of 3 mg $L^{-1}$ BAP on number of shoots and rate of proliferation than other concentrations. Considering this fact, it is highly recommended that the higher concentration of cytokinin component is necessary for the growth and differentiation of tissue culture [8]. It agrees with results obtained by Kadota and Niimi with pear (*Pyrus pyrifolia)* [21]. They suggested that BAP displayed more noticeable effect and it was more suitable for shoot multiplication of pear. Our result showed that the higher concentration of cytokinin growth regulator led to the increase of the proliferation rate, but this issue usually happens with decreasing the length of shoots. Ružić and Vujović declared that even in low concentrations of thidiazuron, elongation was inhibited [8]. Concentration of 3 mg $L^{-1}$ BAP led to more shoots in both TIB (14.33) and solid culture (5.7), but considerably shorter and difficult to handle plants in TIB (4.66 cm) and solid culture (2.74 cm), which is in agreement with other scientific report obtained by Pruski [16].

Throughout the experiments, we observed that the ratio of final to initial fresh weight of carnation shoots in our TIB was 45.65-fold after 15 days, which is higher than the result of low cost mist bioreactor in which the ratio was 10.8 after 14 days [6]. Starting with 10 g inoculum resulted in 450 g in fresh weight at the end of proliferation stage. The reason of increase in multiplication rate and fresh weight may be rely on more uptake of nutrients and hormones over the whole plant surfaces, and daily air exchange which caused ventilation of gaseous compounds in every immersion [34,18].

Two most important steps in micropropagation of plants are rooting and adapting to greenhouse conditions. A great

rooting, which completely rely on the suitable quality of roots like higher length of roots and higher numbers of healthy roots, can be account for signs of perfect acclimatization. In our experiment applying 1 mg $L^{-1}$ IBA in TIB have a suitable effect on enhancing the elongation and root induction of carnation plantlet and improved the roots characteristics in comparison with proliferated plants from solid culture. All the regeneration plantlets from TIB could produce roots after transferred to MS medium. By applying 1 mg $L^{-1}$ IBA in solid culture highest number of healthy roots (4.6 healthy roots) and the highest length of them (6.87 cm) were seen in proliferated plantlets in liquid medium by TIB. Relatively similar result was obtained when IBA was not applied in medium, too (4.6 healthy roots, and 6.56 cm). We believe that rooting induction was induced in TIB, and no more IBA was needed in rooting media. Also, proliferated plantlets from solid culture showed the highest number of healthy roots (2.9) and the highest length of roots (6.12 cm) at the concentration of 1 mg $L^{-1}$ IBA. Plantlets of *Crescentia cujete* exposed to intermittent liquid medium in the temporary immersion system also formed more roots than did the plantlets in the other systems [31]. It was reported that better acclimatization following by stronger plant which showing higher rate of survival, can be observed to be more and more better in TIB [12,7]. By observing more than 90% survival rate of plant material at acclimatization phase, it can be concluded that applying 1 mg $L^{-1}$ IBA (rooting induction agent) in TIB played a significant role in improving rooting related characteristics of proliferated carnation in TIB, and our bioreactor can enhance adaption to *ex vitro* too. Overall, rooting induction which happened in TIB can be considered as one of the method of *in vitro* acclimatization to provide resistance for shoots to tolerate change, and stress produced on the phase of acclimatization to *ex vitro*. Indeed, optimization of aeration in TIB leads to propagules of excellent quality which can be acclimatized easily [36]. The advances obtained via liquid medium by TIB, in the present work, on "Tessino Cherry" cultivar of carnation micropropagation can provide innovation over the conventional propagation systems like solid tissue culture. Large-scale tests, and experiment are recommended to evaluate the efficiency of this protocol for other cultivar of carnation (refer to dependency of protocol for specific cultivar), and compare each steps of micropropagation to ensure the large-scale plantlets regeneration with less labor handling cultures, in a high performance micropropagation strategy through our designed TIB. Finally, some genetic stability test like molecular marker analysis for both solid, and TIB cultures-derived plantlet should be carried out to prevent heterogenous flowering in a case of protocol optimizing for final commercial purposes.

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