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Effect of genotypes and culture medium on shoot regeneration and proliferation of *Gerbera jamesonii*

K. Shabanpour¹, A. Sharifi^{2*}, A. Bagheri³ and N. Moshtaghi³

¹College of Agriculture, Islamic Azad University Science and Research, P.O.Box 14515/755, Tehran, Iran.

²Iranian Academic Center for Education, Culture and Research Branch of Mashhad, P.O. Box 91775-1376, Mashhad, Iran.

³College of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, Iran.

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Gerbera jamesonii is one of the most popular cut flowers in the world and micropropagation is the commercial way for its propagation. This method allows for obtaining large amounts of healthy homogenous plants. Thus, it is necessary to establish efficient micropropagation protocols. The objective of this study was to evaluate the organogenic response of *G. jamesonii*, orange and pink cultivars, under *in vitro* culture. Different levels of N⁶-benzyladenine (BA) (2, 4 and 6 mg/l) and thidiazuron (TDZ) (0.2, 0.4 and 0.6 mg/l) in combination with Indole-3-acetic acid (IAA) (0 and 0.1 mg/l) in MS medium were evaluated for shoot induction. For proliferation, regenerated shoots in TDZ were subcultured in medium supplemented with 0.2, 0.4 and 0.6 mg/l TDZ, 2 mg/l BA or 2 mg/l Kin and regenerated shoots in BA were subcultured on the induction medium. In the second phase, mediums of MS, 1/2 MS, MS with 1/2 NH₄NO₃ and KNO₃ concentration (MS-1/2N), MS with 1/2 micro and iron elements (MS-1/2MI), B₅ and 1/2 B₅ on shoot induction and proliferation of pink cultivar were evaluated. In order to induce rooting in the regenerated shoots, different levels of IAA (1, 2 and 3 mg/l) and 1-Naphthaleneacetic acid (NAA) (1, 2 and 3 mg/l) in combination with sucrose (30 and 40 g/l) were evaluated. Maximum shoot induction, (88.8 % and 44.4 % for orange and pink cultivars, respectively) and multiplication rate (7.6 shoots/explant for orange cultivar and 1.33 shoots/explant for pink cultivar) were obtained in medium with 4 mg/l BA and 0.1 mg/l IAA. The most effective media for shoot induction and proliferation were MS-1/2N and MS, respectively. The best rate of shoot rooting in orange cultivar (4.6 roots/explants with 4.8 cm length) and pink cultivar (5.2 roots/explants with 6.2 cm length) was obtained by using 3 mg/l IAA and 30 mg/l sucrose. The establishment of plantlets was done successfully with 92% of survival in the greenhouse.

Key words: Micropropagation, organogenesis, *in vitro* culture, *Gerbera*, cut flower.

INTRODUCTION

Gerbera jamesonii (Asteraceae family), native to south Africa and Asia, is one of the most famous cut flowers in the world (Parthasarathy and Nagaraju, 1999). In 2001, the production of *Gerbera* in US alone was approximately

220 million \$ with 70 million stems (Broek et al., 2004).

Gerbera can be propagated by both sexual (seed) and asexual (clump division, cutting and micropropagation) methods. The multiplication through *in vivo* methods is too slow to be commercially valuable (Das and Singh, 1989). Therefore, micropropagation is commonly used for its rapid and large-scale multiplication (Kanwar and Kumar, 2008). Over the years, many researchers have studied micropropagation of *Gerbera* and different methods of *in vitro* multiplication and explants including callus induction and regeneration from leaves and ovules (Meynet and Sibi, 1984; Reynoird, 1993; Tyagi and

*Corresponding author. E-mail: a-sharifi@acecr.ac.ir.

Abbreviations: BA, N⁶-Benzyladenine; B₅, Gamborg's medium; IAA, indole-3-acetic acid; MS, Murashige and Skoog; TDZ, thidiazuron; NAA, 1-naphthaleneacetic acid.

Kothari, 2004), adventitious shoot regeneration from petiole (Orlikowska et al., 1999), direct adventitious shoot formation from capitulum (Tyagi and Kothari, 2004; Chakrabarty and Datta, 2008) and direct shoot induction in shoot tips (Aswath and Wazneen, 2004; Thakur et al., 2004).

The effect of medium basal salt, plant growth regulators and environmental conditions on shoot regeneration and propagation of *Gerbera* have been investigated (Kanwar and Kumar, 2008). The nutrient medium is important for successful micropropagation and different media such as Murashige and Skoog (MS), modified MS, Gamborg's medium (B₅), LS and DKW were used (Tyagi and Kothari, 2004; Kanwar and Kumar, 2008). Plant growth regulator composition in the medium is another important factor and generally high ratio of cytokinin/auxin was used (Kanwar and Kumar, 2008). Tyagi and Kothari (2004) used capitulum sections in the medium supplemented with 4 mg/l kinetin and 0.5 mg/l indole-3-acetic acid (IAA) for the rapid *in vitro* multiplication of *Gerbera*. Ray et al. (2005) proposed a protocol for rapid propagation of *Gerbera* with the capitulum explant in the medium containing 7 mg/l Kin and 0.1 mg/l IAA. Chakrabarty and Datta (2008) divided the capitulum explants into 4 to 16 pieces and cultured them in MS medium containing 8.8 µM BA and 2.8 µM IAA. For shoot multiplication, they cultured the apical meristem of regenerated shoots in different combination of cytokinins [N⁶-Benzyladenine (BA), Kin and thidiazuron (TDZ)]. The optimum results were obtained with 8.8 µM BA.

In this work, the effect of genotype, cytokinin and medium on shoot regeneration and multiplication and also the effect of auxin and sucrose on rooting of regenerated shoots were studied.

MATERIALS AND METHODS

Plant material

Capitulum explants (0.5 to 1 cm in diameter) of two *Gerbera* cultivars (orange and pink) were washed under tap water for 20 min and soaked in 1.5% (w/v) solution of Ca(ClO)₂ for 10 min, then submerged in a 0.1% (w/v) solution of HgCl₂ for 10 min and rinsed three times in sterile distilled water. Sterilized capitulum were cut into 4 to 6 pieces and cultured in regeneration medium in 100 ml jars.

Shoot induction and proliferation

In this experiment, the effect of different concentrations of BA (2, 4 and 6 mg/l) and TDZ (0.2, 0.4 and 0.6 mg/l) in combination with IAA (0 and 0.1 mg/l) on shoot regeneration were studied in factorial experiment by completely randomized design with 10 replications in each treatment. Capitulum of two *Gerbera* varieties were placed on MS medium containing 30 g/l sucrose, 9 g/l agar, pH 5.7 and different concentration of growth regulators. Cultures were placed under darkness condition and 25 °C for one month, then subcultured on the same medium and placed in 16 h light photoperiod with cool white fluorescent light at 5000 lux. Explants were subcultured in the same medium and condition until shoot

regeneration. For shoot propagation, regenerated shoots on TDZ were subcultured on the MS medium that contained 0.2, 0.4, 0.6 mg/l TDZ, 2 mg/l BA or 2 mg/l Kin and regenerated shoots on BA were subcultured on the MS medium contained 2, 4 and 6 mg/l BA in combination with IAA (0 and 0.1 mg/l). Then the frequency of shoot regeneration and shoot numbers of both cultivars were determined after 8 weeks in light condition for all treatments.

In the second phase, the effect of basal medium including MS, 1/2 MS, MS with 1/2 NH₄NO₃ and KNO₃ concentration (MS-1/2N), MS with 1/2 micro and iron elements (MS-1/2MI), B₅ and 1/2 B₅ on shoot formation was examined only on pink cultivar by completely randomized design with 10 replications in each treatment. Capitulum of pink cultivar cultured on these media supplemented with 30 g/l sucrose, 9 g/l agar, 4 mg/l BA and 0.1 mg/l IAA at pH 5.7. Cultures were placed in darkness condition at 25 °C. After one month, the explants were subcultured on the same medium under photoperiod (16-h light) and cool white fluorescent light at 5000 Lux. Explants were subcultured at 30 days interval, onto the same medium and condition until shoot regeneration. In the proliferation stage, regenerated shoots were subcultured on MS, MS-1/2N and 1/2 B₅ media. Then the average number of shoot proliferation, shoot number and the frequency of shoot regeneration were determined in three subcultures.

Rooting and acclimatization

Single shoots derived from shoot clusters were rooted on MS medium that contained 1, 2 and 3 mg/l IAA or NAA in combination with 30 and 40 g/l sucrose. Data including the number of roots per shoot were recorded after 4 weeks of culture. Shoots with well-developed roots were transferred to autoclaved sand and perlite (1:1 ratio) and maintained in greenhouse at 25 °C, 80-85 % relative humidity and under natural light.

All of data were analyzed by JMP software and the means were compared by Duncan test in 5% confidential level.

RESULTS

Shoot formation

The results showed that shoot regeneration started in the second week of subculture in the regeneration medium until the sixth week and maximum shoot regeneration per week in media and genotypes were different (Table 1).

Effect of cytokinin and auxin on shoot regeneration

The results of this experiment showed that the best shoot proliferation medium for regenerated shoots in TDZ is 2 mg/l Kin (with 1.6 and 0.6 shoots per subculture in orange and pink cultivars respectively). Also, shoots regenerated on BA showed better proliferation on media containing 4 mg/l BA and 0.1 mg/l IAA for orange cultivar and 2 mg/l BA for pink cultivar (2.5 and 1.03 shoots per subculture respectively) (Tables 3 and 4). It was noticed a positive correlation between subculture frequency and the rate of shoot proliferation, maximum shoot number were obtained in the third and second subcultures of orange and pink cultivars (3.4 and 1.1 shoots respectively) (Tables 3 and 4).

Table 1. The percentage of shoot regeneration in orange and pink cultivars during 8 weeks in light condition.

Cytokinin	Medium		Week after first subculture in regeneration medium								Total regenerated shoot
	Cytokinin concentration (mg/L)	IAA concentration (mg/L)	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	
BA	2.0	0.0	0 (0)	0 (0)	11 (0)	11 (0)	11 (11)	0 (0)	0 (0)	0 (0)	33 (11)
BA	2.0	0.1	0 (0)	0 (0)	11 (0)	22 (11)	0 (11)	11 (0)	0 (0)	0 (0)	44 (22)
BA	4.0	0.0	0 (0)	0 (0)	11 (0)	22 (11)	11 (11)	11 (0)	0 (0)	0 (0)	55 (22)
BA	4.0	0.1	0 (0)	22(0)	33 (0)	22 (22)	11 (11)	0 (11)	0 (0)	0 (0)	88 (44)
BA	6.0	0.0	0 (0)	0 (0)	22 (0)	22 (11)	11 (0)	0 (11)	0 (0)	0 (0)	55 (22)
BA	6.0	0.1	0 (0)	0 (0)	11 (0)	22 (11)	11 (11)	11 (11)	0 (0)	0 (0)	55 (33)
TDZ	0.2	0.0	0 (0)	0 (0)	0 (0)	0 (0)	11 (11)	0 (0)	0 (0)	0 (0)	11 (11)
TDZ	0.2	0.1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	11 (0)	0 (0)	0 (0)	11 (0)
TDZ	0.4	0.0	0 (0)	0 (0)	0 (0)	0 (11)	11 (0)	22 (11)	0 (0)	0 (0)	33 (22)
TDZ	0.4	0.1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TDZ	0.6	0.0	0 (0)	0 (0)	0 (0)	11 (11)	22 (11)	11 (11)	0 (0)	0 (0)	44 (33)
TDZ	0.6	0.1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

BA, N⁶-Benzyladenine; TDZ, thidiazuron; IAA, indole -3-acetic acid.

Table 2. The effect of cultivar and plant growth regulators on the regenerated shoot number.

Cultivar	BA concentration (mg/l)						TDZ concentration (mg/l)					
	2		4		6		0.2		0.4		0.6	
	0.0(IAA)	0.1 (IAA)	0.0 (IAA)	0.1 (IAA)	0.0 (IAA)	0.1 (IAA)	0.0 (IAA)	0.1 (IAA)	0.0 (IAA)	0.1 (IAA)	0.0 (IAA)	0.1 (IAA)
Orange	0.9 ^b	1.3 ^{ab}	1.3 ^{ab}	3.1 ^a	1.3 ^{ab}	1.6 ^{ab}	0.2 ^b	0.2 ^b	1.1 ^b	0.0 ^b	1.1 ^b	0.0 ^b
Pink	0.2 ^b	0.9 ^b	0.4 ^b	1.7 ^{ab}	0.4 ^b	0.9 ^b	0.2 ^b	0.0	0.7 ^b	0.0 ^b	0.9 ^b	0.0 ^b

The means with the same letters have no significant differences by Duncan test ($p \geq 0.05$)

explants) (Tables 1 and 2).

Effect of cytokinin and auxin on shoot proliferation

The results of this experiment showed better shoots proliferation on media with TDZ and 2 mg/l Kin (1.1 shoots per subculture) than other media.

Also, shoots regenerated on BA showed better proliferation on medium that contained 4 mg/l BA and 0.1 mg/l IAA (1.5 shoots per subculture) (Tables 3 and 4). It was noticed that a positive correlation between subculture frequency and the rate of shoot proliferation, for both cultivars; 3.4 and 1.1 shoots in the third and second subcultures were obtained in the medium that contained 4 mg/l BA and 0.1 mg/l IAA and 2 mg/l

BA alone. In addition, these media were the most effective for shoot proliferation of the two genotypes in all the subcultures (Tables 3 and 4).

Effect of basal medium on shoot induction

It was found that for pink cultivar the MS medium with $\frac{1}{2}$ NH₄NO₃ and KNO₃ concentration was the

Table 3. The effect of cultivar, cytokinin and subculture on the average number of shoot proliferation (shoots were regenerated in medium with TDZ).

Cultivar	Subculture	Cytokinin concentration (mg/l)				
		0.2 (TDZ)	0.4 (TDZ)	0.6 (TDZ)	2 (BA)	2 (Kin)
Orange	First	0.22 ^{de}	0.33 ^{de}	0.44 ^{de}	0.66 ^{de}	0.77 ^{de}
	Second	0.33 ^{de}	0.44 ^{de}	0.33 ^{de}	1.66 ^b	1.55 ^{cb}
	Third	0.44 ^{de}	0.66 ^{de}	0.66 ^{de}	2.11 ^{ab}	2.55 ^a
Pink	First	0.11 ^e	0.11 ^e	0.22 ^{de}	0.44 ^{de}	0.44 ^{de}
	Second	0.33 ^{ed}	0.22 ^{de}	0.22 ^{de}	0.44 ^{de}	0.66 ^{de}
	Third	0.77 ^{cd}	0.77 ^{cd}	0.88 ^{cd}	0.88 ^{cd}	0.88 ^{cd}

The means with the same letters have no significant differences by Duncan test ($p \geq 0.05$).

Table 4. The effect of cultivar, cytokinin and subculture on the average number of shoot proliferation (shoots were regenerated in medium with BA).

Cultivar	Subculture	BA concentration (mg/l)					
		2		4		6	
		0 (IAA)	0.1 (IAA)	0 (IAA)	0.1 (IAA)	0 (IAA)	0.1 (IAA)
Orange	First	0.6 ^{f-h}	0.9 ^{d-h}	0.9 ^{d-h}	1.6 ^{c-e}	0.9 ^{d-h}	1.1 ^{-g}
	Second	1.1 ^{d-g}	1.2 ^{c-g}	1.9 ^{bc}	2.6 ^{ab}	1.1 ^{d-g}	1.4 ^{c-f}
	Third	1.2 ^{c-g}	1.4 ^{c-f}	2.6 ^{ab}	3.4 ^a	1.4 ^{c-f}	1.6 ^{bc}
Pink	First	1 ^{d-g}	0.3 ^{gh}	0.7 ^{-h}	0.3 ^{gh}	0.3 ^{gh}	0.6 ^{f-h}
	Second	1.1 ^{d-g}	0.8 ^{d-h}	0.7 ^{e-h}	0.4 ^{gh}	0.4 ^{gh}	0.7 ^{e-h}
	Third	1 ^{d-h}	0.9 ^{d-h}	0.8 ^{d-h}	0.6 ^{f-h}	0.6 ^{f-h}	0.8 ^{d-h}

The means with the same letters have no significant differences by Duncan test ($p \geq 0.05$).

best for shoot induction (86 % shoot regeneration and 2.6 regenerated shoots per explants) (Fig. 1). In the proliferation stage, regenerated shoots have better shoot proliferation in MS medium and it reached as high as 2.5 shoots in second subculture (Fig. 2). In various studies, the basal medium utilized for *Gerbera* micropropagation has varied differently.

Effect of auxin and sucrose on rooting

All of the proliferated shoots were successfully rooted in all hormonal concentrations (100 %), medium with 3 mg/l IAA with producing 4.1 roots per shoot being the most effective medium (Table 5). Increasing the sucrose in the medium from 30 to 40 g/l decreased rooting of regenerated shoots (Table 5). Orange and pink cultivars in the medium supplemented with 3 mg/l IAA and 30 g/l sucrose had optimal rooting (4.6 roots/shoot with 4.8 cm length and 5.1 roots/shoot with 6.2 cm length respectively) (Table 5). The establishment of plantlets was done successfully with 92 percent of survival in the greenhouse.

DISCUSSION

Regeneration of *Gerbera* is dependent on cultivar and hormonal combination, so the procedure of regeneration and media should be optimized for each cultivar. In *Gerbera* micropropagation, cytokinin plays a significant role in the morphogenesis of capitulum explants. In this experiment, addition of BA to the medium increased the adventitious buds formation of all the cultivars at both induction and proliferation stages. The number of adventitious buds and regenerated shoots depends on plant genotype. Most researchers used combination of cytokinin (2 to 15 mg/l BA or Kin) and auxin (0.1 to 0.5 mg/l IAA) for shoot regeneration by capitulum explants as these results (Modh et al., 2002; Zheng et al., 2002; Tyagi and Kothari, 2004; Ray et al., 2008).

The decrease of nitrate in basal medium had positive effect on regeneration but it should be examined in other cultivars. Murashige et al. (1974) used modified MS medium, while Chu and Hung (1983) used medium containing MS macroelements ($1/2$ concentration), Heller's microelement, Na_2FeEDTA (21.4 mg/l), MS organic compound and 0.8% Bacto-agar. Radice and Marconi

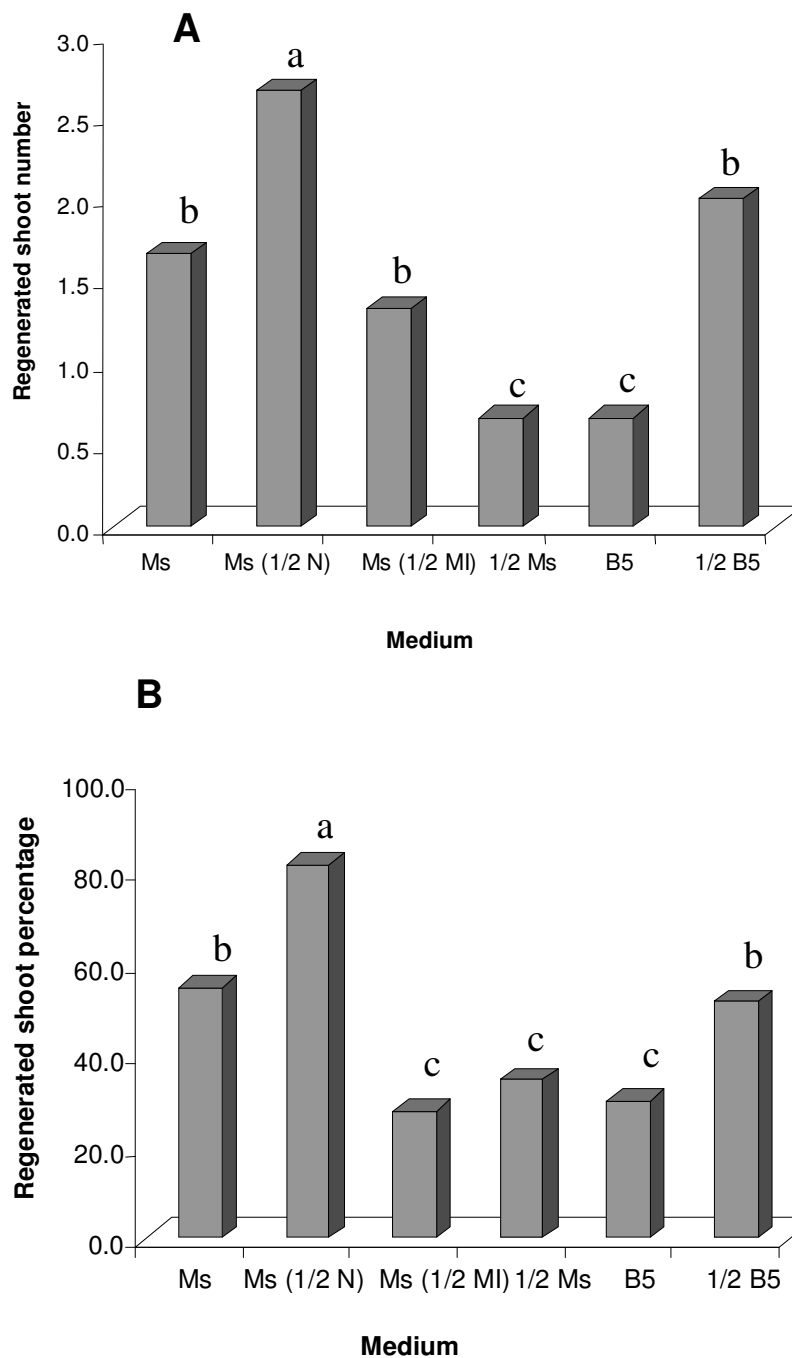


Figure 1. The effect of basal medium on the number (A) and percentage of shoot regeneration (B).

(1998) had a good regeneration of *Gerbera* in $1/2$ MS salt medium with 80 mg/l adenine sulfate. Sato et al. (2001) recommended MS medium with 2.82 $\text{NO}_3^-/\text{NH}_4^+$ ratio for *Gerbera* micropropagation. Topoonyanont and Debergh (2001) utilized MS macro and microelements, 40 mg/l NaFeEDTA and 100 mg/l myoinositol. Mandal and Datta (2002) also used MS and Heller's mineral salts. Tyagi

and Kothari (2004) utilized MS salts with altered concentrations of MnSO_4 , MgSO_4 , ZnSO_4 and thiamin HCl, pyridoxine HCl, nicotinic acid with addition of tyrosine, adenine sulfate and NaH_2PO_4 . So, modification of nitrogen source by using other materials can improved the regeneration of *Gerbera*. In this experiment, MS medium with half nitrate concentration was the most

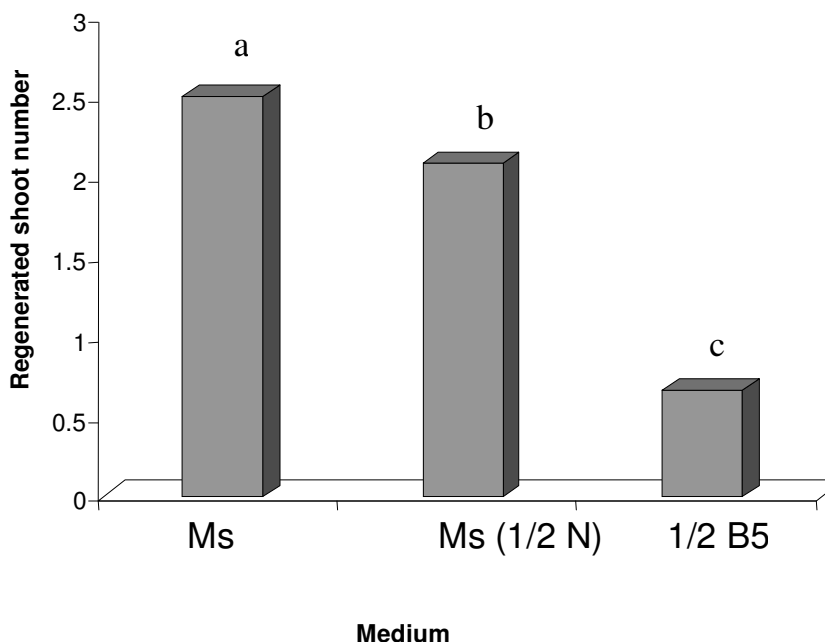


Figure 2. The effect of basal medium on shoot proliferation.

Table 5. Effect of cultivars, auxin and sucrose on the average number of roots per shoot.

Cultivars	Auxin and sucrose concentration (mg/l)													
	Free auxin		IAA				NAA							
	0.0	1	2	3	1	2	3	1	2	3	1	2		
	30	40	30	40	30	40	30	40	30	40	30	40	30	40
Orange	2.2ij	2j	3.5c-f	2.8e-j	3.6c-e	2.9 d-i	4.6ab	3.4d-g	2j	2j	2.5h-j	2.3ij	3.3d-h	3 -i
Pink	2.7f-j	2j	3.6cd	2.3ij	4.2bc	2.8d-j	5.2a	3.2d-h	2.5h-j	2.7g-j	3d-i	3.2 -h	3.4 -g	35 c-f

The means with the same letters have no significant differences by Duncan test ($p \geq 0.05$).

efficient for shoot induction and MS medium was the best for shoot proliferation.

Within *Gerbera* genus, rooting and acclimatization is not difficult to achieve. Different auxins (IAA, NAA and IBA) were reported as efficient for rooting of *in vitro* regenerated shoots as follows: IAA (Soczek and Hampel, 1986), 2.5 mg/l IAA (Maia et al. 1983), 0.5 mg/l NAA (Reynoird et al., 1993), 0.5 mg/l IAA (Tyagi and Kothari, 2004) and 1 mg/l IBA (Nhut et al., 2007). The results suggest that for the proposed cultivars, the most efficient way was the MS medium with 3 mg/l IAA and 30 mg/l sucrose.

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