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Research Report

Interaction Effects of Chitosan, Benzyladenine, and Gibberellic Acid on in Vitro Proliferation of M26 Apple Rootstock

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Abstract. The present study was conducted to evaluate the proliferation ability of M26 apple rootstock on the MS medium supplemented with various concentrations of crab chitosan, gibberellic acid (GA_3), and N6-benzyladenine (BA). Combination of 120 $mg \cdot L^{-1}$ chitosan and 0.5 $mg \cdot L^{-1}$ BA in the absence of GA_3 was most suitable for the proliferation after 12 weeks. Without chitosan, total number of shoots increased by increasing BA concentration, but the shoots led to rosette and dense growth, developing tiny yellowish leaves with soft and watery tissues. Shoots taller than 3 cm were obtained by 0.1 or 0.3 $mg \cdot L^{-1}$ GA_3 in combination with 120 $mg \cdot L^{-1}$ chitosan, but leaves were small with yellowish green color. These results indicated that chitosan can be used as a growth stimulator in combination with a minor amount of plant growth regulators to improve the proliferation rate of in vitro cultured M26 apple rootstock.

***Additional key words*:** *Malus*, micropropagation, plant growth regulators, tissue culture

Introduction

In general, in vitro propagation of tree species is harder than that of herbs, and shoot multiplication is one of the most significant problems encountered in optimization of micropropagation methods for fruit trees. Accelerating this stage successfully by various methods, such as using different chemical compositions, is necessary.

Chitosan $(C_6H_{11}O_4N)n$ is a deacetylated form of chitin $(C_8H_{13}O_5N)n$ that are derived from hard shell crabs and shrimps (Freepones, 1991). Molecular weight and concentration of chitosan are two significant factors affecting the growth of plantlets micropropagated in vitro (Chien et al., 2007; Nge et al., 2006). The positive effects of low molecular weight of chitosan have been reported on in vitro regeneration of somatic embryos and also on reproduction of callus in carrot and branches in strawberry (Kume et al., 2002). It has been reported that the concentration of 5 to 15 $mg \cdot L^{-1}$ of chitosan increased fresh and dry weights of potato tubers and higher concentrations decreased the fresh weight of tubers in the MS medium (Asghari-Zakaria et al., 2009). Chitosan is a growth stimulator and activates plant defense system in stress conditions in vitro and in adapting potato microtuberization (Kowalski et al., 2006).

In agriculture, chitosan has been used in coating seeds, leaves, fruits, and vegetables (Devlieghere et al., 2004), fertilizing and controlled releasing of agrochemicals (Sukwattanasinitt et al., 2001), increasing plant production (Nwe et al., 2004; Wanichpongpan et al., 2001), stimulating the immunity of plants (Hadwiger, 1994), protecting plants against microorganisms (Ait Baraka et al., 2004; Caiqin et al., 2006), and stimulating plant growth. In the later studies, a positive effect of chitosan has been observed on the growth of roots, shoots, and leaves of various plants including gerbera (Wanichpongpan et al., 2001), grapes (Ait Baraka et al., 2004), orchids (Nge et al., 2006), and several crop plants (Chibu and Shibayama, 2001).

In order to stimulate the efficient proliferation of apple plantlets, much effort has been directed to modify culture medium, mainly by inclusion of plant growth regulators such as N6-benzyladenine (BA) (Jones, 1967; Werner and Boe, 1980), gibberellic acid (GA_3), indole-3-butyric acid (IBA) (Jones et al., 1977, 1979), and various brassinosteroids (Schaefer et al., 2002). There have been also investigations on effect of carbon source (Pua et al., 1983), light period, agar concentration, culture container volume, arrangement

of explants on the culture medium, and others (Golosin and Radejevic, 1987; Snir and Erez, 1980). Cytokinins are plant hormones that are essential in tissue culture for cell division and shoot proliferation. In their absence, the metaphase stage is significantly longer. It has been suggested that cytokinins may be required for regulating the synthesis of proteins in forming and function of mitotic spindles (Jouanneau, 1975; Minocha, 1987). Common types of cytokinins include BA, kinetin, isopentenyladenine (2-iP), and zeatin. Among these types of cytokinins, BA was the most active and cheapest, and the only kind that can be autoclaved. Thus, BA is the most widely used, especially in commercial micropropagation because of cost and simplicities (Padhye et al., 2008; Thomas and Blakesley, 1987). The use of high levels of cytokinins produced small shoots that their elongation is not sufficient. This may produced abnormal leaves or vitrified shoots in some species (Ben-Jaacov et al., 1991). By comparing three different types of cytokinins (2-iP, BA, and kinetin) in proliferation stage of some rose varieties, BA was reported to produce more number of shoots per explant than others (Carelli and Echeverrigary, 2002).

Gibberellic acid (GA_3) is the first commercial product and can increase the proliferation rate in the second stage of micropropagation. The benefit of using GA_3 in the second stage of micropropagation often depends on the plant genotype. In proliferation stage, GA_3 has showed antagonistic effect with shoot length in 'McIntosh' apple shoot cultures (Lane, 1992). In contrast, GA_3 is essential for 'Ottawa3' apple rootstock (Lane, 1992). Without adding 5 $mg \cdot L^{-1}$ GA_3, shoots had small deformed leaves with short internodes (Lane, 1992). Among the types of plant growth regulators which were used on proliferation stage of M26 apples rootstock, the combination of 1 $mg \cdot L^{-1}$ BA with 0.1 $mg \cdot L^{-1}$ GA_3 and 1 $mg \cdot L^{-1}$ IBA had better results than kinetin or BA alone (Jones et al., 1977). It was reported that MS medium supplemented with BA and GA_3 or kinetin had the highest shoot proliferation in strawberries (Sakila et al., 2007). Effective and beneficial uses of GA_3 for elongation of short shoots which were produced in large amounts of cytokinins in such plants as apples (Aldwinckle and Gustafson, 1981) and roses (Valles and Boxus, 1987) have been reported. It has been reported that GA_3 had a significant effect on the proliferation stage of Satsuma tangerine or mandarin (Omura and Hidaka, 1992).

In the present study, considering the importance of clonal rootstocks in vegetative propagation and development of regular and homogeneous orchards, the possibility to improve the proliferation methods of in vitro cultured apple rootstock was investigated using the various concentrations of chitosan, GA_3, and BA.

Materials and Methods

Plant Materials

The explants, 0.5-1 cm in length and with two buds, of M26 apple (*Malus domestica*) rootstock were obtained from the in vitro plantings. The in vitro plants were previously produced in Apple Tissue Culture Laboratory of Department of Horticulture, Agriculture College of Urmia University, Orumieh, Iran.

Culture Medium and Conditions

The MS culture medium (Murashige and Skoog, 1962) containing 2.5 $mg \cdot L^{-1}$ thiamine and 208 $mg \cdot L^{-1}$ myoinositol was supplemented with 0, 20, 40, 60, or 120 $mg \cdot L^{-1}$ (designated as CH0, CH20, CH40, CH60, and CH120, respectively) chitosan (MDL, MFCD00167512, Sigma Aldrich, St. Louis, MO, USA) from crab shell with a molecular weight of 20 kDa, 0.5 or 1.5 $mg \cdot L^{-1}$ (designated as BA 0.5 and BA1.5 $mg \cdot L^{-1}$, respectively) BA, and 0, 0.1, or 0.3 $mg \cdot L^{-1}$ (designated as GA 0, GA 0.1, GA 0.3 $mg \cdot L^{-1}$, respectively) GA_3. Following addition of 30 $g \cdot L^{-1}$ sucrose, the culture medium was shaken to have its materials completely dissolved and brought up to a final volume of 1,000 mL with double distillated water. The pH was adjusted to 5.7 prior to autoclaving for 17 min at 121°C and 1.5 $kg \cdot cm^{-2}$. Semi-solid medium was prepared by adding 7 $g \cdot L^{-1}$ agar. All the materials used in the culture medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). In every Petri dish containing 40 mL medium, two shoots were placed horizontally and they were exposed to 40 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPFD cool white fluorescent light in a 16/8 h photoperiod at 24°C for 12 weeks.

Data Collection and Analysis

Factors used for determining the proliferation rate were average plant fresh weight, number of shoots, average shoot length, number of leaves, days needed for starting of new growth in established explants, leaf size and color, and vitrification. Leaf size was measured by scoring 1 to 3 (Arora et al., 2006): 1, 0.5 to 1 cm^2 of leaf area; 2, 1 to 1.5 cm^2 of leaf area, and 3, 1.5 to 2 cm^2 of leaf area. Leaf color was assessed on a 1-5 scale: 1, brownish yellow; 2, yellow; 3, yellowish green; 4, light green; and 5, normal green. Since the intensity of green leaves of proliferated plantlets were strongly influenced by chlorophyll concentration, the chlorophyll content of leaves was measured with SPAD index by chlorophyll meter (502, Konica Minolta, Tokyo, Japan).

Visual observations were made every day. The ordered factors were measured and recorded 12 weeks after cultivation.

The experiment was conducted as a completely randomized design with three factors of chitosan (CH) in five levels, BA in two levels, and GA_3 (GA) in three levels with seven replications. The experiment was triplicated. Data were statistically analyzed and the means were compared using Duncan's multiple range test (DMRT) and MSTATC software (v. 2.10, Michigan State University, E. Lansing, MI, USA).

Results

Variance analysis results of investigated parameters showed that triple interaction of chitosan, BA, and GA_3 significantly affected the quantitative and qualitative characteristics of produced plantlets in probability level of 1%.

Time Need for Starting of New Growth in Established Explants

The M26 explants started to bulge in cut points since they were placed on the culture medium and then buds and new shoots developed gradually in places of these bulges. In the case of the triple interaction among the treatments, the treatments of CH20BA0.5GA0.3, CH20BA1.5GA0.3, and CH120BA1.5GA0.3 showed the shortest time need for starting the new growth in explants, whereas the treatment of CH0BA0.5GA0.1 needed the longest time for starting the new growth in explants (Fig. 1).

Number of Produced Shoots

After the explants started to grow, the buds commenced longitudinal growth and leaf expansion. According to Figs. 2 and 3, the treatment of CH120BA1.5GA0.3 showed the highest shoot proliferation with 12.14 shoots, while the treatment of CH60BA0.5GA0 gave the lowest shoot proliferation of 1.57 shoots

Length of Produced Shoots

Fig. 4 shows the triple interaction between the treatments on the length of shoots. The treatments of CH20BA0.5GA0.3 and CH20BA0.5GA0.1 had developed the longest shoots, while treatments of CH60BA1.5GA0 and CH120BA1.5GA0

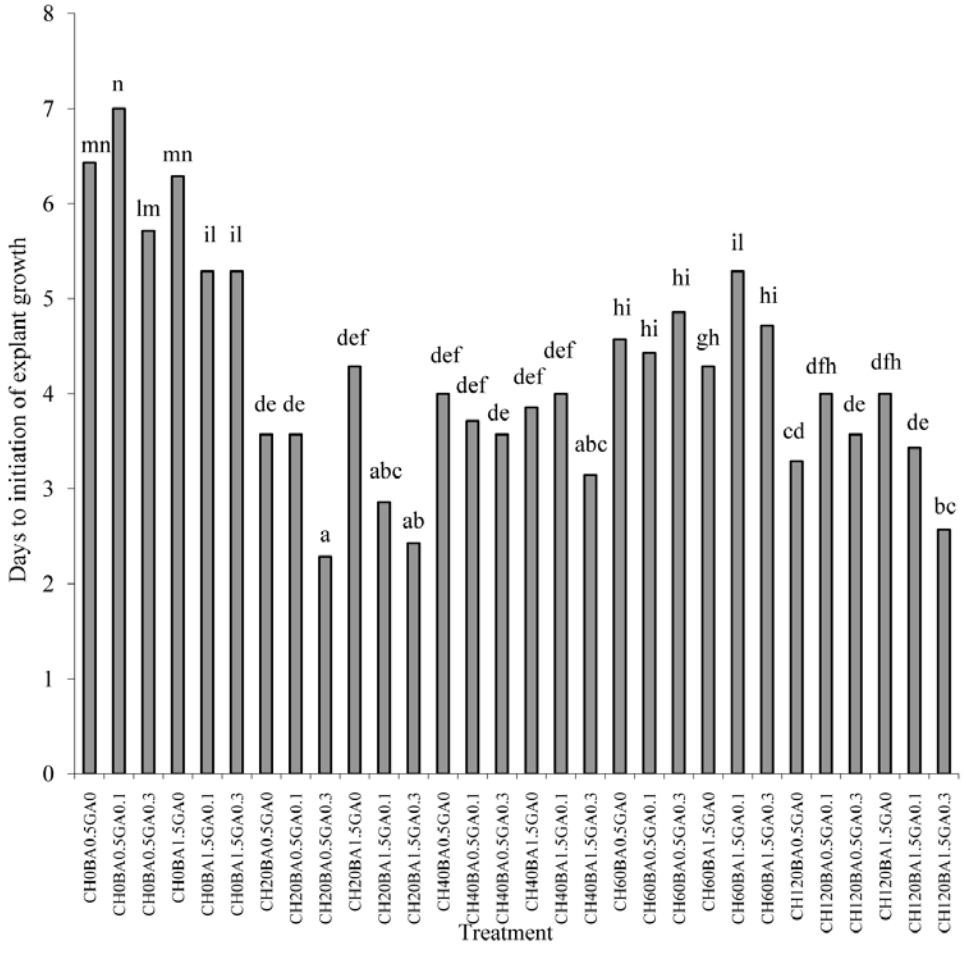

Fig. 1. Interactive effects of chitosan, BA, and GA_3 on the starting of a new growth in explants of in vitro cultured M26 apple rootstock. Means in columns with the same letters are not significant by DMRT at P = 0.01.

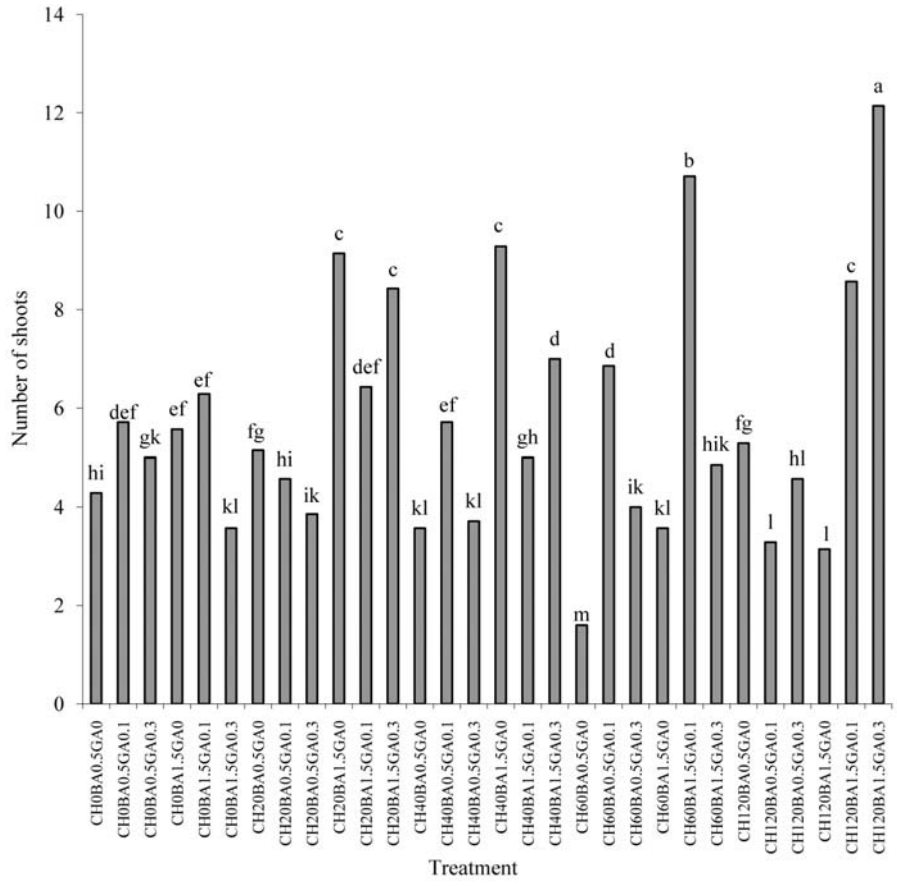

Fig. 2. Interactive effects of chitosan, BA, and GA_3 on the number of produced shoots of in vitro cultured M26 apple rootstock. Means in columns with the same letters are not significant by DMRT at P = 0.01.

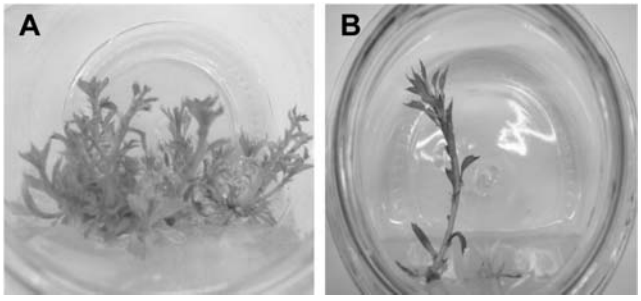

Fig. 3. Comparison of the number of produced shoots related to the maximum (A) (CH120BA1.5GA0.3) and minimum shoot proliferation (B) (CH60BA0.5GA0) of in vitro cultured M26 apple rootstock.

had developed the shortest shoots (Fig. 5).

Interactive Effects of Chitosan, BA, and GA_3 on the Number, Size, and Color of the Produced Leaves

In terms of the triple interactions of the treatments (chitosan, BA, GA) on the number, color, and size of the produced leaves (Table 1), the greatest (91.9) and the least number of leaves (28.0) were obtained from the treatments of CH60BA1.5GA0.1 and CH60BA0.5GA0, respectively. The largest size of the produced leaves was related to the treatments of CH40BA0.5GA0.1, CH20BA1.5GA0.3, CH20BA0.5GA0.3, CH40BA1.5GA0, and CH120BA0.5GA0, and the smallest size of the produced leaves was observed in the treatments of CH0BA1.5GA0 and CH0BA0.5GA0.1.By comparing treatment averages it was found that the best leaf color (normal green) was developed by the treatment of CH120BA0.5GA0 and the worst color (brownish yellow) was related to treatments of CH60BA1.5GA0 and CH60BA0.5GA0.

Interactive Effects of Chitosan, BA and GA_3 on Plant Fresh Weight

According to Fig. 6, the highest plant fresh weight (1.77 g) was observed in the treatment of CH60BA1.5GA0.1 and the lowest was observed in the treatments of CH60BA0.5GA0 and CH40BA0.5GA0 (0.26 and 0.27 g, respectively).

Vitrification of the Produced Plantlets

In this investigation none of the treatments showed vitrification phenomenon. By comparison among all treatments, a combination of 120 $mg \cdot L^{-1}$ chitosan and 0.5 $mg \cdot L^{-1}$ BA in the absence of GA_3 was the most suitable for the proliferation

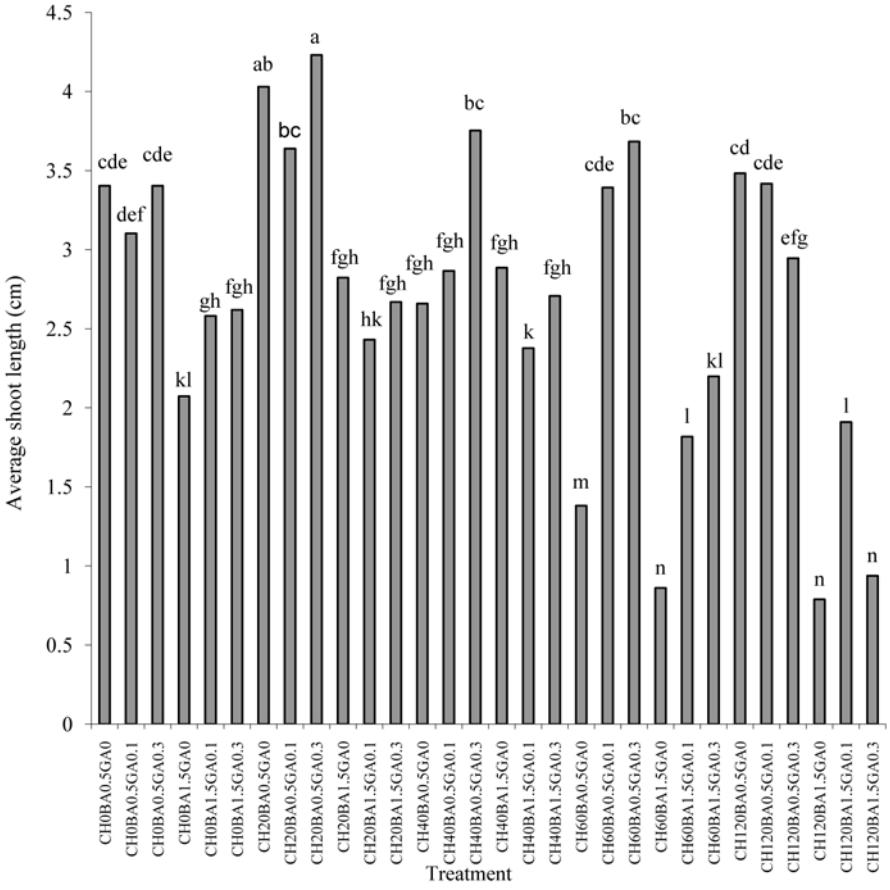

Fig. 4. Interactive effects of chitosan, BA, and GA_3 on the average length of produced shoots of in vitro cultured M26 apple rootstock. Means in columns with the same letters are not significant by DMRT at P = 0.01.

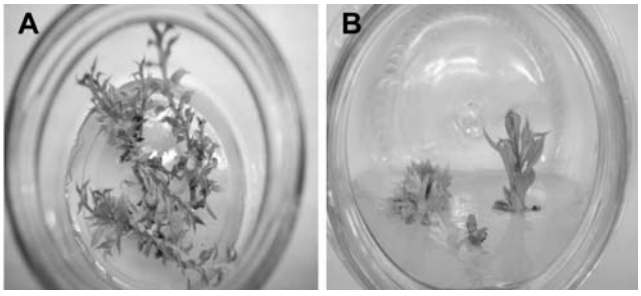

Fig. 5. Comparison of the length of proliferated shoots related to the longest (A) (CH20BA0.5GA0.3) and shortest shoots (B) (CH120BA1.5GA0) of in vitro cultured M26 apple rootstock.

measured after 12 weeks (Fig. 7).

Discussion

Effect of Chitosan on In Vitro Proliferation Stage of M26 Apple Rootstock

In the culture medium with 20 mg·L^{-1} of chitosan, the appearance of green swelling and adventitious buds was observed, and the buds were visible after 2-3 days. Furthermore, application of chitosan at 40 and 120 mg·L^{-1} accelerated the appearance of green swelling buds and adventitious buds that showed a difference of 3-4 days as compared to the control.

The data showed that the average of all measured parameters, except plant fresh weight and number of produced leaves, were greater in the treatment with 20 mg·L^{-1} chitosan than other treatments. This was associated with the results of other various plants (Ait Baraka et al., 2004; Chibu and Shibayama, 2001; Nge et al., 2006). The results showed that low concentrations of chitosan as a non-toxic substance can be used to stimulate plant growth. According to Ait Baraka et al. (2004), formulated chitosan (cytogel) at a concentration of 1.75% increased number of shoots and photosynthetic rate. With increasing concentration, however, the effect of chitosan on grape plantlets was negative.

According to research conducted by Sopalun et al. (2010), 15 mg·L^{-1} chitosan with average molecular weight of 20 kDa increased protocorm-like growth of orchids significantly. With the application of 25 mg·L^{-1} chitosan, the rate decreased. According to the reports, low concentrations of low molecular weight chitosan (less than 60 kDa) increased proliferation rate of orchids. The results of Nge et al. (2006) were consistent in the use of chitosan with average molecular weight of 20

Table 1. Interactive effects of chitosan, BA, and GA_3 on the number, size, and color of produced leaves on shoots of in vitro cultured M26 apple rootstock.

Treatment code	No. of leaves	Leaf size (cm^2)	Leaf color chlorophyll index (SPAD)
CH0BA0.5GA0	54.6 gh[z]	1.3 bc	3.0 cd
CH0BA0.5GA0.1	42.7 ijk	1.0 c	3.7 bc
CH0BA0.5GA0.3	56.7 efg	1.6 b	3.3 bcd
CH0BA1.5GA0	40.4 jkl	1.0 c	3.3 bcd
CH0BA1.5GA0.1	48.3 hi	1.0 c	2.1 hi
CH0BA1.5GA0.3	51.7 gh	1.0 c	3.3 bcd
CH20BA0.5GA0	47.1 hij	1.0 c	2.4 hi
CH20BA0.5GA0.1	42.0 ijk	1.0 c	3.1 bcd
CH20BA0.5GA0.3	37.0 kl	2.0 a	3.1 bcd
CH20BA1.5GA0	62.3 def	1.0 c	3.4 bcd
CH20BA1.5GA0.1	52.9 gh	1.0 c	1.7 1i
CH20BA1.5GA0.3	69.3 bcd	2.0 a	3.4 bcd
CH40BA0.5GA0	33.9 lm	1.0 c	2.6 ef
CH40BA0.5GA0.1	52.9 gh	2.0 a	3.0 cdef
CH40BA0.5GA0.3	39.0 kl	1.6 b	2.9 def
CH40BA1.5GA0	67.5 cd7	2.0 a	3.1 bcf
CH40BA1.5GA0.1	51.9 gh	1.0 c	2.9 dfh
CH40BA1.5GA0.3	66.7 cd	1.3 bc	2.9 dfh
CH60BA0.5GA0	28.0 m	1.0 c	1.0 j
CH60BA0.5GA0.1	52.2 gh	1.6 b	2.6 ef
CH60BA0.5GA0.3	33.6 lm	1.4 bc	2.4 fh
CH60BA1.5GA0	39.9 jkl	1.0 c	1.0 j
CH60BA1.5GA0.1	91.9 a	1.0 c	2.3 ghi
CH60BA1.5GA0.3	64.1 de	1.0 c	2.3 ghi
CH120BA0.5GA0	55.3 fgh	2.0 a	5.0 a
CH120BA0.5GA0.1	33.7 lm	1.0 c	2.6 ef
CH120BA0.5GA0.3	37.3 kl	1.0 c	3.9 b
CH120BA1.5GA0	36.1 kl	1.0 c	2.1 hi
CH120BA1.5GA0.1	72.0 bc	1.0 c	3.6 bcd
CH120BA1.5GA0.3	76.0 b	1.0 c	2.4 ghi

[z]Mean separation within columns by DMRT at $P = 0.01$.

kDa. According to the results of the present study on chitosan, the response of different species of molecular weight of chitosan and its application might be different. According to the reports of Nge et al. (2006), effect of chitosan with molecular weight of 1 kDa in the development of lateral buds of orchids was 4-times more than high molecular weight chitosan (100 kDa). At the concentrations used in this study, the effect of chitosan on the number of leaves was not statistically significant, but significant difference was found from the control that showed the number of produced leaves was not affected by the concentration of chitosan. However, in other measured factors the concentration of the chitosan was important.

The highest plant fresh weight was found in the 60 $mg \cdot L^{-1}$ chitosan treatment and plantlets in the 20 and 120 $mg \cdot L^{-1}$ chitosan treatments had average plant fresh weight and normal growth. Thus, we can conclude that high fresh weight cannot be an excellence, because apple plantlets with high plant fresh weight, but bulky and rosette growth had abnormal growth of stems. These can be problems for the other stages of tissue culture. The results of this study were different from the studies of Nge et al. (2006) on usefulness of high fresh weight. This can be due to differences in culture, plant species, and parts of plants used as the explant.

The results of this study showed that 20 $mg \cdot L^{-1}$ chitosan increased the length and number of shoots in M26 apples and 60 $mg \cdot L^{-1}$ chitosan reduced proliferation. It seems that it is because of the negative effect of high concentrations of chitosan without the use of plant growth regulators. According to Ait Baraka et al. (2004), formulated chitosan (cytogel) at a concentration of 2.5% had a negative effect on the proliferation of explants and it is the similar case in this study. Effect of chitosan on leaf color was significant. Good green color was observed in the treatment of 20 $mg \cdot L^{-1}$ chitosan that was corresponded with studies of Zhang et al. (2009). It also showed that low concentration of chitosan increased the chlorophyll concentration in plant tissues.

In the proliferation stage, increasing the number of shoots was important. Therefore, the use of chitosan had the positive effect on increasing the number of shoots. Positively charged

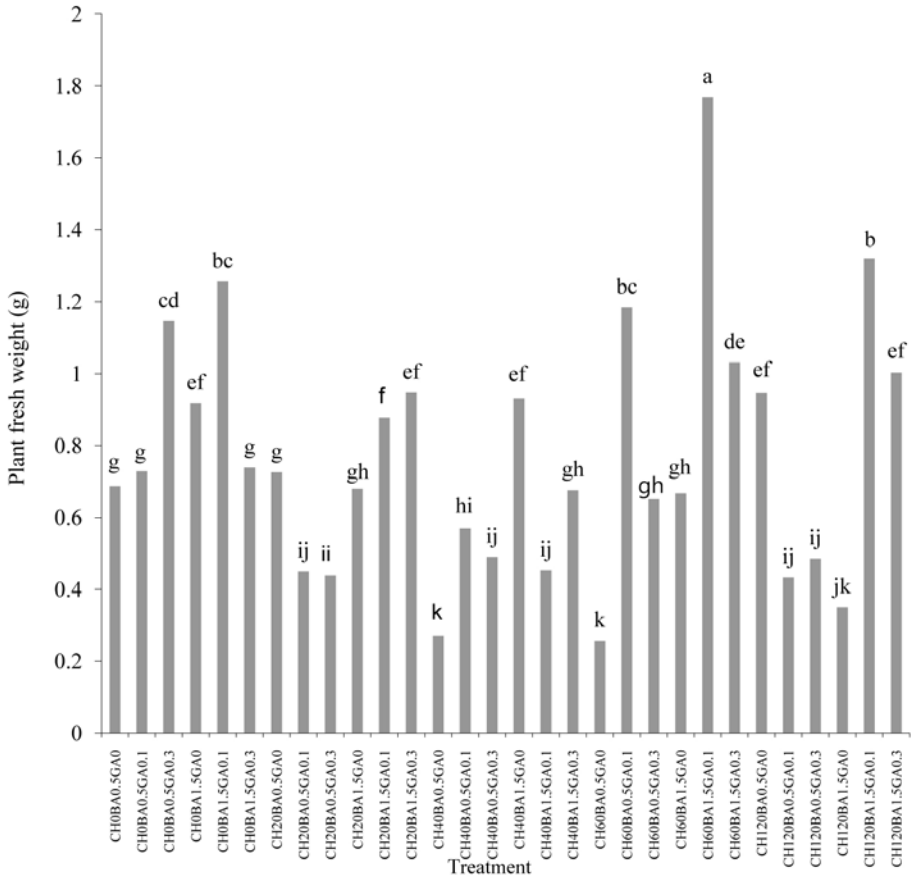

Fig. 6. Interactive effects of chitosan, BA, and GA_3 on plant fresh weight of in vitro cultured M26 apple rootstock. Means in columns with the same letters are not significant by DMRT at P = 0.01.

Fig. 7. Best result obtained from the treatment of CH120BA0.5GA0 of in vitro cultured M26 apple rootstock.

chitosan can cause multiple and exclusive biological and physiological characteristics. Chitosan is known as a growth stimulating substance with high potential (Ren et al., 2001).

Effects of BA and GA_3 on In Vitro Proliferation of M26 Apple Rootstock

In this study, increasing concentration of BA from 0.5 to 1.5 $mg \cdot L^{-1}$ increased the number of shoots at the proliferation stage. Increasing number of shoots resulted in decreased average length of shoots, increased number of leaves, and shortened leaf length. This might be due to competition between shoots in the partitioning of nutrients absorbed from the culture medium.

The results of experiments showed that BA increased number of shoots by reducing the apical dominance and shoot length (Biswas et al., 2007; Carelli and Echeverrigary 2002; Lane, 1992; Owen and Miller, 1996; Yokimova et al., 2000). They also found that increase of BA increased number of shoots and reduced average shoot length. Kapchina-Toteva et al. (2000) on roses, and Grigoriadou et al. (2000) and Kadota and Hirano (2001) on pears also observed increased proliferation rate by increasing BA, which are coincide with our results.

In this study, the results of GA_3 at the proliferation of M26 apple rootstock indicated the usefulness of at low concentrations. Because of its effect on initiation of fast growing explants, bigger leaves with green color, number of leaves, and plant fresh weight, 0.3 $mg \cdot L^{-1}$ of GA_3 is more effective than 0.1 $mg \cdot L^{-1}$ GA_3. And these results coincide with the results of Jones et al. (1977) and He (1996).

Interactive Effects of Chitosan with BA on In Vitro Proliferation of M26 Apple Rootstock

Generally, the treatment of CH120BA0.5 had greater effects on all measured parameters except shoot length and plant fresh weight than the other treatments. The treatment caused initiation of fast growing of explants, producing 5 shoots with appropriate length, more number of leaves, and better leaf color.

Plantlet fresh weight was medium in this treatment. Increasing the concentration of chitosan from 20 to 120 $mg \cdot L^{-1}$ and reducing the concentration of BA from 1.5 to 0.5 $mg \cdot L^{-1}$ accelerated and enhanced the proliferation and improved leaf color and size. Interaction between BA and chitosan in plantlet chlorophyll content was significant. The greatest chlorophyll value was observed in the treatment of 0.5 $mg \cdot L^{-1}$ BA plus 120 $mg \cdot L^{-1}$ chitosan. According to Qiuping and Wenshui (2007), chitosan causes the chlorophyll stability and it might stimulate the expression of genes that are involved in the chlorophyll synthesis pathway. Hadwiger (1994) and Bhaskara et al. (1998) also observed that using chitosan with a small amount of BA accelerated and enhanced the proliferation of explants. Our results coincide with the results of these researchers.

Interactive Effects of Chitosan with GA_3 on In Vitro Proliferation of M26 Apple Rootstock

Generally, the CH60GA0 treatment caused undesirable characteristics on plantlets, and therefore, the importance of using GA_3 and the sensitivity to concentration of chitosan were realized. This phenomenon might be the result of plant growth regulators with their independent and interaction effects on plant metabolism.

Interactive Effects of BA and GA_3 on In Vitro Proliferation of M26 Apple Rootstock

On observations of the interactive effects of BA and GA_3, the maximum proliferation and number of leaves were observed in the treatments of BA1.5GA0.1 and BA1.5GA0.3. In comparison the BA1.5GA0.3 treatment caused initiation of growing of explants in the shortest time and this result was the same as that of Sakila et al. (2007).

The BA0.5GA0 treatment had the longest time for initiating explant growth, the minimum number of shoots (4.0), and the lowest plant fresh weight (0.6 g). This indicated a positive effect of GA_3 at the proliferation stage (Omura and Hidaka, 1992). The BA0.5GA0.3 treatment had the greatest influence on leaf color and size, and produced the medium leaf size with green color. However, the number of leaves in this treatment was less than in other treatments (43.7).The BA1.5GA0.1 treatment gave the worst leaf color and produced the maximum plant fresh weight (1.1 g). Despite this treatment produced the highest number of shoots and leaves, due to the low level of quality indices, such as leaf color and size, and high plant fresh weight with long shoots, this treatment could be useful in the later stages of tissue culture. It has been reported that adding 0.1-0.3 $mg \cdot L^{-1}$ GA_3 to the MS medium with 3 $mg \cdot L^{-1}$ BA and 1 $mg \cdot L^{-1}$ IAA produced longer shoots and smaller leaves in *Duboisia myoporoides* (Kukreja and Mathur, 1985).

The BA1.5GA0 had the shortest shoots (1.9 cm) and internode length (0.2 cm). The results of this experiment were consistent with results obtained by Jones et al. (1977), Pattnaik and Chand (1997), and Sakila et al. (2007).

Interactive Effects of Chitosan, BA and GA_3 on In Vitro Proliferation of M26 Apple Rootstock

The greatest proliferation (12.1 shoots) was observed with the combination of 120 $mg \cdot L^{-1}$ chitosan, 1.5 $mg \cdot L^{-1}$ BA, and 0.3 $mg \cdot L^{-1}$ GA_3. Considering that other quality indicators, such as appearance, and leaf color and size, are important in selecting the appropriate treatment, the treatments with suitable proliferation but with loss of quality indicators should not be considered as the best combination. The results of this study showed that the combination of 120 $mg \cdot L^{-1}$ chitosan, 0.5 $mg \cdot L^{-1}$ BA, and absence of GA_3 is most appropriate treatment for proliferation. This treatment could produce 5.3 shoots with appropriate length (3.5 cm), well-developed leaves and normal green color without callus formation. The required time for the growth of explants was 3 days and plant fresh weight was moderate (0.95 g).

With inexpensive, non-toxic, and biodegradable characteristics of chitosan, it could be considered as a growth stimulator and be applied along with lower concentrations of plant growth regulator to increase in vitro proliferation of apple. It also shortens the duration of proliferation and accelerates the transfer of plantlets to the rooting stage. Plants did not show the same reaction to different concentrations and molecular weights of chitosan. Thus, the different molecular weight and concentration of chitosan should be examined separately for the M26 and other cultivars of apple. With these additional studies, comprehensive understanding of proliferation of different apple cultivars could be obtained. The chitosan which is derived from the skin of Artemia in Urmia Lake is worthy to be tried in tissue culture in comparison with other common chitosan, since extraction of chitin from cyst shells of *Artemia urmiana* and converting it to chitosan may produce materials with high added value of driven cysts to the shores of Urmia Lake (Sorgeloos, 1997).

Based on the results obtained in this study, 120 $mg \cdot L^{-1}$ chitosan in combination with a low concentration of BA was the most suitable treatment for in vitro proliferation of the M26 apple rootstock. This is in accord with the results obtained by other investigators for other plant species (Ait

Barka et al., 2004; Bhaskara et al., 1998; Chibu and Shibayama, 2001; Hadwiger, 1994; Nge et al., 2006). According to these studies, utilizing chitosan as a growth stimulating material accelerated and improved multiplication of the used explant in different plant species.

In this study, explants produced a maximum shoot proliferation with higher concentrations of BA and GA_3 in combination with chitosan by showing the shortest time (2.3 days) for starting of the new growth and also without any increase in qualitative features. Since the other important aspect to be considered in choosing a proper treatment is the qualitative features, such as physical condition, and leaf color and size, treatments with proper shoot numbers, but lower quality features could not be considered as the best composition.Although the maximum number of shoots was observed on the medium containing chitosan alone or high levels of BA and GA_3, it could not be considered as the proper composition for other stages of tissue culture due to production of the shortest shoots (0.96 cm) with tiny denticulate leaves with undesirable color, dense growth, and watery and brittle stems.

In the present study, general reduction in the proliferation rate (the least number of shoots and leaves, shortest shoots, and plant fresh weight) was observed when 60 $mg{\cdot}L^{-1}$ chitosan and 0.5 $mg{\cdot}L^{-1}$ BA in lack of GA_3 were used. The longest shoot (4.2 cm) and internode length (0.7 cm) with normal leaf size was produced with 20 $mg{\cdot}L^{-1}$ chitosan, 0.5 $mg{\cdot}L^{-1}$ BA, and 0.3 $mg{\cdot}L^{-1}$ GA_3. The shortest shoot (0.8 cm) with maximum plant fresh weight (1.8 g) was obtained with 60 $mg{\cdot}L^{-1}$ chitosan and 1.5 $mg{\cdot}L^{-1}$. However, this treatment gave shoots with tiny leaves and undesirable brownish yellow leaves, and it could not be considered as a desirable composition.

Due to the inexpensive, non-toxic, and biodegradable properties of chitosan, it can be recommended as a growth stimulator which can be used in combination with a minor amount of plant growth regulators to promote in vitro proliferation in tissue culture, and that can lead to shortened proliferation time and accelerated transition of produced plantlets to a rooting medium.

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