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STUDY OF MORPHOLOGICAL TRAITS CHANGES IN DIFFERENT MEDIA CULTURE OF TWO APPLE ROOTSTOCKS (M26 AND MM106)

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ABSTRACT

Numerous studies have reported on regeneration from apple somatic tissues, andorganogenesis has been proved to be influenced by several factors including mother shoots (genotype, size, type, and age of explant), *in vitro* conditions (dark period, light intensity, and quality), and others (wounding, orientation of leaf explants). However, one of the most important factors during the regeneration process ismicro and macro elements in media culture and the type and concentration of cytokinin applied. For optimization of the elongation shoot and proliferation medium, two different macro and micro element formulations were tested: MS and DKW. The 2-isopentnyl adenine (2-ip) and benzyl adenine (BA) are the most frequently used cytokinins in the regeneration systems, but their efficiency depends on genotype and other factors. The organogenic ability of explants can also be increased by a properly selected cytokinin treatment. Phytohormones (2 mgl^{-1} BA and 2 mgl^{-1} 2-ip with 0.1 mgl^{-1} IBA) established for the proliferation of M26 and MM106 apple rootstocks. The both of rootstocks, the MS medium with BA (2 mgl^{-1}) yielded in general the best shoot growth and elongation of branch also, DKW with 2-ip (2 mgl^{-1}) showed the best callus formation and branching. Significantly better growth with the MS medium was also favored by BA as the cytokinin.

Key words: Shoot regeneration, rootstock, *Malus* sp., M26, MM106

INTRODUCTION

Apple is the most widely grown fruit in the world, cultivated throughout the temperate zones of both the northern and southern hemispheres (Harris *et al.*, 2002). About 59 species and 7500 cultivars were identified in all of over the world. Considering world fruit production, apple (*Malus* sp.) is the third most important fruit crop (64.3 million ton/year) after banana (81.3 million ton / year) and grape (66.3 million ton/year) (FAO 2009). The apple (*Malus* x *domestica* Borkh.) is believed to have originated from central Asia and from there spread to the rest of the world (Harris *et al.*, 2002).

Numerous clonal apple rootstock belonging to the categories of dwarf, semi-dwarf, and vigorous have been developed. The MM106 (semi-dwarf) and M26 (dwarf) are apple rootstocks used extensively in many countries to produce semi-dwarf and dwarf trees. Therefore, the improvement of micropropagation efficiency of the M26 and MM106 apple rootstocks are very important for commercial practices (Sotiropulos *et al.*, 2005). Breeding of apples by conventional hybridization requires many years because of their long juvenile period, a high level of self-incompatibility, and the concomitant highly heterozygous nature of the genome. The use of biotechnological methods in apple breeding offers a way to bypass the disadvantages of sexual hybridization (Korban and Chen 1992). However, for this to occur, regeneration of adventitious shoots is necessary for breeding plants via nonsexual methods e.g. somaclonal variants and in vitro culture.

A malling growth habit is a goal of many breeding programs and has already contributed to important yield advances (Welander, 1988). Dwarf tress have several advantages: they can be planted close together to give good yields, due to reduce vegetative growth, they require minimal pruning and training so labor cost is reduced, it is easier and less expensive to apply pesticides, and they produce uniform sized and colored fruits (Sarwar *et al.*, 1998). Several factors can influence the success of in vitro

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shoot regeneration such as genotype, mineral substances of media culture and *in vitro* conditions during the regeneration process. Among phyto-hormones, cytokinins have proven to be the most important factor affecting shoot regeneration (Magyar-Tabori *et al.*, 2010).

Dobranszki *et al.*, (2010) researched antioxidant and BA glycosilated effects on "Royal Gala" of regeneration. Bhatti *et al.* (2010) reviewed the in vitro shoot multiplication, rooting, transformation and regeneration methodologies in apple by auxin and cytokinin hormones.

The objective of the present study was to develop an efficient proliferation protocol for *in vitro* establishment, multiplication and elongation of the most interesting rootstocks M26 and MM106 in special region of Iran.

MATERIALS AND METHODS

Two apple rootstocks: M26 and MM106 were used in this study. Actively growing shoots of the year were collected in spring from plants pots in greenhouse. One centimeter of internode sample cut and cultured after sterilization. For sterilization, single nodes were taken from M26 and MM106 rootstocks dipped in ddH2O, NaOCl based on protocol used by Ciccotti *et al.*, 2008. The DKW medium (Driver and Kuniyuki, 1984) and MS medium (Murashige and Skoog, 1962) containing 30 gl^{-1} sucrose, 7.0 gl^{-1} agar and hormones were used for shooting and branching explants production. The MS media used with 2 mgl^{-1} BA and 0.1 mgl^{-1} IBA (SB) and 2 mgl^{-1} 2-ip and 0.1 mgl^{-1} IBA (SP), also DKW used with 2 mgl^{-1} BA and 0.1 mgl^{-1} IBA (DB) and 2 mgl^{-1} 2-ip and 0.1 mgl^{-1} IBA (DP), (IBA concentration was equal in all of media culture) (Table 1). The medium was sterilized by autoclaving for 20 min at 121ºC. All media containing adjusted pH to 5.8 before autoclaving.

Five to six samples were placed into glass jars and maintained at 25±2ºC under a 16/8 h light photoperiodic under a light intensity 3000 lux in a germinator. After 30 days, all of samples transferred to fresh medium and processed till fourth subculture for detection of morphological variations.

Table 1. Medium culture and hormones used in this study

Medium culture	BAP (mgl^{-1})	2-ip (mgl^{-1})	IBA (mgl^{-1})	Abbreviation
MS	2	_	0.1	SB
MS	_	2	0.1	SP
DKW	2	_	0.1	DB
DKW	_	2	0.1	DP

Morphologic characteristics such as size of callus, length of shoots, number of branch in mm, length of branch in mm were evaluated in each subculture for two rootstocks. The experiments were repeated for 3 times for each treatment used and morphological data were analyzed by analysis of variance test (ANOVA) followed by least significant difference test (LSD).

RESULTS AND DISCUSSION

Comparison of Media culture

There is no universal medium for *in vitro* culture, since plant species and cultivars are genetically specific with regard to different components of the medium, which include not only organic substances, but also mineral elements (Saric *et al.*, 1995).

Size of callus

The friable and white of color callus appeared hyperhydric. The cells were highly vacuolated. This callus had some characteristics similar to that of a tumor or habituated organogenic callus as described by Gaspar (1995), and most cells were able to differentiate and reorganize into primary meristematic cells. However, shoot elongation, calli and branch formation were showed in media (SB, DB) and (SP, DP) with different basal media and equal cytokinins.

The M26 rootstock, large calli produced (1.07 mm) in SB medium and did not observe in DB medium, also the largest size of callus were (1.75 and 0.25 mm) in SP, DP media, respectively (Fig. 1a). The MM106 rootstock, calli (0.2 mm) formed in SB and DB media, but the largest size of callus were 0.58 mm and 0.7 mm, in SP and DP media, respectively. Results showed DKW medium was not suitable for production calli (Fig. 1b). The different forms of nitrogen in the culture media alter the endogenous levels of cell metabolites as well as of proteins, organic acids and plant hormones. Moreover, N concentration, its forms and their proportion may influence cell division, differentiation, growth and development of somatic embryos *in vitro* (Mordhorst and Lorz 1993).

Cut edge may provide a way for nutrients and plant growth regulators to be absorbed efficiently from the medium (Sarwar and Skirvin 1997). Moreover, wounding in general causes a stress-related response, which results in the production of a whole series of compounds that could also induce callusing and maybe even differentiation.

In all of media, the size of calli was observed smaller in M26 rootstock and larger in MM106 during subcultures.

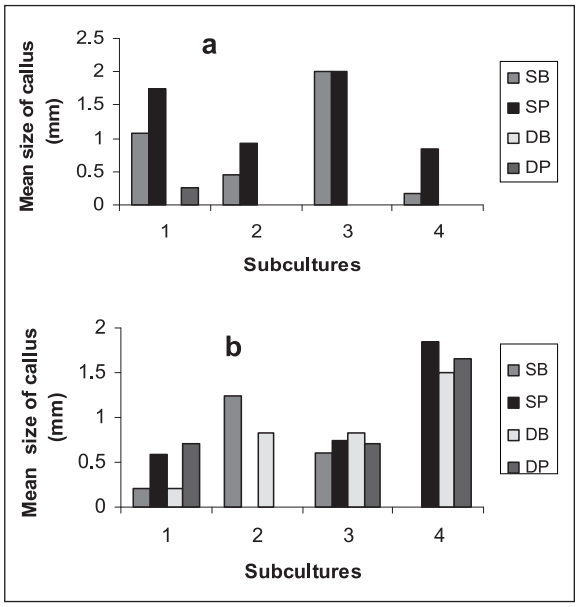

Fig. 1. a) Mean size of callus in M26; b) Mean of size of callus in MM106.

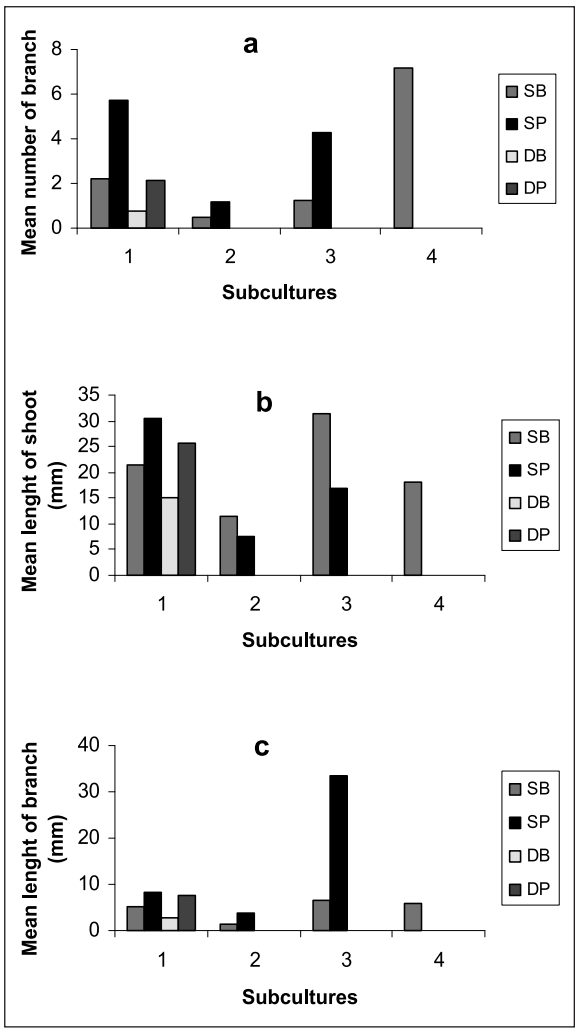

Fig. 2. a) Mean number of branch; b) Mean length of shoot; c) Mean length of branch; during subcultures in M26 rootstock

Length of shoot, number of branch and length of branch

The statistical analysis (ANOVA) of all data revealed that under the same conditions for growth regulators and the salt composition significantly ($P < 0.05$) affected shoot production and elongation. In M26 rootstock, MS medium was significantly ($P < 0.05$) better for propagation (7.1 shoots/explant) than DKW medium (2.1) (Fig. 2a), the best of mean height for shoots were in MS and DKW media respectively (31.2 and 25.7 mm respectively) (Fig. 2b). MS medium was significantly ($P < 0.05$) better for the mean height for branch with 33.4 and 7.4 mm respectively (Fig. 2c).

In MM106 rootstock, MS medium was significantly ($P < 0.05$) better for propagation (5.8 shoots/explant) than DKW (3.5) medium (Fig. 3a), the mean height of the shoots were (17.5 and 20.8 mm, respectively in MS and DKW media (Fig. 3b), also, MS medium was significantly ($P < 0.05$) better for the mean height of the branch, there were the best 6.7 and 19 mm, respectively (Fig. 3c).

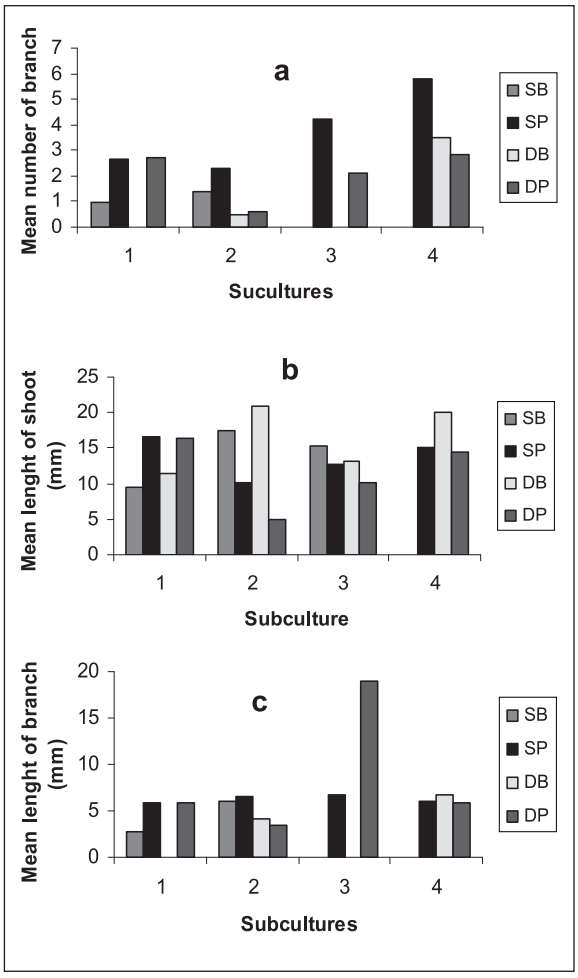

Fig. 3. a) Mean number of branch; b) Mean of length of shoot; c) Mean length of branch; during subcultures in MM106 rootstock.

Significant differences were observed for each genotype when the shoot proliferation rate or the mean shoot height was compared for the two different media. Nevertheless, for each genotype a best suited medium could be defined which enabled a good shoot proliferation rate in combination with a sufficient shoot height. Two standard formulations of macro- and micro- elements were tested to optimize the quality of shoots for shoot elongation and branching. The concentration of micro- and macro- elements salts may play an important role in micropropagation of woody plants (Andreu and Marin, 2005). The media differed mainly in nitrogen content: higher in MS (60mM) and lower in DKW (44mM).

With DKW medium a reduced shoot height was observed for most of the genotypes, possibly due to the low nitrogen content. On the contrary, the MS medium with the highest content of nitrogen yielded the highest proliferation rates and the best shoot growth for two genotypes. The MS medium commonly used for micropropagation of M26 and MM106rootstocks.

Two ionic forms of inorganic nitrogen, ammonium NH_4^+ and nitrate NO_3^- are generally included in tissue culture media. Standard media generally provide nitrogen in the form of a mixture of NO_3^- and NH_4^+. The level and form in which nitrogen is supplied to cultures not only influences the growth rate and metabolic activity of plantlets, but also interferes with cell morphology and tissue development (Hyndman *et al.,* 1982; Thorpe *et al.,* 1989). The form of inorganic nitrogen and ammonium nitrate ratio has been reported to greatly affect the growth and differentiation of cultured tissues (Grimes and Hodges 1990; Abu-Qaoud *et al.,* 1991; Niedz 1994; Matsubayashi and Sakagami 1998). For this reason, standard media may yield less than maximal growth in plant cell cultures if the tissue cannot cope with the level and form of nitrogen provided. It has been shown that MS medium (Murashige and Skoog 1962), routinely applied for fruit tree cultures, does not provide the best nutrition source for differentiating adventitious buds of pears (Chevreau *et al.,* 1989; Abu-Qaoud *et al.*, 1991; Leblay *et al.*, 1991) and apples (Fasolo *et al.*, 1989; Predieri and Fasolo Fabri Malavasi 1989; Yepes and Aldwinckle 1994). The most successful adventitious bud regeneration from leaf explants of sweet cherries was noted on media containing basalt salts of QL or DKW and WPM media mixed (Matt and Jehle 2005).

The MS medium has high level potassium than DKW medium, and three different forms potassium-salts are in MS medium. The potassium is not a direct substrate for biomass production., it has an important function in osmotic regulation of the cell, thus contolling the influx of other compounds, for example, it acts as counter ion of nitrate (Kopcewicz and Lewak 2002, Ramage and Williams 2002). As a consequence of potassium uptake, nitrate utilization increases, promoting growth. The promoting effect of potassium on number of regenerated buds was particularly pronounced on media with lower pH of 5.7. This could be ascribed to the stronger effect of potassium cations is more acidic medium, causing a more distinct shift in plasma lemma polarization, which in turn can result in higher nitrate uptake or could be alteration in water infiltration inside the tissues, as the potassium is characterized by high osmotic activity (Nowak *et al.*, 2007).

Comparison of hormones in Media culture

Several factors can influence the success of *in vitro* conditions during the regeneration process. Among phytohormones, cytokinins have proven to be the most important factor affecting shoot regeneration, and their significant effects may be related to the histological changes in induced tissues.

Size of callus

The calli formation, shoot and branch regeneration were observed in media (SB, SP) and (DB, DP) with the same basal media and different cytokinins. The friable and cream color callus appeared hyperhydric. In M26 rootstock, the largest size of calli (1.07 mm, 1.75 mm) in SB and SP media and the lowest in DB and DP media (0.25 and 0 mm).

In M106 rootstock, the largest as, size of calli were in SP (1.8 mm), DP (1.6 mm) than SB and DB media (0.2 and 0.8 mm, respectively).

Length of shoot, number of branch and length of branch

Morphogenesis in vitro can be manipulated by controlling *in vitro* conditions such as light, temperature, vessel humidity and osmotic potential via mineral nutrients, carbohydrates and plant growth regulator (cytokinins and Auxins) content of the medium. These conditions interact with intrinsic factors of explants (Hazarika 2006, Ziv and Chen 2008).

There are two main classes of cytokinins according to the chemical structure of the side chain: isoprenoid (e.g. 2-ip) and aromatic cytokinins (e.g. BA), which differ in their biochemistry, their receptors, biological activity and metabolism (Strnad *et al.*, 1997, Werbrouck *et al.*, 1996, van Staden *et al.*, 2008). In M26 rootstock, MS and DKW media with BA (2 mgl^{-1}) and as the same of basal media with 2-ip (2 mgl^{-1}) were significantly ($P < 0.05$) the best propagation (7.1 and 2.1 shoots/explant) respectively (Fig. 4a), the mean height of shoots

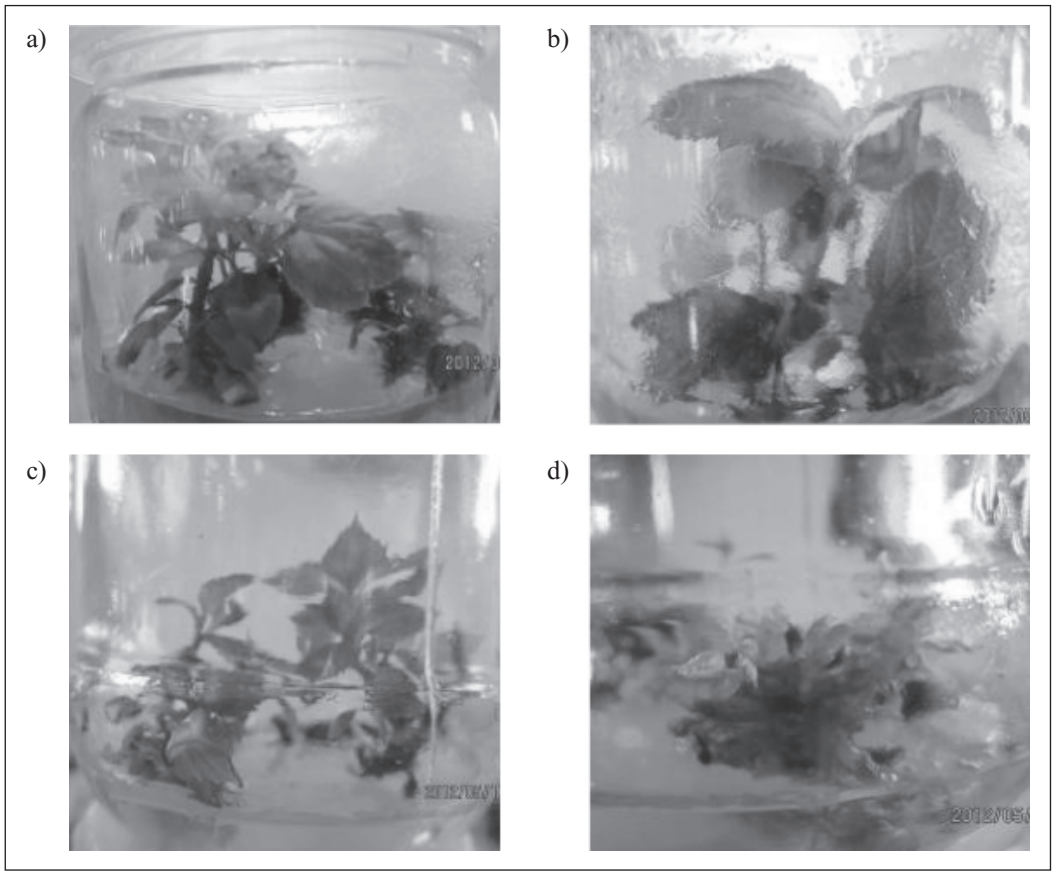

Fig. 4. Regeneration shoot in M26 rootstock: a) in MS medium with BA; b) DKW medium with BA; c) MS medium with 2-ip; d) DKW medium with 2-ip.

were 31.2 mm and 25.7 mm (Fig. 4b). The best of mean height of the branches were (6.4 and 33.4 mm (Table 2, 3).

In MM106 rootstock, MS and DKW media with BA (2 mgl^{-1}) and as the same of basal media with 2-ip (2 mgl^{-1}) were significantly ($P < 0.05$) the best propagation (5.8 and 2.8 shoots/explant) respectively (Fig. 5a), the mean height of shoots were 20.8 mm and 16.6 mm (Fig. 5b). The best mean height of the branches were (6.8 and 19 mm), respectively. (Table 4, 5).

Treatment of shoots in MS medium culture containing BA also significantly hastened the regeneration process and increased the number of shoots. The both of rootstocks, length of shoots were larger in Media culture with BA, but mean size of callus and branching were better in Media culture supplemented 2-ip cytokinin. Morphogenesis *in vitro* can be manipulated by controlling *in vitro* conditions such as light, temperature, vessel humidity and osmotic potential via mineral nutrients, carbohydrates and plant growth regulator (cytokinins and Auxins) content of the medium. These conditions interact with intrinsic factors of explants (Hazarika 2006, Ziv and Chen 2008).

Shoots from media culture containing BA was more elongation shoot and had smaller leaves with

Table 2. Representative mean difference test (LSD) for morphological characters among M26 rootstock treatments in the first subculture

Dependent Variable	(I) (M26)	(J) (M26)	Mean Difference (I-J)	Sig.
Size of callus	SB	SP	-.6786*	.015
		DB	1.0714*	.000
		DP	.8214*	.000
	SP	SB	.6786*	.015
		DB	1.7500*	.000
		DP	1.5000*	.000
	DB	SB	-1.0714*	.000
		SP	-1.7500*	.000
	DP	SB	-.8214*	.000
		SP	-1.5000*	.000
	SP	DB	15.3889*	.017
	DB	SP	-15.3889*	.017
		DP	-10.6389*	.017
	DP	DB	10.6389*	.017
Number of branch	SB	SP	-3.5357*	.005
	SP	SB	3.5357*	.005
		DB	4.9722*	.000
		DP	3.6250*	.003
	DB	SP	-4.9722*	.000
	DP	SP	-3.6250*	.003
Length of branch	DB	DP	-4.7160*	.038
	DP	DB	4.7160*	.038

* The mean difference is significant at the 0.05 level.

Table 3. Representative mean difference test (LSD) for morphological characters among M26 rootstock treatments in the second subculture

Dependent Variable	(I) (M26)	(J) (M26)	Mean Difference (I-J)	Sig.
Size of callus	SP	DB	.9259*	.047
		DP	.9259*	.047
	DB	SP	-.9259*	.047
	DP	SP	-.9259*	.047
Length of shoot	SB	DB	11.4500*	.027
		DP	11.4500*	.027
	DB	SB	-11.4500*	.027
	DP	SB	-11.4500*	.027
Number of branch	SB	DP	.5000	.461
Length of branch	SB	SP	-2.3620*	.042
	SP	SB	2.3620*	.042

* The mean difference is significant at the 0.05 level.

thick petioles. Not only did the presence of cytokinin affect the shoot regeneration capacity of apple explants but also its type and concentration in the medium culture. Moreover, an interaction could be observed between cytokinin content of treatment and regeneration media, especially

Table 4. Representative mean difference test (LSD) for morphological characters among MM106 rootstock treatments in the first subculture

Dependent Variable	(I) (MM106)	(J) (MM106)	Mean Difference (I-J)	Sig.
Size of callus	SB	DP	-.4917*	.039
	DP	SB	.4917*	.039
Length of shoot	SB	SP	-7.0833*	.010
		DP	-6.8167*	.019
	SP	SB	7.0833 *	.010
	DP	SB	6.8167*	.019
Number of branch	SB	SP	-1.7083*	.007
		DP	-1.7417*	.009
	SP	SB	1.7083*	.007
		DB	2.6667*	.005
	DB	SP	-2.6667*	.005
		DP	-2.7000*	.006
	DP	SB	1.7417*	.009
		DB	2.7000*	.006
Length of branch	SB	SP	-3.1333*	.016
		DP	-3.0800*	.026
	SP	SB	3.1333*	.016
		DB	5.9333*	.003
	DB	SP	-5.9333*	.003
		DP	-5.8800*	.004
	DP	SB	3.0800*	.026
		DB	5.8800*	.004

* The mean difference is significant at the 0.05 level.

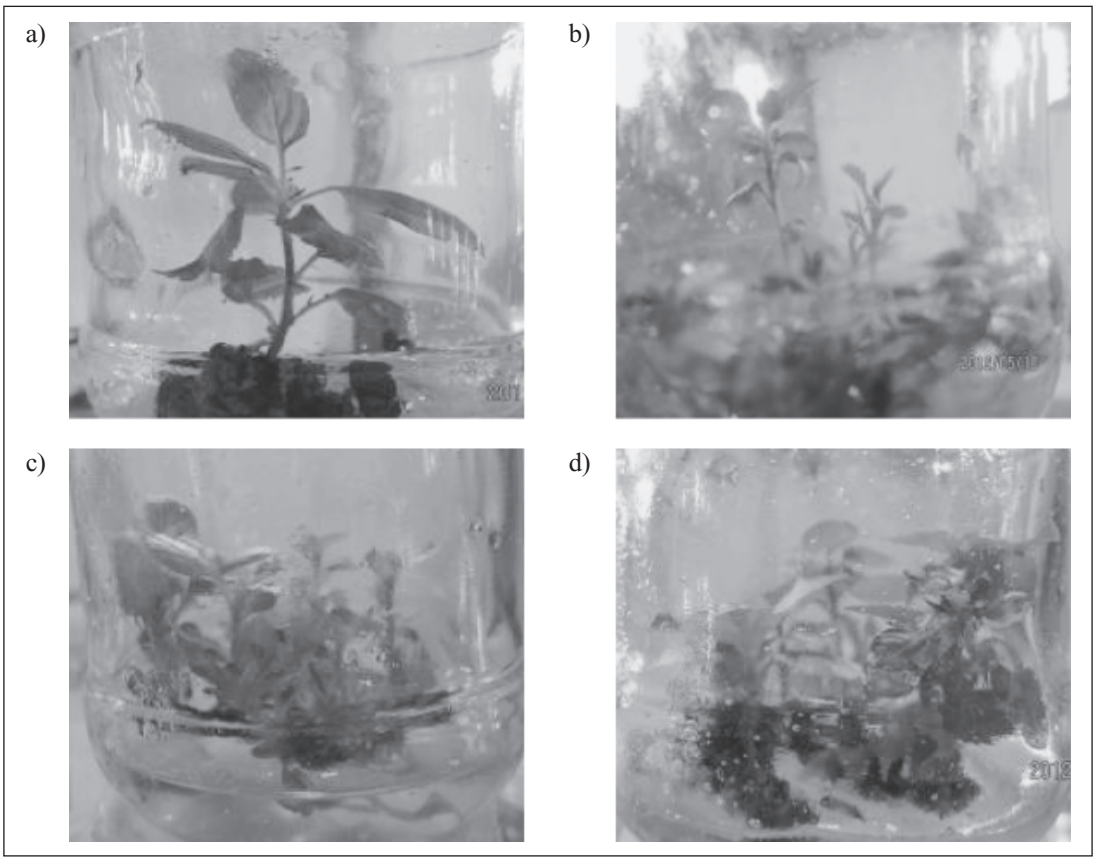

Fig. 5. Regeneration shoot in MM106 rootstock: a) in MS medium with BA; b) DKW medium with BA; c) MS medium with 2-ip; d) DKW medium with 2-ip.

Table 5. Representative mean difference test (LSD) for morphological characters among MM106 rootstock treatments in the second subculture

Dependent Variable	(I) (MM106)	(J) (MM106)	Mean Difference (I-J)	Sig.
Size of callus	SB	DP	1.2500*	.000
	SP	SB	-1.2500*	.000
		DB	-.8333*	.007
		SP	.8333*	.007
		DP	.8333*	.008
	DP	DB	-.8333*	.008
Length of shoot	SB	SP	7.2692*	.001
		DP	12.5833*	.000
	SP	SB	-7.2692*	.001
		DB	-10.6026*	.000
		DP	5.3141*	.005
	DB	SP	10.6026*	.000
		DP	15.9167*	.000
	DP	SP	-5.3141*	.005
		DB	-15.9167*	.000
Number of branch		DB	1.8077*	.002
	DB	SP	-1.8077*	.002
	DP	SP	-1.6410*	.001

* The mean difference is significant at the 0.05 level.

considering the physiological state of regenerated shoots. Since then, cytokinins have been shown to regulate a variety of biological activities in whole plants and in tissue cultures. They promote outgrowth of axillary buds, stimulate leaf expansion and suppress leaf senescence in whole plants (Mok 1994). BA is the most frequently used cytokinin in apple regeneration and it was compared in several studies. Theiler-Hedtrich and Theiler-Hedtrich (1990) observed that M9, Golden Delicious and Florina regenerated better on media with TDZ, while Priscilla, M26 and M27 showed best results on media with BA.

The 2-ip is an isoprenoidcytokinin that 2-ip localization plays a role in both phases of active cell division, one leading to callus formation and later on to the differentiation of vegetative buds (D'Angeli *et al.*, 2001, Magyar-Tabori *et al.*, 2010). The 2-ip hormone failed to promote stem elongation. Sarwar *et al.* (1998) showed that shoots did not proliferate or grow well beyond 20, 25 and 25 μM 2-ip for McIntosh, Macsppur and Wijcik, respectively. BA concentration can have various effects on regeneration of adventitious shoots depending on other factors. These include the use in combination with different levels of IBA. Fasolo *et al.* (1990) showed, when indole-3-butyric acid (IBA) was used as auxin, 'Gala' also needed a low level of BA (4.4 mM) for best regeneration.

CONCLUSIONS

According to Williams (1995), the in vitro chemical micro environment can be regarded as having three main phases, the medium, the atmosphere or head space and the plant material. Under in vitro conditions, plant growth depended on the mineral elements and organic components of the medium, due to a very low level of photosynthesis and small leaf area of the plants. Hence, the choice of mineral and organic components is very important (Lumsden *et al.*, 1990). For apple, both the amount and type of cytokinin has proved to be the one of the most important factors during the regeneration phase (Fasolo and Predieri 1990; Dufour 1990; Yepes and Aldwinckle 1994; Dobra´nszki *et al.,* 2002). The optimal type and level of cytokinins is largely dependent on genotype, and interactions between genotype and cytokinin (BA) concentration have also proved to be significant (Yepes and Aldwinckle 1994). The effectiveness of natural and synthetic cytokinins is different and endogenous BA is one of the most frequently used in regeneration. Applying 2-ip as a cytokinin-like substance can also be very effective, especially in the case of recalcitrant genotypes, but 2-ip can have undesirable side effects such as hyperhydricity or dwarfing.

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