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Research rate of *in vitro* proliferation malling apple (*Malus domestica*) rootstocks cv. M26 and cv. MM106

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Abstract

Apple belong to Malus Miller genus. Today, more of 10000 varieties known in the world, some of varieties are economic , resistance to diseases and sexual proliferation of ability. The vegetative rootstock (M26 and MM106) are early harvesting, yield is high and effect on quality of fruits. The subject of project is research kinds vegetative rootstock of varieties and number of subculture on rate of *in vitro* proliferation. The basal medium culture MS supplemented with BAP (2 mg/l) and IAA (0.5 mg/l) have prepared. In M26 rootstock, length of stem (1.11 cm) increased during the second and third subcuktures. In MM.106 and M26, maximum number of leaves (18.7 and 9.2) produced in two subculture and such as, length of stem elongated during the first subculture. The length of shoot and number of leaves increased with repetitive subcultures but on effected on branching.

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Introduction

Micro-propagation has been used in research laboratories as a research tool in genetic, gardening and phytology for many years. Many studies have been performed about apple reproduction through somatic tissues and organogenesis depends on factors such as genotype, size, type and age of explant, but one of the most important factors is the amount and type of cytokinin. Micro-propagation of apple results in plants lacking microbes, and the plant could be produced at any time of year(Dobranszki &Silva). Apples are popular fruits, not only for their good taste, but also as a suitable snack. Since apple has a long adolescence period, so it has been tried to reproduce this fruit through asexual ways, therefore reedy stalks production is essential in asexual methods(Gupta *etal,*2011). Many factors are involved in asexual reproduction, but the role of cytokinin hormone is demonstrated(Bairu *etal2007).* In 2005, in a research done on M. 26 cultivar, plant's response to different concentrations of BA was investigated. The highest shooting was obtained for M. 26 with BA = 5mm(lane & Mc,1992).Scientits used leaf segments of MM. 106 for stalk generation and they observed that the leaves being near the top of stalk have more ability for reproduction(Modgil *et al*,2006). Different stages of micro-*propagation such as stalk propagation, stalk elongation, root generation and stalk* propagation and even reproduction are related to treatment mixture (Zimmerman and Karp, 1991; Debergh, 2005; Zhu *et al,* 1995). A certain micro-propagation method in a certain treatment could not be applied for other genotypes (George and Debergh, 2008). Different effects of BA would be obtained during propagation of branches of apple in order to increase the branches and prevent side effects of BA such as reduction and difficulty of later root generation (Jones and Webster, 1991) or toxicity after several cultivations (Warner and Boe, 1980). Better reproduction in MM. 106 could be obtained when BA is applied in 0.5 to 1 µm dose (De Klerk *et al.,* 2001). The best value for growth regulators in first phase of tissue culture depends on genotype (Ziv and Halevy, 1983; Wang *et al*, 1991; Webster and Jones, 1994 and Hofer, 1997). The purpose of present project is optimizing the treatment or culture environment for micro- propagation of different genotypes of apple. Different mixtures of BA, TDZ and auxines (IBA, NAA, 2, 4-D) and GA3 were investigated in propagation and reproduction of branches length and formation of roots and callus. Results indicated that reproduction speed of different bases of apple is significantly related to cytokinins. MM. 106 compared to M. 27 produced more branches. The highest speed of growth (2.86) was observed in MM. 106. All factors (shoot regeneration, length of branches, leaf regeneration, and root production) of vegetative bases of apple are significantly under the influence of culture mixture. This issue was confirmed by other researchers (Lane and Mc.Douglad, 1982; Caboni and Tonelli, 1999; Kovalchuk *et al*, 2009). In most works done on apple branches, BA was applied as uxine source in the range of 0.5 to 0.2 mg/l (Baraldi *et al*, 1991; Caboni *et al*, 1994; Yepes and Aldwinckle, 1993; Marin *et al*, 2001; De Klerk *et al*, 2000; Kausal *et al;* Sharma *et al*, 2005). As our results offer, regeneration of apple branches was based on treatments containing cytokinins especially BA in the treatment, although the speed of genotypes propagation in their response to BA in treatment was different (Yepas and Aldwinkle, 1994).

In this research we studied some morphological characteristics of two cultivars of apple(M.26 &MM.106) on BAP treatment during three vegetative period.The main aim of this study was to develop an efficient protocol about micropropagation of these two cultivars.In fact we decided to improve lateral shoot formation (height of trees,number and length of lateral shoot)under BAP treatment.

Materials and methods

Plant materias ,chemicals

In this research we prepared strilized explants of cvM.26 and cv MM.106 from agriculture research institute in karaj city.all routin chemicals were prepared by lab stuff .chemical materials had been purchased from Sigma.

Medium prepration

Apple rootstocks of M.26 and MM.106 were mentained on MS(Murashige&skoog,1962)medium consisting of macro and micro elements and supplemented with MS vitamins, different hormones such as IBA,BAP,7.5 g l-1 agar and 30 g l-1 sucrose .The stock solution of all ingredients were stored under refrigeration.

Culture conditions

The pH of media was adjusted to 5.8 before autoclaving at 121°C for 40min.All cultures were incubated under flourscent lights with 16 h lightness and the temperature was 25+_2.

Expriment

First of all,steril explants of two cultivars were cultured on MS treatment with BAP=2mg/l ,IBA=0.1mg/l with 3 replications per treatments and 5 explant(shoot)per replication.every treatment was cultured for 30days .cultures were prepared in laminar air flow aseptically ,after prepration ,cultures were taken into incubator and after regeneration, morphological characteristics (such as the length and number of stems and roots, and color and number leaves) of Maling 26 and 106 was studied during three growth periods(30 days).

Statistical analysis

Analysis of the data was carried out by using Analysis of Variance (ANOVA) technique, SPSS and means were compared by using Least Significance Difference (LSD) Test at 5%probability level (Steel *et al.*, 1997).

Results

Investigating morphological characteristics in two base cultivars MM.106&M.26 during three sub-cultures.

Table 1. Average of length and number of stem and number of leaf in M.26 in first subculture.

Mean	1.5000	2.4167	15.0000
Std.Error of Mean	0.13762	0.37856	2.87360
Std.Deviation	0.47673	1.31137	9.95444
variance	0.227	1.720	99.091
Statistical analysis	Lenght of stem of MM.106 first subcalture	Number of stem of MM.106 first subcalture	Number of leaves of MM.106 first subcalture

Investigating length and number of stems and number of leaves in MM. 106.

The maximum average of length of stem was 1.5cm .It was observed in first subcutureThe maximum number of stem was obtained in first subculture.The average of number of leaves in this cultivar in second subculture was 18.7.It was maximum between three subcultures. (Graph2).

Table 2. Average of length and number of stem and number of leaf in MM.106 in second subculture

Statistical analysis	Lenght of stem of MM.106 Second subcalture	Number of stem of MM.106 Second subcalture	Number of leaves of MM.106 Second subcalture
Mean	1.2500	2.2917	18.7083
Std.Error of Mean	0.0793	.0.20394	1.50420
Std.Deviation	0.39009	0.99909	7.36903
variance	.152	0.998	54.303

Investigating length and number of stems and number of leaves in M. 26 between three subcultures In M.26 in first subculture.the average of length of stem was 1.15cm and the number of stem 2.37 and 7.75 for number of leaves.(table 4)The maximum length and number of stem was obtained in first subculture but the third subculture had first grade in view of number of leaves.

Table 3. Average of length and number of stem and number of leaf in MM.106 in third subculturStatistics MM106-sub3.

Statistical analysis	Lenght of stem of MM.106 third subcalture	Number of stem of MM.106 third subcalture	Number of leaves of MM.106 third subcalture
Mean	.9771	1.8571	14.7714
Std.Error of Mean	0.08200	0.14286	1.00091
Std.Deviation	0.48512	0.84515	5.92147
variance	.235	.714	35064

Comparision between morphological characteristics of M.26 and MM.106.

In investigating morphological characteristics of seedlings of malyng base, cultivar M. 26 and cultivar MM. 106 during sub-cultures, the length of stem and number of stems were reduced. The number of leaves in cultivar M.26 during third sub-culture and in cultivar MM. 106 during second sub-culture increased.

Table 4. Average of length and number of stem and number of leaf in M.26 in first subculture.

Statistical analysis	Lenght of stem of M.26 first subcalture	Number of stem of M.26 first subcalture	Number of leaves of M.26 first subcalture
Mean	1.1500	2.3750.	7.7500
Std.Error of Mean	0.10856	.37500	0.52610
Std.Deviation	0.30706	1.06066	1.48805
variance	0.094	1.1250	2.214

Based on decomposition table, mean variance of stem number, stem length and leaves number among malyng apple bases of two cultivars M.26 & MM. 106 and during sub-cultures in probability level less than 0.05 was significant. The mean length of stem according to Duncan's grouping indicated that malyng base of M. 26 cultivar in second and third sub-culture and cultivar MM. 106 in first sub-culture in probability level of 0.05 was significant. Lateral buds were cultivated in MS treatment with BAP (2 mg/l) and IBA (0.1 mg/l) hormones. Morphological characteristics of apple seedlings, malyng base, M. 26 cultivar were studied in sub-cultures in growth room under 25°C and 16.8 photo-period. Comparing stem length, number of stems and number of leaves in two base cultivars M. 26 & MM. 106 during three sub-cultures. Length of stem in M. 26 and MM.106 bases in second sub-culture increases and it was reduced in third sub-culture (diagram 3). Number of stems in M. 26 base in second sub-culture reduced and it was increased in third sub-culture; it was gradually reduced in MM. 106 base (diagram 4).Number of leaves in M. 26 base increased and it was increased in MM. 106 base in second sub-culture (diagram 5).

Disussion

Apple is an important fruit tree in commerce and food industry planted in different parts of the world. Iran is one of the main producers and exporters of apple in the world and its local and foreign cultivars are planted in suitable locations. Since modification and production of new cultivars by hybridization is time consuming due to long seedling period of apple trees, high self-incompatibility level and high heterozygotisity of genome, for obtaining new cultivars, lateral stems regeneration by new biotechnical methods such as production of somatic embryos and artificial seeds are suggested.

Table 5. Average of length and number of stem and number of leaf in M.26 in second subculture.

Statistical analysis	Lenght of stem of M.26 Second subcalture	Number of stem of M.26 Second subcalture	Number of leaves of M.26 Second subcalture
Mean	1.0500	1.5000	7.2000
Std.Error of Mean	0.15723	.16667	1.05198
Std.Deviation	0.49721	.52705	3.32666
variance	0.247	.278	11.067

Table 6. Average of length and number of stem and number of leaf in M.26 in third subculture.

Statistical analysis	Lenght of stem of M.26 Second subcalture	Number of stem of M.26 Second subcalture	Number of leaves of M.26 Second subcalture
Mean	0.6846	1.5714	10.8333
Std.Error of Mean	0.11813	.20203	1.87420
Std.Deviation	0.42592	.75593	6.49242
variance	0.181	.571	42.152

In present research, we used two apple base cultivars, M. 26 and MM. 106 in MS treatment with two hormones, IBA + BAP in order to regenerate and produce embryonic callus for producing artificial seeds. In apple base, M. 26 and MM. 106 cultivars in first sub-culture, the length of stems were longer and more number of branches and leaves were formed. Gradually, during sub-cultures, the stems became shorter and number of branches and leaves reduced that could be attributed to weakening of apple bases in in -vitro environment.

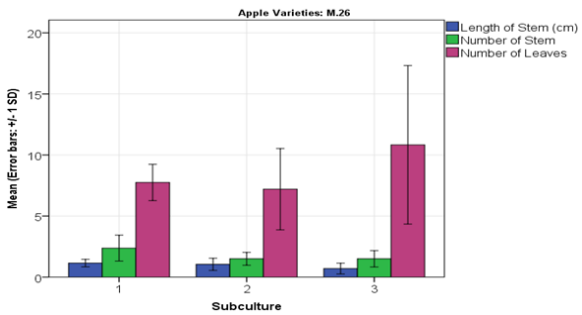

Fig. 1. Average of length and number of stem and number of leaf in M.26 in three subculture.

Many cultivars produce few lateral shoots because of apical dominance (Cline *etal* 2000).A balance between auxin and cytikinin level is effective in formation of lateral shoots(Elfing *etal,* 1984).cytokinins such as BA and BAP have been used overcome apical dominance and development of leteral branches.(Jaumien *etal*,2002) Cytokinin regulates shoot proliferation ,cell division and differentiation(Gross *etal*,1994).BAP is the most used cytikinin in micropropagation (Bairu *etal*,2007).

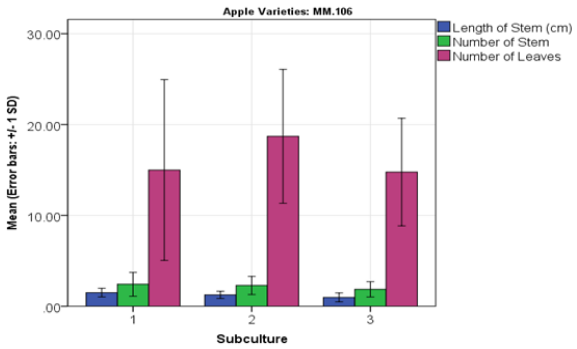

Fig. 2. Average of length and number of stem and number of leaf in MM.106 in three subculture.

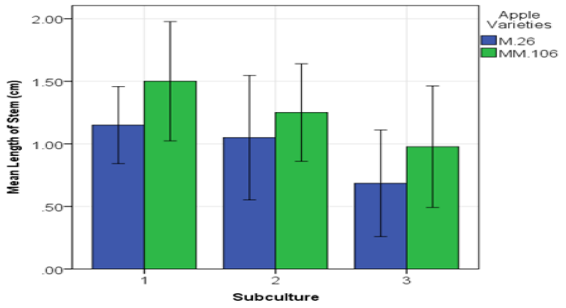

Fig. 3. Comparison between length of stem of two apple cultivar MM.106,M.26 during 3 subculture.

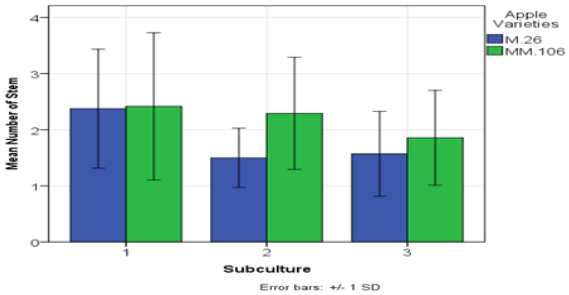

Fig. 4. Comparison between number of stem of two apple cultivar MM.106,M.26 during 3 subculture.

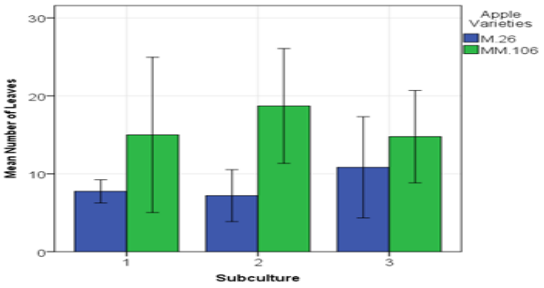

Fig. 5. Comparison between number of leaf of two apple cultivar MM.106,M.26 during 3 subculture.

Hazarika *et al* (2006) and Ziv *et al* (2008) reported that morphogenesis under in vitro conditions could be manipulated by controlling environmental conditions including the light, temperature, moisture of dish and adjustment of osmotic potential through minerals, carbohydrates and hormones of treatment. These conditions impact internal factors of explant. M. 26 and MM. 106 apple bases are semi-short and they have high commercial importance and they are commonly micro-propagated in vitro in many countries (Sotiropoulos *et al,* 2005). Jones *et al*, (1979) reported propagation of 5 hybrid apple cultivars through tissue cultivation. The cultures propagated every three weeks by five times and it is important since it facilitates availability of new cultivars obtained through modification programs. Moreover, propagation of these cultivars through imping is difficult and or they need intense care and many instruments. Erez and Snir (1980) reported propagation of Merton malyng bases (MM. 109, MM. 106, MM. 104) through tissue cultivation. Grimes *et al.* (1990) reports that the form of mineral nitrogen and ratio of ammonium to nitrate is effective in the growth and differentiation of cultivates tissues and MS treatment was applied as standard treatment in cultivating fruit trees tissues and apple tree, but it did not provide maximum growth in cultivation of plant cells which is due to the amount and form of nitrogen. Fasolo *et al* (1990), Yepez *et al* (1994) and Dobranszki *et al* (2002) reported that the amount and type of cytokinins is very important before and after regenerative stage. Suitable type and level of cytokinin depends on the genotype and interactions between genotype and amount of cytokinin (Yepez *et al*, 1994).

Effects of natural cytokinins and synthetic are different. BAP is cytokinin of endogen that is highly important in regeneration. BAP hormone has been commonly used in regeneration and micro-propagation of apple bases and it has various consequences such as: hard rooting, toxicity, hydrated tissues, aerial organs stop growing and callus is not formed. In 1967, Jones selected his micro- samplefrom meristem of the top of the branch of M. 26 base. He investigated the effect of benzyl adenine (BA) on branch shooting. Jacoboni and Standardi (1982) devised the protocol of apple vegetative buds cultivation and regeneration for malyng bases in MS treatment together with BAP and IBA hormones. Propagation of apple bases from lateral buds was high and regeneration rate was also high that was consistent to the results obtained by Famiani *et al.* (1994) who reported that lateral buds propagation is higher than micro-propagation. Werbrouck *et al* (1995) reported that cytokinin BAP is chemically stable but its biological activity is low due to its attachment to alanine or glycosylation. This stability has made it to be used in tissue cultivation systems. Aldwinckle *et al.* (1994) reported that optimal amount of cytokinin hormones is different in

regeneration of stems in terms of genotypes and different genotypes of apple shoe different biologic responses. M. 26 genotype with 22.2 micromullar BAP (Famiani *et al.*, 1994) showed optimum regeneration.

Conclusion

Since the maximum of number of leaf in two cultivars was produced during second subculture and these cultivars produced longer branches in first subculture.in view of number of stem two cultivars had more branches in second and third subculture .we conclueded type of bases and number of subcultures were effective on rate of growth of malling apple.Magor-Tabori *et al* (2010)reported cytokinin is very important in cell division and cytokinin can increases number of stems very well ,on the other hand we must not forget the rol of genotype in morphological changes.

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