

Full Length Research Paper

# Optimization of medium composition for apple rootstocks

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**Impact of medium composition (plant growth regulators, mineral nutrients) on multiplication rate, shoot elongation, callusing and rooting of apple rootstocks ('M9', 'M27', and 'MM106') cultured on gelled basal Murashige and Skoog (MS) medium were investigated. Multiplication rate was mainly dependent upon kind of plant growth regulators especially, 6-benzylaminopurine (BA), mineral concentration and genotypes. The best shoot production in terms of shoot number and shoot quality was obtained using 4.4  $\mu\text{M}$  BA and 2.27  $\mu\text{M}$  thidiazuron (TDZ) during the shoot multiplication phase, but 8.8  $\mu\text{M}$  BA + 1.14  $\mu\text{M}$  TDZ and 2.8  $\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ) during the shoot elongation phase for all genotypes. Application of high (2.8  $\mu\text{M}$ ) concentration of  $\text{GA}_3$  increased the elongation of adventitious shoots than low concentrations. The highest multiplication rate (5.7 No./shoot) and the highest amount of total fresh weight (2.25 g/jar), as growth rate, were produced by applying 4.4  $\mu\text{M}$  BA + 2.27  $\mu\text{M}$  TDZ for 'M27' genotype. Micropropagation potential of 'M27' genotype was higher than other genotypes. 'MM106' genotype had the lowest multiplication rate (0.7 No./month), when 0  $\mu\text{M}$  BA+9.08  $\mu\text{M}$  TDZ was applied. Multiplication of explants from the 1st subculture was more sensitive to BA than that from the 3th or 4th subculture. The rooting of explants was promoted by indole-3-butyric acid (IBA) significantly and the best result for rooting was achieved in the half-strength MS medium containing 5.4  $\mu\text{M}$  IBA and 1.2  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2, 4-D). The highest percentage (64%) rooting was produced for 'MM106' genotype and the lowest (11%) for 'M9' after 3 months. Root formation was increased with decreasing concentrations in cytokinins, but increasing auxins (IBA). Rooting percentage of shoot cultures in the low 1/2X-MS medium was significantly more than shoot cultures in the high 2X-MS medium.**

**Key words:** Apple rootstocks, medium composition, multiplication rate, plant growth regulators (PGRs).

## INTRODUCTION

Micropropagation of apple rootstocks depends upon new areas of research and fruit tree propagation allowing the problems of conventional methods to be overcome and enabling rapid multiplication of disease-free fruit plants at

a commercial scale. Different stages of apple micropropagation such as shoot multiplication, shoot elongation, rooting of microshoots, acclimatization and even regeneration were related to medium composition (Zimmerman and Debergh, 1991; Karp, 1995; Zhu et al., 2005). A micropropagation procedure developed for an apple genotype on a specific medium could not always be extrapolated with the same success for another genotype (George and Debergh, 2008). Successes are commonly found between cultivars, mainly in the rate of multiplication or in the demand for the composition of medium (Modgil et al., 1999; Dobránszki et al., 2005). Ciccotti et al. (2008, 2009) compared the effect of MS (Murashige and Skoog, 1962), QL (Quorin and Lepoivre, 1977), WPM (Lloyd and McCown, 1981) and DKW (Driver and Kinyuki, 1984) media on the shoot multiplication in *Magnolia sieboldii* and 10 *M. sieboldii*

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**Abbreviations:** 2,4,-D, 2,4-Dichlorophenoxy-acetic acid; BA, 6-benzylaminopurine;  $\text{GA}_3$ , gibberellic acid; IBA, indolebutyric acid; NAA,  $\alpha$ -naphthalene acetic acid; NOA,  $\gamma$ -naphthoxy-acetic acid; PGRs, plant growth regulators; TDZ, thidiazuron; IAA, indoleacetic acid; MS, Murashige and Skoog; QL, Quorin and Lepoivre; DKW, Driver and Kinyuki; WPM, woody plant medium.

derived hybrid genotypes. A very strong genotype-dependence was detected regarding the mineral composition of the best propagation media; the level of minerals in the MS medium obtained the best propagation, with multiplication rates 3.3 in *M. sieboldii* and 5.7 in 'C1907'. However, in the case of other two genotypes ('D2212', 'H0801'), QL salts led to significantly better multiplication rates (3.3 and 3.9) and in two others. Apparently, the formulation of mineral composition of media has been largely an empirical process. Even the most important work of MS medium was also empirical and was developed to optimise the mineral composition of the medium for growth of tobacco callus. A subsequent development such as Woody Plant Medium (WPM) (Lloyd and McCown, 1981) was based on this previous process.

6-Benzylaminopurine (BA) and thidiazuron (TDZ) are the most frequently used cytokinins in apple shoot multiplication and they were compared in several studies (Yepes and Aldwinckle, 1994). The effects of different BA-derivates were studied during shoot multiplication of apple in order to increase the efficacy of shoot multiplication and avoid the side effects of BA, such as difficulties in subsequent rooting (Webster and Jones, 1991), or toxicity after several subcultures (Werner and Boe, 1980). Better multiplication could be achieved in 'MM106' when BA was used at 0.5-1.0  $\mu\text{M}$  (De Klerk et al., 2001). The optimal plant growth regulator (BA) concentration in the first phase of culture initiation depends on genotype (Ziv and Halevy, 1983; Webster and Jones, 1991; Wang et al., 1994; Hofer 1997). For example, BA at 22.0  $\mu\text{M}$  was found optimal to obtain shoot multiplication of 'M26' (Welander, 1989), 'Jork9' (Pawlicki and Welander, 1994), 'MM106' (Modgil et al., 2005), while the optimal range of BA was from 22.2 to 44.4  $\mu\text{M}$  in 'Florina' (Bartish and Korkhovoi, 1997). Furthermore, some workers reported that the optimum BA concentration for apple rootstock multiplication is less than 22.0  $\mu\text{M}$ . For example, the optimum concentrations of BA for efficient multiplication rate for 'Toscan', 'M9', and 'M25' was 8.8  $\mu\text{M}$  (Chakrabarty et al., 2003) for 'Marubakaido' was 11.0  $\mu\text{M}$  (Camargo et al., 1998; Schaefer et al., 2002); for 'Malling Greensleaves' (54% regeneration rate and 1.8 shoots/explants) was 8.8  $\mu\text{M}$ , while 'M26' required a higher concentration (22.0  $\mu\text{M}$ ) for a similar regeneration rate (Ongjanov, 1988). A higher concentration of BA (31.1  $\mu\text{M}$ ) proved to be optimal for 'Golden Delicious' and 'Empire' (Yepes and Aldwinckle, 1994). Similarly, a high BA concentration (22.2  $\mu\text{M}$ ) in regeneration media produced a higher percentage of regenerating leaves in 'M26' rootstock compared to those cultured on media with 11.1  $\mu\text{M}$  BA, but the number and length of shoots were not affected (Famiani et al., 1994).

The efficacy of different types of auxin such as indolebutyric acid (IBA), indoleacetic acid (IAA), and  $\alpha$ -naphthalene acetic acid (NAA) was investigated on rooting of nine apple cultivars by Zimmerman and Fordham (1985). In 'Royal Red Delicious', 'Gala', 'Golden Delicious' and

'Spur McIntosh', there were no differences in the effects of different auxins. IBA significantly induced the most rooting in 'Delicious', 'Redspur Delicious', 'McIntosh', 'Mutsu' and 'Spartan'. Welander (1985) examined the rooting potential of 'Akeru' under different IBA concentrations (0.5-10.0  $\mu\text{M}$ ) and found 2.5  $\mu\text{M}$  IBA to be optimal (up to 100% rooting). In three cultivars ('Delicious', 'McIntosh', 'Spartan'), NAA resulted in better rooting than IAA. When root development was induced in 'Liberty' by the addition of IBA or NAA (0.1 mg/l to 1.0 mg/l), NAA induced callus formation at the base of shoots even at a low concentration (0.3 mg/l) and poor root development was observed (Yepes and Aldwinckle, 1994). The effect of NAA on rooting was studied at a concentration range between 0.1 to 33  $\mu\text{M}$  for 21 days in 'M.27', 'M.26', 'MM.111', 'M.9' and 'Macspur'. Rooting responses of each genotype were different (Lane and McDougald, 1982). Some others used  $\gamma$ -naphthoxy-acetic acid (NOA) at (0.4 mg/l to 5.5 mg/l) for rooting of several apple cultivars. The results were completely different (James and Thurbon, 1979, 1981; Zimmerman and Fordham, 1985; Webster and Jones, 1989; Modgil et al., 1999; Sharma et al., 2000). Rooting of 'Granny Smith' was investigated by Sriskandarajah and Mullins (1981) using different auxins, such as IBA, NOA, and 2,4-dichlorophenoxy-acetic acid (2,4-D) at 0-40  $\mu\text{M}$ . 2,4-D inhibited rooting even at low concentrations but 59-69% rooting was achieved by the application of 10  $\mu\text{M}$  NOA and IBA. There was an interaction between the NOA and IBA content of the medium (James and Thurbon, 1979).

The aim of this project is to optimize medium composition for micropropagation of different apple genotypes ('M9', 'M27', 'MM106'). The combination of different concentrations of plant growth regulators including cytokinins (BA, TDZ), auxins (IBA, NAA, 2, 4-D) and  $\text{GA}_3$  in the multiplication and elongation phases, and different concentration of mineral concentration with auxin in the root formation and callusing were tested.

## MATERIALS AND METHODS

### Plant materials

One year-old apple rootstocks ('M9', 'M27' and 'MM11'), which were kept in greenhouse, were used as source materials. All plant materials were surface-sterilized in 75% ethanol for 30 s, and rinsed three times with sterile distilled water. Subsequently they were immersed in a 0.1%  $\text{HgCl}_2$  solution containing 2-3 drops of Tween-20 for 6 min and rinsed five times in sterile distilled water. The sterilized buds were placed in the cultural medium using node (single or multiple) in early spring for culture. The subculture medium consisted of MS medium supplemented with PGRs (2.0  $\mu\text{M}$  BA and 1.50  $\mu\text{M}$  IBA). Subculture was performed every 30 days on the same medium.

### Culture media and culture conditions

Four experiments were carried out in this study, separately. The first experiment aimed at improving shoot multiplication by using different combinations of growth regulators BA, TDZ, IBA, NAA,

**Table 1.** Culture media and their compositions used during shoot proliferation and adventitious shoot regeneration.

| Item                     | Mult.-M ( $\mu\text{M}$ ) <sup>a</sup> | Elon.-M ( $\mu\text{M}$ ) <sup>b</sup> | Rooting-M ( $\mu\text{M}$ )   | Callusing-M ( $\mu\text{M}$ ) |
|--------------------------|--|--|-------------------------------|-------------------------------|
| Basic medium             | 1/2MS, MS, 2MS                         | 1/2MS, MS, 2MS                         | 1/2MS, MS, 2MS                | 1/2MS, MS, 2MS                |
| <b>PGRs<sup>c</sup>:</b> |  |  |                               |                               |
| BA                       | 0, 2.2, 4.4, 8.8                       | 2.2                                    | 0                             | 4.4                           |
| TDZ                      | 1.14, 2.27, 4.5, 9.08                  | 0                                      | 0                             | 4.5                           |
| IBA                      | 0, 0.5, 1.0, 1.5                       | 0.5                                    | 4.9                           | 0                             |
| NAA                      | 0, 0.66, 1.22, 1.88                    | 0                                      | 2.7                           | 0                             |
| 2, 4-D                   | 0, 0.45, 0.90, 1.35                    | 0                                      | 0                             | 0                             |
| GA <sub>3</sub>          | 0, 1.40                                | 2.8                                    | 0                             | 0                             |
| Agar                     | 7000 (mg L <sup>-1</sup> )             | 7000 (mg L <sup>-1</sup> )             | 6500 (mg L <sup>-1</sup> )    | 6500 (mg L <sup>-1</sup> )    |
| Sucrose                  | 30000 (mg L <sup>-1</sup> )            | 30000 (mg L <sup>-1</sup> )            | 30000 (mg L <sup>-1</sup> )   | 30000 (mg L <sup>-1</sup> )   |
| Activated charcoal       | 0                                      | 0                                      | 10 – 20 (mg L <sup>-1</sup> ) | 0                             |

<sup>a</sup>Mineral concentrations: 1/2X (low), 1X (equal), 2X (double) basal MS-medium; <sup>b</sup>elongation medium; <sup>c</sup>BA (6-Benzylaminopurine); TDZ (thidiazuron); IBA (indolebutyric acid); NAA (a-Naphtalene acetic acid); 2,4,-D (2,4-dichlorophenoxy-acetic acid); GA<sub>3</sub> (gebblerlic acid).

and 2,4,-D on different mineral concentration (1/2X, 1X, and 2X) (Murashige and Skoog, 1962) (Table 1). Based on experiment 1, the second was conducted which aimed at optimising concentrations of growth regulators (especially GA<sub>3</sub>) for shoot elongation (Table 1). In the third experiment, the interaction of growth regulators (cytokinins: BA, TDZ with auxins: IBA, NAA, and 2, 4,-D) shoots were cultured on different mineral concentration (1/2X, 1X, and 2X) of MS-medium on rooting. In the fourth experiment, influence of chemical medium composition on callusing on basal shoots was studied. The medium used contained Murashige and Skoog's medium salts, vitamins, 3% (w/v) sucrose and solidified with 0.65%/0.70% (w/v) agar (Solarbio, Beijing, China). All media were adjusted to pH 5.8±0.2 with 1 N KOH or 1 N HCl before autoclaving at 120°C, 1 atm for 20 min. The detailed treatments and conditions used in all experiments are presented in Table 1. All plant growth regulators were added before autoclaving. All cultures were kept in growth room at 25±2°C in the first 6-7 days and later under a 16/8 h (day/night) photoperiod with a light intensity of 50  $\mu\text{m}^{-2} \text{s}^{-1}$  provided by cool white fluorescent tubes.

### Shoot multiplication and elongation

Uniform explants from the 1st to 4th subculture about 2-3 cm high were separated, and then transferred to the multiplication medium. The details and conditions used in the multiplication medium experiment are presented in Table 1. Average of shoot multiplication rate (shoot explants<sup>-1</sup> month<sup>-1</sup>), total fresh weight (g jar<sup>-1</sup>) (as growth index), shoot elongation (mm), leaf production (No. explants<sup>-1</sup> month<sup>-1</sup>) were measured after 30 days.

### Root formation and callusing

The uniform isolated shoots with 2-4 cm long collected from subculture medium were harvested for each genotype ('M9', 'M27', 'MM106') and transferred to the three relative concentrations of minerals (X0.5, X1 and X2 of basal MS-medium) media. According to Table 1, each rooting medium was supplemented with combinations of auxins. Rooting percentage, number of roots, root length and callusing percentage were determined after 50 days of

treatment.

### Data collection and statistical analysis

Each experiment was conducted complete block design and 30 explants were considered for each treatment. The Statistical Analysis System (SAS) software program (SAS Institute Inc. 1999) was used for the analysis of variance (ANOVA) (statistical analysis) and least significant differences (LSD).

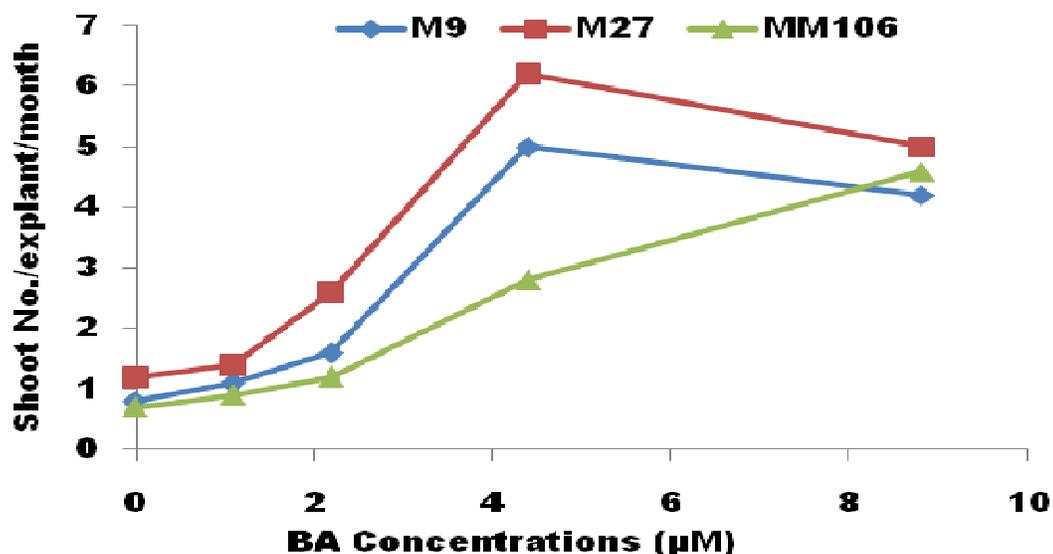
## RESULTS

The results revealed that the multiplication rate of apple rootstocks was significantly dependent on cytokinin (especially, BA) and mineral concentrations. Using combination of 4.4  $\mu\text{M}$  BA + 2.27  $\mu\text{M}$  TDZ with auxins (1.0, 1.22  $\mu\text{M}$  IBA + NAA) in the shoot multiplication medium resulted in a significantly greater multiplication rate (5.7 No./explants/month) than other treatments (Table 2). Without BA, multiplication rates were less than 1 No./explants in all treatments (Table 2). BA stimulated shoot multiplication in a concentration range of 2.2 up to 4.4, but increasing BA decreased multiplication rate (Figure 1). Applying high concentration of auxins (IBA, NAA, 2, 4-D) decreased multiplication and produced short shoot clumps, which were thick, and unfavorable (date not shown). On the other hand, increasing mineral concentration to 2X strength, multiplication rate increased. Different rootstock genotypes 'M9', 'M27', and 'MM106' had different potential of micropropagation (Figure 2). Comparison to 'MM106' genotype, 'M27' produced more number of shoots. The highest growth rate (2.86 g/jar FW) of plantlet was observed for 'MM106' rootstock which was grown on medium with 8.8  $\mu\text{M}$  TDZ plus 1.4  $\mu\text{M}$  BA. Application of higher concentration (2.8

**Table 2.** Effects of combination of PGRs on multiplication rate (No./month), shoot elongation (mm), total fresh weight (g/jar) and leaf production (No./explant) of apple rootstocks (M9, M27, MM106) cultured on basal MS medium.

| Genotype | Combination of PGRs ( $\mu\text{M}$ ) |      |      |      |       |                 | Multiplication rate <sup>a</sup> (No./month) | Shoot elong. <sup>b</sup> (mm) | T-FW <sup>c</sup> (g/jar) | Leaf prod. <sup>d</sup> (No./explant) |
|----------|---------------------------------------|------|------|------|-------|-----------------|--|--------------------------------|---------------------------|---------------------------------------|
|          | BA                                    | TDZ  | IBA  | NAA  | 2,4-D | GA <sub>3</sub> |  |                                |                           |                                       |
| 'M9'     | 0                                     | 9.08 | 0    | 0    | 1.35  | 0               | 0.8  | 9.5                            | 1.42                      | 1.3                                   |
|          | 2.2                                   | 4.54 | 0.50 | 0.66 | 0.90  | 0               | 1.4  | 13.9                           | 1.75                      | 1.5                                   |
|          | 4.4                                   | 2.27 | 1.0  | 1.22 | 0.45  | 1.4             | 4.9  | 15.5                           | 1.86                      | 2.2                                   |
|          | 8.8                                   | 1.14 | 1.5  | 1.88 | 0     | 2.8             | 4.5  | 17.8                           | 1.81                      | 1.9                                   |
| 'M27'    | 0                                     | 9.08 | 0    | 0    | 1.35  | 0               | 1.2  | 10.2                           | 1.56                      | 1.8                                   |
|          | 2.2                                   | 4.54 | 0.50 | 0.66 | 0.90  | 0               | 2.6  | 15.5                           | 1.75                      | 2.6                                   |
|          | 4.4                                   | 2.27 | 1.0  | 1.22 | 0.45  | 1.4             | 5.7  | 17.6                           | 2.25                      | 3.3                                   |
|          | 8.8                                   | 1.14 | 1.5  | 1.88 | 0     | 2.8             | 4.6  | 19.0                           | 1.68                      | 3.1                                   |
| 'MM106'  | 0                                     | 9.08 | 0    | 0    | 1.35  | 0               | 0.7  | 19.8                           | 1.52                      | 2.1                                   |
|          | 2.2                                   | 4.54 | 0.50 | 0.66 | 0.90  | 0               | 1.2  | 22.6                           | 1.87                      | 2.6                                   |
|          | 4.4                                   | 2.27 | 1.0  | 1.22 | 0.45  | 1.4             | 2.8  | 24.5                           | 1.62                      | 3.5                                   |
|          | 8.8                                   | 1.14 | 1.5  | 1.88 | 0     | 2.8             | 3.9  | 28.0                           | 2.86                      | 4.5                                   |
| LSD      | -                                     | -    | -    | -    | -     | -               | 0.72   | 2.65                           | 0.45                      | 0.65                                  |

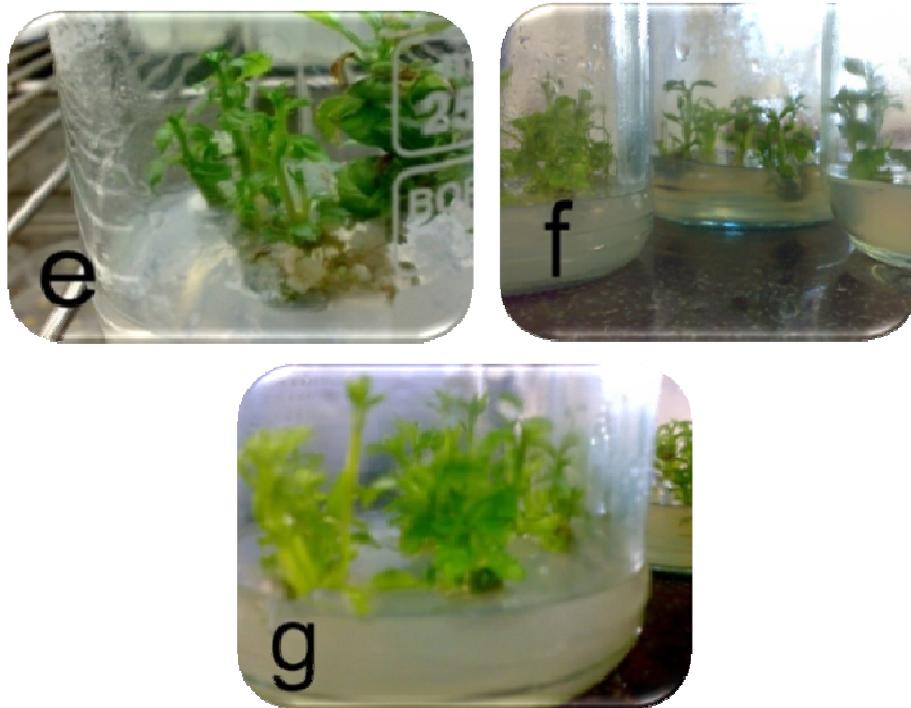
<sup>a</sup>Multiplication rate (explants/month); <sup>b</sup>mean of shoot elongation (mm); <sup>c</sup>total fresh weight(g/jar); <sup>d</sup>leaf production (No/explant). Data were collected after 6 weeks of culture on basal MS medium supplemented with the indicated growth regulators. Values are the means of at least three replicates.

**Figure 1.** Influence of BA concentrations on shoot production per explant of apple rootstocks ('M9', 'M27', 'MM106') cultured on basal MS medium.

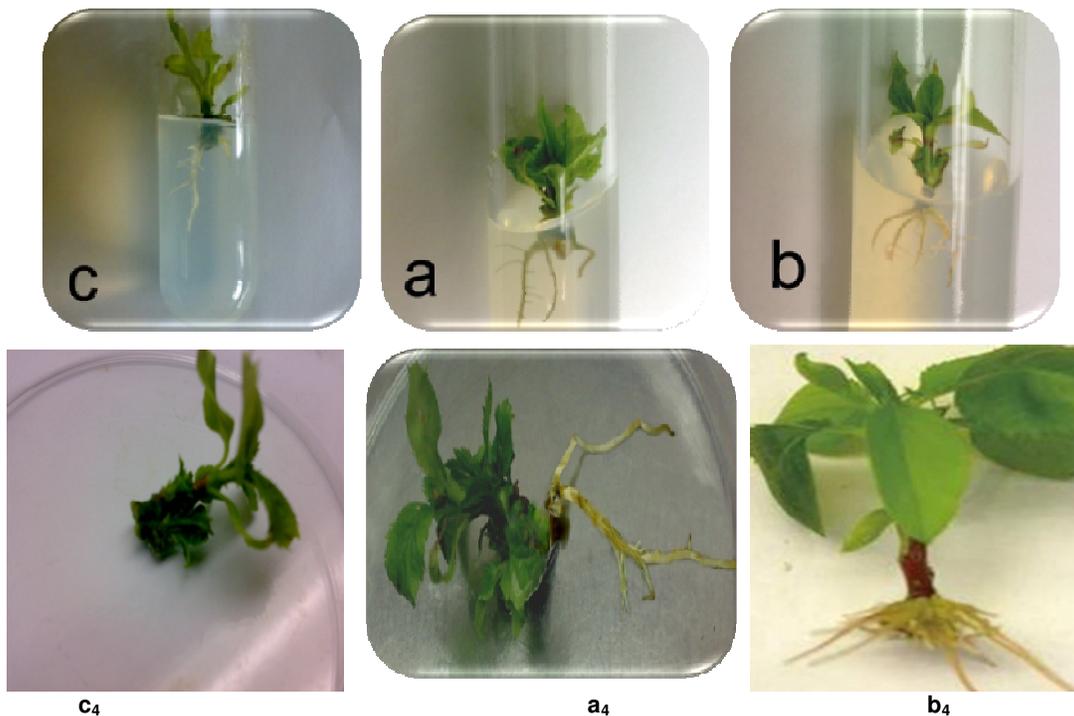
$\mu\text{M}$ ) GA<sub>3</sub> increased the elongation of adventitious shoots compared with lower (1.4  $\mu\text{M}$ ) concentration (Table 2). On the other hand, high concentrations of cytokinins (BA and TDZ) during the shoot multiplication phase inhibit rooting after transferring to rooting medium (date not shown).

Shoot growth, root formation and multiplication of apple rootstocks were dependent on the level of mineral concentration in the medium (Table 4). The best shoot growth was observed in the high (2X) concentration mineral treatment whereas, the most rooted explants

were obtained in the low (1/2X) mineral concentration treatment. Explant fresh weight increased as a result of increasing the level of mineral concentration from low to high during 6 weeks of culture (Table 4). Increasing mineral concentrations up to 2X MS-medium increased multiplication rate (5.9 explants/month), shoot elongation (33.6 mm), total fresh weight (4.6 g/jar) and leaf production (3.1 No/explant). Different genotypes ('M9', 'M27', and 'MM106') had different potential of rooting. Comparing to 'M9' genotype, 'MM106' produced more roots (Figure 3). The highest percentage (64%) of



**Figure 2.** Different potential of shoot multiplication (No./explants) and shoot elongation in apple genotypes (e, 'M27'; f, 'MM106'; g, 'M9') were cultured on the basal MS medium contain BA 4.4  $\mu\text{M}$  + TDZ 2.7  $\mu\text{M}$ , + IBA 1.0  $\mu\text{M}$  + NAA 1.22  $\mu\text{M}$  and GA<sub>3</sub>  $\mu\text{M}$  for shoot elongation six months after culture.



**Figure 3.** Different potential of root formation and root number per explant in apple genotypes (c, 'M9'; a, 'M27'; b, 'MM106') were cultured on the 1/2X basal MS medium contain IBA 4.9  $\mu\text{M}$  +NAA 2.7  $\mu\text{M}$ , and growth of root four months after culture (c<sub>4</sub>, 'M9'; a<sub>4</sub>, 'M27'; b<sub>4</sub>, 'MM106').

**Table 3.** Effects of combination of PGRs on rooting (%), root number (No./explants), root length (mm) and callusing (%) of apple rootstocks ('M9', 'M27', 'MM106') cultured on basal MS medium.

| Genotype | Combination of PGRs ( $\mu\text{M}$ ) |      |     |     |       | Rooting (%) | Root No. (No./exp.) | Root length (mm) | Callusing (%) |
|----------|---------------------------------------|------|-----|-----|-------|-------------|---------------------|------------------|---------------|
|          | BA                                    | TDZ  | IBA | NAA | 2,4-D |             |                     |                  |               |
| 'M9'     | 0                                     | 1.1  | 0   | 8.1 | 0     | 8           | 0.4                 | 52               | 14            |
|          | 0                                     | 1.1  | 1.5 | 5.4 | 0.6   | 16          | 1.2                 | 46               | 19            |
|          | 0.4                                   | 0    | 4.9 | 2.7 | 1.2   | 32          | 1.5                 | 33               | 25            |
|          | 0.4                                   | 0    | 5.4 | 0   | 1.2   | 38          | 1.8                 | 30               | 21            |
| 'M27'    | 0                                     | 1.14 | 0   | 8.1 | 0     | 10          | 0.8                 | 65               | 18            |
|          | 0                                     | 1.14 | 1.5 | 5.4 | 0.6   | 18          | 1.6                 | 58               | 24            |
|          | 0.4                                   | 0    | 4.9 | 2.7 | 1.2   | 34          | 2.1                 | 42               | 32            |
|          | 0.4                                   | 0    | 5.4 | 0   | 1.2   | 40          | 3.0                 | 41               | 18            |
| 'MM106'  | 0                                     | 1.14 | 0   | 8.1 | 0     | 13          | 1.3                 | 45               | 28            |
|          | 0                                     | 1.14 | 1.5 | 5.4 | 0.6   | 20          | 2.1                 | 40               | 36            |
|          | 0.4                                   | 0    | 4.9 | 2.7 | 1.2   | 33          | 3.4                 | 32               | 19            |
|          | 0.4                                   | 0    | 5.4 | 0   | 1.2   | 43          | 4.2                 | 29               | 13            |
| LSD      | -                                     | -    | -   | -   | -     | 4.5         | 1.6                 | 6.5              | 8.4           |

Data were collected after 6 weeks of culture on MS basal medium supplemented with the indicated growth regulators (see Table 1)

rooting was obtained in the medium with low (1/2X MS minerals) plus IBA 4.9  $\mu\text{M}$  +NAA 2.7  $\mu\text{M}$  for 'MM106' genotype, and the lowest percentage (11%) was observed for 'M9' genotype (Table 4). The root number and root length were also lower when the previous medium contained higher concentrations of BA and IBA.

## DISCUSSION

Each aspect (shoot multiplication, shoot elongation, leaf production or rooting) of apple rootstocks were significantly influenced by medium composition. This has been described by several authors (Lane and McDougald, 1982; Caboni and Tonelli, 1999; Kovalchuk et al., 2009). In most work on shoot multiplication of apple, BA was used as the cytokinin source mainly in a concentration range between 0.5 and 2 mg/l (Baraldi et al., 1991; Marin et al., 1993; Yepes and Aldwinckle, 1994; Caboni et al., 2000; Sharma et al., 2000; De Klerk et al., 2001; Kausal et al., 2005). As our result indicated, shoot multiplication of apple rootstocks was based on media containing cytokinins (especially, BA) as the major PGR, 4.4  $\mu\text{M}$  concentration in medium, also auxins and GA<sub>3</sub> in low concentration (Table 2). It can be said that multiplication rates of all genotypes ('M9', 'M29', 'MM106') were influenced by BA and mineral concentrations than other medium chemical composition (that is, auxins); however, multiplication rates of the genotypes were different in their response to the concentration of BA in the medium. Yepes and Aldwinckle (1994) found that at a constant BA level (2.2 mg/l), the balance between GA<sub>3</sub> and IBA affected shoot elongation; when the level of IBA was increased from 0.01 up to 1.0 mg/l, shoot elongation was stimulated but only in the presence of

GA<sub>3</sub>.

Results suggested that the optimum concentration of cytokines for shoot multiplication phase is 4.4  $\mu\text{M}$  BA + 2.27  $\mu\text{M}$  TDZ, and the optimum concentration of auxins for root formation phase is 5.4  $\mu\text{M}$  IBA + 1.2  $\mu\text{M}$  2, 4-D cultured on low level of minerals (1/2X MS medium) (Tables 2, 3 & 4). This was reported for other cultivars by Welander (1985) and Marin et al. (1993). Lower shoot growth suppression (30 mm) was observed using more than 5.4  $\mu\text{M}$  BA, while the highest (65 mm) stunting effect was caused by GA<sub>3</sub>. There was a negative correlation between shoot length and shoot number in all genotypes (Table 2). BA at 4.4  $\mu\text{M}$  was optimal for the maximum shoot multiplication rate.

Optimal IBA concentration for rooting was genotype-dependent. The effects of combination of auxin (IBA, NAA, 2, 4-D) on the rooting of 'M.27', 'M9' and 'MM106' were not the same. The effect of IBA on the rooting was more effective than others. The optimal concentration of IBA necessary for rooting depended on genotype (Table 3) and it was lower in 'M9' compared to other genotypes, possibly, due to indigenous auxin concentration (De Klerk et al., 1999; Magyar-Tábori et al., 2002). James (1983) examined IBA uptake and distribution in a difficult-to-root rootstock ('M.9') and in an easy-to-root rootstock 'M.26'. He found that differences in rooting reflect the metabolism of exogenous IBA rather than differences in its uptake or distribution. Later, Alvarez et al. (1989) and Harbage and Stimart (1996) found that conjugated auxin levels were significantly higher in 'M.9' than in 'M.26' while free IAA levels in apical shoot sections of both rootstocks were comparable (298 and 263.7 mg/FW) but in basal sections, the free level of IAA was 2.8-times higher in 'M.26' than in 'M.9'.

Rooting ability of *in vitro* shoots can also be influenced

**Table 4.** Effects of mineral concentrations (0.5, 1X and 2X MS-medium strength plus BA 4.4  $\mu$ M + TZD 2.27  $\mu$ M) and genotype on multiplication rate of apple rootstocks 6 weeks after culture.

| Genotype | Mineral concentration <sup>a</sup> MS-strength | Multiplication rate <sup>b</sup> (No./month) | Shoot Elongation (mm) | T-FW <sup>d</sup> (g/jar) | Leaf production <sup>e</sup> (No./explant) | Rooting <sup>f</sup> (%) |
|----------|--|--|-----------------------|---------------------------|--|--------------------------|
| 'M9'     | 1/2X   | 2.2  | 9.7                   | 1.3                       | 1.5  | 42                       |
| //       | 1X   | 4.7  | 13.9                  | 1.9                       | 2.2  | 37                       |
| //       | 2X   | 5.1  | 16.7                  | 4.2                       | 2.6  | 11                       |
| 'M27'    | 1/2X   | 1.8  | 13.3                  | 1.2                       | 1.8  | 51                       |
| //       | 1X   | 2.6  | 19.0                  | 1.8                       | 2.6  | 39                       |
| //       | 2X   | 5.9  | 22.8                  | 4.0                       | 3.1  | 13                       |
| 'MM106'  | 1/2X   | 1.4  | 19.6                  | 1.3                       | 1.8  | 64                       |
| //       | 1X   | 2.2  | 28.0                  | 1.9                       | 2.6  | 43                       |
| //       | 2X   | 3.8  | 33.6                  | 4.6                       | 3.1  | 22                       |
| LSD      | -  | 0.72   | 5.2                   | 0.8                       | 0.65                                       | 8.4                      |

<sup>a</sup>Mineral concentrations: Low (1/2X), equal (1X), double (2X) basal MS-medium; <sup>b</sup>multiplication rate (explants/month); <sup>c</sup>shoot elongation (mm); <sup>d</sup>total fresh weigh(g/jar); <sup>e</sup>leaf production (No/explant); <sup>f</sup>root formation in the medium contain IBA 4.9  $\mu$ M +NAA 2.7  $\mu$ M. Data were collected 6 weeks after culture.

by the hormone-content of the proliferation medium. Potential of rooting between rootstocks genotypes varied (Table 3). It can be said that rooting is associated with rooting frequencies in the field. If it is, the results can be useful for breeding purposes. Lane and Mc Dougald (1982) compared the response of 'M.27', 'M.9', 'M.26', 'MM.111' and 'Macspur' to different concentrations (0.1, 0.33, 1.0, 3.3, 10 and 33  $\mu$ M) of NAA and found that the optimal level of NAA for rooting differed between cultivars. 'M.27', 'M.26' and 'Macspur' rooted best at 1  $\mu$ M while 'MM.111' at 3.3  $\mu$ M NAA.

Interaction effect of BA and IBA on the root formation of apple was reported by De Klerk et al. (2001). Rooting percentage was not modified by the BA content of the medium. Rooting ability of 'Red Fuji' decreased by up to 55% when the BA content of the medium increased from 1 to 3 mg/l (Magyar-Tábori et al., 2002). Moreover, rooting ability improved from 51 to 85%, when proliferation medium contained 1 mg/l BA and 0.1 mg/l IBA (Magyar-Tábori et al., 2002). When a higher IBA level (0.3 mg/l) in the medium was applied together with BA, higher root number was developed in 'M26', than a lower (0.1 mg/l) level of IBA alone (Ricci et al., 2001). Increasing IBA (from 0.15 up to 15  $\mu$ M) increased root number. Caboni et al. (2000) stated that increasing levels of minerals and cytokinins in the medium decreased root formation, but increased callusing; this is shown in Table 4. The high salt media such as B5 (Gamborg et al., 1968) and N6 (Chu, 1978) are suitable for callus growth and morphogenesis (Caboni et al., 2000), but these media are not suitable for the growth of excised roots, anthers and other floral organs (George et al., 1987; Ozias-Akins and Vasil, 1985). The lower ionic strength media such as Heller (1965) and White (1943) are more suitable for rooting Theriou, 1995). Other physical factors, such as relative humidity in culture vessels, ethylene and carbon dioxide and culture of excised roots (Street, 1967; Dimassi-

content (CO<sub>2</sub>) of gas phase in vessels can have an important influence on shoot growth and multiplication (Hicks, 1987; Welander and Pawlicki, 1993; George and Davies, 2008).

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