Establishment of an efficient micropropagation system in enhancing rooting efficiency via stem cuttings of apple rootstock M9T337

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Abstract: Rootstocks play a vital role in regulating the environmental adaptability and controlling the growth and development of apple trees. M9T337, an excellent apple rootstock widely used in commercial orchards, could confer dwarf tree architectures, early fruiting and suitability for high-density planting. However, the rooting ability of M9T3337 is low when it is vegetatively propagated, and researchers have not yet established an efficient micropropagation system. The present study systematically evaluated the multiplication in adventitious shoots and the *in vitro* formation of adventitious roots to determine the effects of the culture media and plant growth regulators of M9T337 and a rapid micropropagation system was developed. For the shoot multiplication, the highest multiplication index of 3.93 was obtained on Murashige and Skoog (MS) media supplemented with 2.0 mg/L 6-BA, 0.1 mg/L NAA and 0.3 mg/L GA$_3$ from 12 combinations of 6-BA and NAA. Stronger and taller adventitious shoots were grown on MS supplemented with 1.8 mg/L 6-BA and 0.5 mg/L NAA. The optimal media with 100% rooting was obtained using 1/2 MS supplemented with 0.3 mg/L IBA or MS supplemented with 0.6 mg/L IBA for the rooting induction, resulting in mean rooting numbers of 13.00 and 11.33, respectively. Additionally, the effect on rooting of adding 0.3 mg/L IBA or not on the 1/2 MS and MS media was compared; the results suggested that an appropriate IBA concentration was the key to successful rooting. The rooted plantlets were acclimatised in a shaded greenhouse with an 84% survival rate. The established micropropagation system could be used for the rapid propagation of M9T337 for commercial production.

Keywords: regeneration; tissue culture; rooting induction; IBA

The apple (*Malus domestica* Borkh.), an economically important fruit crop, is widely planted in temperate zones (Dobránszki, Teixeira da Silva 2010). China has become a major apple-producing country, accounting for nearly half of the world's total apple production and 45% of the world's apple planting area in 2017 (Food and Agriculture Organization). Apple trees are primarily produced by grafting scions onto rootstocks. Different rootstocks can be used to confer desirable traits in the grafted apple trees, such as improved growth and productivity (Sotiropoulos 2008; Kosina 2010; Teixeira da Silva et al. 2019), vigour and dwarfing architecture (Van-Hooijdonk et al. 2010; Lordan et al. 2017) and nutrient uptake. Mod-

ern orchards are primarily established on dwarf and semi-dwarf rootstocks. Dwarfing rootstocks enable high-density planting and are, therefore, widely used in apple production worldwide (Gjamovski, Kiprijanovski 2011; Mao et al. 2019).

M9T337, an excellent apple rootstock widely used in commercial orchards, confers dwarf tree architectures and early fruiting and is suitable for high-density plantings (Mao et al. 2019; Wang et al. 2019). Due to the highly heterozygous apple genome, vegetative propagation is the only way to maintain a consistent genetic background in a desirable apple cultivar, rootstock, or species (Teixeira da Silva et al. 2019). Apple M9T337 rootstocks are traditionally propagated by stem cuttings. However, the rooting percentages of cutting propagation is low and disease-free plants cannot be ensured. Therefore, the micropropagation technique is the most effective method to rapidly produce healthy and disease-free rootstocks on a commercial scale.

Micropropagation techniques can be used for conservation and producing healthy and disease-free plants (Wang et al. 2016; Khamushi et al. 2019). In addition, compared to conventional cloning methods, micropropagation has a much higher capacity to multiply the target plant material all-year round (Dobránszki, Teixeira da Silva 2010; Fan et al. 2017). Furthermore, regenerating transgenic lines successfully requires micropropagation and determines if a transformation protocol is effective in plants (Aroonpong, Chang 2015; Teixeira da Silva et al. 2019). Multiple studies on apple micropropagation have been reported (Modgil et al. 1999; Sicurani et al. 2001; Nagy et al. 2005; Li et al. 2014; Amirchakhmaghi et al. 2019). Different explants in the apple regeneration have been selected and studied, such as the axillary bud, thin cell layers, leaf shoot apices, stem cuttings or shoot tips (Yepes, Aldwinckle 1994; Caboni et al. 2000; Dobránszki, Teixeira da Silva 2010; Modgil et al. 2012). In recent years, apple rootstock propagation has achieved great progress, and the rapid propagation by tissue culture has been successful in some rootstocks (Sicurani et al. 2001; Kermani et al. 2009; Dai et al. 2014). Studies suggested that different genotypes of apple rootstock have different micropropagation systems, such as the explant choice, the establishment of *in vitro* culture, sterilisation, and rooting media (Sarwar et al. 1997; Aroonpong et al. 2015; Khamushi et al. 2019). These different responses of the genotypes might be associated with varied levels of endogenous growth regulators (Teixeira da Silva et al. 2019).

Plant hormones provided from the media can be continuously supplied by artificial control to promote plant tissue growth and survival. The key factors promoting organogenesis during the micropropagation process are the combinations and concentrations of growth regulators (Machakova et al. 2008). Plant growth regulators, such as auxins, play a major role in the micropropagation process and are essential for adventitious root formation and development (Zhang et al. 2014; Lei et al. 2018). Most studies on auxins focus on the indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA). IBA is considered to be more stable and efficient in inducing adventitious roots in *in vitro* cultured explants than IAA and is widely used in clonal propagation (Bellini et al. 2014; Yang et al. 2019). Previous studies showed that exogenous IBA significantly improved the adventitious root formation of apple cultures (Rafael et al. 1989; Amiri and Elahinia 2011; Lei et al. 2017). However, the effect of IBA on adventitious rooting depended on the apple genotypes and media constituents, as well as the applied concentration (Zimmerman et al. 1995; Magyar-Tábori et al. 2002; Sedira et al. 2007).

The objectives of the present study were to develop reliable protocols for the micropropagation of M9T337 through a systematic evaluation of different culture media; the effects of the combinations and concentrations of plant growth regulators on the adventitious shoot multiplication and adventitious root formation.

MATERIALS AND METHODS

Plant materials and culture condition. Apple rootstock M9T337 was used as the plant material. M9T337 is a superior line of virus-free and dwarfing rootstock selected from the M9 apple rootstock. The *in vitro* cultures were maintained in a controlled-environment chamber at 25/18 °C (day/night), with a 16-hour photoperiod (photosynthetically active radiation = 160 μmol/m^2/s), and the relative humidity was approximately 70–80%.

Adventitious shoot multiplication. The M9T337 rootstocks were cultured on Murashige and Skoog (MS) media (Murashige and Skoog 1962) supplemented with 7 g/L agar (Sangon Biotech Co. Ltd., Shanghai, China), 30 g/L sucrose, 1.5 mg/L 6-BA and 0.5 mg/L NAA. For all the media, the pH value was adjusted to 5.8 with 1 M NaOH before autoclaving at 121 °C for 20 minutes. For the adventitious

shoot induction, until the cultured shoots reached a height of 2.0 cm, stem cuttings of 0.5–1.0 cm were cut as explants and transferred to the MS media containing twelve concentration combinations of 6-BA and NAA (0.1, 0.2 or 0.3 mg/L), as well as 0.3 mg/L GA$_3$, 6.5 g/L agar and 30 g/L sucrose. Four stem cuttings were transferred into each sterile 100 mL triangular flask containing 50 mL media. Each treatment contained ten flasks. NAA and 6-BA were added before the autoclaving sterilisation, while GA$_3$ was added at 60 °C after the autoclaving sterilisation in the clean bench. Each experiment included three replicates with forty samples (four stem cuttings or cultures in each triangular flask). The shoot multiplication number and multiplication index were calculated in three repetitions after 50 days culturing, and the morphological characteristics were recorded. The formulas were as follows in this study: Multiplication number = number of shoots generated – number of stem cuttings inoculated; Multiplication index = number of shoots generated/number of stem cuttings inoculated.

To obtain virus-free and strong adventitious shoots, nine combinations of 6-BA and NAA were set to optimise the concentrations of the plant growth regulators (Table 1). The multiplied shoots following 60-days of culturing were transferred to the MS media supplemented with 6.5 g/L agar, 30 g/L sucrose, 1.5 mg/L 6-BA and 0.5 mg/L NAA, and cultured for 30 days. Shoots 0.5–1.0 cm tall were inoculated on the MS media with different concentration combinations of 6-BA (0, 0.6, 1.2, 1.8 or 2.4 mg/L) and NAA (0, 0.5 or 1.0 mg/L) for a 60-day culture, and then the growth status of the shoots was surveyed.

Rooting induction. Until the regenerated shoots were 1.5 cm high, the callus around the stem base was removed and the shoots were transferred to a 1/2 MS media containing IBA (0, 0.3, 0.6, 0.9 or 1.2 mg/L) in the dark for 1 week. Then, the cultures were transferred onto two schemes of the 1/2 MS and MS media (with 0, 0.3, 0.6, 0.9 or 1.2 mg/L IBA), to explore the effect of the media types on the rooting induction. The 1/2 MS media and MS media both included 6.5 g/L agar; but differed in containing 0 g/L sucrose and 30 g/L sucrose, respectively. The mean number of roots, rooting percentage, root length and diameter were calculated after 40 d culturing, and the morphological traits were observed to clearly distinguish the growth status of the root system. The formulas used were as follows: Rooting percentage = number of cuttings with adventitious roots/total number of cuttings × 100%; Mean number of roots = total rooting number/number of inoculated stem cuttings.

Transplant acclimatisation. The transplanting and acclimatising of the tissue culture plantlets are the last stages of the tissue culture. The 40-day-old rooted plantlets were transferred into a greenhouse (at 20 °C, under a 16-hour photoperiod light and an 8-hour dark photoperiod) and grown for 3 days. All the media around the roots was removed by washing thoroughly. Then, the rooted plantlets with 3 or 4 leaves were surface-disinfested in 2% (w/v) carbendazim for 5 s and then transferred into plastic pots containing sterile soil, vermiculite, and a perlite mixture (1 : 1 : 1). Finally, the plantlets were covered with transparent plastic caps and shaded with three-layers of newspaper, and one newspaper layer was removed every two days. Every seven days, a 1 000 times solution of 50% carbendazim was sprayed onto the plantlets to prevent diseases spreading. During the transplant acclimatisation, the culture substrates were sterilised completely and maintained at 30–60% relative hu-

Table 1. Growth vigour comparison of the shoots on the different combinations of 6-BA and NAA in the MS media

6-BA (mg/L)	NAA (mg/L)	Growth vigour	Plant height	Yellow leaves	Lateral branch
0	0	strong	low	less	less
0.6	0.5	strong	high	less	less
1.2	0.5	strong	high	less	less
1.8	0.5	strong	high	no	much
2.4	0.5	strong	high	less	no
0.6	1.0	weak	low	much	no
1.2	1.0	weak	low	much	no
1.8	1.0	weak	low	much	no
2.4	1.0	weak	low	much	no

midity. The plantlets were checked in the morning and evening each day, and the unhealthy plantlets and leaves were discarded. After 60 days of growth, the survival rates of the plantlets were calculated.

Statistical analysis. SPSS 19.0 statistical software (Chicago, USA) was used for all the analyses. A standard factorial analysis of variance was conducted and presented as the means ± standard deviation according to Duncan's new multiple range test (at $P < 0.05$).

RESULTS

Effects of 6-BA and NAA on the adventitious shoot regeneration. ifferent combinations of 6-BA and NAA in the MS media were used to obtain healthy adventitious shoots. After 50 days, the multiplication data were observed (Table 2). There were significant differences among the treatments, except for the combinations 2.0 mg/L 6-BA plus 2.0 mg/LNAA and 3.0 mg/L 6-BA plus 1.0 mg/L NAA. The media containing 2.0 mg/L 6-BA plus 1.0 mg/L NAA had the highest multiplication number at 118 and the highest multiplication index of 3.93, while the values for the media containing 2.0 mg/L 6-BA and 3.0 mg/L NAA were only 6 and 0.22, respectively. The multiplication number did not increase with the 6-BA concentration

Table 2. Effects of the different combinations of 6-BA and IBA on the shoot multiplication of the M9T337 apple rootstock on the MS media

6-BA (mg/L)	NAA (mg/L)	Multiplication number	Multiplication index
1.0	0.1	76[b]	2.53 ± 0.08[b]
1.0	0.2	10[j]	0.33 ± 0.16[j]
1.0	0.3	31[e]	1.03 ± 0.05[e]
2.0	0.1	118[a]	3.93 ± 1.25[a]
2.0	0.2	16[i]	0.53 ± 0.25[i]
2.0	0.3	6[k]	0.22 ± 0.10[k]
3.0	0.1	17[i]	0.63 ± 0.20[i]
3.0	0.2	36[d]	1.20 ± 0.45[d]
3.0	0.3	22[g]	0.81 ± 0.30[g]
4.0	0.1	19[h]	0.63 ± 0.15[h]
4.0	0.2	26[f]	0.87 ± 0.30[f]
4.0	0.3	41[c]	1.37 ± 0.20[c]

The small letters within a column indicate significant differences by Duncan's new multiple range test ($P < 0.05$)

or the NAA concentration. The induced shoots grew regularly between 1.0–2.0 mg/L 6-BA. When the 6-BA was above 2.0 mg/L, the induced shoots appeared weaker and were not up-right (Figure 1), regardless of the NAA concentration. Fewer adventitious shoots came up from the stem cuttings at 4.0 mg/L 6-BA, and calluses were formed at the stem base (Figure 1D). In summary, the optimal media for the multiplication was the MS media containing 2.0 mg/L 6-BA, 0.1 mg/L NAA and 0.3 mg/L GA_3, which resulted in a multiplication index of 3.93 (Table 2).

Effects of 6-BA and NAA on the adventitious shoot growth. To obtain healthy. and strong adventitious shoots, different combinations of 6-BA and NAA in the MS media were used to improve the rooting percentage. We found that the shoots had obvious differences in growth among the nine treatments (Figure 2 and Table 1), of which the plants grown on the MS media containing 1.8 mg/L 6-BA and 0.5 mg/L NAA had green leaves and more twigs, and the plant's height was about 5.5 cm. Plants grown on the MS media containing 0, 0.6 1.2, 1.8 6-BA mg/L and 0, 0.5, NAA mg/L were more vibrant and taller than the others. The shoots were weaker with many yellow leaves and had no lateral branches on the media supplemented with 2.4 mg/L 6-BA and 0.5, 1.0 mg/L NAA. Therefore, 0.5 mg/L NAA was more suitable for the adventitious shoots of the M9T337 apple rootstock,
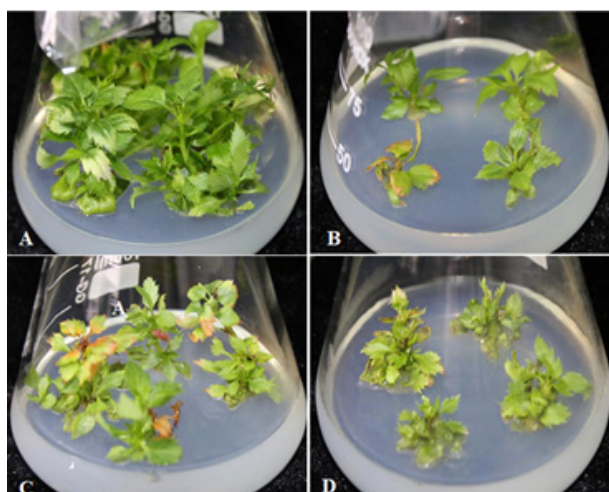

Figure 1. Shoot multiplication of M9T337 on the MS media supplemented with the different combinations of 6-BA and NAA. (A) MS + 2.0 mg/L 6-BA + 0.1 mg/L NAA; (B) MS + 2.0 mg/L 6-BA + 0.3 mg/L NAA; (C) MS + 3.0 mg/L 6-BA + 0.2 mg/L NAA; (D) MS + 4.0 mg/L 6-BA+0.1 mg/L NAA
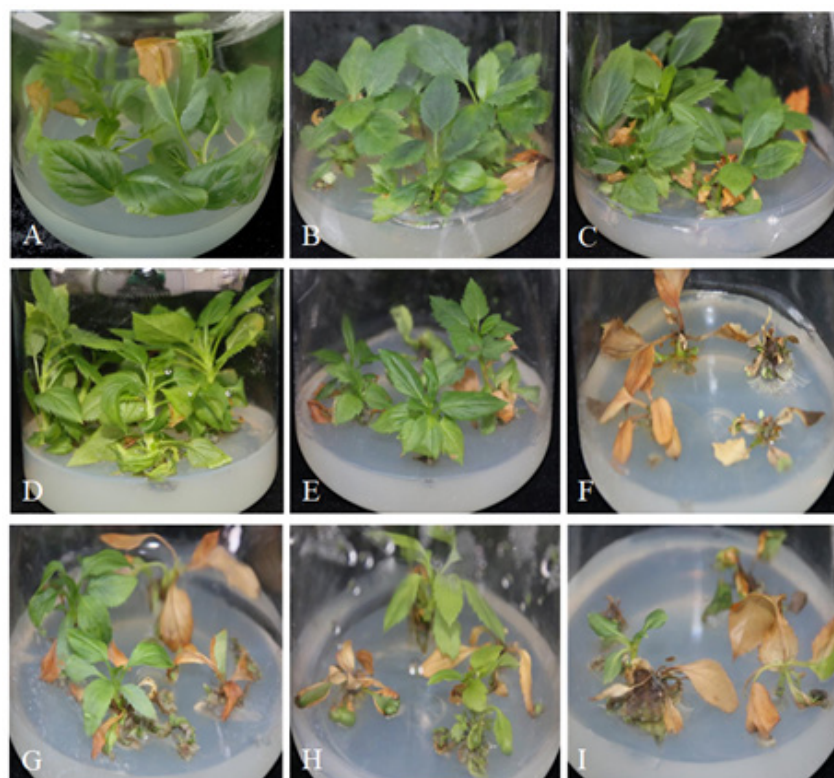
Figure 2. Effects of the various combinations of 6-BA and NAA on the adventitious shoot growth in the MS media of M9T337. (A) 0 mg/L 6-BA+0 mg/L NAA; (B) 0.6 mg/L 6-BA + 0.5 mg/L NAA; (C) 1.2 mg/L 6-BA + 0.5 mg/L NAA; (D) 1.8 mg/L 6-BA + 0.5 mg/L NAA; (E) 2.4 mg/L 6-BA + 0.5 mg/L NAA; (F) 0.6 mg/L 6-BA + 1.0 mg/L NAA; (G) 1.2 mg/L 6-BA + 1.0 mg/L NAA; (H) 1.8 mg/L 6-BA + 1.0 mg/L NAA; (I) 2.4 mg/L 6-BA + 1.0 mg/L NAA

when combined with the 6-BA concentrations ranging from 0.6–2.4 mg/L designed in this study. Furthermore, the comparison analysis indicated that the best concentration of plant growth regulators for the shoot growth was 1.8 mg/L 6-BA and 0.5 mg/L NAA in the MS media (Figure 2D).

Effects of the IBA concentration and two media types on the rooting. Two media types (1/2 MS media and MS media) were supplemented with IBA (0, 0.3, 0.6, 0.9 or 1.2 mg/L) to obtain the optimal media with the suitable IBA concentration. Regarding the 1/2 MS media, the rooting percentage ranged from 0% (1.2 mg/L IBA) to 100% (0.3, 0.6 or 0.9 mg/L of IBA), showing that an IBA above 1.2 mg/L in the ½ MS media inhibits rooting (Table 3). The maximal mean rooting number per adventitious shoot was 13.00 at 0.3 mg/L IBA, and the root length and diameter were longer than the other IBA concentrations. Furthermore, the morphological comparison of the roots indicated that many long and strong roots were present at 0.3, 0.6 and 0.9 mg/L IBA, while fewer lateral roots were

present at 0.9 mg/L IBA (Figure 3). In conclusion, the best media for rooting in this scheme was the 1/2 MS + 0.3 mg/L IBA.

Compared with the 1/2 MS media, the highest rooting percentage of 100% on the MS media was also achieved at 0.3, 0.6 and 0.9 mg/L IBA, whereas the lowest rooting of 33.33% occurred at 0 mg/L IBA (Table 4). The highest rooting number with 11.33 roots per adventitious shoot occurred at 0.6 mg/L IBA while the lowest was at 0 mg/L IBA. However, the longest root also appeared on the MS media without the IBA. Moreover, as the concentration of the IBA increased, the root length shortened while the root diameter increased, that is, higher IBA concentrations resulted in thicker and shorter roots (Table 4, Figure 3). The lateral roots formed at 0, 0.3 or 0.6 mg/L IBA, but no lateral roots were produced above 0.6 mg/L IBA. Therefore, adding 0.6 mg/L IBA into the MS media was most suitable for the rooting. In addition, the morphological comparison of the roots on the best performance of the two media showed that the roots appeared thinner and longer on the 1/2 MS media than

Table 3. Effect of the different IBA concentrations in the 1/2 MS media on the rooting of M9T337

IBA mg/L	Rooting (%)	Mean number of rooting	Root length cm)	Root diameter (mm)	Number of lateral roots
0	33.33 ± 5.16^b	0.67 ± 0.14^c	3.38 ± 0.55^a	0.96 ± 0.12^a	0.00 ± 0.00^b
0.3	100.00 ± 0.00^a	13.00 ± 1.25^a	3.50 ± 0.46^a	0.97 ± 0.09^a	0.00 ± 0.00^b
0.6	100.00 ± 0.00^a	8.00 ± 2.56^b	3.40 ± 0.48^a	0.90 ± 0.10^a	6.40 ± 0.17^a
0.9	100.00 ± 0.00^a	5.67 ± 0.89^b	3.30 ± 0.48^a	0.92 ± 0.12^a	0.00 ± 0.00^b
1.2	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^b	0.00 ± 0.00^b

The mean rooting number was calculated from the per adventitious shoot. The small letters within a column indicate significant differences by Duncan's new multiple range test ($P < 0.05$)

on the MS media (Figure 4). The above results suggested that the IBA in two media types could inhibit the root elongation and the higher IBA concentration (above 0.9 mg/L IBA in this study) also suppressed the formation of the lateral roots for M9T337. It was, therefore, inferred that the media type had less of an effect on inducing the root formation than the IBA levels, and the IBA concentration was essential in inhibiting the root elongation.

Transplant acclimatisation. The 40-day-old rooted plantlets were transferred into the greenhouse and grown for 3 days, followed by transplanting into plastic pots (Figure 5A). The morphological response of the M9T337 plantlets was observed on 40 and 60 days after transplanting (Figure 5B, 5C). After 60 days, the survival rate reached 84%.

DISCUSSION

Micropropagation is essential for producing disease-free apple trees and in the rapid multiplying of desirable scions and rootstocks (Dobránszki, Teixeira da Silva 2010). Different scions and rootstocks have different requirements during *in vitro* propagation, including suitable media, hormone types and concentrations; establishment of the *in vitro* cultures; adventitious shoot regeneration and/or multiplication; and rooting induction (Li et al. 2014; Teixeira da Silva et al. 2019). In this study, an effective protocol was developed for the successful *in vitro* micropropagation of the M9T337 apple rootstock by optimising the culture media and plant growth regulator concentrations. To avoid tissue browning and to minimise the incidence of contam-
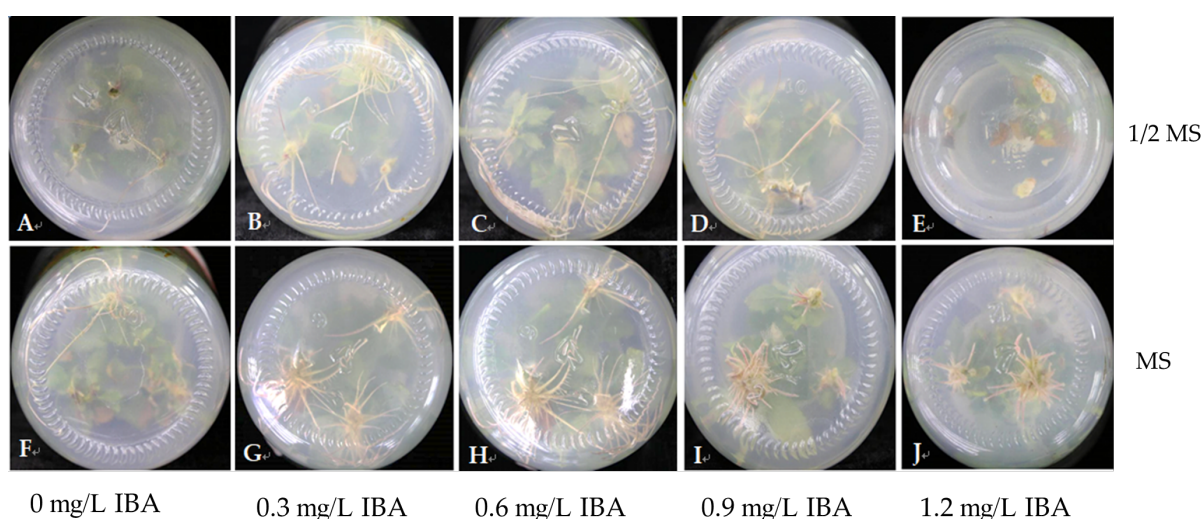

Figure 3. Morphological observation of the adventitious root of M9T337. (A) 1/2 MS medium; (B) 1/2 MS + mg/L IBA; (C) 1/2 MS + 0.6 mg/L IBA; (D) 1/2 MS + 0.9 mg/LIBA; (E) 1/2 MS +1.2 mg/L IBA; (F) MS medium; (G) MS + 0.3 mg/L IBA; (H) MS + 0.6 mg/L IBA; (I) MS + 0.9 mg/L IBA; (J) MS + 1.2 mg/L IBA

Table 4. Effect of the different IBA concentrations on the rooting in the MS medium of M9T337

IBA (mg/L)	Rooting (%)	Mean number of rooting	Root length (cm)	Root diameter (mm)	Number of lateral roots
0	33.33 ± 0.16^{c}	0.67 ± 0.30^{e}	4.50 ± 0.63^{a}	0.95 ± 0.05^{c}	12.00 ± 1.50^{c}
0.3	100.00 ± 0.00^{a}	5.56 ± 2.10^{b}	3.50 ± 0.39^{b}	1.20 ± 0.03^{b}	15.20 ± 1.25^{b}
0.6	100.00 ± 0.00^{a}	11.33 ± 2.55^{a}	2.80 ± 0.66^{c}	1.25 ± 0.45^{b}	17.20 ± 2.55^{a}
0.9	100.00 ± 0.00^{a}	4.22 ± 1.20^{c}	1.90 ± 0.37^{d}	1.40 ± 0.25^{a}	0.00 ± 0.00^{d}
1.2	88.89 ± 12.00^{b}	3.22 ± 1.05^{d}	1.75 ± 0.25^{d}	1.41 ± 0.24^{a}	0.00 ± 0.00^{d}

The mean rooting number was calculated from the per adventitious shoot. The small letters within a column indicate significant differences by Duncan's new multiple range test ($P < 0.05$)

Figure 4. Phenotype of M9T337 on the different MS media IBA concentrations. (A) 1/2 MS + 0.3 mg/L IBA; (B) MS + 0.6 mg/L IBA

ination, stem cuttings were selected as the explants of M9T337 grown in a controlled-environment chamber. The results showed that using 0.5–1.0 cm stem cuttings as explants was successful in avoiding tissue browning and minimising the contamination incidence. Previous studies revealed that various explants could be used in apple micropropagation, such as the leaf, meristems and shoot tips (Modgil et al. 1999; Dobránszki, Teixeira da Silva 2010;

Modgil et al. 2012; Zhang et al. 2014; Wang et al. 2016). Modgil et al. (1999) reported that using nodal cuttings as explants resulted in the highest number of new shoots compared to shoot tips and the basal mass.

The rate of regeneration and multiplication is crucial for apple micropropagation (James et al. 1988; Dobránszki, Teixeira da Silva 2010). Previous studies showed that the efficacy of the shoot regeneration and multiplication is influenced by the genotypes, media composition, hormones and the *in vitro* environment, etc. Regarding the shoot regeneration of M9T337, the optimal media was determined from twelve combinations of 6-BA and NAA with different concentrations. Our results indicated that the highest multiplication index of 3.93 occurred on MS + 2.0 mg/L 6-BA + 0.1 mg/L NAA + 0.3 mg/L GA_3. The level of plant hormones is a crucial factor in determining the organogenic capacity of explants. Obviously, 6-BA serves as the primary cytokinin, but the optimum concentrations for shoot regeneration varied according to genotype in the M.26, MM.111, M.9, JTE-H, G.41 apple rootstocks (Sarwar, Skirvin 1997; Kaushal et al. 2005; Magyar-Tábori et al. 2010; Zhang et al. 2014).
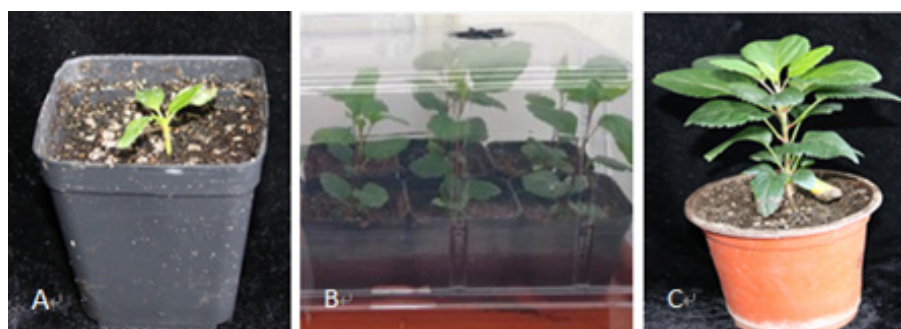

Figure 5. Different stages of the micropropagation of M9T337. (A) 0 day after transplanting; (B) 40 days after transplanting; (C) 60 days after transplanting

Culturing strong adventitious shoots is a necessary precondition for rooting. During this stage, it is the auxins, cytokinins and their interactions that have the greatest influence on shoot multiplication (Ward, Leyser 2004). Therefore, adjusting the concentration of 6-BA and NAA to meet the demand of shoot growth might further improve the shoot multiplication. Among nine combinations, MS + 1.8 mg/L 6-BA + 0.5 mg/LNAA was optimal for the adventitious shoots of M9T337 rootstock. Additionally, the effect of NAA on the growth vigour was greater than that of 6-BA. The growth vigour of the adventitious shoots was weaker at 1.0 mg/L NAA than at 0.5 mg/L NAA, and relatively higher concentrations of NAA led to more yellow leaves and no lateral branches.

For the vegetative and micropropagation of woody plants, an adventitious root formation is a key step (Ďurkovič, Lux 2010). IBA have been used to stimulate the rooting of cuttings in many plant species that are difficult to root (Yang et al. 2019). Previous studies have shown that IBA efficiently induced the adventitious root formation of both greenwood and hardwood cuttings (Machakova et al. 2008; Lei et al. 2018; Yang et al. 2019). The present study compared the effects of different concentrations of IBA on the rooting in 1/2 MS and MS media. Our results showed that the maximum root number per adventitious shoot, 13.00, was achieved on the 1/2 MS supplemented with 0.3 mg/L IBA, reaching a 100% rooting percentage. Compared with the 1/2 MS media, the highest root percentage of 100% on the MS media was also achieved at 0.3, 0.6 and 0.9 mg/L IBA, whereas, the highest rooting number with 11.33 per adventitious shoot was at 0.6 mg/L IBA. The results suggest that there are great differences in the rooting on the 1/2 MS and MS media during the *in vitro* rooting phase. Moreover, it was suggested that the presence of IBA was essential for the rooting of M9T337 and an appropriate IBA concentration was key to the successful rooting, which was in agreement with the previous result reported by Amiri and Elahinia (2011). Furthermore, different IBA levels also required different media types and similar findings were reported in apples (Zimmerman et al. 1995; Magyar-Tábori et al. 2002; Sedira et al. 2007)

In conclusion, an efficient micropropagation system of the M9T337 apple rootstock was established and several critical media for the M9T337 micropropagation were identified. First, using 0.5–1.0 cm stem cuttings as explants was successful in avoiding tissue browning and minimising the contamination incidence. Second, the shoot multiplication was cultivated over 60 days on the MS media containing 6.5 g/L agar, 30 g/L sucrose, 2.0 mg/L 6-BA, 0.1 mg/L NAA and 0.3 mg/LGA3. Third, the highest rooting percentage (100%) was achieved on the 1/2 MS containing 6.5 g/L agar, 20 g/L sucrose and 0.3 mg/L IBA in a dark culture for 7 days and then in a 16-hour light and 8-hour dark photoperiod for 40 days. The rooted plantlets were then transferred into the greenhouse for acclimation and grown for 3 days, followed by transferring into plastic pots with saucers containing sterile water and sterilised culture substrates with 30–60% relative humidity. The established micropropagation system provides a solid foundation for the rapid multiplication of the M9T337 apple rootstock on a commercial scale. It is also an essential step in the success of transformation research with this apple rootstock for desirable traits.

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