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Research on the Optimization of Tissue Culture Technology Applied to the Production of Several Apple Dwarf Rootstocks

Man Cheng[1], Huijun Liu[2,*], Tianhao Liu[3]

[1]*Shandong Guanfu Holdings Group Co., Ltd., Jinan, Shandong, 250300, China*
[2]*Northeast Forestry University, Harbin, Heilongjiang, 150006, China*
[3]*Henan Finance University, Zhengzhou, Henan, 451464, China*
[*]*Corresponding author*

Abstract: *In this study, the branches of apple dwarf rootstock variety T337 that had not yet germinated during the dormant period and the young branches of the virus-free mother plant with native M26, GM256 and M9 were used as the test materials, a variety of methods for establishing aseptic systems were designed, and the best ways and methods were selected. We found that the most suitable method for disinfecting explants is a mixture of 70% alcohol for 12 seconds, 0.1% mercuric chloride, and Tween for 7 minutes, followed by 10 washes with sterile water; The optimal subculture medium for T337 is MS+IBA 0.6 mg/L+6-BA 0.4 mg/L. MS+6-BA 0.7 mg/L+IBA 0.6 mg/L is suitable for M9 and GM256. The differentiation effect of M26 is good when MS+6-BA 0.9 mg/L+IBA 0.7 mg/L; 1/2 WPM+IBA 0.6 mg/L+NAA 0.02 mg/L has the best rooting effect; Domesticated substrate perlite: vermiculite: peat=1:1:3, temperature controlled at 23-28 ℃, humidity gradually decreased from high humidity, and light gradually increased from low light to adapt to natural environment. It is most suitable for seedling growth and is more conducive to production application and variety promotion.*

Keywords: *Apple dwarf rootstock; Aseptic system; Proliferation and differentiation; Rooting and domestication*

1. Introduction

Dwarf apple dense planting cultivation is the trend of apple development, and excellent dwarf rootstock is the key to high yield and quality. With the expansion of apple planting areas and the increasing demand for fruit in society, the application and promotion of apple dwarf rootstock dense planting cultivation technology have gradually formed the characteristics of short tree body, fast formation, early flowering, early fruiting (starting to bear fruit after 2 years of planting, forming yield after 3 years, 4-5 years for early high yield, and 6-7 years for abundant production), early high yield, easy management, and high income. The economic benefits are obvious, and it has also played an important role in promoting the stable development of the apple industry[1].

The commonly used apple dwarfing rootstock varieties in production include M26, M9, SH40, SH6, and M9-T337, all of which have the characteristics of drought resistance, cold resistance, and easy flowering[2]. As an important resource, the tissue culture technology of apple dwarfing rootstock has been successfully studied both domestically and internationally. Most of the research is focused on articles and patents related to asexual reproduction technology[3]. However, when applied in production, it encounters browning, vitrification, proliferation coefficient, domestication and transplantation, which seriously affect production costs. It is urgent to optimize tissue culture and rapid propagation technology and apply it on a large scale for production promotion.

Therefore, this study selected apple dwarfing rootstock T337, M26, GM256 series, and M9 as experimental materials. Apple dwarfing rootstock T337 is an excellent variety promoted and applied in enterprise production, and in recent years, there is an urgent need for a large number of uniform and consistent propagation materials.This experiment involves various tests such as pre culture of explants, surface disinfection, inoculation to obtain a sterile system, differentiation, proliferation, rooting, domestication, and transplantation. After obtaining experimental data, repeated experiments are conducted for validation, pilot testing, and large-scale application, among other steps and research methods.

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2. Materials and methods

2.1 Test materials

This experiment introduced T337, M26, GM256, and M9 apple asexual rootstocks and grafted them onto the *M. robusta* rootstock. This study established a germplasm resource collection area of 2 acres and a scion nursery of 5 acres, respectively, to provide the necessary breeding materials for research.

2.2 Treatment of explants

This study collected T337 branches that had already passed dormancy but had not yet germinated, as well as young branches of M26, GM256, and M9 that were born in the same year on virus-free mother plants from the cutting nursery. After rinsing thoroughly with running water, they were kept moist and stored in a refrigerator at 4℃ for future use. To minimize pollution and enhance survival rates, it is essential to change the water every 24 hours. Additionally, trimming approximately 2cm from the lower part of the branches every 3 days facilitates better water absorption. When the bud grows to 2cm, remove the scales and unfolded leaves from the bud prior to disinfection. Wash the cut bud thoroughly with a detergent, and then rinse it with clean water. Move the buds to the ultra-clean workbench for sterilization, rinse them with sterile water 8-10 times, and sterilize the culture medium used in the experiment via high-pressure sterilization. The cultivation conditions are 25℃, 12 hours of light exposure, and a light intensity of 2000 lx.

2.3 T337 subculture differentiation formula experiment

The sterile explants that survived on the blank medium were transferred to the following MS media, each supplemented with different hormone ratios, as detailed in Table 1. The agar concentration of the culture medium is 6 g/L, and the sucrose concentration is 30 g/L. The cultivation conditions include a temperature of 25 ± 2 ℃, 12 hours of light exposure per day, and a light intensity of 2000 lx. Each treatment consists of 25 bottles, with 5 plants per bottle. The differentiation coefficient and growth status of all materials are recorded.

Table 1: Experimental Design of Apple T337 Differentiation Formula

Code name	Culture Media Formulation (mg/L)
M0	MS(contrast)
M1	MS+IBA0.8+6-BA0.3
M2	MS+IBA0.6+6-BA0.4
M3	MS+IBA0.7+6-BA0.5
M4	MS+IBA0.5+6-BA0.6

2.4 Optimization experiment of M26, GM256, and M9 subculture media

Based on the above cultivation, it can be concluded that increasing BA concentration and decreasing IBA concentration can promote their differentiation. Therefore, the M26, GM256, and M9 differentiation media were optimized and designed accordingly, as shown in Table 2. MS basic medium (with additional agar 6.0 g/L and sucrose 30 g/L) was used, and the cultivation conditions were the same as above.

Table 2: Experimental Design for Optimization of Apple M26, GM256 and M9 Differentiation Formulas

Code name	Culture Media Formulation (mg/L)
CK	MS+6-BA 0.4+IBA0. 6
1	MS+6-BA 0.5+IBA0. 4
2	MS+6-BA0. 6+IBA0. 6
3	MS+6-BA 0.7+IBA 0.6
4	MS+6-BA 0.9+IBA0. 7
5	MS+6-BA 1.1+IBA0. 6

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2.5 T337 rooting culture formula orthogonal experiment

According to data reports, the hormones and their concentration ranges that have a significant effect on rooting of apple dwarfing rootstock T337 are as follows: IBA (0.1-1.5 mg/L)+NAA (0-0.1 mg/L), and sucrose concentration of 15-30 g/L. Using 1/2WPM as the basic culture medium, an orthogonal experiment L9 (33) was designed with IBA, NAA, and sucrose as the three orthogonal influencing factors, as shown in Table 3. This experiment selected seedlings with uniform growth conditions and cultivated them using 1/2WPM basic medium (containing 6.0 g/L of agar and 30 g/L of sucrose) under the aforementioned cultivation conditions. Each treatment involved 25 bottles, with 5 seedlings in each bottle. The rooting rate and root quality were observed and recorded.

Table 3: Orthogonal experimental design of rooting formula for apple T337 L_9 (3^3)

Factor / Horizontal	IBA(mg/L)	NAA(mg/L)	Sucrose (mg/L)
I	0.6	0.2	20
II	0.8	0.4	25
III	1.0	0.6	30

2.6 Optimization experiment of rooting medium for M26, GM256 and M9

The experimental design was based on the rooting formula of T337, adjusting the IBA concentration (0.6, 0.5, 0.4, 0.3 mg/L), using 1/2WPM basic medium (additional agar 6.0 g/L, sucrose 30 g/L), with the same cultivation conditions. 25 bottles were made for each treatment, and 5 plants were placed in each bottle to observe the rooting rate and number of roots.

2.7 Experimental Design of Apple Dwarf Rootstock Domestication Technology

In order to adapt the tissue culture seedlings of apple dwarfing rootstocks T337, M26, GM256, and M9 to changes in the environment, the transplanting substrate should be mainly made of materials that are loose and permeable, have excellent water retention and dehydration systems, and are not prone to mold and mold growth, supplemented by nutrient rich materials to achieve the best survival rate. The key factors affecting the microenvironment of greenhouse domestication technology include temperature, humidity, light, and substrate. Taking the above four technical steps as four factors, an orthogonal experiment L9 (34) was designed for optimal combination or treatment, as shown in Table 4. Six plug trays were planted for each substrate combination, with 50 plants per tray. The survival rate was calculated, and various indicators of growth traits were analyzed, as shown in Table 5.

Table 4: Comprehensive Experimental Design for Greenhouse Microenvironment Factor Control Table L_9 (3^4)

Factor / Horizontal	Base material	Temperature	Humidity	Intensity of illumination
I	Sder: peat: vermiculite =1:1:1	high temperature	High wet	low light level
II	Gravel: peat: peanut shell =0.5:1.5:1	Temperature control	To control the wet	Control light
III	Vermiculite: peat: perlite =0.5:1.5:0.5	blank space	blank space	blank space

Note: The matrix ratio in the table is the volume ratio

Table 5: Form and Quality Index Standards

Fraction	Leaf colour	In order	Growth potential
3	hispid arthraxon	More than 95%	stubborn
2	centre	80-95%	centre
1	acid green	Under 80%	weak

3. Results and analysis

3.1 Establishment of aseptic system for apple dwarfing rootstock

The experiment adopts indoor hydroponic method, which not only avoids large-scale pollution, but

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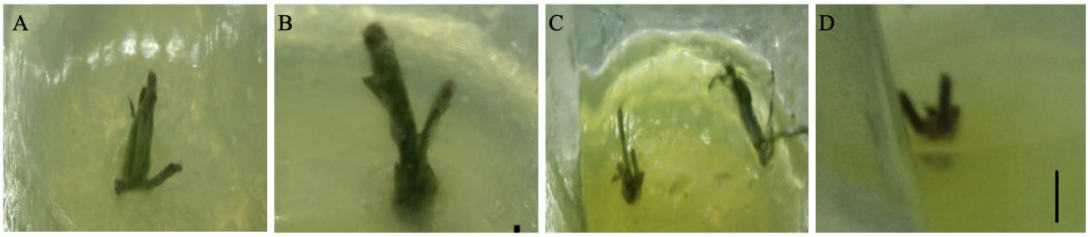
also makes the disinfection time relatively short. According to the results in Table 6 and Figure 1, the second group showed the best sterilization effect, with a sterile rate of 65.4% for the explants. The method was to soak the material in 70% alcohol for 12 seconds, then soak it in a mixture of 0.1% mercuric chloride and Tween for 7 minutes, rinse it with sterile water 10 times, re cut the stem segments, and transplant them into the basic culture medium. Next is the third group, with a sterile rate of 70% for explants, but high browning and mortality rates. The sterilization effect of the first and fourth groups was the worst. The first group had a too short sterilization time and all the materials were contaminated. The fourth group had a sterilization rate of 92%, but a too long sterilization time could harm the materials, inhibit the sprouting and growth of the explants, and almost all of them turned brown and died. So combination 2 is considered a good combination, but the success rate is very low at 13%, which shows that it is very difficult to establish a sterile system for apple rootstock T337.

The establishment of sterile systems for apple rootstocks M26, GM256, and M9, and the use of T337 as the optimal sterilization reagent and method for external colonies, were carried out by planting in blank culture medium (with an agar concentration 30% higher than usual), placed at 25°C, exposed to light for 12 hours, and a light intensity of 2000 lx. The survival rate reached about 40%. As is shown in Table 6.

Table 6: Sterilization test results of hydroponic explants in a light incubator

The sterilization reagent	Assemble	The sterilization time	Death, browning rate (%)	Sterility rate (%)	Mission success rate (%)
And 7 0% alcohol + 0.1% liters of mercury	1	Alcohol for 10s and 5 min liters of mercury	89.6	30.2	1.8%
	2	Alcohol for 12s, and 7 min for the liter of mercury	87.6	65.4	13%
	3	Alcohol for 15s and a liter of mercury for 8 min	96	70	1.6%
	4	Alcohol for 2 0s and a liter of mercury for 10 min	99.7	80.1	0.8%

Note: 3 drops of Tween were added for all combinations



A. Alcohol for 10 seconds, mercuric chloride for 5 minutes; B. Alcohol for 12 seconds, mercuric chloride for 7 minutes; C. Alcohol for 15 seconds, mercuric chloride for 8 minutes; D. Alcohol for 20 seconds, mercuric chloride for 10 minutes. Bar:1 cm.

Figure 1: Sterilization of apple dwarfing rootstock explants (10d)

3.2 Research on the formula for subculture and differentiation of T337 apple

The higher the concentration of cytokinin, the lower the auxin, and the more it promotes differentiation. Only by combining the two in a reasonable way can the ideal differentiation effect be achieved. According to the experimental results in Table 7 and Figure 2, T337 began to differentiate in combination 2 with MS+NAA 0.6+6-BA 0.4, and the differentiation was slightly more pronounced than in other groups. At this time, the number of bud proliferation remained stable at 4.34, and the seedlings were tender green. Combination 3 has a differentiation coefficient of 3.14, which can also meet the production requirements, but its differentiation rate is too low; The differentiation coefficient of combination 1 and combination 4 is too low, and the optimal bud differentiation medium is MS+IBA 0.6 mg/L+6-BA 0.4 mg/L+sucrose 30g/L+agar 6.0g/L. Through experiments, it was found that after 4 weeks, the color of the seedlings tends to dark green and grows rapidly. It is necessary to transfer the plants for cultivation in a timely manner. Transferring too early not only affects the proliferation coefficient but also the growth of offspring. It is more appropriate to transfer and subculture at 28-30 days, when the proliferation coefficient of the transferred seedlings is high and the offspring grow robustly.

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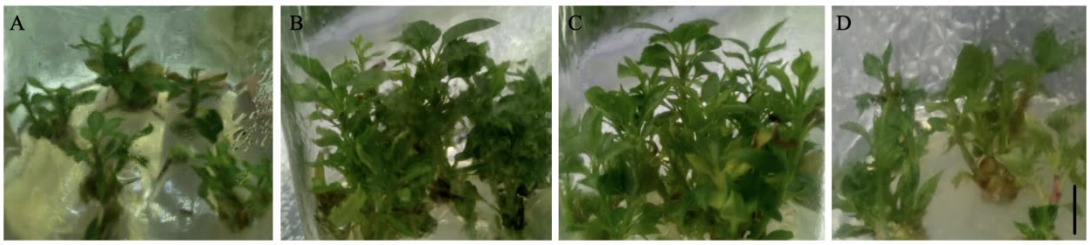
Table 7: Results of hormone adjustment test for apple T337

Assemble	Make up a prescription (mg/L)	Growth	Coefficient of differentiation
CK	MS(blank space)	The reproductive cycle is 20 – 25 d, with robust stems and yellow leaves	No differentiation
1	MS+IBA0.8+6-BA0.3	The reproductive cycle is 20 – 25 d, with robust stems, green leaves and 20% differentiation	1.86
2	MS+IBA0.6+6-BA0.4	The reproductive cycle was 23 – 28 d, with robust stems, leaves pale green and 80% differentiated	4..34
3	MS+IBA0.7+6-BA0.5	The reproductive cycle was 23 – 28 d, with robust stems, yellow-green leaves and 50% differentiation	3.13
4	MS+IBA0.5+6-BA0.6	The reproductive cycle is 23 – 28 d, with robust stems, slightly yellowish leaves and 40% differentiation	2.17



A.MS+IBA0.8mg+6-BA0.3mg; B. MS+IBA0.6mg+6-BA0.4mg; C. MS+IBA0.7mg+6-BA0.5mg; D. MS+IBA0.5mg+6-BA0.6mg. Bar: 1cm.

Figure 2: Growth of Apple T337 on Media with Different Hormones Added

3.3 Optimization selection of apple M26, GM256, and M9 offspring formulas

According to Table 8 and Figure 3, it can be seen that combination 3 of MS+BA 0.7 mg/L+IBA 0.6 mg/L is more suitable for M9 and GM256 subculture, with larger and stronger callus growth, and a differentiation coefficient of 4.11; Combination 4 of MS+BA 0.9 mg/L+IBA 0.7 mg/L is more suitable for M26 subculture, with good differentiation effect, strong growth potential, and a differentiation coefficient of 4.6.

Table 8: Statistical Results of Apple M26, GM256, and M9 Differentiation Formulas

Order number	Make up a prescription	Coefficient of differentiation		Growth potential		Callus	
		M26	GM256,M9	M26	GM256,M9	M26	GM256,M9
CK	MS+6-BA 0.4+IBA0. 6	2.04	2.54	weak	weak	centre	small
1	MS+6-BA 0.5+IBA0. 4	2.57	2.81	centre	weak	centre	small
2	MS+6-BA0. 6+IBA0. 6	2.94	3.16	centre	centre	big	centre
3	MS+6-BA 0.7+IBA 0.6	3.12	4.11	stubborn	stubborn	big	big
4	MS+6-BA 0.9+IBA0. 7	4.60	3.58	stubborn	stubborn	centre	centre
5	MS+6-BA 1.1+IBA0. 6	3.27	2.89	stubborn	centre	centre	centre

A.M9; B. GM256; C. M26. Bar: 1cm.

Figure 3: Growth of the optimal formula for M9, GM256, and M26 apples

3.4 Study on rooting formula of apple T337

According to the orthogonal experiment results in Table 9, from the perspective of key rooting rate, the K value shows that the optimal combination is IBA0.6 mg/L+NAA 0.02 mg/L+sucrose 25 g/L; The R value also indicates the degree of influence of factors on the indicators, with the size of the influencing factors being: NAA>IBA>sucrose; The optimal rooting formula is 1/2 WPM medium+IBA

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0.6 mg/L+NAA 0.02 mg/L+sucrose 25 g/L+agar 6.0 g/L.

Table 9: Results of orthogonal experiment on rooting culture of apple T337

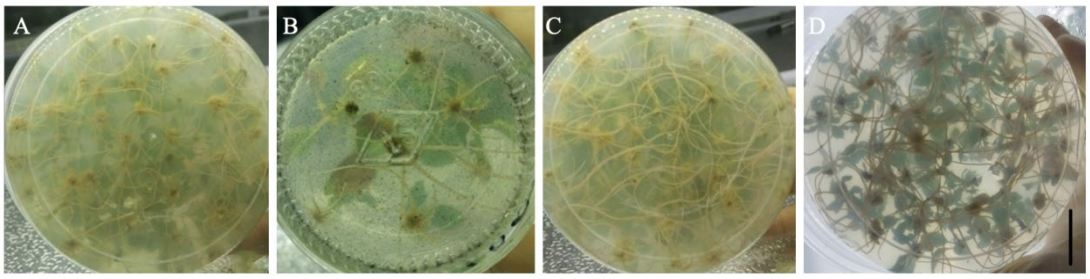
Order number	IBA(mg/L)	NAA(mg/L)	Saccharose (g/L)	The root rate is (%)	Equal root number (article)
1	0.60	0.02	20.00	92.12	12.81
2	0.60	0.04	25.00	89.56	10.47
3	0.60	0.06	30.00	60.14	8.86
4	0.80	0.02	25.00	69.38	9.69
5	0.80	0.04	30.00	71.53	12.23
6	0.80	0.06	20.00	65.35	10.06
7	1.00	0.02	30.00	73.09	11.52
8	1.00	0.04	20.00	64.87	8.71
9	1.00	0.06	25.00	68.85	8.53
K 1	241.82	234.59	222.34		
K2	206.26	225.96	227.79		
K3	206.81	193.84	204.76		
k1	80.61	78.20	74.11		
k2	68.75	75.32	75.93		
k3	68.94	64.61	68.25		
R	11.86	13.59	7.68		

3.5 Optimization of rooting formula for apple M26, GM256, and M9

Taking the rooting formula of T337 as a reference, the NAA concentration was very low, and only IBA was chosen for the experiment. Table 10 and Figure 4 shows that the rooting rates of combination 1 and combination 4 are 99.1% and 98.7%, respectively. The rooting quality is good, with an average of 6.3 and 6.9 roots, respectively. Other combinations also perform well. The optimal formula for production based on cost considerations is 1/2 WPM+IBA 0.3.

Table 10: Root rooting test results of apple M26, GM256, and M9

Assemble	Make up a prescription (mg/L)	The root rate is (%)	Number of root bars (bars)
1	1/2WPM+IBA0.6	99.1	6.3
2	1/2WPM+IBA0.5	85.2	3.4
3	1/2WPM+IBA0. 4	96.8	5.7
4	1/2WPM+IBA0.3	98.7	6.9



A.T337; B. GM256; C. M26; D. M9. Bar: 1cm.

Figure 4: Rooting situation of four apple dwarfing rootstocks in 1/2WPM+IBA0.3

3.6 Research on the domestication technology characteristics of apple dwarfing rootstock

The results of the orthogonal experiment on the domestication of apple dwarfing rootstock are shown in Table 11 and Figure 5. From the K value, it can be seen that the optimal combination for each observed trait is K1 survival rate: III-II-II; K2 growth trend: II-II-III and II-II-I-III; K3 neatness: II-I-II-II; K4 leaf color: I-II-II, II-II-I-II, II-I-II, and I-II-II; K5 angle, wax thickness: III-II-II; Production of K6 new roots: III-II-I-II.

The R value also indicates the degree of influence of each factor on each indicator. The survival rate of R1 indicates that the size of the influencing factors is: temperature>light>humidity>substrate. The R2 growth potential value indicates that the size of the influencing factors is: humidity>light intensity>substrate>temperature. The R3 uniformity value indicates that the size of the influencing factor is: substrate>light intensity>temperature>humidity. The R4 leaf color value indicates that the size of the influencing factors is: light intensity>humidity>substrate and temperature. The R5 angle and

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wax thickness values indicate that the size of the influencing factors is: light intensity>humidity>substrate>temperature. The production value of R6 new roots indicates that the size of the influencing factors is: light intensity>temperature>substrate>humidity.

Due to the different optimal combination values of each indicator, emphasis is placed on the three indicators of survival rate, uniformity, and new root production. In addition, because survival rate is more important than new root production and uniformity in selection, the best combination selected is III-II-II-II, which is vermiculite: peat: perlite=0.5:1.5:0.5. The temperature control is most suitable for seedling growth at 23-28 ℃. The humidity control gradually decreases from high humidity to quickly adapt to the natural blank environment, and the light control gradually increases from low light to better adapt to the natural blank environment for cultivation. Other factor levels can be addressed according to the problems that arise in production.

Table 11: Results of Orthogonal Experiment on Comprehensive Domestication of Apple Dwarf Rootstock

Assemble	Base material	Temperature	Humidity	Light intensity	Rate of survival%	Growth potential	In order	Leaf colour	The horns and wax are thick	New root production
①	I	I	I	I	37	3.8	2.0	2.7	1.5	3.4
②	I	II	II	II	90	4.4	2.0	2.8	1.6	3.9
③	I	III	III	III	41	2.7	1.5	2.2	1.2	2.5
④	II	I	II	III	58	4.2	1.9	2.5	1.3	3.5
⑤	II	II	III	I	63	3.0	1.8	2.3	1.4	2.8
⑥	II	III	I	II	57	4.5	2.0	2.9	1.7	3.8
⑦	III	I	III	II	64	3.1	2.0	2.5	1.6	2.8
⑧	III	II	I	III	75	4.0	1.8	2.6	1.5	3.6
⑨	III	III	II	I	60	3.7	1.7	2.3	1.5	3.8
K1	56%	53%	56%	53%						
	59%	76%	69%	70%						
	66%	53%	56%	58%						
K2	3.63	3.7	4.1	3.5						
	3.9	3.8	4.1	4						
	3.6	3.63	2.93	3.63						
K3	1.83	1.97	1.70	1.83						
	1.90	1.87	1.87	2.00						
	1.83	1.73	1.77	1.73						
K4	2.57	2.57	2.73	2.43						
	2.57	2.57	2.53	2.73						
	.47	2.47	2.67	2.43						
K5	1.43	1.47	1.57	1.47						
	1.47	1.5	1.47	1.63						
	1.53	1.47	1.4	1.33						
K6	3.27	3.23	3.63	3.33						
	3.37	3.43	3.73	3.53						
	3.40	3.40	3.70	3.20						
R1	0.11	0.23	0.13	0.17						
R2	0.30	0.17	1.07	0.5						
R3	0.67	0.23	0.17	0.27						
R4	0.10	0.10	0.20	0.30						
R5	0.10	0.03	0.17	0.30						
R6	0.13	0.20	0.10	0.33						

A.T337; B. GM256; C. M26; D. M9. Bar: 1cm.

Figure 5: Domestication of Four Apple Dwarf Rootstock Combinations III-II-II

4. Conclusion and discussion

The selection of explants is one of the key factors for the success of tissue culture. The age,

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developmental stage, growth environment, selection site, and time of explants may all lead to differences in their physiological and biochemical status, thereby affecting the experimental process and tissue culture seedling morphology in subsequent stages. This experiment selected non germinated branches that had passed the dormancy period as explants, and the result showed a high contamination rate. The main reason for this may be that they belong to woody plants, and the stem segments contain a large number of endophytic bacteria. The sterilization time and degree are crucial for tissue culture experiments. If the sterilization time is too short, the endophytic bacteria in the explant cannot be removed, which affects the growth and development of the material. However, if the sterilization time is too long, it is easy to cause the explant to die. In this experiment, a mixture of 70% alcohol for 12 seconds, 0.1% mercuric chloride, and Tween was washed 10 times with sterile water for 7 minutes. The success rate of T337 was 13%, while the success rates of M26, GM256, and M9 were 40%. The same sterilization method resulted in different genotypes and sterilization effects depending on the variety.

Basic culture medium can only ensure the minimum physiological activity for the survival of the culture, and only with the use of appropriate plant growth regulators can the growth of explants be induced. In this experiment, the effect of IBA+6-BA on bud proliferation was significantly higher than that in MS medium, which is consistent with the conclusion of Han Xiuqing [4] that the combination of cytokinin and auxin has a good proliferation effect on apple 'M9-T337'.

Rooting cultivation is the key to in vitro rapid propagation, which directly affects the success or failure of factory breeding. During the rooting culture of 'M9T337' test tube seedlings, Han Xiuqing found that IBA0.1 mg/L was beneficial for the rooting and growth of 'M9T337'. This study found that at 1/2 WPM+IBA 0.3-0.6 mg/L, the rooting effect of test tube seedlings was good. At IBA 0.3 mg/L, the rooting rate reached 98.7, with an average of 6.3 roots, which is consistent with previous studies.

This study found that the domestication substrate perlite: vermiculite: peat=1:1:3, temperature controlled at 23-28 ℃, humidity gradually decreased from high humidity, and light gradually increased from low light to adapt to natural blank environment, is most suitable for seedling growth and is more conducive to production application and variety promotion. The main factors affecting the domestication of tissue culture seedlings are temperature, humidity, light, and substrate, which are basically consistent with the domestication and transplantation process of apple dwarfing rootstocks' M9-T337 'and' M26 'by Wang [5] and Liu [6]. This study provides technical support for better transplantation domestication and industrial production.

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