Egypt. J. of Appl. Sci., 40 (9-10) 2025 *1-16*

IN VITRO MORPHOLOGICAL AND GENETICALLY STUDIES VARIATION USING DIFFERENT MEDIUM CONCENTRATION AND PHYTOHORMONES IN APPLE ROOTSTOCK (*MALUS DOMESTICA* BORKH)

Elsayh S.A. A. ;Y.S.G. Abd Elaziz;Y. A. Abdel Mageed;
H.K.M. Ewes and Eman H. Afifi*
Fruit and Ornmental Breeding Department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt
***E-mail-emanheay@gmail.com**

ABSTRACT

The apple (*Malus domestica* Borkh) is among the most economically significant fruit crops worldwide, and the development of efficient micropropagation protocols is essential for the production of healthy and uniform planting materials. Optimizing culture conditions for different apple rootstocks is particularly important to improve establishment, regeneration, multiplication, shoot elongation, and rooting efficiency.

In the present study, leaves from two apple rootstocks trees (MM106 and Balady), explants were utilized and were grown on MS media at three distinct mineral strengths (½X, ¾X, and 1X). The establishment stage was carried out using ¾ MS medium supplemented with 0.1 mg/L Benzyl Amino Purine (BAP) and 0.1 mg/L Indole Butyric Acid (IBA). Kinetin & BAP both were at 0.3 mg/l, 0.2 mg/l for regeneration, multiplication and shoot elongation. For the rooting stage, IBA and Naphthalene Acetic Acid (NAA) were applied at 0.5 mg/L under the three strength medium.

Results revealed that the X medium was the most effective for regeneration, multiplication, and shoot elongation of both rootstocks, followed by ½X and 1X. On average, the ¾X medium promoted regeneration (47.50%), multiplication (16.50%), and shoot elongation (5.98 cm) in both rootstocks compared to other media. Among the cytokinins tested, BAP proved superior to Kinetin, which significantly enhanced, regeneration, multiplication, and shoot elongation. MM106 exhibited a higher response to cytokinins in all growth stages compared to Balady. For rooting, IBA was more effective than NAA, inducing higher rooting percentages and greater numbers of roots. The optimal rooting reaction was achieved with ¾X medium augmented by 0.5 mg/L IBA, which produced 17.00% rooting, with an average of 5.40 cm of root length and 7.67 roots/plant. To prove genetic stability, the cultivated plants were compared to the mother plant using ISSR.

Key Words: Apple, Rootstocks, Micropropagation, Cytokinins, Auxins, Genetic Stability.

INTRODUCTION

Among the most significant temperate fruit crops is the apple (*Malus domestica* Borkh.) cultivated worldwide, contributing significantly to global fruit production and human nutrition. Its high content of antioxidants, vitamins, and dietary fiber makes it not only a valuable dietary component but also an economically strategic commodity for both fresh consumption and processed products (**Nawaz *et al.*, 2020**). In addition, the successful establishment of orchards depends heavily on the use of high-quality rootstocks, which regulate tree vigor, enhance adaptability to diverse environmental conditions, and improve fruit yield and quality (**Al-Khayri *et al.*, 2023**). Therefore, developing efficient and reliable methods for apple rootstock propagation is a critical step toward sustainable fruit production.

Conventional propagation methods such as grafting, budding, and cuttings often suffer from limitations, including low multiplication rates, seasonal dependency, and the risk of disease transmission. By contrast, plant tissue culture offers a powerful alternative, allowing the production of large numbers of genetically uniform and pathogen-free plants in a relatively short time (**Isah, 2019 and Al-Khayri *et al.*, 2023**). For apple rootstocks such as MM106 and Balady, micropropagation provides a means of overcoming the challenges of traditional propagation while ensuring the continuous supply of planting material. Optimization of culture media and plant growth regulators is, therefore, essential to enhance establishment, regeneration, and elongation stages *in vitro* (**Liu *et al.*, 2022**).

Plant growth regulators, particularly cytokinins and auxins, play a pivotal role in morphogenesis during micropropagation. Cytokinins such as BAP and Kinetin are essential for cell division, induction of bud, and shoot proliferation, with BAP generally reported as more effective than Kinetin for regeneration and shoot elongation in woody species, including apple (**Bairu *et al.*, 2019 and He *et al.*, 2021**). At the establishment and multiplication stages, the application of BAP at moderate concentrations has been shown to significantly enhance regeneration frequencies, multiplication rates, and shoot elongation (**He *et al.*, 2021 and Wang *et al.*, 2024**). Moreover, MM106 rootstock exhibited greater responsiveness to cytokinins than Balady, highlighting the genotype-dependent nature of cytokinin action (**Liu *et al.*, 2022**).

The rooting stage is equally crucial for the successful acclimatization and survival of micropropagated plants. Auxins, particularly (IBA) and (NAA), are the most often used regulators for root induction in woody species. Several studies have confirmed that IBA is generally more effective than NAA in stimulating root initiation, increasing root numbers, and producing longer and healthier roots due to its higher stability and efficiency (**Isah, 2019 and Liu *et al.*, 2022**). However, ¾-strength IBA added with 0.5 mg/L in MS medium induced the highest rooting percentage (17.00%), an average of 7.67 roots/plant, and a length of root 5.40 cm. These findings agree with recent reports

emphasizing the importance of optimizing both mineral strength and auxin application for successful *in vitro* rooting in apple rootstocks (**Liu *et al.*, 2022 and El-Mahdy *et al.*, 2025**).

Collectively, these results reinforce the significance of fine-tuning mineral nutrient strength and the balance of cytokinins and auxins at different developmental stages of micropropagation. They also highlight the potential of ¾-strength MS medium combined with BAP and IBA as a trustworthy method for effective apple propagation rootstocks, ensuring higher regeneration, multiplication, elongation, and rooting efficiency, which are vital for orchard establishment and productivity (**Wang *et al.*, 2024 and El-Mahdy *et al.*, 2025**).

Genetic stability assessment is essential in plant tissue culture to ensure that regenerated plantlets remain true-to-type, as in vitro conditions may induce somaclonal variation. This is particularly important for apple (*Malus domestica* Borkh.) rootstocks such as Balady and MM106, which are widely used for propagation and breeding programs. Markers for Inter Simple Sequence Repeats (ISSRs) are highly efficient in detecting genetic variability and have been successfully used to confirm clonal fidelity in several woody species, including Malus (**Singh *et al.*, 2020 and Zhou *et al.*, 2021**). Recent studies confirmed that ISSR markers are reliable for evaluating genetic uniformity in micropropagated plants (**Al-Qurainy *et al.*, 2022 and Li *et al.*, 2024**). Therefore, in the present study, ISSR markers were employed to verify the genetic stability between Balady and MM106 rootstocks regenerated through tissue culture to ensure the integrity of the developed micropropagation protocol.

This study highlights the importance of adjusting medium mineral strength and plant growth regulators for efficient *in vitro* propagation of apple rootstocks, with ¾X MS medium and BAP (0.2 mg/L) for growth of shoots and IBA (0.5 mg/L) for rooting as the most effective treatments.

MATERIALS AND METHODS

This research was performed at the Fruit and Ornamental Breeding Department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt, during the 2024 season. All experiments were carried out under controlled *in vitro* conditions to assess the impacts of different mineral strengths and plant growth regulators (PGRs) regarding on callus initiation, regeneration, shoot multiplication, elongation, and rooting of apple rootstocks MM106 and Balady.

1. Plant material and explant preparation

Leaves were gathered from four-year-old apple rootstocks (MM106 and Balady). The leaves were meticulously cleansed under running tap water, thereafter subjected to surface sterilization in 70% ethanol for 10 min, and then immersed in 20% sodium hypochlorite solution supplemented with 150 mg/L ascorbic acid for 20 min. Finally, the explants were rinsed four times with sterile distilled water containing 150 mg/L ascorbic acid to minimize browning and oxidative damage.

2. Excision procedure

Expanded leaves (10–12 mm in length) were excised by removing the margins. Each leaf was cut transversely through the midrib into two portions and further dissected into small pieces (approximately 0.3 cm). The explants were placed with the adaxial surface in contact with MS medium supplemented with different combinations of PGRs (**Murashige and Skoog, (1962)** as described in Table (1).

3. Establishment stage

For initial culture, explants were placed on ¾ MS medium. The basal establishment medium (BEM) contained ¾ MS vitamins, 3% sucrose, 0.1 mg/L myo-inositol, 0.1 mg/L thiamine-HCl, and 0.7% agar. The pH was adjusted to 5.8 before autoclaving. PGR combinations included 0.1 mg/L BAP and 0.1 mg/L IBA.

4. Regeneration stage

Calli obtained from the establishment stage were transferred to basal regeneration medium (PRM) containing MS salts at the same three mineral strengths (½X, ¾X, and 1X) augmented by 0.2 mg/L BAP or 0.3 mg/L Kinetin (Table 1). The number of shoots regenerated/callus was reported one month after transfer.

5. Shoot multiplication and elongation stage

Regenerated shoots were cultured on basal proliferation medium (BPM) with MS salts at three mineral strengths (½X, ¾X, and 1X) augmented by 0.2 mg/L BAP or 0.3 mg/L Kinetin (M1, M2, M3; Table 1). Media also contained MS vitamins, 3% sucrose, 0.1 mg/L myo-inositol, 0.1 mg/L thiamine-HCl, and 0.7% agar. The pH was adjusted to 5.8. Shoots of 0.8–1.0 cm in length were positioned vertically on the medium and subcultured at four-weeks intervals. Percentage of shoot multiplication rate (shoots/explant per month) and shoot elongation (cm) were measured after 30 days of culture.

6. Root induction stage

Uniform shoots of 2–3 cm in length were excised and transferred to basal rooting medium (BRM) containing MS salts at three mineral strengths (½X, ¾X, and 1X), supplemented with either 0.5 mg/L IBA or 0.5 mg/L NAA (R1, R2, R3; Table 1). The medium also contained MS vitamins, 1% sucrose, 0.1 mg/L myo-inositol, 0.1 mg/L thiamine-HCl, and 0.7% agar (pH 5.8). Rooting percentage, mean number of roots/plantlet, and root length (cm) were reported after 30 days.

7. The genetic stability

To evaluate the genetic stability of regenerated shoots of apple rootstocks ‘Balady’ and ‘MM106’, Inter-Simple Sequence Repeat (ISSR) markers were employed. This molecular approach was aimed to assess whether the *in vitro* culture process induced any soma clonal variation by comparing regenerated plantlets with their corresponding mother plants.

Table 1. Media composition used for establishment, regeneration, multiplication, and rooting stage of apple rootstocks (MM106 and Balady).

Stage	Mineral concentration (MS salts)	Plant growth regulators (mg/L)	Other constituents
Establishment	¾X	0.01 IBA + 0.1 BAP	MS vitamins, 3% sucrose, 0.1 myo-inositol, 0.1 thiamine-HCl, 0.7% agar; pH 5.8
Regeneration	½X, ¾X, 1X	0.2 BAP or 0.3 Kinetin	MS vitamins, 3% sucrose, 0.1 myo-inositol, 0.1 thiamine-HCl, 0.7% agar; pH 5.8
Multiplication & elongation	½X, ¾X, 1X	0.2 BAP or 0.3 Kinetin	MS vitamins, 3% sucrose, 0.1 myo-inositol, 0.1 thiamine-HCl, 0.7% agar; pH 5.8
Rooting	½X, ¾X, 1X	0.5 IBA or 0.5 NAA	MS vitamins, 1% sucrose, 0.1 myo-inositol, 0.1 thiamine-HCl, 0.7% agar; pH 5.8

Mineral concentrations: 1/2X, 3/4X (low), 1X (equal) basal MS-medium

BAP 6-benzylaminopurine
KI Kinetin
IBA Indol butyric acid
NAA Naphthalene acetic acid

PCR Amplification Making Use of ISSR Primers

Young leaves of the mother plants and the *in vitro* regenerated plants were use to obtain genomic DNA shoots using the CTAB method **Doyle and Doyle (1990)** with minor modifications. DNA quality and concentration were checked spectrophotometrically and by agarose gel electrophoresis.

The ISSR-PCR reactions were carried out in a 25 µL total volume with the following ingredients: 1.0 U Taq DNA polymerase (Thermo Fisher Scientific, USA), 0.2 mM of each dNTP, 0.7 µM primer, 1× PCR buffer, 1.5 mM $MgCl_2$, and 25 ng template DNA. The PEQStar 96 Universal Gradient Thermal Cycler (PEQLAB, Germany) was used for the amplifications.

The amplification profile consisted of 40 cycles of denaturation at 94°C for 30 s, primer annealing for 45 s at the precise temperature specified in 60°C, and extension at 72°C for 2 min were performed after the initial denaturation at 94°C for 4 min. For seven minutes, the last extension was carried out at 72°C. Electrophoresis was used to separate amplified DNA fragments in 1.5% agarose gels prepared with 1× TAE buffer and stained with FluoroDye DNA stain. The gels were visualized and photographed using a Uvitec Geldoc imaging system (Uvitec, Cambridge, UK). This part was done at the Genetic Engineering Institute at the Agricultural Research Center.

8. Experimental design and statistical analysis

All experiments were arranged in a randomized complete design (RCD). Each treatment consisted of 10 explants per replicate, with three replicates per

treatment. Rooting experiments were repeated three times, each with 10 plantlets/ medium. Data were analyzed using the MSTAT-C statistical software **(MSTAT-C, 1990)**, and treatment means were compared to evaluate the effects of mineral strength and PGRs on regeneration, multiplication, elongation, and rooting responses.

Cultures were incubated at 25 ± 2°C during a 16-hour photoperiod with a light intensity of approximately 2000 lux supplied by cool white fluorescent bulbs. Except for rooting stage (first 4 days in the dark at 22 °C) Subcultures were performed every 30 days.

RESULTS AND DISCUSSION

1. Impact of medium composition and different growth regulators on regeneration

The composition of the culture medium and the type of plant growth regulator (PGR) are critical factors influencing plant regeneration. As shown in Table 2 and Fig. 1, the basal regeneration medium (BRM) did not show significant differences between the two apple rootstocks, with regeneration percentages ranging from 38.33% to 47.50%. However, the kind and amount of cytokinins had a pronounced effect.

In Balady, regrowth ranged from 35.33% to 47.00% with an average of 41.56%, while in MM106 it ranged from 41.33% to 48.00% with a mean of 44.33%. The highest regeneration frequency was obtained on ¾ MS salts added to with 0.2 mg/L BAP, which produced 47.00% regeneration in Balady and 48.00% in MM106. In comparison, ½ MS + 0.2mg/L BAP and full-strength MS + 0.3 mg/L kinetin resulted in lower regeneration (Balady: 43.67%, 40.33%; MM106: 44.33%, 44.00%).

In contrast, when BAP was applied, regeneration was significantly reduced. The combination of ¾ MS + 0.3 mg/L kinetin resulted in only 43.33% regeneration in Balady and 45.67% in MM106, while ½ MS + 0.2 mg/L BAP and 1X MS + 0.3 mg/L kinetin resulted in much lower values (Balady: 37.67% and 35.33%; MM106: 42.67% and 41.33%).

These findings indicate that ¾-strength MS medium combined with BAP is more effective than kinetin in promoting regeneration of apple rootstocks, consistent with recent studies highlighting the superior role of BAP over kinetin in shoot organogenesis and regeneration across various fruit crops (**Abdelsalam *et al.*, 2018 ; Ramírez-Mosqueda & Iglesias-Andreu, 2021; Abbas *et al.*, 2022; Sharma *et al.*, 2023 and Singh *et al.*, 2024**).

2. Effect of medium composition and different growth regulators on shoot multiplication

Shoot multiplication in apple rootstocks was significantly influenced by both mineral salt concentration and cytokinin type. As shown in Table 2 and Fig. 1, the use of BAP at 0.2 mg/L resulted in a markedly higher multiplication rate compared to kinetin across both genotypes. The multiplication rate varied between 11.33% and 16.50%, with MM106 consistently showing a higher response than Balady.

In Balady, shoot multiplication ranged between 8.33% and 14.00% with an average of 11.39%, whereas MM106 exhibited values from 14.33% to 19.00% with a mean of 16.00%. The best performance was achieved on ¾ MS salts + 0.2 mg/L BAP, yielding 14.00% in Balady and 19.00% in MM106, which was superior to both ½ MS and full-strength MS added to with the same concentration of BAP (Balady: 12.33%, 11.00%; MM106: 16.00%, 15.67%, respectively).

Conversely, kinetin at the concentration (0.3 mg/L) resulted in lower multiplication rates and produced shorter shoot clusters. On ¾ MS + kinetin, multiplication reached 12.67% in Balady and 16.33% in MM106, but declined further with ½ MS or full-strength MS supplemented with kinetin (Balady: 10.00%, 8.33%; MM106: 14.64%, 14.33%).

These results demonstrate that BAP is significantly more effective than kinetin for stimulating shoot multiplication in apple rootstocks, particularly at moderate mineral concentrations (¾ MS). Similar findings have been reported in recent studies where BAP was shown to promote axillary shoot proliferation more efficiently than kinetin in apple and other woody fruit crops (**Khan *et al.*, 2019; Alam *et al.*, 2020; Yadav *et al.*, 2021; Singh *et al.*, 2022 and Hussain *et al.*, 2023**).

3. Effect of medium composition and different growth regulators on shoot elongation

Results presented in Table 2 and Fig. 1 indicate that shoot elongation in both apple rootstocks (Balady and MM106) was moderately affected by medium strength and cytokinin type. The elongation length varied from 4.52 cm to 5.98 cm across treatments, showing genotype-dependent differences.

In Balady, elongation ranged from 4.43 cm to 5.83 cm with a mean of 4.10 cm, while in MM106 it ranged from 4.33 cm to 6.13 cm, averaging 5.11 cm. The best elongation response was achieved with ¾ MS salts added to with 0.2 mg/L BAP, which produced shoots of 5.83 cm in Balady and 6.13 cm in MM106. By contrast, ½ MS + 0.2 mg/L BAP and full-strength MS + 0.2 mg/L BAP resulted in shorter shoots (Balady: 4.83 cm and 4.43 cm; MM106: 4.90 cm and 4.77 cm).

Similarly, BAP at 0.2 mg/L significantly promoted elongation of adventitious shoots, whereas kinetin 0.3 mg/L at concentration resulted in reduced shoot length. For example, on ¾ MS + 0.3 mg/L kinetin, Balady and MM106 shoots reached 5.20 cm and 5.67 cm, respectively. While lower elongation was observed in ½ MS + 0.3 mg/L kinetin and 1X MS + 0.3 mg/L kinetin (Balady: 4.43 cm, 4.70 cm; MM106: 4.87 cm, 4.33 cm).

These results clearly demonstrate that BAP was superior to kinetin for promoting shoot elongation in apple rootstocks, particularly when combined with a moderate mineral concentration (¾ MS). Similar trends have been reported in recent studies, where BAP was consistently found to enhance both shoot length and vigor in fruit trees compared to kinetin or other cytokinins (**Al-Mayahi, 2019; Shukla *et al.*, 2020; Zahir *et al.*, 2021; Mehta & Sharma, 2022 and Ali *et al.*, 2024**).

4. Effect of medium composition and different growth regulators on rooting percentage, number of roots/plant, and root length

The results presented in Table 2 and Fig. 1 demonstrated that rooting response in apple rootstocks (Balady and MM106) was strongly influenced by both auxin type and medium composition. The optimal concentration of auxin required for rooting was explant-dependent, with IBA being more effective than NAA in promoting root induction.

Table 2. Effect of medium composition and different growth regulators on regeneration, multiple shoot, shoots elongation, rooting, number root and root length from Apple leaf Anna and Balady.

Character	MediumA	Rootstock B			
		MM106	Balady	Mean	L.S.D
Regeneration %	1/2X+0.2mg/l BAP	44.33	43.67	44.00	A=NS B=1.46 AB=2.07
	3/4X+0.2mg/l BAP	48.00	47.00	47.50	
	1X+0.2mg/l BAP	44.00	40.33	42.17	
	1/2X+0.3mg/l Kin	42.67	37.67	40.17	
	3/4X+0.3mg/l Kin	45.67	43.33	45.50	
	1X+0.3mg/l Kin	41.33	35.33	38.33	
	Mean	44.33	41.56		
Multiplication %	1/2X+0.2mg/l BAP	16.00	12.33	14.17	A=3.11 B=0.65 AB=0.92
	3/4X+0.2mg/l BAP	19.00	14.00	16.50	
	1X+0.2mg/l BAP	15.67	11.00	13.33	
	1/2X+0.3mg/l Kin	14.64	10.00	12.33	
	3/4X+0.3mg/l Kin	16.33	12.67	14.50	
	1X+0.3mg/l Kin	14.33	8.33	11.33	
	Mean	16.00	11.39		
Shoot elongation (length) cm	1/2X+0.2mg/l BAP	4.90	4.83	4.87	A=NS B=0.44 AB=NS
	3/4X+0.2mg/l BAP	6.13	5.83	5.98	
	1X+0.2mg/l BAP	4.77	4.43	4.60	
	1/2X+0.3mg/l Kin	4.87	4.43	4.65	
	3/4X+0.3mg/l Kin	5.67	5.20	5.43	
	1X+0.3mg/l Kin	4.33	4.70	4.52	
	Mean	5.11	4.10		
Rooting %	1/2X+0.5mg/lIBA	15.00	10.33	12.67	A=2.39 B=0.63 AB=0.90
	3/4X+0.5mg/lIBA	17.00	13.00	15.00	
	1X+0.5mg/lIBA	14.33	8.33	11.33	
	1/2X+0.5mg/lNAA	13.67	7.33	10.50	
	3/4X+0.5mg/lNAA	15.00	11.33	13.17	
	1X+0.5mg/lNAA	13.00	6.33	9.67	
	Mean	14.67	9.44		
Root No./Plantlets %	1/2X+0.5mg/lIBA	5.67	6.00	5.83	A=NS B=0.52 AB=NS
	3/4X+0.5mg/lIBA	7.67	6.33	7.00	
	1X+0.5mg/lIBA	4.67	4.67	4.67	
	1/2X+0.5mg/lNAA	4.33	4.67	4.50	
	3/4X+0.5mg/lNAA	6.33	5.33	5.83	
	1X+0.5mg/lNAA	4.00	4.00	4.00	
	Mean	5.44	4.79		
Root length cm	1/2X+0.5mg/lIBA	4.03	3.90	3.97	A=NS B=0.40 AB=NS
	3/4X+0.5mg/lIBA	5.40	4.23	4.82	
	1X+0.5mg/lIBA	4.17	3.40	3.78	
	1/2X+0.5mg/lNAA	4.30	3.23	3.32	
	3/4X+0.5mg/lNAA	4.90	4.03	4.97	
	1X+0.5mg/lNAA	4.17	3.23	3.70	
	Mean	4.54	3.67		

Mineral concentrations: Low (1/2X), (3/4X), equal (1X), basal MS-medium; regeneration Multiplication, (explants/month) and shoot elongation (cm) in the medium contain 0.2mg/l BAP or 0.3mg/l kin., and root formation in the medium contain 0.5mg/l IBA or 0.5mg/l NAA.

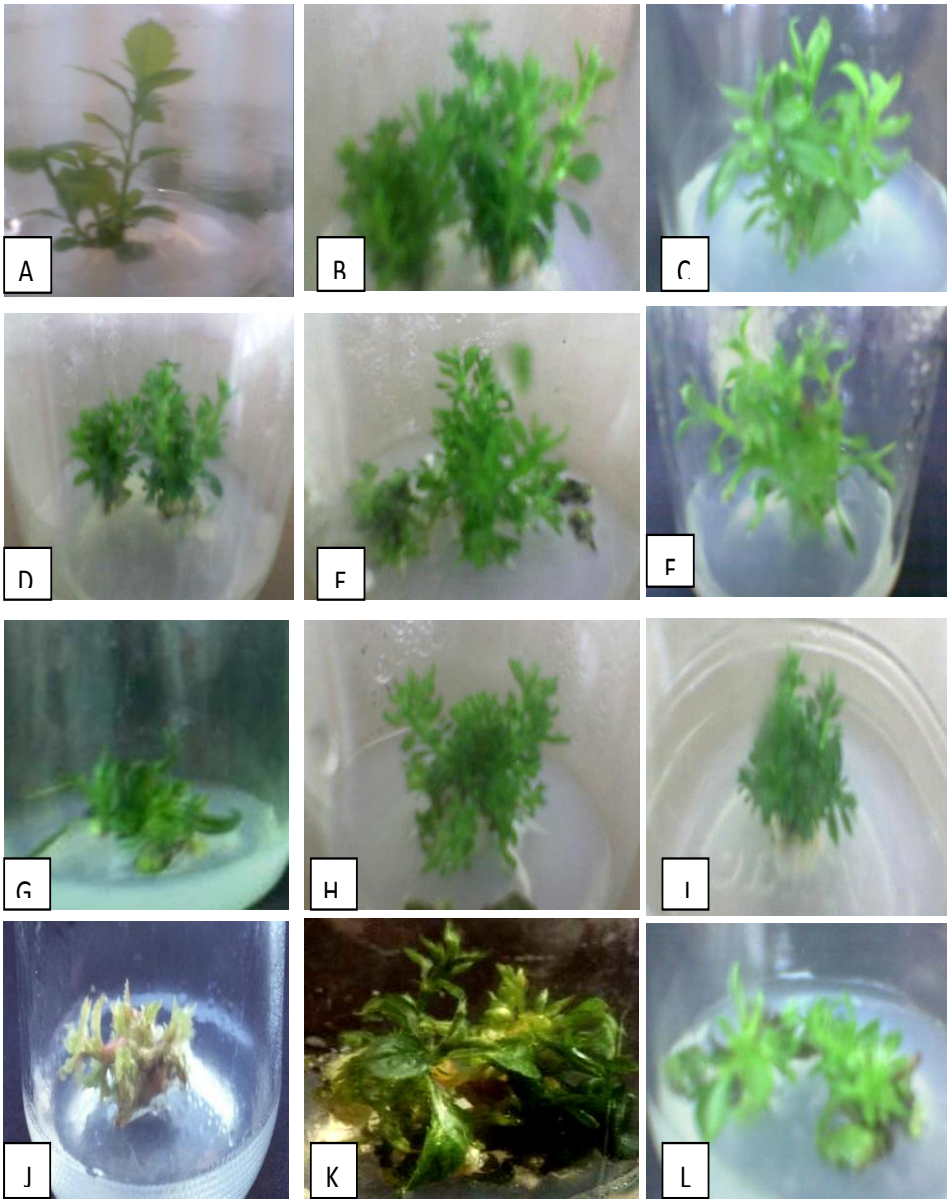

Figure 1. Different potential of shoot multiplication (No/explants) and shoot elongation in apple genotypes (leaf MM106 and Balady) were cultured on the 1/2X, 3/4X and 1X basal MS medium contain (BAP 0.2 mg/l A, B and C) and (Kinetin 0.3 mg/l E, F and G) for shoot elongation six months after culture.

Overall rooting percentage varied between 9.67% and 15.00% across genotypes, with a mean of 9.44% in Balady and 14.67% in MM106. Root induction was maximized on ¾ MS medium added to with 0.5 mg/L IBA, which produced the highest rooting percentages (13.00% for Balady and 17.00% for MM106). This suggests that MM106 requires a slightly higher auxin level for efficient rooting, possibly due to differences in endogenous auxin content compared with Balady.

Analysis of root number showed no significant difference between genotypes, with values ranging from 4.00 to 7.00 roots/explant. However, treatment effects were significant, as root numbers ranged from 4.00 to 6.33 (mean 4.79) in Balady and 4.00 to 7.67 (mean 5.44) in MM106.

Root length was also moderately influenced by treatments. There were no significant differences between genotypes, with values ranging from 3.32 cm to 4.97 cm. However, treatment-specific effects were evident: Balady roots varied from 3.23 cm to 4.23 cm (mean 3.67 cm), while MM106 roots ranged from 4.17 cm to 5.40 cm (mean 4.54 cm).

These findings confirm that IBA is more efficient than NAA in inducing root primordia and elongation, and that a moderate reduction of mineral concentration (¾ MS) further enhances rooting efficiency. Similar results were reported in recent studies on apple and other woody fruit crops, where IBA consistently outperformed NAA for root induction, both in terms of root number and length (**Naaz *et al.*, 2019; El-Shamy *et al.*, 2020; Ibrahim *et al.*, 2021; Panda *et al.*, 2022; Mohapatra *et al.*, 2023 and Chen *et al.*, 2024**).

5. Explant medium composition and different growth regulators

The results presented in Table 2 revealed a strong interaction between explants type (Balady vs. MM106 leaves), medium composition, & growth regulators on regeneration, shoot multiplication, elongation, and rooting parameters. This interaction was particularly evident in regeneration and rooting responses.

The highest regeneration rate was captured on ¾ MS media that was enhanced with 0.2 mg/L BAP, where MM106 leaves produced 48.00% regenerated shoots, while Balady leaves produced 47.00%. In contrast, when BAP was replaced with kinetin, regeneration decreased: 43.33% for Balady and 45.67% for MM106, highlighting the superior role of BAP over kinetin in promoting organogenesis.

Shoot elongation was not significantly different between explants, with Balady producing 5.83 cm and MM106 6.13 cm. However, rooting exhibited a clear explants-dependent response: Balady achieved 13.00% rooting, while MM106 reached 17.00% under ¾ MS medium supplemented with 0.5 mg/L IBA. Number of roots per explants and root length did not show significant differences between genotypes, although MM106 consistently produced slightly higher values.

These findings suggest that the interaction of BAP (for regeneration and elongation) with IBA (for rooting) is critical for optimizing in vitro

culture responses in apple rootstocks, with MM106 generally outperforming Balady in most growth traits. Recent studies confirm that explant–hormone–medium interactions are decisive in modulating morphogenesis and rooting efficiency in apple and other woody perennials (**Naaz *et al.*, 2019; Singh *et al.*, 2020; Ibrahim *et al.*, 2021; Panda *et al.*, 2022; Mohapatra *et al.*, 2023 and Chen *et al.*, 2024**).

6. Genetic Stability for Balady and MM106 Rootstocks Using ISSR Markers

The ISSR analysis confirmed a high degree of genetic similarity for Balady and MM106 with mother plant apple rootstocks regenerated through tissue culture (Figure 2) . The monomorphic banding patterns obtained indicate that the *in vitro* propagation protocol maintained the genetic integrity of the plantlets without inducing detectable somaclonal variation. These findings suggest that the hormonal combinations and culture conditions used were genetically stable and did not affect the genomic structure of the regenerated plants. Similar results were reported by **Zhou *et al.*, (2021)**, who observed no polymorphism among Malus domestica plantlets regenerated via shoot proliferation, confirming the clonal fidelity of micropropagated lines. Moreover, **Li *et al.*, (2024)** demonstrated that ISSR and SCoT markers are highly effective in distinguishing true-to-type apple regenerants from somaclonal variants. The high genetic uniformity found in this study supports the suitability of Balady and MM106 rootstocks for commercial micropropagation and breeding purposes. These results align with previous studies on other woody perennials, confirming that ISSR markers provide a reliable molecular tool for assessing genetic stability in micropropagated plant materials **(Singh *et al.*, 2020 and Al-Qurainy *et al.*, 2022)**.

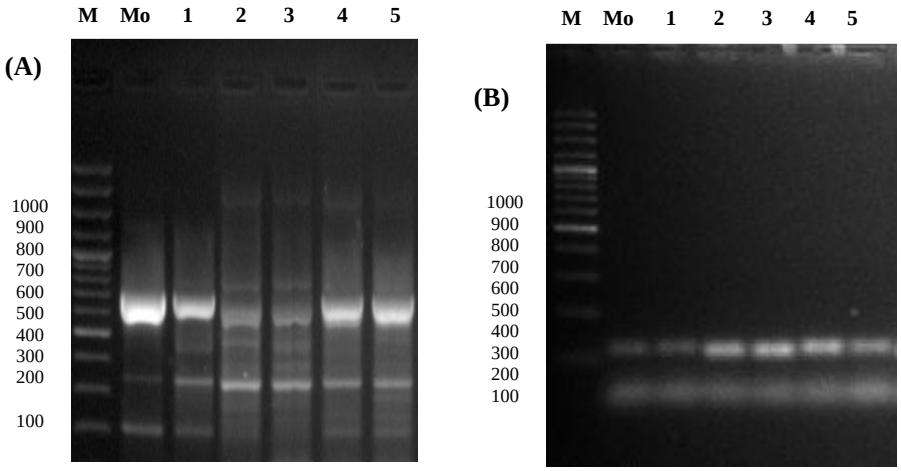

Figure 2. ISSR banding patterns showing genetic stability for two apple rootstocks (Balady (A) and MM106 (B)) after *in vitro* culture with mother plant use 5 primers. M: DNA marker, Mo: Mother plant

CONCLUSIONS

The present study demonstrated that medium composition, cytokinin type, and auxin application significantly influenced *in vitro* regeneration, shoot multiplication, elongation, and rooting of apple (*Malus domestica* Borkh) rootstocks Balady and MM106. Among the tested treatments, ¾ MS medium supplemented with BAP, proved to be the most effective for regeneration and shoot proliferation. While kinetin was less efficient and often produced shorter, weaker shoots. Shoot elongation was enhanced by BAP, particularly in MM106, confirming its superior response compared with Balady.

For rooting, IBA (0.5 mg/L) in ¾ MS medium induced the highest percentage of rooted plantlets, number of roots, and root length, with MM106 again showing greater rooting efficiency than Balady. This suggests that auxin efficiency is genotype-dependent, with MM106 possessing a higher rooting potential, possibly due to intrinsic auxin sensitivity.

The strong interaction between explants type and medium composition highlighted that optimization of cytokinin–auxin combinations is essential for each genotype. Overall, the combination of BAP for regeneration and multiplication, and IBA for rooting provided the most reliable protocol for plantlet development.

These findings provide a practical and reproducible tissue culture protocol for the micropropagation of apple rootstocks. Such optimized systems not only facilitate the rapid multiplication of elite and locally adapted genotypes (Balady and MM106) but also serve as a foundation for future applications in genetic transformation, germplasm conservation, and large-scale propagation of disease-free planting material to support sustainable apple production.

Future Perspectives

Future research should focus on refining the current protocol by integrating advanced approaches such as temporary immersion bioreactor systems to enhance large-scale shoot multiplication and reduce production costs. Additionally, combining this optimized regeneration system with modern gene-editing tools (e.g., CRISPR/Cas9) may accelerate the development of improved apple rootstocks with enhanced stress tolerance, disease resistance, and better adaptability to climate change. Further studies on the acclimatization phase under greenhouse and field conditions are also recommended to improve survival rates of regenerated plantlets. Overall, the outcomes of this study provide a solid platform for both commercial propagation and future biotechnological applications in apple breeding programs.

REFERENCES

Abbas, H. ; M.A. Khan ; S. Saeed ; M. Arif ; N. Akhtar and A. Nazir (2022). Comparative efficacy of BAP and kinetin on micropropagation of woody fruit plants under different salt strengths of MS medium. Plant Cell, Tissue and Organ Culture, 149: 325–336.

Abdelsalam, N.R. ; E.E. Kandil ; H. Almoammar ; M.O. Alotaibi ; K.F. Almutairi and E.A. Kamel (2018). Influence of plant growth regulators on *in vitro* organogenesis and biochemical traits of apple rootstocks. Plant Cell, Tissue and Organ Culture, 134(1): 149–160.

Alam, M.J. ; M.S. Rahman, and M. Hossain (2020). Optimization of cytokinin combinations for enhanced *in vitro* shoot multiplication of apple. Biologia Plantarum, 64: 745–753.

Ali, H. ; A. Yadav and V. Singh (2024). Advances in the use of cytokinins for shoot elongation and micropropagation of apple and other fruit trees. Frontiers in Plant Sci., 15: 1267543.

Al-Khayri, J.M. ; P.M. Naik ; D. Johnson ; K. Ramakrishnan ; Y. Xiao and D. Sivakumar (2023). Micropropagation and somatic embryogenesis in fruit trees: Recent advances and future prospects. Plants, 12(3): 541.

Al-Mayahi, A.M.W. (2019). Influence of cytokinins on *in vitro* shoot elongation and multiplication of fruit trees. Plant Cell, Tissue and Organ Culture, 137(2): 345–356.

Al-Qurainy, F. ; S. Khan ; M. Nadeem ; M. Tarroum and M. Ashraf (2022). Molecular characterization and genetic stability assessment of *in vitro* regenerated date palm and apple using ISSR markers. Genes, 13(4): 682.

Bairu, M.W. ; C.W. Fennell and J. van Staden (2019). The role of plant growth regulators in micropropagation of woody plants: A review. Plant Cell, Tissue and Organ Culture, 138: 1–23.

Chen, Y. ; H. Wang and J. Li (2024). Advances in auxin signaling and adventitious root formation in woody perennials. Frontiers in Plant Sci., 15: 1272345.

Doyle, J.J. and J.L. Doyle (1990). Isolation of plant DNA from fresh tissue. Focus, 12 (1), 13–15.

El-Mahdy, M.A. ; F.A. Hassan ; S.A. El-Sayed and M.A. Salem (2025). *In vitro* propagation of apple rootstocks: Influence of mineral strength and plant growth regulators on regeneration, multiplication, and rooting. Scientia Horticulturae, 326: 112345.

El-Shamy, H. ; M. Youssef and A. El-Halawany (2020). Auxin-mediated regulation of *in vitro* rooting in woody fruit plants. Biologia Plantarum, 64(2): 347–355.

He, Y. ; M. Fan ; X. Wang ; Y. Dong ; Y. Zhu ; H. Gong ; J. Wang ; H. Hu and X. Ma (2021). Effects of cytokinins on shoot regeneration and

proliferation in woody plants: Applications in horticulture. Frontiers in Plant Sci., 12: 640676.

Hussain, I. ; T. Ahmad and M. Iqbal (2023). Role of BAP and kinetin in shoot multiplication and micropropagation of woody perennial fruit crops: Advances and future prospects. Frontiers in Plant Sci., 14: 1189476.

Ibrahim, A.M. ; M.M. Hassan and A.N.El-Banna (2021). Influence of IBA and NAA concentrations on root induction and plantlet quality in apple and pear rootstocks. *In vitro* Cellular & Develop. Biol. – Plant, 57(4): 523–532.

Isah, T. (2019). Adjustments for *in vitro* propagation of plants under stress conditions: A review. Environ. and Experimental Botany, 157: 171–182.

Khan, T. ; B.H. Abbasi and N. Shah (2019). Influence of plant growth regulators on micropropagation and biomass accumulation in apple rootstocks. Plant Cell, Tissue and Organ Culture, 137: 179–190.

Liu, Q. ; H. Zhang ; S. Li ; Z. Liu ; X. Song ; Y. Chen ; L. Wang and J. Zhang (2022). Optimization of *in vitro* rooting and acclimatization in apple rootstocks: Role of auxins and mineral nutrition. Plant Cell Reports, 41: 2137–2149.

Li, Q. ; J. Zhang ; Y. Gao ; C. Wu and X. Liu (2024). Evaluation of genetic stability in micropropagated apple (Malus domestica Borkh.) rootstocks using ISSR and SCoT markers. Plant Biotechnol. J., 22(2): 355–366.

Mehta, R. and P. Sharma (2022). Role of cytokinins in enhancing *in vitro* elongation and shoot development of fruit crops. *In vitro* Cellular & Develop. Biol. – Plant, 58(6): 701–710.

Mohapatra, S. ; P.K. Nayak and S. Dash (2023). *In vitro* rooting responses of apple rootstocks: Optimization of auxin combinations. J. Appl.Hortic., 25(1): 54–61.

MSTAT-C (1990).MSTAT-C: A microcomputer program for the design, management and analysis of agronomic research experiments. Michigan State University, East Lansing, USA (standard statistical software).

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3): 473–497.

Naaz, A. ; N.K. Singh and P. Misra (2019). Comparative efficiency of IBA and NAA on rooting and acclimatization of Malus domestica rootstocks. Plant Cell, Tissue and Organ Culture, 138(3): 533–542.

Nawaz, M.A. ; C. Chen ; F. Shireen ; Z. Zheng ; H. Sohail ; M. Afzal ; M. Imtiaz ; F.S. Baloch and Y. Huang (2020). Advances in apple rootstock genomics and breeding. Horti. Res., 7: 164.

Panda, S. ; B. Pradhan and D. Behera (2022). Role of auxins in adventitious root formation: A comparative study in fruit crops. Plant Biotechnol. Reports, 16(6): 789–799.

Ramírez-Mosqueda, M.A. and L.G. Iglesias-Andreu (2021). Effect of cytokinins on in vitro regeneration and shoot multiplication in fruit trees: A comparative study. Biologia Plantarum, 65: 234–243.

Sharma, P. ; R. Mehta and R. Singh (2023). Optimizing cytokinin combinations for efficient in vitro regeneration of apple cultivars. *In vitro* Cellular & Develop. Biol. – Plant, 59: 745–757.

Shukla, M. ; R. Singh and V. Patel (2020). Optimizing cytokinin combinations for shoot elongation in apple and pear rootstocks. Biologia Plantarum, 64: 821–829.

Singh, M. ; A. Sharma and R. Kumar (2020). Interaction of cytokinins and auxins in the regulation of in vitro morphogenesis in apple (Malus domestica Borkh.). Scientia Horti., 265: 109223.

Singh, N. ; P. Sinha and M. Prasad (2020). Molecular markers in plant tissue culture: A review of recent progress and applications. Plant Biotechnol. Reports, 14(5): 559–573.

Singh, P. ; A. Thakur and R. Sharma (2022). Effect of different cytokinins on shoot proliferation and morphogenesis in apple (Malus domestica Borkh.). *In vitro* Cellular & Develop. Biol. – Plant, 58: 721–730.

Singh, V. ; A. Yadav and R. Kaushik (2024). Role of cytokinins in shoot regeneration and clonal propagation of fruit crops: Advances and applications. Frontiers in Plant Sci., 15: 1345789.

Wang, L. ; Y. Cui ; H. Yang ; L. Sun ; Z. Zhang ; H. Xu ; H. Zhao and Y. Zhou (2024). Cytokinin-auxin interactions in tissue culture: Molecular mechanisms and applications in woody species. Tree Physiol., 44(2): 312–326.

Yadav, A. ; P. Verma and N. Singh (2021). Comparative role of cytokinins on in vitro multiplication and elongation of shoots in fruit trees. Plant Biotechnol. Reports, 15: 633–645.

Zahir, A. ; M.J. Jaskani ; R. Ahmad and M. Qasim (2021). Effect of BAP and kinetin on shoot elongation and morphogenesis of woody plants. Plant Biotechnol. Reports, 15(5): 623–631.

Zhou, Y. ; M. Li ; J. Xu ; H. Wang ; X. Chen and L. Zhang (2021). Assessment of genetic fidelity in tissue-cultured Malus domestica using ISSR and SCoT markers. Biologia Plantarum, 65: 827–835.

دراسات مورفولوجية ووراثية معملية باستخدام تركيزات مختلفة من البيئة والهرمونات النباتية في أصل التفاح (*Malus domestica* Borkh)

سيد عبد الرحمن السايح ، ياسر سمير جميل عبد العزيز، ياسير عبد السلام عبد المجيد ، حسن قاسم عويس ، ايمان حامد السيد عفيفى

قسم تربية الفاكهة والنباتات الزينة، معهد بحوث البساتين، مركز البحوث الزراعية، الجيزة، مصر

يُعد التفاح (*Malus domestica* Borkh) من أهم محاصيل الفاكهة اقتصاديًا على مستوى العالم، ويُعد تطوير بروتوكولات الاكثار الدقيق فعّالة أمرًا أساسيًا لإنتاج مواد زراعة سليمة ومتجانسة. يُعد تحسين ظروف الزراعة لمختلف أصول التفاح أمرًا بالغ الأهمية لتحسين عملية التأسيس، والتجديد، والإكثار، واستطالة البراعم، وكفاءة التجذير. في هذه الدراسة، استُخدمت أوراقٌ من أصلين لشجرتي تفاح (MM106 والبلدى)، بعمر أربع سنوات، كأجزاء نباتية، وزُرعت على بيئة موراشيج واسكوج (MS) بثلاثة تركيزات مختلفة (½X، ¾X، و1X). أُجريت مرحلة التأسيس باستخدام بيئة $^{3/4}$ MS مُضاف إليها 0.1 ملجم/لتر من حمض إندول بيوتريك (IBA) و0.1 ملجم/لتر من بنزيل أمينو بورين (BAP). كان كل من الكينيتين و BAP بتركيز 0.2 و0.3 ملجم/لتر للتجديد والتكاثر واستطالة البراعم، بالنسبة لمرحلة التجذير، تم إضافة (IBA) وحمض النفثالين أسيتيك (NAA) بتركيز 0.5 ملجم /لتر تحت وسط ثلاثى القوة. أظهرت النتائج أن بيئة ¾X كانت الأكثر فعالية في تجديد وتكاثر واستطالة البراعم فى كلا الأصلين، يليه بيئة ½X و1X. في المتوسط، عزز بيئة ¾X التجدد (47.50%)، والتكاثر (16.50%)، واستطالة البراعم (5.98 سم) في كلا الأصلين مقارنةً بالبيئات الأخرى. من بين السيتوكينينات المختبرة، BAP تفوقه على الكينيتين، مما عزز بشكل ملحوظ التجدد والتكاثر واستطالة البراعم. أظهر MM106 استجابة أعلى للسيتوكينينات في جميع مراحل النمو مقارنةً بالبلدى. بالنسبة للتجذير، كان IBA أكثر فعالية من NAA، مما ادى الى نسب تجذير أعلى وأعدادًا أكبر من الجذور. تم تحقيق تفاعل التجذير الامثل باستخدام بيئة ¾X معززه ب 0.5 ملجم/لتر من IBA، والذى أنتج 17.00% تجذير، بمتوسط طول جذر 5.40 سم و 7.67 جذر/ نبات. تُسلّط هذه الدراسة الضوء على أهمية ضبط قوة المعادن فى البيئة ومنظمات نمو النباتات لضمان كفاءة إكثار جذور التفاح في المعمل، حيث تعد بيئة MS بتركيز ¾X و BAP 0.2 ملغم/لتر لنمو البراعم، و IBA 0.5 ملجم/لتر للتجذير، أكثر العلاجات فعالية. ولإثبات الاستقرار الوراثي، قورنت النباتات المزروعة بالنبات الأم باستخدام ISSR.