The Open Biotechnology Journal

Content list available at: https://openbiotechnologyjournal.com

RESEARCH ARTICLE

Optimization of Hormonal Compositions of Media for *In vitro* Propagation of Apple (*Malus ×domestica* Borkh.) Cultivars

Bona Abdella[1], Zekeria Yusuf[1,*] and Yohannes Petros[1]

[1]*School of Biological Sciences and Biotechnology, Haramaya University, Dire Dawa, Ethiopia*

Abstract:

Background:

Apple *(Malus × domestica* Borkh.) is one of the most important fruit crops in the world. Traditionally, vegetative propagation methods (including cutting, budding, and layering) are time-consuming (about three years), with low production rates and low success in obtaining virus-free plants.

Objective:

The present study was planned to investigate the *in vitro* propagation of apple (*M. domestica*) cultivars from nodal segments.

Methodology:

The Murashige and Skoog (MS) medium supplemented with sucrose and different concentrations of plant growth regulators (PGRs) were used for shoot proliferation and root induction. The optimal concentrations of PGRs in the MS medium were assessed. The effect of full and half-strength MS medium on root induction was investigated.

Results:

Examination of the effects of MS medium supplemented with various concentrations of indole-3-acetic acid (IAA) and kinetin revealed that the significantly highest shoot response was recorded for the 'Princess' cultivar with maximum shoot proliferation rate (65.25%), shoot number per explant (2.57), shoot length (7.28cm), and leaf number per shoot (6.15) after four weeks of culture. The root induction in micro shoots of three apple cultivars was observed after 20 days of culturing. The strength MS medium (full and half) containing 1.5 mg/L IAA significantly affected (at $P<0.05$, chi-square test) root induction in all three apple cultivars, especially rooting rate. However, there was no significant difference in root number and root length per micro shoot among the apple cultivars. Among the cultivars, significantly the highest rooting rate(48.30%), root number (6.25), and root length (4.15cm) were recorded for cultivar 'Princess' on full-strength MS medium.

Conclusion:

PGR combination of IAA (1.0 mg/L) and kinetin (3.0 mg/L) was found to be the best for shoot proliferation. The shoot responses were found to increase with an increase in kinetin concentration combined with IAA at 1.00 mg/L.

Keywords: IAA, Kinetin, Shoot proliferation rate, Shoot number, Shoot length, Rooting rate.

Article History	Received: September 12, 2022	Revised: November 24, 2022	Accepted: January 03, 2023

1. INTRODUCTION

Traditionally, vegetative propagation methods (including cutting, budding, and layering) are time-consuming (about three years), with a low production rate. An additional consequence of these techniques is that the resulting germplasm cannot be guaranteed virus- and disease-free [1, 2].

* Address correspondence to this author at the School of Biological Sciences and Biotechnology, Haramaya University, Dire Dawa, Ethiopia;
E-mail: Zakoyusuf@yahoo.com

The tissue culture of the domesticated apple (*Malus × domestica* Borkh.) has a rich and extensive history spanning, approximately 60 years [3]. Micropropagation is a high-speed propagation method under aseptic conditions that has been effectively used for the production of thousands of healthy and disease-free apple cultivars in a limited space and short period [4]. The lack of a suitable regeneration system has been a major limitation to the production of transgenic plants. Therefore, the availability of a reliable regeneration system is a significant advance for the apple tree industry to allow it to

utilize multiplication, genetic improvement, and cryopreservation processes. However, one of the challenges with *in vitro* regeneration of apples is its recalcitrant nature [3, 4].

A consistent genetic background in a given cultivar can be maintained only by vegetative propagation, *i.e*., cloning since the apple genome is highly heterozygous. This would be important in the production of genetically uniform scions and rootstocks for commercial apple production [5]. Regeneration from the root under aseptic conditions is a biotechnological approach to producing large numbers of true-to-type, young plants in a short period of time without seasonal interruption. A root-based regeneration procedure is important because roots are: ideal organs to be preserved during cryopreservation, easy to culture and maintain, genetically stable over a long time, and produce true-to-type plants. A rapid *in vitro* vegetative propagation method offers an accelerated method of production of new tree fruit cultivars/rootstocks that may be extremely valuable for commercial and private nursery growers [6].

Apple has wider consumer acceptability in Ethiopia both as a fresh table fruit and value-added processed product. The scarcity of apple planting materials, the absence of large-scale apple orchards, and other factors hampered consumers' need for apple fruit despite the huge potential for apple production in Ethiopia. The productivity is very low compared to horticulturally advanced countries where orchards are established using apple trees grafted on dwarfing rootstocks predominantly. The main bottleneck for the adoption of high-density plantations is the limited availability of clonally propagated dwarfing rootstocks. Therefore, the present study was planned to investigate the *in vitro* propagation of apple (*Malus* × *domestica* Borkh.) cultivars from nodal segments.

2. MATERIALS AND METHODS

2.1. Sample Collection

The nodal segment explants were collected from three apple cultivars like ‘Anna’, ‘Princess’, and ‘Dorsett Golden’ from Girawa Research Site, Haramaya University, Ethiopia. The nodal segments close to the shoot tip were with sterile scissors at about 30cm including the meristematic shoot tip during the vegetative onset of the fall season (on 20th October 2020). The actively growing shoot tips were used as the explant's sources for the successful organogenesis of the explants. The explant samples were brought to Plant Biotechnology Laboratory, Haramaya University, Ethiopia.

2.2. Explant Preparation and Surface Sterilization

The shoot tip nodal segment explants of mature apple cultivars were used as explant sources. Following the removal of leaves with sterile scissors, the shoot tip nodal segments 16 cm in length were washed with distilled water in 1000 ml beakers for 5 min. Then the explants were successively surface sterilized in 75% (v/v) ethanol (30 sec), and 0.1% (w/v) mercuric chloride with two drops of Tween-20per100mL of disinfectant solution(10min) in a laminar hood. To remove all traces of detergents and Tween-20 from the surface, the explants were rinsed in sterile double distilled water three times [7, 8]. The exposed cut ends of the explants were trimmed off with sharp secateurs to eliminate all toxic effects of mercuric chloride. Four explants were used per treatment. All experiments were conducted in two replications.

2.3. Media Preparation

The Murashiege and Skoog (MS) medium was prepared as per the standard procedure [7]. The pH of the media was adjusted to 5.8 using 0.1NHCl and 0.1NNaOH, and finally, 0.8% (w/v)agar-agar (Merck, Germany) was added as a gelling agent. The prepared MS medium was autoclaved at 121°C and 2.2kpa pressure for 15-20min in 150mL Erlenmeyer flasks by dispensing 40mL of molten media into each flask plugged with aluminum foil. The sterilized medium was kept for two days before inoculation so as to check for possible contamination. The filter-sterilized vitamins and hormone supplements were poured into autoclaved MS basal medium.

2.4. Shoot Regeneration

The culture media for *in vitro* shoot induction consist of the basic salts and vitamins of basal MS medium at full strength supplemented with kinetin (KIN) at 1.0, 1.5, 2.0, and 3.0 mg/L; indole-3-acetic acid (IAA) at 0.5, 0.8, and 1.0 mg/L according to the protocol described by Dalal *et al.* [9] and James and Thurbon [10] with slight modifications in concentrations of PGRs. The media were also supplemented with 3.5%(w/v) sucrose and 8%(w/v) bactoagar. Ascorbic acid at 2g/L was used to minimize explants browning by adsorbing phenolics, in activating polyphenol oxidase and peroxidases. About 16 cm nodal segment explants were inoculated into culture jars containing 50 ml MS medium.

The cultured jars were sealed with non-absorbent cotton and placed in a growth chamber room at 26 ± 2°C, 16 h photoperiod, and high light intensity being 600 lux. The experiment was replicated twice. Two weeks later, the following parameters of shoot response were recorded-average number of shoots/explant, the average length of proliferated micro shoots/explant and the average number of leaves/micro shoots.

2.5. Rooting of Regenerated Shoots

The basic salts and vitamins of MS medium at full and half strength were used for rooting according to the procedure described by James and Thurbon [10] with slight modifications in concentrations of PGRs. At the end of the growing period, healthy regenerated shoots were excised, and transferred individually in glass jars (9 × 4.5 cm), each containing 30 ml basal medium supplemented with 0.2, 0.5, 1.0, 1.5 mg/L naphthalene-3 acetic acid (NAA), 3% (w/v) sucrose, and 1g/L bactoagar and cultured vertically. The pH of the rooting media was adjusted to 5.8 before the addition of agar. The culture jars were capped with aluminum foil and autoclaved at 121°C for 20 min, then left to cool and harden for five days before being used. The cultured jars were incubated at 25 ± 1C, with 16h photoperiod and high light intensity being 500 lux [2]. Each experiment was replicated two times. Data, including rooting rate, root number per plantlet, and root length per plantlet were recorded after five weeks of culturing.

2.6. Data Analysis

The data pertaining to shoot regeneration and rooting were subjected to one-way analysis of variance (ANOVA) and the differences among means were compared based on the least significance difference (LSD) test. Analyses were conducted using SAS software version 9.2 [11].

3. RESULTS AND DISCUSSION

3.1. Optimization of MS Medium for Shoot Proliferation of Apple Cultivars

The effects of MS medium supplemented with various concentrations of IAA and KIN on shoot proliferation rate, shoot number per explant, shoot length, and leaf number are shown in Table **1**. The hormone-free basal MS medium was used as a control. In all experiments, no growth was observed on the basal MS medium. Significantly, the highest shoot response was recorded for cultivar 'Princess' with maximum shoot proliferation rate (65.25%), shoot number per explant (2.57), shoot length (7.28cm), leaf number per regenerated shoot (6.15) after four weeks of culture. The least shoot responses were recorded in cultivar 'Anna' with shoot proliferation rate (51.25%), shoot number per explant (2.15), shoot length (3.26 cm), and leaf number per shoot (4.40).

PGR combination of IAA (1.0mg/L) and KIN (3.0mg/L) was found to be the best for shoot proliferation. In the presence of IAA at 1.0mg/L, the shoot response was found to increase with an increase in KIN concentration. A similar study was conducted by Bhatt *et al.* [12] who reported that axillary shoot tips of M9 rootstock formed multiple shoots on MS medium containing 3mg/L 6-benzyladenine (BA), 2mg/L KIN, 3% (w/v) sucrose and 0.8% (w/v) agar-agar, with 85.7% of shoot apices forming multiple shoots. The best shoot production in terms of shoot number and shoot quality was obtained using 4.4μM BA and.27μM thidiazuron(TZD) during the shoot multiplication phase, while 8.8 μM BA combined with 1.14μM TZD and 2.8μM gibberellic acid (GA_3) was found to perform the best during the shoot elongation phase for all genotypes [13].

3.2. Optimization of MS Medium for Root Initiation of Apple Cultivars

The root induction in micro-shoots of three apple cultivars was conducted by supplementing full or half-strength basal MS medium with IAA at different concentrations (Table **2**). Root induction was observed after 20 days of culturing. Significant differences in rooting rate, root number, and root length were observed among treatments. Significantly highest rooting rate (48.30%), root number (6.25), and root length (4.15cm) were recorded for the 'Princess' cultivar on full-strength MS medium.

The 'Dorset' cultivar displayed the lowest rooting rate (38.35), root number (4.10), and root length (2.45cm) on full-strength MS media; and rooting rate (45.80), root number (4.34) and root length (3.90 cm) on half-strength MS media. As the concentration of IAA within the medium increases, the root response including root initiation rate, root number, and root length also increased with maximum values observed at 1.5 mg/L. No root induction was observed in the control medium (HF MS medium). It was also noticed that the root response increased as the concentration of the growth regulator increased from 0.2 to 1.5 mg/L. The half-strength MS medium was better for root induction than full strength MS medium.

Table 1. The effects of various combinations of plant growth regulators in full-strength Murashige and Skoog (MS) medium on shoot proliferation of three apple cultivars.

Cultivar	IAA+KIN (mg/L)	Shoot Proliferation Rate (%)	Shoot Number/ Explants	Microshoot Length (cm)	Leaf Number/Microshoot
'Anna'	/	0.00 g	0.00 e	0.00 f	0.00 h
	0.5+1.0	22.00 f	1.10 d	1.45 e	3.10 g
	0.8+ 1.5	36.00 de	1.15 d	2.05 d	3.05 g
	1.0+ 2.0	47.50 c	2.15 b	3.26 c	4.40 d
	1.0+ 3.0	51.25 bc	2.05 b	3.20 c	4.38 d
'Princess'	/	0.00 g	0.00 e	0.00 f	0.00 h
	0.5+1.0	35.50 e	1.10 d	3.26 c	4.11 e
	0.8+ 1.5	47.50 c	1.47 c	3.46 c	4.83 c
	1.0+ 2.0	64.00 a	2.57 a	7.28 a	6.12 a
	1.0 + 3.0	65.25 a	2.47 a	7.25 a	6.15 a
'Dorsett Golden'	/	0.00 g	0.00 e	0.00 f	0.00 h
	0.5+1.0	25.65 f	1.11 d	2.06 d	3.16 g
	0.8 + 1.5	41.00 d	1.45 c	2.26 d	3.65 f
	1.0 + 2.0	54.20 b	2.07 b	4.11 b	5.10 b
	1.0 + 3.0	62.25 a	2.11 b	4.10 b	4.90 bc

Note: Means followed by the same letter within a column were not significantly different at 0.05 according to the Least Significant difference (LSD) test. IAA: Indole-3-acetoc acid; KIN: Kinetin

Table 2. The effect of strength of Murashige and Skoog (MS) medium supplemented with different concentrations of IAA on the rooting ability of micro-shoots in different apple cultivars.

Cultivars	1MS + IAA	Rooting Rate (%)	Root Number	Root Length (cm)	1/2MS + IAA	Rooting Rate (%)	Root Number	Root Length (cm)
'Anna'	0.0	0.00 j	0.00 i	0.00 h	0.0	0.00 g	0.00 h	0.00 h
	0.2	10.25 i	3.10 g	1.45 g	0.2	45.25 e	3.55 g	2.48 g
	0.5	15.40 h	3.45 ef	1.75 f	0.5	51.10 d	3.99 f	3.05 ef
	1.0	32.50 e	3.65 e	2.90 c	1.0	59.45 c	4.70 cd	3.25 de
	1.5	44.00 b	4.46 b	3.16 b	1.5	64.85 b	5.05 bc	4.74 b
'Princess'	0.0	0.00 j	0.00 i	0.00 h	0.0	0.00 g	0.00 h	0.00 h
	0.2	25.25 f	4.05 d	2.20 e	0.2	50.10 de	4.29 ef	2.90 f
	0.5	40.10 c	4.35 bc	2.79 c	0.5	65.40 b	4.61d e	3.35 d
	1.0	40.25 c	6.05 a	3.35 b	1.0	70.90 a	5.10 b	4.75 b
	1.5	48.30 a	6.25 a	4.15 a	1.5	73.65 a	5.70 a	5.64 a
'Dorsett Golden '	0.0	0.00 J	0.00 i	0.00 h	0.0	0.00 g	0.00 h	0.00 h
	0.2	21.50 g	2.68 h	1.25 g	0.2	36.30 f	3.26 g	2.48 g
	0.5	27.40 f	3.11 g	2.05 e	0.5	39.25 f	3.58 g	2.88 f
	1.0	35.80 d	3.35 fg	2.10 e	1.0	47.60 de	4.11 f	3.12 e
	1.5	38.35 cd	4.10 cd	2.45 d	1.5	45.80 e	4.34 ef	3.95 c

Means followed by the same letter within a column are not significantly different at 0.05 according to the Least Significance difference (LSD) test. 1 MS: full strength MS medium; ½ MS: half strength MS medium; IAA: Indole-3-acetic acid.

For successful *in vitro* rooting, micorshoots were cultivated on basal MS medium supplemented with different IBA concentrations. Indeed, root development was apparent in the course of as brief as 5 days of exposure of microshoots to a rooting medium [14]. Afterroot initiation, the micro-shoots were re-implanted on half-strength MS medium for root development [14, 15]. Renu *et al.* [14] reported the highest number of roots (7.66) as well as root length (22cm) in the medium containing 0.05μMNAA for the 'Golden Delicious' apple, while 'Red Fuji' showed no root induction in presence of NAA. On the other hand, successful *in vitro* propagation of apple rootstock 'G. 814' was achieved using 1 mg/L BAP at the multiplication stage and 1.5 mg/L IBA at the rooting phase [16].

CONCLUSION

The protocols developed in this study have the potential to be utilized for the generation of disease-free, quality planting materials and in crop improvement programs. PGR combination of IAA (1.0 mg/L) and kinetin (3.0 mg/L) was found to be the best for shoot proliferation of examined apple cultivars. The shoot responses were found to increase with an increase in kinetin concentration when it is combined with IAA at 1.00 mg/L. The root induction was observed after 20 days of culturing in all cultivars. Significant differences in rooting rate, root number, and root length were observed for all treatments. The comparison of the effect of full and half-strength MS medium on root induction at 1.5 mg/L IAA revealed significant differences ($P < 0.05$) in rooting rate among apple cultivars. Additional studies are required to identify the most efficient and cost-effective micropropagation protocol for studied apple cultivars.

LIST OF ABBREVIATIONS

KIN = Kinetin

ANOVA = Analysis of Variance

LSD = Least Significance Difference

TZD = Thidiazuron

AUTHORS' CONTRIBUTION

Zekeria Yusuf: initiation and design of the study, Lab experiment, data analysis; Bona Abdella: Lab experiment, data collection, and write-up of the document; Yohannes Petros: Analysis and interpretation of data. All authors contributed to the article and revised it critically for important intellectual content.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data will be available on request from the corresponding author [Z.Y].

FUNDING

This project was funded by a Haramaya University Research grant, under project code: HURG_2020_06_01_22.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or

otherwise.

ACKNOWLEDGEMENTS

The authors are grateful to Haramaya University Research Office for their financial support and laboratory facilities.

REFERENCES

[1] Hartmann HT, Kaster DE, Davies FT, Geneve RL. Plant Propagation: Principles and Practices. 2004.

[2] Magyar-Tábori K, Dobránszki J, Teixeira da Silva JA, Bulley SM, Hudák I. The role of cytokinins in shoot organogenesis in apple. Plant Cell Tissue Organ Cult 2010; 101(3): 251-67. [http://dx.doi.org/10.1007/s11240-010-9696-6]

[3] Dobránszki J, Teixeira da Silva JA. Micropropagation of apple — A review. Biotechnol Adv 2010; 28(4): 462-88. [http://dx.doi.org/10.1016/j.biotechadv.2010.02.008] [PMID: 20188809]

[4] Bhatti S, Jha G. Current trends and future prospects of biotechnological interventions through tissue culture in apple. Plant Cell Rep 2010; 29(11): 1215-25. [http://dx.doi.org/10.1007/s00299-010-0907-8] [PMID: 20697717]

[5] Teixeira da Silva JA, Gulyás A, Magyar-Tábori K, Wang MR, Wang QC, Dobránszki J. *In vitro* tissue culture of apple and other *Malus* species: recent advances and applications. Planta 2019; 249(4): 975-1006. [http://dx.doi.org/10.1007/s00425-019-03100-x] [PMID: 30788577]

[6] Bommineni VR, Mathews H, Samuel SB, Kramer M, Wagner DR. A New method for rapid *in vitro* propagation of apple and pear. HortScience 2001; 36(6): 1102-6. [http://dx.doi.org/10.21273/HORTSCI.36.6.1102]

[7] Murashige T, Skoog F. A revised medium for rapid growth andbioassays with tobacco tissue cultures. Physiol Plant 1962; 15(3): 473-97. [http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x]

[8] Lane WD, Bhagwat B, Wahlgren S, Armstrong JD. Apple micrografting protocol to establish transgenic clones on field ready rootstock. Horttechnology 2003; 13(4): 641-6. [http://dx.doi.org/10.21273/HORTTECH.13.4.0641]

[9] Dalal MA, Das B, Sharma AK, Mir MA, Sounduri AS. *In vitro* cloning of apple (*Malus domestica* Borkh) employing forced shoot tip cultures of M9 rootstock. Indian J Biotechnol 2006; 5: 543-50.

[10] James DJ, Thurbon IJ. Shoot and root initiation *in vitro* in the apple rootstock M.9 and the promotive effects of phloroglucinol. J Hortic Sci 1981; 56(1): 15-20. [http://dx.doi.org/10.1080/00221589.1981.11514960]

[11] SAS Institute. SAS enterprise guide, Version 92. Cary, NC, USA: SAS Inst. 2011.

[12] Bhatt A, Kansal S, Chauhan RS, Sood H. Low-cost tissue culture procedures for micropropagation of apple root stocks. Int J Plant Develop Biol 2012; 6(1): 67-72.

[13] Amiri EM, Elahinia A. Optimization of medium composition for applerootstocks. Afr J Biotechnol 2011; 10(18): 3594-601.

[14] Renu M, Bhattacharya A, Singh R. Optimization of micropropagation from nodal segments of apple (Malus × domestica. Borkh) cultivars Golden Delicious and Red Fuji. Curr Appl Sci Technol 2018; 31(3): 1-9.

[15] Purohit SD, Kukda G. Micropropagationof an adult tree – *Wrightia tinctoria.* Indian J Biotechnol 2004; 3: 216-20.

[16] Meneguzzi A, Gonçalves MJ, Camargo SS, Grimaldi F, Weber GC, Rufato L. Micropropagation of the new apple rootstock 'G.814'. Ciência Rural,Santa Maria 2017; 2017: e20160615.