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In vitro CULTURE OF NODAL CUTTINGS IN KIWIFRUIT

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ABSTRACT

This study aimed to determine the effects of plant growth regulators and different medium on *in vitro* propagation of 'Hayward' and 'Zespri Gold' kiwifruits. Micro-cuttings were used as explant sources and cultured in MS and WPM medium containing different concentrations of BA and GA_3. Subcultures were carried out at intervals of 4-6 weeks. Contamination rate (%), blackening rate (%), callus development rate (%), shoot rate (%), and propagation coefficient (plantlet/explant) were determined. The micropropagation experiment was carried out according to the randomized plot design. The arc-sin transformation was applied to the percentage values before analysis. Significance levels of the means were compared with the LSD test. It was determined that 0.1% mercury chloride application increased the sterilization efficiency. As the BA concentration in the medium increased, the rate of callus formation of the explants increased. While there was no difference in shoot rates of explants cultured on MS and WPM medium, the propagation rate was higher in MS medium. The propagation coefficient of the 'Hayward' kiwi cultivar was 3.08 in MS medium containing 1.0 mgL^{-1} BA + 0.5 mgL^{-1} GA_3. MS medium can be recommended as a basic medium for *in vitro* kiwi propagation.

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1. Introduction

Commercially produced kiwifruits consist of 1-2 species within the genus *Actinidia*. The genus *Actinidia* includes 55 species and approximately 76 taxa (Wang and Gleave, 2012). The kiwifruits originated in Eastern China, and they grow spontaneously in the forest foothills, in regions with high relative humidity (70-80%) at an altitude of at least 300, mostly 800-1400 m above sea level (Üçler et al., 2000). According to paleobiological studies, *Actinidia* species are estimated to be 20-26 million years old (Ying-qian and Yu, 1991). Although the homeland of kiwi fruit is China, the first commercial garden was established in New Zealand in the 1930s. The cultivation studies conducted on kiwifruit growing have shown that the cultivation in the Black Sea, Marmara, and Aegean Regions of Türkiye is suitable for cultivation (Alp, 2017). Today, it is also grown economically in the Mediterranean Region. While world kiwifruit production was over 4.4 million tons in an area of approximately 270 thousand hectares in 2020, it was approximately 73.8 thousand tons in 3261 hectares in Türkiye (FAO, 2021).

Like other plant products, the kiwi plant is propagated by generative or vegetative methods. However, generative propagation is not preferred for commercial production purposes. A major problem is the high variation in propagated plants due to the genetic expansion in generative production. In addition, due to the dioecious plant structure, 80% of the seedlings obtained from propagation by seed are male, and 20% are female. Therefore, vegetative propagation methods are preferred in commercial seedling production (Tanimoto, 1994; Sivritepe and Tuğ, 2011).

Studies on the propagation of the kiwi plant by tissue culture started with Harada's study in 1975, and this research was followed by many studies using different species and different explant types (Gui, 1979; Monette, 1986; Lin et al., 1994; Kumar and Sharma, 2002; Kim et al., 2007). In the studies, the medium developed by Murashige and Skoog (1962) was mostly used for shoot and callus stimulation. In addition, Gamborg B5 (Barbieri and Morini, 1987) and N6 medium (Lin et al., 1994) are other medium that have been used successfully. The literature has reported that regeneration can be achieved in kiwifruit by the indirect organogenesis method using different explant sources (Kumar and Sharma, 2002; Rugini and Gutierrez-Pesce, 2003). However, since regeneration is achieved from callus in this way, there are drawbacks in the mass production of plants, and the most important of these is the inability to control genetic stability (Kumar and Sharma, 2002). Prado et al. (2007) found genetic variation between donor plants in their AFLP analysis of male plants obtained by indirect organogenesis technique. Therefore, it is seen that single-node cuttings with axillary buds and shoot tips are used as starting materials in *in vitro* propagation of kiwis (Monette, 1986; Wang and Gui, 1988; Marino and Bertazza, 1990; Wiyaporn et al., 1990; Pedroso et al., 1992; Ding et al., 1997; Kumar et al., 1998; Souad et al., 1998).

This study aimed to determine the effect of MS and WPM medium containing different concentrations of BA and GA_3 on the *in vitro* propagation of single-node micro steel explants taken from 'Hayward' and 'Zespri Gold' kiwifruit cultivars.

2. Material and methods

2.1. Plant materials

'Hayward' and 'Zespri Gold' kiwi cultivars planted in the research area of Ondokuz Mayıs University Faculty of Agriculture were used as explant sources. Young shoots were taken from the plants in the spring and brought to the laboratory environment (Figure 1A). Micro cuttings containing a single node, prepared by cutting 3-5 cm long from these shoots (Figure 1B, C), were used as explants.

2.2. The surface sterilization of explants

During sterilization, the explants taken from the field were kept under running tap water for 60 min in all sterilization experiments to remove surface contaminants. Four different sterilization protocols were tested. The explants were placed in a sterile environment and soaked in 70% ethyl alcohol (EtOH) for 1-2 min and then in 20% commercial NaOCl (Domestos) containing 1-2 drops of Tween-20 for 20 min. Finally, explants were rinsed with sterile pure water in the first protocol.

Figure 1. Preparation of micro cuttings, A: Shoots, B: A cutting with leaf, C: Explants

In the second sterilization protocol, only the commercial NaOCl concentration was increased to 30%. In the third, unlike the first protocol, explants were treated with alcohol, and they were kept in a 10% H_2O_2 solution for 10 min. In the last protocol, unlike the first, the explants were kept at 0.1% $HgCl_2$ for 20 min before being treated with alcohol. After all sterilization procedures, the explants were rinsed again with sterile pure water in the final stage.

2.3. *In vitro* culture of micro cuttings

After sterilization, the single-node explants were taken into the laminar flow hood and prepared for *in vitro* culture by cutting the upper and lower parts of the node, leaving approximately 3 cm of material. In order to obtain shoots from micro cuttings, WPM (Lloyd and McCown, 1980) and MS (Murashige and Skoog, 1962) medium containing BA (0, 0.5, 3.0 mgL^{-1}) and GA_3 (0, 0.3, 0.5 mgL^{-1}) concentrations were used in the experiment. While preparing the medium, 20 gL^{-1} sucrose was added as a carbon source and 7 gL^{-1} agar was used as a solidifier. The pH of the medium was adjusted to 5.7-5.8 before autoclaving. 50 mL of the medium was poured into 660 cc glass jars. The medium and other equipment were autoclaved under 1.5 p.s.i at 121 °C for 15 min.

2.4. Examined criteria and statistical analyses

Surface sterilization experiments were conducted using a factorial randomized trial design with ten replications in both cultivars, using three explants in each replication. Observations were taken four weeks after the culture initiation, and the contamination rate (%) and blackening rate (%) were determined. An experiment was set up for micropropagation according to the random plot design. The experiment was conducted in two nutrient medium with four different plant growth regulators (PGRs) concentrations, including a control group. Each combination had four replications, and four explants were used in each replication. Subcultures were carried out in 4-6 weeks periods. In the experiment, callus development rate (%), shooting rate (%) and propagation coefficient (plantlet/explant) were determined. The number of plantlets was observed after three subcultures.

The arc-sin transformation was applied to the percentage values before analysis. The data were subjected to variance analysis using the JMP (version 8.01) package program, and the significance levels of the means were compared with the LSD ($P<0.05$) test.

3. Results and discussion

The contamination rates in surface sterilization experiments were evaluated statistically, and no significant difference was found among protocols ($P>0.05$).

The highest contamination rate in the culture medium was observed in the first protocol. A 79.4% contamination rate was observed in the 'Zespri Gold' cultivar and a 73.9% contamination rate in the 'Hayward'. The lowest contamination rates were 52.4% and 43% in the 'Zespri Gold' and 'Hayward' cultivars, respectively (Figure 2). Although there were no statistically significant effects of different sterilization protocols, it was observed that hydrogen peroxide (H_2O_2) and mercuric chloride ($HgCl_2$) applications relatively reduced the contamination rate (Figure 2). Observations showed that the contamination was caused by bacterial and mostly fungal contamination.

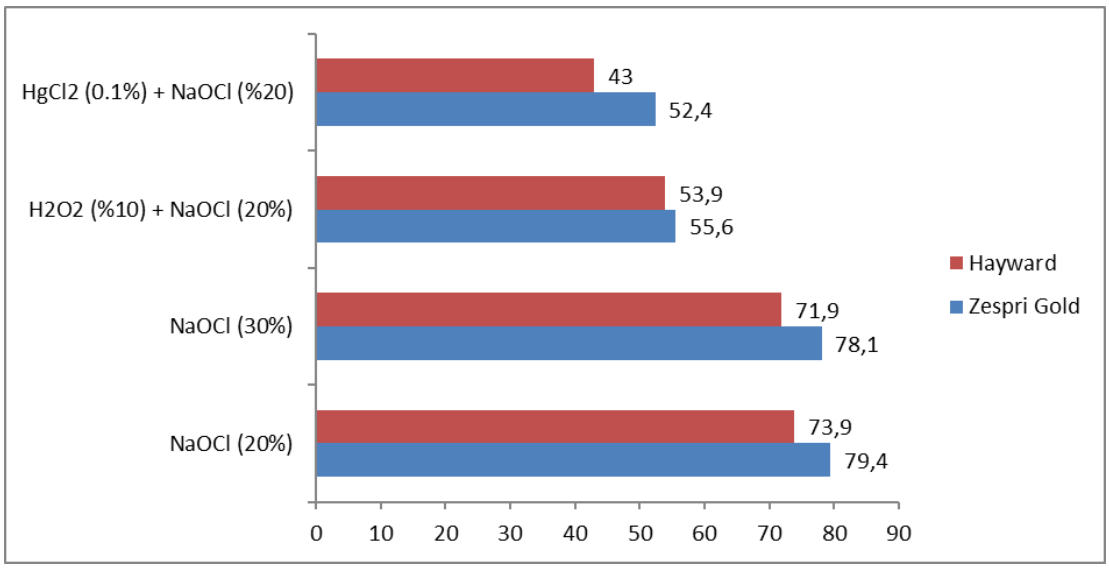

Figure 2. Contamination rate (%) in explants subjected to different sterilization agents.

Although no contamination problems were observed in the first weeks of culture, in the following weeks, fungal or bacterial contaminations occurred on the explants, suggesting that the source of contamination may be internal. Debenham et al. (2016) used nodal segments in *in vitro* propagation studies on *Actinidia arguta* (Siberian kiwi), *A. chinensis*, *A. deliciosa* (kiwi) and *A. polygama* (Silver vine) species and explants subjected to 4.2 mgL^{-1} sodium hypochlorite for 30 min. They observed intense contamination in the *in vitro* culture. Researchers reported that even biocide and fungicide applications used in surface sterilization applications seem beneficial in the first weeks, but contaminations are observed in the later stages of culture. Similarly, Cassells (1991) and Leifert et al. (1994) indicated that the reason for the contamination observed in explants taken from plants grown under field conditions was endogenous. Sivritepe and Tuğ (2011) found that contamination rates ranged from 10 to 30% in their study on *in vitro* propagation of 'Hayward' and 'Matua' kiwifruit cultivars. However, the means of contamination rates were not statistically significant, and absolute sterilization success was not achieved in their studies. In the current study, due to the contamination observed in the culture environment, it became clear that there was fungal and bacterial contamination in the donor plants. Therefore, the limited number of donor plants of 'Zespri Gold' cultivars continued the study with a few sterile explants. Similar results were observed in explants from two commercial plantations of 'Hayward' cultivars. However, the results were affected by contamination problems, so the experiment was continued using non-contaminated explants.

No statistically significant difference was found in the blackening rate in micro cuttings placed in the culture medium. When the blackening rates were examined according to the nutrient medium, blackening occurred in 14.87% of the explants cultured in the MS medium and 13.68% cultured in the WPM medium (Table 1). It was observed that the blackening rate increased as the PGR concentrations increased, although it was not statistically significant. It is thought that the reason for the blackening of explants in the culture medium is related to surface sterilization practices. Although chemicals are necessary to prevent microbial contamination in surface sterilization processes, they can also lead to phytotoxicity.

On the other hand, the oxidation of phenolic compounds secreted from the cut surface of explants by polyphenoloxidases and peroxidases can also be the reason for blackening (Singh (1996). Therefore, optimizing surface sterilization processes and using clean materials is necessary for *in vitro* propagation of kiwifruit.

Table 1. Blackening rate in cultured explants

Medium No	PGR (mgL^{-1}) ($BA+GA_3$)	MS 'Hayward'	MS 'Zespri Gold'	WPM 'Hayward'	WPM 'Zespri Gold'	Medium x PGR
0	0 + 0	24.98 (22.57)	8.33 (8.94)	8.33 (8.94)	0.0 (0.0)	10.41 (10.16)
1	0.5 + 0.3	8.33 (8.94)	16.65 (13.80)	0.0 (0.0)	8.33 (8.94)	8.33 (7.97)
2	0.5 + 0.5	24.98 (22.57)	16.65 (17.71)	16.65 (17.71)	8.33 (8.94)	16.65 (16.73)
3	1.0 + 0.3	8.33 (8.94)	24.98 (22.57)	24.98 (22.57)	16.65 (17.71)	18.73 (17.95)
4	1.0 + 0.5	16.65 (13.80)	8.33 (8.94)	24.98 (22.57)	24.98 (22.57)	18.73 (16.97)
5	3.0 + 0.3	24.98 (22.57)	16.65 (17.71)	24.98 (22.57)	8.33 (8.94)	18.73 (17.95)
6	3.0 + 0.5	8.33 (8.94)	0.0 (0.0)	16.65 (13.80)	8.33 (8.94)	8.33 (7.97)
	Cultivar x Medium	16.65 (15.48)	13.08 (12.84)	16.65 (15.48)	10.71 (10.89)	
	Mean of Medium	14.87 (14.16)		13.68 (13.18)		

There was no statistically significant difference between the averages (P>0.05), and the values obtained from arc-sin transformation are given in parentheses.

Callus structures are formed from explants placed in the culture medium, and callus formation rates are affected mainly by genotype, culture medium and plant growth regulators. According to the results, the effects of PGR concentrations and the nutrient medium on callus formation were statistically significant. In contrast, the interaction of cultivars and factors was not statistically significant. The highest callus formation rate was 66.62% in the 'Hayward' cultivar placed on MS medium containing 3.0 mgL^{-1} BA and 0.5 mgL^{-1} GA_3 growth regulator, and 58.30% was in the 'Zespri Gold' cultivar. Similarly, callus formation in the WPM medium containing the same concentration of PGR was 41.65% in the 'Zespri Gold' cultivar, while it was 24.98% in the 'Hayward' cultivar. It has been determined that the callus formation rate decreases as the PGR concentration decreases. When PGR concentrations were evaluated individually, the highest callus formation was observed in the medium containing 3.0 mgL^{-1} BA and 0.5 mgL^{-1} GA_3, with a rate of 47.88%. The lowest callus formation rate was observed in the control group. Compared to the nutrient medium, it was determined that the rate of callus formation in MS nutrient medium was higher than in WPM medium (Table 2).

Table 2. Callus formation rates in explants

Medium No	PGR (mgL^{-1}) ($BA+GA_3$)	MS 'Hayward'	MS 'Zespri Gold'	WPM 'Hayward'	WPM 'Zespri Gold'	Medium x PGR**
0	0 + 0	16.65 (17.90)	8.33 (9.24)	8.33 (9.24)	0.0 (0.57)	8.33 (9.24) c
1	0.5 + 0.3	8.33 (9.24)	33.30 (31.43)	0.0 (0.57)	16.65 (17.90)	14.57 (14.79) bc
2	0.5 + 0.5	8.33 (9.24)	16.65 (17.90)	16.65(17.90)	16.65 (17.90)	14.57 (15.74) bc
3	1.0 + 0.3	24.97 (26.57)	24.98 (22.77)	24.97(26.57)	24.98 (22.77)	24.97 (24.67) bc
4	1.0 + 0.5	41.62 (36.30)	0.0 (0.57)	16.65(17.90)	24.98 (22.77)	20.81 (19.38) bc
5	3.0 + 0.3	41.65 (40.26)	41.65 (40.26)	24.98(22.77)	16.65 (17.90)	31.23 (30.30) ab
6	3.0 + 0.5	66.62 (58.65)	58.30 (53.79)	24.98(22.77)	41.65 (40.26)	47.88 (43.87) a
Cultivar x Medium*		29.74 (28.31)	26.17 (25.14)	16.65 (16.82)	20.22 (20.01)	
Mean of Medium**		27.95 (26.72) a		18.44 (18.41) b		

* There was no statistically significant difference between the averages (P>0.05),

** The difference between the means is statistically significant (P<0.05); the values obtained from angle transformation are given in parentheses. LSD PGR=16.20, LSD Medium=8.25

When micropropagation studies on kiwifruit are examined, callus formation from explants generally emerges as undesirable. Debenham et al. (2016) reported that excessive callus formation causes unstable growth and a decreased proliferation rate in explants. Sivritepe and Tuğ (2011) used single node cuttings in 'Hayward' and 'Matua' kiwifruit cultivars, similar to our study, and reported that callus formation occurred at a rate of 40-90%. Researchers reported that callus formation may depend on the explant type. Our study showed that callus formation from explants was similar to this study, and excessive callus development was observed in explants. However, callus formation may be caused by the explant source and the effect of the plant growth regulators used. The effect of the growth regulator concentrations used in our study was statistically significant.

After culture initiation, the dormant buds on the explants formed shoots. Except for BA and GA_3 concentrations, the effects of cultivars, mediums, and their interactions on shooting were not statistically significant ($P>0.05$). The mean shoot formation percentage was approximately 55%. The effects of PGR concentrations on shoot formation were statistically significant, and it was determined that the shoot formation rate differed by 41-62%. The highest shoot formation rate, 62.46%, was observed in medium containing 0.5 mgL^{-1} BA + 0.5 mgL^{-1} GA_3 and 1 mgL^{-1} BA + 0.3 mgL^{-1} GA_3. The lowest shoot formation rate was determined in medium without PGR (Table 3).

Table 3. Shooting rate in single node cuttings cultivated

Medium No	PGR (mgL^{-1}) (BA+GA_3)	MS 'Hayward'	MS 'Zespri Gold'	WPM 'Hayward'	WPM 'Zespri Gold'	Medium x PGR**
0	0 + 0	49.95 (44.97)	33.3 (35.24)	41.62 (40.10)	41.62 (40.10)	41.62 (40.10) c
1	0.5 + 0.3	49.95 (44.97)	41.62 (40.10)	41.62 (40.10)	49.95 (44.97)	45.78 (42.53) bc
2	0.5 + 0.5	66.62 (58.20)	66.62 (58.20)	58.3 (53.34)	58.3 (53.34)	62.46 (55.77) a
3	1.0 + 0.3	58.3 (53.34)	74.97 (66.57)	49.9 (44.97)	66.62 (58.20)	62.46 (55.77) a
4	1.0 + 0.5	58.27 (49.83)	49.95 (44.97)	58.3 (53.34)	58.27 (49.83)	56.20 (49.50) abc
5	3.0 + 0.3	41.62 (40.10)	66.62 (58.20)	58.3 (53.34)	58.3 (53.34)	56.21 (51.25) ab
6	3.0 + 0.5	58.3 (53.34)	58.3 (53.34)	66.6 (58.20)	58.3 (53.34)	60.38 (54.55) ab
	Cultivar x Medium*	54.71 (49.25)	55.92 (50.95)	53.5 (49.06)	55.91 (50.44)	
	Mean of Medium*	55.31 (50.10)		54.72 (49.75)		

* There was no statistically significant difference between the averages ($P>0.05$).
** The difference between the means is statistically significant ($P<0.05$). The values obtained from the arc-sin transformation are given in parentheses. LSD PGR = 12.71

Sivritepe and Tuğ (2011) reported that the shoot emergence rate in single node cuttings taken from 'Hayward' and 'Matua' cultivars was between 3-10%. According to the results of our study, the percentage of shoot emergence from dormant buds was higher than in the previous study. This may be due to the difference in genotype, the period in which the explants were taken, and the effects of other internal and external factors. However, our study may suggest that the difference in the nutrient medium at the initial stage and the use of GA_3 increased the shoot emergence. On the other hand, they obtained a similar shoot emergence (50%) only from shoot tip explants.

After shoot formation occurred in micro cuttings, the shoots were cut and subcultured in the same medium to ensure propagation. Among the kiwi explants, especially in the 'Hayward' cultivar, the growth and development of the explants differed to the extent that they were visually distinguishable according to the nutrient medium (Figure 3).

The propagation was not observed in the first subculture of explants, while it was determined that the leaves were visually enlarged instead of having shoot growth. It is thought that the disproportionate growth of the leaves is due to the increase in the concentration of cytokinin used in the nutrient medium since the developmental difference was observed visually in explants cultured on medium containing constant GA_3 concentrations (MS-2, MS-4, MS-6) (Figure 3).

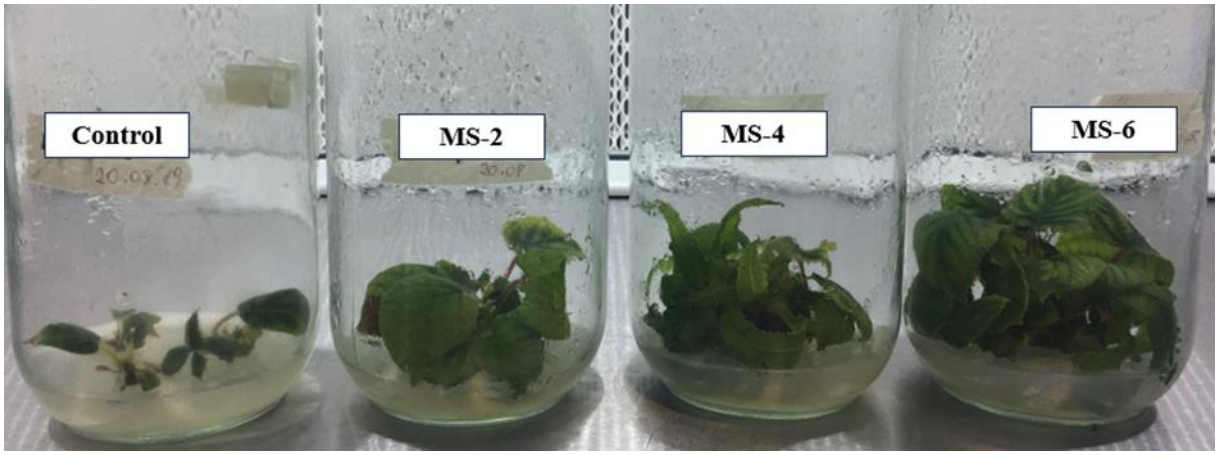

Figure 3. Developmental differences in explants in the 'Hayward' cultivar

As a result of the statistical analysis, an average of 1.82 plantlets per explant was obtained in the 'Hayward' cultivar. The effect of the medium on the propagation coefficient was not statistically significant (P>0.05). However, it was determined that the propagation coefficient in the MS medium (2.00) was higher than in the WPM medium (1.64). The effect of BA and GA_3 concentrations on the propagation coefficient was statistically significant (P<0.05). The highest propagation coefficient was 2.62, obtained from the medium containing 1.0 mgL^{-1} BA + 0.5 mgL^{-1} GA_3. The lowest propagation coefficient was observed in the medium without PGR (Table 4).

Table 4. Propagation coefficient in Hayward kiwi cultivar

Medium No	(BA+GA_3) (mgL^{-1})	'Hayward'		Medium *x* PGR**
		MS	WPM	
0	0 + 0	1.33	1.16	1.25 b
2	0.5 +0.5	1.83	1.66	1.75 b
4	1.0 + 0.5	3.08	2.16	2.62 a
6	3.0 + 0.5	1.75	1.58	1.66 b
	Mean of Medium*	2.00	1.64	
	Mean of Cultivar	1.82		

* There was no statistically significant difference between the averages (P>0.05),
** The difference between the means is statistically significant (P<0.05), LSD PGR=0.753

As a result of the observations during *in vitro* culture, limited shoot propagation was observed in the WPM medium. Shoot growth and development in the WPM medium were restricted (Figure 4A). MS medium supplemented with 0.5 mgL^{-1} BA + 0.5 mgL^{-1} GA_3, which contained a lower cytokinin concentration, plants were similarly observed to be smaller but larger in volume (Figure 4B). It was determined that increasing the concentration of cytokinin further increased volumetric growth in leaves (Figure 4C). However, this growth and development has not been fully reflected. Although the effect of the nutrient medium on shoot formation and propagation is not statistically significant, the visual weakness observed in the plant's growth and development suggests that the nutrient medium's content is important in obtaining healthy plantlets. Considering the medium components, it is known that the nitrogen (N), potassium (K), and calcium (Ca) content of the MS medium is more than three times higher than that of the WPM medium.

Therefore, the higher volume growth of explants placed in the MS medium may be due to the richer medium content. In the later stages of the culture, it was observed that some shoots spontaneously rooted as the subculture time increased (Figure 4D). No spontaneous rooting was observed from any explants on the WPM medium.

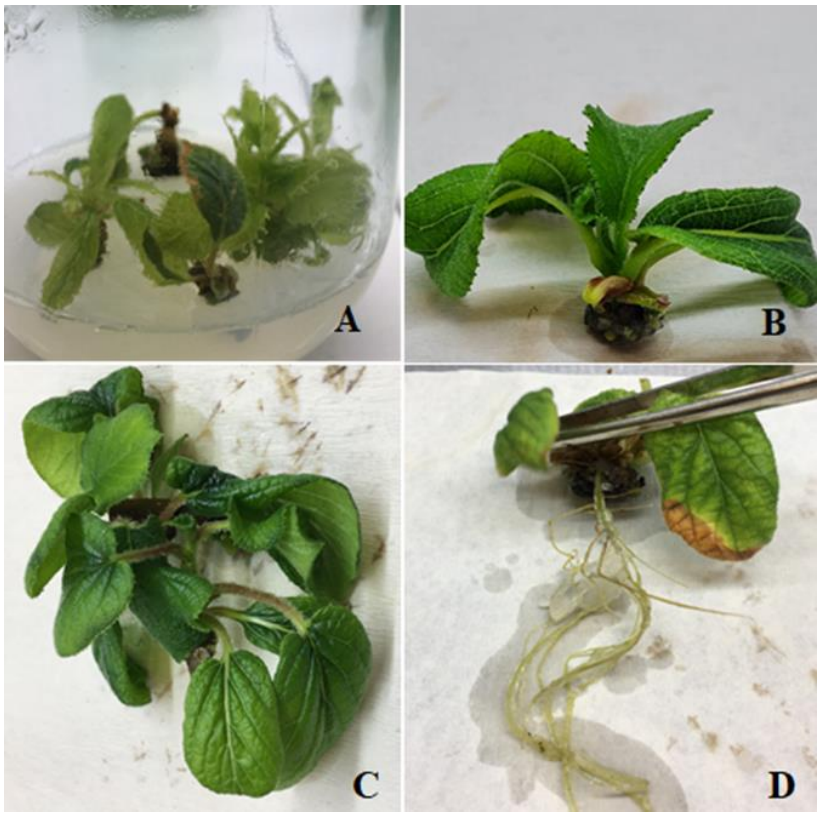

Figure 4. Different developmental stages of Hayward kiwi cultivar (A: Plantlets developed from explants cultured on WPM medium + 1.0 mgL^{-1} BA + 0.5 mgL^{-1} GA_3, B: MS medium + 0.5 mgL^{-1} BA + 0.5 mgL^{-1} GA_3, C: MS medium + 3.0 mgL^{-1} BA + 0.5 mgL^{-1} GA_3, D: rooting of plantlets in culture medium).

4. Conclusion

Propagation by tissue culture has disadvantages, such as constant research and development, high initial costs, skilled labor, and protocols based on plant material. However, when the necessary protocols for plant material are optimized, mass production can be made cheaply and quickly with the automation system. At the beginning of our study, which aimed at micropropagation of 'Hayward' and 'Zespri Gold' kiwifruit cultivars in *in vitro* culture, different experiments were set up to overcome the contamination problems observed in plant materials. However, the donor plant was contaminated with internal contaminants, which did not allow the 'Zespri Gold' cultivar micropropagation. On the other hand, testing different sterilization protocols in the study gives an idea about which sterilization is more successful for future studies. The propagation coefficient of the 'Hayward' cultivar was determined. Although researchers could not agree on the starting medium in previous studies, using MS medium as the basic medium can be recommended. However, testing different cytokinin derivatives in future studies suggests that micropropagation success may be increased.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Mehmet TÜTÜNCÜ: Investigation, Methodology, Data curation, Validation, Writing - original draft. **Muharrem ÖZCAN**: Investigation, Conceptualization, Data curation, Review and editing.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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