OPTIMIZATION OF KINETIN CONCENTRATIONS AND MEDIUM COMPOSITIONS FOR CITRUS SHOOT MULTIPLICATION FROM COTYLEDONARY NODES

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ABSTRACT

Shoot multiplication plays an important role in tissue culture activities; therefore, it should be optimized via several factors, such as the composition of the medium and the concentration of plant growth regulators. The aim of this study is to optimize the kinetin concentrations and medium compositions for shoot multiplication originating from cotyledonary node explants of several local citrus cultivars for genome editing activities. The cotyledonary nodes from three citrus cultivars (Batu55, Siam Madu, and Proksi-1 Agrihorti) were incubated in MS medium with Morel and Wetmore vitamins (VMW) supplemented with several kinetin concentrations (0; 0.2; 0.4; 0.6; 0.8; and 1 mg/L). The best kinetin concentrations for the number of shoots variable were then combined with Murashige and Tucker (MT) medium. The results showed that the combination of VMW medium and kinetin concentration at 0.8 mg/L produced the best number of shoots in Batu55 cultivar as well as 1 mg/L concentration in Siam Madu and Proksi-1 Agrihorti cultivars. Therefore, those two concentrations were then chosen to be combined with MT medium in the second experiment. The results showed that combination of 1 mg/L kinetin with MT medium showed the best number of shoots, the highest percentage of shoot formation, the biggest number of leaves, the biggest number of nodes, and the longest shoot length in this study. This medium composition could be further used for shoot multiplication in genetic transformation in those three citrus cultivars, including genome editing activities in the development of new improved citrus varieties.

Keywords: citrus, cotyledonary nodes, kinetin, murashige and tucker medium, shoot multiplication

INTRODUCTION

Citrus is a high-value horticultural crop that is beneficial to our health because its fruits contain high levels of vitamin C and antioxidants. However, one of the challenges faced in citrus cultivation programs not only in Indonesia, but also globally is the attack of Huanglongbing disease or citrus vein phloem degeneration (CVPD) caused by *Candidatus* Liberibacter asiaticus (CLas) transmitted through the insect *Diaphorina citri* (Killiny *et al.* 2018, Wang 2019). The symptoms include leaf chlorosis resembling manganese or zinc deficiencies, stunted plant growth, smaller number of fruit, smaller fruit size, bitter fruit taste, and asymmetrical locules/segments within the fruit (Bove 2006). Such symptoms can result in staggering yield losses in citrus plants, ranging from 30% to as high as 100% (Iftikhar *et al.* 2016).

The development of a newly improved citrus cultivar resistant to Huanglongbing disease is necessary to prevent yield losses due to the disease. Unfortunately, the majority of the commercial citrus cultivars are susceptible to this disease (Pandey *et al.* 2022). Several citrus species and their relatives, including *Citrus latipes*, *Poncirus trifoliata*, *Murraya paniculata*, *Severinia buxifolia*, *Carrizo citrange*, rough lemon, and *Microcitrus austrasica* are known to have resistance to

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Huanglongbing infections (Killiny *et al.* 2018, Rao *et al.* 2018). However, efforts to transfer resistance genes to commercial citrus through interspecies crosses have been hindered by various challenges, such as incompatibility, apomixis, and polyembryony (Zhang *et al.* 2018, Montalt *et al.* 2021). CRISPR/Cas9 is one of the new promising technologies that could be used to knock out the activity of genes responsible for the susceptibility to Huanglongbing disease in citrus. Among the targeted genes for silencing is the callose synthase gene, which is responsible for callose accumulation in phloem tissue – a physiological response observed in citrus plants infected by this disease.

Genetic transformation is one of the crucial steps in genome editing activities. Information regarding plant regeneration methods is very necessary because it will affect the successful process of the genetic transformation itself. This information can be obtained through an optimization process which include several factors such as the type of explant, the composition of the medium, and the concentration of plant growth regulators.

The utilization of different explants from epicotyl for genetic transformation of Taroco blood orange has been previously reported by Peng *et al.* (2015) with the transformation efficiency range from 21.4-22%. In contrast, the use of embryogenic callus originating from unfertilized ovules in sweet orange (*Citrus sinensis*) showed transformation efficiency ranging from 26.3-36.5% (Dutt *et al.* 2020). The use of explants from nucellar and zygotic embryos in genetic transformation activities of *Japansche Citroen* has been reported by Aisyah (2021) with transformation efficiencies of 13.80 and 21.17% respectively. The utilization of cotyledonary nodes as explants in genetic transformation activities of citrus has not been widely reported despite their high regeneration ability. These nodes offer the advantage of direct organogenesis for shoot multiplication without callus formation (Nwe *et al.* 2014, Fatonah *et al.* 2018).

The composition of the medium plays an important role in facilitating successful plant regeneration in citrus species. Several studies have shown that Murashige and Skoog (MS) media modified by Morel & Wetmore vitamins (VMW) are suitable for *in vitro* growth of citrus, as reported in somatic embryogenesis of mandarin (Purwito *et al.* 2015), somatic embryogenesis from endosperm tissue of *C. nobilis* (Kosmiatin *et al.* 2014), and in micropropagation and micrografting of *Japansche Citroen* (Putra *et al.* 2015). Conversely, Murashige & Tucker (MT) medium is also widely utilized in citrus plants, including *in vitro* propagation of Thomson navel sweet oranges (Esmaeilnia & Dehestani 2015) and embryo rescue in progenies resulting from crosses between mandarin and Carrizo citrange (Kurt & Ulger 2019).

In addition to the type of explant and medium composition, the concentration of plant growth regulators also determines the success of *in vitro* regeneration processes. Excessive concentrations of plant growth regulators could impede growth and result in abnormal shoots, while insufficient concentration will result in a low number of plantlets produced (Shankar *et al.* 2014). Therefore, the concentration of plant growth regulators also needs to be optimized. Kinetin is a plant growth regulator from cytokinin group that is useful in the process of shoot formation and regeneration. The use of powerful cytokinin such as Benzyl Adenine (BA) or 6-benzylaminopurine (BAP) in shoot formation can lead to rapid cell division and the formation of undesirable callus as previously reported by Kosmiatin *et al.* (2014) and Prameswari *et al.* (2019). Furthermore, Kumar *et al.* (2014) also reported that kinetin treatment in 1 mg/L showed the best survival rate in shoot tip grafting of Kinnow Mandarin. The use of kinetin in shoot proliferation have been previously reported in several studies (Shankar *et al.* 2014, Putra *et al.* 2015, Khiem *et al.* 2022). The aim of this study is to optimize the kinetin concentrations and medium compositions for shoot multiplication derived from cotyledonary node explants of several citrus cultivars in order to obtain the best combination that will be used in genome editing activities.

MATERIALS AND METHODS

Genetic Materials

The immature citrus fruits, approximately 11-15 weeks after anthesis, were collected from three cultivars; Batu55 (*C. reticulata* Blanco.), Siam

Madu (*C. nobilis* L.), and Proksi-1 Agrihorti (a cross between Siam Madu and Mandarin Satsuma). The fruits were obtained from Balai Pengujian Standar Instrumen Tanaman Jeruk dan Buah Subtropika (BSIP Jestro) Malang and Balai Besar Pengujian Standar Instrumen Bioteknologi dan Sumber Daya Genetik Pertanian (BBPSI Biogen). The study was conducted at the Plant Cell and Tissue Culture Laboratory, BBPSI Biogen Bogor, from September 2022 to July 2023.

Isolation and Sterilization of Citrus Seeds

The seeds were isolated from the fruits following the method outlined by Kosmiatin & Husni (2018). Initially, the citrus fruit was sterilized by dipping it in 96% alcohol and passing it over the Bunsen five times. The sterilized fruit was then placed in a sterile petri dish, where the skin was peeled using a scalpel, and the seeds were removed using tweezers. The seed coats were then removed, and the cotyledons were planted in MS medium supplemented with 3 mg/L GA_3 (Kosmiatin *et al.* 2014).

Optimization of kinetin concentrations in MS medium modified with Morel and Wetmore vitamins (VMW)

The cotyledonary nodes, measuring 0.5-0.7 cm in length, were isolated from one-month-old seedlings of the three citrus cultivars (Figure 1A). These nodes were then incubated in MS media modified with Morel and Weitmore vitamins (VMW) (Morel & Wetmore 1951) supplemented with 30 g/L sucrose and 7 g/L of agar. The pH of the medium was adjusted to 5.8 by adding 0.1 N NaOH before autoclaving for 15 min at 121°C. There were several levels of kinetin concentration used in this study e.g., 0; 0.2; 0.4; 0.6; 0.8; and 1 mg /L (Figure 1B). This study was designed on a completely randomized design (CRD). Each kinetin concentration treatment consisted of three cotyledonary nodes with ten replications. The explants were cultured at room temperature (25±2°C) using TL 60 W 220 V (±1000 lux) lighting with 16 hours per day over an 8-week period. Observations were carried out for 8 weeks, focusing on four variables: namely the number of shoots, the leaves and nodes, the shoot length, and the percentage of shoot formation. The data were analyzed using analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) with a significance level of 5% using the Statistical Tool for Agricultural Research (STAR) version 2.0.1 (Gulles *et al.* 2014).

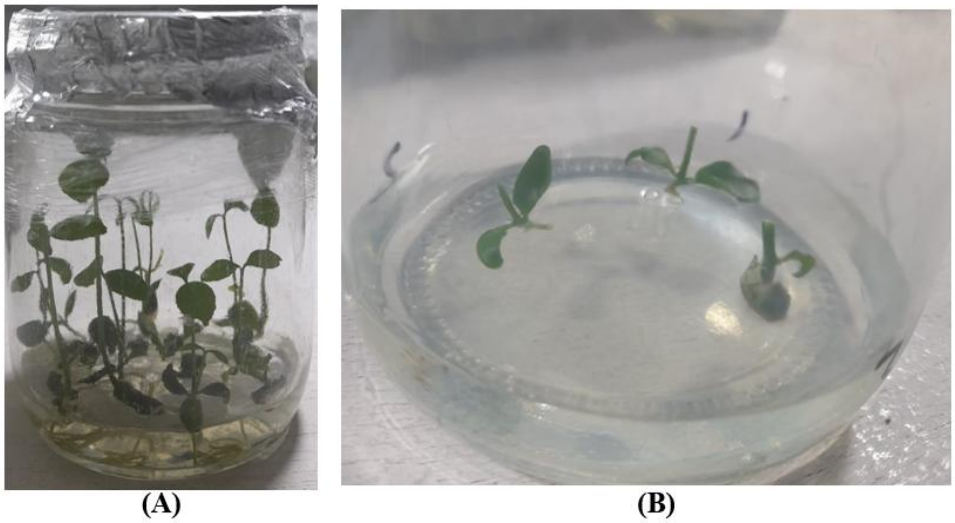

Figure 1 The explants that were used in this study consisted of (A) one-month citrus seedling in MS + 3 mg/L GA_3 medium, and (B) cotyledonary nodes cutting from one-month seedling

Combination of the optimum kinetin concentration with Murashige and Tucker (MT) medium

The optimal kinetin concentrations identified from the previous treatment were incorporated into Murashige and Tucker (MT) medium (Murashige & Tucker 1969), using cotyledonary node explants from similar citrus cultivars. Each treatment consisted of three cotyledonary nodes with ten replications. Observations were carried out over an 8-week period, focusing on four variables: namely, the number of shoots, the leaves and nodes, the shoot length, the and percentage of shoot formation. The data were then compared to the previous treatment using VMW medium and analyzed using analysis of variance (ANOVA) followed by Least Significant Difference (LSD) with a significance level of 5% using the Statistical Tool for Agricultural Research (STAR) version 2.0.1 (Gulles *et al.* 2014).

RESULTS AND DISCUSSION

Optimization of Kinetin Concentration in VMW Medium

The effects of kinetin supplementation in VMW medium on shoot multiplication showed varied responses across the citrus cultivars used in this study, as shown in Table 1. Interestingly, all kinetin concentrations treated in this study showed no significant differences in the number of shoots, leaves and nodes, and shoot length variables across all cultivars examined. In Batu 55 cultivar, the optimal number of shoots was observed at a kinetin concentration of 0.8 mg/L, while the optimal number of leaves occurred at 0.6 mg/L kinetin. Additionally, the optimal number of nodes was observed at kinetin concentrations of 0.4 and 0.6 mg/L respectively. The optimum shoot length was obtained with the control treatment. In Siam Madu cultivar, the optimal number of shoots, leaves, and shoot length were identified from 1 mg/L kinetin concentration, while the optimal number of nodes was identified from 0.6 mg/L kinetin concentration. Conversely, in Proksi-1 Agrihorti cultivar, the optimal number of shoots, leaves, and nodes were identified from 1 mg/L kinetin concentration, while the optimal shoot length was identified from 0.8 mg/L kinetin concentration.

Table 1 The effect of several kinetin concentrations on citrus shoot multiplication after 8 weeks cultivation

Citrus cultivars	Kinetin concentrations (mg/l)	Number of shoots	Percentage of shoot formation	Number of leaves	Number of nodes	Shoot length
Batu 55	0	1.00±0.17	76.67±35.31	3.73±0.90	1.57±0.52	**0.88±0.35**
	0.2	0.90±0.0	60.00±30.63	3.00±0.73	1.42±0.39	0.74±0.24
	0.4	1.00±0.0	53.33±28.11	3.18±0.64	**1.60±0.52**	0.82±0.21
	0.6	0.90±0.17	70.00±42.89	**3.82±1.16**	**1.60±0.59**	0.76±0.18
	0.8	**1.07±0.34**	60.00±37.84	3.48±1.23	1.23±0.56	0.66±0.25
	1.0	1.00±0.0	**76.67±16.10**	3.63±0.84	1.45±0.53	0.83±0.23
Siam Madu	0	0.68±0.22	43.33±44.58	1.87±0.72	0.83±0.39	0.37±0.10
	0.2	0.73±0.13	60.00±46.61	2.18±0.12	1.13±0.49	0.53±0.17
	0.4	0.83±0.12	53.33±39.13	2.93±1.28	1.35±0.38	0.64±0.19
	0.6	0.90±0.0	66.67±35.14	2.80±0.83	**1.60±0.55**	0.73±0.16
	0.8	0.80±0.0	56.67±38.65	2.05±0.68	0.97±0.31	0.51±0.14
	1.0	**1.08±0.20**	**73.33±34.43**	**3.40±1.08**	1.48±0.29	**0.75±0.18**
Proksi-1 Agrihorti	0	0.63±0.14	56.67±49.81	2.27±0.66	1.43±0.33	0.56±0.22
	0.2	0.73±0.24	66.67±38.49	2.70±0.81	1.37±0.49	0.72±0.31
	0.4	0.67±0.13	63.33±45.68	2.43±0.74	1.42±0.44	0.64±0.37
	0.6	0.87±0.43	70.00±42.89	3.23±1.70	1.50±0.71	0.68±0.41
	0.8	0.87±0.35	**73.33±43.89**	3.60±1.75	1.63±0.74	**0.82±0.29**
	1.0	**1.07±0.40**	**73.33±40.98**	**3.63±0.80**	**1.67±0.56**	0.72±0.29

Note: The numbers in bold show the highest average value for each variable

The kinetin concentrations used in this study also did not show significant difference in the percentage of shoot formation variable after eight weeks of cultivation. The highest percentage of shoot formation was obtained with 1 mg/L kinetin concentration in Batu55, Siam Madu, or Proksi-1 Agrihorti cultivars (Table 1). Masekesa *et al.* (2016) reported that supplementation of 0.4 mg/L kinetin gave the best shoot formation percentage in sweet potato regeneration using petiole explants. Meanwhile, Foo *et al.* (2018) reported that the highest percentage of shoot formation (65%) in eggplant was obtained with kinetin treatment at 2.0 mg/L from the cotyledon explants. A previous study by Kumar *et al.* (2014) showed that supplementation of 1 mg/L kinetin produced the best result in shoot length from shoot tip grafting of Kinnow mandarin (*Citrus deliciosa*). Kumar *et al.* (2014) also reported that the increasing level of kinetin higher than 1 mg/L showed the inhibitory effect for citrus. On the other side, Abu-Romman *et al.* (2015) reported that 1 mg/L kinetin gave the best result in regeneration rate, number of shoots, and length of shoots in cucumber. In good agreement with those studies, Dincer (2023) reported that a 1 mg/L kinetin concentration showed the best result for shoot number, shoot length, and number of nodes in *Sorbus aucuparia* and was also the optimal concentration for number of shoots and percentage shooting efficiency in regeneration of barley from calli (Abbas *et al.* 2023).

Shoot multiplication is an important stage in micropropagation, wherein new shoots are induced from the axil as presented in Figure 2. The presence of cytokinins such as kinetin will disrupt the apical dominance and promote lateral shoot formation (Kane 2011). The type and concentration of cytokinin play a critical role in the shoot multiplication rate, shoot length, and frequency of genetic variation (Kane 2011). A previous study from Abu-Romman *et al.* (2015) in cucumber showed that kinetin had a more influential effect on shoot multiplication compared to other cytokinin types such as 6-benzylaminopurine (BAP), thidiazuron, and zeatin.

In this study, the number of shoots became the most crucial variable to consider, given that all citrus cultivars that used in this study are classified as scion type. Scion is a type of citrus cultivar with high production and commercially appealing, favored by consumers (Kosmiatin *et al.* 2010), which is in contrast to rootstocks which are tolerant to dry condition and have a strong stem and root, but are less attractive and commercially viable (Jayanti *et al.* 2015). The shoots will be utilized in genetic transformation mediated by *A. tumefaciens* and selected using antibiotic medium for genome editing activities. The selected shoots will then be grafted ex vitro to citrus rootstock in the greenhouse. The reason why the root induction variable was not observed in this study is that the acclimatization process will not be carried out. Even though the kinetin concentration used in this study did not show a significant difference in the number of shoot variables, the kinetin concentration at 0.8 mg/L showed the highest number of shoots in Batu 55 as well as 1 mg/L concentration at Siam Madu and Proksi-1 Agrihorti cultivar. Therefore, those two concentrations were chosen to be combined with Murashige and Tucker medium in the second experiment.

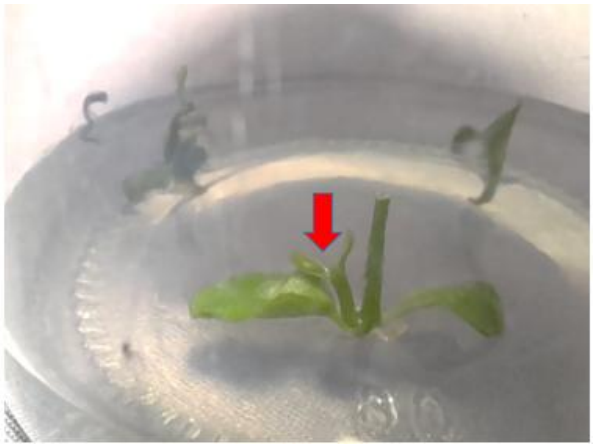

Figure 2 New shoot induced from lateral shoot on cotyledonary node explant used in this study

Combination of the Optimum Kinetin Concentrations with Murashige and Tucker (MT) Medium

The effects of combining the best kinetin concentrations from the previous experiment (0.8 and 1 mg/L) with Murashige and Tucker medium are presented in Table 2. Overall, the results indicate that this combination led to an increase in citrus shoot multiplication variables compared to the previous treatment using VMW medium. In Batu55 cultivar, the optimal number of shoots, percentage of shoot formation, number of nodes, and shoot length were obtained at 1 mg/L kinetin, while the optimal number of leaves was obtained at 0.8 mg/L kinetin. The use of MT medium in Batu55 cultivar showed significant difference to VMW medium at 1 mg/L kinetin concentration in terms of the number of shoots, percentage of shoot formation, number of nodes, and shoot length variables. In the Siam Madu cultivar, all of the optimal variables were identified at 1 mg/L kinetin, and the use of MT medium showed significant differences to VMW medium at 1 mg/L kinetin concentration across all variables. In line with the findings for Siam Madu, all optimal variables were also identified at 1 mg/L kinetin in Proksi-1 Agrihorti cultivar, and the use of MT medium showed significant differences to VMW medium at 1 mg/L kinetin concentration in the number of leaves, number of nodes, and shoot length variables.

Basal medium composition plays an important role in plant micropropagation. While the Murashige and Skoog (MS) medium is commonly used for *in vitro* culture of citrus, reports suggest that MT medium has shown better results in some instances (Carimi & de Pasquale 2003). Basically, MT medium composition is similar to MS basal medium, with increased concentration of several vitamins such as thiamine HCl, pyridoxine HCl, and nicotinic acid. Vitamins present in tissue culture medium serve as cofactors for enzymes in plant metabolisms (Abrahamian & Kantharajah 2011). Together with other media components, vitamins have direct and indirect effects on several physiological responses such as somatic growth, rooting, callus formation, and embryonic development (Kadhimi *et al.* 2014). The utilization of MT medium in citrus micropropagation has been reported in several previous studies. These include micropropagation of Thomson navel sweet oranges (Esmaelnia & Dehestani 2015), *in vitro* regeneration of *C. limon* from leaf explants (Kasprzyk-Pawelec *et al* 2015), somatic embryogenesis of Mexican lime (*C. aurantifolia*) from juice vesicles (Amin & Shekafandeh 2015), somatic embryogenesis of mandarin Batu 55 from nucellus (Februyani *et al* 2016), embryo rescue in progenies from crossing between

Table 2 The effect of the combination between the best kinetin concentration from the previous experiment with Murashige and Tucker medium on citrus shoot multiplication after 8 weeks

Citrus cultivars	Kinetin concentrations (mg/L)	Medium composition	Number of shoots	Percentage of shoot formation	Number of leaves	Number of nodes	Shoot length
Batu55	0	MS + VMW	1.00	76.67	3.73	1.57	0.88
		MT	1.27	90.00	4.30	1.77	1.21
	0.8	MS + VMW	1.07	60.00[a]	3.48	1.23[a]	0.66[a]
		MT	1.25	93.33[b]	**4.90**	2.38[b]	1.20[b]
	1.0	MS + VMW	1.00[a]	76.67[a]	3.63[a]	1.45[a]	0.83[a]
		MT	**1.30[b]**	**100.00[b]**	4.60[b]	**2.43[b]**	**1.43[b]**
Siam Madu	0	MS + VMW	0.68	43.33	1.87	0.83[a]	0.37[a]
		MT	1.17	76.67	2.60	1.65[b]	1.14[b]
	0.8	MS + VMW	0.80	56.67[a]	2.05[a]	0.97[a]	0.51a
		MT	1.03	90.00[b]	3.57[b]	2.27[b]	1.39b
	1.0	MS + VMW	1.08	73.33[a]	3.40	1.48[a]	0.75[a]
		MT	**1.27**	**100.00[b]**	**4.57**	**2.30[b]**	**1.49[b]**
Proksi-1 Agrihorti	0	MS + VMW	0.63[a]	56.67	2.27	1.43	0.56
		MT	1.13[b]	76.67	3.67	1.93	0.83
	0.8	MS + VMW	0.87[a]	73.33	3.60[a]	1.63	0.82
		MT	1.43[b]	83.33	1.43[b]	2.37	1.19
	1.0	MS + VMW	1.07	73.33	3.63[a]	1.67[a]	0.72[a]
		MT	**1.57**	**86.67**	**5.55[b]**	**2.48[b]**	**1.32[b]**

Note: Numbers followed by different letters show significant differences based on LSD test. The numbers in bold show the highest average value for each variable.

mandarin and *Carrizo citrange* (Kurt & Ulger 2019).

The VMW medium uses similar inorganic salt to the MS basal medium but incorporates modifications to the Morel & Wetmore (VMW) vitamins such as nicotinic acid, pyridoxine HCl, calcium pantothenate, and biotin. The addition of biotin and calcium pantothenate in VMW medium is crucial for cell membrane formation, the mitosis process, and the multiplication of shoots and roots (Sukmadjaja et al. 2022). On the contrary, in this study VMW medium generally showed lower results in terms of shoot number, percentage of shoot formation, number of leaves, number of nodes, and shoot length compared to MT medium as evidenced in Table 2, Figure 3.

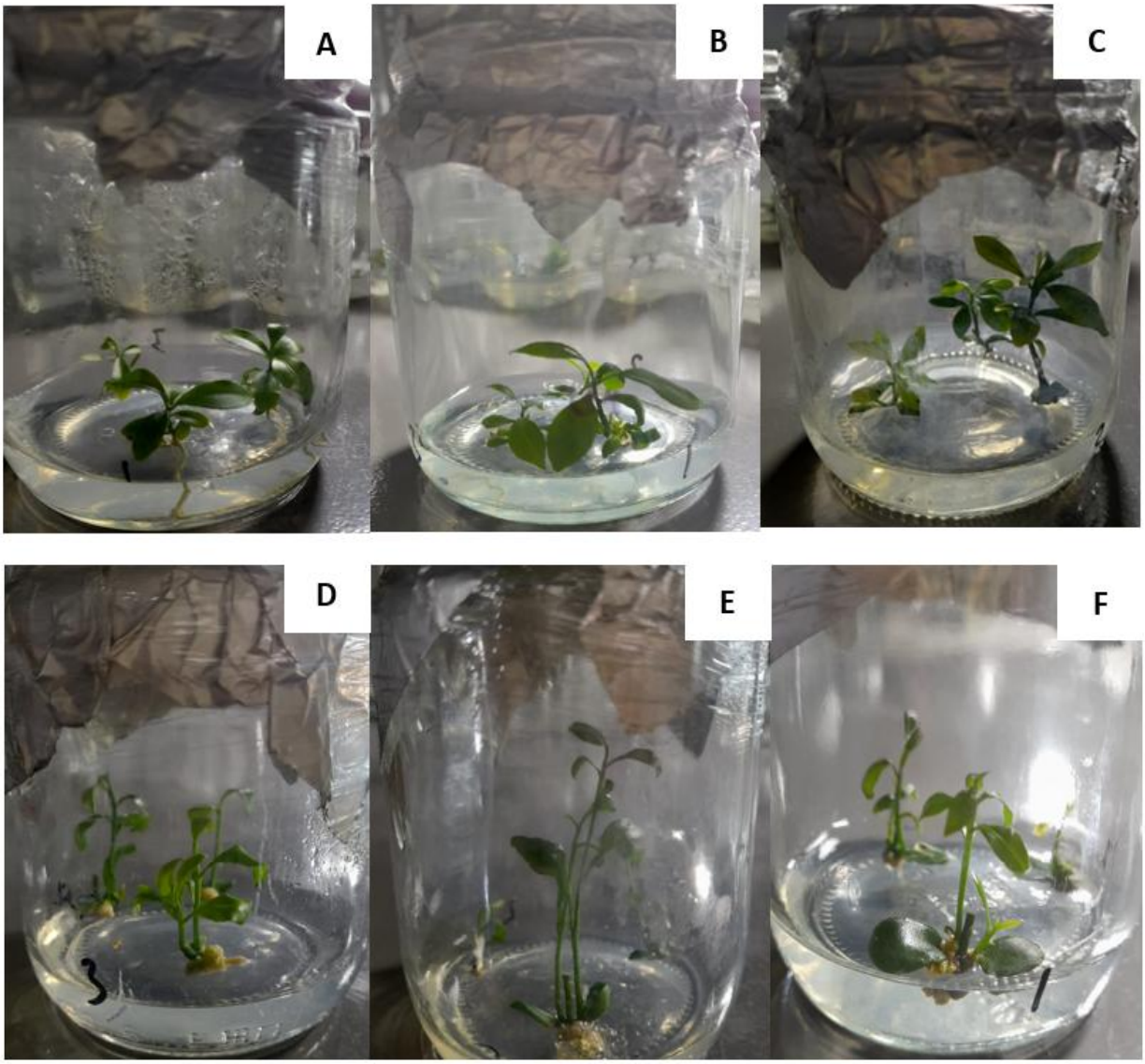

Figure 3 Citrus shoot formed after eight weeks derived from combination of 1 mg/L kinetin with VMW medium (A, B, C) and MT Medium (D, E, F). A and D: Batu 55 cultivar, B and E: Siam Madu cultivar, C and F: Proksi-1 Agrihorti cultivar

The absence of thiamine HCl from VMW medium composition significantly affects citrus shoot multiplication. According to Al-Khayri (2001), thiamine serves an important function as cofactor in metabolism of carbohydrate, while biotin plays an important role in carboxylation reactions. Al-Khayri (2001) reported that improving thiamine concentration from 0.1 to 0.5 mg•/L gave the maximum callus growth while increasing of biotin from 0 to 1 mg/L gave the maximum callus weight respectively. In a previous study by Kazemiani *et al.* (2018),

supplementation of 10 mg/L thiamine-HCl in MS medium, combined with 50 mg/L nicotinic acid, 50 mg/L pyridoxine-HCl, and 4 mg/L BAP, resulted in the maximum number of lateral shoots in potato *in vitro* culture. Additionally, Vollmeer *et al.* (2023) reported that the addition of 0.1 mg/L thiamine to modified MS medium significantly increased plant height, root length, and number of nodes of *in vitro* sweet potato shoot culture. The regeneration medium is one of the key factors in plant genetic transformation besides construct design, explant selection, transformation method, and antibiotic selection method (Altpeter *et al.* 2016). Thus, the selection of a suitable regeneration medium is very important to ensure the success of the genetic transformation process, as mentioned by Zhang *et al.* (2021). They highlighted that, despite advances in genome editing systems, establishing a plant tissue culture or regeneration system for the targeted plant species remains challenging.

CONCLUSIONS

The optimization of kinetin concentration combined with VMW media on three citrus cultivars in this study showed that a kinetin concentration of 0.8 mg/L yielded the highest number of shoots in Batu55 cultivar, while a concentration of 1 mg/L concentration was optimal for Siam Madu and Proksi-1 Agrihorti cultivar. Overall, the supplementation of kinetin in VMW medium did not show significant differences in the number of shoots, percentage of shoot formation, number of leaves, number of nodes, and shoot length variables on three citrus cultivars investigated in this study. In contrast, the combination of the best kinetin concentration on number of shoots variables (0.8 and 1 mg/L) with MT medium showed that 1 mg/L kinetin gave the best number of shoots, percentage of shoot formation, number of leaves, number of nodes, and shoot length on three citrus cultivars used in this study. This medium composition could be used for shoot multiplication in genetic transformation processes in these three citrus cultivars, in order to knock out the activity of callose synthase gene and to increase citrus resistance to Huanglongbing disease.

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