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EFFECT OF CCC ON GROWTH RETARDATION DURING MEDIUM-TERM SLOW-GROWTH STORAGE FOR *IN VITRO* CULTURE OF 'RAJA SEREH' BANANA (*MUSA* SPP., AAB, SUBGROUP SILK)

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ABSTRACT

This study aimed to find a method for 16-week *in vitro* storage of 'Raja Sereh' banana using chlormequat chloride (Cycocel, CCC) as a growth retardant. Shoot multiplication was carried out using kinetin to obtain aseptic shoots. The results showed effective shoot multiplication on Murashige and Skoog (MS) medium supplemented with 6.0 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA). *In vitro* storage in media containing CCC at concentrations of 5.0 mg·L^{-1} and 7.5 mg·L^{-1} without subculturing inhibited growth, reducing plantlet height and the number of shoots and leaves, inhibiting leaf length, while leaf width increased. Subculturing the plantlets on fresh medium without CCC showed that the banana plantlets retained their regenerative potential and formed new shoots and roots. This study concluded that CCC could be a potential growth retardant for the slow-growth storage of 'Raja Sereh' banana and its application in agricultural practices for banana propagation and preservation.

Key words: chlormequat chloride, kinetin, medium-term storage, shoots multiplication

INTRODUCTION

Banana and plantain, as the leading fruit commodities, are widely grown and used in almost all regions in Indonesia. The dessert 'Raja Sereh' banana (*Musa* spp., AAB, subgroup Silk) is triploid and male sterile and its propagation is usually carried out vegetatively through suckers (Valmayor et al. 2000). However, the traditional method of banana plant propagation is labor-intensive (Benelli et al. 2022). Hence, the potential loss of banana germplasm is quite significant, especially since bananas are herbaceous giant plants that require large areas. To address these challenges, developing solutions for *in vitro* propagation and *in vitro* storage is crucial to reduce the potential loss of germplasm.

In vitro propagation of banana plants has been extensively studied. Notable cultivars include 'Raja Bulu' (Elma et al. 2017), 'Kepok' (Masykuroh et al. 2016), 'Barangan' (Indrayanti et al. 2018; Riono 2019), and 'Tanduk' (Abdulhafiz et al. 2018; Indrayanti et al. 2021). The success of *in vitro* propagation methods relies on several factors, including the genotype of the plant, the nutrient medium used, the synthetic plant growth regulators (PGRs), their concentrations, and the type of culture used, all of which can lead to different growth responses (Le Bris 2017; Koti et al. 2024). Kinetin (KIN), a synthetic cytokinin, has proven to be effective in banana propagation (Muhammad et al. 2007; Habib et al. 2016).

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The term subculture refers to the transfer of plants to new, fresh medium. The frequency and timing of subcultures can affect the success of the *in vitro* culture process, emphasizing the importance of optimized protocols to achieve large-scale propagation (Hesami et al. 2023). In long-term culturing, repeating subcultural stages poses several challenges, including the need for time, cost, labor, risk of contamination, and potential loss of morphogenetic potential and somaclonal variability (Hao & Deng 2003; Li et al. 2022; Benelli et al. 2022). Currently, medium-term *in vitro* storage is an encouraging technique to reduce plant maintenance costs and genetic variability due to repeated subculturing and to facilitate the distribution of plant material (Benelli et al. 2022; Li et al. 2022). This technique can be developed and implemented for banana plants, including storage in a growing state (short-term), with minimal growth (medium-term), and by freezing or cryopreservation (long-term) (Engelman 1991; Chauhan et al. 2019).

Slow growth of plant germplasm through medium-term storage based on *in vitro* culture presents several cost-effective advantages over traditional methods (Ramírez-Pérez et al. 2020; Benelli et al. 2022). Slow-growth method, often carried out by using alternative gelling agents and carbon sources (Agrawal et al. 2010; Sintim & Akromah 2015), lowering the culture temperature (de Oliveira et al. 2000; de Lacerda et al. 2021), providing osmotic agents to the culture medium (Negash et al. 2001; El-Bahr et al. 2016), and adding growth inhibitors such as paclobutrazol (Ribeiro et al. 2011; Indrayanti et al. 2018) and chlormequat chloride (Chang et al. 2019; Taha et al. 2019; Buldakov 2021) or removing growth promoters from the medium (Oseni et al. 2018). These methods have been effective in the conservation of several plant germplasms such as ginger, potato, and sweet potato (Li et al. 2022).

Chlormequat chloride or chlorocholine chloride (CCC), trade name Cycocel, is an organic salt containing equal numbers of chlormequat and chloride ions (Lucho et al. 2021; NCBI 2024). CCC is used as a plant growth retardant, known for its anti-gibberellin activity that affects plant physiology and morphology (Kulkarni et al. 2018; Lucho et al. 2021; Koti et al. 2024). This study addresses the challenges of medium-term *in vitro* storage processes and potentially sustainable and cost-effective plant propagation. However, for each plant species or even genotype, factors such as the composition of salts in the culture media, plant growth regulators, and transplantation period must be determined to maximize storage efficiency. According to de Oliveira et al. (2000), monitoring conserved materials for genetic stability, viability, and phytosanitary conditions is crucial.

MATERIALS AND METHODS

The research was conducted at the Plant Tissue Culture Laboratory of the Biology Study Program, FMIPA, Universitas Negeri Jakarta, Indonesia. A 4–5-month-old 'Raja Sereh' banana (*Musa* spp., AAB, subgroup Silk) plantlet was obtained from the Centre for Seed Development and Plant Protection Lebak Bulus in Jakarta. The chemicals used included MS mineral salt medium (Murashige & Skoog 1962), 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine HCl, 0.5 $mg \cdot L^{-1}$ thiamine HCl, 2.0 $mg \cdot L^{-1}$ myo-inositol, 100 $mg \cdot L^{-1}$ $FeSO_4 \cdot 2H_20$, 28.7 $mg \cdot L^{-1}$ NaEDTA, 6-furfurylaminopurine (KIN), indole-3-acetic acid (IAA), CCC (Sigma-Aldrich), commercial agar, and sugar. Three experiments were conducted in a completely randomized design (CRD): 1 – effect of KIN on 'Raja Sereh' shoot multiplication, 2 – effect of CCC on 'Raja Sereh' medium-term slow-growth *in vitro* storage, 3 – plantlet regeneration after *in vitro* storage.

Effect of 6-furfurylaminopurine (KIN) concentration on *in vitro* growth and multiplication

This experiment aimed at obtaining aseptic shoots in media supplemented with KIN and IAA for further experimental stages. Two media were evaluated: MS with 3.0 mg·L^{-1} KIN + 0.175 mg·L^{-1} IAA and 6.0 mg·L^{-1} KIN + 0.175 mg·L^{-1} IAA. Each treatment had 16 replicates (culture jars) and four explants per replicate. Plantlets were cultured at 24 ± 2°C under moderate fluorescent light intensity (1000 lux) for a 16-h photoperiod. Growth was evaluated after four weeks based on the number of shoots, roots, leaves, and leaf length and width.

Effect of chlormequat chloride (CCC) on minimal growth *in vitro*

This stage of the research aims to determine the optimal concentration of CCC needed to induce slow growth of banana plantlets. In this experiment, aseptic shoots from the previous trial were subcultured on MS medium containing 6.0 mg·L^{-1} KIN and 0.175 mg·L^{-1} IAA. The medium was supplemented with different concentrations of CCC: 0 (control), 2.5, 5.0, and 7.5 mg·L^{-1}. Each CCC treatment was replicated six times, with four shoot explants per replicate. Plantlets were stored *in vitro* in these media for 16 weeks without subculturing. The evaluation included the number of shoots and leaves, leaf length, leaf width, root formation, and plantlet height.

Regeneration of banana plantlets after *in vitro* storage

The ability to propagate was evaluated after 16 weeks of storage. Banana plantlets were subcultured on fresh MS medium supplemented with KIN and IAA without CCC. The parameters observed 1–4 weeks after subculture on fresh medium included the number of shoots, leaves, roots, and the height and leaf shape of plantlets.

Data analysis

Data were analyzed using descriptive statistics, independent Student t-test, and analysis of variance (ANOVA) using SPSS version 26 to determine the effect of treatment, followed by Duncan's multiple range test at the 5% level.

RESULT AND DISSCUSSION

Effect of 6-furfurylaminopurine (KIN) on banana growth and multiplication *in vitro*

Shoot growth was affected by KIN concentration. Significant differences were visible 2–4 weeks after transplanting (Table 1). The number of shoots increased with KIN concentration from 3 mg·L^{-1} to 6 mg·L^{-1} by 45% in the second and third week and by 34% in the fourth week, reaching 5.8 shoot per explant (Table 1). The number of leaves was non-significantly lower at higher KIN concentration. Previous studies by Gana (2011) reported that high concentrations of cytokinins (1–10 mg·L^{-1}) could induce the formation of adventitious shoots while inhibiting root development. Similarly, Ferdous et al. (2015) found that higher concentrations of cytokinin increased shoot production in 'Sabri' and 'Amritasagar' banana.

Table 1. Effect of 6-furfurylaminopurine (KIN) in combination with 0.175 mg·L^{-1} indole-3-acetic acid (IAA) on the number of shoots and leaves of 'Raja Sereh' banana (mean ± SE)

KIN (mg·L^{-1})	Week of culture			
	1	2	3	4
	Number of shoots			
3	2.50^{a} ± 0.16	2.90^{a} ± 0.25	3.59^{a} ± 0.27	4.28^{a} ± 0.33
6	2.87^{a} ± 0.25	4.21^{b} ± 0.33	5.09^{b} ± 0.37	5.75^{b} ± 0.38
	Number of shoots			
3	1.06^{a} ± 0.06	1.78^{a} ± 0.25	3.59^{a} ± 0.65	5.59^{a} ± 0.59
6	0.93^{a} ± 0.07	1.68^{a} ± 0.31	3.25^{a} ± 0.41	5.28^{a} ± 0.68

Means followed by the same letter in the same column are not significantly different based on the independent Student t-test at the 5% level

Research by Muhammad et al. (2007) on 'Basrai' banana showed that 6.0 mg·L^{-1} KIN produced 4.4 shoots during in four weeks of culturing. On the other hand, a study on 'Grande Naine' banana culturing in media containing 1.0 mg·L^{-1} KIN produced an average of 5.4 shoots. Plantlets grown in media containing 3.0 mg·L^{-1} KIN produced 5.59 leaves in four weeks of culturing (Habib et al. 2016). Elma et al. (2017) reported that 'Raja Bulu' banana cultured in media containing 2.0 mg·L^{-1} KIN produced an average of 5.6 leaves in 12 weeks of culture. According to the above reports and Agbadje et al. (2021), the optimal cytokinin concentration for shoot proliferation is highly dependent on genotype.

Banana plantlets grown on 3.0 mg·L^{-1} KIN were taller than those grown in media with higher KIN concentration (8.0 versus 6.3 cm) (Fig. 1). Our results confirmed those obtained by Riono (2019) for 'Barangan'. Higher KIN concentration would induce cell division but inhibit cell elongation, resulting in a shorter plantlet. This combination of growth regulators is necessary for *in vitro* culture media, and the auxin to cytokinin ratio must be considered to effectively modify the growth pattern of plant material during culture (Dixon & Gonzales 1994).

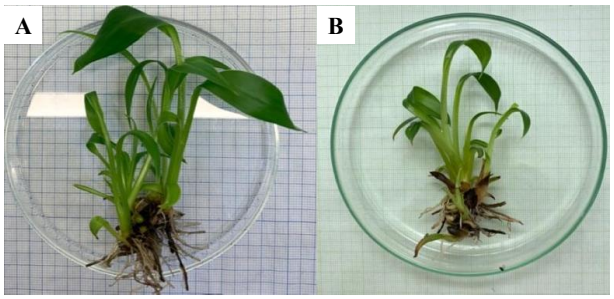

Figure 1. Effect of 6-furfurylaminopurine (KIN) concentration and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) on the height of plantlets in media containing 3.0 mg·L^{-1} (A) and 6.0 mg·L^{-1} (B) KIN after 4 weeks of culturing

Effect of chlormequat chloride (CCC) on minimal growth *in vitro*

Subculturing of banana shoots on MS medium containing the growth retardant CCC showed a reduction in shoot production over a 16-week period by 76% (at 5 mg·L^{-1}) and 62% (at 7.5 mg·L^{-1}) (Table 2). Under the same conditions, the number of roots was reduced by 15%. The highest number of leaves was obtained at 2.5 mg·L^{-1} CCC, which was different from the other treatments. Plantlets grown without CCC developed necrotic leaves (Fig. 2), which may indicate nutrient deficiencies or imbalances in the media, a phenomenon that can lead to plantlet necrosis and eventual plant death (Teixeira da Silva et al. 2020). The presence of CCC also affected leaf shape. After 16 weeks, plantlets grown in media with higher CCC concentrations exhibited shorter but wider leaves compared with the control and the 2.5 mg·L^{-1} CCC treatment (Table 3, Fig. 3).

Slowing down the growth of *in vitro* plantlets, which is implemented in commercial production or *in vitro* plant collections, is mainly achieved by lowering the temperature, but also by growth inhibitors (Benelli et al. 2022). Culturing banana plantlets for 16 weeks at 5.0 mg·L^{-1} and 7.5 mg·L^{-1} CCC in the medium can significantly minimize shoot and leaf growth and root development (Table 2). CCC is a plant growth retardant that is a highly stable inhibitor of gibberellin (GA) biosynthesis, resulting in plants with thicker and more vigorous stems (Pirasteh-Anosheh et al. 2021; NCBI 2024). By inhibiting GA production, CCC effectively reduces stem growth by retarding elongation and affecting root growth and overall plant development (Emam & Moaicd 2000; Kulkarni et al. 2018; Li et al. 2022).

In some plant species, GA-induced cell elongation resulted from a shift in the orientation of cell growth from random to longitudinal. GA promotes the transverse alignment of cortical microtubules, which mediates the deposition of cell wall microfibrils. Furthermore, GA promotes cell enlargement, probably by loosening the cell wall and altering the turgor pressure within the cell, leading to leaf expansion and changes in leaf shape (Hedden 2016). Gibberellins are isoprenoid plant hormones that share biosynthetic pathways with abscisic acid (ABA). This pathway leads to the formation of five classes of plant hormones: cytokinins, brassinosteroids, gibberellins, abscisic acid, and strigolactones (Bajguz & Piotrowska-Niczyporuk 2023). Recent discoveries have shown direct molecular links between GA and ABA signaling components, indicating their antagonistic regulation (Liu & Hou 2018).

Table 2. Effect of chlormequat chloride (CCC) in combination with 6.0 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) on the number of shoots, leaves, and roots of 'Raja Sereh' banana after 16 weeks of *in vitro* storage

CCC (mg·L^{-1})	Number of shoots	Number of leaves	Number of roots
0	$7.33^{b} \pm 1.66$	$12.75^{a} \pm 2.16$	$21.83^{b} \pm 1.20$
2.5	$7.25^{b} \pm 0.72$	$16.91^{b} \pm 1.07$	$20.08^{ab} \pm 1.00$
5.0	$4.16^{a} \pm 0.35$	$12.50^{a} \pm 1.31$	$18.75^{a} \pm 0.78$
7.5	$4.50^{a} \pm 0.46$	$14.08^{a} \pm 1.22$	$18.41^{a} \pm 0.15$

Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test (DMRT) at the 5% level

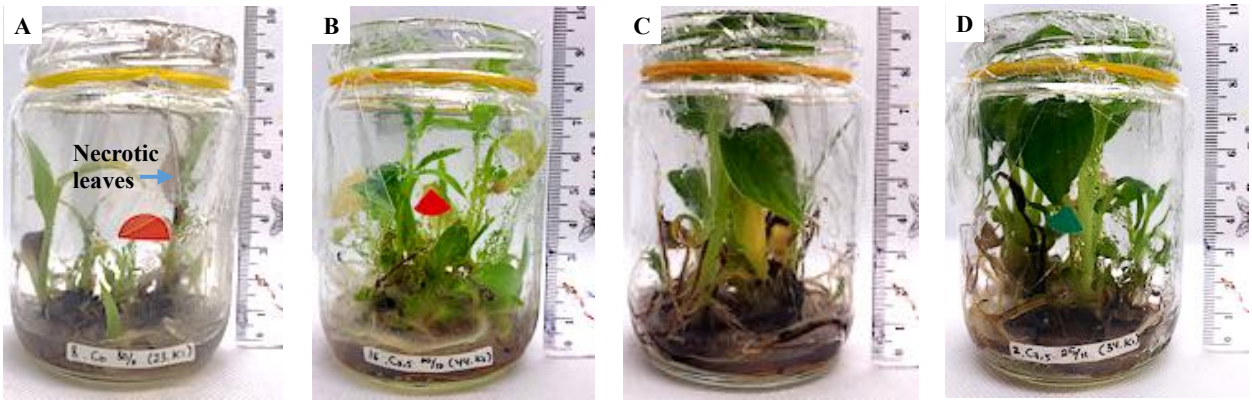

Figure 2. Effect of chlormequat chloride (CCC) concentration in medium containing 6 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA): 0 mg·L^{-1} (A), 2.5 mg·L^{-1} (B), 5.0 mg·L^{-1} (C), and 7.5 mg·L^{-1} (D) CCC after 16 weeks of *in vitro* storage

Table 3. Effect of chlormequat chloride (CCC) in combination with 6.0 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) on leaf length (cm) and width (cm), leaf length to width ratio, roots number, and plantlet height (cm) of banana after 16 weeks of storage

CCC (mg·L^{-1})	Leaf length	Leaf width	Leaf length to width ratio	Plantlet height
0	$3.69^{b} \pm 0.21$	$0.79^{a} \pm 0.15$	$4.67^{c} \pm 0.18$	$7.29^{b} \pm 0.53$
2.5	$3.50^{b} \pm 0.26$	$0.90^{a} \pm 0.05$	$3.89^{b} \pm 0.25$	$7.26^{b} \pm 0.54$
5.0	$2.81^{a} \pm 0.16$	$1.34^{b} \pm 0.05$	$2.10^{a} \pm 0.21$	$6.74^{ab} \pm 0.82$
7.5	$2.82^{a} \pm 0.41$	$1.24^{b} \pm 0.07$	$2.27^{a} \pm 0.24$	$6.28^{a} \pm 0.38$

Note: see Table 2

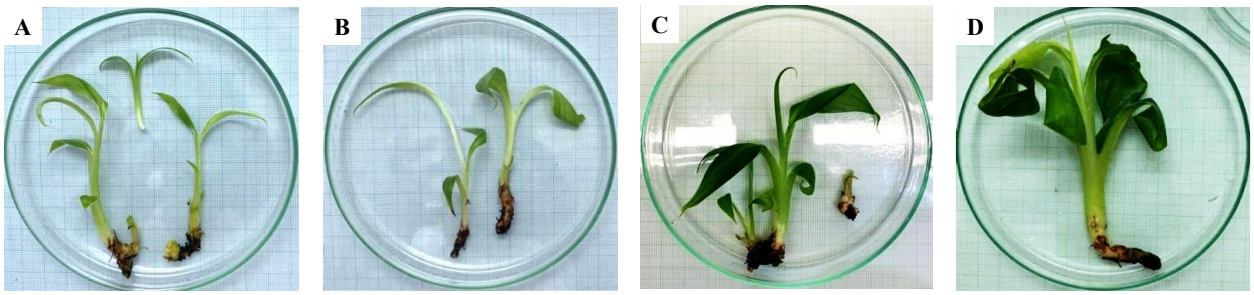

Figure 3. Effect of chlormequat chloride (CCC) concentration in media containing 6 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) on single shoots: 0 mg·L^{-1} (A), 2.5 mg·L^{-1} (B), 5.0 mg·L^{-1} (C), and 7.5 mg·L^{-1} (D) CCC after 16 weeks of *in vitro* storage

ABA modulates various metabolic processes in plants, including shoot growth and development. It is a typical auxin-induced inhibitor to limit lateral bud growth (Le Bris 2017; Agehara & Leskovar 2015). GAs are derived from geranylgeranyl diphosphate (GGPP), produced via the methylerythritol phosphate (MEP) pathway, whereas ABA is synthesized directly from carotenoids, which are also derived from GGPP (Bajguz & Piotrowska-Niczyporuk 2023). When CCC inhibits GA synthesis, it can increase ABA synthesis, suggesting inhibition of shoot growth. This study showed that CCC application could reduce the number of shoots (Table 2). Shukla et al. (1992) observed that CCC application could increase the ABA content in *Cosmos caudatus* (L.) cultures.

Previous studies have shown that different concentrations of CCC can significantly affect plant growth. Studies by Taha et al. (2019) showed that CCC at 5 mg·L^{-1} inhibited new leaf and root growth and plantlet height in pineapple (*Ananas comosus* 'Queen'). In potato, Li et al. (2022) reported that the combination of 1/4 MS medium with a high concentration of CCC (100 mg·L^{-1}) almost completely arrested growth, reducing the number of leaves and roots and stem height in 'Zihuabai' without causing malformations. Furthermore, Sallam et al. (2023) showed that CCC at 2 mg·L^{-1} extended seed storage to 24 weeks, enabling germination of encapsulated seeds 45 days after storage. In this study, CCC application at 5.0 mg·L^{-1} and 7.5 mg·L^{-1} also modified leaf characteristics, making them shorter, wider, dark green, and thicker (Table 3, Fig. 3). Shorter leaves are probably due to inhibition of GA synthesis, which limits cell elongation while allowing further cell division. Youssef and Abd El-Aal (2013) noted that the growth-inhibiting effects of CCC vary with concentration, application method, location, species, and cultivar. According to Moore (1979), plant growth retardants are generally organic compounds that inhibit growth without stimulating effect at any concentration.

Regeneration of banana plantlets after *in vitro* storage in media containing chlormequat chloride (CCC)

Subculturing of banana plantlets in fresh medium without retardant was aimed at restoring their growth capacity after 16 weeks of storage in medium containing CCC. In this study, all plantlets regenerated successfully, indicating no further retardation (Table 4; Fig. 4). After 4 weeks in the regeneration medium, plantlets stored in 5.0 mg·L^{-1} and 7.5 mg·L^{-1} CCC formed more shoots (55%) and roots (77%). This suggests that CCC application during storage resulted in more shoots and roots compared to storage without growth retardant.

Table 4. Regeneration of shoots, leaves, and roots of 'Raja Sereh' banana after *in vitro* storage in media containing 6 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) after 1–4 weeks of culture

CCC (mg·L^{-1})	Number of shoots		Number of leaves		Number of roots	
	1 week	4 weeks	1 week	4 weeks	1 week	4 weeks
0	1.41^{a} ± 0.20	1.50^{a} ± 0.18	3.33^{a} ± 0.27	4.75^{a} ± 0.77	0.75^{a} ± 0.17	2.16^{a} ± 0.45
2.5	1.33^{a} ± 0.16	1.58^{a} ± 0.30	4.58^{a} ± 0.39	5.91^{a} ± 0.59	0.83^{a} ± 0.10	2.91^{a} ± 0.42
5.0	1.58^{a} ± 0.15	2.33^{b} ± 0.44	3.75^{a} ± 0.52	5.66^{a} ± 0.57	1.33^{a} ± 0.30	3.83^{b} ± 0.50
7.5	1.60^{a} ± 0.21	2.08^{b} ± 0.27	4.33^{a} ± 0.57	5.50^{a} ± 0.81	1.25^{a} ± 0.30	3.25^{ab} ± 0.44

Note: see Table 2

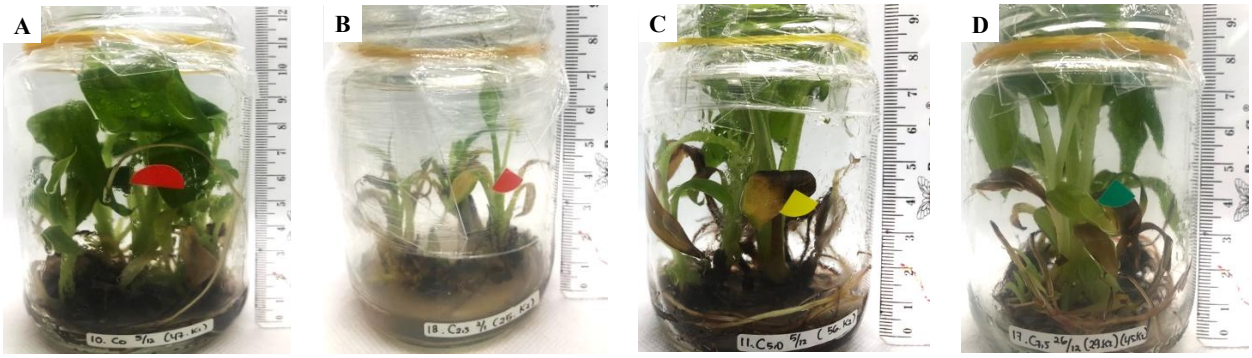

Figure 4. Effect of chlormequat chloride (CCC) concentration on banana regrowth in media supplemented with 6 mg·L^{-1} 6-furfurylaminopurine (KIN) and 0.175 mg·L^{-1} indole-3-acetic acid (IAA) after 16 weeks of *in vitro* storage: 0 mg·L^{-1} (A), 2.5 mg·L^{-1} (B), 5.0 mg·L^{-1} (C), and 7.5 mg·L^{-1} (D) CCC after 4 weeks of culture

In vitro cultured plants can be easily retrieved to produce new plants while maintaining full genetic integrity (Ahmad et al. 2012; Bhatia 2015). Regeneration or regrowth of plantlets after *in vitro* storage is essential for evaluating the effectiveness of storage methods and determining the ability of plantlets to grow after storage for extended periods. The potential for banana shoots to grow and develop after storage will help validate the effectiveness of *in vitro* storage methods. In this study, 'Raja Sereh' banana successfully regenerated on MS medium supplemented with 6.0 mg·L^{-1} KIN and 0.175 mg·L^{-1} IAA for four weeks after *in vitro* storage (see Fig. 4). Benelli et al. (2022) reported that potato regeneration after storage in 100 mg·L^{-1} CCC supplemented with 1/4 MS medium in vacuum-packed plates led to significantly lower new tissue regrowth compared to MS medium. Indrayanti et al. (2024) report that encapsulated nodule-like meristems of 'Barangan' banana could form new shoots after regrowth on MS medium containing 0.22 mg·L^{-1} TDZ and 1.75 mg·L^{-1} IAA after six months of *in vitro* storage. The ability of the encapsulated meristem to grow depends on its source. The presence of new shoots and roots indicated that the growth inhibition by CCC could be reversed in the regeneration medium. This study confirms that CCC effectively manages the growth of banana plantlets during *in vitro* storage without harming future regeneration.

CONCLUSION

Research has shown that shoot multiplication of 'Raja Sereh' banana is successful on Murashige and Skoog medium supplemented with 6.0 mg·L^{-1} 6-furfurylaminopurine and 0.175 mg·L^{-1} indole-3-acetic acid. The lower level of 3.0 mg·L^{-1} 6-furfurylaminopurine was more effective in elongating plantlets, showing that the ideal concentration of a plant growth regulator may differ based on the desired growth outcome. The ability of banana plantlets to grow after *in vitro* storage concluded that chlormequat chloride at concentrations of 5.0 mg·L^{-1} and 7.5 mg·L^{-1} effectively induced slow-growth conditions. Maintaining minimal plant growth is beneficial for *in vitro* storage, allowing plantlets to control their viability and developmental capacity. This practice also improves the effectiveness of chlormequat chloride in preserving banana germplasm.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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