Research Article

Effects of medium components on *in vitro* multiplication efficiency of Siam tulip

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Abstract

Siam tulip (*Curcuma alismatifolia*) is a significant economic ornamental plant in Thailand. A commercial production of Siam tulip should mainly use disease-free rhizome to avoid disease accumulation, which could affect the export of Siam tulip's rhizomes. Therefore, the enhancing efficiency of tissue culture of Siam tulip for disease-free and quality-consistent in plant production is another approach to improve the quality of Siam tulip production for export capacity. This study aimed to evaluate the effects of cytokinins and nutrient concentrations in MS medium, by using shoots harvested from cv. Red Shadow and cv. Khao Yai as explant for the experiments. The results showed that the 4 mg/L of N6-benzylaminopurine (BAP) resulted in the highest shoot multiplication of cv. Khao Yai and 5 mg/L of BAP gave the highest shoot multiplication of cv. Red Shadow. While Kinetin resulted in the increasing of length of explants. Moreover, the reduction of nutrient concentration in MS medium to 0.5 times and the increase of sucrose concentration to 2 times or 6% provided the highest shoot multiplication. While reducing the nutrient concentration in MS medium to 0.25 times decreased the growth of both cv. Red Shadow and cv. Khao Yai.

Keywords: Cytokinins, Shoot multiplication, Concentration of medium

Introduction

Curcuma alismatifolia, commonly known as Siam tulip or Phatumma, is a beautiful and valuable ornamental plant. The species belongs to the Zingiberaceae family due to its attractive colorful bracts [1]. It can be cultivated as a potted plant, used for decoration, or grown in the field for tourism, such as Phatumma fields. Siam tulip is a tropical perennial species native to countries in Southeast Asia such as Thailand, Cambodia or Laos. Thailand is one of the leaders for exporting flowers and rhizomes of *C. alismatifolia* to several countries, i.e. the United States, the Netherlands, New Zealand and Japan [1, 2]. Increasing the economic value of this plant in Thailand has started from the domestic market to the international market, resulting in higher income for the Siam tulip farmers [1, 2]. Typically, the underground part of Siam tulip consists of rhizomes and storage roots [2]. The conventional technique for rhizomes production is to grow rhizomes harvested from the previous season in soil, which can cause problems such as low-quality plant stocks or contamination with soil borne pathogens [2, 3]. Nowadays, several growers or farmers in Thailand cultivate Siam tulip for rhizome production using soil-less media to produce high quality and disease-free rhizomes [3]. The most critical disease found in Siam tulips is bacterial wilt, caused by

Ralstonia solanacearum [3]. Although farmers use many chemicals to control wilt disease, this approach fails to provide a sustainable solution for long-term cultivation. Besides, applying unnecessary or ineffective chemicals can cause soil pollution in fields. Using clean and aseptic rhizomes for crop production with integrated pest management is a solution for the contamination problems because healthy mother plants yield high quality of Siam tulip rhizomes for exporting [3, 4].

Micropropagation is an appropriate technique to produce healthy, disease free, uniform, and massive rhizomes in a short period of time [5, 6, 7]. This study focused on the efficient and repeatable micropropagation protocol for *C. alismatifolia*. The growth of plantlets in sterile conditions is influenced by several factors. One of them is the balance of cytokinins and auxins hormones, which affect the growth and morphological change of the shoots [8]. Typically, the application of cytokinins alone has been used for shoot multiplication in many plant species [8, 9, 10]. BAP and kinetin are effective compounds used as plant growth regulators for shoot regeneration [8, 9, 10]. Nevertheless, the adjustment of mineral salts was one of a crucial factor for improving the micropropagation in many plants, such as stevia and mockorange [11, 12, 13]. Moreover, sucrose is used as an energy source for plant tissue culture. Optimizing the sucrose supplement enhanced the *in vitro* growth of plants [14, 15, 16]. Therefore, the objective of this study was to investigate the types of cytokinins and optimize the concentration of BAP and kinetin for increasing shoot multiplication and *in vitro* growth of *C. alismatifolia* cv. Red Shadow and cv. Khao Yai.

Materials and Methods

Preparation

Clean culture plantlets derived from inflorescences of two Siam tulip cultivars: cv. Red Shadow and cv. Khao Yai. were proliferated in the plant tissue culture laboratory at the University of Phayao, Thailand (Fig. 1A). These plantlets were cultured on the Murashige and Skoog (MS) medium [17] with 2 mg/L N6-benzylaminopurine (BAP) in glass bottles. All cultures were maintained in a culture room at 25±1^{o}C with a 16 h photoperiod and 40-50 μM/m^{2}s irradiance provided by cool white, fluorescent bulbs (Zeberg LED T8, 22W, BKK, Thailand). Subcultures with four-week intervals were required to produce adequate plant materials. Before culturing in each experiment, plantlets were transferred to free-plant growth regulator medium.

The effects of cytokinins on *in vitro* growth and multiplication of Siam tulip cv. Red Shadow and cv. Khao Yai

For investigating the effects of cytokinins, BAP and Kinetin were tested at different concentrations (1, 2, 3, 4 and 5 mg/L). The experimental design was completely randomized with 11 treatments, and MS basal medium without cytokinins was a controlled treatment. Explants for the experiment were cut pseudostems about 3-5 cm shown in Fig. 1B. All cultured vessels were incubated in the culture room described above. In this experiment, plants were grown for 5 weeks. The length of new shoots, new shoots number, new roots number, and length of explants were recorded to evaluate the growth (Fig 1B).

The combinatory effects of concentration strength of MS basal medium and sucrose concentrations on the *in vitro* growth of Siam tulip cv. Red Shadow and cv. Khao Yai

Fifteen semi-solid media were designed using 0.25-, 0.5-, 1-, 1.5- and 2-time MS strength (xMS) with 3%, 6% and 9% sucrose. These combinations were used to investigate the effect of MS mineral strength and sucrose concentration on *in vitro* growth and micro-rhizome production. The experimental design was completely randomized with 5 replicates. New shoots number (shoots), length of new shoots (cm), new root length (cm) and length of explant shoots (cm) were evaluated after cultured for 6 weeks.

Statistics

The results were presented as mean ± standard error (SE). Analysis of variance (ANOVA), a collection of statistical models and their associated estimation procedures used to analyze the differences among means, was employed for this study. The mean values were compared using Duncan's Multiple Range test (DMRT) and analyzed by Statistical Package for Social Sciences (SPSS) version 28.0 software (IBM Corp.; Armonk, NY, USA).

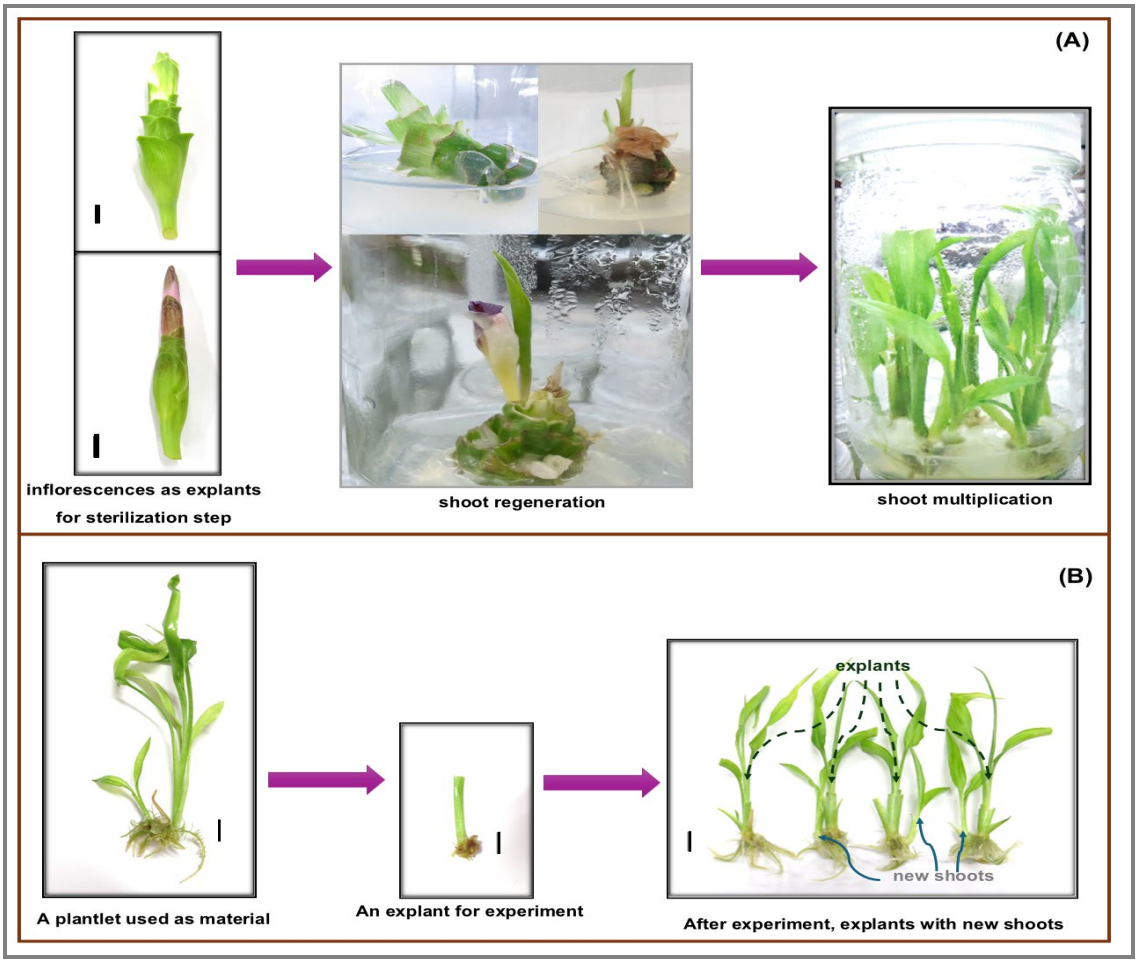

Fig 1 Scheme for (A) plantlets preparation from inflorescences of *C. alismatifolia* or Siam tulip cultivars (cv. Red Shadow and cv. Khao Yai) and (B) definition of plantlets, explants and new shoots.

Results

The effects of cytokinins on *in vitro* growth and multiplication of Siam tulip cv. Red Shadow and cv. Khao Yai

The effects of two types of cytokinins (BAP and Kn) were presented in Fig. 2 and 3. Typically, BAP was more effective in increasing the length of new shoots and new shoot number in both cultivars. Any concentrations of BAP exhibited a positive effect on the length of new shoots (Fig. 2A and 3A). For cv. Red Shadow, the effect of BAP on the length of new shoots derived from explants was significantly different. The highest concentration of BAP provided the highest new shoots number. However, explants grown on 5 mg/L BAP were not significantly different from explants grown on 2, 3 or 4 mg/L BAP (Fig. 2B). Moreover, for new roots number of cv. Red Shadow, explants grown on any BAP concentration were not significantly different compared to the control. However, explants grown on any of the Kn concentrations were significantly different compared to the control. They had lower new roots numbers in any concentrations of Kn (Fig. 2C). The length of explants in any Kn concentrations were not significantly different compared to control (MS without BAP or Kn). Nevertheless, explants grown on MS medium supplemented with any BAP concentration were less length of explant significantly from control, except the 0.1 mg/L of Kn (Fig. 2D).

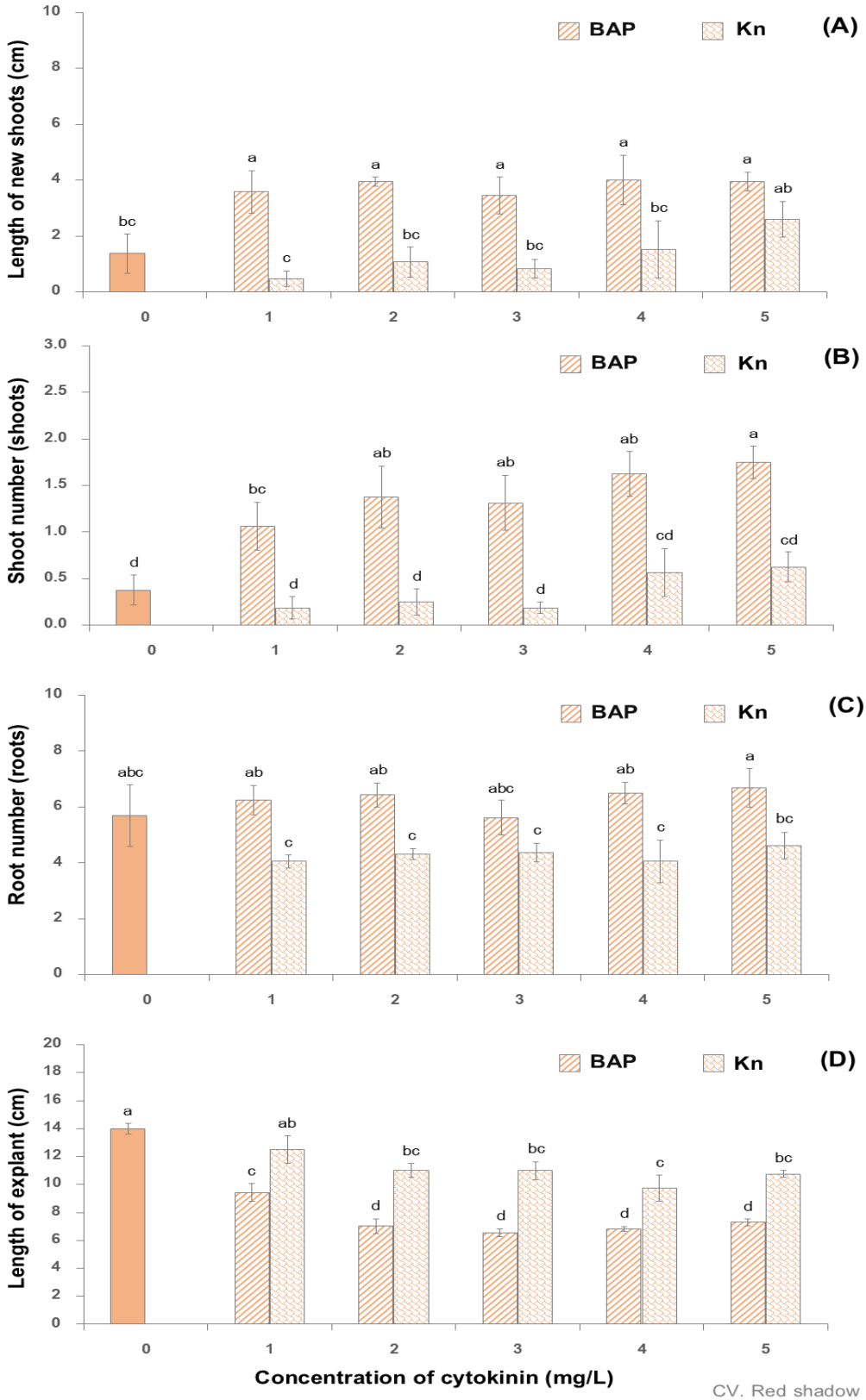

Fig 2 The effects of cytokinins on (A) length of new shoots, (B) new shoots number, (C) new roots number, and length of explant of *C. alismatifolia* cv. Red Shadow cultured for 5 weeks. Each bar graph represents the mean (n = 12) and error bar indicates ± SE. The same letter at each treatment indicates not significantly ($p > 0.05$).

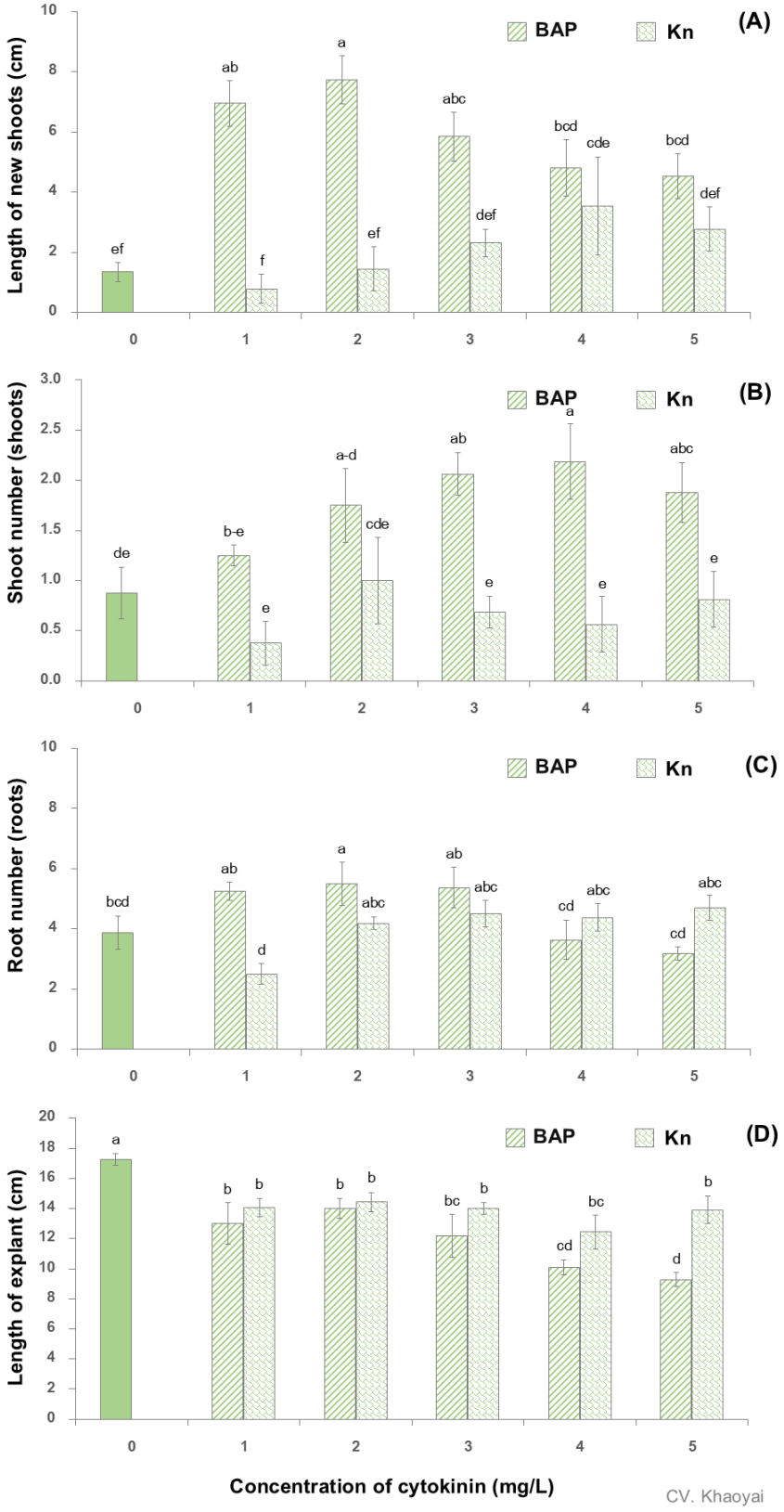

Fig 3 The effects of cytokinins on (A) length of new shoots, (B) new shoots number, (C) new roots number, and length of explant of *C. alismatifolia* cv. Khao Yai cultured for 5 weeks. Each bar graph represents the mean (n = 12) and error bar indicates ± SE. The same letter at each treatment indicates not significantly ($p > 0.05$).

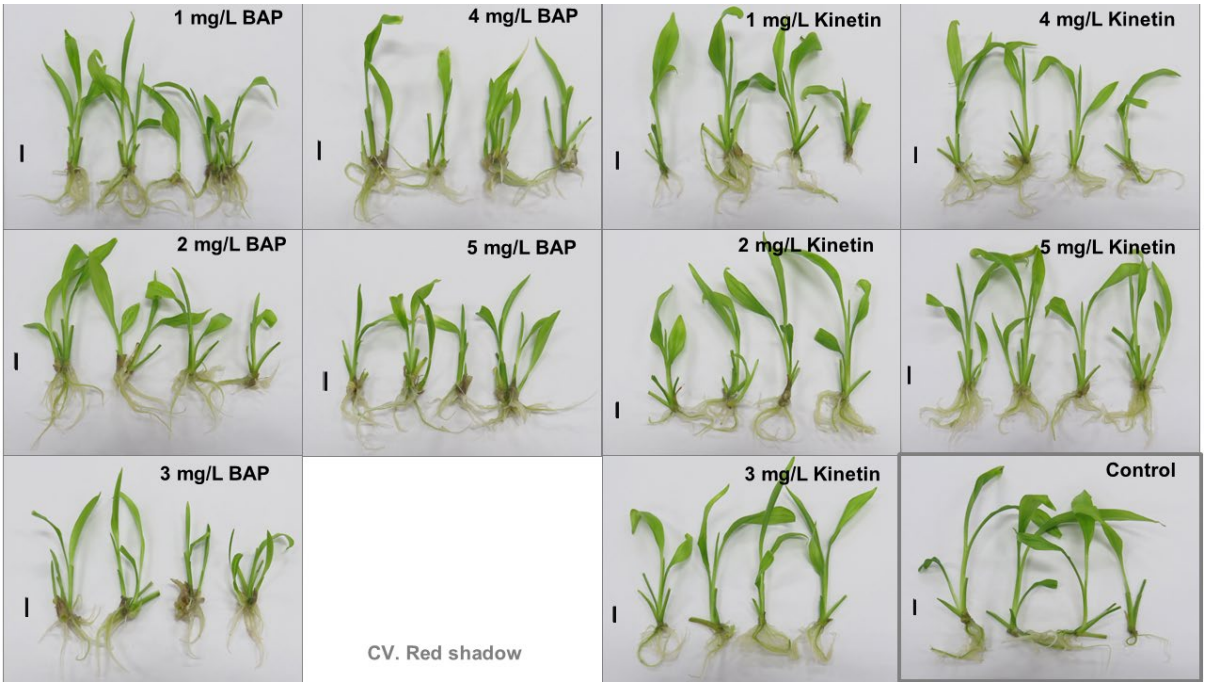

Fig 4 Growth appearance of *in vitro* shoots of *C. alismatifolia* cv. Red Shadow cultured on MS medium supplemented with various cytokinins for 6 weeks (bar = 1.0 cm).

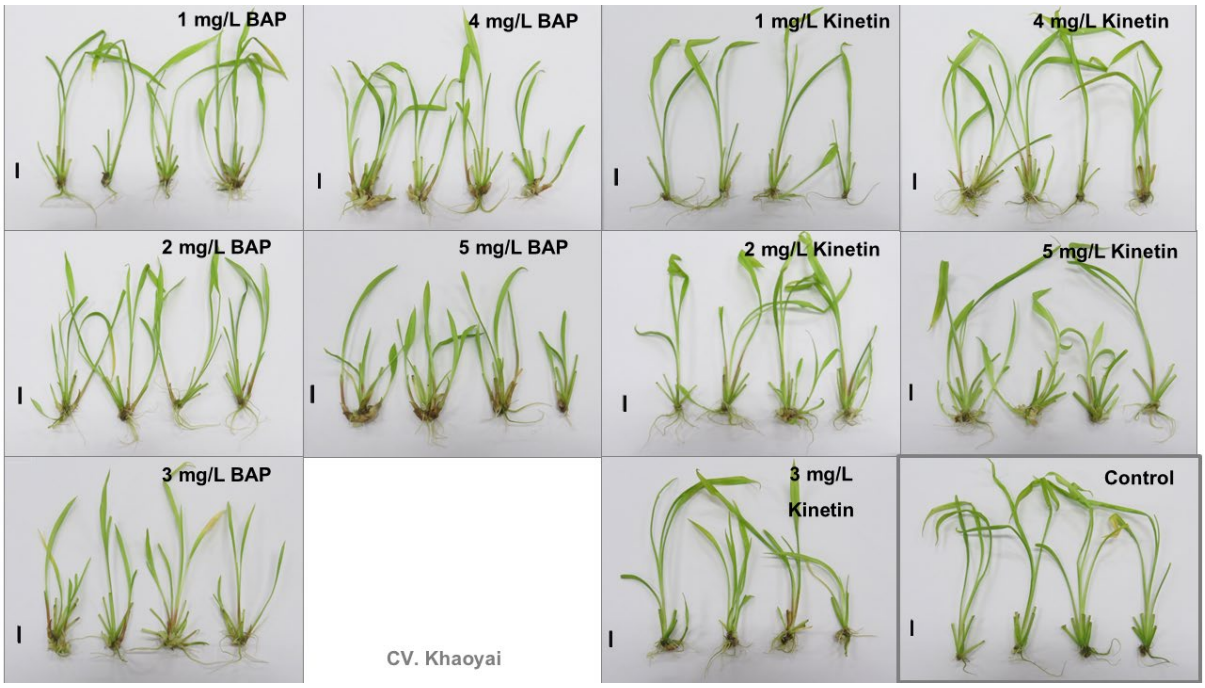

Fig. 5 Growth appearance of *in vitro* shoots of *C. alismatifolia* cv. Khao Yai cultured on MS medium supplemented with various cytokinins for 6 weeks (bar = 1.0 cm).

For cv. Khao Yai, the response of the new shoots number was the same trend as the response of the cv. Red Shadow. The length of new shoots of cv. Khao Yai, explants grown on any BAP concentrations had longer new shoots compared to explants grown on any Kn concentrations and control (Fig. 3A and B). Explants grown on 2 mg/L BAP had the highest length of new shoots, but it was not significantly different from explants grown on 1 or 3 mg/L BAP (Fig. 3A). For the new shoots number of cv. Khao Yai, the response of this cultivar was the same trend as cv. Red Shadow (Fig. 3B). For new roots number, explants grown on 2 mg/L BAP had the highest new roots number, but it was not significantly different from explants grown on 1 or 3 mg/L BAP including 2, 3, 4 or 5 mg/L Kn (Fig. 3C). For the length of explants, explants grown on control medium had the highest length compared to other treatments (Fig. 3D). The growth appearance of both cultivars had been shown in Fig. 4 and 5. Shoots multiplication could be observed in explants grown on the MS media supplemented with cytokinins (Fig. 4 and 5).

The combinatory effects of concentration strength of MS basal medium and sucrose concentrations on the *in vitro* growth of Siam tulip cv. Red Shadow and cv. Khao Yai

The study examined the effects of different sucrose concentrations (3, 6, and 9%) combined with five MS basal medium concentration strengths (0.25, 0.5, 1, 1.5, and 2x) on the shoots multiplication and growth of *C. alismatifolia* varieties cv. Red Shadow and cv. Khao Yai in Table 1 and 2. The highest new shoots number was observed at 0.5x MS + 6% sucrose for both varieties: cv. Red Shadow (2.42 shoots) and cv. Khao Yai (2.21 shoots). The longest new shoots were recorded at 1x MS + 9% sucrose for cv. Red Shadow (1.66 cm) and 1x MS + 6% sucrose for cv. Khao Yai (3.41 cm) (Table 1 and 2). Sucrose levels (6–9%) with 1x MS significantly promoted shoots elongation. Optimal shoots multiplication occurred at 0.5x MS + 6% sucrose. Optimal shoots elongation was promoted at 1x MS with 6–9% sucrose, particularly benefiting cv. Khao Yai. Excessively high MS concentrations (1.5x and 2x) negatively affected both new shoots number and length, likely due to excessive nutrients or osmotic stress. Higher MS concentrations (1.5x and 2x) resulted in shorter shoot lengths.

Table 1 The combinatory effect of MS strength (expressed as xMS) and sucrose concentration on shoots growth and development of Siam tulip after 6 weeks of culture (Trt = treatment).

Trt	MS	Sucrose	New shoots number (shoots)		Length of new shoots (cm)	
	(x)	(%)	cv. Red Shadow	cv. Khao Yai	cv. Red Shadow	cv. Khao Yai
1	0.25	3	0.21±0.1^{f}	0.67±0.3^{cde}	0.17±0.1^{e}	1.67±0.6^{cde}
2	0.25	6	1.08±0.1^{cde}	1.29±0.3^{a-d}	0.64±0.1^{cde}	1.88±0.4^{b-e}
3	0.25	9	0.88±0.2^{def}	0.96±0.2^{b-e}	0.32±0.1^{e}	1.44±0.6^{cde}
4	0.5	3	1.25±0.6^{b-e}	0.50±0.1^{de}	0.57±0.1^{de}	1.73±0.5^{cde}
5	0.5	6	2.42±0.1^{a}	1.50±0.3^{a-d}	0.88±0.1^{cd}	3.32±0.7^{ab}
6	0.5	9	1.92±0.3^{ab}	1.17±0.4^{b-e}	0.63±0.1^{cde}	2.01±0.4^{a-d}
7*	1	3	0.83±0.2^{def}	0.63±0.2^{cde}	1.13±0.2^{bc}	1.63±0.5^{cde}
8	1	6	1.75±0.4^{abc}	1.92±0.4^{ab}	1.13±0.2^{bc}	3.41±0.4^{a}
9	1	9	1.79±0.2^{abc}	1.75±0.3^{ab}	1.66±0.2^{a}	1.62±0.2^{cde}
10	1.5	3	0.13±0.1^{f}	0.17±0.1^{e}	0.22±0.1^{e}	0.40±0.2^{e}
11	1.5	6	1.54±0.2^{bcd}	2.21±0.6^{a}	1.16±0.2^{bc}	3.30±0.7^{ab}
12	1.5	9	1.92±0.2^{ab}	0.58±0.2^{cde}	1.42±0.2^{ab}	0.87±0.3^{de}
13	2	3	0.29±0.1^{f}	0.17±0.1^{e}	0.49±0.3^{de}	0.32±0.2^{e}
14	2	6	0.75±0.2^{ef}	1.58±0.5^{abc}	0.88±0.2^{cd}	2.78±0.6^{abc}
15	2	9	0.67±0.2^{ef}	0.67±0.3^{cde}	0.66±0.3^{cde}	0.85±0.3^{de}
F-test			Sig	Sig	Sig	Sig

Values (mean ± Standard Deviation: SE) in each column superscripted with different lowercase letters are significantly (p < 0.05) different; sig = significantly (p < 0.05) different, * = controlled treatment.

Table 2 The combinatory effect of MS strength (expressed as xMS) and sucrose concentration on root growth and development of Siam tulip after 6 weeks of culture (Trt = treatment)

Trt	MS	Sucrose	Length of explant shoots (cm)		New roots length (cm)	
	(x)	(%)	cv. Red Shadow	cv. Khao Yai	cv. Red Shadow	cv. Khao Yai
1	0.25	3	5.45±0.4^{ef}	3.17±0.6^{d}	3.75±0.2^{bc}	0.86±0.3^{c-f}
2	0.25	6	4.21±0.2^{f}	4.19±0.4^{bcd}	3.36±0.4^{bc}	1.15±0.2^{b-f}
3	0.25	9	6.50±1.6^{def}	3.45±0.1^{d}	1.08±0.3^{ef}	0.47±0.2^{f}
4	0.5	3	5.52±0.5^{ef}	5.83±0.3^{ab}	3.50±0.2^{bc}	1.45±0.1^{a-d}
5	0.5	6	6.56±0.5^{c-f}	4.44±0.3^{a-d}	4.46±0.4^{b}	1.89±0.4^{ab}
6	0.5	9	5.57±0.4^{ef}	3.43±0.2^{d}	2.13±0.5^{de}	0.53±0.1^{ef}
7*	1	3	12.16±0.3^{a}	6.31±0.4^{a}	4.38±0.3^{b}	1.99±0.4^{a}
8	1	6	9.04±1.0^{bc}	5.86±0.3^{ab}	4.29±0.8^{b}	1.59±0.3^{abc}
9	1	9	8.74±0.6^{bcd}	3.51±0.4^{cd}	5.96±0.3^{a}	0.65±0.1^{def}
10	1.5	3	11.74±1.1^{a}	5.57±0.3^{ab}	4.04±0.5^{bc}	1.26±0.1^{a-f}
11	1.5	6	10.17±0.7^{ab}	5.03±1.2^{a-d}	3.34±0.4^{bc}	1.26±0.3^{a-f}
12	1.5	9	8.27±0.7^{bcd}	3.83±1.0^{bcd}	2.94±0.3^{cd}	0.76±0.4^{def}
13	2	3	10.20±0.8^{ab}	4.05±0.9^{bcd}	3.33±0.2^{bc}	0.56±0.2^{ef}
14	2	6	7.71±1.2^{cde}	5.51±0.8^{abc}	2.06±0.2^{de}	1.32±0.3^{a-e}
15	2	9	6.97±0.3^{cde}	3.51±0.6^{cd}	0.38±0.2^{f}	0.56±0.2^{ef}
F-test			Sig	Sig	Sig	Sig

Values (mean ± Standard Deviation: SE) in each column superscripted with different lowercase letters are significantly (p < 0.05) different; sig = significantly (p < 0.05) different, * = controlled treatment.

Discussion

Cytokinins play a crucial role in shoots multiplication by promoting cell division and shoots formation in plant tissue culture [18]. This study looked at how two cytokinins, 6-benzylaminopurine (BAP) and Kinetin (Kn), affect the growth of shoots, their length, and the formation of new roots in two types of *C. alismatifolia*: cv. Red Shadow and cv. Khao Yai. BAP was shown to be more effective than Kn in increasing shoots proliferation and shoots elongation in both cultivars. The positive response of BAP across all tested concentrations aligns with previous findings that BAP is a strong promoter of axillary shoots formation in Zingiberaceae species [19, 20, 21]. In cv. Red Shadow, the highest new shoots number was observed at 5 mg/L BAP, but no significant difference was found between 2–5 mg/L BAP. This suggests that a BAP concentration of 2–3 mg/L may be the optimal concentration, as the higher concentrations may not significantly improve shoots proliferation. A similar trend was observed in cv. Khao Yai, where 2 mg/L BAP promoted the longest new shoots, consistent with findings in *Curcuma longa* [21]. Kn was generally less effective in stimulating shoots elongation and multiplication. This supports earlier studies showing that BAP is more efficient than Kn in promoting *in vitro* shoot proliferation in *Curcuma* species [20, 22]. The higher efficacy of cytokinins could be attributed to each plant species and its tissue compared to BAP. The most effective cytokinins for inducing shoots from nodal explants of cucumber were Kn, and the highest number of shoots (7.93 shoots/explant) and the longest shoots (3.61 cm) were obtained on MS medium supplemented with 1 mg/L Kn [23]. New roots development varied between cytokinins treatments. In cv. Red Shadow, BAP had no significant effect on new roots number compared to the control, while Kn significantly reduced new roots formation. Similar trends were observed in cv. Khao Yai, where 2 mg/L BAP promoted the highest new roots number, but it was not significantly different from other BAP or Kn treatments. These results align with previous studies in *Curcuma spp.*, where BAP at moderate concentrations (1–3 mg/L) promoted roots formation, but higher cytokinins levels suppressed rooting due to cytokinins-auxins interactions [24]. High cytokinins concentrations have been shown to reduce roots induction by inhibiting auxins transport, a key hormone in roots development [24]. Explant length was significantly influenced by cytokinins supplementation. In both cultivars, explants grown on BAP-containing medium were shorter than the control, indicating that BAP stimulates lateral shoots proliferation but inhibits internode elongation. This is consistent with findings in *Zingiber officinale*, where BAP promoted compact shoot clusters [21]. Interestingly, the combination of 4 mg/L BAP and 3 mg/L Kn resulted in the maximum number of shoots [21].

The present study examined the effects of Murashige and Skoog (MS) medium strength and sucrose concentration on the shoot multiplication, elongation, and roots development of *C. alismatifolia* (cv. Red Shadow and cv. Khao Yai). The findings highlighted the influence of nutrient concentration and carbohydrate availability on *in vitro* plant growth, supporting previous research on micropropagation of Zingiberaceae species. Shoots multiplication was significantly influenced by MS strength and sucrose concentration. The highest number of shoots was observed at 0.5x MS + 6% sucrose, particularly in cv. Red Shadow (2.42 shoots) and cv. Khao Yai (2.21 shoots). This is consistent with previous studies that suggest an optimal salt concentration promotes shoot growth and development in Zingiberaceae [25, 26]. The highest shoot elongation was recorded in 1x MS + 3% sucrose, with 12.16 cm in cv. Red Shadow and 6.31 cm in cv. Khao Yai. This suggests that a balanced nutrient supply with suitable cytokinins favors shoots elongation and roots

growth [27]. The highest percentage of proliferation of *Curcuma caesia* was found in full strength MS medium supplemented with 3 mg/L BAP and 0.5 mg/L NAA. Micropropagation of strawberry 'Pharachatan 80', by using the modified MS basal medium at half strength of KNO3, higher Mesos, Minors (Zn, Cu, B, Mo, Cl, Mn) and Iron significantly improved growth of micro-shoots [28]. The best roots development was observed in 1x MS + 9% sucrose for cv. Red Shadow (5.96 cm) and 1x MS + 3% sucrose for cv. Khao Yai (1.99 cm). This confirms that sucrose plays a crucial role in root elongation, increased carbohydrate supplies enhanced roots length for *in vitro* ginger (*Zingiber officinale* Rosco) [29]. Roots growth of ginger was highly affected by increased sucrose due to treatment effects in which the maximum number and length of new roots were recorded from mediums adding 60 g/L (or 6%) sucrose supplemented with 3, 6, and 9 mg/L BAP [29]. Carbon sources have a direct bearing on the frequency and quality of roots, which ultimately determines *ex vitro* growth. The sugar types and concentrations, including their interactions, have significant effects on root thickness [30]. Sucrose in the culture medium results in the production of high levels of reducing sugars: glucose and fructose [31]. Those reducing sugars may accelerate cell division and consequently lead to an increase in the plant weight and growth at a suitable pH of the culture medium.

Conclusion

The results suggest that BAP is the preferred cytokinins for *in vitro* multiplication of *C. alismatifolia*, with optimal shoots proliferation occurring at 2–5 mg/L BAP. Kn was less effective in promoting shoots multiplication and caused a reduction in roots formation. The study confirms that optimal MS strength and sucrose concentration vary depending on the specific growth parameter being targeted. For shoots multiplication, 0.5x MS + 6% sucrose was the most effective, whereas shoots elongation was best achieved with 1x MS + 3% sucrose. Roots development benefits from 1x MS + 9% sucrose, though excessively high MS and sucrose levels negatively affect overall growth.

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