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Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of *Chlorophytum borivilianum* Sant. & Fernandez

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ABSTRACT

Background: Chlorophytum borivilianum is a rare medicinal plant originally distributed throughout the forest of India. The tubers of *C. borivilianum* are used as an aphrodisiac and impotence supplement. The propagation of *C. borivilianum* is possible through seeds and tubers, but conventional methods may take several months. Hence *in vitro* technique of shoot regeneration could be an efficient alternative means of propagating the species. Latest study reported microtuberization of *C. borivilianum* but there is no sufficient study on a rapid method for shoot multiplication and elongation.

Results: Young shoot buds of *C. borivilianum* were cultured on MS medium containing 6-benzylaminopurine (BAP) and Kinetin (Kn), both at 0, 8.88, 17.8 and 26.6 μM, either individually or in combinations. Proliferated shoots were subcultured on fresh medium of the same constituents on week 3 of culture for further shoot multiplication and elongation. BAP alone (8.88–26.6 μM) was significantly effective on shoot multiplication, while Kn alone (8.88–26.6 μM) was significantly effective on shoot elongation compared to the control containing MS basal medium without any plant growth regulator. However, combination of both cytokinins stimulated an interaction producing higher shoot number and shoot length compared to their individual application.

Conclusions: The most suitable combination was 8.88 μM BAP + 8.88 μM Kn, reaching a mean shoot number of 10.83 and shoot length of 6.85 cm.

1. Introduction

Chlorophytum borivilianum commonly known as safed musli is a rare medicinal plant originally distributed throughout the forest of India [1] but is becoming important and being cultivated in other parts of the world including Malaysia. The tubers of *C. borivilianum* contain proteins (8–9%), carbohydrates (42%), root fibers (4%), saponins (2–17%), minerals and vitamins [2]. The genus *Chlorophytum* is famous for saponin and other compounds [3]. Saponin extracted from the tuberous roots of *C. borivilianum* is known for its therapeutic effect in the Ayurvedic medicinal system [4]. Conventionally, the tubers of *C. borivilianum* are used as an aphrodisiac and impotence supplement. The powdered form of the tubers can be used daily as a general health tonic. Reports have shown the impact of safed musli on arthritis, diabetes, rheumatism and joint pain [5]. It is believed that the leaves of safed musli, consumed as a vegetable once in a season, will provide immunity from diseases for the whole year. Studies have shown that the polysaccharides found in safed musli can raise the immune system [6]. Other pharmacological studies on safed musli tubers have revealed their antiviral, anticancer, anti-oxidant, anti-stress, antimicrobial, hypolipidemic and anti-inflammatory properties [7].

The propagation of safed musli is possible through seeds and tubers, but such conventional methods may take several months [8]. Moreover, with the low rate of seed germination (11–24%), the plants are traditionally regenerated through tubers [9]. However, the indiscriminate use, increased demand and abused harvesting of the tubers have led to decrease availability for replanting. Hence *in vitro* technique of shoot regeneration could be an efficient alternative means of propagating the species [10]. Purohit et al. [11] used young shoot bases as explants, even though shoot regeneration through immature inflorescence cultures was reported by Samantaray et al. [12]. Latest study reported microtuberization of *C. borivilianum* by using RITA system with saponin enhancement of *in vitro* tubers [13,14], but there is no sufficient study on a rapid method for shoot

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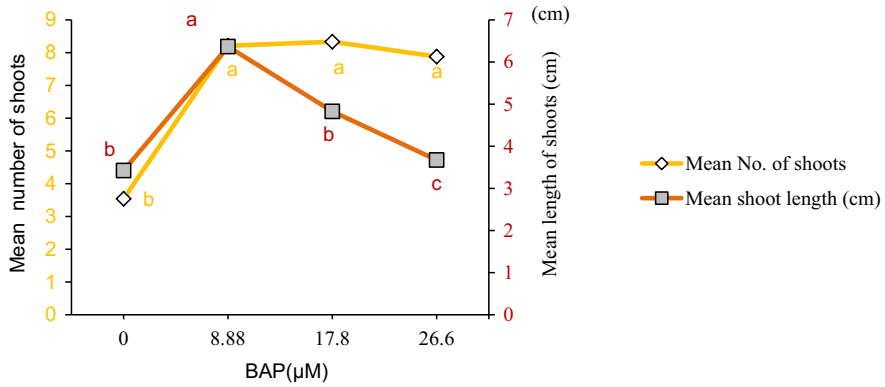

Fig. 1. Effect of BAP on shoot multiplication and elongation of *C. borivilianum* after 6 weeks of culture. Means followed by the same letter (s) are not significantly different.

multiplication and elongation. This study reports on the attainment of improved shoot multiplication and elongation of *C. borivilianum* based on the interaction between the cytokinins BAP and Kn.

2. Materials and methods

2.1. Experimental material

The tubers of *C. borivilianum* were collected from Felda field in Lanchang, Pahang, Malaysia. Young shoot buds that emerged from the tubers were used as explants. The explants were initially washed and surface disinfected with benlate (0.2%, w/v) for 1 h under constant agitation. The young shoots were then surface sterilized by treating with 0.1% (w/v) mercuric chloride for 7 min followed by three rinses in sterile distilled water. Finally the cut ends of the buds were trimmed and the buds were cultured on 40 mL of Murashige and Skoog (MS) medium [15] containing 3% (w/v) sucrose, 0.4% (w/v) Gelrite™ (Duchefa, Haarlem, The Netherlands) and different combinations of BAP with Kn. The pH of the media was adjusted to 5.8 ± 0.1 using 0.1 N HCl and/or 0.1 N NaOH prior to autoclaving at 121°C and 1.05 kg cm^2 of pressure for 20 min. The cultures were incubated at 25 ± 2°C under 16 h photoperiod of 45 μmoL m^{-2} s^{-1} light intensity provided by cool white fluorescent tubes.

2.2. Treatments

In this study, 16 different hormonal treatments containing BAP at 0, 8.88, 17.8 and 26.6 μM in combination with 0, 8.88, 17.8 and 26.6 μM Kn were assessed for shoot induction from young shoot buds of *C. borivilianum*. The experiment was replicated three times. Each treatment per replication consisted of 30 explants. The proliferated shoots were subsequently subcultured on fresh medium of the same constituents on week 3 of culture for further shoot multiplication and elongation. The multiplication and elongation rates were measured based on the number of shoots produced per explant and the length of shoots attained at the end of week 6 of culture, respectively. Finally, all regenerated and elongated shoots were successfully transferred to rooting medium [16] supplemented with 1.0 mg/L indole-3-butyric acid (IBA) and 30 g/L sucrose. For *ex vitro* establishment, well-rooted plantlets were transferred in potting medium containing vermiculite: organic matters (1:1).

2.3. Statistical analysis

The experiment was factorial arranged in a Completely Randomized Design (CRD). Data were subjected to analysis of variance (ANOVA) using the SPSS software version 16. Prior to data analysis, normality test of all variables was done using Kolmogorov–Smirnov method and based on the result all data were normal. Treatment means were compared using the Duncan's New Multiple Range Test (DNMRT), at $\alpha = 0.01$ where the F-value was significant.

3. Results and discussion

3.1. Effect of BAP on shoot multiplication and elongation

Shoot multiplication in the presence of BAP was significantly higher (8.3) compared to the control containing only MS basal salt (3.5) after 6 weeks of culture (Fig. 1). However, there was no significant difference observed between the various concentrations of BAP on the

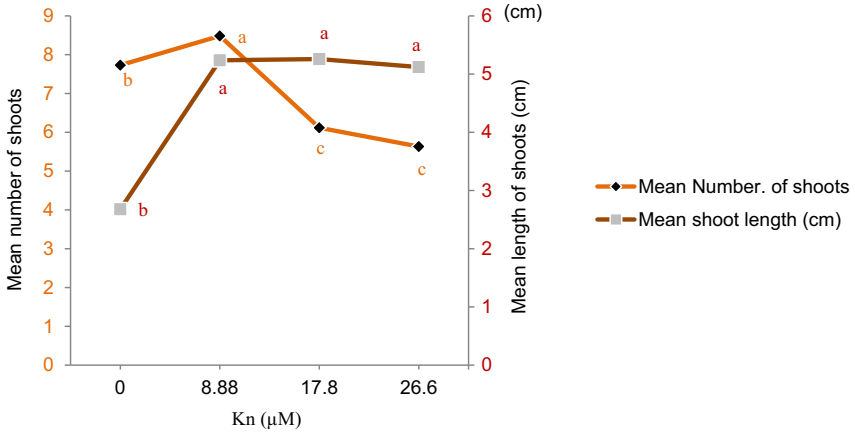

Fig. 2. Effect of Kn on shoot multiplication and elongation of *C. borivilianum* after 6 weeks of culture. Means followed by the same letter (s) are not significantly different.

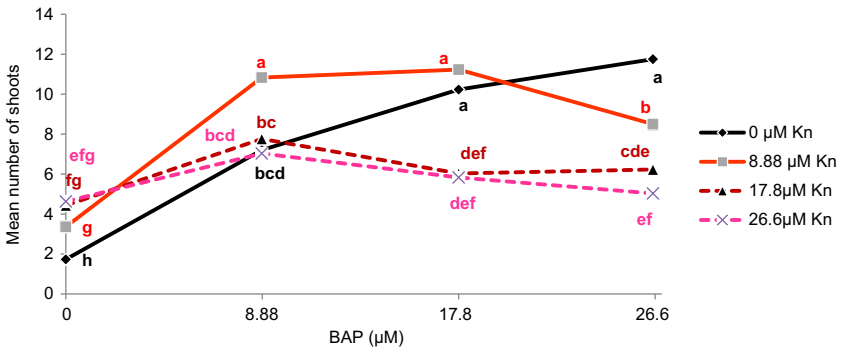

Fig. 3. Interaction between BAP and Kn on shoot multiplication of *C. borivilianum* after 6 weeks of culture. Means followed by the same letter (s) are not significantly different.

number of shoots produced per explant. It can be seen that the addition of BAP as a cytokinin is certainly essential for shoot induction and multiplication of *C. borivilianum*. Meanwhile, the highest length of shoot (6.36 cm) was achieved on medium with 8.88 μM BAP which differed significantly from the treatment containing 17.8 and 26.6 μM BAP as well as the control (Fig. 1). With increase in BAP concentration above 8.88 μM, the shoot length plummeted to 4.82 cm and 3.67 cm at 17.8 μM and 26.6 μM respectively. In addition, the shoot length attained at the higher concentration of 26.6 μM BAP did not differ significantly compared with the control.

3.2. Effect of Kn on shoot multiplication and elongation

After 6 weeks of culture, the maximum number of shoots (8.48) was obtained on medium containing 8.88 μM Kn which differed significantly from other concentrations of Kn tested as well as the control (Fig. 2). Although there was a but significant increase in shoot number from 0 μM Kn reaching a peak at 8.88 μM Kn but a steep slump followed at 17.8 (6) and 26.6 μM (5.6) Kn. It is seen that high concentrations of Kn (17.8 and 26.6 μM) significantly inhibited shoot production compared to the control. Meanwhile, the length of shoots (5 cm) was enhanced in the presence of all tested concentration of Kn compared to the control. The shoot length did not differ significantly among the Kn concentrations but they differed significantly from the control.

3.3. Interaction between BAP and Kn on shoot multiplication and elongation

Based on Fig. 3 and Fig. 4, an interaction was observed between BAP and Kn on shoot multiplication and elongation of *C. borivilianum* after 6 weeks of culture. It shows that with increasing concentration of BAP alone up to 26.6 μM, the shoot number significantly increased (from 2 to 11.75 cm). The combination of BAP and Kn showed an increase in shoot numbers and decreased at higher concentration of BAP (26.6 μM). All treatments without BAP showed low rate of multiplication even at the highest Kn concentration of 26.6 μM. The MS media containing 8.88 μM Kn in combination with 8.88 and 17.8 μM BAP showed the best shoot multiplication producing 10.9 and 11.2 shoots respectively. The media containing only BAP at 26.6 μM showed the highest shoot numbers (11.75) whereas an inhibitory effect on shoot multiplication was observed in combinations of BAP (26.6 μM) with 8.88 and 17.8 μM Kn. Based on the results, treatments that significantly produced among the highest shoot proliferation rates were 26.6 μM BAP, 17.8 μM BAP, 17.8 μM BAP + 8.88 μM Kn and 8.88 μM BAP + 8.88 μM Kn. Fig. 5 shows the stages in shoot regeneration of *C. borivilianum* on medium containing BAP and Kn.

The result shows an interaction between BAP and Kn on the length of shoots (Fig. 4). The highest shoot length was significantly observed on MS media containing 8.88 μM BAP in combination with 8.88, 17.8 and 26.6 μM Kn. It was significantly evident that higher concentrations of BAP (17.8 and 26.6 μM) inhibited the shoot length compared to BAP at 8.88 μM with or without Kn.

Primarily, cytokinins have a major role on plant development, such as the regulation of shoot formation and multiplication and the promotion of cell division and expansion [17]. In general, BAP increases shoot multiplication of several medicinal plant species [18], which is in conformity with the results of this study. George [19] stated that BAP enhances shoot formation and releases lateral buds from dormancy. In the present study, although BAP alone at 26.6 μM could induce the highest shoot number (11.75) as shown in Fig. 3, the treatment had the lowest impact on shoot length (1.98 cm) (Fig. 4) of *C. borivilianum*. This indicates that BAP at higher concentration has an

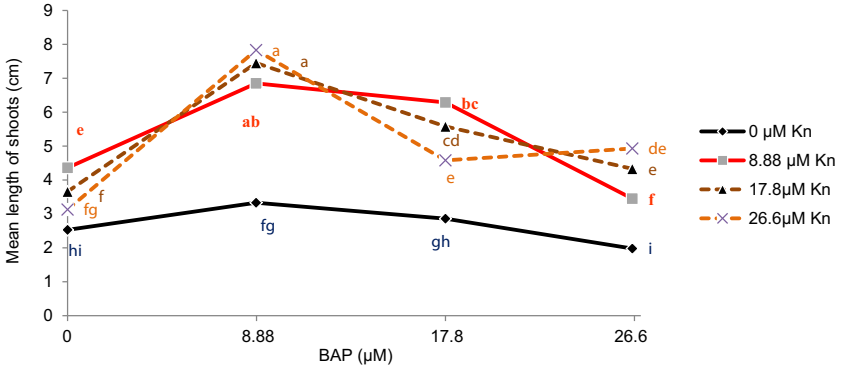

Fig. 4. Interaction between BAP and Kn on shoot elongation of *C. borivilianum* after 6 weeks of culture. Means followed by the same letter (s) are not significantly different.

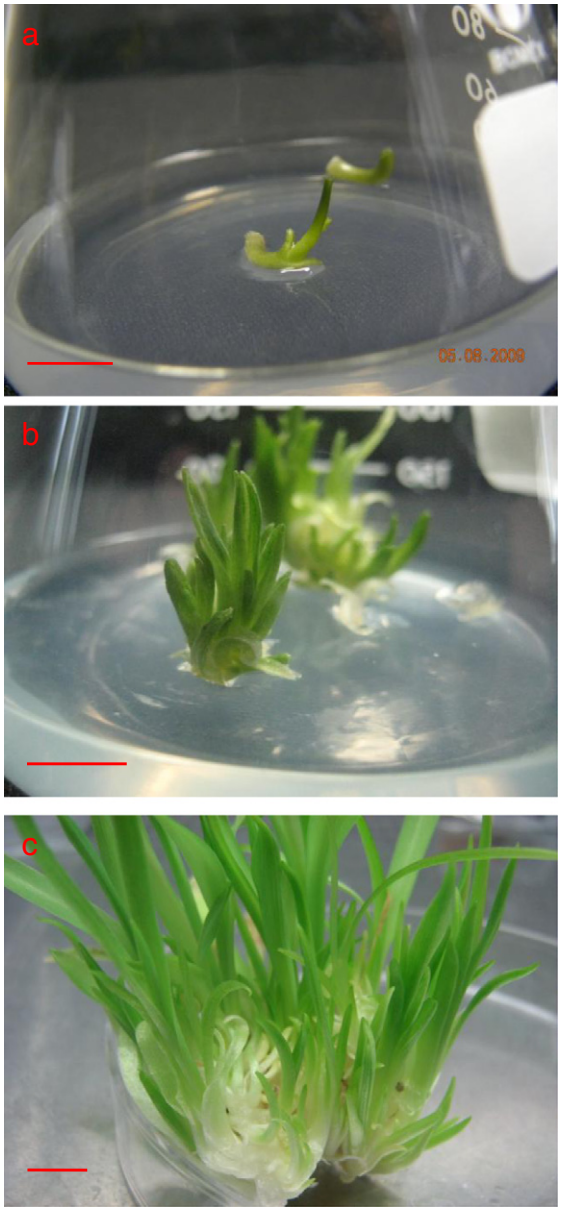

Fig. 5. Shoot regeneration of *C. borivilianum* on medium with BAP and Kn. (a) Young shoot bud after 1 week of culture, (b) shoot emergence after 3 weeks of culture, and (c) proliferation of shoots after 6 weeks of culture. Bar = 10 mm.

enhancing role on bud proliferation followed by higher shoot multiplication but less effective on shoot elongation and each explants taken for study showing dormancy.

The effectiveness of BAP on shoot induction and multiplication but less effective on shoot elongation compared to Kn is supported by earlier reports on *in vitro* propagation of *Gentiana kurroo* [20] and *C. borivilianum* [10]. Sharma et al. [20] reported that Kn was less effective on shoot multiplication of *G. kurroo* compared to BAP. However, MS media supplemented with Kn enhanced the *in vitro* shoot elongation of *Asparagus racemosus* and *Chlorophytum arundinaceum* [10,21]. Samantaray and Maiti [22] have used 3 mg L^{-1} BAP, 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA), 150 mg L^{-1} adenine sulfate and 3% saccharose instead of for shoot induction of *C. borivilianum*.

Our results indicated that 8.88 μM BAP and above led to a high number of shoot production (Fig. 1) while increasing the Kn concentration to 8.88 μM increased the shoot number but at 17.8 and 26.6 μM Kn, the shoot number dropped abruptly (Fig. 2), thus indicating the inhibitory effect of higher Kn on shoot multiplication of *C. borivilianum*.

The combined effect of BAP and Kn has been reported in previous studies [23,24] on other crops which are in accordance with our results on the interaction of BAP and Kn obtained on *C. borivilianum* in this study. BAP combined with Kn showed a synergistic effect producing high rate of shoot multiplication and elongation in *Bambusa glaucescens* Willd [23] and Bottle Gourd [24] compared with BA or Kn when applied separately. Kumar et al. [25] reported achieved maximum shoot regeneration from nematode tolerant grape rootstock 1613C shoot tips on MS medium supplemented with 1 mg L^{-1} BAP and 0.5 mg L^{-1} Kn. Srivastava and Joshi [26] reported that BAP tested individually was more effective than Kn alone on shoot multiplication of *Portulaca grandiflora* but their combination was most advisable for shoot multiplication. They elaborated that Kn stimulated faster BAP-dependent shoot growth and 8 μM Kn with a low concentration of BAP (2 μM or 4 μM) were most suitable for multiple shoot formation and elongation of *P. grandiflora*.

According to Goba [27], endogenous production of ethylene by explants in plant tissue culture vessel in some ethylene sensitive species led to less elongation. Ozden-Tokatli et al. [28] and Saha et al. [24] noted that higher amount of ethylene was released in medium containing BAP in Pistachio and Bottle Gourd. Saha et al. [24] further noticed the stimulatory effect of Kn on shoot elongation in Bottle Gourd which indirectly indicated its critical inhibitory role on the production of ethylene. Similarly in this study on *C. borivilianum*, the effectiveness of Kn on enhancing shoot elongation could likely be due to its inhibitory effect on ethylene released by BAP in the medium. However, the role of ethylene in plant tissue culture studies is complicated. In some crops the positive effect of ethylene on shoot formation rate was reported. For example in rice callus, ethylene has a remarkable effect on shoot morphogenesis stimulation [29], but in other crops such as *Zea mays* [30] and *Brassica* [31] ethylene showed inhibitory effect on shoot regeneration. Bleecker et al. [32] described various inhibiting ethylene function of endogenous plant regulator on morphogenic processes.

In this study, it can be concluded that BAP enhanced shoot number whereas Kn promoted shoot elongation, but when in combination they worked synergistically to produce optimal shoot multiplication and elongation of *C. borivilianum*. Besides, the *in vitro* propagation of *C. borivilianum* was evaluated using treatment combinations of BAP and Kn. BAP was shown to be more effective on shoot induction and multiplication whereas Kn was more effective on shoot elongation. The MS media containing 8.88 μM BAP with 8.88 μM Kn was the optimal combination for shoot multiplication and elongation of *C. borivilianum*.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Author contribution

Proposed the theoretical frame: MFA, MAA, II; Conceived and designed the experiments: MFA; Software development: MFA; Contributed reagents/materials/analysis tools: MFA, NK, MAA, II;

Wrote the paper: MFA; Performed the experiments: MFA; Analyzed the data: MFA.

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