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Effect of different concentrations of 2,4-D and BAP on callus and shoots induction of Thai Cannabis (*Cannabis sativa* L.) cv. Hang Kra Rog Phu Phan

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Abstract

The Thai Cannabis cultivar (*Cannabis sativa* L.) cv. Hang Kra Rog Phu Phan is highly demanded in the pharmaceutical, cosmetic and various industries. This study investigates the effects of different concentrations of 2,4-D on callogenesis and BAP on shoot multiplication to enhance the propagation of this valuable cultivar. The results show that the MS medium supplemented with 3 mg/L of 2,4-D induces the highest callogenesis, while the MS medium supplemented with 3 mg/L BAP promotes the maximum shoot multiplication. These findings have the potential to significantly impact the cultivation of the Thai Cannabis cultivar "Hang Kra Rog Phu Phan," ensuring a sufficient supply to meet the increasing demand in various applications.

Keywords

callogenesis; *Cannabis sativa* L.; micropropagation; shoot multiplication

Introduction

Cannabis sativa L. (Cannabis, hemp, or marijuana) is in the Cannabaceae family and an annual herb (1, 2). Cannabis is an essential economic crop that has turned the world's attention due to its wide range of applications. There is scientific confirmation of the pharmacological properties of cannabis. Nowadays, cannabis is used in the food, fibre, medical and recreational industries (3, 4). Cannabinoids (CBD) are the primary chemical compounds present in cannabis (5, 6). The main known psychoactive substances are Δ9-tetrahydrocannabinol (Δ9-THC), which has psychoactive, addictive, analgesic and antifungal effects.

Additionally, cannabidiol (CBD) is a non-psychoactive substance. However, it has clinical effects and was used to treat medical conditions such as depression, epilepsy, glaucoma, etc. It also has the potential to resist infection in the form of anti-inflammatory, which is a severe respiratory group (7). Thai Cannabis cultivar "Hang Kra Rog Phu Phan" (*Cannabis sativa* L. cv. Hang Kra Rog Phu Phan) originates in the Phu Phan Mountain range in Sakon Nakhon and Kalasin provinces in the northeastern region of Thailand. It has a unique characteristic and a high Δ9-THC. The word Hang Kra Rog means a squirrel's tail and Phu Phan is the name of the mountain range from which it originated (8). Fig. 1 shows the morphology of *Cannabis sativa* L. cv. Hang Kra Rog Phu Phan.

Currently, trade and cultivation of cannabis have been relaxed to allow the use of cannabis in medicine and various fields, causing a significant demand for the plant. Planting seeds results in an uncertain ratio of sexes in plants. Most can determine their gender during flowering. In cannabis, the importance of each gender's plant is different. Gender influences economic value

and commercial exploitation (9). To get consistent yields, the micropropagation technique is a technique that can increase the number of plants consistently and adequately to meet demand. *In vitro* propagation has excellent potential in plant production. They are fast to produce using synthetic media in a controlled environment (10, 11). There have been many research reports on experiments to find suitable conditions for tissue culture to multiply cannabis (12-15). Most studies have succeeded in inducing calluses using different auxins and cytokinins (11, 16). Plant induction is an essential step for *in vitro* culture techniques. The sativa and indica subspecies of *C. sativa* are mainly culture using leaves, axillary nodes, cotyledons, shoot tips and epicotyls to achieve high multiplication rates, produce disease-free plants and reduce factors from heterozygosity of crossing (16, 17).

Although some cultivars of cannabis are successful in micropropagation, more studies are needed with other cultivars. Many studies revealed that each cultivar of cannabis requires different and specific components of the growing medium (13, 16). Auxin hormones such as 2,4 Dichlorophenoxyacetic Acid (2,4-D) and cytokinins such as Benzyl Adenine (BAP), meta-tooling (mT) and Thidiazuron (TDZ) are widely used in propagated cannabis to develop callus and increase the number of shoots through tissue culture. However, some strains have some limitations (16, 18). Tissue culture is a technique that has the potential to produce large quantities of cannabis that are suitable for industrial use and can produce enough cannabis plants to meet the needs of farmers. However, the previous study was a study of foreign strains of cannabis, which have strains and ecology that are different from strains in Thailand. Therefore, the appropriate conditions for cultivating Thai Cannabis tissue should be studied for effective cultivation. This research studies the tissue culture of the Thai Cannabis cultivar "Hang Kra Rog Phu Phan" by comparing suitable plant tissue culture media for increasing callus and shoots. This study will serve as a guideline for economic cannabis cultivation and will be knowledge for developing the cultivation of other Thai Cannabis cultivars in the future.

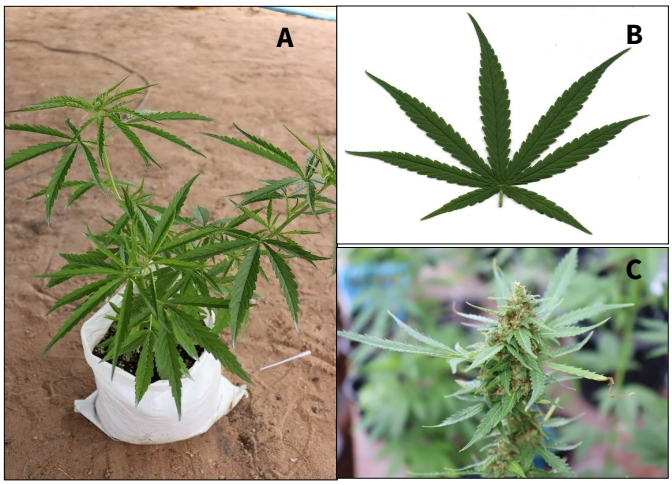

Fig. 1. Morphology of *Cannabis sativa* L. cv. Hang Kra Rog Phu Phan (A) Female plant, 2 months old (B) Characteristics of leaves (C) Characteristics of inflorescence.

Materials and Methods

Plant material

The plant in this study was a native Thai cannabis cultivar named Hang Kra Rog Phu Phan, which is a cultivar with high THC. Morphological characteristics of the plant are as follows: stem 150-421 cm in height, canopy width 55-82 cm, leave the compound, palmate 10.5-12.8 cm wide, leaflet: 3-9 linear-lanceolate, 0.2-1.5 cm wide, 1.0-11.5 cm long, attenuate leaf base, tip caudate, margin serrate, Inflorescences: Inflorescence clusters tightly at the tip of the branch. The base of the long branches is tightly packed like a squirrel's tail. There are many of them and they have a unique smell. The fruits are achene and the seeds are oval-shaped, cream-brown, with brown longitudinal stripes. The tip is circular, 3.9-4.5 mm wide and 3.0 -3.5 mm long. The plant was from a 2-month-old female, showing a plant grown under controlled conditions.

Growth conditions and callus induction

Using young leaf samples to wash the surface with tap water, callogenesis was studied. They were surface sterilized with a 2 % v/v sodium hypochlorite solution with 1 drop of Tween 20 for 10 min. They followed with a 1 % v/v sodium hypochlorite solution for 10 min and a rinsing period with sterile distilled water for 10 min three times. The leaves were cut about 1x1 cm. They were then transferred into Murashige and Skoog medium (MS medium) (19) containing 30 g/L sucrose and 8 g/L agar pH 5.8, supplemented with different concentrations of 2,4 -D (0, 1, 3, 5 mg/L). The cultures were grown under controlled conditions at 25±2 °C in the dark for 60 days.

Shoot induction

Young shoots were washed with tap water to clean the surface and surface sterilized with 2 % v/v sodium hypochlorite solution with 1 drop of Tween 20 for 10 min, followed by 5 % hypochlorite solution for 10 min and finally washed three times in sterile distilled water for 10 min. Shoot explants were cut in pieces of 1.5 cm. The explant was cultured on an MS medium containing 30 g/L sucrose and 8 g/L agar pH 5.8, supplemented with different concentrations of BAP (0, 1, 3, 5 mg/L). Explants were grown under controlled conditions at 25 ± 2 °C and 16/8 light darkness photoperiod for 60 days.

Data analyses

A factorial experiment based on a completely randomized design (CRD). The number and length of shoots and the width and length of the callus were investigated after 60 days. The mean of ten replications and standard error (SE) were analyzed using SPSS software. Data normality was tested before conducting an Analysis of Variance (ANOVA). The significant differences were compared using Duncan's multiple range test ($p < 0.05$).

Results and Discussion

Effect of 2,4 D on callus induction

Effects of cultivating Hang Kra Rog Phu Phan leaves on medium supplemented with different concentrations of 2,4 -D in the dark for 2 months. The results showed no callus formation in the control medium without adding 2,4-D. Adding 3 mg/L and 1 mg/L of 2,4-D resulted in the maximum

callus width significantly different from the other experimental sets, with widths of 4.85 and 4.19 mm, respectively. In addition, this condition showed the highest effect on callus length, significantly different from other groups, which was 9.07 mm. The size of the callus decreased with the concentration of 2,4-D increased (Table 1, Fig. 2). Callus development is the result of a process better known as de-differentiation or re-differentiation to stimulate callus induction and development. Many growth hormones induce and develop callus that can be increased using 2,4-D, NAA and kinetin, resulting in a callus that can regenerate effectively (16, 18). The study results found that the leaves that developed callus in 30 days have the characteristics of a loose callus. The hormone of 2,4-D is commonly used to stimulate callus formation in cannabis. It has been reported that use alone or in combination with other substances can also promote callus formation (20, 21). This 2,4-D hormone plays a vital role in cell division through protein production and affects the efficiency of enzymes, respiration and cell division (22). Furthermore, dark cultures of tissues supplemented with the 2,4-D hormone have been found to enhance callus formation in some plant species, such as tobacco and maize (23, 24). This situation is thought to result from the photodegradation of auxin (25).

Effect of BAP on shoot induction

Effects of cultivating Hang Kra Rog Phu Phan shoots on MS medium supplemented with different concentrations of BAP for 2 months showed that shoots grown on MS medium containing 1 and 3 mg/L BAP had significantly different shoots (Table 2). Explant shoot tips on MS medium containing 1 and 3 mg/L BAP showed 100 % forming and an average of 4.50 and 3.20 shoot production per experiment, respectively. The results showed that culture on high BAP medium did not increase the number of shoots in the explant, with MS medium containing 5 mg/L BAP showing the lowest shoot formation (Fig. 3). The response to the BAP of each cannabis cultivar was different. This research was different from studies on the MX-CBD-11 strain of cannabis with high THC, which has a good response with high doses of BAP. When cannabis was grown on MS medium with 5 mg/L of BAP, the results showed that the highest average shoots were 1.3 shoots per explant and the highest shoot length was found to be an average of 11 mm at 30 days in the medium with 2.5 mg/L of BAP (26). However, this research gave more shots, possibly due to differences in species and culture periods. Moreover, the number of shoots in this study was lower than that reported for cultivating cannabis in media supplemented with mT in cultivars CS-01 and CS-02, with 11.89 and 13.44 shoots, respectively (27). This effect may be due to differences in species and the type of hormone used. The use of BAP in some cultivars is also suitable for specific cultivars of cannabis. Increasing the number of medical cannabis cultivars when comparing the use of MS + BAP (1.0, 2.0, 4.0 and 8.0 µM) with TDZ (1.0, 2.0, 4.0 and 8.0 µM), the results were found that both full and half formulas of MS + 4.0 µM BA results in the highest number of shoots being 3.63 shoots (28). BAP is a widely used cytokinin in plant propagation and has good efficacy on many plant species (29 -31).

A factor for the success of *in vitro* multiplication was the basal nutritional. MS was the most used basal medium for medicinal and aromatic plants for *in vitro* multiplication. Callus induction and callus-mediated plant regeneration depend on different types of nutrients, their concentration and the combination of phytohormones, especially auxins and cytokinin (29). In general, auxin in the culture medium is necessary for callus induction. The type and concentration of auxin varies among species (33, 34). Additionally, the age of the culture and the number of subcultures favour the appearance of soma clonal variations in some plants. Therefore, the increased frequency of polyploid cells may be due to prolonged callus culture (35). However, each cultivar of cannabis was variable and responded differently to hormones and media (36, 37). Genotypic and tissue-dependent regeneration characteristics also affect the responses of cannabis (16, 38, 39). Studying the appropriate media formula for each cultivar was necessary for increasing the efficiency and appropriateness of increasing the number of each

Table 1. Influence of 2,4-D on callus induction

Medium	% Response	Callus wide (mm)	Callus length (mm)
MS (control)	0 %	0.00 ± 0.00^{c}	0.00 ± 00.0^{d}
MS + 2,4-D 1 mg/L	100 %	4.19 ± 0.53^{a}	5.42 ± 0.73^{b}
MS + 2,4-D 3 mg/L	100 %	4.85 ± 0.29^{a}	9.07 ± 0.92^{a}
MS + 2,4-D 5 mg/L	80 %	2.80 ± 0.71^{b}	3.23 ± 0.86^{c}

*Values with different superscripts in the same column are statistically significantly different as determined by ANOVA and Duncan's Multiple Range Test ($p < 0.05$). The data are presented as mean ± standard error (SE).

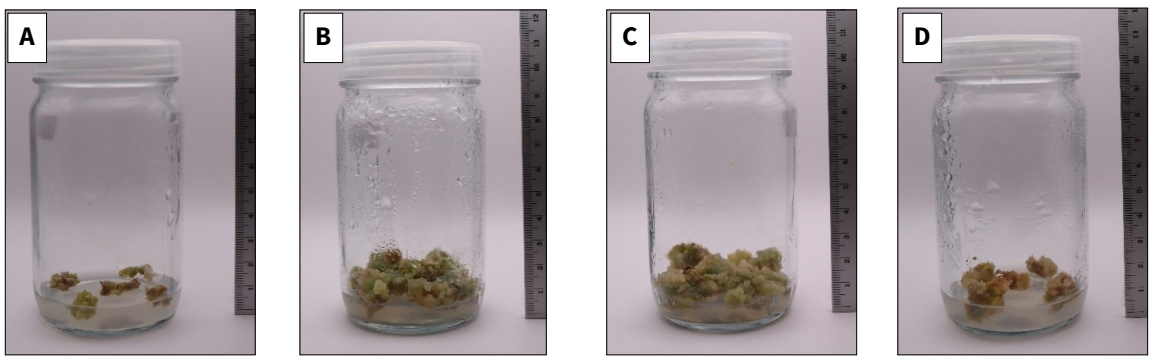

Fig. 2. Callus formation on Hang Kra Rog Phu Phan grown on MS + 2,4-D medium in various concentrations, (A) 2,4-D 0 mg/L, (B) 2,4-D 1 mg/L, (C) 2,4-D 3 mg/L, (D) 2,4-D 5 mg/L.

Table 2. Influence of BAP on shoot induction

Medium	% of explants forming of the shoot	Shoot length (mm)	No. of shoot per explant
MS (control)	80 %	24.92 ± 7.14^{ab}	2.30 ± 0.59^{bc}
MS + BAP 1 mg/L	100 %	33.66 ± 6.55^{a}	3.20 ± 0.64^{ab}
MS + BAP 3 mg/L	100 %	36.64 ± 4.88^{a}	4.50 ± 0.60^{a}
MS + BAP 5 mg/L	80 %	13.19 ± 1.87^{b}	0.80 ± 0.32^{c}

*Values with different superscripts in the same column are statistically significantly different as determined by ANOVA and Duncan's Multiple Range Test ($p < 0.05$). The data are presented as mean ± standard error (SE).

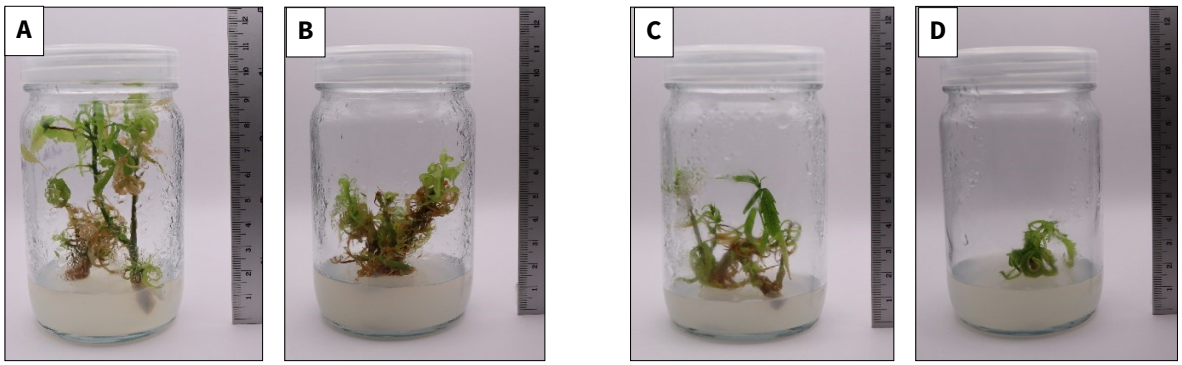

Fig. 3. Hang Kra Rog Phu Phan shoot growth on the medium of MS + BAP at various concentrations, (A) BAP 0 mg/L, (B) BAP 1 mg/L, (C) BAP 3 mg/L, (D) BAP 5 mg/L.

Conclusion

In sumy, micropropagation of *Cannabis sativa* L. cv. Hang Kra Rog phu phan cultivar using -2, 4 D and BAP found the highest callus induction in MS medium supplemented with 2,4-D 3 mg/L. There was a 100 % response. The callus width was 4.85±0.29 mm and the width was 9.07±0.92 mm. The highest germination rate on MS medium with BAP 3 mg/L induced a maximum of 4.50 shoots. This research is a guideline for conservation. Increasing the number of Thai Cannabis cultivars, "Hang Kra Rog Phu Phan," and providing a guideline for similar cannabis cultivars to produce large quantities to meet market demand and utilization in various fields.

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Authors' contributions

TT conceived of the study and participated in its design and coordination. SP participated in the design of the study and performed the statistical analysis. TT and SP participated in the study's design, conducted the experiments, performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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