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Callus Induction and Cell Suspension Culture from Leaves of *Kadsura coccinea*

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Abstract *Kadsura* spp. is a vine glabrous woody which belongs to Schisandraceae that is rare plant found in highland. It is an ancient plant group that low adaptation for living. Shape and scent of fruits like sugar apple, and edible when ripping. It contains the nutrition value and high antioxidants. It is a valuable medicine to prevent the tumor HIV resistant and hepatitis. In presently *Kadsura* spp. become extinct. The calli from leaves of *Kadsura* spp. was induced by this study. The leaves were sterilized and cultured on solid synthetic medium, Murashige and Skoog (MS) medium supplement with Plant Growth Regulators (PGRs) were used as 0.5, 1, 2, 3 and 5 mg/L concentrations of 6-benzylaminopurine (BAP), *meta*-Topolin (*m*T), 2, 4-dichlorophenol-xyacetic acid (2,4-D) and 0.5 mg/L of BAP combined with 0.5, 1, 2, 3 and 5 mg/L of 2,4-D. The maximum number of leaves were induced calli of 55.55 % and 350.16 mm^3 averaged area on medium with 0.5 mg/L of BAP with 0.5 mg/L of 2,4-D, but 0.5 mg/L of BAP with 2 mg/L of 2,4-D gave the highest average area (1,079.53 mm^3) after 4 weeks. The growth rate of cell suspension cultured in liquid MS medium supplement with 0.5 mg/l of BAP and 0.5 mg/l of 2,4-D resulted the fresh weight and dry weight of cell suspension with the best grown for 15 days of 1.12 g/10 mL, 0.14 g/10 mL fresh and dry weight respectively. Cell suspension was rapidly grown during the period of 6-15 days. The cell suspension was cultured on MS solid medium combine with 0.5 mg/l of BAP and 0.5 mg/l of 2,4-D and 0.2% (W/V) AC after 4 weeks. The best result for calli induction and perfectly for plant regeneration were the MS medium without AC. This research work is developed an optimized protocol for plant breeding.

Keywords: Callus induction, Cell suspension, *Kadsura coccinea* (Lem.)

Introduction

The *Schisandraceae* family composed of 25 species in East and Southeast Asia and North America (Krussmann, 1978). *Kadsura coccinea* (Lem.) A.C.Sm is a deciduous, woody stem liana native in East Asian countries. It is an important medicinal plant, high medicinal value are used full as an important component of various traditional Asian pharmacies used for HIV resistant ,exhibit quite potent antiviral (Lewis and Davin,

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1999), anticancer (Liu *et al.*, 1984), and antibiotic effects (An *et al.*, 1997) its low toxicity and now was applicable in Western medicine (Weinberg *et al*., 1999). However the seed germination of *Schisandraceae* requires the period cyclic stratification and germination rate is low (Saunders, 2000).

An origin of Plant Genetic Conservation Project Under The Royal initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) was established in 1992 at the Royal Chitralada Projects. The ultimate goal to develop the personal and plant genetics resources for the keeping of plant varieties, and for the development to be helpful for the farmer and business section of the country. *Kadsura coccinea* (Lem.) A.C.Sm (Kad 001) is 1 of 9 conservation plants were precious and become extinct in some activities of this projects want to protection, planting, preservation, conservation and utilization of plant genetic. That activities are operated with researchers from universities, academic institutes, research centers and experimental stations (Plant Genetic Conservation Project Office, 1996) was started of this study and the aims are induce callus from leaves an *in vitro* culture that could recognize on low seed germination rate and study on growth rate by cell suspension of *Kadsura coccinea* (Lem.) A.C.Sm (Kad 001).

Materials and methods

Plant materials

The leaves of *Kadsura coccinea* (Lem.) A.C.Sm, var Kad 001 was explants were sampled from Plant Genetic Conservation Project Under The Royal initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) at Lampang province, Thailand under RSPG at Royal Chitralada Projects Bangkok, Thailand.

Sterilization of leaves

Leave samples were sterilized by 1 min rinse in 70% (V/V) ethanol, followed by shook on 250 rpm for 30 min of 0.2% (W/V) mercuric chloride ($HgCl_2$) solution combine with 0.1% (V/V) Cefotaxime (Nida Pharma incorporation, Thailand), 0.1% (V/V) Antibiotic Antimycotic Solution [100X] (Sigma) and 0.1% (V/V) Preservative for Plant Tissue Culture Media Active (PPM)(Plant cell technology), a further shook on 250 rpm for 10 min of sterile distilled water with 0.1% (V/V) Cefotaxime, 0.1% (V/V) Antibiotic Antimycotic Solution [100X] and 0.1% (V/V) PPM then shook on 250 rpm for 10 min again of sterile distilled water with 0.1% (V/V) Antibiotic Antimycotic Solution [100X] and 0.1% (V/V) PPM and last one washed in sterile distilled water on 250 rpm for 5 min and then surface dried with sterilized absorbent paper before culture on medium.

Culture medium and conditions

Different concentrations (0.5, 1, 2, 3 and 5 mg/L) of 6-benzylaminopurine (BAP), *meta*-Topolin (*m*T), 2,4-dichlorophenol-xyacetic acid (2,4-D) and 0.5 mg/L of BAP combine with 0.5, 1, 2, 3 and 5 mg/L of 2,4-D were added into Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Phytotech) appended with 30 g/L sucrose and 2.6 g/L Gellan Gum powder (Phytotech), pH was changed to 5.8. The media were autoclaved at 121 ℃ for 15 min.

The sterilized leaves were cut into 1 × 1 cm per piece, which were placed on callus induction medium. The culture temperature was 25 ℃ in darkness. Callus induction rate was counted and recorded 4 weeks later. The callus composition and structures were observed under scanning electron microscopy (SEM), and Field Emission Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer (FESEM-EDS (7610F)).

$$\text{Callus induction rate (\%)} = \frac{\text{No.of explants generating callus}}{\text{No.of total explants}} \times 100$$

Growth rate by cell suspension materials and conditions

Calli from previous experiment were the explants MS medium appended with 30 g/L sucrose without Gellan Gum powder combine with 0.5 mg/L of BAP and 0.5 mg/L of 2,4-D, pH was adjusted to 5.8 and then added 10 ml into 50 ml flask's size. The medium was autoclaved at 121 ℃ for 15 min. Added 0.15 g of callus into flask use sterilized spatula for mashed it to small pieces culture on shaker at 100 rpm temperature was 25 ± 2 ℃, 16 h lightness and 8 h darkness. Fresh and dry weight cell were made after 0, 3, 6, 9, 12, 15, 18, 21 and 24 days. Cells were collected by used the vacuum filtration for 1 min with No.1 Whatman's filter paper, rinsed flask with 10 ml water balanced of fresh weight cell after that incubated at 110 ℃ for 1 h and moved to desiccator 1 h balanced of dry weight cell (Poeaim, 2007). The average fresh and dry weight cells were calculated analysis of variance (ANOVA) and were compared treatment means by using Ducan's Multiple Range Test. The data were analyzed by using the SPSS (Statistical Package for the Social Sciences) software (IBM Corporation and other(s) 1989, 2017). The calli were staining with 0.5% Fluorescein Diacetate (FDA) (Poeaim et.al., 2012) and observed under fluorescent microscope, Nikon 80i Fluorescence microscope alive cells were green color (Kvach and Veras, 1982). The cell suspension was treated for 30 days then transferred calli to MS solid medium combined with 0.5 mg/L of BAP and 0.5 mg/L of 2,4-D with 0.2% (W/V) Activated Charcoal (AC) and without AC, and kept at 25 ± 2 ℃, 16 h lightness and 8 h darkness. The callus induction rate was observed at 4 weeks later. The calli were moved to plant regeneration

medium, MS combine with 0.5 mg/L of BAP and 0.5 mg/L of 2,4-D with 0.2% (W/V) AC after cultured for 4 weeks.

$$\text{Average fresh weight cell (g.)} = \frac{\text{fresh weight plusing all of repetitive}}{\text{No. of repetitive}}$$

$$\text{Average dry weight cell (g.)} = \frac{\text{dry weight plusing all of repetitive}}{\text{No. of repetitive}}$$

Results

Callus induction

In this study the callus induction was defined by the size of the spot on explant less than 2 mm (Fig 1A). The bigger size more than 5 mm (Fig 1B) was observed by using Venire caliper to measure width, length and height in mm. The explants were cultured on MS medium with PGRs, the callus induction was recorded to select the optimal PGRs in medium combination after 4 weeks (Table 1). Callus induction on the different concentration of BAP, 2,4-D individually and in combination 0.5 mg/L of BAP with 0.5, 1, 2, 3 and 5 mg/L of 2, 4-D were affected callus induction while *m*T was not respond. The combination of 0.5 mg/L of BAP with 0.5 mg/L of 2,4-D showed the maximum percentage of callus induction (55.56%) and average area was 244.23 mm^3(Fig 2A), however 0.5 mg/L of BAP combined with 2 mg/L of 2,4-D which made the biggest average area of callus (1,079.95 mm^3)(Fig 2B). The callus structure was observed by SEM showing friable callus (Fig 3).

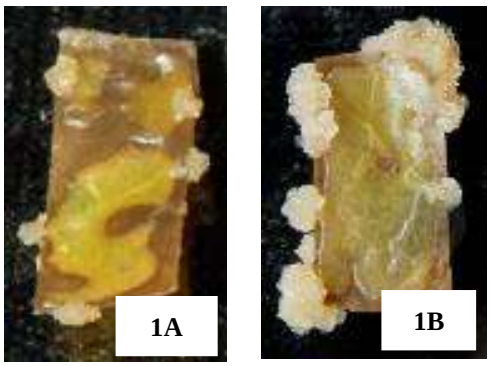


Figure 1A. The size of callus induction was measured less than 2 mm.
Figure 1B. The spot bigger size than 5 mm was recorded and measured.

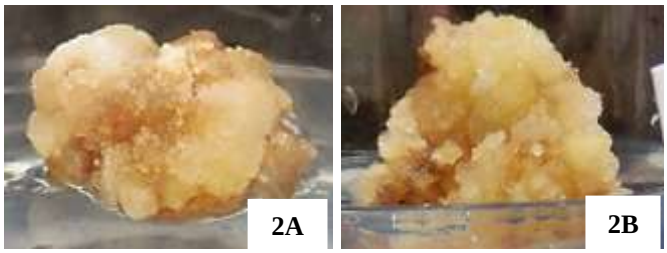


Figure 2A. Average callus area (244.23 mm^3) by 0.5 mg/L of BAP and 0.5 mg/L of 2,4-D on MS medium at 4 weeks after cultured.
Figure 2B. Average the biggest callus area by 0.5 mg/L of BAP and 2 mg/L of 2,4-D on MS medium at 4 weeks after cultured.

Table 1. Effect of PGRs on the callus induction and the average area (mm^3) from leaves of *Kadsura coccinea* (Lem.) A.C.Sm (Kad 001) after 4 weeks cultured.

PGRs (mg/L)		Callus induction[/1] (%)	Average area[/2,3] (mm^3)
BAP 0.5		2 (11.11)	-
1		-	-
2		-	-
3		-	-
5		-	-
*m*T 0.5		-	-
1		-	-
2		-	-
3		-	-
5		-	-
2,4-D 0.5		3 (16.67)	-
1		1 (5.56)	-
2		-	-
3		2 (11.11)	-
5		-	-
BAP 0.5 combine with	2,4-D 0.5	10 (55.56)	$244.23^{c} \pm 22.13$
	1	5 (27.78)	$743.27^{b} \pm 103.63$
	2	5 (27.78)	$1{,}079.95^{a} \pm 74.77$
	3	-	-
	5	-	-

[1]/The number of total explants in each treatment are 18 explants.
[2]/Each value presents the mean ± SE of three repeats per treatment.
[3]/The data were statistically analyzed by Duncan's multiple range test. In the same column, significant differences in accordance with the least significant difference (LSD) at the $P \leq 0.05$ level are referred by different letters.

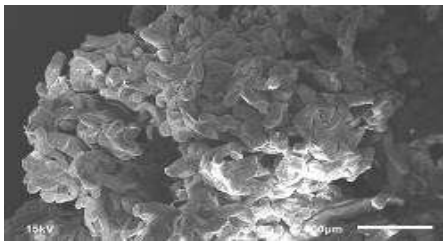


Figure 3. Callus structure from leaves of Kadsura *coccinea* (Lem.) A.C.Sm (Kad 001) by SEM resolution 500 μm at 15 kV on x40.

Growth rate by cell suspension materials and conditions

The growth rate of cell suspension cultured in MS medium appended with (0.5 mg/L) BAP and (0.5 mg/L) 2,4-D for 24 days all of the results shown in Table 2. The best grown of cell suspension for 15 days of 1.12 g/10 mL of medium and 0.14 g/10 mL of medium fresh and dry weight cell respectively. Cell fresh weigh (Fig 4A) and dry weight (Fig 4B) were strongly increased between 6 and 15 days. The cells were observed under bright fields that showed all calli (Fig 5A). The color under fluorescent microscope was stained with FDA alive cells showed green and death cells were black (Fig 5B). In one callus can be observed alive and death cells. In the part of cell suspension (Fig 6) that transferred callus to MS solid medium combine with (0.5 mg/L) BAP and (0.5 mg/L) 2,4-D used to compare that effect of AC and without AC. The black medium (with AC) gave the best result showing a compact and many green spots all-around piece. Callus can be able to develop for plant regeneration (Fig 7A) after transferred to solid medium for 4 weeks. While without AC medium for 4 weeks, calli were still weakness with yellow to light brown color (Fig 7B), no more green spot, imperfectly like treated on medium with AC. Sub-cultured to same condition of medium with 0.2% (W/V) AC gave perfectly calli and young leave tissue at 12 weeks (Fig 8).

Table 2. Average fresh and dry weight cell of *Kadsura coccinea* (Lem.) A.C.Sm (Kad 001) cell suspension from 0 to 24 days.

Days	Average fresh weight cell[1,2] (g/10 mL of medium)	Average dry weight cell[1,2] (g/10 mL of medium)
0	$0.2229^{f} \pm 0.01$	$0.0107^{d} \pm 0.00$
3	$0.2579^{f} \pm 0.01$	$0.0118^{d} \pm 0.00$
6	$0.3465^{ef} \pm 0.01$	$0.0209^{cd} \pm 0.00$
9	$0.6084^{cd} \pm 0.09$	$0.0704^{bc} \pm 0.01$
12	$0.9549^{ab} \pm 0.06$	$0.1236^{ab} \pm 0.01$
15	$1.1217^{a} \pm 0.16$	$0.1454^{a} \pm 0.04$
18	$0.8283^{bc} \pm 0.10$	$0.0960^{ab} \pm 0.01$
21	$0.6805^{cd} \pm 0.01$	$0.0708^{bc} \pm 0.01$
24	$0.5355^{de} \pm 0.01$	$0.0290^{cd} \pm 0.01$

[1]/Each value presents the mean ± SE of three repeats per treatment. [2]/The data were statistically analyzed using Duncan's multiple range test. In the same column, significant differences in accordance with the least significant difference (LSD) at the $P \leq 0.05$ level are referred by different letters.

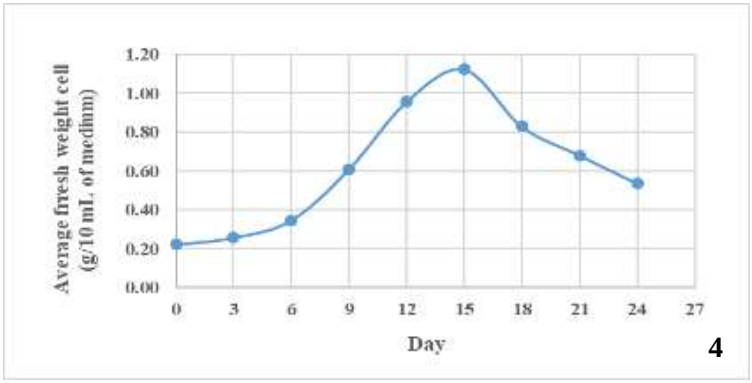


Figure 4A. Average fresh weight cell (mg/10 mL of medium) of *Kadsura coccinea* (Lem.)A.C.Sm (Kad 001) cell suspension from 0 to 24 days.

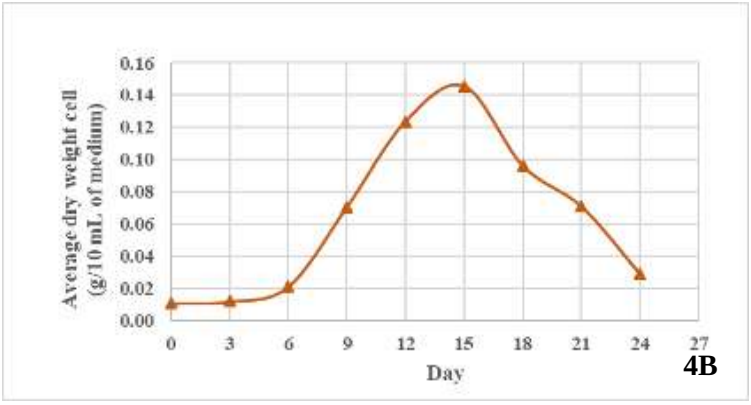
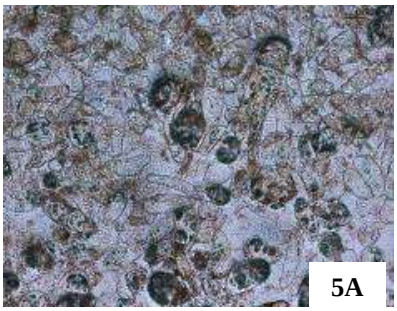


Figure 4B. Average dry weight cell (mg/10 mL of medium) of *Kadsura coccinea* (Lem.)A.C.Sm (Kad 001) cell suspension from 0 to 24 days.



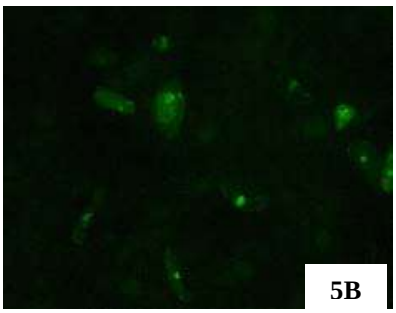


Figure 5A. The cells of callus under bright filed mode at 10X.
Figure 5B. The green color of live cells under fluorescent microscope by FDA staining at 10X.

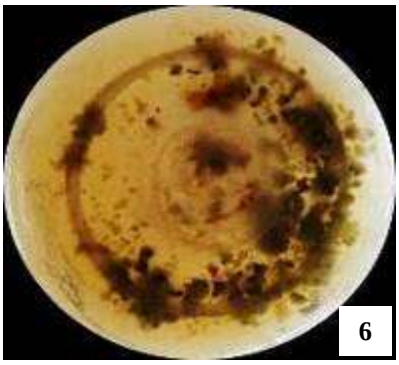
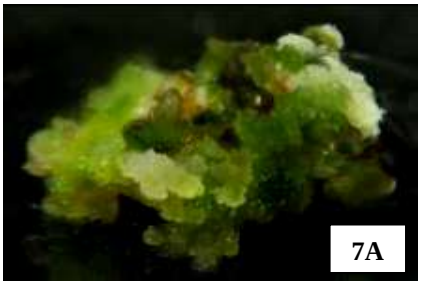


Figure 6. The cell suspension culture was the explant for transferred to compared effect of AC and without AC on callus induction.



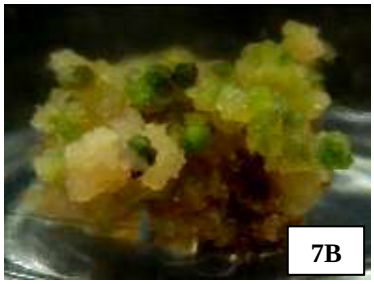


Figure 7A. The compact callus with yellow, light brown color and few of green spot on surface at 4 weeks on medium with AC.
Figure 7B. Yellow to light brown color and few of green spot at 4 weeks on medium without AC.

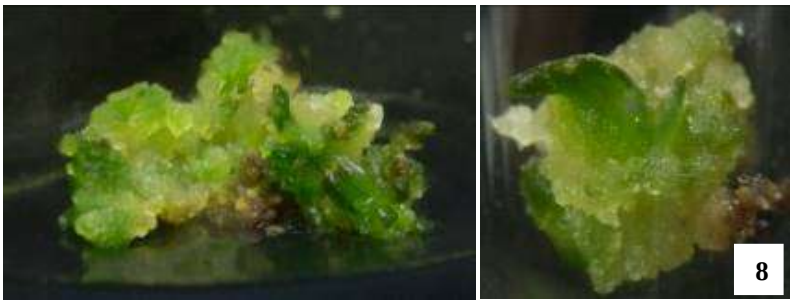


Figure 8. The green perfectly callus with young leave tissue at 12 weeks after transferred to MS solid medium with 0.5 mg/L of BAP, 0.5 mg/L of 2,4-D with 0.2% (W/V) AC.

Discussion

The present on callus induction and cell suspension from leaves of *Kadsura coccinea* was investigated. The optimum percentage of callus induction was on MS medium appended in two groups of PGRs, cytokinin (0.5 mg/L BAP) and auxin (0.5 mg/L 2, 4-D) accordingly with the

embryogenic callus from seed of *Schisandra chinensis* induced on Merkle's and Sommer's medium supplemented with 2, 4-D (9.04 μ*M*) and zeatin (0.09 μ*M*) showed the maximum number of embryogenic callus (KIM et.al., 2004). From the result of the optimal medium for *Schisandra chinensis Baill* callus induction both tender leaf and petiole was MS + 3 mg/L BAP (Jia *et al.*, 2011) and the results on MS+2, 4-D 2.0 mg/L+6-BA 0.5 mg/L medium for callus induction of SANDITI *Medicago sativa L.* was the best highest induction frequency (De-ping *et al.*, 2008) are the same result in the kind of PGRs but different concentration.

On the cell suspension culture of *K. coccinea* was shown the maximum of fresh and dry weight cell at 15 days as a review of cell suspension cultures from friable callus of *Gladiolus x grundiforus cv.* Peter Pears. was dispersed and doubled in packed cell amount in 2 weeks on MS media with zeatin or with 0.25 μM BAP (Remotti, 1995) apropos of PGRs in medium, 0.5 mg/L BAP and 0.5 mg/L 2,4-D was representative with the cell suspension cultures from callus of *Eysenhardtia polystachya (Fabaceae)* on MS medium with picloram plus kinetin grew appropriately and the maximum dry biomass accumulation (14 g L^{-1}) which occurred at 12 days of culture (Antonio *et al.*, 2017). Picloram can activated like 2,4-D while kinetin worked like BAP were in auxin and cytokinin group respectively. As a result of AC effect in the medium combine with 0.2% (W/V) gave the best efficiency for plant regeneration that shown compact callus, green color and young leave tissue according with of WV5 medium combined with 1.5% sucrose, 0.1% activated carbon and 0.05 μM of IBA.

It is demonstrated that callus induction and cell suspension cultured of *K. coccinea* was enhanced by increasing MS media with PGRs and adjusted cell suspension culture.

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