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In Vitro Laticifer Identification in Young Shoot-Derived Callus of *Hevea brasiliensis* Muell. Arg.

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Abstract

In vitro laticifer in young shoot-derived callus of *Hevea brasiliensis* Muell. Arg. (RRIM600 cultivar) was examined histologically and histochemically. Calluses were induced on a 2,4-D (1 mgL^{-1}) supplemented Murashige and Skoog (MS) culture medium containing different strengths of MS-micronutrients (quarter (¼), half (½) and full) and kinetin (0, 1 and 2 mgL^{-1}). The highest percentage of callus induction (52.78 ± 5.01 %) exhibiting hard and compact calluses was achieved using ½-strength MS-micronutrients without kinetin. Friable calluses were obtained on full-strength MS-micronutrients supplemented with 1 mgL^{-1} kinetin. Histological details indicated that calluses initiated from the sub-epidermal layers. One-month-old calluses presented thick cell wall laticifers which were mainly randomly arranged and located in separately and aggregately. All *in vitro* laticifers in the callus were non-elongated which were different from those in the stem. Some callus cells exhibited secondary wall thickening which had structural features similar to those of the vessel.

Keywords: *Hevea brasiliensis*, laticifers, callus, tracheary element, development

Introduction

Rubber trees (*Hevea brasiliensis* Muell. Arg.) are the most important source of natural rubber (cis-1, 4-polyisoprene) which is needed at the global level and shows increasing demand in many developing countries around the world [1,2]. Many researchers have tried to develop latex productivity by genetic manipulation such as conventional breeding (leading to creation of various cultivars) and biotechnology (transgenic plants). However, these techniques take a very long time to obtain a new cultivar and spend around 6 - 8 years for the cultivar selection by measuring the amount of latex per year in adult plants [3]. Because of the long period taken to select the best cultivar producing more latex, a novel technique for *in vitro* selection has been determined and evaluated. From histochemical and immunohistochemical studies, it was reported that the pattern of laticifer distribution between *in vivo* laticifers and *in vitro* secondary laticifers of anther-derived calluses of *H. brasiliensis* was different. However, the laticifer frequency and plant regeneration efficiency were significantly different among the anther-derived callus lines of distinct *H. brasiliensis* cultivars which had different latex yield [4]. Moreover, *in vitro* laticifers of anther-derived calluses of *H. brasiliensis* was age-dependent. Thus, this explant source was suggested to be a new model to screen suitable phytohormones and to improve latex yield [5]. Therefore, studying laticifer development *in vitro* is an alternative research for laticifer differentiation. Consequently, the successful establishment of callus cultures will open up the possibilities of mutant selection at the cellular level, providing knowledge involved in rubber biosynthesis process under controlled conditions [6]. Laticifer

cells were found in stem-derived calluses of *C. procera* (Ait) R.Br. cultured on a MS medium supplemented with 1.5 mgL^{-1} 2,4-D, 0.5 mgL^{-1} kinetin and polyvinylpyrrolidone (PVP) [7]. Datta and De [8] reported that laticifer cells were detected in 4-week-old calluses of *Calotropis gigantean* R.Br. ex Ait. when they were cultured on a MS medium containing 1 mgL^{-1} IAA with laticifer development age-dependent. *In vitro* laticifers were also observed in the calluses of papaya (*Carica papaya* L.) on a LS medium containing 0.1 µM 2,4-D and 1 µM kinetin. They found that the differentiation of *in vitro* laticifer of papaya was related to the production of papain-like enzymes [9].

For *H. brasiliensis*, *in vitro* laticifer cells were observed in stem-derived calluses cultured on a MS medium added with 2 mgL^{-1} 2,4-D and 0.5 mgL^{-1} kinetin [10] and in anther-derived calluses cultured on a MS medium supplemented with 1 mgL^{-1} 2,4-D, 1.5 mgL^{-1} kinetin, 2 mgL^{-1} NAA, 5 % coconut water (v/v) and 2.2 gL^{-1} phytagel [4,5]. However, there are no reports of *in vitro* laticifer development of young shoot-derived calluses of *H. brasiliensis*. Therefore, this study aims to identify *in vitro* laticifers and their features of the *H. brasiliensis* (RRIM600 cultivar).

Materials and methods

Plant materials

The stems and young shoots were collected from the bud-grafted rubber tree, *H. brasiliensis* (RRIM600 cultivar), Songkhla Rubber Research Center plantation, Thailand. Both stem and young shoot-derived calluses were examined for the presence of laticifers. This experiment was carried out during October 2011 - June 2012.

Preculture

Young shoots were collected and cut into 3-5 mm long shoots using a sharp sterilized blade. The explants were surface-sterilized with 70 % (v/v) ethanol for 5 min, soaked with 20 % (v/v) clorox for 20 min and washed in sterilized distilled water 3 times. They were then sterilized with 10 % and 2 % clorox for 10 min and 2 min, respectively. The explants were inoculated on solidified agar medium (6.8 gL^{-1} agar) supplemented with 30 gL^{-1} sucrose and maintained for 5 days in darkness.

Callus induction

The obtained explants were transferred to a basal Murashige and Skoog (MS) [11] medium containing macronutrients of MS, 1 mgL^{-1} 2,4-dichlorophenoxy-acetic acid (2,4-D), 30 gL^{-1} sucrose and 2.2 gL^{-1} phytagel at pH 5.7. These basal media were supplemented with micronutrients of MS at various concentrations (¼-strength, ½-strength and full strength) and kinetin (0, 1 and 2 mgL^{-1}). There were 7 replicates per treatment, each replicate contained 4 explants. The cultures were maintained in a culture room at 25 ± 2 ºC in darkness for a month. The percentage of callus induction was calculated by the following formula.

$$\%\text{callus induction} = \frac{\text{Number of explants forming callus}}{\text{Number of explants inoculated}} \times 100 \quad (1)$$

$$\%\text{browning explant} = \frac{\text{Number of explants browning}}{\text{Number of explants inoculated}} \times 100 \quad (2)$$

Statistical analysis

Statistical analysis was done by one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT) at a significance level of $P < 0.05$ using the Statistical Package for Social Science (SPSS) Programme. Data were expressed as mean ± S.E.

Identification and development of *in vitro* laticifers

Young shoot-derived calluses which were induced on MS medium supplemented with full-strength MS-micronutrients and 1 mgL^{-1} kinetin in the dark (the best result of the previous experiment) were collected at 7-day intervals for 28 days (0, 7-, 14-, 21- and 28-day-old culture) and a 60-day-old culture.

The stem of epicormic shoots of *H. brasiliensis* [12] were used as a positive control of laticifer staining. Samples were collected and fixed in FAA I (formalin, glacial acetic acid, 50 % ethanol; 5:5:90) at room temperature for 48 h, and treated with iodine and bromine in glacial acetic acid at 60 °C for 48 h [5]. They were then washed with glacial acetic acid, dehydrated in *n*-butyl alcohol series and embedded in histoplast PE (ThermoScientific). The embedded tissues were sliced into 10 µm thick sections with a rotary microtome, dewaxed and stained with fast green (5 % fast green in 95 % ethanol) for studying the general structure and laticifer. The sections were also stained with periodic acid-Schiff's (PAS) reaction to observe the carbohydrate accumulation [13]. The samples were photographed under a light microscope (Olympus BX51) with photographic apparatuses (DP 12) linked to a computer.

Results and discussion

Callus induction

The strength of MS-micronutrients and kinetin affecting on callus induction of *H. brasiliensis* Muell. Arg. were examined. It was found that callus formation could be observed from all media tested within 4 weeks. The highest percentage of callus induction (52.78 ± 5.01 %) was found on a 2,4-D - containing basal medium supplemented with ½-strength MS-micronutrients without kinetin (T4) (**Table 1**). However, the calluses obtained were compact and pale yellow (**Figure 1A**). The compact and hard calluses of the root explant of *H. brasiliensis* became brown and died while friable calluses appeared to have considerable morphogenic growth and somatic embryogenesis [14]. It is possible that the compact calluses from this present experiment provided embryogenic calluses, eventually developing into somatic embryos with difficulty. However, the compact embryogenic calluses were revealed to be highly regenerable in a hybrid bermudagrass (*Cynodon dactylon* (L.) Per. × *Cynodon transvaalensis* Burtt-Davy.) [15]. While, the medium containing full-strength MS-micronutrients and 1 mgL^{-1} kinetin produced soft, yellow and friable callus of crumbly appearance (**Figure 1B**, T2) which gave 36.11 ± 7.34 % of callus induction. Dhiya *et al*. [16] revealed that the clumbly structure was very suitable for breaking up for sub-culturing and produced a suspension culture in *Barringtonia racemosa* (L.) Spreng. The decrease in MS-micronutrients concentration increased the ability of callus formation of this *H. brasiliensis*. The present study agrees with Abe and Sasahara [17] who reported that low concentrations of mineral elements could increase the callus formation of *Japonica* sp. The decreased concentration of MS-micronutrients also provided a low quality compact callus. The highest percentage of callus browning (33.33 ± 5.89 %) was observed in basal MS medium containing ½-strength MS-micronutrients and 2 mgL^{-1} kinetin (T6) while calluses cultured on ½-strength MS-micronutrients provided the lowest percentage of callus browning (8.33 ± 4.17 %). Accordingly, the yellow and friable callus (**Figure 1C**) from T2 treatment (MS medium containing full strength of MS-micronutrients and 1 mgL^{-1} kinetin) was selected for the study of *in vitro* laticifer development.

In vitro laticifer development

Laticifer development between young shoot-derived calluses and stem (control) were histologically compared. The elongated primary laticifers (**Figure 2A**, arrow) and the enlargement of the subepidermal layers (**Figure 2A**, bracket zone) were observed at day 7 of the young shoot culture. It was also found that the enlarged parenchyma cells at the cut surface led to calluses exhibiting meristematic characteristics (**Figure 2B**, square zone). These meristematic cells were similar to those revealed by Park *et al*. [18] who reported the meristematic cells were converted from the elongated and the enlarged parenchyma and epidermal cells of mung beans (*Vigna radiata* (L.) R. Wilc.). Callus cells (**Figure 2C**, ca) were small, had a dense cytoplasm and large nucleus in the center of the cells indicating the meristematic site, while the explant cells were large with no nucleus (**Figure 2C**, ex). The laticifer stained brown with iodine-bromine staining could be observed in the callus after culturing for a month (**Figure 2D**, arrow head).

Table 1 Effect of strength of MS-micronutrients and kinetin on callus induction of *H. brasiliensis* (RRIM600 cultivar).

Media code	Strength of MS-micronutrients	kinetin (mgL^{-1})	% callus induction (mean ± S.E.)	% browning (mean ± S.E.)	Callus texture*
T1	full-strength	0	33.33 ± 10.26 ^{abc}	12.50 ± 8.54 ^{ab}	yc, fc
T2	full-strength	1	36.11 ± 7.34 ^{abc}	27.78 ± 7.73 ^{ab}	yc, fc
T3	full-strength	2	19.44 ± 5.55 ^{cd}	22.22 ± 6.51 ^{ab}	yc, fc
T4	½ strength	0	52.78 ± 5.01 ^{a}	8.33 ± 4.17 ^{b}	py, cc
T5	½ strength	1	19.44 ± 9.10 ^{cd}	30.56 ± 6.94 ^{ab}	yc, cc
T6	½ strength	2	22.22 ± 6.51 ^{bcd}	33.33 ± 5.89 ^{a}	yc, cc
T7	¼ strength	0	44.44 ± 10.85 ^{ab}	19.44 ± 8.09 ^{ab}	py, cc
T8	¼ strength	1	30.56 ± 5.56 ^{abcd}	22.22 ± 6.51 ^{ab}	yc, cc
T9	¼ strength	2	8.33 ± 4.17 ^{d}	27.78 ± 6.51 ^{ab}	yc, cc

Calluses were induced on 2,4-D - MS basal medium in dark condition for 1 month. Data were recorded after 4 weeks of culture on basal MS medium supplemented with different concentrations of MS-micronutrients and KN.
Values represent the means ± S.E. and the same letters within the same columns are not significantly different according to Duncan's multiple range test (DMRT) ($P < 0.05$).
* yc, yellow callus; py, pale yellow callus; fc, friable callus; cc, compact callus

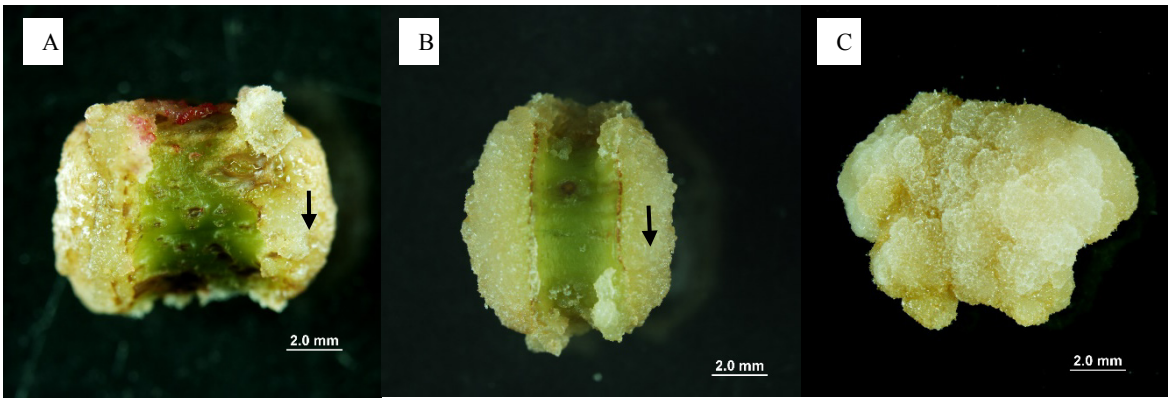

Figure 1 Young shoot-derived callus of *H. brasiliensis* exhibiting (A) compact callus and (B) friable callus on 2,4-D-MS media containing ½-strength MS-micronutrients and full strength MS-micronutrients with 1 mgL^{-1} kinetin, respectively. In the latter case, (C) 2- month-old callus presenting yellow and friable structure was used as initial callus for the experiment of *in vitro* laticifer development.

One-month-old calluses and young stems showed laticifers which were randomly distributed in both individual and group locations (**Figure 3**). These results were similar to those of the distribution of *in vitro* laticifers in the anther-derived callus of *H. brasiliensis* [4,5]. The laticifers from both calluses and young stems of *H. brasiliensis* could be classified into 3 types; 1) primary laticifer, 2) starch granule laticifer and 3) latex wiped off laticifer (**Figures 3A** and **3B**). These laticifers carried the thick cell walls

which play a role in preventing the cells from high latex pressure [19]. In addition, non-elongated laticifer cells were noticed in calluses which were similar to those of primary laticifers observed in the stem and anther derived-calluses of *H. brasiliensis*, possibly due to inhibition because of a lack of polarity of the callus and the suppression of cell elongation [4,5]. Laticifers containing starch granules could be observed in the young shoot-derived callus (**Figure 3A**), the stem (**Figure 3B**) and the anther-derived callus of *H. brasiliensis* [5]. These starch granule laticifers might serve as a storage system to protect plants from the insects [20]. In the present study, starch granule laticifers were produced at the early stage of callus formation. It could be inferred that this starch granule laticifer type was important for callus growth of *H. brasiliensis*. This result agrees with previous research showing the starch granule accumulation in callus was closely related to somatic embryogenesis and growth of sweet potato (*Ipomoea cordatotriloba* Denn.) [21].

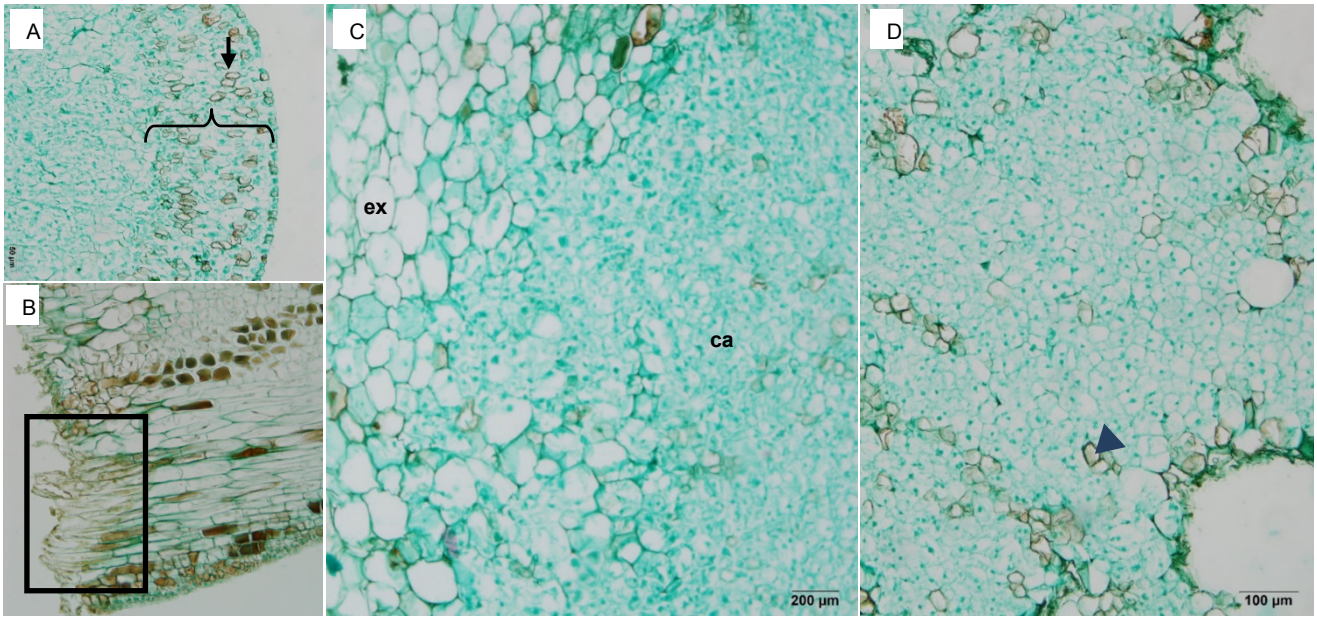

Figure 2 Histology of callus formation from young shoot explant of *H. brasiliensis*. A: Transverse and B: longitudinal sections of shoot explant at day 7 showing callus initiated from the subepidermal cell layers (A, bracket), elongated laticifer cell (A, arrow) and cell dedifferentiation along the cutting surface (B, square) C: Transverse section through the callus (ca) involved in the explant (ex) exhibiting small callus cells and explant parenchyma cells of explant D: Laticifer cell (arrow head) in callus.

The morphological observations also presented similar laticifer features among the different explant sources; namely, the young shoot-derived callus, the primary laticifers in stem (control) as well as the anther-derived calluses. In the last case, it was planned to be a model for studying laticifer differentiation and improving latex yield [4,5]. However, more advantages could be gained by using young shoot explants than anther explants due to having the convenience to collect and having actual genetics of their own.

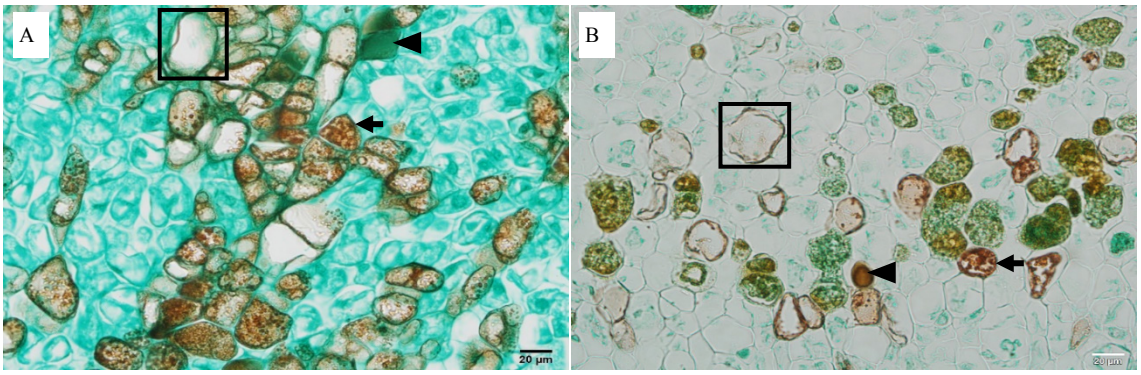

Figure 3 Different laticifer types, namely, primary laticifer (arrow head), starch granule laticifer (arrow) and latex wiped off laticifer (square) observed in A: one-month-old callus derived from young shoot and B: stem of *H. brasiliensis*.

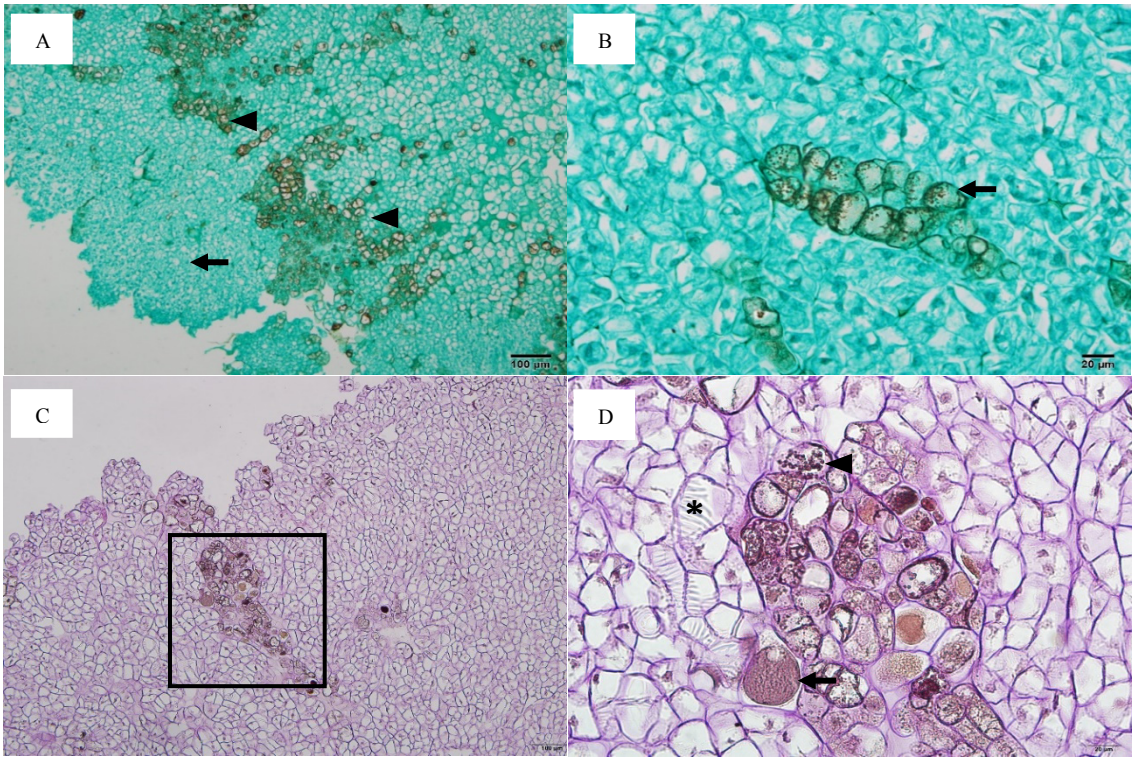

Figure 4 Histology of laticifer development in 2-month-old calluses A: New callus mass (arrow) without the laticifer originating from the old callus tissue presenting laticifers (arrow heads) B: Enlargement of laticifers cluster (arrow) C - D: callus section stained with PAS reaction exhibiting laticifer containing starch granule (arrow head), tracheary element-like structure (✱) and the laticifer with the rubber content (arrow). D is the enlargement of the square region of C (at 40× magnification).

After 2 months of culture, the new callus tissues presenting no laticifer were formed on the old callus mass (**Figure 4A**) in which the non-elongated laticifers remained stable (**Figure 4B**). The latex and starch granule-containing laticifers were stained purple by PAS reaction (**Figure 4C**). In addition, the tracheary elements (TE) like structures (**Figure 4D**, *), known as xylem vessels, were characterized by an elongated shape and lignified secondary cell wall. The TE differentiation was also characterized by cytological changes, lignin deposition and programmed cell death both in plant and *in vitro* culture [22]. The presence of TE in the callus is a complex process which may be associated with many factors including hormonal induction, wounding and shoot meristem development [23]. Gatz and Kawalski [24] revealed that the TE appearance was also observed in the early stages of *in vitro* organogenesis and the shoot formation of pepper (*Capsicum annuum* L.) and the accumulation of carbohydrate might be a factor stimulating TE formation particularly in the medium supplemented with auxin. Moreover TE differentiation was involved with secondary cell wall development by cortical microtubules in *Arabidopsis* cell suspension [25]. This process has been studied in many plant species such as *Cupressus sempervirens* L. [26].

Conclusions

Results from this study revealed that the best callus induction medium which can produce friable calluses was a MS medium containing full-strength MS-micronutrients and 1 mgL^{-1} kinetin. These induced calluses initiated from sub-epidermal cells. Moreover, the callus contained 3 types of laticifers as observed in stems. These results also indicated that the presence of laticifers in young shoot-derived callus is useful new knowledge for further applications such as comparing between *in vitro* laticifers of interesting *H. brasiliensis* cultivars. The latex yield indicator and improvement of latex yield needs to be done in the next research.

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