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Research article

กายวิภาคเปรียบเทียบของเซลล์ท่อน้ำยางในระบบ *in vivo* และ *in vitro* ของยางพารา (*Hevea brasiliensis* Muell. Arg.) 2 สายพันธุ์ (RRIM600 และ RRIT402)

Comparative Anatomical Study of *In Vivo* and *In Vitro* Laticifers in Two Rubber Tree (*Hevea brasiliensis* Muell. Arg.) Cultivars (RRIM600 and RRIT402)

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บทคัดย่อ

ศึกษาเปรียบเทียบกายวิภาคของเซลล์ท่อน้ำยางในยางพารา (*Hevea brasiliensis* Muell. Arg.) ทั้งในลำต้น (*in vivo*) และในแคลลัสภายในหลอดแก้ว (*in vitro*) ระหว่างพันธุ์ที่ให้ปริมาณน้ำยางสูง (พันธุ์ RRIM600) และพันธุ์ที่ให้เนื้อไม้สูงแต่ให้ปริมาณน้ำยางน้อย (พันธุ์ RRIT402 หรือ พันธุ์ฉะเชิงเทรา 50) โดยใช้วิธีการทางเนื้อเยื่อวิทยา ทั้งนี้เพื่อค้นหาลักษณะทางกายวิภาคของท่อน้ำยางที่สัมพันธ์กับปริมาณน้ำยาง - โดยเก็บตัวอย่างลำต้นยางพาราในระบบ *in vivo* บริเวณฉัตรที่ 1 จากกิ่งยางพาราที่มี 5 ฉัตร และในระบบ *in vitro* ได้เก็บตัวอย่างแคลลัสที่ชักนำจากยอดอ่อนของยางพาราที่เพาะเลี้ยงเป็นเวลา 1 เดือน รวมถึงเปรียบเทียบค่าความหนาแน่น พื้นที่ และเส้นผ่านศูนย์กลางของเซลล์ท่อน้ำยางของทั้ง 2 พันธุ์ ผลการศึกษาพบว่า ชิ้นส่วนยางพาราจากระบบ *in vivo* และ *in vitro* ของทั้ง 2 พันธุ์ ประกอบด้วยเซลล์ท่อน้ำยางปฐมภูมิ (primary laticifer) 3 ชนิด ได้แก่ เซลล์ท่อน้ำยางทั่วไป (latex-contained laticifers) เซลล์ท่อน้ำยางที่มีเม็ดแป้ง (starch granule laticifers) และเซลล์ท่อน้ำยางที่มีการหลุดออกของน้ำยาง (latex-wiped off laticifers) ลักษณะร่วมของเซลล์ท่อน้ำยางคือ มีผนังเซลล์หนาและติดสีน้ำตาลเมื่อย้อมด้วยสี Iodine-bromine เมื่อตัดตามยาวของลำต้นพบว่า ยางพาราพันธุ์ RRIT402 มีการเชื่อมต่อของเซลล์ท่อน้ำยางก่อนพันธุ์ RRIM600 ซึ่งการเชื่อมต่อของท่อน้ำยางที่เกิดขึ้นเร็วอาจเป็นสาเหตุให้พันธุ์ RRIT402 มีการผลิตน้ำยางได้น้อย และพบว่าพันธุ์ RRIM600 มีความหนาแน่นของเซลล์ท่อน้ำยางมากกว่าพันธุ์ RRIT402 อย่างมีนัยสำคัญทางสถิติทั้งในระบบ *in vivo* และ *in vitro* ผลการศึกษาแสดงให้เห็นว่าความหนาแน่นของเซลล์ท่อน้ำยางทั้งในระบบ *in vivo* และ *in vitro* สามารถใช้เป็นเครื่องหมายบ่งชี้ปริมาณน้ำยางพาราในแต่ละสายพันธุ์ได้
คำสำคัญ: แคลลัส, สายพันธุ์ยางพารา, ยางพารา, เซลล์ท่อน้ำยาง

Abstract

Comparative anatomy of laticifer cells in rubber tree (*Hevea Brasiliensis* Muell. Arg.) in both stem (*in vivo*) and callus (*in vitro*) between a high latex-yielding cultivar (RRIM600) and a high woody-quality presenting low latex-yielding cultivar (RRIT402 or Chachoengsao 50) was histologically studied. This study aimed to achieve whether anatomical features of laticifers were closely related to the latex yield. The stem of the uppermost extension unit 1 (EU1) of the epicormic shoot having 5 EUs and 1-month-old young shoot-derived calli of both cultivars were collected for *in vivo* and *in vitro* studies, respectively. The density, area, and diameter of laticifers of both cultivars were also measured and compared. The result showed that 3 types of primary laticifers including latex-contained laticifers, starch granule laticifers, and latex-wiped off laticifers were observed in *in vivo* and *in vitro* samples of both cultivars. Common feature of the laticifers was a thick cell wall with stained brown due to the iodine-bromine staining. Longitudinal sections through stems exhibited the anastomosed laticifers in RRIT402 were formed earlier than those of RRIM600. The early laticifer connection might be the cause of low latex yield in RRIT402 cultivar. It was

also found that the laticifer density of the high latex yielding cultivar (RRIM600) was significantly higher than that of the RRIT402 in both *in vivo* and *in vitro* suggesting that the laticifer density of both *in vivo* and *in vitro* could be used as a latex yield indicator for each cultivar.

Keywords: Callus, cultivars, *Hevea brasiliensis*, laticifers

Introduction

Latex productivity is the major focus in rubber cultivar selection of rubber tree (*Hevea brasiliensis* Muell. Arg.) since the latex demand has been increased in developed countries (Hayashi, 2009). The conventional breeding exhibiting time consuming for breeders and biotechnology have been applied to acquire a new high latex-yielding cultivar. Therefore efficient techniques for early selection of new rubber cultivars need to be developed. The research that gains the latex yield indicators associated with early selection of high latex-yielding cultivars during breeding program is the interesting subject.

Laticifers are specialized cells containing cytoplasmic fluid called latex which is used to describe as milk plant exudates (Hagel *et al*., 2008). Three types of laticifers; 1) latex-contained laticifers 2) starch granule laticifers and 3) latex-wiped off laticifers, were observed both *in vivo* and *in vitro* study (Tan *et al*., 2011). Two types of laticifer cells in rubber tree divided by the origin of cells, the primary laticifers which are originated from both procambium and ground meristem at early stage of growth and the secondary laticifers from vascular cambium, could be found. Regarding the secondary laticifers, these cells are anastomosed from the contiguous cells to form the reticulated chains arranging in rings parallel to the vascular cambium (d' Auzac *et al*., 2000). Hao and Wu (2000) revealed that number and rings of the secondary laticifer cells in rubber bark could determine the ability of the vascular cambium in latex production. Goncalves *et al*. (1995) revealed that the laticiferous system in young *H. brasiliensis* is useful criterion for selection of different latex-yielding cultivars. Hence, the anatomical study of laticifer development is important to understand the process of latex production in the rubber tree, as well as the obtained knowledge may facilitate the cultivar selection in breeding program. Moreover, investigating laticifer anatomy in proper parts of the plant can give insight into the parameter which indicates latex yield in different cultivars and can be used as an early selection indicator.

Previous study reported that *in vitro* laticifers could be observed in two-month-old callus of rubber tree (Tan *et al*., 2011, 2014). Tan *et al*. (2011) found the significant difference of laticifer frequency and plant regeneration efficiency among anther-derived calli of 5 rubber tree cultivars. Consequently, *in vitro* laticifer development of *H. brasiliensis* anther-derived callus was proposed as a model for improvement of latex yield in future research (Tan *et al*., 2014). However, the anther-derived calli were gained from anther explants which spent a long time during anther harvesting. Recently, young shoot-derived calli exhibiting the *in vitro* laticifers, which were genetically similar to their own parents, were conveniently obtained from our study (Yodyotee *et al.,* 2017). To evaluate the proposed indicator for latex yielding, the anatomical characteristics were also compared between RRIM600 and RRIT402 cultivars of *H. brasiliensis*. These 2 cultivars were the representative cultivars of high latex-yielding cultivar (RRIM00) and low latex-yielding cultivar (RRIT402). Accordingly, the main objective of this present study was to examine these characteristics relating the latex yield in stem (*in vivo*) and young shoot-derived callus (*in vitro*).

Materials and methods

Plant materials

Two cultivars of rubber tree – RRIM600 (high latex-yielding cultivar, produced 875 kg/ha/year of latex product) and RRIT402 (high woody-quality and low latex-yielding cultivar, produced 162.5 kg/ha/year of latex product) – were collected from Songkhla Rubber Research Center plantation, Songkhla province for the study. Both cultivars are grown on Kho Hong series (Kh) soil (Coarse-loamy) pH 4.5-6 under natural light and controlled by Songkhla Rubber Research Center plantation. This work was done during March-December 2012.

Plant stem – The uppermost extension unit (EU1) stems of the epicormic shoot having 5 EUs (4-month-old) of the two cultivars were applied for investigating the laticifer development. These stems grew from buds of rubber trees

which were pruned every year and used as source parents. Both cultivars grew under the same climatic and soil conditions.

Callus – Young shoot-derived calli of both cultivars were induced on modified Murashige and Skoog (1962) medium (preliminary result) for one month in darkness at 25 °C. The modified MS medium was composed of 1 mgL^{-1} 2,4 D and 1 mgL^{-1} KN, 30 gL^{-1} sucrose and 2.2 gL^{-1} phytagel at pH 5.7. The RRIM600 calli were yellow, compact and unsmooth with a small spherical protuberance on its surface. While a pale yellow and compact calli were observed from RRIT402.

There were 6 replicates of plant stems and 3 replicates of calli of each cultivar.

Histochemical preparation for laticifer study

Stems and young shoot-derived calli of both cultivars were fixed in FAA I (formalin, glacial acetic acid, 50% ethanol; 5:5:90) at room temperature for 48 h, treated with iodine and bromine in glacial acetic acid at 60°C for 48 h (Hao and Wu, 2000). The samples were washed with glacial acetic acid, dehydrated in *n*-butyl alcohol series and embedded in histoplast PE (Thermo scientific). These embedded tissues were sliced into 10 µm thick sections with a rotary microtome, dewaxed and stained with fast green (5% of fast green in 95% ethanol) for laticifer localization. Sample sections were photographed under light microscope (Olympus BX51) with photographic apparatuses (DP-72) linked to the computer. The sections were also stained with periodic acid-Schiff's (PAS) reaction to observe the carbohydrate accumulation.

Measurement of laticifers and starch granule size

The distribution patterns and types of laticifer cells were investigated and described. The density, area, and diameter of laticifers in 30 randomly chosen locations from each stem and callus section were photographed and measured in a light microscope. All three types of laticifers including latex-contained laticifer, starch granule laticifers and latex-wiped off laticifers were measured. The details of collecting data were as follows:

1) Laticifer density (cells/mm^2): Number of laticifer cells per mm^2
2) Laticifer cell diameter (µm): Average of cell diameter taken from 30 cells measured (ImageJ software version 1.33)
3) Laticifer area (%): The presented area of laticifer cells in the visual view (ImageJ software version 1.33)

Starch granules size in the stem sections were also measured by ImageJ software version 1.33 from 30 starch granules observed.

Statistical analysis

A paired sample T-test was performed for statistical comparison between means of laticifer density, area, diameter and starch granule size of two cultivars (RRIM600 and RRIT402). The letters 'a' and 'b' indicate the significant difference between two *H. brasiliensis* cultivars.

Results and discussion

1. In vivo and In vitro laticifer characteristics of RRIM600 and RRIT402 cultivars

The laticifers of stem barks of RRIM600 and RRIT402 cultivars were stained brown and had thick cell walls. In longitudinal sections of the EU1, RRIM600 cultivar exhibited non-anastomosing articulated laticifers in phloem region (Figure 1A) while RRIT402 cultivar presented anastomosing articulated laticifers (Figure 1B). This result revealed that the anastomosing laticifers of RRIT402 were formed eariler than those of RRIM600. We found that laticifers walls of RRIT402 dissolved and presented network cells, while RRIM600 laticifers wall still persist and exhibited seperated cells. These results revealed why higher numbers of laticifers were observed in RRIM600 than in RRIT402. However, distribution patterns and types of laticifers in EU1 of both RRIM600 (Figure 1C) and RRIT402 (Figure 1D) were similar. In addition, both cultivars exhibited only primary laticifers in primary phloem. Some primary laticifers distributed both separately and aggregately in the phloem. Three types of laticifers: 1) latex-contained laticifer 2) starch granule and 3) latex-wiped off laticifers, were observed in both RRIM600 and RRIT402 (Figure 1E and 1F). However, no secondary laticifer could be observed in the developing secondary phloem of EU1. Absence of secondary laticifers in EU1 could be explanined by the result of Hao and Wu (2000) who reported that secondary laticifers did not emerge until the shoot produced 5 EUs in which a large number of secondary phloem elements had differentiated.

The results also presented the accumulation of starch granules in the area of pith and primary xylem of stem sections of RRIM600 (Figure 2A) and RRIT402 (Figure 2B). These starch granules from both areas of RRIM600 (Figure 2C, E) were similar to those of RRIT402 (Figure 2D, F). The presence of identical feature of starch granule in both cultivars was not related to the difference of latex yield. However, this result showed that the starch granules of RRIT402 were significant larger than RRIM600 (Table 1). Chantuma *et al.* (2007) reported that the increase of carbohydrate accumulation could promote the latex yield in *H. brasiliensis*. Moreover, the availability and metabolism of sugar during latex regeneration were some of the major factors affecting latex yield (Mesquita *et al.,* 2006). In contrast to the present study, the different latex yields between 2 cultivars were not depended on the appearance of carbohydrate. However, the different sizes of laticifers were depended on cultivars and might involve with latex yield. Dedeh and Sackey (2002) reported that the different sizes of starch granules and different rheological properties of *Xanthosoma sagittifolium* (red-flesh) and *X. sagittifolium* (white-flesh) might be involved with the genetic control of starch deposition and these findings also aided to characterize the different cocoyam cultivars. In the present study, therefore, the size of starch granule might be able to indicate the latex yield. Thus, a smaller size of starch granule might be observed in high latex yielding cultivar.

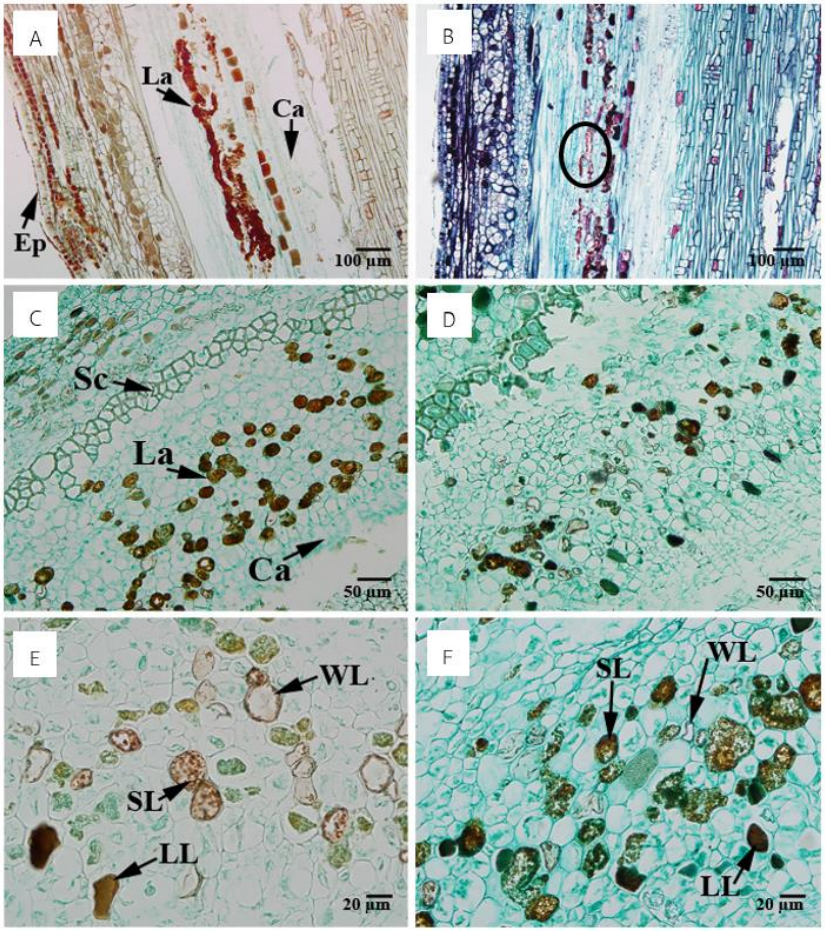

Figure 1 Distribution and various types of laticifers in the stem of the uppermost extension unit (EU1) of *H. brasiliensis* **(A, C, E)** RRIM600 and **(B, D, F)** RRIT402. Longitudinal sections presenting **(A)** non-anastomosing articulated laticifer in RRIM600 and **(B)** anastomosing articulated laticifer in RRIT402, circle, and connected laticifer walls. Transverse sections showing laticifer distribution in **(C)** RRIM600 and **(D)** RRIT402. Three laticifer types; starch granule laticifer (SL), latex-contained laticifer (LL) and, latex-wiped off laticifer (WL), shown in **(E)** RRIM600 and (**F**) RRIT402. Ca, cambium, Ep, epidermis; La, laticifers; Sc, sclerenchyma

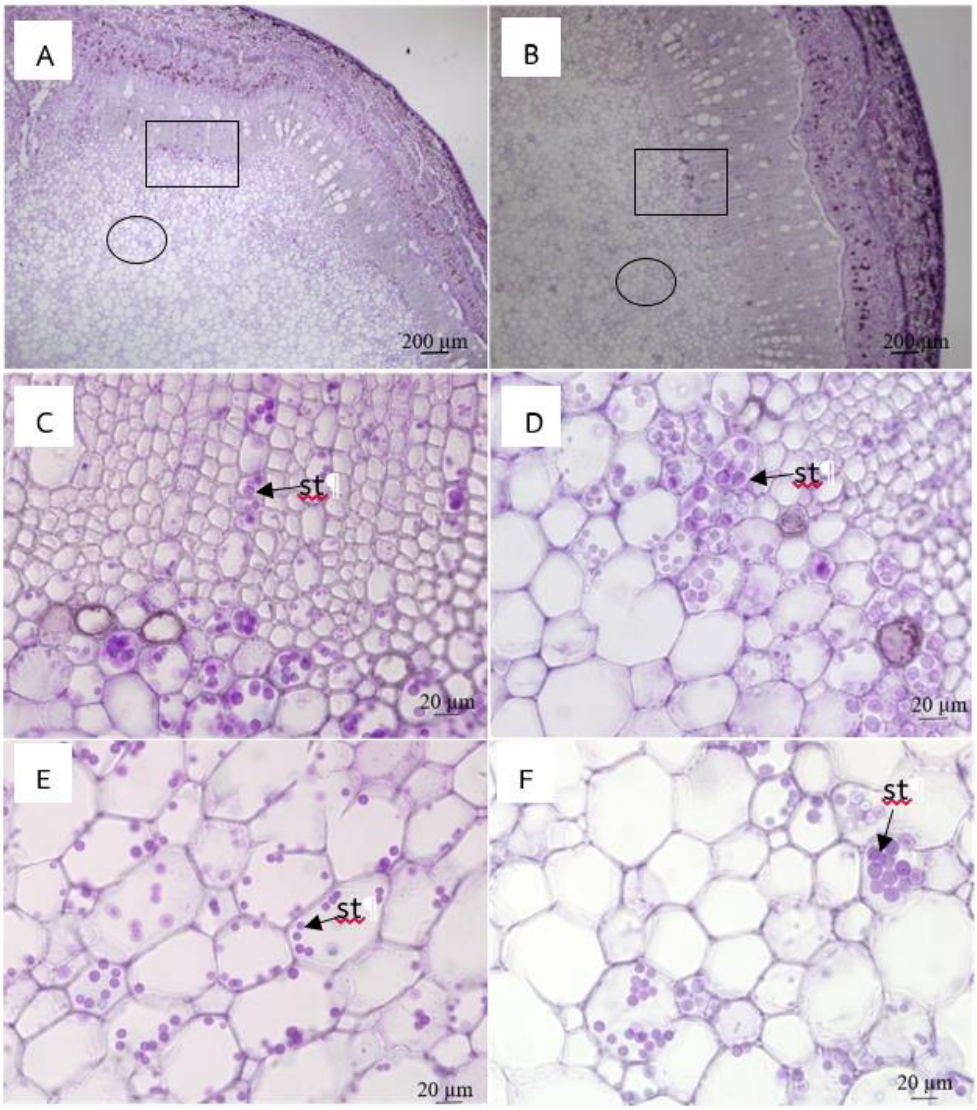

Figure 2 Transverse sections of *H. brasiliensis* stem regions presenting (**A, B**) accumulation of starch granules in pith (circle) and primary xylem (rectangular) of **(Left)** RRIM600 and **(Right)** RRIT402. Different sizes of starch granules in (**C**, **D**) xylem and (**E**, **F**) pith. St, starch granule

Table 1 Starch granules size in rubber tree stem of differences rubber cultivars (RRIM600 and RRIT402)

Rubber cultivars	Starch granules size (µm) ± S.E.
RRIM600	6.01 ± 0.26 [b]
RRIT402	8.21 ± 0.34 [a]

Values with the different letters within the column are significantly different according to a paired sample T-test ($P < 0.05$).

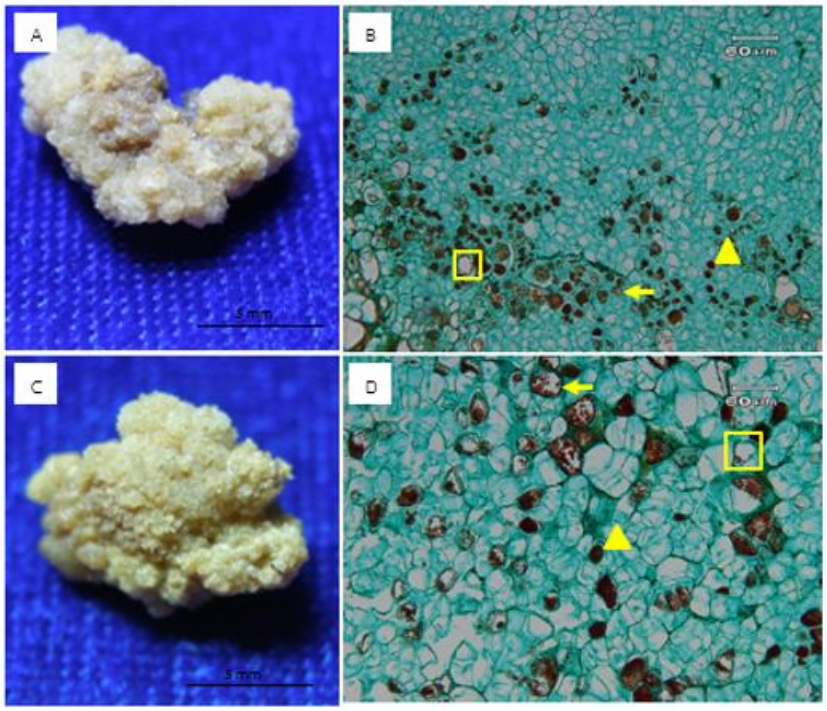

Figure 3 Morphological and histological features of callus mass of **(A, B)** RRIM600 and **(C, D)** RRIT402. Both cultivars presenting latex-contained laticifer (arrow head), starch granule laticifer (arrow) and latex-wiped off laticifer (square).

Young shoots of RRIM600 and RRIT402 cultivars were induced to form callus for 2 months. The yellow calli presenting friable and compact were obtained from RRIM600 (Figure 3A) and RRIT402 (Figure 3C), respectively. Histological studies revealed that three types of laticifers including latex-contained laticifer (Figure 3B, D, arrow), starch granule laticifer (Figure 3B, D, arrow head) and latex-wiped off laticifer (Figure 3B, D, square) were also observed in both cultivars. All three types of laticifer cells were similarly found in anther-derived callus (Tan *et al*., 2011). *In vitro* laticifer features and location of RRIM600 resembled to those of RRIT402.

2. Comparative anatomical evaluation of laticifer development between RRIM600 and RRIT402 cultivars

Both *in vivo* and *in vitro* laticifers including diameter, density and area of laticifers of RRIM600 (high latex-yielding cultivar) and RRIT402 (low latex-yielding cultivar) were examined. It was found that the highest laticifer density (548.07 ± 22.51 cells/mm^2) was observed in EU1 stem of RRIM600 while the lowest laticifer density (169.97 ± 9.31 cells/mm^2) was found in callus of RRIT402 (Figure 4). The laticifer density in stem of RRIM600 was significantly higher than that of RRIT402. The result coincided with Laosombut *et al*. (2016) who found that the number of laticiferous vessel of RRIM600 was higher than that of RRIT402. The number of laticifer cell is one of the most important factors influencing rubber yield of *H. brasiliensis* (Gomez, 1982). Wu (1998) also reported that the early selection in *H. brasiliensis* has benefited to reduce the number of field testing and to increase the selection efficiency of mature plants. Therefore, from the present study, it was confirmed that laticifer density in young stem of *H. brasiliensis* could be used and applied as an early indicator of latex yield.

Similar to the pattern in stem, the laticifer density of RRIM600 callus was significantly higher than that of RRIT402. Therefore, the density of laticifer (cells/mm^2) in callus could also be used as an indicator required for the early selection of high latex-yielding cultivar. Surprisingly, this research is the first presented that laticifer density in callus could be applied for early selection of *H. brasiliensis*. This result was conformed with Tan *et al.* (2011) who found that the laticifer frequency (%) of anther-derived callus of RRIM600 was higher than Haiken2 (low latex-yielding cultivar). However, the different explant used and laticifer number calculated between Tan *et al.* (2011) and this report provided different value of laticifer number. Our results could be as the basic knowledge for callus applying in the process of cultivar selection.

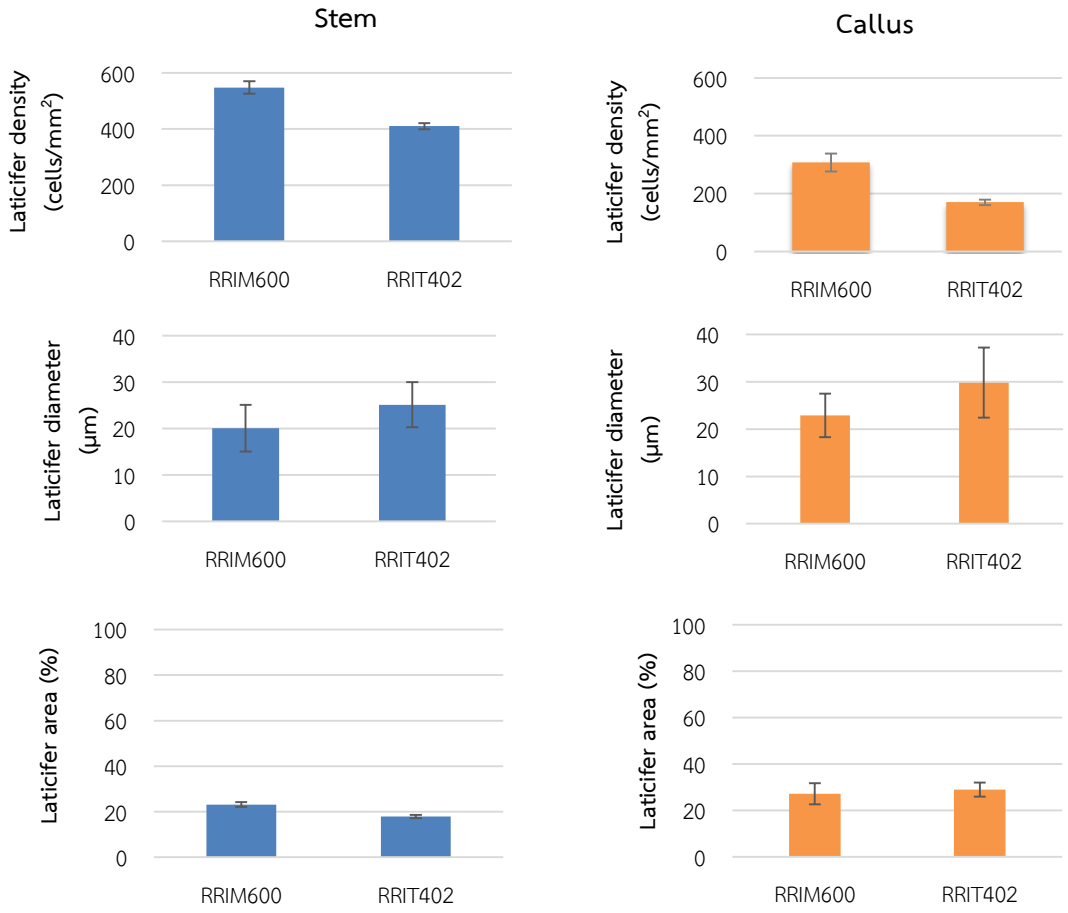

Figure 4 Measurements of primary laticifers in stem of epicormic shoot (EU1) and 1-month-old callus derived from young shoot of RRIM600 and RRIT402. *ns, non-significant difference

The application of plant tissue culture on cultivar selection process was also reported in the salt and drought tolerant of wheat (Barakat, 1996) and sugarcane callus (Srinath and Jabeen, 2013), respectively.

The largest (29.83 ± 7.42 µm) and smallest (20.10 ± 5.03 µm) laticifer diameters were observed in RRIT402 stem and RRIM600 callus, respectively (Figure 4). The laticifers (in both stem and callus) of RRIT402 were larger in diameter than those of RRIM600, and the large RRIT402 laticifers might be caused by early development of laticifer anastomosing. Demarco *et al.* (2013) reported that the differentiation of broad laticifers of *Sapium haematospermum* Muelll. Arg. was occurred before starting small laticifers. Thus, the production of laticifer cells in RRIM600 at early stage before the anastomosing was higher in a number of cells than that of RRIT402. This was the reason why RRIM600 exhibiting the highest latex-yielding cultivar provided the great number of laticifer cells. Moreover, since a small size and slow laticifer anastomosing in RRIM600, this cultivar could produce high volume of latex from several laticiferous branches. Accordingly, laticifer diameter, known as clonal character with genetically determined, and rate of anastomosing in *H. brasiliensis* bark (virgin and renewed bark) were positive correlation (Thomas *et al*., 1995). The increasing of laticifer diameter was depended on anastomosing rate and also time and age of plant (Mahlberg, 1993). Thus, the different size of laticifers in both stem and callus of *H. brasiliensis* were depended on rate of laticifer anastomosing.

The highest laticifer area (28.97 ± 3.04 %) was found in RRIT402 callus, when the lowest (17.91 ± 0.70 %) was found in RRIT402 stem (Figure 4). Moreover, the average laticifer area in callus was larger than that of stem of both cultivars due to the toxic metabolites could be released from the large laticifers more than the small laticifers (Tan *et al*., 2011). Some reports revealed that latex contained several metabolites such as enzymes, mineral, organic acids in which

some of them were toxic metabolites (d' Auzac, 2000). Therefore, this was the reason why the callus from young shoot was certainly brown after culture for a long period. The present result also displayed that the laticifer area in stem of RRIM600 was significantly higher than that of RRIT402. According to this result, the large laticifer area was produced from high laticifer density cultivar (RRIM600). In summary, therefore, this pattern of laticifer area from the present study was suggested to be as latex yield indicator in *H. brasiliensis* stem. However, only laticifer density in callus was confirmed to be a latex yield indicator because the laticifer area in callus of both cultivars was not significantly different.

Conclusion

Based on the findings of anatomical study of laticifer of *H. brasiliensis* cultivars, density of *in vitro* laticifer taken from callus could be an indicator of latex production and would be considered to apply for the cultivar selection program. The advantage of this method was reduction of time-consuming than other selection techniques. We suggested that an *in vitro* laticifer anatomy should be examined in other *H. brasiliensis* cultivars, as well as expression of genes that are related to latex production should be investigated in order to be used as one of the reliable latex yield indicators.

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