THE GROWTH PERFORMANCE OF CITRUS DERIVED FROM SOMATIC EMBRYOGENESIS PLANTLET AND SCION STOCK

Keragaan Pertumbuhan Jeruk dari Planlet Hasil Perbanyakan Embrio Somatik dan Mata Tunas Batang Atas

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ABSTRACT

Somatic embryogenesis (SE) of callus culture *in vitro* is one of citrus propagation ways for producing free virus and genetically true-to-type plantlets. To induce growing of plantlets derived from this technology, they should be grafted *ex vitro* onto a citrus rootstock. The research aimed to evaluate the growth performance of citrus plants cv. Siam Kintamani (*Citrus nobilis* L.) that used both plantlets and scions as their stocks. The research was conducted at Tlekung Research Station, Indonesian Citrus and Subtropical Fruit Research Institute from June 2011 to December 2012. The treatments were done at nursery house by grafting a plantlet and budding a scion onto an eight-month-old Japanese Citroon (JC) rootstock plant. The grafted and budded plants of one-year old were maintained at nursery house then transplanted into the field. In the field, the research was arranged in a randomized block design with three replications and used 15 plants as unit samples. The results showed that the vegetative growth of Siam Kintamani seedling derived from SE or grafted plant was faster than that of budded plant started from 10 to 12 months after treatment in the nersery house. In the field, the growth of SE grafted plant was only significantly different up to 6 months after transplanting. Plantlets produced from SE *in vitro* propagation can be used as a good alternative stock material for producing healthy citrus plants. Therefore, a further research is required especially on varieties used, reproductive growth and massive planlets production.

[***Keywords*****:** *Citrus nobilis*, somatic embryogenesis, plantlet, scion, grafting]

ABSTRAK

Embrio somatik (ES) yang berasal dari kultur kalus in vitro *merupakan salah satu cara perbanyakan jeruk untuk memproduksi plantlet bebas virus dan secara genetik bersifat sama dengan induknya. Untuk mempercepat pertumbuhannya, planlet asal SE harus disambung secara* ex vitro *pada batang bawah. Penelitian ini bertujuan untuk mengevaluasi keragaan pertumbuhan tanaman jeruk cv. Siam Kintamani (*Citrus nobilis *L.) yang batang atasnya berasal dari planlet dan mata tunas entres. Penelitian dilakukan di Balai Penelitian Tanaman Jeruk dan Buah Subtropika pada Juni 2011-Desember 2012. Perlakuan dilaksanakan dengan cara menyambung plantlet dan menempel mata tunas pada batang bawah Japanese Citrun (JC) berumur delapan bulan. Tanaman hasil sambungan/tempelan dipelihara di pembibitan sampai berumur satu tahun, kemudian tanaman tersebut ditanam di lapangan. Penelitian di lapangan disusun dengan menggunakan rancangan acak kelompok dengan tiga ulangan dan menggunakan 15 tanaman sebagai unit sampel. Hasil penelitian menunjukkan bahwa pada fase pembibitan, pertumbuhan vegetatif tanaman yang berasal dari planlet lebih baik dibandingkan dengan tanaman yang berasal dari mata tunas pada umur 10-12 bulan. Pada fase di lapangan, pertumbuhan tanaman yang batang atasnya berasal dari planlet tetap lebih baik dan berbeda nyata sampai umur 6 bulan setelah tanam. Planlet yang berasal dari ES hasil perbanyakan* in vitro *dapat digunakan sebagai bahan batang atas alternatif yang baik untuk memproduksi tanaman jeruk sehat. Oleh karena itu, penelitian lebih lanjut diperlukan terutama mengenai varietas yang digunakan, pertumbuhan pada fase produktif, dan produksi planlet secara massal.*

[***Kata kunci*****:** *Citrus nobilis*, embrio somatik, planlet, batang atas, penyambungan]

INTRODUCTION

Most of the commercially healthy citrus orchards are produced from scions taken from protected budwood multiplication block (BMB), while BMB itself is developed from foundation block (FB). In Indonesia, production of commercially healthy citrus plants has been started since 1985 using the shoot tip grafting method. This method is primarily used to produce virus free plants that may serve as a mother plant (FB) through grafting a meristem tip of scion onto a rootstock *in vitro* (Supriyanto and Whittle 1991).

According to the data of Indonesian Citrus and Subtropical Fruit Research Institute (ICISFRI), both FB and BMB have been built in 19 and 25 provinces, respectively, in Indonesia to provide healthy scion sources for growers as well as farmers. Recently the demand for healthy budwoods of superior citrus cultivars including *Citrus nobilis* L. cv. Kintamani tends to increase significantly. In 2012 and 2013, distributed healthy budwoods of all cultivars from BMB were 7,917 and 8,555 budwoods, respectively to fulfill 13 provinces of citrus production centers (ICISFRI 2012, unpubl.), while the availability of healthy citrus budwoods produced from BMB is relatively limited. Therefore, an alternative method to produce healthy citrus plants is required to support BMB program.

The use of somatic embryogenesis (SE) for producing virus free citrus plant is very limited. This method is very useful to produce mass plants that are genetically similar with its parent or true to type and virus free as well. SE is the embryo formation derived from somatic organ non-zygotic cells. Moreover, the nucellus tissue is used as a callus source in mass citrus propagation *in vitro*. The cells grow by periclinal and anticlinal division both in the primary parietal layer and in the epidermis of developed seed. This tissue has somatic embryo characteristic, in which somatic cells develop into different plants through embryological stages (Tomaz *et al*. 2001; Han *et al*. 2002). Mendes-da-Gloria *et al*. (1999) and Ricci *et al*. (2002) reported that the capability of the explants to grow, multiply and develop depends on some factors, such as the culture media, varieties, species and conditions of callus used. That the final product (plantlets) that can be utilized optimally is highly depended on several stages, such as the rooting induction phase, hardening phase and acclimatization. However, it is finally determined by the percentage of plants that optimally grow and develop *ex vitro* (Kadlecek *et al*. 2001).

Nas and Read (2003) mentioned that the growth of plantlets derived from SE can be induced by grafting it onto a rootstock *ex vitro*. In addition, the embryos of Kinnow citrus can be grafted on 2-18-month old healthy seedling, whereas the maximum and minimum survivals of normal healthy embryos are 76% and 46%, respectively (Altaf and Iqbal 2003). According to Devy *et al*. (2011), both cotiledonary embryos and plantlets of Calamondin citrus derived from SE can be used as scions, and they grow satisfactorily when they are grafted onto Japanese Citroon (JC) rootstock *ex vitro*. Even after one year in the field, those plants still produce flowers and fruits normally (Devy *et al*. 2013).

The aim of this research was to evaluate the growth performance of citrus plants cv. Siam Kintamani that used both plantlets and scions as their stocks.

MATERIALS AND METHODS

This experiment was conducted at Indonesian Citrus and Subtropical Fruit Research Institute (ICISFRI) in June 2011 to December 2012. Siam Kintamani (*Citrus nobilis* L. cv. Kintamani) was used in this research because it is a of commercially superior cultivar, while rootstock used was JC because it is a highly adaptable and compatible rootstock commonly used in all Indonesian citrus production centers. The scions and plantlets of Siam Kintamani were used as stocks, and they were derived from BMB and SE *in vitro* propagation, respectively (Fig. 1).

BMB was built in an insect proof screen house (100 mess). The sizes of beds were 80-100 cm width; the

Fig. 1. Citrus scion from budwood multiplication block (a), plantlet from somatic embryogenesis propagation method (b), and eight-month old JC rootstock (c).

distances between beds were 50-60 cm; and the lengths were adjusted to the size of the screen house. The free-disease citrus mother plants (BMB citrus plants) were planted with plant spacing of 25-30 cm x 40-50 cm, and every bed contained two rows of plants.

Somatic Embryogenesis *In Vitro* Propagation

Nucellus tissue was used as explants in this method. It was excised from a young fruit seed and cultured *in vitro* on an initiation medium, i.e. a solid standard MS that consisted of macro- and micro-MS salt, 50 g l^{-1} sucrose, 0.2 mg l^{-1} thiamin-HCl, 1 mg l^{-1} pirydoksin-HCl, 1 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 4 mg l^{-1} glysin and 7 g l^{-1} bacto agar. The pH of the medium was adjusted to 5.7. The calli and embryos that grew from them were maintained on MS medium + 500 mg l^{-1} ME + 1.5 mg l^{-1} BA + 30 g l^{-1} sucrose + 7 g bacto agar. The plantlets (small plants) would grow from embryos after 8-10 months at culture (Fig. 2).

Budding the Scions and Grafting the Plantlets

The treatments were done at nursery house by budding a scion of Siam Kintamani on an eight-month-JC rootstock plant by using chip budding method. Budding was done at a 15-20 cm height of rootstock base (Fig. 3). For the grafting one, we used a plantlet derived from SE *in vitro* propagation.

Plantlets were acclimatized by placing the culture bottle at open space for three days prior to graft onto a rootstock. Before being grafted, a JC rootstock was

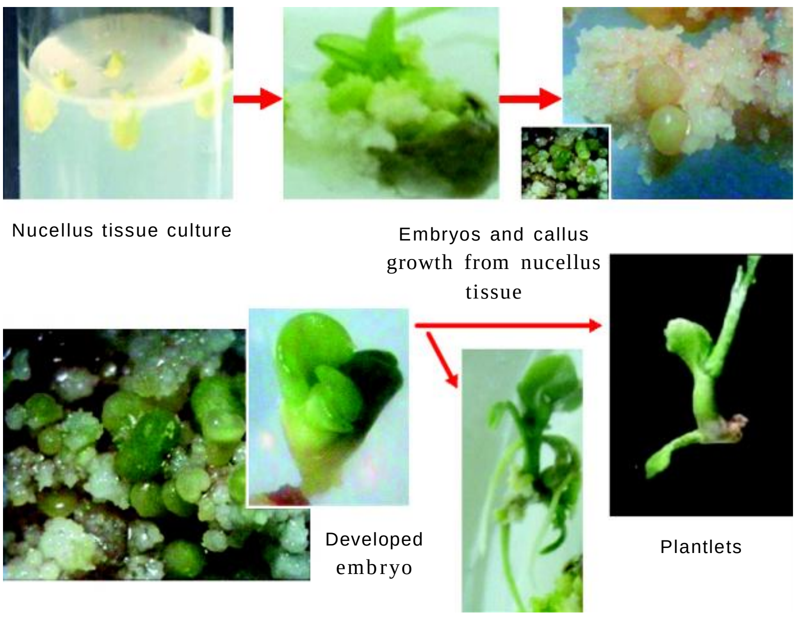

Fig. 2. Flow chart of plantlet production on citrus derived from somatic embryogenesis method.

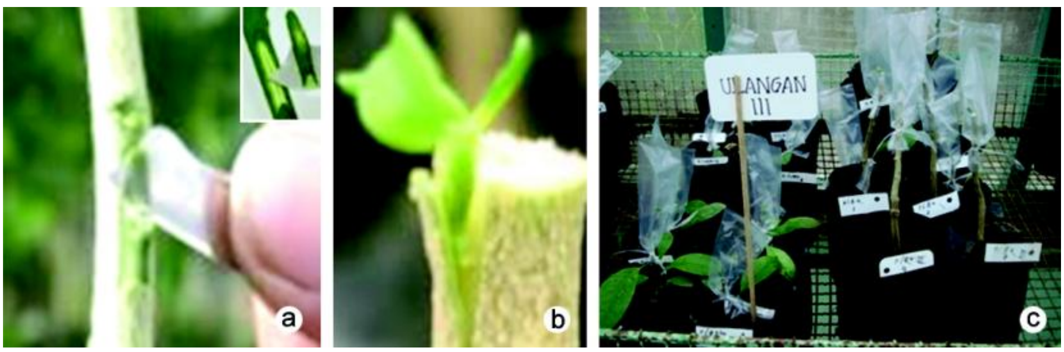

Fig. 3. The budding (a), grafting *ex vitro* (b), and covered grafted plant (c) of citrus cv Siam Kintamani.

cut at a height of ± 15 cm from the base of the stem, then the bark was peeled or cut with the size of 0.1-1.0 cm length and 0.1-0.3 cm width on the cut section. The next step, the sliced-plantlet was inserted into a rootstock and tied with plastic rope. After that, the grafted plant was covered by using a small plastic bag (Fig. 3).

The budded and grafted plants were maintained optimally at nursery house up to one-year old based on ICISFRI recommendation. After that, the plants were transplanted into the field at a plant spacing of 1 m x 1 m.

Histology Assessment of Graft Development

Two samples of seven-month old grafted plants were harvested to study their graft union anatomy. Histological observation was done at Botany Laboratory of Brawijaya University, Malang, East Java, using the standard method (Fig. 4).

Observation was done since plants were grafted or budded up to one year old at the field. The parameters measured were plant height and total leaves of nursery house-plants (1-12 months after grafting/ budding). In the field, plant parameters observed were morphology of one-year old plant, plant height, and rootstock and stock diameter (started from 4 up to12-month old, with one month interval). However, the total plant leaves could not be counted because the minor branches of plants had already been pruned. The plant height was measured to 5 cm above and below the graft union for stock and rootstock, respectively

A randomized block design was used in this research consisted of two treatments with three replications, and it used 15 trees as a unit sample. The comparison between means was carried out according to DMRT at $P < 0.05$. Histological observation was done to examine the level of connection between scion and rootstock parts of SE-grafted plant.

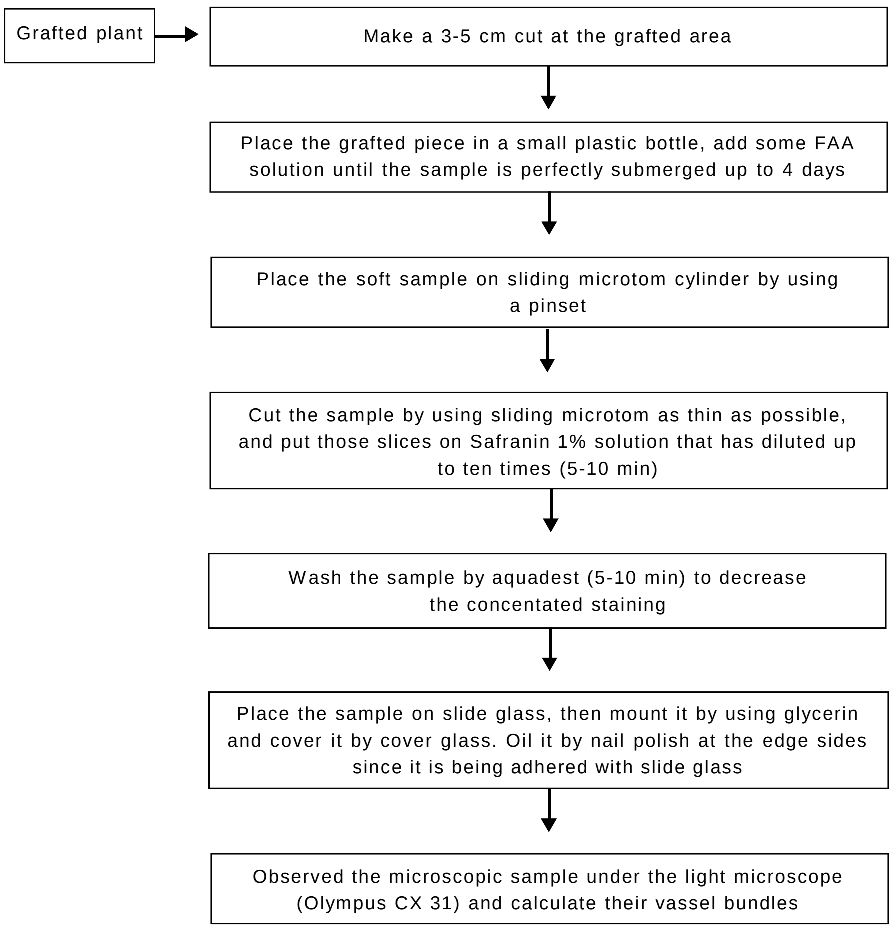

Fig. 4. Flow chart of histology assessment procedure on the of somatic embryogenesis-grafted plant.

RESULTS AND DISCUSSION

Growth Performance of Seedling in Nursery

One-year-old Siam Kintamani seedlings produced from planlets (SE) showed the normal growth, and it was significantly different to budded plants obtained from BMB especially for plant height at 10-12 months after grafting and budding in nursery. The height of SE grafted plant reached 54.3 cm, whereas budded plant was only 35.9 (Fig. 5). In terms of leaf number, it was not significantly different between treatments from 1 to 12 months after grafting or budding (Table1). The growth performance of citrus derived from planlets or budwood is apparently influenced by varieties used. Devy *et al.* (2011) reported that plant height of Calamondin (*Citrus mitis* Blanco) derived from planlets that was grafted on JC rootstock was significantly higher than that of Siam Kintamani at 10 months after grafting.

Plantlet is a small plant; it develops from embryo *in vitro*. Like other plants, during acclimatization or before it is grafted, this plant becomes anatomical, physiological and biochemical adapted. According to Ranasinghe *et al*. (2013), during acclimatization of *in vitro*-grown coconut plants, the plantlets exhibited the progress of improvement in stomatal regulation, photosynthetic capacity, and related anatomical, physiological and biochemical characters that are comparable to that of nursery-raised seedlings. It indicates that they can adapt well to the conditions of changeable environment during acclimatization, and the plants can be considered fully acclimatized at the time of field grafting.

In this research, the percentage of grafted and budded plants were about 90%. It was thought that plantlets were easier and faster to unite in graft phase because of their morphological and anatomical characters. Even though it was small (1-1.5 cm length and 0.2-0.4 mm stem diameter), plantlet had leaves, stem and root with its vascular bundle that was perfectly formed than scion. According to Aloni *et al*. (2010), grafted plants will grow well when their vascular tissues are connected perfectly. At the linkage process, vascular regeneration is a very complex process included the stages of differentiation

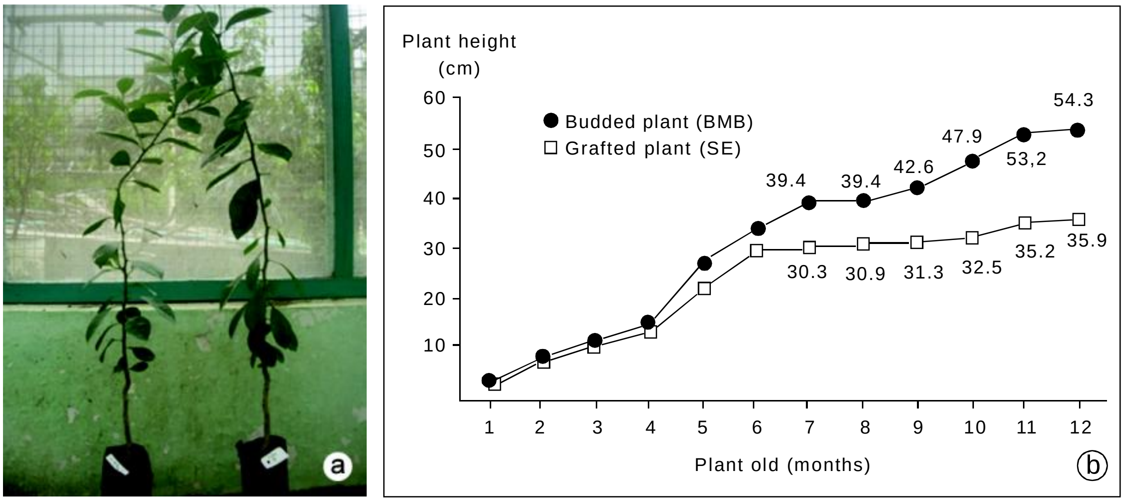

Fig. 5. The budded (left) and grafted (right) citrus plants (a), and the average of budded/grafted citrus plant height at nursery house (b).

Table 1. The average of leaf number of citrus cv Siam Kintamani at 1-12 months after budding/grafting.

Treatments[1)]	Leaf number											
	1	2	3	4	5	6	7	8	9	10	11	12
BMB	3.9 a[*)]	8.7 a	14.4 a	16.1 a	16.2 a	19.5 a	27.3 a	28.8 a	34.5 a	34.6 a	35.3 a	36.7 a
SE	6.0 a	9.9 a	14.4 a	17.4 a	17.6 a	20.7 a	27.5 a	31.8 a	34.3 a	34.6 a	35.7 a	37.3 a
CV (%)	25.7	16.5	16.8	11.2	10.5	9.4	11.6	10.9	3.9	9.4	8.5	7.2

[1)] BMB = budwood multiplication block, SE = somatic embryogenesis
Means in the same column followed by the same letter are not significantly different based on DMRT at 5% level.

of parenchymal tissue on the both sides and development to be the xylem and phloem; process begins with the growth of the calli (bridge calli) on both of the cross-sectional slices (Hartman and Kester 1983). This is consistent with the results of Kinnow citrus embryo grafted onto Rough Lemon rootstock (Altaf and Iqbal 2003). It shows that the physical characteristics of scion stem definitely influence graft union and survival; the healthy green round stem scions give the highest compatibility with their rootstock. Martinez-Ballesta *et al.* (2010) *in* Trinchera *et al.* (2013) also considered that the success of graft union occurs when both phloem and xylem connect across the graft interface via cytoplasmic cell connections.

On grape plant, Gokbayrak *et al.* (2007) stated that compatibility between the rootstock and stock is supposedly determined by total protein profiles and acid phosphatase (AcPH). Meanwhile, the growth of grafted plants is not optimal due to the parenchymal tissue that is not formed normally, and it causes impaired vessel formation in the graft area. Those spots contained in the xylem will hinder the development of vascular tissue, cause changes in the normal flow of endogenous hormones and lead to disruption of water and nutrient transport to the upper trunk.

Plant Performance in the Field

After one year in the field, transplanted plants were still in the vegetative phase, so that they had no flower yet. Nevertheless, seedling derived from SE-grafted plant produced thorny branches (Fig. 6).

Citrus plants derived from embryo *in vitro* had morphological character similar to seedling one. They tended to have thorny stem and upright branch than trees produced from scion budding. According to Frost (2011), the embryos on citrus plants could be resulted from sexual process or apomictic one of maternal nucellar tissues. The somatic embryos that grew in the nucellus tissue sac surrounded the developing zygotic embryos (Koltunow *et al.* 1996). Both of these embryos had the same character, i.e. the plants would have thorny stems and a long vegetative phase (12-15 years). It is known as a juvenile phase (Ligeng *et al.* 1995), and it is considered as a major obstacle in citrus propagation through SE technique (Ollitrault 1990). Furthermore, Pillitteri *et al.* (2004) stated that this vegetative phase is a quantitative character controlled by multigenes.

The stock and rootstock diameters of 4-12-month old plants derived from both treatments were not significantly different at all observations, even though the SE-derived plants had bigger size than another plants (Table 2). On the other hand, plant heights were only significantly different from 4 to 6 months after transplanting, after that there was no difference between them. However, the growth of SE-derived plant was still relatively better than that of budding treatment (Fig. 7).

The use of different scion sources in citrus propagation would affect the growth of plants. One-year old plants transplanted from nursery into field had the different performances between two treatments, especially on plant height. The citrus plants that used plantlets as their scions had significantly taller performance than another citrus plant. This favorable condition might lead to better growth than plants used BMB scion as their stocks.

Fig. 6. Performance of one-year-old grafted plant (a) and budded plant (b) of citrus cv. Siam Kintamani.

Table 2. The average of stock and rootstock diameter of citrus cv. Siam Kintamani at 4-12 month old.

Treatment[1)]	Plant diameter (cm)				
	4	6	8	10	12
	Stock				
SE	0.89a	0.91a	0.98a	1.02a	1.11a
BMB	0.56a	0.62a	0.68a	0.75a	0.86a
CV (%)	18.0	18.6	17.9	18.2	18.6
	Rootstock				
	4	6	8	10	12
SE	0.95a	1.00a	1.13a	1.22a	1.36a
BMB	0.79a	0.88a	0.91a	0.99a	1.12a
CV (%)	10.5	14.6	13.0	14.5	15.1

[1)]SE = somatic embryogenesis, BMB = budwood multiplication block

Means in the same column followed by the same letter are not significantly different based on DMRT at 5% level.

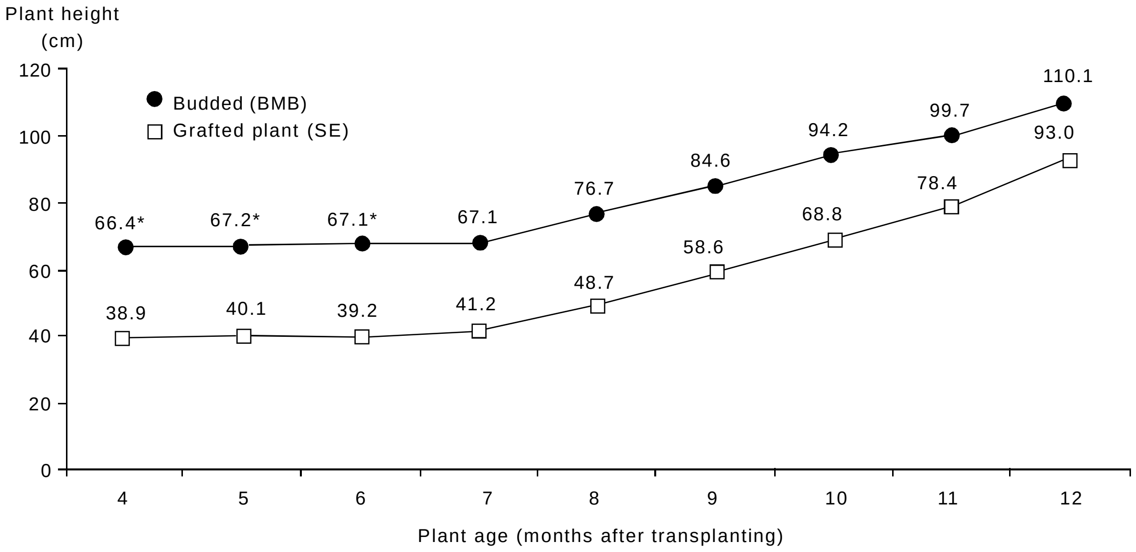

Fig. 7. The average plant height of Citrus cv. Siam Kintamani at 4-12 months after transplanting. *significantly different between SE grafted plant and budded plant based on DMRT at 5% level.

The embryonic grafting techniques need to be applied to shorten the juvenile period of the SE-derived plants. According to Ollitrault (1990), this technique is considered to be easier and faster than the budding one. On cocoa plant, application of this technique the plant cocoa to obtain the first flowers in one or two year earlier (Couturon 1982 *in* Ollitrault 1990). This technique has also been applied by Altaf and Iqbal (2003) by using the embryos of Kinnow mandarin as stock which is grafted onto 2-18-month old RL rootstock and produces individual plant that grows well. Moreover, Ligeng *et al.* (1995) successfully grafted one-year old nucellar seedling onto a mature plant that has been fruitful. This way, a long juvenile phase can be reduced to 2-4 years.

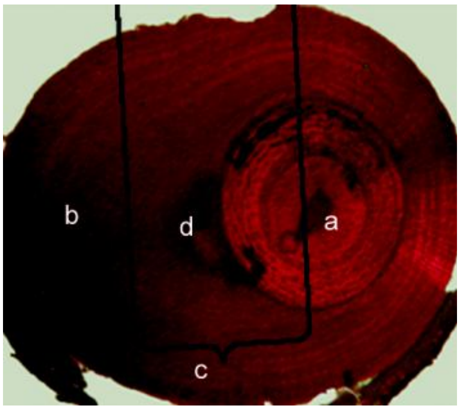

Fig. 8. Cross-section of successfully grafted JC + planlet citrus; a = perfect round stem, b = unperfect round stem, c = among margin stem, d = small xylem.

Histology of Graft Development

Based on histology analysis of SE-grafted plants, the graft union of epidermis, phloem and xylem of vascular plants belonging to both sides was done perfectly. It was indicated by the merger of the skin and cambial continuity observed between the stock and the scion cambium. The similar result was occurred on budded Soe Mandarin citrus onto a JC rootstock (Budiyati *et al.* 2012) (Fig. 8).

Hystology observation on the SE-grafted sections showed that the epidermis, phloem and xylem have perfectly united (Fig. 8). This means the grafting is successful and the grafted plant would normally develop. This condition would support normal growth in those plants.

CONCLUSION

Plantlets of citrus cv. Siam Kintamani as the product of SE *in vitro* propagation can be used as an alternative virus free stock material to support the citrus BMB program in Indonesia. A further research is required especially on varieties used, reproductive growth and massive produced plantlets.

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