EVALUATION AND CHALLANGES IN IMPLEMENTATION OF CITRUS SHOOT TIP GRAFTING (STG) TECHNOLOGY IN INDONESIA

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ABSTRACT

For the past three decades, the main problem of citrus development in Indonesia has been the Huanglongbing (HLB), Citrus Tristeza Virus (CTV) andand other virus diseases. Due to the severity, the government since 1987 has started to produce virus-free mother plants through Shoot Tip Grafting (STG) followed by indexing. This present research aims to evaluate the implementation of STG technology in Indonesia from 2008 to 2018. The activity was carried out in the tissue culture laboratory of ICSFRI by grafting 0.14-0.18 mm shoot tips as scions onto a 2-week-old JC rootstocks *in vitro*. To accelerate the growth, a one-month old micro grafted plant was re-grafted onto an 6-month old rootstock at the nursery house. Four to six months later, they were indexed using indirect ELISA and RT-PCR. The total number of micro grafting activities was 10,882, consisted of 9 citrus species (71 cultivars) with 9.8% success rate. The highest was on *C. microcarpa* (15%), whereas the lowest was on *C. grandis* Osbeck (3.4%) and *C. sinensis* (5.7%). Among 391 indexed plants, 75.2% of them were virus-free; 21.7% and 4.1% were still infected by CTV and HLB respectively, and 1% infected by both of the diseases. Although the success rate achieved is low, this technology is still an effective way to produce virus-free mother plants. From its propagation, in the period 2009-2014 at least more than 5 million virus-free plants have been produced by citrus farmers.

Keywords: Citrus, Huanglongbing, CTV, STG, Indexing

1. INTRODUCTION

In Indonesia, citrus is one of the horticulture commodities that has high economic value. Although it is not a native tropical plant, it has a wide genetic diversity and is grown in many developing areas (Martasari *et al.*, 2012; Devy & Hardiyanto, 2017; Yulianti *et al.*, 2016). According to the commercial harvested area in the year of 2018, from about of 46,922 ha, 91.9% of them were mandarin and tangerine types areas with an average production of 55.86 tons/ha (Ministry of Agriculture, 2019). Almost all of them are grown exclusively on Japanese Citroen (JC) rootstock.

In the 1980s, Huanglongbing (HLB), Citrus Tristeza Virus (CTV) and others diseases were common and this had an impact through severe crop damage that caused loss. To restore them, the Indonesia government launched the Citrus Rehabilitation Program in 1987, with the aim to produce disease-free plants, especially from HLB and CTV using the STG method (Supriyanto *et al.*, 2017). This technique is also widely adopted in the world's citrus producing countries, including China, Pakistan, Italy, Cyprus, and India (Ruilin *et al.*, 1996; Naz *et al.*, 2007; Continella *et al.*, 1997; Kapari-Isaia *et al.*, 2002; Chand *et al.*, 2013).

To date, *in vitro* shoot tip grafting has been cited as the most reliable method to obtain virus free citrus mother trees from infected parental source (Fifaei *et al*., 2007; Sharma *et al*., 2007; Meziane *et al*., 2009). According to Carimi *et al*. (2001), it can effectively eliminate all citrus diseases originating from pathogens carried during the grafting process, although the success rate varies between 60% (Tatterleaf, Psorosis) to 100% (Citrus viroid).

The implementation of this technique in Indonesia is carried out by the Indonesian Agency for Agriculture Research and Development (IAARD) and assigned to the Indonesian Citrus and Subtropical Fruit Research Institute (ICSFRI). The plant materials are cleaned from viruses of HLB and CTV based on protocols described by past researches (Navarro *et al*., 1974) with little modification (Devy, 2014; Supriyanto & Whittle, 1991; Devy *et al*., 2014). The virus free STG plant acts as a nucleus plant and becomes the source materials for foundation seeds at Foundation Blocks (FB). These are then propagated to be Budwood Multiplication Block (BMB) plants. BMB itself is a source of scions for certified commercial citrus seeds. To prevent re-infection in the field, the FB and BMB parent plants are managed in an insect proof screen house. The purpose of this study is to evaluate the STG activities that have been carried out from 2008 to 2018.

2. MATERIALS AND METHODS

Evaluation was performed on activities carried out from January 2008 to December 2018 at the ICSFRI Tissue Culture Laboratory. The number of cultivars and total of in vitro grafted plants via the STG method are listed in Table 1.

Table 1. Total cultivars and plants derived from STG year 2008-2018

No	Year	Total Cultivars	Total of in vitro grafted plants
1	2008	18	1372
2	2009	10	809
3	2010	7	343
4	2011	13	1281
5	2012	12	810
6	2013	14	1265
7	2014	19	1223
8	2015	15	1046
9	2016	6	849
10	2017	16	1043
11	2018	13	841
Total			10,882

Principally, the STG method done was based on the technique adopted by the researchers as in (Navarro *et al*., 1974). It consisted of grafting in vitro 0.1 - 0.2 mm long shoot tips composed of the apical meristem plus three leaf primordia on to a 2-week-old seedling rootstocks. The Japanese Citroen (JC) was used as rootstocks to graft all scion shoot tips. The cultivars were generally identified as a potential citrus varieties originating from various regions in Indonesia.

2.1. Preparation of *in vitro* rootstocks

Seeds of JC were extracted from fresh or stored fruit. Before planting, the outer shell of the seed was peeled and sterilized with a 1% fungicide. Inside the Laminar Air Flow Cabinet (LAFC), the seeds were peeled and re-sterilized, and planted in vitro on solid MS media. The test tube containing seed culture was incubated in a dark cupboard at 27 °C for 2-3 weeks until seedlings grew to 5-7 cm in height, with an ideal diameter of 1-1.5 mm.

2.2. *In vitro* grafting

Grafting was done on 2-3 week-old seedlings of JC. Shoot tips were obtained directly from the field-grown trees or from potted plants established in the glasshouse. The shoots were sterilized. Under a stereomicroscope, lateral leaves were removed from the shoot, and the meristem tip with two primordia leaves (about 0.2 mm in size) was excised. The shoot tip was inserted into an inverted-T incision made on an in vitro JC seedling. Grafted plants were placed into a test tube containing a MS liquid medium and incubated at 27 °C under 16- hour light exposure.

2.3. Acclimatization and re-grafting

About one month after grafting, plants were grown in greenhouse conditions for acclimatization, after which were re-grafted in vivo onto six-month-old JC seedlings. Four to five months after re-grafting, the leaves of re-grafted plants were ready to be used as indexing material, which is a process to ensure that the result of STG is free from CTV and HLB by using ELISA and PCR methods. Observations were made on the number of in vitro grafted plants, survival of grafted plants, duration of plants to grow, and number of re-grafted plants that were free from virus.

3. RESULTS

3.1. Citrus species, cultivars, and number of STG plants

To determine the graft union in the STG method, the number of grafted and surviving plants from year 2008 - 2018 was recorded. There were 10,882 grafted plants derived from 71 cultivars belonging to 9 species of citrus with an average 9.8% of survival rate (Table 2).

Table 2. Species, cultivars, and survival rate of STG

Species	Cultivar	Σ STG plants	Survival rate (%)
I. Mandarins (*C. reticulata* Blanco)	22	4,272	10.0
II. Lemon (*C. limon* L.)	12	1,761	7.2
III. Sweet Oranges (*C. sinensis* L. Osbeck)	4	474	5.7
IV. Calamondin (*C. microcarpa*)	1	419	15.0
V. Pummelo (*C. grandis* Osbeck)	21	2,592	3.4
VI. Citron (*C. medica*)	2	188	14.4
VII. Tangerine (*C. nobilis* Lour)	4	275	11.3
VIII. Kaffir Lime (*C. hystrix*)	2	238	10.1
IX. Key Lime (*C. aurantifolia*)	3	663	10.9
Total	71	10,882	9.8

Among all species, the STG method was done mostly on mandarin (*Citrus reticulata* Blanco), followed by pummelo (*C. grandis* Osbeck), and lemon (*C. limon* (L.) Burm. f.). This corresponded with the distribution of local varieties of citrus in Indonesia in which the major species are mandarin and pummelo (Martasari *et al*., 2012; Devy & Hardiyanto, 2017; Yulianti *et al*., 2016).

In general, pummelo is relatively difficult to graft in vitro compared to others. For this species, there were 143 grafted plants derived from four cultivars that failed to grow. This is consistent with the results of the 1997-2007 activities (Devy *et al.*, 2015). During that period, the percentage of survival rate was 7.82%. The low percentage is thought to be caused by the relatively large size of the meristem tip compared to the others, so the joining process between meristem and the rootstock is relatively more difficult. In addition, it is suspected that their compatibility is low with the JC rootstock, as also stated in the research of (He *et al.*, 2018).

The ability of survival rate between cultivars among a species is also not significantly different ($p<0.05$) (Table 3). This illustrates that the low percentage of survival is due to conditions that are not favourable for the graft union process using very small material.

Table 3. Percentage survival rate of STG on four main citrus species

Sp. Mandarin		Sp. Lemon		Sp. Pummelo		Sp. Sweet Orange	
Local name	**%**	**Local name**	**%**	**Local name**	**%**	**Local name**	**%**
'Brasil'	1.8^{ns}	'T.Varigata'	17.2^{ns}	'Jeruk Besar'	2.5^{ns}	'Kisar'	7.2^{ns}
'Kisar'	16.5	'Swanggi'	4.6	'Jeruk Kelapa'	5.1	'M. Variegata'	2.7
'Madu'	16.0	'Bali'	4.2	'Nambangan'	0.6	'S O. Local'	5.8
'Santang'	12.0	'Jumbo'	18.0	'Baco'	2.8		
'Soe'	4.1	'Nipis Kecil'	6.4	'Bona'	3.6		
'Terigas'	6.4	'UB'	5.2	'India'	2.8		
'Topo Hitam'	7.1			'Pekalongan'	1.9		
'Topo Putih'	8.2			'Pamindo'	2.3		
p	**0.05**		**0.34**		**0.95**		**0.45**
R2	**53.2**		**34.3**		**11.0**		**23.6**

3.2. Acclimatization and regrafting

The duration required for STG plants to reach the ideal size and to be ready for acclimatization and regrafting is highly varied. After in vitro micro grafting, almost all plants (83.8%) required between 2-8 weeks to grow, and the rest do more than 9 weeks (Figure 1).

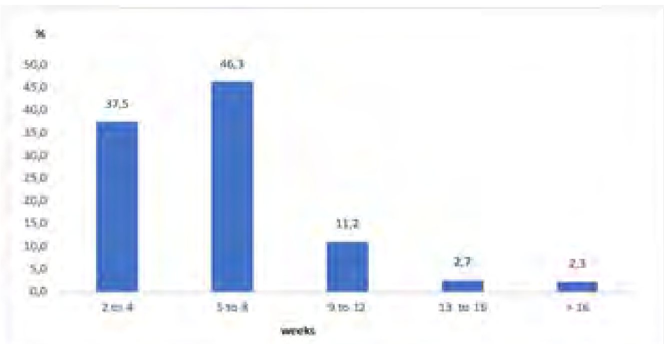

Figure 1. Percentage of in vitro grafted plants acclimated at 2-16 weeks

The differences in cultivars also affect their growth abilities. Among 37 cultivar samples, pummelo cv. 'Pekalongan' is the slowest. It took about 10.4 weeks to acclimatize, followed by lime cv. 'Kutai', lemon cv. 'UB', and 'Jari Budha'. They are significantly different from Lemon cv. 'Swanggi' and 'Manis', and lime cv. 'N. Red Center' which only took between 3.7-4 weeks ($p < 0.05$; $R2 = 81.3\%$) (Table 3).

Table 3. Time needed of *in vitro* grafted plant to be acclimated

Species	Cultivars	Duration (weeks)	Species	Cultivars	Duration (weeks)
C. reticulata	'K. Madu'	8.4 abcd*)	*C. grandis*	'P. Pekalongan'	10.4 a
	'K. Kacang'	6.8 abcde		'P. Nambangan'	6.8 abcde
	'K. Chokun'	6.6 abcde		'P. Baco'	5.6 bcde
	'K. SoE'	6.5 abcde		'P. Kelapa'	5.6 bcde
	'K. Thay Ayam'	6.5 abcde		'PA Chuan'	5.1 bcde
	'K. Dekopon'	6.4 abcde	*C. aurantifolia*	'Nipis Kutai'	9.1 ab
	'K. Kisar'	6.3 abcde		'Jr Nipis Kecil'	6.2 abcde
	'K. Komun'	7.3 abcde		'N. Red Center'	3.7 e
	'K. Terigas Bsr'	5.8 bcde		'Lemon UB'	8.7 abc
	'K. Selwasa'	5.7 bcde	*C. limon*	'L. Tea Varigata'	7.4 abcde
	'K. Santang'	5.6 bcde		'Lemon Kuit'	6.9 abcde
	'K. Emperor'	5.4 bcde		'Lemon Jumbo'	5.6 bcde
	'K. Topo Putih'	5.1 bcde		'L. Swanggi'	4.0 e
	'K. G. Lebong'	5.0 bcde		'Lemon Manis'	4.0 e
	'K. Kendari'	4.9 bcde		'L. Cina Lamo'	5.3 bcde
	'K. Topo Hitam'	4.2 bcde	*C. microcarpa*	'Calamansi'	4.9 bcde
C. medica	'Jari Buda'	8.5 abcd	*C. histryx*	'Monte Hondu'	4.1 de
C. sinensis	'M. Varigata'	5.1 bcde			
C. nobilis Lour	'S. Mahang'	5.1 bcde			
	'S. Gn Omeh'	4.8 bcde			

***) Means that do not share a letter are significantly different (p<0.05)**

3.3. Indexing

The time interval required by in vitro grafted plants to be indexed varies greatly between cultivars. All the stages of that process needs about 20-89 weeks or 5-22 months. Cultivars of 'S. Mahang', 'K. Kendari', 'L. Jumbo', and 'K. Dekopon' were faster than others, they needed only 20-23 weeks. While 'K. Brasil', 'M. Variegata', 'L. Tea Variegata', and 'N. Red Center Lime' needed more than 1.5 years (Table 4).

Table 4. Duration up to indexing phase of *in vitro* grafted plant

Species	Cultivar	Duration (weeks)	Species	Cultivar	Duration (weeks)
C. reticulata	'K. Brasil'	73.7 ab *)	*C. grandis*	'P. Pamindo'	27.5 cde
	'K. SoE'	46.3 abcde		'P. Nambangan'	34.8 bcde
	'K. Santang'	31.5 bcde	*C. limon*	'L. Bali'	28.5 cde
	'K. Dekopon'	22.5. cde		'L.T.Varigata'	85,5 a
	'K. Terigas Bsr'	34.3 bcde		'L. Jumbo'	22.3 cde
	'K. Santang'	88.4 abcde	*C. nobilis*	'S. Mahang'	20.5 e
	'K. Kendari'	21.6 de	*C. sinensis*	'M. Varigata'	83.3 a
	'K. Topo Hitam'	61.2 abcde		'SO. Local'	64.8 abc
C. aurantiifolia	'Jr Nipis Kecil'	53.5 abcde		'M. Kisar'	51.8 abcde
	'N. Red Center'	88.4 a			

***) Means that do not share a letter are significantly different (p<0.05)**

Among 391 re-grafted plants that were indexed during2008-2018, 75.2% of them were virus free; 21.7% and 4.1% were still infected by CTV and HLB respectively, and 1% were infected by both of diseases (Table 5). A relatively similar result was obtained by (Muharam & Whittle, 1992), 80% and 60% of the STG plants indexed were CTV-free for mandarin and sweet oranges, respectively. The STG plants without preliminary heat treatment consisted negative CTV of mandarins and sweet oranges were 80% and 60% respectively. The percentage of virus free plants seemed to increase if an addition of thermotherapy treatment was performed before micrografting (Tianmiao, 1996).

Table 5. Total re-grafted plants infected by CTV, HLB, and both of CTV & HLB

Species	Σ Cultivar	Σ re-grafted plants	Σ CTV		Σ HLB		Σ CTV & HLB	Σ Infected	Σ Virus-free
			-	+	-	+	+		
C. medica	1	5	5	0	5	0	0	0	5
C. reticulata	22	156	109	47	150	4	0	49	105
C. limon	6	35	25	10	30	5	2	13	22
C. sinensis	4	14	8	6	12	2	2	6	8
C. histryx	2	12	12	0	12	0	0	0	12
C. aurantifolia	3	15	15	0	14	1	0	1	14
C. grandis	13	48	43	5	43	4	0	9	39
C. nobilis	4	14	11	3	11	0	0	3	11
Others	23	92	78	14	78	0	0	14	78
total	78	391	306	85	355	16	4	95	294
%				21.7		4.1	1.0		75.2

4. DISCUSSION

The challenges of implementing STG in Indonesia are various. The skills of the technicians are the most important. During the decade of 1997-2007, the total grafted plants in vitro was 2,616 with an average survival rate of 21.5% (Devy *et al.*, 2010). In the following decade, the decline of success was due to limited skilled technicians. The relatively low result also occurred in Cyprus and others (Kapari-Isaia *et al.*, 2002); the average success was 17% and 2.5 - 50 % respectively, depending on the cultivars of scion and rootstocks and the size of the shoot tip (Chand *et al.*, 2013).

Low rates of success can also be due to the very minute size of the grafted meristem. A rough sliced surface will cause problems in that the two sections cannot fuse properly (Hussain *et al.*, 2014). Besides this, the slice made on the seedling usually stimulates rapid callus growth, thus inhibiting the growth of the meristem in it (Navarro *et al.*, 1974).

The high percentage of dead grafted plants could be due to low incompatibility between the two parts; as reflected by their vascular systems that affects the flow of the growth hormones and others. This disrupts cellular growth and development (Wang *et al.*, 2016; Thomas & Frank, 2019).

Another problem is the duration needed for grafted plants to be re-grafted and indexed. Almost 84% of grafted plants can be acclimated at 2-8 weeks and require approximately 20-89 weeks to be indexed after STG process. Various ways have been done to induce the growth, including by using some tested rootstocks (Navarro *et al.*, 1974) and increasing the ambient temperature on the re-grafted plants (Hussain *et al.*, 2014).

However, during the years 2008 to 2018, a total of 78 virus-free citrus cultivars were produced. Meristematic tissue from the apical shoots can be free of pathogens because the vascular system of the stem is not yet interconnected. One virus-free mother plant is very valuable for the continuity of the citrus development program in Indonesia. During the period of 2009-2014, ICSFRI produced and distributed 4,437 and 27,647 plants of FB and BMB respectively derived from 31 virus-free cultivars to most Indonesian provinces. There were at least 5,529,400 virus-free citrus plants produced by farmers with a planted area of 11,058 hectares.

5. CONCLUSION

The success rate of STG method implemented in Indonesia is relatively low (9.8%). However, until now this method is still used because it is considered feasible and effective to produce disease-free mother plants. By using this, at least 78 cultivars have been produced as virus-free mother citrus plants during the last decade.

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