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IN VITRO PROPAGATION OF THREATENED BRASTAGI CITRUS VARIETY BRASTEPU (*CITRUS NOBILIS* BRASTEPU) CVPD FREE THROUGHSHOOT TIPS SUBCULTURE

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Abstract

In vitro propagation of threatened Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu) through shoot tip subculture was carried out to obtain good quality citrus seedling free from CVPD. Propagation of citrus plants via cutting bud technique was firstly conducted to rescue rare plant as well as to provide adequate shoot tip explants for *In vitro* propagation. Shoot tip explants of *Citrus nobilis* Brastepu were cultured in MS basal media supplemented with combinations concentrations (0-2 mg/L) of 2,4 dichlorophenoxyacetic acid (2,4-D) and (0-3 mg/L) of benzyl-amino purine (BAP) followed by subculture techniques. The results showed that subculture frequency and combination variations of growth stimulator become key role to success of citrus growth development. The best culture was obtained in D1B2 treatment of the second subculture, that was with supplementation of 0.5 mg/L 2,4-D and 1.0 mg/L BAP, resulted in the average weight of callus (2.30 g), number of embryosomatic (22.30), and the number of shoot (8.60) respectively. Subculture technique was chosen to provide nutrients for long period of incubation time. Subculture technique has improved the quality of citrus culture and be able to produced good quality citrus plantlets. Citrus culture developed normal plant in which the stem, leaves, and roots were generated. The plantlets of *Citrus nobilis* Brastepu were all free from CVPD. The method was efficient for mass production of *Citrus nobilis* Brastepu seedling to conserve threatened local Brastagi citrus.

Key words: *In vitro*, Brastagi citrus, *Citrus nobilis* Brastepu, Threatened citrus, CVPD, Subculture, Shoot tips.

Introduction

The production of citrus seedling by *In vitro* propagation is a good alternative, particularly for plant under threat of extinction such as Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu). The technique is believed to be the fast strategy for mass production to conserve the citrus from extinction. *Citrus nobilis* Brastepu has high genetic potential having large size, sweet taste, and the feel contains bioactive that can be used in traditional medicine made Brastepu citrus popular many years ago. Brastagi citrus become one of well known comodity from Brastagi North Sumatra (Nurwahyuni, 2016). However, mass destruction by Citrus Vein Phloem Degeneration (CVPD) forced the *Citrus nobilis* Brastepu becomes wild plant. Brastepu citrus nowdays has not been planted properly, and has been replaced with imported citrus. The eruption of Mount Sinabung at Kabupaten Karo as impact area in 2013-2017 made the existence of local citrus be destroyed faster. Therefore, *Citrus nobilis* Brastepu has to be conserved by providing citrus seedling to protect the citrus diminishing from the ecosystem.

In vitro propagation is a powerful tool for mass production of high quality seedling of valuable plants (Ahmad *et al.,* 2011; Abbassi *et al.,* 2010). Propagation of citrus by *In vitro* technique has successfully applied for various type of citrus (Maggon, 1995; Duran-Vila *et al.,* 1989), such as Rough lemon, Cleopatra mandarin Pectinifera and Troyer citrange (Sharma *et al.,* 2009), Pokan mandarin (Zeng *et al.,* 2009; Dutt *et al.,* 2010), pummelo (Paudyal & Haq, 2000), Ginger Lime (Yaacob *et al.,* 2014), and Brastagi citrus (Nurwahyuni & Sinaga, 2014). *In vitro* propagation of various types of citrus species by using different explants such as as stem, leaf, nodal, root segments, epicotil segment, ovaries from flower buds, nucellar tissues and cotyledons from seeds, and juice vesicles have been described to produce citrus seedlings (Duran-Vila *et al.,* 1989; Raman *et al.,* 1992; Usman *et al.,* 2005; Ali & Mirza, 2006; Khan *et al.,* 2009; Zeng *et al.,* 2009; Singh & Rajam, 2010; Pérez-Tornero *et al.,* 2010; Savita *et al.,* 2012; Yaacob *et al.,* 2014; Savita *et al.,* 2015). Shoot tip explant was very effective for callus induction of citrus rootstocks (Sharma *et al.,* 2009; Wang *et al.,* 2002) and in the propagation of complete plants (Paudyal & Haq, 2000). Micropropagation of nodal explants from mature trees of varius type of citrus have also been studied (Tallón *et al.,* 2012; Savita *et al.,* 2012). *In vitro* propagation techique to eliminate Huanglongbing disease (HLB) from infected plants for several citrus species has been carried out (Ding *et al.,* 2008).

In vitro growth of higher plants are commonly conducted for long period of incubation time and needed sufficient nutrient in culture medium to support cell proliferation (Hussain *et al.,* 2013; Nurwahyuni, 2015). Subculture technique is a good strategy to extend success of *In vitro* propagation to overcome the limitation of nutrients in medium culture (Tsay 1998; Yang *et al.,* 2012; Khan *et al.,* 2014). Several studies on *In vitro* propagation with subculture technique have been reported (Cheruvathur & Thomas, 2014; Leva *et al.,* 2013; Noodezh *et al.,* 2012; Chauhan & Kothari, 2004; Cao & Earle, 2003, Khan *et al.,* 2016). The method has successfully applied on improving multiplication of the plant to produce normal plants. Successive subculture of explant materials for a long period of time could lead to increase the number of plantlets (Quiala *et al.,* 2014).

Production of Brastagi citrus seedling generally takes place through seed, budding, cutting, or layering (Nurwahyuni, 2015). Attempts to preserve Brastagi citrus have been conducted by cutting bud propagation (Nurwahyuni *et al.*, 2012). Citrus seedlings produced through cutting bud techniques are free from CVPD, and are the source of explants for *In vitro* propagation of *Citrus nobilis* Brastepu (Nurwahyuni & Sinaga, 2014). Cutting bud propagation has been successfully combined with *In vitro* propagation to obtain good quality seedling (Nurwahyuni & Sinaga, 2014; Nurwahyuni 2013). The study has intensively been carried out to propagate Brastepu citrus through subculture to produce good quality citrus seedling free from CVPD infection (Nurwahyuni & Sinaga, 2016; Nurwahyuni, 2016). The present study was aimed to propagate Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu) through shoot tips subculture to obtain good quality citrus seedling free from CVPD.

Materials and Method

Research procedures are consisted of preparation of plant materials, culture medium, sterilization of explants, *In vitro* propagation with subculture, and the analysis of CVPD in the citrus culture as illustrated in Fig. 1. Short descriptions of the procedures are as follows:

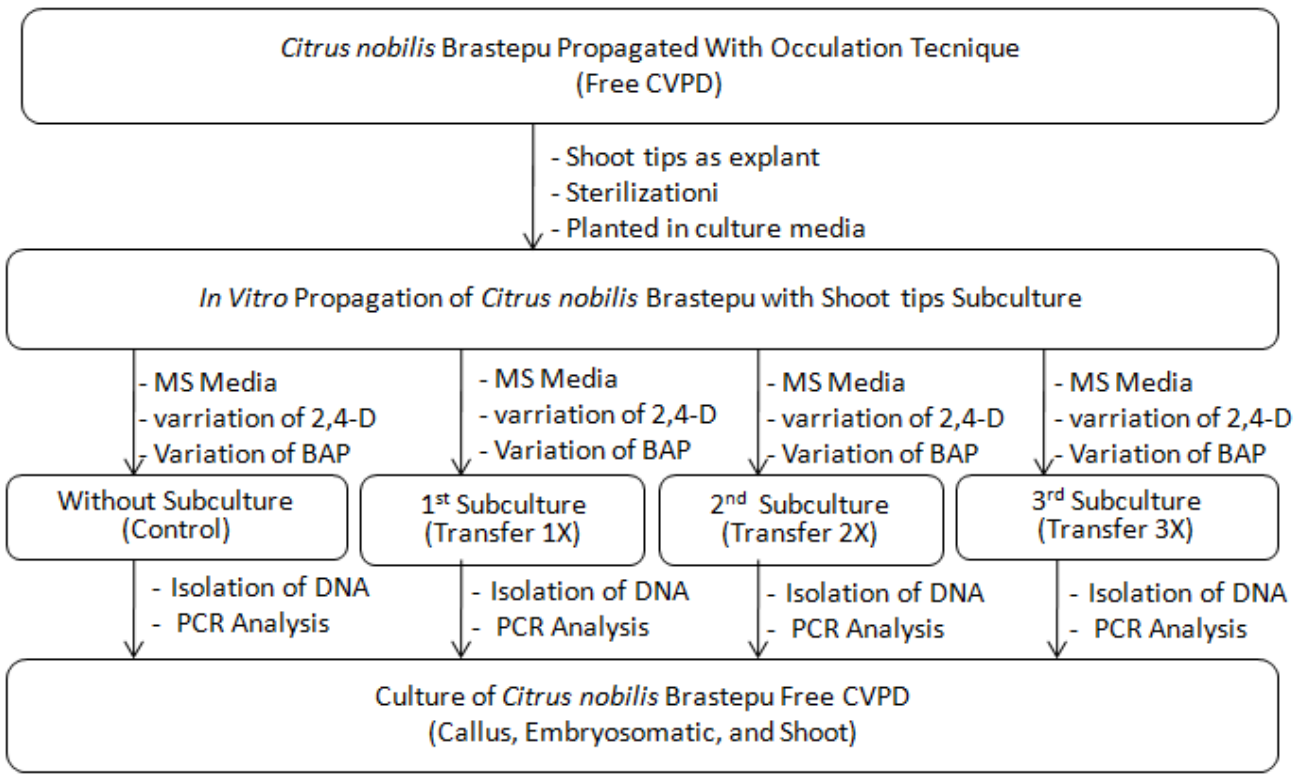

Fig. 1. Procedure for *In vitro* propagation of Brastagi citrus variety Brastepu (*Citrus nobilis* Brastepu) free from CVPD through shoot tips subculture.

Preparation of plant material: Mother plant of *Citrus nobilis* Brastepu was selected from survived old citrus and used as a source of scion for cutting bud propagation to rescue threatened Brastepu citrus (Nurwahyuni & Sinaga, 2014). The preparation of plant material was carried out following the cutting bud technique explained previously (Nurwahyuni *et al.*, 2012). The steps to prepare adequate citrus explants for *In vitro* propagation was illustrated in Fig. 2. A survive healthy mature tree of Brastepu citrus, aged abot 30 years, grown in Desa Bukit Brastagi Kabupaten Karo, North Numatera Indonesia was chosen (Fig. 2a). The citrus was productive with high quality fruits (Fig. 2b). The citrus tree has been tested and confirmed free from CVPD (Nurwahyuni *et al.*, 2015). The branch of Brastepu citrus was taken and the scion in the bud was cut. It was then stick on to the bark stem of sour citrus root stock, followed by incubation to securely join the scion onto the rootstock for two weeks. The upper stem of sour citrus were then cut and allowed the sprout of the citrus to grow on sour citrus root stock (Fig. 2c). The plants were then developed into good quality citrus seedling (Fig. 2d) to provide sufficient shoot tips explants for *In vitro* propagation. This study has demonstrated that cutting bud propagation was the fastest and simple techniques to produce healthy citrus seedlings (Nurwahyuni & Sinaga, 2014). The plants provide good quality shoot tips with minimum contamination explants for *In vitro* propagation (Nurwahyuni, 2016).

Preparation of medium culture: Basal medium used for shoot tips culture was Murashige & Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with various combination concentrations of 2,4 dichlorophenoxyacetic acid (2,4-D) and of Benzyl-amino purin (BAP). The medium was adjusted to pH 5.8-6.0, and it was then sterilized at 121ºC, 15 lb for 20

minutes. Earlier experiments with different 2,4-D and BAP have demonstrated the most beneficial effects on the rate of multiplication for *Citrus nobilis* Brastepu (Nurwahyuni & Sinaga, 2014). Thus, a two complete factorial experiment with (0-2 mg/L) 2,4-D and (0-3 mg/L) BAP was conducted to study their effects on *In vitro* propagation through subculture. Variation composition combinations of 2,4-D and BAP are presented in Table 1 with ten replicates.

Explant preparation: Shoots tips explants were prepared from the tips of Brastepu citrus plants using standard procedures described in the references (Nurwahyuni & Sinaga, 2016). The procedure on the preparation of explants for *In vitro* propagation is shown in Fig. 3. Healthy citrus plants were used as explants sources (Fig. 3a). The shoots were taken from good quality citrus branches (Fig. 3b), and were then cut (0.5 - 1.0 cm long), washed carefully with water and detergent, immersed for 5 min in 70% (v/v) ethanol, then for 20 min in a 20% (v/v) solution of sodium hypoclorate (0.8% w/v NaClO), followed by rinsing three times with sterile water. After sterilized, the shoots tip explant was then cut to provide wound site (Fig. 3c) followed by inoculating in culture medium (Murashige & Skoog, 1962) supplemented with 2,4-D and BAP (Fig. 3d). The cultures were then incubated at a temperature of 25±2 °C under 16-h daily illumination with UV light.

Result and Discussion

***In vitro* propagation of citrus nobilis brastepu without subculture:** *In vitro* propagation of *Citrus nobilis* Brastepu were carried out by using explants from healthy young shoots prepared as explained above. The experiments were carried out in series, starting from culture initiation without subculture (control). It was reported that cytokinin and auxin were the two most important plant growth regulators to promote growth development of Citrus species (Kotsias & Roussos, 2001; Carimi & De Pasquale, 2003; Tallón *et al.,* 2012). With this study, the combination of 2,4-D and BAP were chosen followed the success in previous study (Nurwahyuni, 2016). The growth of citrus cultures in MS medium was observed. Shoot tips culture on media MS medium enriched with 2,4-D and BAP showed low mortality. The explants 95% were survived beyond one month initial culture. The development of the culture to become plantlets is shown in Fig. 4, and the results for weight of callus, the number of embryosomatics, and number of shoots are presented in Table 2. Induction of callus occurred at the wound site of the explant was cut. It was observed that white callus was produced and covered the explants surfaces after one month incubation (Fig. 4a). Somatic embryo and shoots of *Citrus nobilis* Brastepu were developed after two months. The morphology of 2-month-old explants was observed as shown in Fig. 4b. The quality of calli resulted from the variation in concentration combination of 2,4-D and BAP. Most of calli were white, and some were yellowish and greenish color with big, rough texture and nodular formation. Citrus plant was grown well, where the stem, leaves, and roots have developed and became citrus plantlets (Fig. 4c-d).

It has been observed that variation in the combination concentration of 2,4-D and BAP in medium culture influenced the culture development. The number of calli, embriosomatics, and shoots per-explants increased when 2,4-D was included in the medium. The highest callus was obtained in D1B1 with combination of 0.5 mg/L 2,4-D and 0.5 mg/L BAP, where 1.83 g callus was produced. This experimental condition yieded greenish callus formation and rough texture. Interaction combination of 2,4-D and BAP have significant effects on the rate production of embryosomatic and shoots. The greatest multiplication of citrus culture was observed in D1B2 with combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP, where 20.60 embryosomatics and 2.70 shoots were obtained respectively. When the concentration of (0-2 mg/L) BAP increase at constant concentration of 0.5 mg/L 2,4-D results in high number embryosomatics and shoots. The combination of 2,4-D and BAP resulted in a 515x increase in the rate of embryosomatics multiplication as well as 67x increase in the rate of shoots over the culture D0B0 without growth stimulator. However, growth development of citrus culture decreased gradually when increasing the concentration combination exceeded 0.5 mg/L 2,4-D and 1.0 mg/L BAP. The BAP had also significantly influenced the development of the culture. The growth development of calli, embryosomatics and shoots were improved when the concentration of BAP was increased, but the growth decreased when increasing concentration to 3.0 mg/L BAP (see results in Table 2).

***In vitro* propagation of citrus nobilis brastepu with subculture:** *In vitro* propagation of *Citrus nobilis* Brastepu with subculture had been carried out to overcome slow growth plant multiplication due to limitation of the nutrient in culture medium. Transferring the explants into new fresh culture medium with the same variation concentration combination of 2,4-D and BAP had been carried out successively for first subculture, second subculture, and third subculture. Subculture was found to be able to support growth and development of *Citrus nobilis* Brastepu. The development of *Citrus nobilis* Brastepu for *In vitro* propagation through subculture are explained as follows.

Table 1. Composition combination of 2,4-D (D) and BAP (B) supplemented into MS basal medium for *In vitro* propagation of *Citrus nobilis* Brastepu.

2,4 D concentration (D/mg/L)	BAP Concentration (B/mg/L)			
	0	**1**	**2**	**3**
0	D0B0	D0B1	D0B2	D0B3
1	D1B0	D1B1	D1B2	D1B3
2	D2B0	D2B1	D2B2	D2B3

D0 = 2,4-D 0.0 mg/L; D1 = 2,4-D 0.5 mg/L; D2 = 2,4-D 1.0 mg/L; B0 = BAP 0.0 mg/L; B1 =BAP 0.5 mg/L; B2 = BAP 1.0 mg/L; B3 = BAP 3.0 mg/L

Table 2. Effect of 2,4-D and BAP interactions on growth and develpment of calli, embryosomatics, and shoots of *In vitro* propagation of *Citrus nobilis* Brastepu through shoot tips in control after four months of inoculation. Variations of experimental conditions are presented in Table 1. Data shown are mean of ten experiments followed by notation letter are significant according to Duncan's multiple range analysis (P = 0.05).

Experiment Treatments	Weight of callus (g)	Number of embryosomatic	Number of shoot	Discription observations of culture growth
D0B0	0.04[a]	0.50[a]	0.20	White callus cover the explant
D0B1	0.35[d]	1.20[b]	0.30	Greenish callus formation with big texture
D0B2	0.25[c]	3.10[d]	0.60	Green explant big texture
D0B3	0.16[b]	2.30[c]	0.50	Green explant big texture
D1B0	1.14[i]	9.00[i]	1.50	White callusand rough texture
D1B1	1.83[l]	11.70[j]	1.80	Greenish callus formation and rough texture
D1B2	1.51[k]	20.60[l]	2.70	Greenish callus formation, rough texture and nodularr
D1B3	1.37[j]	15.50[k]	2.10	Greenish callus formation, rough texture and nodularr
D2B0	0.51[e]	4.20[e]	0.80	Yellowish callusand rough texture
D2B1	0.94[h]	4.90[f]	0.90	Yellowish callusand rough texture
D2B2	0.86[g]	7.90[h]	1.30	Yellowish callusand rough texture
D2B3	0.62[f]	6.10[g]	1.10	Greenish callus formation, rough texture and nodularr

Table 3. The effect of 2,4-D and BAP interactions on growth development of calli, embryosomatics, and shoots for *In vitro* propagation of *Citrus nobilis* Brastepu through shoot tips for first subculture after four months of inoculation. Experimental variation conditions are in Table 1. Data shown are mean of ten experiments followed by notation letter are significant according to Duncan's multiple range analysis (P = 0.05).

Experiment Treatment	Weight of callus (g)	Number of embryosomatic	Number of shoot	Discription observations of culture growth
D0B0	0.46[a]	1.40[a]	0.30	Formation of aggregat cell culture suspension
D0B1	1.26[d]	3.60[b]	0.40	Green explant and big texture
D0B2	0.99[c]	4.30[c]	0.70	Green explant and big texture
D0B3	0.74[b]	3.20[b]	0.50	Green explant and big texture
D1B0	1.99[i]	12.20[h]	2.80	Aggregat suspension cell
D1B1	2.82[l]	16.20[i]	2.90	Callus developed and proliferation to become noduler
D1B2	2.39[k]	24.80[k]	4.00	Callus developed and proliferation to become noduler
D1B3	2.16[j]	20.50[j]	3.50	Callus developed and proliferation to become noduler with shoots
D2B0	1.34[e]	5.10[d]	0.90	Formation of aggregat cell culture suspension
D2B1	1.78[h]	6.80[e]	1.50	Formation of aggregat cell culture suspension
D2B2	1.51[f]	10.10[g]	2.20	Formation of aggregat cell culture suspension
D2B3	1.65[g]	8.40[f]	1.80	Formation of aggregat cell culture suspension

First subculture: The explant of Brastepu citrus obtained from culture initiation was treated for first subculture. One month-old explants were transferred into new medium with variation combination concentrations of stimulator (Table 1). Growth development of *Citrus nobilis* Brastepu culture in first subculture is shown in Fig. 5. Typical 1-month-old explants covered with callus obtained from callus initiation was let to grow for first subculture (Fig. 5a). Proliferation of the explants in subculture medium was observed after one month incubation, where some culture produced calli and proliferated to become noduler, another culture were green explants with big texture and just aggregate suspension cell. These explants were developed further to become plantlets (Fig. 5b-c). Normal citrus plant with leaves, stem and roots were obtained (Fig. 5d).

The effects of 2,4-D and BAP on weight of callus, number of embryosomatic and number shoots of Brastepu citrus in first subculture are summarized in Table 3. The culture without supplementation of stimulator produced aggregate cell culture suspension with very low rate intensity. Addition of low concentration of 0.5 mg/L 2,4-D, in the absence or presence of BAP, resulted in high growth rate for calli, embryosomatics and shoots in all experimental treatments. Growth and development trend for citrus culture in respond to the combination concentration of growth stimulator in first subculture were almost similar to that obtained in the control. Suplementation of low concentration of 2,4-D and BAP improved the development rate of calli, embryosomatics and shoots. The highest growth and development rate were obtained in D1B2 treatment using combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP. Maximum formations of callus (2.39 g), embryosomatic (24.80) and 4.00 shoots were noticed in this condition. However, increase in the concentration of auxin up to 1.0 mg/L 2,4-D did not improve development rate intensity of the cultures. These results were in agree with that of the experiment conducted in the propagation of *Citrus grandis* L. Osbeck (Paudyal & Haq, 2000). Cytokinin at certain concentration was commonly used for *In vitro* development of citrus (Carimi & De Pasquale 2003; Usman *et al.,* 2005), and therefore the effect of BAP in this study was also investigated. The results showed that growth development of Brastepu citrus increased significantly with addition of low to medium concentration of BAP, although at the high concentration 3.0 mg/L BAP resulted in the decrease of the development rate of citrus culture. It seems that high concentrations BAP in the medium has to be avoided for *In vitro* propagation of *Citrus nobilis* Brastepu.

Fig. 2. Preparation of healthy *Citrus nobilis* Brastepu seedlings by cutting bud propagation onto sour citrus (*Citrus aurantium*) rootstock to be used as sources of explants for *In vitro* propagation: (a) A survive, mature and productive *Citrus nobilis* Brastepu, (b) Typical fruit of Brastepu citrus, (c) The scion and the root stock have securely join for growing the sprout of Brastepu citrus in polybag, (d) Good quality of Brasepu citrus plants, age 4 months.

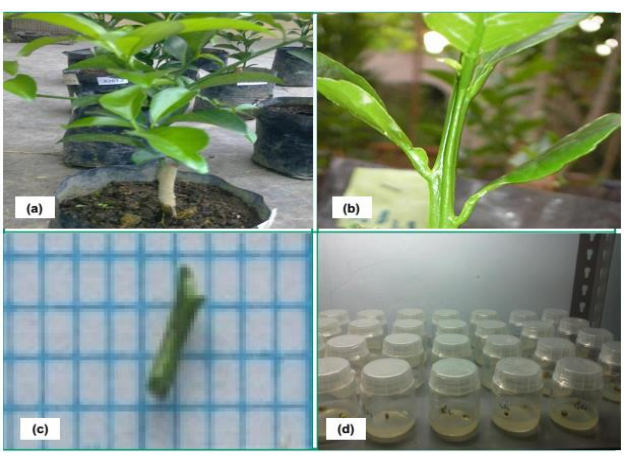

Fig. 3. Preparation of explant from *Citrus nobilis* Brastepu plants: (a) Healthy citrus seedling age 4 months, (b) Shoot tips of Brastagi citrus, (c) Explant of shoot tips (mst) with wound site ready to be planted in cultur media, and (d) The mst planted in cultur media and placed at room temperature for incubation.

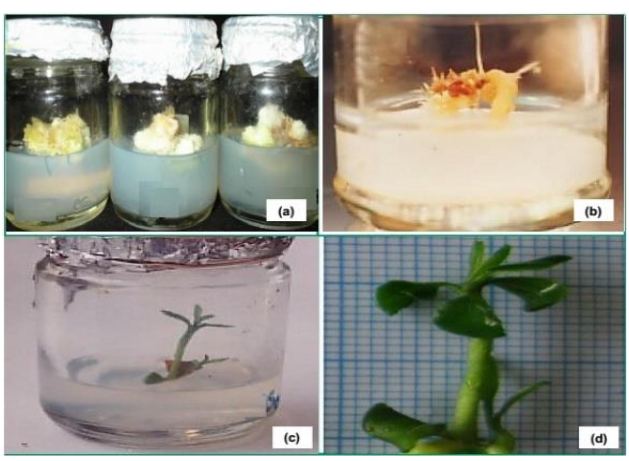

Fig. 4. *In vitro* development of culture shoot tips of *Citrus nobilis* Brastepu in MS medium enriched with 2,4-D and BAP without subculture (control): (a) Typical 1-month-old callus in culture medium enriched with stimulator, (b) Morphology of 2-month-old explants in medium culture, (c) Embryosomatic was developed to become citrus plant inculture media, (d) Plantlets of *Citrus nobilis* Brastepu taken from culture medium after four months.

Fig. 5. Growth and development of culture shoot tips of *Citrus nobilis* Brastepu in medium supplemented with 2,4-D and BAP for first subculture: (a) Typical 1-month-old explants covered with callus in new culture medium for first subculture, (b) The development of citrus plants in medium culture after 4 months, (c) Closed view of citrus plants resulted from first subculture, (d) Normal plants with leaves, stem and roots obtained from first subculture.

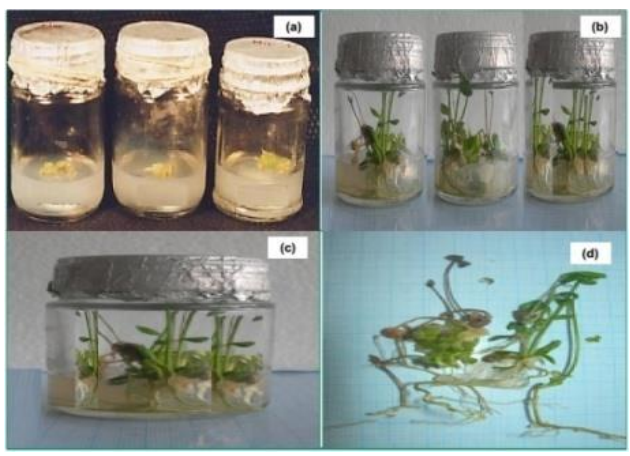

Fig. 6. Growth and development of culture shoot tips of *Citrus nobilis* Brastepu in MS medium containing of 2,4-D and BAP in second subculture: (a) Typical 1-month-old explants were transfered into new culture medium for subculture, (b) Growth development of citrus plants in medium culture after incubation for 4 months, (c) Closed view of citrus plantlets resulted from *In vitro* propagation in second subculture, (d) Plantlets obtained from second subculture containing leaves, stem and roots.

Fig. 7. Growth and development of culture shoot tips of *Citrus nobilis* Brastepu in medium culture of third subculture: (a) Typical one month explants were transfered into new fresh culture medium for third subculture, (b) The development of citrus plants in medium culture after four months, (c) Closed view of plants in third subculture, (d) Plantlets of *Citrus nobilis* Brastepu obtained from medium culture of third subculture.

Second subculture: Further subculture was carried out to overcome slow growth development of citrus culture when the nutrient was not sufficient in the culture media. Plantlets of *Citrus nobilis* Brastepu from one month old of first subculture onto the new fresh culture medium were transferred in second subculture with similar concentration of stimulator in the medium. The results demonstrated that the growth development of Brastepu citrus culture was significantly improved in second subculture. All cultures produced high rate intensity of growth development. Supplementation of 2,4-D and BAP in the media resulted in green calli, rough textureand nodular, also green explant and big texture, and some were yellowish calli with rough texture. Formation of yellowish low intensity calli was obtained in the control in the absence of growth stimulator. Further development with high multiplication of explants to become embryosomatic and shoots were obtained (Fig. 6). The nutrient in the medium culture was sufficient to support growth development of Brastepu citrus to become plantlets (Fig. 6b-c). High quality plantlets of *Citrus nobilis* Brastepu were obtained in second subculture experimentation with leaves, stem and roots after four months (Fig. 6d).

The effect of growth stimulator on to the growth and development of *Citrus nobilis* Brastepu in second subculture was observed as presented in Table 4. Interaction of 2,4-D and BAP had significantly influenced the growth rate of calli, embryosomatics and shoots. Increase in the concentration of 2,4-D and BAP was directly propotional to growth rate of citrus culture, but when the two were incorporated at high concentration, the development rates were reduced significantly (see results in Table 4). Growth and development trends of citrus culture insecond subculture were similar to that observed in the first subculture and the control. The best condition was observed in D1B2 with combination concentration of 0.5 mg/L 2,4-D and 1.0 mg/L BAP. The callus (2.30 g), embryosomatic (22.30), and shoots (8.60) were produced respectively. Green callus, rough texture and nodular types were observed at this experimental condition. The results revealed that interaction of 2,4-D and BAP contributed to the growth of citrus culture. Second subculture produced good quality plantlets at high intensity and developed into normal plant with stem, leaves, and roots. However, very high concentration of stimulator did not stimulate further to the growth of the culture, and probably caused severe toxicity problems to the explants (Kaji *et al.*, 2013; De Klerk *et al.*, 1997).

Third subculture: Growth and development of *Citrus nobilis* Brastepu in culture medium has been carried outfor third subculture. The explants from second subculture were then transferred into new fresh culture medium and incubated for another month. The growth of citrus culture was monitored and typical plantlets were shown in Fig. 7. The presence of various concentration of growth stimulator resulted in the typical of plantlets, where most of the culture were green plantlets, rough texture and nodular, some were found as white plantlets and yellowish with rough texture. The explants were developed to become plantlets and the medium was sufficient to support the development of the culture and became normal plantlets. It was observed that culture multiplication did not improve in the medium of third subculture (Fig. 7b). Typical citrus plants with low intensity growth, green plantlets, rough texture and nodular were observed after four months (Fig. 7c-d). Good quality plantlets developed into normal citrus plant with stem, leaves, and roots(Fig. 7d).

Transfering the culture into new fresh medium for third subculture did not improve growth development of the culture. The effects of stimulator on the growth development of Brastepu citrus were summarised in Table 5. The effect of 2,4-D and BAP interaction on the growth development of citrus culture in third subculture was similiar to those observed in first and second subcultures. However, the growth intensity of Brastepu culture in the third subculture was lower than that in the second subculture. The combination concentration of 0.5 mg/L 2,4-D and 0.5 mg/L BAP in D1B1 was found to produce the heaviest callus (1.53 g) compared to another experimental treatments, where typical culture was greenish plantlets with rough texture. Furthemore, high rate formation of embryosomatics (17.60) and shoots (6.50) were obtained in group treatment D1B2 with interaction combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP. Typical culture of green, rough textureand nodular plantlets was observed during the development accompanied with development of normal plant with shoots, leaves and roots. However, supplementation of high concentration of 3 mg/L BAP in the culture did not improve the number of embryosomatic and shoots. The number of embryosomatic and shoots decreased with increasing the combination concentration above 0.5 mg/L 2,4-D and 1.0 mg/L BAP. This results was in agreement with that *In vitro* propagation of Pummelo (*Citrus grandis L.* Osbeck) where proliferation rate of shoots did not improve when relatively high concentration of BAPwere added in the culture medium (Paudyal & Haq, 2000).

Optimization of culturedevelopment: *In vitro* propagation was effective when two or more different types of growth regulators were used such as the combination of cytokinin-auxin interactions for regulating plant growth (Ozudogru *et al.*, 2011). Optimization of culture conditions to obtain the highest growth development of callus, embryosomatic, and shoots had been carried out in this study with the inclusion of interaction concentration of 2,4-D and BAP in the medium, similar to the results obtained in the previous study (Nurwahyuni & Sinaga, 2016). It was observed that the explants from shoot tips are all generated calli, embryosomatics and shoots. Growth developments of Brastepu citrus culture significantly varied in subculture. The effects of interaction concentration of 2,4-D and BAP in medium on to the growth development of calli, embryosomatics, and shoots are presented in Fig. 8. The presence of 2,4-D in the medium culture had the most influence on the growth of citrus culture. Combination concentration of 2,4-D and BAP at low to medium level were found very effective to promote the development of calli, embryosomatics, and shoots, where the very high concentration of stimulator did not improve the growth intensity of citrus culture.

Table 4. The effect of 2,4-D and BAP interactions on growth development of calli, embryosomatics, and shoots *In vitro* propagation of *Citrus nobilis* Brastepu through shoot tips of second subculture. Experimental combinations are listed in Table 1. Data shown are mean of ten units followed by notation letter are significant according to Duncan's multiple range analysis (P = 0.05).

Experiment treatment	Weight of callus (g)	Number of embryosomatic	Number of shoot	Description observations of culture growth
D0B0	0.17[a]	1.20[a]	0.30[a]	Formation of yellowish low intensity plantlets
D0B1	0.87[d]	1.80[b]	0.40[a]	Green explant and big texture
D0B2	0.55[c]	4.30[d]	0.70[ab]	Green explant and big texture
D0B3	0.37[b]	2.70[c]	0.50[ab]	Green explant and big texture
D1B0	1.89[i]	11,50[i]	4,20[e]	Yellowish plantlets with rough texture
D1B1	2.17[j]	12.80[j]	5.40[f]	Greenish plantlets with rough texture
D1B2	2.30[k]	22.30[l]	8.60[h]	Green callus, rough texture and nodular
D1B3	1.92[i]	18.10[k]	6.80[g]	Green callus, rough texture and nodular
D2B0	1.34[g]	5.10[e]	0.90[b]	White callus with rough texture
D2B1	1.59[h]	6.80[f]	1.50[c]	Yellowish plantlets with rough texture
D2B2	1.25[f]	8.30[h]	2.80[d]	Yellowish plantlets with rough texture
D2B3	0.94[e]	7.40[g]	1.90[c]	Green plantlets, rough texture and nodular

Table 5. Effect of 2,4-D and BAP interactions on growth and development of citrus plants in propagation of *Citrus nobilis* Brastepu through shoot tips on third subculture. Combination variation of stimulator are listed in Table 1. Data shown are mean of ten experiments followed by notation letter are significant according to Duncan's multiple range analysis (P = 0.05).

Experiment treatment	Weight of callus (g)	Number of embryosomatic	Number of shoot	Discription observations of culture growth
D0B0	0.03[a]	0.40[a]	0.2[a]	Explant with low intensity yellowish plantlets
D0B1	0.05[ab]	0.70[a]	0.40[ab]	Green explant and big texture
D0B2	0.07[b]	2.00[c]	0.50[ab]	Green explant and big texture
D0B3	0.04[a]	1.30[b]	0.40[ab]	Green explant and big texture
D1B0	0.95[g]	7.90[h]	2.10[d]	White callus with rough texture
D1B1	1.53[j]	9.70[i]	4.30[e]	Greenish plantlets with rough texture
D1B2	1.45[i]	17.60[k]	6.50[g]	Green plantlets, rough texture, nodular
D1B3	1.16[h]	14.40[j]	5.20[f]	Green plantlets, rough texture, nodular
D2B0	0.19[c]	2.70[d]	0.80[b]	White plantlets with rough texture
D2B1	0.85[f]	3.80[e]	0.90[bc]	Yellowish plantlets with rough texture
D2B2	0.66[e]	6.30[g]	1.80[d]	Yellowish plantlets with rough texture
D2B3	0.35[d]	5.20[f]	1.30[c]	Green plantlets, rough texture, nodular

Callus development: The calli were grown in explants in almost all experimental treatments. The effect of 2,4-D and BAP interaction on to weight of calli have been compared for subculture and control (Fig. 8a). First subculture generate calli more than that in another subculture. The best treatment condition to generate calli were found in first subculture, followed by second and third subculture. Low intensity calli were obtained in the culture medium without stimulator. The weight of calli increased linearly with increasing concentration of 2,4-D and BAP in culture medium. However, high concentration of stimulator did not improve growth intensity of citrus callus. The optimization process showed that the heaviest callus was obtained with the treatment of D1B1 of first subculture, that was with the combination of 0.5 mg/L 2,4-D and 0.5 mg/L BAP in the medium. High growth intensity calli were obtained in all cultures at this treatment condition. The weight of calli was successively obtained in the first, second, third subculture, and the control. This indicated that the presence of low concentration of growth stimulator in medium culture was very efficient to regenerate callus.

Development of embryosomatic: Inclusion of stimulator in culture medium influenced the development rate of embryosomatic of subculture and control. Embryosomatics intensity generated in respect to the variation combination of 2,4-D and BAP in culture medium had been compared for subculture and control (Fig. 8b). Growth rate intensity for embryosomatic was the highest in first subculture, followed by second subculture, and third subculture respecticely. Embryosomatic was obtained very low in control. The best condition to generate embryosomatic of Brastepu citrus was D1B2 with supplementation of 0.5 mg/L 2,4-D and 1.0 mg/L BAP in culture medium. The presence of low concentration of 0.5 mg/L2,4-D was efficient to generate embryosomatic. Increase of high concentration of 1.0 mg/L 2,4-D in the medium did not improve the growth rate of embryosomatic. The study revealed that 2,4-D at certain concentration played the key role to generate embryosomatic of *Citrus nobilis* Brastepu.

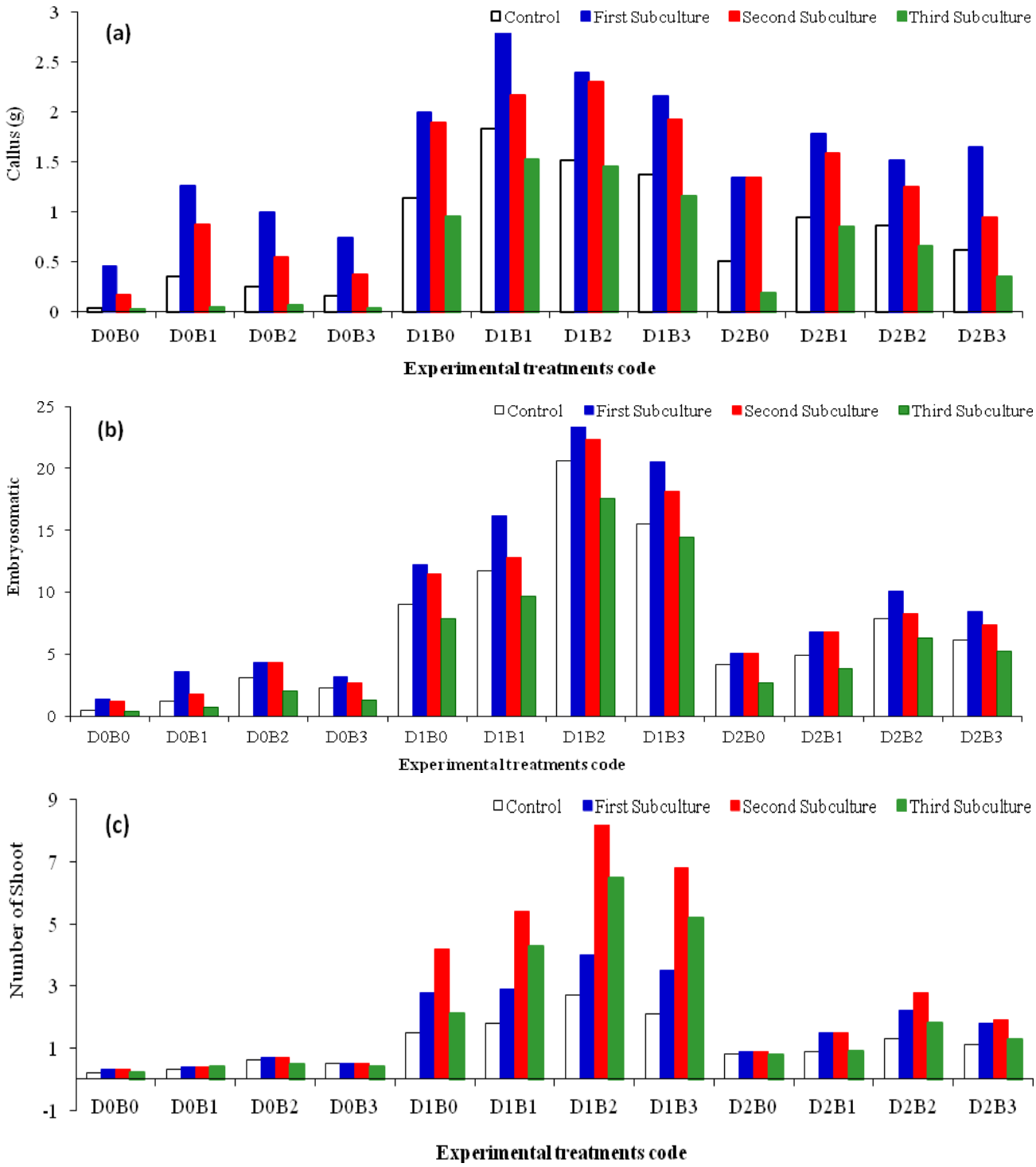

Fig. 8. Optimization ofgrowth stimulator suplementedin medium culture for *In vitro* propagation of *Citrus nobilis* Brastepu through shoot tips subculture at various experimental treatments. The resuls are subject to ten replicates and harvested after four months: (a) Weigh of calli, (b) Number of embryosomatics, and (c) Number of shoots. Variation in the concentration combination of 2,4-D and BAP are shown in Table 1.

Shoots induction: The effect of 2,4-D and BAP on to the rate induction of shoots of Brastepu citrus in subculture and control have been compared (Fig. 8c). Effective shoots multiplication was observed when low concentration of 2,4-D are supplemented in the medium. The combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP generated high number of shoots in all experiments. Hence, few shoots were generated in the absence of 2,4-D, whether it was combined with low or high concentration of BAP in the medium. It was found that shoot proliferation was enhanced in second subculture, but the shoots did not generate further in the third subculture. High number of shoots generated in culture medium successively in second subculture, followed by third subculture, first subculture, and the control. The results demonstrated that new fresh medium with frequency of subculture was necessary to generate shoot of *Citrus nobilis* Brastepu in culture medium but the shoots multiplication rate did not improve further when third subcuture was applied.

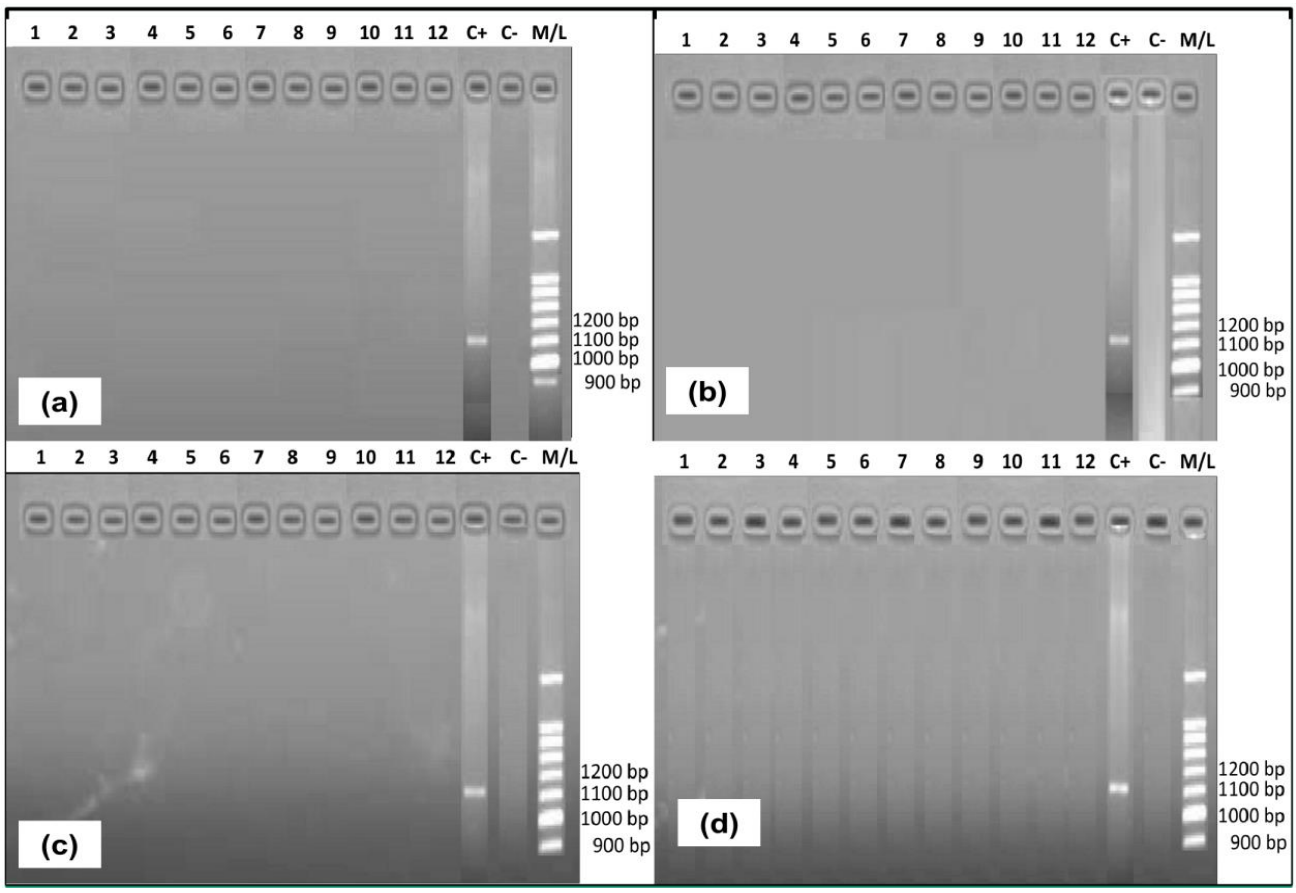

Fig. 9. PCR profiles of 12 plantlets samples randomly selected form *In vitro* propagates of *Citrus nobilis* Brastepu for CVPD determination: (a) control without subculture, (b) first subculture, (c) second subculture, and (d) third subculture. (C+) is CVPD control positive, (C-) is CVPD control negative, and (M/L) is a marker.

Screening cultures for CVPD: The PCR analysis was carried out to determinethe presence of CVPDinfection in *Citrus nobilis* Brastepu plantlets that were regenerated through *In vitro* propagation. Twelve plantlets were randomly selected from the population of citrus regenerated through shoot tips subculturefollowed by analysed for CVPD. The PCR profilesof Brastepu samples are shown in Fig. 9. The PCR profile under the same amplification conditions for citrus Brastepu plantlets propagated in first subculture (Fig. 9b), second subculture (Fig. 9c), third subculture (Fig. 9d),and the control (Fig. 9a) all had band patterns similar tothat control negative (C-), indicated that all samples were free from CVPD. There was no band pattern found similar toCVPD control positive (C+) in all plantlets samples. These results implythat plantlets of *Citrus nobilis* Brastepu regenerated through shoot tips subculturewere all free from CVPD infection (Nurwahyuni *et al.,* 2015).

Discussion

Brastagi citrus variety *Citrus nobilis* Brastepu is a threatened citrus plants from North Sumatera Indonesia, and is need to preserve and to avoid the diminishing of the local citrus plant. Brastepu citrus is rarely found now days because it is not planted properly anymore due to CVPD infection that has destroyed citrus in Indonesia, including Brastagi North Sumatera many years ago (Titrawidjaja, 1984). The existence of Brastepu citrus is categorized as unintended plants that are only grown wild and survived in the field. The eruption of Mount Sinabung in 2013-2017 in the impact area where the plants were grown speed up the extinction of local citrus (Nurwahyuni & Sinaga, 2014). Preservation of the local plant has been conducted *via* cutting bud propagation (Nurwahyuni *et al.,* 2012; 2015). The technique was applied in this study to provide sufficient good quality shoot tips explants for *In vitro* propagation. Cutting bud technique has been demonstrated to be the very efficient to provide high quality explant with minimum contamination (Nurwahyuni, 2013; Nurwahyuni & Sinaga, 2014). Propagation of citrus by using the explants from shoot tips have high survival rate, rapid growth, and great number of shoots were produced (Nurwahyuni, 2016). The results was similar to that for micropropagation of *Prunus* sp., Rootstocks (Dorić *et al.,* 2014).

Previous studies have reported that the combination of two or more different types of growth regulators are required for successful *In vitro* propagation of citrus (Savita *et al.,* 2012; Paudyal & Haq, 2000). Cytokinin-auxin interaction was considered to be the most important for regulating plant growth to stimulate callus, induce somatic embryogenesis, and to initiate shoots (Panicker *et al.,* 2007). The combination of 2,4-D and BAP was chosen in this study following the success for *Citrus nobilis* Brastepu reported previously (Nurwahyuni, 2016; Nurwahyuni & Sinaga, 2014). It was known that the presence of cytokinin or auxin, and the mixture of cytokinin/auxin, had significant effect on

improving the development rate of citrus culture (Nurwahyuni & Sinaga, 2016). Number of studies have reported the effect of stimulator on the growth development of callus (Thomas & Michael, 2007, Janarthan & Seshadri, 2008), embryosomatic (Chung *et al.,* 2007) and shoots (Hossain *et al.,* 2010; Diaz & Alvarez, 2009). It was found in the present study that low concentration (0.5 mg/L) 2,4-D had significantly influenced the growth development of callus, embryosomatic and shoots. However, lower rates multiplication was obtained when high concentration (1.0 mg/L) 2,4-D combined with medium to high concentration (1,0 mg/L - 3,0 mg/L) BAP was applied. This result agreed with previous report on various types of citrus (Yaacob *et al.,* 2014; Savita *et al.,* 2012; Tallón *et al.,* 2012; Sharma *et al.,* 2009) and other plants such as *Tectona grandis* (teak) trees (Shirin *et al.,* 2005), *Dendrobium* (Zhao *et al.,* 2008). Therefore, cytokinin-auxin interaction at low concentration was chosen to propagate *Citrus nobilis* Brastepu.

Subculture technique was applied to improve growth development of citrus culture for long period of incubation time to overcome limitation of nutrients in the medium culture (Tsay, 1998; Yang *et al.,* 2012). Subculture frequency to supply the availability of nutrient and growth regulator in the medium culture was found effective to support growth and development of *Citrus nobilis* Brastepu. It was expected that increasing the frequency subculture would lead to the improvement of growth and development of culture Brastepu citrus. With this experiment, the highest growth and development of plantlets were obtained in second subculture, followed by first subculture, third subculture and control respectively. *In vitro* propagation of *Citrus nobilis* Brastepu through second subculture was found to be very efficient to provide good quality plantlets. The decline in the growth rate of citrus obtained in the third subculture was probably due to occurrence of hyperhydricity (Quiala *et al.,* 2014; Kevers *et al.,* 2004; Vieitez *et al.,* 1985). The optimal cytokinin/auxin balance subsequently controlled the development of citrus culture to produce normal citrus seedling. *In vitro* propagation by using shoot tips explants through subculture were able to regenerate citrus plantlets free from CVPD.

Conclusion

In vitro propagation of *Citrus nobilis* Brastepu with shoot tips subculture was developed with 95% survival, and be able to generate callus, embryosomatic, and shoots. Supplementation of 2,4-D and BAP in medium culture have been examined to maximise the growth development of citrus culture. The presence of 2,4-D in the medium culture has the most influence on the growth and development of citrus culture. Combination concentration of 2,4-D and BAP at low to medium level were found significantly effective to promote growth intensity of citrus culture. The growth intensity of citrus culture vary in respect to the frequency subculture. Second subculture in D1B2 supplemented with 0.5 mg/L 2,4-D and 1.0 mg/L BAP was found to be the best to propagate *Citrus nobilis* Brastepu. The culture in this condition produced good quality plantlets and with high multiplication intensity. Normal plant with leaves, stem and roots was developed. The PCR profiles confirmed that plantlets of *Citrus nobilis* Brastepu regenerated through shoot tip subculture were all free from CVPD infection. The present study has provided *In vitro* protocol for production of *Citrus nobilis* Brastepu seedlings, and become good strategy to conserve local threatened Brastagi citrus.

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