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IN VITRO PROPAGATION FOR BIOCONSERVATION OF THREATENED BRASTAGI CITRUS IN NORTH SUMATRA INDONESIA

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ABSTRACT

In vitro propagation for bioconservation of threatened Brastagi citrus, local citrus in North Sumatra Indonesia is explained. The aims of bioconservation is to protect Brastagi citrus from diminish in the ecosystem due to the eruption of Mount Sinabung. The studies were conducted through cuting bud propagation to prepare explant sources followed by in vitro propagation to obtain plant seedling. The propagation was carried out by cutting the bud from branch of survive Brastagi citrus and stick onto the bark of sour citrus (*Citrus aurantium*) root stock. In vitro propagation was conducted experimentally with treatment combinations to obtain seedling of Brastagi citrus for bioconservation. The results showed that cutting bud propagation has successfully conducted to rescue three varieties of Brastagi citrus and to provide explant material for in vitro propagation. The callus were grown in all explants for all treatments. Two types of callus were observed, one in brown color, watery and non embryogenic, and the other was embryogenic callus with green color. The embryogenic callus is nodular that was develops and directly regenerate to plantlet. Growth stimulator in medium culture was significantly influenced callus induction, the growth of somatic embryo, plantlets, leaves, and roots.

KEY WORDS: **In vitro propagation, cutting bud, Brastagi citrus, bioconservation, threated plant**

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INTRODUCTION

In vitro propagation for bioconservation of threatened species of local citrus in Brastagi North Sumatra is very important to obtain good quality seedling of citrus as a strategy in the bioconservation of an endanger local variety of citrus due to the eruption of Mount Sinabung in North Sumatra. The propagation technique is a fast strategy to conserve local variety citrus from demolished. Brastagi is the regency situated in Kabupaten Karo North Sumatra Indonesia that well known with production of citrus which is called as "Brastagi citrus". Various local citrus at Brastagi North Sumatra were not planted properly and has tend to be replaced by imported citrus with some reasons, the production quantity and their resistance to some diseases such as CVPD virus. Most of the local Brastagi citrus nowadays are grown in the field as unintended plant even though they were very popular many years ago and become a well known comodity from Brastagi North Sumatra. The eruption of Mount Sinabung in 2013-2014 where Brastagi is the impact areas make the existence of local citrus be demolished faster. If the Brastagi citrus are not conserved properly, it is predicted that these citrus varieties will disappear in the short time. It has been known that many potential genetic from local citrus have to be preserved, such as the quality of fruits and the content of medicinal bioactive compounds in the plants that have been proven cure some deseases[1,2]. Therefore, the local variety of citrus in Brastagi North Sumatra has to be conserved to protect Brastagi citrus from diminishing from the ecosystem.

Plant Conservation

Plant conservation is a biodiversity strategy to sustain the potential of the present plant for future generation[3]. Conservation of plant is carried out to protect endanger plant from species diminish in the ecosystem, to promote environmental sustainability, to reduce species and habitat loss, and to avoid specific plant from extinction[4-6]. To conserve plant can be conducted in various ways, including collection, propagation, characterization, evaluation, disease indexing and elimination, storage and distribution[7]. Plant conservation can be done by *in situ* or *ex-situ* conservation to safeguard populations in danger of destruction, replacement or deterioration[8]. There are several techniques can be applied to conserve plant genetic such as in vitro conservation, in vitro propagation, and re-introduction of plants to their natural habitats, and molecular marker technology[7]. In vitro conservation of rare and threatened ferns has been reviewed explaining the importance of collection measures, in vitro culture procedures and cryopreservation and methods for the integrated conservation of threatened ferns[9]. In vitro technique for bioconservation of rare medicinal plant has also been reported[10-12]. The vitro techniques to storage vegetative propagated and recalcitrant seed producing species has also introduced to slow the growth of the plant and by cryopreservation to store the plant in liquid nitrogen[7].

Propagation of Citrus

In vitro technique has been applied to the propagation of many *dicotylae* plants, and has been conducted for many years to provide seedling of the plants with high economic value[13]. Various works have been conducted on the use of plant tissue culture to propagate new seedling[14] by using various explants such as roots[15], leaves, tuber[16], ovary[17], stem and *epicotyls*[18], and the protoplast[19]. The production of transgenic plant has also been reported[20] by using *Agrobacterium* as transformed media[21]. Genetic variation of transgenic plant for citrus has also performed[22-23] to obtain high quality citrus[24]. The propagation of *Citrus sinensis* L. Osbeck have been reported[25]. The effect of various type of growth stimulator into the growth of the callus have also been explained[26,27]. Some factors have to be considered in the tissue culture techniques to obtain good seedling such as the genetic of the plant, the enzymatic reaction, and the metabolism process[28,29].

Local Variety of Sweet orange in North Sumatra

Various variety of local sweet orange planted and developed in Brastagi North Sumatra have been identified, they are variety Boci (*Citrus nobilis* Lour), variety Brastepu (*Citrus nobilis* Brastepu), and variety Rimokeling (*Citrus*

nobilis), they are known as “Jeruk Brastagi” or Brastagi citrus. The variety Brastagi citrus has superior in the genetic as it has very sweet taste compare with honey citrus, the color of a raped feel is reddish, and the fruit is large. The survey has shown that Brastagi citrus is very popular in North Sumatra because the citrus could be used as sources of fruit and the plant leaves and the feels contains bioactive that are used for Karo traditional medicine to cure some diseases. The seedling for Brastagi citrus is provided by cutting and budding technique to obtain the good quality seedling. However, those propagation techniques only produced very limited seedling and therefore it is difficult to provide large number of seedling for plantation. To overcome the problem, in vitro propagation is the best choice for mass production of good quality seedling at a short time. The propagation of local citrus has been conducted by using of stem and young leaves as explants followed by micro propagation[30]. The seedling obtained in the medium culture has been tested for its resistance and tolerance of the *Citrus nobilis* Brastepu into salinity[31], their resistance to fungus, and their ability to adapt with extreme season. All of those studies were conducted to obtain a good quality seedling of citrus. The aim of the research is to propagate Brastagi citrus to obtain good quality seedling of variety of local citrus in order to provide mass production of citrus seedling as a strategy in the bioconservation of local variety of citrus to protect the local citrus from diminish in North Sumatra. This work describes cutting bud propagation followed by in vitro propagation to preserve the potential genetics of local citrus from diminished due to the eruption of Mount Sinabung in North Sumatra and to stop Brastagi citrus lost of plant diversity.

MATERIALS AND METHODS

Research methods are consisted of collection of Brastagi citrus, cutting bud propagation to rescue threatened citrus, and in vitro propagation. Collection of Brastagi citrus was conducted to obtain threatened local citrus in the villages around Mount Sinabung at Brastagi, Kabupaten Karo, Province North Sumatra, Indonesia. Citrus collection was focused on to rare citrus which have genetic potential to be preserved in plant diversity. Cutting bud propagation was carried out to stick Brastagi bud onto sour citrus (*Citrus aurantium*) as root stock followed the procedures explained in the previous study[32,33]. In vitro propagation of Brastagi citrus was conducted followed the procedures explained previously[34,35]. In vitro propagation procedures are consisted of preparation of culture medium, preparation and sterilization of explant, and plantation of the explants in culture medium. In vitro propagation was set up experimentally with 10 replications with combination of various variable, they are (A) Name of local citrus: (A-1) Citrus variety Boci (*Citrus nobilis* Lour), (A-2) Citrus variety Brastepu (*Citrus nobilis* Brastepu), and (A-3) Citrus variety Rimokeling (*Citrus nobilis*); (B) Variation in explants sources: (E-1) embryo, (E-2) stem, (E-3) cotyledon; (C) Variation in growth stimulator: (M0) Basal medium MS[36] without growth stimulator, (M1) Basal medium MS containing of 1,0 mg/L 2,4 dichlorophenoxyacetic acid (2,4-D), (M2) Basal medium MS containing of 1,0 mg/L benzyl amino purine (BAP), (M3) Basal medium MS containing 1,0 mg/L 2,4-D and 1,0 mg/L BAP. The pH of the medium is adjusted to pH 5.8 – 6.0, and it is then sterilized at 121 ^{o}C, 15 lb for 20 minutes.

RESULTS AND DISCUSSION

1. Rescue of Brastagi Citrus

Brastagi citrus are collected from farmers in villages closed to Mount Sinabung and from the Department of Agriculture in Kabupaten Karo, North Sumatra Indonesia. The information related to potential genetic of the local citrus was obtained from local farmer based on their experiences on planting and carrying the citrus. There are three varieties of Brastagi citrus have been rescued in this study, those are: (1) Citrus variety Boci, (2) Citrus variety Brastepu, and (3) Citrus variety Rimokeling. These Brastagi citrus are rarely found in the field this time because the farmer is not interested to plant the citrus for some reason, mainly due to low production and low resistant to plant diseases compare with imported citrus Washington navel orange. The ecosystem where the Brastagi citrus grown are situated in impact area of Mount Sinabung which has errupted recently (September 2013 -

February 2014) makes local citrus become endanger species. Most of survive plants are old, undomesticated, unintended, and suffer with various plant diseases. Therefore, Brastagi citrus has to be rescued to obtain explant for bioconservation study.The first step to rescue local citrus was carried out by using cutting bud propagation technique. The propagation technique is aimed to provide good seedling from threatened local citrus to obtin enough material for in vitro propagation in the bioconservation study. The steps to rescue Brastagi citrus are illustrated for citrus variety Boci presented in Figure 1. The bud from branch of survive Brastagi citrus (Figure 1a) was taken, and young sour citrus was prepared as root stock (Figure 1b). The bark on the stem of sour citrus root stock was cut and prepared for cutting bud propagation (Figure 1c). The scion in bud of Brastagi citrus was cut and stick onto the bark of sour citrus root stock, followed by wrapping tightly with plastic to avoid infection and to make it free from water (Figure 1d). After the incubation has been proceed for 2 weeks, the plastic wrap was released, and the scion and the root stock have securely joined, followed by cutting of the upperstem of sour citrus abot 5 cm from the cutting bud to allow the sprout in the scion bud grow (Figure 1e). The development of shoot in root stock has successfully obtained and the sprouts were grown well for three varieties of Brastagi citrus (Figure 1f). It has been demonstrated that cutting bud propagation is the fastest and simple technique to rescue Brastagi citrus to provide good quality plants seedling. The sprout of three varieties of Brastagi citrus have successfully grown on sour citrus root stock to provide sufficient explant material for in vitro propagation.

Figure 1

Cutting bud propagation of local citrus into sour citrus (Citrus aurantium) to rescue endanger Brastagi citrus as a source of explants for in vitro propagation: (a) The appearance of citrus variety Boci (Citrus nobilis Lour), (b) Sour citrus plant for rood stock, (c) The bark of Sour Citrus was cutted for occulation, (d) Wrapping cut and budding occulation with plastic to join rood stock with the bud of local citrus, (e) Sprouting of local citrus in sour citrus rood stock, and (f) The development of shot in three varieties of Brastagi citrus.

2. In Vitro Propagation of Brastagi Citrus

In vitro propagation of Brastagi citrus has been carried out by taking the embryo, stem, and cotyledon as explants in culture medium. It was known that the ability of plant tissue to produce callus was effected by the composition and the concentration of culture medium, the presence of growth stimulator, and the light intensity[37]. Therefore, the basal MS medium is used to initiate callus in this study. The propagation of Brastagi citrus starting from callus initiation in the explants for three local varieties of citrus, culture regeneration become new plants and the formation of multiple plantlets is presented in Figure 2.

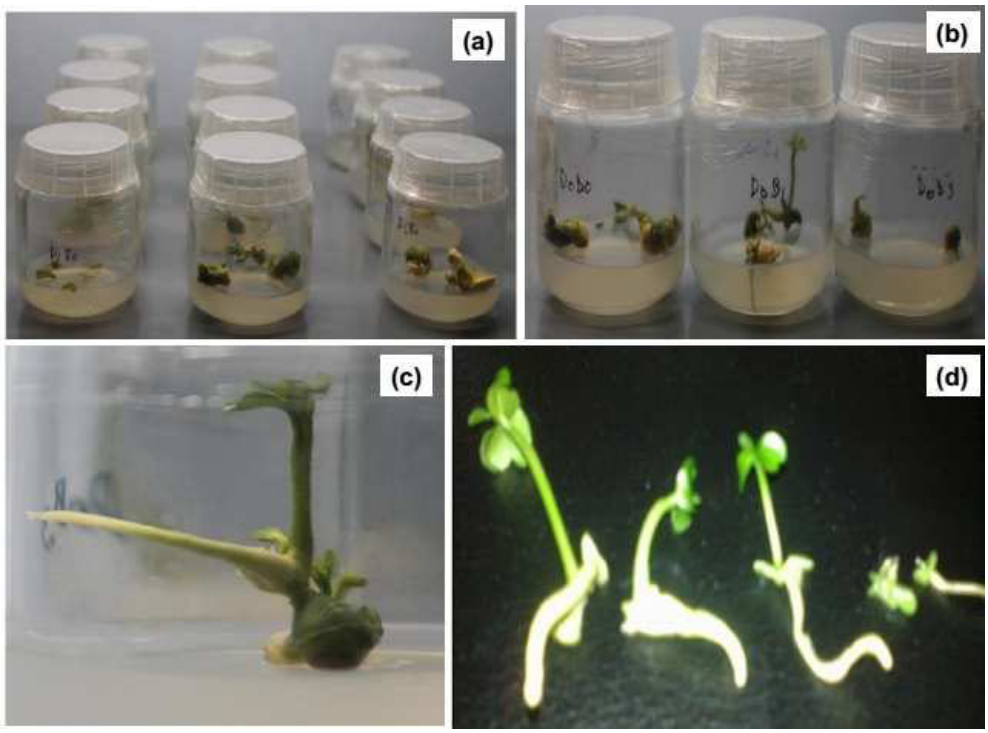

Figure 2

In vitro propagation of Brastagi citrus in cultur medium: (a) Callus initiation in the explants for three local varieties of citrus, (b) Culture regeneration become new plants, (c) The growth of multiple plantlets, (d) 5 plantlets are separated from culture.

It was observed that the callus was grown in the all explants (embryo, stem, and cotyledon) for all varieties of Brastagi citrus (Figure 2a). The embryo, stem, and cotyledons of all three varieties of Brastagi citrus were initiated to callus with high growth intensity. The callus was dominated by the parenchyma cell that was the active cell in the multiplication process. The anatomy of shoot tip of Citrus variety Boci is presented in Figure 3. There was a callus stain in the surface and the nodule that is potentially become regenerated or morphogenesis become plantlet. The apical meristem (Figure 3a) was clearly shown (arrow in the figure) in the shoot tip (Figure 3b), and the globular somatic embryo (Figure 3d) was also observed in the anatomy of the callus (Figure 3c). The type of the callus was meristemic cell (Figure 3c). The potential of the cell become somatic embryo has also observed which was developed as globular somatic embryo as shown in Figure 3d.

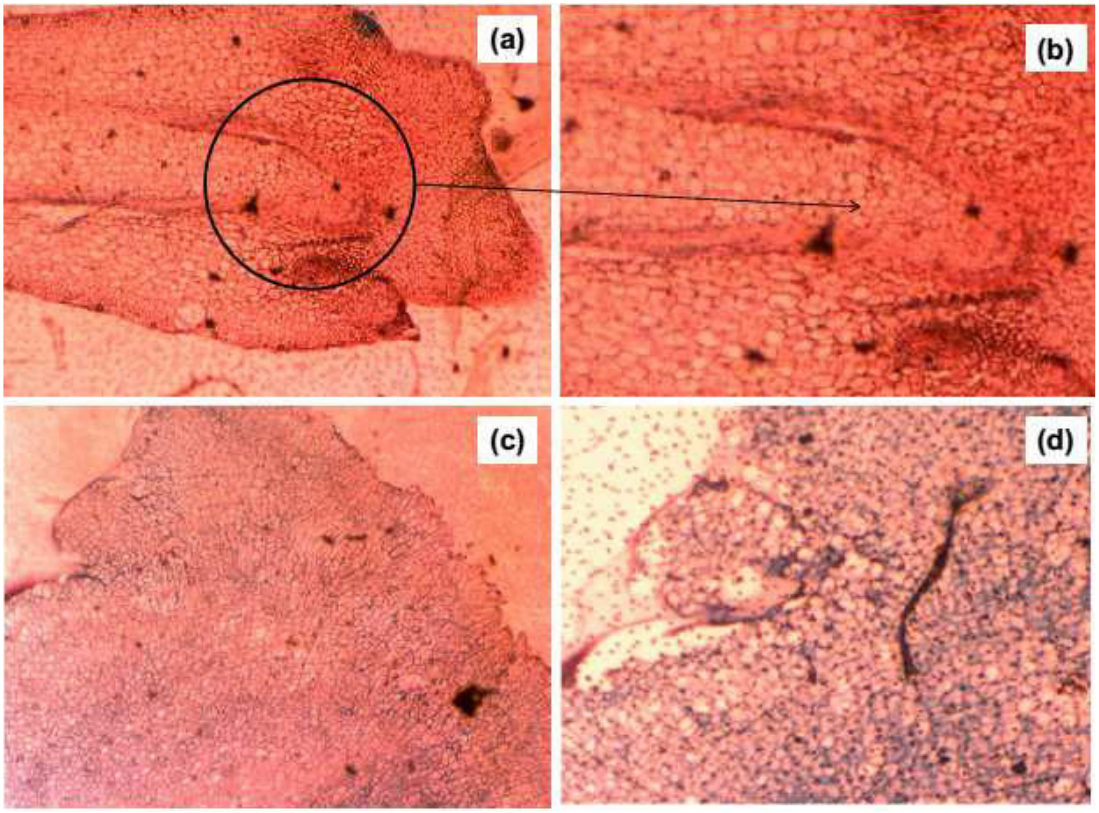

Figure 3

Shoot tip as an explants source and the morphogenesis of the callus for Citrus variety Boci (Citrus nobilis Lour): (a) The anatomy of shoot tip, (b) apical meristem (showed by arrow), (c) the anatomy of the callus, and (d) globular somatic embryo.

2.1. The growth of Callus of Brastagi Citrus in Medium Culture

It was observed that the callus of local citrus grow well in the medium culture. The callus was grown starting from the edge of the explants that was direct contact with culture medium. The growth was continued until the explants surface was covered with callus. The growth intensity of the callus based on the avarage of wet callus weight after 4 weeks was summarized in Table 1. The weigh of the callus are vary depend on the treatment conditions. The avarage weigh of the callus for Citrus variety Boci lies between 0.10 - 1.37 g callus, and the heaviest was obtained in E1M0 (1.37 g). for Citrus variety Brastepu, the avarage weight of the callus were obtained between 0.10 - 0.39 g callus, and the heaviest was found in E2M3 (0.39 g). In culture of Citrus variety Rimokeling, the callus were grown and developed in most of the culture where the avarage weight of the callus were found between 0.10 - 0.25 g callus, except for E2M0 and E3M0 (0,0 g callus), and the heavies was observed in E2M3 (1.71 g). The results shown that basal medium MS stimulate the formation of callus in all explants of Citrus variety Boci and Citrus variety Brastepu. In culture of Citrus variety Rimokeling, the basal medium MS stimulate the formation of callus explant from embryo, but were not able to generate the callus in explant of stem and cotyledon. The presence of 1,0 mg/L 2,4-D and 1,0 mg/L BAP in basal medium MS was able to improve the weight of callus in most of explants. The growth formations of the callus were seen as callus with brown color, watery, non embryonic, and some of them were green color and embryonic callus. The callus was starting to develop and differentiate become planlets after four weeks in culture medium.

Table 1

The growth of callus of Citrus variety Boci (Citrus nobilis Lour), Citrus variety Brastepu (Citrus nobilis Brastepu), and Citrus variety Rimokeling (Citrus nobilis) in culture medium at various experimental parameters after 12 weeks incubation time.

	Weight of Culture (g)		
Experimental Treatment	Citrus variety Boci	Citrus variety Brastepu	Citrus variety Rimokeling
E1M0	1.37a	0.10e	1.37a
E1M1	0.92ab	0.16bcde	0.51a
E1M2	0.84abc	0.27ab	0.89a
E1M3	0.85abc	0.37a	0.64a
E2M0	0.39abc	0.1e	0.00b
E2M1	0.14c	0.14cde	0.72a
E2M2	0.82abc	0.2bc	0.25a
E2M3	1.32ab	0.39a	1.71a
E3M0	0.10c	0.1e	0.00b
E3M1	0.2bc	0.2bc	0.39
E3M2	0.10c	0.12de	0.34a
E3M3	0.20bc	0.18bcd	1.32a

Note: The number followed by notation is significant level (α = 0.05)

2.2. The Development of Somatic embryo of Brastagi Citrus Culture

The callus was consistently grown in culture medium and regenerate morphogenically become somatic embryo. Regeneration intensity of somatic embryo in cultue medium at various conditions were vary for different species of Brastagi citrus due to variation of growth stimulator in the medium. The growth of the somatic embryo in a medium culture has been observed and the avarage number of somatic embryo in a culture medium is presented in Table 2. The results showed that most of the cultures produce somatic embryo with high intensity. The basal media MS without growth stimulator could not stimulate somatic embryo in almost all of variety of Brastagi citrus, only embryo explant of Citrus variety Boci (E1M0) with 0.02 somatic embryo per culture. The avarage number of somatic embryos grown for Citrus variety Boci were lies between 0.02 - 33.86 somatic embryo per culture, where the higest was observed in E2M3 (33.86). High number of somatic embryos were found in Citrus variety Brastepu with the avarage lies between 3.67 - 33.86 somatic embryo per culture, and the hihest was obtained in E1M3 (33.86). For Citrus variety Rimokeling, the avarage number of somatic embryos were obeserved between 2.24 - 12.85 somatic embryo per culture, where the highest was obtained in E1M2 (12.85). It can be concluded that basal medium MS containing 1,0 mg/L BAP as well as basal medium MS containing both 1,0 mg/L 2,4-D and 1,0 mg/L BAP were effective to stimulate the growth of somatic embryo for Brastagi citrus.

Table 2

The growth of somatic embryo of Citrus variety Boci (Citrus nobilis Lour), Citrus variety Brastepu (Citrus nobilis Brastepu), and Citrus variety Rimokeling (Citrus nobilis) in culture medium at various experimental parameters after 12 weeks incubation time.

	Number of Somatic Embryo in a Culture Medium		
Experimental Treatment	Citrus variety Boci	Citrus variety Brastepu	Citrus variety Rimokeling
E1M0	0.02	0.00b	0.00
E1M1	6.94	6.94ab	5.51
E1M2	27.74	7.34ab	12.85
E1M3	18.97	33.86a	5.30
E2M0	0.00	0.00b	0.00
E2M1	4.69	3.67b	2.24
E2M2	6.94	4.69b	6.53
E2M3	33.86	4.69b	3.26
E3M0	0.00	0.00	0.00b
E3M1	1.02	4.69	2.24b
E3M2	10.40	12.85	4.28b
E3M3	6.12	7.14	7.14ab

Note: The number followed by notation is significant level (α = 0.05)

2.3. The Development of Plantlet in Brastagi Citrus Culture

The explants have been covered by callus and were consistently grown well until they were starting to regenerate become new plantlet at week five followed by development morphogenically become plantlet (Figure 2b). The regeneration intensity of plantlets were vary for different species of Brastagi citrus. The basal medium MS with growth stimulators were able to stimulate the development of the callus onto plantlets in cultue medium for all variety of Brastagi citrus. The growth and the development of the callus onto plantlet after six weeks incubation time is summarized in Table 3. Some explant from stem of citrus variety of Boci (E2M0 and E2M1) and Rimokeling (E2M1) did not produce plantlets. The development of plantlet in a medium culture was categorized as good in most of the cultures, where some of them are with multiple plantlets. The explants were consistently grown linearly with the incubation time, and the plantlets were then initiated directly to become leaf, stem and roots. Typical growth of multiple plantlets for Citrus variety Brastepu (*Citrus nobilis* Brastepu) is presented in Figure 2c. The cultur of Citrus variety Brastepu produce higher number of plantlets compare to culture of Citrus variety Boci and Citrus variety Rimokeling. The average plantlet was observed for Citrus variety Brastepu were lies beteween 1.01 - 3.03 plantlets per culture, where the highest was found in E1M3 (3.03 plantlets). For Citrus variety Boci the highest average plantlet in the groups was found in E1M2 (2.02 plantlets), and the highest average for Citrus variety Rimokeling was observed in E2M0 (2.4 plantlets). Extending the incubation time up to ten weeks did not improved the growth intensity of the plantlet, however, the differentiation of the callus become plants were clearly observed. After the incubation time has been conducted for 12 weeks, the plantlet was develop become real plants in medium culture, where the root, leaves, the stem and bud were developed well. The example of for the growth and development of plantlets in medium culture is presented in Figure 2c, where leaves, stem and the roots were clearly showed when the plantlets has been taken from medium culture (Figure 2d). With this condition, the new plants could be transferred into other media for acclimatization.

Table 3

The growth of plantlet of Citrus variety Boci (Citrus nobilis Lour), Citrus variety Brastepu (Citrus nobilis Brastepu), and Citrus variety Rimokeling (Citrus nobilis) in culture medium at various experimental parameters after 12 weeks incubation time.

	Number of Plantlet/Culture		
Experimental Treatment	Citrus variety Boci	Citrus variety Brastepu	Citrus variety Rimokeling
E1M0	1.41	1.01b	1.84ab
E1M1	1.82	1.01b	1.84b
E1M2	2.02	2.02ab	1.43bc
E1M3	1.01	3.03a	1.43bc
E2M0	0.00	1.01b	2.45a
E2M1	0.00	1.01b	0.00
E2M2	0.40	1.41ab	0,41d
E2M3	0.61	2.42ab	1.02bc
E3M0	1.01	1.01ab	2.04ab
E3M1	1.01	1.62ab	1.02bc
E3M2	1.01	2.22ab	1.84b
E3M3	1.01	2.83a	1.84b

Note: The number followed by notation is significant level (α = 0.05)

CONCLUSION

In vitro propagation for bioconservation of threatened Brastagi citrus has successfully conducted followed by rescuing the plants from diminished by using cutting bud propagation. The development of shot of Brastagi citrus in sour citrus (*Citrus aurantium*) root stock has successfully grown become sprouts for three varieties of Brastagi citrus, to provide adequate materials to be used as explant sources for in vitro propagation. In vitro regeneration of Citrus variety Boci (*Citrus nobilis* Lour), Citrus variety Brastepu (*Citrus*

nobilis Brastepu), and Citrus variety Rimokeling (*Citrus nobilis*) have been done well to produce new plants. The conditions for basal medium with growth stimulator to initiate the callus, and to generate plantlets become plants have been obtained. The growth formations of the callus were callus with brown color, watery, non embryonic, and some of them were green color and embryonic callus. After the incubation time has been conducted for 12 weeks, the plantlets were developing become real plants in medium culture, where the root, leaves, the stem, and bud were developed well. The new plants were ready for acclimatization process in the next experiment to produce Brastagi citrus good seedling.

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REFERENCES

1. Simatupang, S., Karakterisasi and Pemanfaatan Plasma Nutfah Jeruk In Situ oleh Masyarakat Lokal Sumatra Utara, *Buletin Plasma Nutfah* 15(2): 70-74, (2009).
2. Nurwahyuni, I., Napitupulu, J.A., Rosmayati, and Harahap, F., Pertumbuhan Okulasi Jeruk Keprok Brastepu (*Citrus nobilis Var.* Brasitepu) Menggunakan Jeruk Asam Sebagai Batang Bawah, *Jurnal Penelitian Saintika* 12(1): 24-35, (2012).
3. Rajeswara, R.B.R., Syamasundar, K.V., Rajput, D.K., Nagaraju, G. and Adinarayana, G., Biodiversity, Conservation And Cultivation Of Medicinal Plants, *Journal of Pharmacognosy* 3(2): 59-62, (2012).
4. Huang, H., Plant diversity and conservation in China: planning a strategic bioresource for a sustainable future, *Botanical Journal of the Linnean Society* 166: 282–300, (2011).
5. Blackmore, S., Gibby, M., and Rae, D., Strengthening the scientific contribution of botanic gardens to the second phase of theGlobal Strategy for Plant Conservation, *Botanical Journal of the Linnean Society* 166: 267–281, (2011).
6. Jackson, P.W., and Sharrock, S., The context and development of a global framework for plant conservation, *Botanical Journal of the Linnean Society* 166: 227–232, (2011).
7. Kasagana, V.N., and Karumuri, S.S., Conservation Of Medicinal Plants (Past, Present & Future Trends), J. Pharm. Sci. & Res. 3(8): 1378-1386, (2011).
8. Kasagana, V.N., and Karumuri, S.S., Conservation Of Medicinal Plants (Past, Present & Future Trends), J. Pharm. Sci. & Res. 3(8): 1378-1386, (2011).
9. Barnicoat, H., Cripps, R., Kendon, J., and Sarasan, V., Conservationin vitro of rare and threatened ferns—case studies of biodiversity hotspot and island species, *In Vitro Cell. Dev. Biol. Plant* 47: 37–45, (2011).
10. Yogananth, N., Bhakyaraj, R., Chanhuru, A., and Palanivel, S., In vitro Regeneration and consevation of Rare Medicinal Plant Dregea volubilis Benth, *J. Plant Sciences* 6(6): 225-228, (2011).
11. Krishnan, P.N., Decruse, S. W., and Radha, R. K., Conservation of medicinal plants of Western Ghats, India and its sustainable utilization throughin vitro technology, *In Vitro Cell. Dev. Biol. Plant* 47: 110–122, (2011).
12. Lone, S,A., Yadav, A.S., Badkhane,Y., Sharma, A.K., Bakhshi, S.H., and Raghuwanshi, D.K., Effect Of Different Plant Growth Regulators On In-Vitro Propagation of *Barleria Prionitis L.* — A Threatened Medicinal Plant., International *Journal of Pharma and Bio Sciences* 2(1): 438-444, (2011).
13. Chaturvedi, H.C., and Mitra, G.C., Clonal propagation of citrus from somatic callus culture, *HortScience* 9: 118-120, (1974).
14. Niedz, R.P., and Evens, T.J., The effects of nitrogen and potassium nutrition on the growth of nonembryogenic and

embryogenic tissue of sweet orange (*Citrus sinensis* (L.) Osbeck), *BMC Plant Biol.* 8: 126-131, (2008).
15. Grosser, J.W., and Chandler, J.L., Somatic hybridization of high yield, cold-hardy and disease resistant parents for citrus rootstock improvement, *Journal Horticultural Science Biotechnology* 75: 641-644. (2000).
16. Costa, M.G.C.; Alves, V.S.; Lani, E.R.G; Mosquim, P.R.; Carvalho, C.R., and. Otoni, W. C.,., Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of *Citrus, Scientia Horticulturae* 100(1-4): 63-74, (2004).
17. Carimi, F.; Pasquale, F.D., and Crescimanno, F.G., Somatic embryogenesis in *Citrus* from styles culture, *Plant Science* 105: 81-86, (1995).
18. Edriss, M.H., and Burger, D.W., In Vitro propagation of troyer citrange from epicotyl segment, *Scientia Horticulturae* 23: 159-162, (1984).
19. Das, A.; Paul, A.K. and Chaudhuri, S., Micropropagation of sweet orange, Citrus sinensis Osbeck. for the development of nucellar seedlings, *Indian Journal of Experimental Biology* 38: 269-272, (2000).
20. Hunt, P.W.; Watts, R.A.; Trevaskis, B.; Llewelyn, D.J.; Burnell, J.; Dennis, E.S. and Peacock, W.J., Expression and evolution of functionally distinct haemoglobin genes in plants, *Plant Molecular Biology* 47: 677-692, (2001).
21. Casanova, E.; Trillas, M.I.; Moysset, M. and Vainstein, A., Influence of *rol* genes in floriculture, *Biotechnology Advances* 23(1): 3-39, (2005).
22. Hao, Y.J.; Cheng, Y.J, and Deng, X.X., Stable maintenance and expression of a foreign gene in transgenic pear shoots retrieved from *in vitro* conservation, *Journal of Plant Physiology* 162(2): 237-243, (2005).
23. Li, W., Song, Q., Brlansky, R.H., and Hartung, JH., Genetic diversity of citrus bacterial canker pathogens preserved in herbarium specimens, *Proc. Natl. Acad. Sci. USA* 104(47): 18427–18432, (2007).
24. Terol, J., Naranjo, M.A., Ollitrault, P., and Talon, M., Development of genomic resources for *Citrus clementina*: Characterization of three deep-coverage BAC libraries and analysis of 46,000 BAC end sequences, *BMC Genomics* 9: 423-429, (2008).
25. Duran-Vila, N.; Ortega, V., and Navarro, L., Morphogenesis and tissue cultures of three citrus species, *Plant Cell and Organ Culture* 16: 123-133, (1989).
26. Maggon, R., and Singh, B.D., Promotion of adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyl explants sweet orange (*Citrus sinensis* L.Osbeck), *Scientia Horticulturae* 63: 123-128, (1995).
27. Hidaka, T., and Kajiura, I., Plantlet differentiation from callus protoplasts induced from *Citrus* embryo, *Scientia Horticulturae* 34(1-2): 85-92, (1988).
28. Terol, J., Conesa, A., Colmenero, J.M., Cercos, M., Tadeo, F., Agustí, J., Alós, E., Andres, F., Soler, G., Brumos, J., Iglesias, D.J., Götz, S., Legaz, F., Argout, X., Courtois, B., Ollitrault, P., Dossat, C., Wincker, P., Morillon, R., and Talon, M., Analysis of 13000 unique *Citrus* clusters associated with fruit quality, production and salinity tolerance, *BMC Genomics* 8: 31, (2007).
29. Vu, J.C.V.; Niedz, R.P., and Yelenosky, G., Activities of sucrose metabolism enzymes in glycerol grown suspension cultures of sweet orange (*Citrus sinensis* L. Osbeck), *Environmental Experimental Botany* 35: 455-463, (1995).
30. Nurwahyuni, I., Kultur jaringan daun jeruk manis (*Citrus nobilis* Brasitepu) untuk mikropropagasi, *Jurnal Sain Indonesia* 24(1): 17-20, (2003).
31. Nurwahyuni, I., Uji ketahanan kultur jeruk manis (*Citrus nobilis* Brasitepu) terhadap salinitas menuju bibit unggul, *Jurnal Scientia* 3(2): 75-84, (2003).
32. Nurwahyuni, I., and Sinaga, R., Bioconservation Of Brastagi Citrus (*Citrus nobilis* Brastepu) A Local Citrus Of Sumatra Utara Through Cutting Propagation, *Proceeding of SEMIRATA BKSPTN-B,* IPB Bogor, Indonesia, (2014), in press.
33. Nurwahyuni, I., Napitupulu, J.A., Rosmayati, and Harahap, F., Teknik Okulasi Jeruk Keprok Brastepu (*Citrus nobilis Var.* Brasitepu) Untuk Menghasilkan Bibit Bebas Penyakit

CVPD, *Proceeding of SEMIRATA BKSPTN-B,* UNIMED Medan, Indonesia, (2012), pp. 594-599.

34. Nurwahyuni, I., Optimalisasi Teknik *In Vitro* Perbanyakan Jeruk Keprok Brasitepu, *Jurnal Sains Indonesia* 36(1): 8-15, (2012).
35. Nurwahyuni, I., and Rahayu, S., Teknik *In Vitro* Jeruk Keprok Brastagi (*Citrus nobilis* Brastepu) Sebagai Strategi Biokonservasi Mengatasi Kepunahan Jeruk Lokal Sumatra Utara, Pros, *Proceeding of SEMIRATA BKSPTN-B,* UNILA Bandar Lampung, Indonesia, (2013), pp. 419-427.
36. Murashige, T., and Skoog, F., A revised media for rapid grouth and bioassay with tobacco tissue culture, *Physiol. Plant.* 15: 473-496, (1962).
37. Nurwahyuni, I., and Tjondronegoro, P., Induksi kalus dan regenerasi tanaman Dioscorea composita Hemls, *Hayati* 1: 15-17, (1994).