Meristematic shoot tip culture obtain good quality seedling of citrus (*Citrus nobilis var.* brastepu) free from citrus vein phloem degeneration

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Abstract. Meristematic shoot tip culture to obtain good quality seedling of citrus (*Citrus nobilis Var.* Brastepu) free from citrus vein phloem degeneration (CVPD) is explained in this research. The method is conducted by using a diseased plant and is then propagated in optimum conditions. The research is conduted in Tissue Culture Laboratory Department of Biology, University of North Sumatra, Indonesia. Optimum conditions for citrus propagation have been obtained to produce good quality of *Citrus* seedlings that is free from CVPD. The analysis of the DNA using PCR method has confirmed that the seedlings are healthy and free from CVPD. Genomic analysis using 10 RAPD markers show the planlets regenerated from the cultures are 100% similar with the mother plant.
Keywords: Optimization, in vitro propagation, citrus, CVPD free, seedling

Introduction

The propagation of citrus Brastepu are commonly conduced by generative and vegetative methods (Sanford, 1992; Rieger, 2006; Nurwahyuni, *et.al*, 2012). The production of citrus seedling are mostly with vagetative method such as cutting and grafting, (Williamson dan Jackson, 1994; Prastowo, *et..al*., 2006). However the problem to obtain good root are often found and results in low performance of seedling. The occulation method by joining the bud with lemonade citrus as a mother plant has successfully conducted (Nurwahyuni, *et.al*, 2012). However, the production of the good quality of seedling free from CVPD for mass production is difficult to conduct. Therefore, it is good to find another method to obtain good seedling for mass production by using in vitro propagation. The application of in vitro propagation technique is compulsory to be done as a strategy to conserve local variety citrus in North Sumatra. The genetic potential from local citrus have to be preserved in the quality development of the citrus, such as resistance to plant diseases, quantity and quality of fruits and the content of medicinal bioactive compounds in the plants (Nurwahyuni, 2011). Therefore, the local variety of citrus in North Sumatra has to be conserved. The aim of the research is to apply meristematic shoot tip culture to obtain good quality seedling of citrus free from CVPD. The success in the production of good quality seedling of citrus that is free from CVPD could save the variety local citrus in North Sumatera and will motivate the farmer to grow the citrus in Karo regency, North Sumatera, Indonesia.

In vitro technique has been applied for the propagation of many *dicotyl* plants, and has been conducted for many years to provide seedling of the plants with high economic value (Chaturvedi, *et.all*., 1974). Various works have been conducted on the use of plant tissue culture to propagate new seedling (Niedz and Evens, 2008) by using various explants such as roots (Grosser, *et.all*., 2000), leaves, tuber (Costa, *et.all*., 2004), ovary (Carimi, *et.all*., 1995), stem and *epicotyl* (Edriss and Burger, 1984) and the protoplast (Das, *et.all*., 2000). The propagation of *Citrus sinensis* L. Osbeck have been reported (Duran-Vila, *et.all*., 1989). The effect of various type of growth stimulator into the growth of the callus have also been explained (Maggon and Singh, 1995)). 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 (Terol, *et.all*., 2007).

Various variety of local sweet orange planted and developed in Brastagi North Sumatra have been identified, they are Brastepu, Boci, and Rimokeling. The variety Brastepu is known as "*Jeruk Brastagi*". The variety Brastepu 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 size fruit. The survey has shown that Citrus Brastepu is very popular in North Sumatra because

the citrus could be used as sources of fruit and the leaves and the feels contains bioactive that is used as medicine to cure some diseases. The seedling for Brastepu is provided by cut and budding technique to obtain the good quality seedling. However, propagation by using cut and budding techniques only produced very limited seedling and therefore it is difficult to provide lage 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 the local citrus has been conducted by using of stem and young leaves as explants followed by micropropagation (Nurwahyuni, 2003). Variation of growth stimulator in culture medium were very effective to initiate callus. Culture medium containing of 2,4-D was found more effective to initiate the callus compare with other growth stimulator such as NAA, ZI and KI or the combination of IAA, BA and KI. It was found that the MS medium enriched with 2,4-D and KI are able to develop the callus to a plantlet. The seedling obtained in the medium culture has been tested for its tolerance into salinity (Nurwahyuni, 2003), 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 nobilis Var.* Brastepu.

Materials and Methods

Research methods are consisted of identification and characterization of *Citrus nobilis Var.* Brastepu, and experimental procedures are explained in details (Nurwahyuni, et.all., 2012). Experimantal procedures are consisted of preparation citrus explant, preparation of culture medium, sterilization and plantation of the explants, and shoot tip culture of *Citrus nobilis Var.* Brastepu. Diseased plants that still grow well, are chosen as the sources of explants, that were obtained from Bukit Village, Kabupaten Karo, Province North Sumatra. In vitro propagation is set up experimentally with 10 replications by using combination of various variable of D_0 is 2,4 dichlorophenoxyacetic acid or 2,4-D 1,0 mg/l; B_0 is Benzyl Amino Purine or BAP 0,0 mg/l; B_1 is BAP 0,5 mg/l; B_2 is BAP 1,0 mg/l; B_3 is BAP 3,0 mg/l. The pH of the medium is adjusted to pH 5.8 – 6.0, and it is then sterilized at 121 ^oC, 15 lb for 20 minutes. The meristem used as plant material for the culture shown in Figure 1.

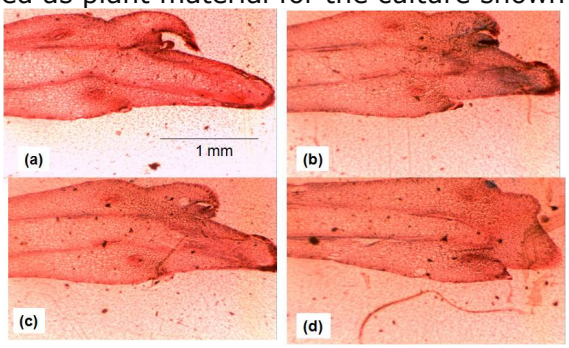

Figure 1. The microscopy slides of the meristematic shoot tips of *Citrus nobilis Var.* Brastepu as explants: a,b,c,d are a series of cuttings.

Results and Discussion

Description of *Citrus nobilis Var.* Brastepu

The local citrus that has been studied is *Citrus nobilis Var.* Brastepu. It was collected from farmers in many villages, and from The Department of Agriculture in Kabupaten Karo. All informations about the orange are obtained from the farmer based on their experiences on planting and carrying the citrus. The citrus Brastepu are rarely found in the field because the farmer did not interested in planting it for some reason. The performance of *Citrus* Brastepu and appearance of its fruit is presented in Figure 2.

Callus induction of *Citrus nobilis Var.* Brastepu

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 (Nurwahyuni and Tjondronegoro, 1994). Therefore, the basal MS medium is used to initiate callus in this study. The basal MS consisted of growth stimulators are use as reported in the previous study. It was observed that the callus was grown in the culture where the texture of the callus in the medium is almost the same as young shoot. The

variation of the growth stimulator in MS solid media enriched with 2,4-D: 0; 0.5; and 1.0 mg/l and BAP; 0; 0.5; 1.0, and 1.50 mg/l results in the variation of the culture (Figure 3b). Low intensity of callus is observed when using growth stimulator 2,4-D 0 mg/L and BAP 0.5 mg/L (D_0B_1), where when the culture is enriched with 2,4-D 1.0 mg/L without BAP (D_2B_0) produce high intensity of callus. Callus induction in D_2B_0 is found very good, however it does not produce shoot. The MS medium consisted of 2,4-D 0.5 mg/L without BAP (D_1B_0) could initiate the callus in medium intensity and could directly produce shoot. Therefore, the medium culture that is enriched with 2,4-D 0 mg/L and BAP 0.5 mg/L (D_0B_1) is found effective to initiate the shoot in the shoot tip culture of *Citrus* Brastepu.

Figure 2. The plant appearance of *Citrus nobilis Var.* Brastepu and it's fruit

The growth of Callus in Medium Culture

The culture development in various condition of culture is observed based on the growth of callus, the development of embryosomatic, the intensity of shoot, and the characteristic of the explant in the culture is presented in Table 1. The morphogenesis of the callus is observed from its preparative of the culture (the picture not showed). The microscopic preparation of the callus for meristematic propagation of a shoot is showed in where the shape of the callus is a meristematic cell. The differenciation of a meristem become a nodule of shoot is clearly shown, as could be seen clearly . The callus is dominated by the parenchyma cells that is the active cell in the multiplication process. There is a callus stain in the surface and the nodule that is potentially become regenerated or organogenesis become a plant.

The Development of Embryosomatic of *Citrus nobilis Var.* Brastepu

The formation of embryosomatic of the *Citrus nobilis Var.* Brastepu were conducted by transferring the embryogenic callus into MS medium enriched with 2,4-D and BAP. Thevariation of the 2,4-D and BAP in the MS medium is sufficient to grow the embryosomatic. The growth and the development of embryosomatic influenced by the variation of growth stimulator in medium is summarized in Table 1. It is known that MS0 medium could not initiate the embryosomatic. The addition of 2,4-D is not also regenerate the embryosomatic until the concenntration of the growth srtimulator of 1,0 mg/L 2,4-D and 1,0 mg/L BAP, where the best condition is found in D_1B_2 with the texture of embryosomatic is green color, rough texture with noduls.

The Development of Plantlet of *Citrus nobilis Var.* Brastepu

The development of plantlet in a medium culture was categorized as good in all experiments. The explants are consistently grown linearly with the incubation time. The observation for the development of the callus after six weeks incubation time is presented in Figure 3. Extending the incubation time up to seven weeks did not improved the growth intensity of the plantlet. The same phenomena were also found after extending the incubation time up to ten weeks. After the incubation time has been conducted for12 weeks, the plantlet is develop become a real plants in medium culture, where the root, leaves, the stem and bud are

developed well. The example of for the growth and development of plantlets in medium culture is presented in Figure 4, where the leaves, stem and bud are clearly differentiated become citrus plants. The development of leaves, stem and the roots is clearly showed when the plantlet is taken from medium (Figure 4b) and the variation of the plantlets for a single plant is also presented. With this condition, the plant could be transferred into other media for acclimatization.

Tabel 1. The development and the characteristic of shoot tip culture of *Citrus nobilis Var.* Brastepu in MS media enriched with 2,4-D and BAP. The number represent the Duncan test (P 0.05).

Experiment Treatment	Callus (g)	Embryosomatics	Shoot	The description and the characteristic of shoot culture
D_0B_0	0.04 a	0.50 a	0.20	Explant with low intensity callus
D_0B_1	0.35 d	1.20 b	0.30	Green explant and big texture
D_0B_2	0.25 c	3.10 d	0.60	Green explant and big texture
D_0B_3	0.16 b	2.30 c	0.50	Green explant and big texture
D_1B_0	1.14 i	9.00 i	1.50	White callus and rough texture
D_1B_1	1.83 l	11.70 j	1.80	Green callus, rough texture
D_1B_2	1.51 k	20.60 l	2.70	Green callus, rough with nodul
D_1B_3	1.37 j	15.50 k	2.10	Green callus, rough with nodul
D_2B_0	0.51 e	4.20 e	0.80	White callus, rough texture
D_2B_1	0.94 h	4.90 f	0.90	Brown callus, rough texture
D_2B_2	0.86 g	7.90 h	1.30	Brown callus, rough texture
D_2B_3	0.62 f	6.10 g	1.10	Green callus, rough texture

D_0 = 2,4-D 0.0 mg/l; D_1 = 2,4-D 0.5 mg/l; D_2 = 2,4-D 1.0 mg/l; B_0 = BAP 0.0 mg/l; B_1 = BAP 0.5 mg/l; B_2 = BAP 1.0 mg/l; B_3 = BAP 3.0 mg/l.

Figure 3. The growth and development of plantlets of local citrus in culture medium enriched with growth stimulator: (a) The plantlet emerged from calli (b) Plenty of plantlet in various sizes.

Isolasi of DNA of *Citrus nobilis Var.* Brastepu and Analysis for CVPD

Isolation of the DNA of from the culture of the *Citrus nobilis Var.* Brastepu have been conducted. An amount of 5 µL DNA has been taken followed the analysis on gel electrophoresis. The results showed that pure DNA has been produced from the culture which is then be used in PCR analysis for the CVPD screening. The profile of the electrophoresis gel have confimed that all samples are free from CVPD. It is known that from the marker (forward and reverse primers) is specific for *C. Liberibacter asiaticus* which show a single band for the sDNA. It also can be seen from Figure 4. that there are no band for the 24 samples cultures , mean all of them are free from CVPD and the samples with a single band are the CVPD positive.

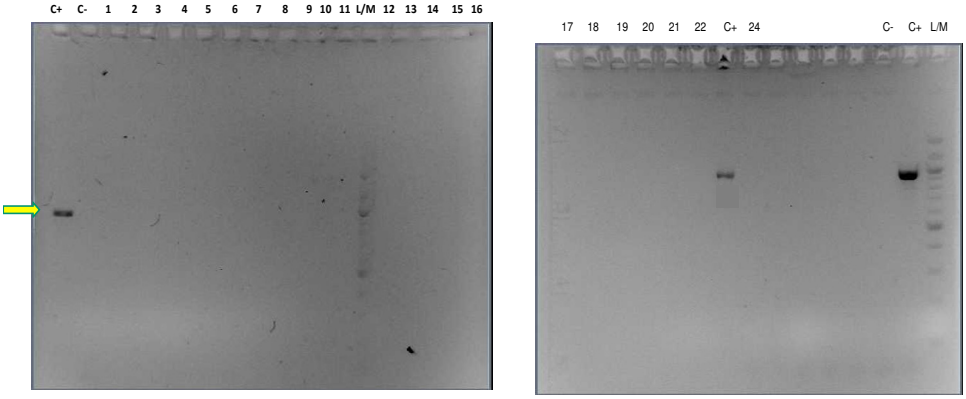

Figure 4. The electrophoresis of the 24 PCR DNA samples from shoot tip cultures of *Citrus nobilis Var.* Brastepu using primer HLB 65/66

Conclusions

Meristematic shoot tip culture to obtain good quality seedling of citrus (*Citrus nobilis Var.* Brastepu) free from CVPD) have successfully conducted. The best condition by using 0.5 mg/L 2,4-D and 0.5 mg/L BAP (D1B1) in medium culture has been obtained that could initiate the callus, embryosomatic, plantlets, and the plants.

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