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Development of *In Vitro* Conservation Medium of *Carica pubescens* Lenne & K. Koch through Nutrients Concentration Reduction and Osmoregulator Addition

Pengembangan Medium Penyimpanan Carica pubescens Lenne & K. Koch Secara In Vitro dengan Reduksi Konsentrasi Nutrisi dan Penambahan Osmoregulator

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Abstract

Carica pubescens Lenne & K. Koch is a rare species that need to be conserved. The research aim was to develop a slow growth method of *in vitro* conservation medium through determining some effects of nutrition decreasing availability in the conservation medium on growth and survival of explants. Establishing epicotyls reached from *in vitro* seed germination was grown on diluted basic medium of 75% MS (Murashige and Skoog), 50% MS, 25% MS, while osmoregulator compound of mannitol and sorbitol was added to the full MS medium in several concentrations. The treatments were arranged in a completely randomized design with three replications. The epicotyls were grown at storage medium for 12 and 16 weeks, then their survival were evaluated at regeneration medium and rooting medium. The diluted basic medium and osmoregulator addition were evaluated for its influence in retarding the culture growth in terms of improved survival over the period of 16 weeks. Data analyzed by one way analysis of variance and Duncan's multiple range test. The results showed that the decreasing of nutrition concentration suppressed the growth of the epicotyls until 16 weeks after conservation. Epicotyls taken from 16 weeks after conservation could grow on the regeneration medium. The best survival was shown by the 75% MS, 50% MS and supplementing of 20 g/l mannitol treatments. Based on these results, 50% MS medium is recommended for storage C. pubescens for 16 weeks with no sub-culture.

Abstrak

Carica pubescens Lenne & K. Koch (karika dieng) merupakan tanaman yang langka sehingga perlu dilestarikan. Penelitian ini bertujuan untuk memperoleh medium penyimpanan in vitro dengan teknik pertumbuhan minimal dengan mengamati pengaruh penurunan ketersediaan nutrisi dalam medium terhadap penurunan pertumbuhan dan daya tumbuh eksplan.Eksplan berupa epikotil kecambah in vitro. Perlakuan penurunan ketersediaan nutrisi dilakukan melalui reduksi konsentrasi nutrisi dari medium Murashige & Skoog (MS) dan penambahan osmoregulator (manitol dan sorbitol) dengan berbagai konsentrasi. Penelitian dilakukan dengan rancangan acak lengkap satu faktor dengan tiga ulangan. Epikotil dipelihara dalam medium penyimpanan selama 12 dan 16 minggu, kemudian dievaluasi daya tumbuhnya dengan memelihara dalam medium regenerasi dan medium pengakaran. Data dianalisis dengan analisis varians satu arah dan uji Duncan. Hasil penelitian menunjukkan bahwa penurunan kecepatan penyerapan nutrisi berpengaruh terhadap pertumbuhan eksplan. Epikotil yang telah disimpan selama 12 minggu dan 16 minggu dan ditumbuhkan kembali pada medium regenerasi masih dapat tumbuh dengan intensitas tertinggi pada perlakuan pengenceran 50% MS dan 75% MS, serta penambahan manitol 20 g/l. Komposisi medium ini dapat dimanfaatkan untuk penyimpanan karika dieng selama 16 minggu tanpa sub-kultur. Konsentrasi medium MS 50% direkomendasikan untuk digunakan dalam penyimpanan C. pubescens selama 16 minggu tanpa sub-kultur.

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INTRODUCTION

Carica pubescens Lenne & K.Koch is a species belonging to the Caricaceae . Unlike *Carica papaja L.* (papaya) that grows and spreads in various regions, C. *pubescens* only grow in certain plateau. In Java, this species only grows optimally in Dieng Mountains, Central Java, therefore known as *dieng papaya* . At this time, *C. pubescens* is processed into various types of food and drinks, sweets, syrup, *dodol,* and it is potential to be developed into a leading commodity. However, the cultivation of *C. pubescens* is relatively limited compared to potato cultivation that is done very intensively in the area of Dieng. *C. pubescens* only grow on the roadside, land borders, or less productive land (direct observation 2008-2013). So, if the cultivation of *C. pubescens* is not developed optimally, it is concerned that this germplasm will be increasingly rare and extinct. Therefore it is necessary to do some conservation efforts because it is not cultivated specifically.

As endemic plant that produce recalcitrant seeds, the plant is suitable to be conserved in *in vitro* storage, especially with minimal growth technique. The technique is done by maintaining the plant material in a culture medium that inhibits the rate of plant growth and development. The success of *in vitro* storage method, according Uyoh et al. (2003) and Paunesca (2009) depends on the ability to 1) suppress plants' growth and development to extend the time interval between sub-cultures, 2) maintain the viability and stability of plant genetic material that is kept as much as possible, and 3) significantly save energy resource, time and cost in the conservation activities.

Culture conditions that allow minimal growth can be achieved through the use of not optimal culture medium and environmental conditions culture, by reduction of medium concentration, osmoregulator addition, plant growth inhibitor addition, and manipulation of environmental condition culture (i.e. decreasing of temperature and light duration). These factors can be combined. Planting medium recipes generally used in *in vitro* storage is Murashige and Skoog 1962 or MS (Uyoh et al. 2003). Medium nutrient reduction can be done by lowering the concentration to be ¾, ½, ¼, or even up to 1/10 of an optimal concentration. The optimal concentration for *in vitro* storage differs between one species to others (Gopal et al., 2002, Bermawie & Kristina 2003, Seswita et al., 2003, Tyagi et al., 2009, Yunpeng et al., 2012).

The giving of osmoregulator substance aims to reduce water potential in the culture medium. Osmoregulator substance often used in *in vitro* storage is sugar alcohol, such as mannitol and sorbitol. Those substances are high-weight molecular that dissolves in the water and will increase the concentration of the solution or decreasing the availability of water in the growing medium (Rajashekaran 2008, Paunesca 2009). As a result, the rate of diffusion of nutrients from the culture medium to plant material decreases, the need to grow is not sufficient and the growth declines. Osmoregulator using for *in vitro* conservation has also been widely reported, for example, the conservation of sugarcane (Sarwar & Siddique 2004), garlic (Hassan 2007), and *Coleus* (Dube et al., 2011). However, the storage medium for *C. pubescens* has never been developed.

This study aims to develop *C. pubescens in vitro* conservation medium with minimal growth techniques by nutrient concentration reduction and osmoregulator addition. Protocol of storage medium is useful as a basis for *C. pubescens* conservation as an alternative to *in situ* conservation done in the area of Dieng.

METHODS

The study was conducted in the Laboratory of Plant Tissue Culture, Department of Biology, Semarang State University, for 24 weeks. The plant materials were seedlings derived from *in vitro* germination of seeds. Epycotyl of 10 mm length was cut from seedling and used as explants.

The research was carried out experimentally by using completely randomized design of the nutrient concentration reduction and osmoregulator addition. Nutrient concentration reduction consisted of four level, i.e. concentration of 25%, 50% and 75% MS medium (Murashige and Skoog 1962), while osmoregulator addition consisted of five-level, they were mannitol 20%, mannitol 40%, sorbitol 20%, sorbitol 40%, and a mixture of sorbitol and mannitol consisting 20% of each substance. Osmoregulator addition was applied to the 100% MS medium. As the control 100% nutrient concentrations without the osmoregulator addition was used. Each treatment was repeated three times. Experimental units were five culture bottles each planted by one explant.

Conservation medium with various treatments made by standard techniques were poured into culture bottle size of 100 cc consisting 30 cc of each bottle. One epicotyl was planted into each culture bottle, then it was put randomly based on experimental design, in a closed incubation room

in temperature of 15 2 ° C with 40 watt light for 24 hours continuously. Partial cultures were maintained for 12 weeks, and some others were maintained up to 16 weeks with no sub-culture.

After storage for 12 and 16 weeks, the survival testing was done. In this case suvival is the explant ability to develop into complete plantlets. To test the survival, all epicotyls that have been maintained in the storage medium in the previous stage were transferred to the regeneration medium (MS + BA 2 mg / l) and root induction medium (MS + NAA 10 mg / l).

The survival was evaluated by observing the number of epicotyls that were capable to develop into normal plantlet with more than 10 mm height, consisting at least two open leaves and normal root. The parameters observed were the height of explants, the percentage of explants that formed the roots, the number of root and its length. An explant was declared formed roots when it formed at least three roots with length of ≥ 3 mm. The number of roots was determined by counting the number of roots with length ≥ 3 mm. In addition, morphological of plantlets include etiolation, browning and necrosis was also observed.

Quantitative data were analyzed by one-way Analysis of Variance and Duncan Analysis by using statistical analysis program of SAS System for Windows 9.0. Optimal conservation medium was determined by selecting the composition of the conservation medium resulting the growth of explants with minimum rate but was still be able to retain its survival to be normal plantlets.

RESULTS AND DISCUSSION

The nutrient concentration reduction and osmoregulator addition significantly affected the survival (visually it looks green and rigid) and height of epicotyls. After 12 weeks of storage, the percentage of survivable epicotyls in the MS nutrient concentration of 75% and 50% and the addition of sorbitol 20 % did not differ significantly from the control one (MS 100%), but in the other treatments of osmoregulator addition the percentage of survivable epicotyls were significantly lower than that the control. There was a tendency of the higher concentration of osmoregulator, the survivable epicotyl percentage decreases. The height of epicotyls of all nutrient concentration reduction and osmoregulator addition treatments were significantly lower compared to the control one (Table 1).

The nutrient reduction treatment also significantly influenced the survival and the height of explants after 16 weeks of storage. The survival of explants in the 75% medium concentration did not differ significantly from the control one, while in the 50% medium concentration and all treatments of osmoregulator addition was significantly lower than that the control. As in the 12-week storage period, the height of epicotyls on all treatments of nutrient concentration reduction and osmoregulator addition significantly lower than that the controls (Table 1).

All of epicotyls maintained in the 75% and 100% medium were able to grow, while in the 25% MS they were not able to grow (Table 1). Explants performance in the conservation medium of 75% MS (Figure 1B) was similar to the control (Figure 1A). Otherwise, in the medium of 50%, the chlorosis happened and some leaves fell (Figure 1C). This fact occurs as a result of non-optimal nutrient availability interferes with metabolic processes of plant, that turns to inhibit their growth. Growth is an expression of the integration of the various biochemical reactions, biophysical phenomenons and physiological processes in plant cells with external factors. Optimal plant growth can be achieved when environmental factors (for example nutrients) are in adequate number. If a factor is not balanced with other factors, this factor can reduce or even sometimes stops the growth of plants (Taiz & Zeiger 2010).

The observation results showed that the *C. pubescens* explants had relatively slow growth and proliferation properties, so it could be stored in concentration of 50% or 75% without sub-culture. This result is consistent with the research result on vanilla that can be stored in ¾ MS medium without sub-culture (Seswita et al. 2003); and the cardamom that can be conserved in the medium of ½ MS (Tyagi et al., 2009).

The addition of mannitol and sorbitol in many concentrations also affected the percentage of the growth of healthy explants (characterized by morphological that is fresh and green) and the height of epicotyls. The data showed that the higher of the osmoregulator concentration, the lower the value of both parameters. The shortest epicotyls resulted from the mannitol treatment of 20 g/l + sorbitol 20 g/l were not significantly different from mannitol treatment 40 g/l (Table 1). The osmoregulator addition is able to lower osmotic potential in the medium (Serraj & Sinclair, 2002) that slows the absorption of nutrients and lower the growth rate (Taiz & Zeiger, 2005). On two wheat genotypes, decrease of osmotic potential due to the osmoregulator addition negatively affected on the growth of callus (Javed & Ikram,

Table 1. The survival and the height of epicotyls in 12 and 16 weeks of conservation period from various conservation medium treatments

Conservation medium	Conservation periods			
	12 weeks		16 weeks	
	Survival percentage (%)	Height(mm)	Survival percentage(%)	Height(mm)
Nutrients concentration (%)				
100	100 a	33,4 a	100 a	36,4 a
75	100 a	22,2 b	100 a	24,6 b
50	87 ab	15,7 c	67 b	16,2 c
25	0d	0 e	0 d	0 f
Osmoregulator addition(g/l) on MS 100%				
mannitol 20	80 b	14,8c	53 c	16,0 cd
mannitol 40	67 c	12,5 cd	53 c	13,1 de
sorbitol 20	87 ab	15,6 c	53 c	16,2 cd
sorbitol 40	67 c	13,2 cd	53 c	14,3d
manitol 20 + sorbitol 20	67 c	11,5d	33 d	13,0 de

* Numbers followed by the different letter in a column indicate significantly different based on Duncan test with significance level of 5%

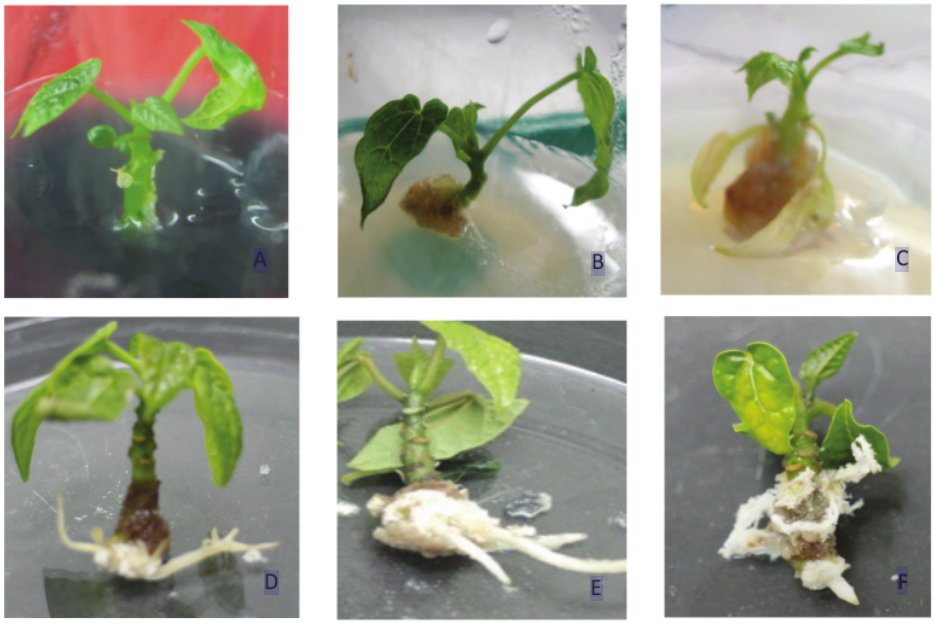

Figure 1. Performance of explants in the conservation medium and after root induction. Explant in conservation medium of 100% MS (A), 75% MS (B) and 50% MS (C). Roots formed from explants were stored in the conservation medium of 100% MS (D), 75% MS (E) and 50% MS (F).

2008).

Several previous studies have also concluded that the addition of osmoticum material in the medium can inhibit the growth of explants. Medium added by 0.1M sorbitol inhibits the growth of shoots, root length, and the number of garlic roots (*Allium sativum* L.) cv *peds-40* which was stored for 18 months. During the storage time there are no seedling growing, then after 18 months the percentage of survived tubers reach

up to 100% (Hassan, 2007). Research result of Dube et al. (2011) showed that the optimal concentration of mannitol for *Coleus forskohlii* Briq. storage is 4 M.

To determine the effectiveness of the conservation medium, epicotyls that had been grown in the conservation medium during a certain time was tested its growing potency by planted it in regeneration medium, i.e. MS + BA 2 mg / l for 4 weeks. Growing potency was shown by the growth of stems and leaves, both in quantity and morphology. Result showed that the decreasing of the nutrients availability in the conservation medium affected the growing potency after being transferred to the regeneration medium. Treatment of nutrient concentration decreasing of 75% and 50% did not result in decreasing growing potency in the regeneration medium, either after the storage of 12 weeks or 16 weeks. This was indicated by the parameters of increasing of height and leaves after being maintained in the regeneration medium; both those parameters did not differ significantly between the treatment of nutrient concentration of 100%, 75% and 50%. The addition of 20% mannitol and 20% sorbitol resulting in increasing of height and leaves was not different from the control one; whereas another treatment significantly lowered both parameters (Table 2). The results showed that the concentration of nutrients conservation medium of 75% and 50%, and the addition of 20% mannitol and 20% sorbitol did not reduce the growth potency or regeneration ability after the plantlet was taken from conservation medium.

Epicotyls successfully grew, were induced to form roots maintaining them in the medium of MS + 10 mg / l NAA for 4 days, then they were transferred to MS medium without PGR for 1 week. Result showed that the lowering of nutrient absorption in the conservation medium affected the root growth after being transferred to root induction medium. The treatment of nutrient concentration of 75% and 50% did not decrease the ability of explants to form roots, both of after the storage of 12 and 16 weeks. This statement was indicated by the parameters of explants percentage that can form roots and the average number of formed roots. There was no significant difference in those parameters of the treatment of nutrient concentrations of 100%, 75% and 50% (Table 3, Figure 1D, 1E, 1F).

Osmoregulator addition inhibited root formation; epicotyls percentage that was able to form roots and number of root of all treatment of osmoregulator addition was significantly lower than the control, except for the addition of 20% mannitol and 20% sorbitol for 12 weeks. Epicotyls stored for 16 weeks in the various treatment of osmoregulator addition was able to form roots and number of root was significantly lower than that the control, except for the addition of 20% mannitol (Table 3).

Reduction of nutrient concentration of 75% and 50% significantly decreased the height of epicotyls during conservation period (Table 1), but when it was returned to the regeneration medium epicotyls grew, formed normal leaves (Table 2) and roots (Table 3). This condition did not

Table 2. Epicotyls growth response after being conserved for 12 and 16 weeks in regeneration medium.

Conservation medium	Conservation period			
	12 weeks		16 weeks	
	Height (mm)	Leaves number	Height (mm)	Leaves number
Nutrients concentration (%)				
100	9,2 [a]	2,3 [a]	11,4 [a]	2,0 [a]
75	10,0 [a]	2,7 [a]	10,6 [a]	2,3 [a]
50	10,2 [a]	2,0 [a]	11,2 [a]	2,3 [a]
25	-	-	-	-
Osmoregulator addition (g/l) on MS 100%				
mannitol 20	9,8 [a]	2,3 [a]	6,0 [b]	2,3 [a]
mannitol 40	3,5 [c]	0,7 [c]	2,1 [d]	0,0 [c]
sorbitol 20	7,6 [ab]	1,7 [b]	6,2 [b]	2,7 [a]
sorbitol 40	2,2 [d]	0,0 [d]	3,3 [cd]	1,0 [c]
mannitol 20 + sorbitol 20	1,5 [d]	0,3 [d]	3,0 [d]	0,0 [c]

* Numbers followed by the different letter in a column indicate significantly different based on Duncan test with significance level of 5%

Table 3. The growth of root after being conserved for 12 and 16 weeks in root induction medium

Conservation medium	Conservation period			
	12 weeks		12 weeks	
	Percentage of rooted epicotyls	Number of root	Percentage of rooted epicotyls	Number of root
Nutrients concentration (%)				
100	100 [a]	6,6 [a]	100 [a]	5,3 [a]
75	100 [a]	6,1 [a]	100 [a]	5,8 [a]
50	100 [a]	5,5 [a]	100 [a]	4,9 [ab]
25	-	-	-	-
Osmoregulator addition (g/l) on MS 100%				
mannitol 20	100 [a]	6,3 [a]	85 [b]	5,4 [a]
mannitol 40	40 [c]	4,0 [b]	30 [c]	3,3 [c]
sorbitol 20	87 [b]	5,5 [a]	50 [c]	3,4 [c]
sorbitol 40	40 [c]	3,7 [b]	40 [c]	3,2 [c]
mannitol 20 + sorbitol 20	30 [c]	3,1 [bc]	40 [c]	2,4 [cd]

* Numbers followed by the different letter in a column indicate significantly different based on Duncan test with significance level of 5%

significantly different with the control. Based on the data it can be stated that the nutrient concentration of 75% or 50% MS was effectively used for *in vitro* conservation of *C. pubescens* for 16 weeks with no sub-culture, so it can save energy and cost, while negative influence of genetic such as somaclonal due to frequent sub-culture can be avoided.

Osmoregulator addition with concentration total of 60%, 40% and 20% resulted in the withered of explant of 100%, 33%, and 13-20%, respectively, in 16 weeks conservation period; and 100%, 67% and 47%, respectively, in 12 weeks conservation period (Table 1). The survival after the conservation period had the same tendency. The survival of explants grown in medium added by 20% mannitol was not significantly different from the control (Table 2, Table 3). This result is in line with the finding on pepper (*Capsicum chinense* Jacq.), where the addition of 2% mannitol results minimum growth of plantlets and it does not negatively affect its physiology and quality; whereas the addition of sorbitol decreases the quality of plantlets (Montalvo-Peniche et al. 2007).

Because osmoregulator compounds can inhibit the growth of explants and in certain concentration it can maintain the explants growing potency, it can be stated that the addition of osmoregulator can be used as a compound of efficient *in vitro* conservation. Osmoregulator additon also has many advantages, such as stored culture is alive and growing very slow, so it can save people work for sub-culturing and save the cost of medium production. Besides, the decreasing of growth keep viability and genetic stability , because somaclonal variation can be avoided. Somaclonal variation can occur when cultures are sub-cultured repeatedly (Pontaroli & Camadro 2005). Therefore the recommended addition of osmoregulator for *in vitro* conservation of *C. pubescens* is 20% mannitol concentration.

CONCLUSION

Based on the research it can be concluded that nutrient concentration reduction of 75% and 50% of the MS basic formulation and mannitol addition of 20g/l decreased the growth of *C. pubescens* in *in vitro* conservation for 16 weeks, and maintained its survival after being returned to the regeneration medium. Based on the results, the nutritional composition of 50% and 75% of MS as well as the addition of mannitol 20 g/l on MS medium can be used for *in vitro* conservation of *C. pubescens* without sub-culture for 16 weeks. Medium concentration of 50% MS is recommended for storage *C. pubescens* efficiently.

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