American Journal of Biochemistry and Biotechnology

Original Research Paper

The Effect of Explant Type and 6-Benzyl Adenine (BAP) in Sapodilla (*Achras zapota*) Micropropagation

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Article history
Received: 22-08-2016
Revised: 28-09-2016
Accepted: 29-09-2016

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Abstract: Sapodilla (*Achras zapota*) is one type of tropical fruits with high economic value that are easily found in Indonesia. However, the number of productive plants has been decreased and affected the rate of fruit production. The objective of this research is to develop in vitro propagation of *Achras zapota* through meristem induction to provide great quality seedling. Type of explants (apical and lateral meristem) are cultured on Woody Plant Medium (WPM) and combined with different concentrations of BAP (0 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm). Variables observed are shoot, leaf, root (emergence, length, number) and callus formation. The results show that all treatments able to induce shoot and leaf, but only several treatments able to induce root and callus formation. Lateral meristem is an appropriate explant to induce high number of leaves significantly and lateral meristem has the highest rate of multiplication 1.7 with application of BAP 2 ppm. The results can be used as reference to promote shoot multiplication. But further research is needed to determine proper combination to induce shoot multiplication significantly.

Keywords: Sapodilla, Tissue Culture, Shoot Multiplication, Lateral Meristem, BAP

Introduction

Sapodilla (*Achras zapota*) is one type of tropical fruits with high economic value species of the *Sapotaceae* family native to Mexico and Central America and has been introduced to many tropical areas of the world (Morton, 1987).

The tree is has an extensive root system, it reaches a height of more than 30 m and a diameter of up to 1.5 m (Orwa *et al*., 2009). The canopy is dense, generally with a rounded crown, ovate-elliptic to elliptic-lanceolate leaves, which are glossy and dark green and clustered at the tips of the branches (Mickelbart, 1996). The fruit is a fleshy berry, ellipsoidal, conical or oval and contains one or several shiny black seeds. Its weight is about 70 to 300 g and its size ranges from 5 to 9 cm in diameter. Immature fruit are hard, gummy and very astringent (Morton, 1987).

According to Peiris (2009), sapodilla has been used for many purposes including:

- Sapodilla is primarily a source of the latex called chicle. This kind of latex has been produced on a commercial scale in Mexico and certain parts of Central America.
- Sapodilla wood is very strong and durable. It is very good to make furniture
- Sapodilla has tannin content which is very good for medicinal ingredient. Such as:
    - Boiled and decoction of young fruits can be used to stop diarrhea.
    - An infusion of young fruits and flowers can be drunk to relieve pulmonary complaints.
    - A decoction of old, yellowed leaves can be used to remedy for coughs, colds and diarrhea.
    - "Tea" of the bark can be used to halt diarrhea and dysentery.
    - The crushed seeds have a diuretic action and are claimed to expel bladder and kidney stones.

On the basis of the above quotation, it is very valuable to grow Sapodilla in more economic and productive way. This is the main objective of this study in the long run. That is develop *in vitro* propagation of Achras zapota through meristem induction to provide great quality seedling.

Table 1. Total of productive plants and fruits production

Year	Plant	Productive plant	Fruit production
2013	3,714	2.265	1.344 quintals
2014	4,931	2.267	1.924 quintals

Kepuh Sari Village, Manyaran, Wonogiri, Central java is one of Sapodila producer area in Indonesia. According to Central Bureau of Statistic survey (SCBWR) 2013-2014, the total of Sapodilla plants increase significantly from 2013 to 2014, but the production is decreasing. The complete data is presented in the following table.

Table 1 shows clearly that the increase of Sapodilla plants in number does not correspond to the increase in productivity. According to the author's observation, this is due to lack of intensive maintenance and the use of bad quality of seedling.

A proper breeding technique is therefore needed to provide high quality seedling. Vegetative propagation is one alternative. This technique is able to produce seedlings which are genetically identical with the productive mother plant.

Tissue culture is a popular method for vegetative propagation of plants. The most significant advantage of this method for clonal propagation is that it can be conducted in a relatively short time and limited space. Moreover, a large number of plants can be produced starting from a single individual (Bhojwani and Razdan, 1996). Apical and lateral meristem are chosen as a single individual. Those are cultured on Woody Plant Medium (WPM). Because of some woody plant species have salt sensitivity. To counteract it, Lloyd and McGown (1980), developed the Woody Plant Medium (WPM). Application of BAP is needed as a plant growth regulator to promote cell division and shoot growth.

Materials and Methods

Plant Materials:

- *Achras zapota* seedlings are collected from Kepuh Sari Village, Manyaran, Wonogiri, Central Java
- The seedlings are raised in the screen house of Sebelas Maret University, as the experiment logistic, for about two months
- Taking nodes (apical and lateral meristem). The nodes are cleaned thoroughly using disinfectant solution and then washed under running tap water
- Immersing the nodes into bactericide and fungicide solution for 15 minutes and then washed under running tap water
- The nodes then are briefly rinsed with sterile distilled water for three times in Laminar Air Flow (LAF). Surface sterilization of the nodes are carried out aseptically by immersing in 20% Clorox for 45s and 70% alcohol for 15s.
- The nodes are immersed by ascorbic and citric acid solution for 5 min

Culture Condition

The nodes are cultured in the Woody Plant Medium (WPM) with various concentration of BAP (0 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm). For each experiment, there are 3 replicates and repeated 3 times. These samples are moved to the culture room and placed on the shelf. The culture room is maintained at 24°C by regulating the room air conditioner. White fluorescent irradiate the samples on shelf for 24 h.

Shoot, Leaf, Root and Callus Induction

The variables are measured every day since the first until ninety days after planting.

Statistical Analysis

The data are analyzed using descriptive analysis because the value of normality test is below 5% while the number of leaf is analyzed using variance analysis by F test level at 5% level of probability. The variance analysis of leaf number shows that the explant significantly affect on leaf number and continued by descriptive analysis. This is due to Post hoc tests are not performed for explant because there are fewer than three groups. SPSS software version 23.0 (SPSS Inc. USA) is used to compute the collected data.

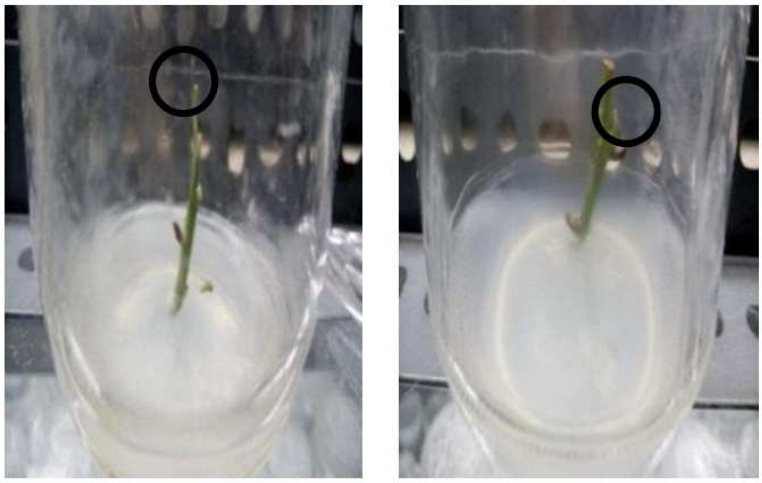
Results

Shoot Induction

Apical shoot grows in the growing tip (Fig. 1A) and lateral shoot grows along the sides of a shoot (Fig. 1B). The result shows that the mean of lateral shoots emerges faster than apical shoots (Fig. 2). Apical shoot is induced on apical meristem without BAP application (17.7 days after planting) and lateral shoot is induced on lateral meristem + BAP 4 (15.7 days after planting). This result indicates that BAP application promote axillary shoot formation.

The highest mean of lateral shoot length on lateral meristem + BAP 3 ppm is 1.3 cm (Fig. 3B), while the highest mean of apical shoot length on apical meristem + BAP 5 ppm is 1,2 cm (Fig. 3A). The highest mean of apical shoot is little bit lower than lateral shoot. This is due to the concentration of cytokinin is not able to inhibit apical dominance due to high endogenous auxin in apical shoot.

Shoots number is one of important parameter in determining the success of shoot multiplication. Based on Fig. 4, apical and lateral meristem have highest shoot multiplication rate are 1.7 that treated with application of BAP 2 ppm (Fig. 4). But this result is belong to low shoot multiplication rate. High shoot multiplication rate can be achieved at the proper concentration of cytokinin to stimulate cell division.



(A) (B)

Fig. 1. Shoot emergence of (A) apical shoot and (B) lateral shoot

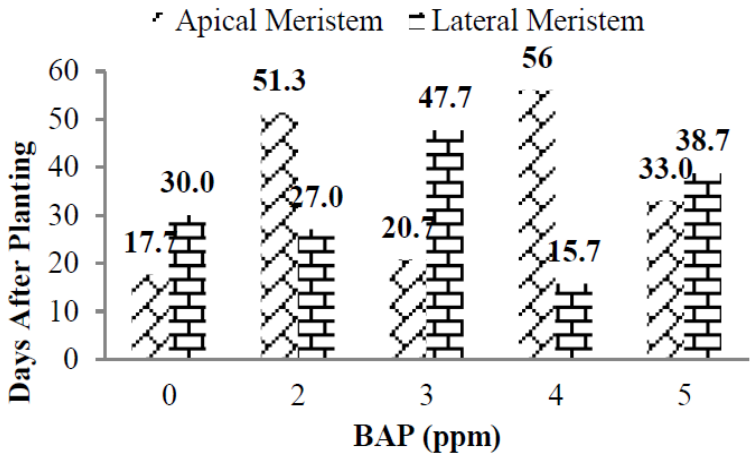
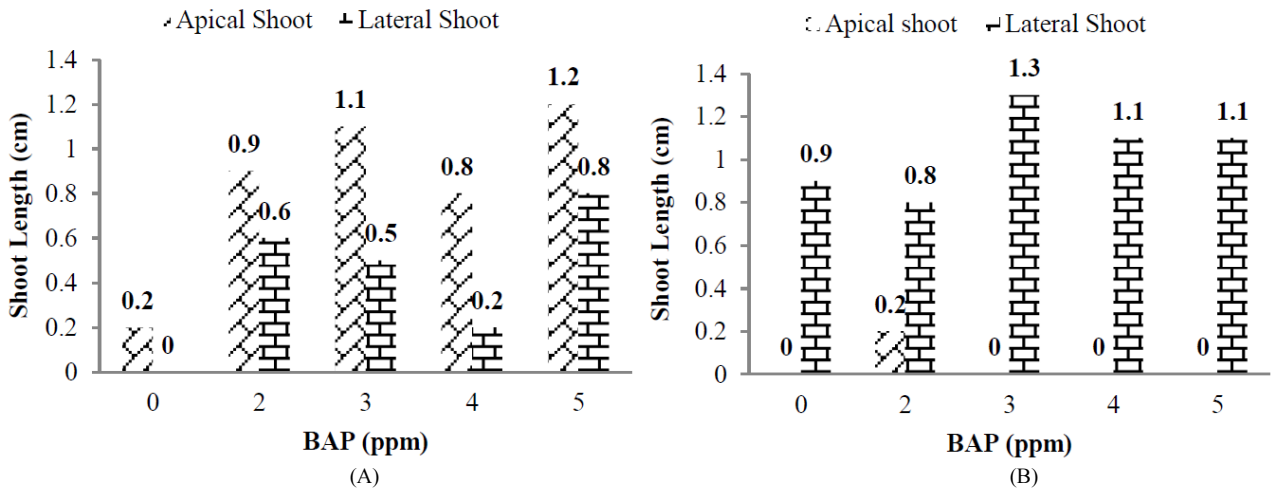


Fig. 2. The effect of explant type and BAP in sapodilla toward shoot emergence (days after planting)



(A) (B)

Fig. 3. The effect of explant type and BAP in sapodilla toward shoot length (cm) using (A) apical meristem and (B) lateral meristem)

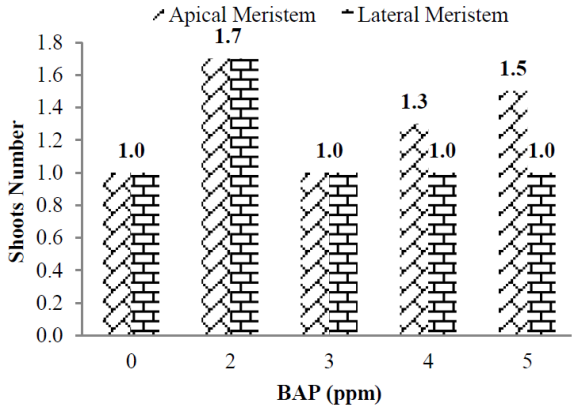


Fig. 4. The effect of explant type and BAP in sapodilla toward number of shoot

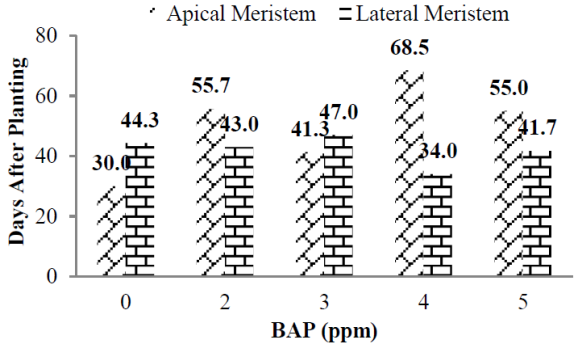


Fig. 5. The effect of explant type and BAP in sapodilla toward leaf emergence (days after planting)

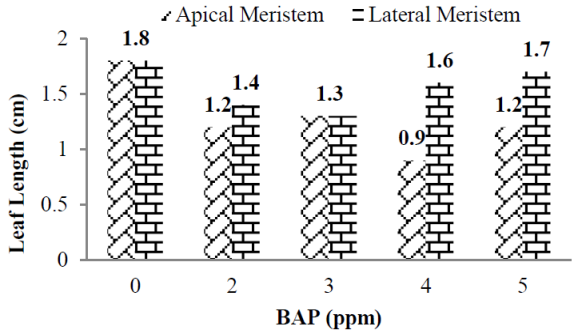


Fig. 6. The effect of explant type and BAP in sapodilla toward leaf length (cm)

Leaf Induction

Leaf emergence is one of variables observed that indicate the occurrence of organogenesis. Based on the Fig. 5, shows that on apical meristem without application of BAP has the fastest mean of leaf emergence is 30 days after planting. This is due to the endogenous auxin and cytokinin in apical meristem are able to stimulate organogenesis.

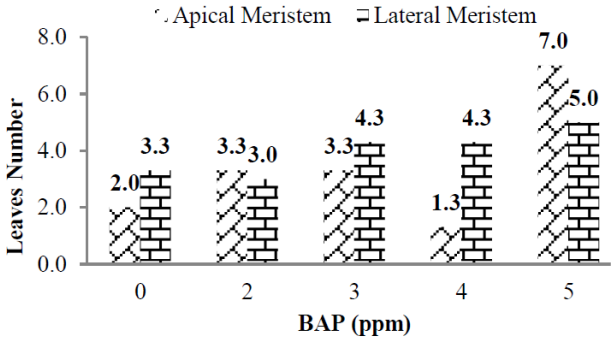


Fig. 7. The effect of explant type and BAP in sapodilla toward leaves number

(A) (B)

Fig. 8. Main root emergence on treatment combination of (A) M1B2 and (B) M2B3

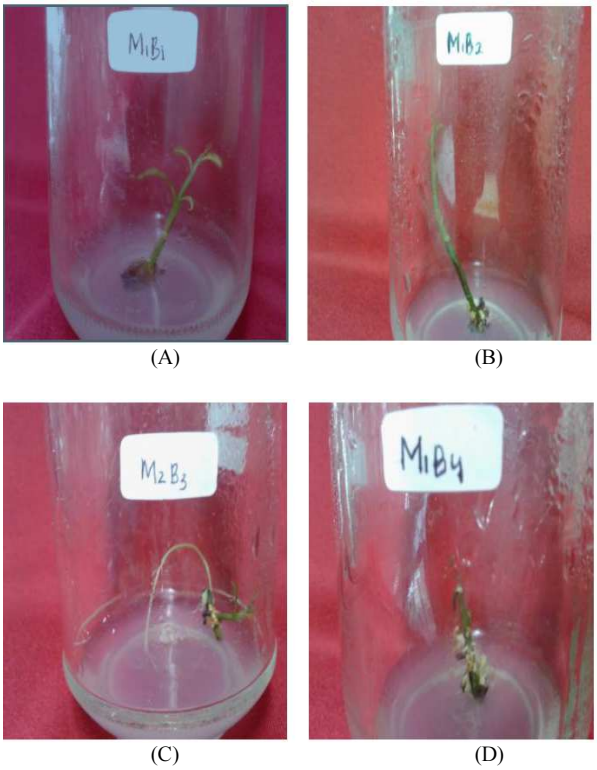
Table 2. Variance analysis of leaves number

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Explant	17.633	1	17.633	4.975	0.039*
BAP	6.200	4	1.550	0.437	0.780^{ns}
Block	0.867	2	0.433	0.122	0.886^{ns}
Explantx BAP	10.867	4	2.717	0.766	0.561^{ns}
Error	63.800	18	3.544		
Total	413.000	30			

Information: * 0.01-0.05 significant; ** <0.01 very significant; ns > 0.05 non significant

Leaf length is measured using graph paper from base to the tip leaf. The highest mean of leaves length, without application of BAP, either for apical or lateral meristem is 1.8 cm (Fig. 6). It is assumed that endogenous auxin and cytokinin ratio effect on leaf emergence and length.

Based on the variance analysis (Table 2), type of explant affect on leaves number significantly. However, application of BAP and combinatian between explant type and BAP application do not affect on leaves number significantly. Figure 7 shows clearly that lateral meristem has highest mean of leaves number.



(A) (B) (C) (D)

Fig. 9. Induction of (A, B, C) embryogenic callus and (D) non-embryogenic callus

On lateral meristem with application of BAP 5 ppm able to induce more leaves than other treatments.

Root Induction

The main root only induced on two combinations treatment. It is due to application of exogenous cytokinin generally inhibit root formation whereas in certain research has been founded that promote root induction of *Arabidopsis*. Apical meristem with application of BAP 3 ppm take 65 days after planting to induce root with 1.5 cm in length (Fig. 8A). Lateral meristem with application of BAP 4 ppm take 67 days after planting with 6.2 cm in length (Fig. 8B). It is assumed that an sufficient amount of endogenous auxin able to promote root formation. So that no need for application of exogenous auxin eventhough will take longer time.

Callus Induction

Callus formation is one of plant growth indicator in vitro. There are two types of callus formed during this research. First, embryogenic callus is callus which have potential to regenerate into shoot, leaf and even root through organogenesis. This callus only induced on apical meristem with application of BAP 2 ppm (Fig. 9A), apical meristem with application of BAP 3 ppm (Fig. 9B) and lateral meristem with application of BAP 4 ppm (Fig. 9C). Embryogenic callus is appeared in the base of explant.

Second, non embryogenic callus is callus which have no ability to regenerate into plant that only induced on apical meristem with application of BAP 5 ppm (Fig. 9D) is appeared in the explant surface. The color of embryogenic callus generally yellowish white to yellow, while non embryogenic callus is white. The compact callus has a dense texture and hard, which is composed of tiny cells that are very tight as embryogenic callus. A soft texture is composed of cells with chamber intercellular is non embryogenic callus.

Discussion

Shoot multiplication can be considered as the "standard methodology" of micropropagation. The method starts with growing shoot tip and uses media with high cytokinin concentrations to promote growth and to overcome apical dominance (Einset, 1986). Vegetative plant parts especially leaves, nodes and shoot tips are desirable explants for *in vitro* cultures because plant regeneration from these explants would preserve the genetic identity of the parent genotype (Sridhar and Naidu, 2011). The explant can be derived from apical meristem is located at the tip of a shoot and measures 0.1 mm in diameter and 0.25 to 0.30 mm in length. Apical meristem culture has recently become an important technique for virus elimination in plant tissue culture (Grout and Brian, 1999). Meanwhile, shoot tips and nodal culture were shown able to produce pathogen-free plants (Morel, 1960; Senula *et al*., 2000; Quak, 1977) and has led to a large scale propagation and improvement of tree species (Bajaj, 1986; Boulay, 1987).

The successful of micropropagation is organogenesis occurence. Organogenesis is formation and development of organs such as shoot, leaf and root either directly from the explants or via callus differentiation. In this research, the first organ has been developed is shoot. Lateral shoot emerges faster than apical shoot (Fig. 2). This is due to BAP is the most effective class of cytokinin. It is suitable to be applied because it is cheapest, more stable and able to be transported by plants without degradation due to oxidation of cytokinin. Application of exogenous cytokinin, capable of activating the lateral buds under the influence of apical dominance and proven effective in stimulating metabolic activity and growth of shoots on woody plants for example in woody plant species such as *Picea abies*, *Abies balsamea*, *Macadamia teteraphylla*, *Pinus strobus* and *Pinus sylvesteris* (Bhojwani and Razdan, 1996; Jafari *et al*., 2011: Eldoma *et al*., 2015). Nagarathna *et al*. (2010) explain that, endogenous cytokinin is increase in the axillary node which is far from the top and application of exogenous cytokinin will encourage rapid shoot proliferation.

Cell elongation occurs because enlargement process and division of new cells. This process occurs in meristem tissue (Gardner *et al*., 1985). Tissue response

of shoot lengthening and plantlet proliferation belongs to the effect of BAP (Reddy *et al*., 2011). This is in line with Jafari *et al*. (2011), that combination of cytokinin and auxin in proper ratio is very effective in stimulating shoot elongation. The result has been shown in Fig. 3 that the different mean of shoot length between lateral and apical shoot is 0,1 cm. Lateral shoot length little bit longer than apical shoot length.

BAP is considered to be one of the most useful cytokinin for achieving the multiplication and micropropagation of plants and showed highest effect in respect of multiplication of axillary buds (Martin 2002; Joshi and Dhar, 2003). That statement corresponds with the result in Fig. 4 that application of BAP 2 ppm has the highest shoot multiplication rate. Tyagi and Tomar (2013) reported that apical and basal portion of *Tecomella undulata* produced more or less same 2 fold compare to middle part 1.3 fold. Meanwhile, Purohit and Singhvi (1998) reported that, application of BAP 2 ppm on SH medium able to stimulate highest shoot multiplication rate of *Achras zapota* with six shoots per node. This shoot multiplication rate is three times higher than shown in Fig. 4. It is assumed that physiological age of explant and diffrent composition of culture medium affect on shoot multiplication rate. On the other hand, higher concentration of BAP decline and stagnation of shoot number. Jafari *et al*. (2011) reported that, high concentration of BAP after bud initiation is not essential for shoot propagation due to cause reduction in shoot number and high incidence of abnormality.

Shoot meristems produce leaves on their flanks called phyllotaxy. Meristem cells divide and replace the cells that have just been committed to initiating a leaf primordium. The regular pattern of leaf initiation allows one to predict where the next leaf will appear (Micol and Hake, 2003). Leaf formation is indicate that organogenesis has been occured. George *et al*. (2008) report that, appropriate balance ratio of auxin and cytokinin are effective in triggering organogenesis especially for leaf formation. The leaf will expand through cell enlargement and cell division process are closely related to cytokinin (Gardner *et al*., 1985). According to George *et al*. (2008), auxin plays a role in cell elongation whereas cytokinin play a role in cell division, so that combination both of them able to affect leaf length. In addition, the nutrient contained on the media can be optimized by explant such as nitrogen, phosphorus and potassium are needed in photosynthesis which affect on leaf elongation (Hossain *et al*., 2010). The statement above, in line with the results are shown in Fig. 5 and 6. The highest mean of leaves number on lateral meristem with application of BAP 5 ppm on WPM has same result were observed by Rostika *et al*. (2005). On the medium MS + BA 5 mg/l able to induce highest shoot and leaf number in mangosteen. Endogenous cytokinin on lateral meristem able to inhibit apical dominance and stimulate axillary buds proliferation. Increasing number of axillary buds are directly proportional to increase the number of leaves.

Root is a main vegetative organ which supplies water, mineral and materials that are essential for plant growth and development (Samanhudi, 2010). According to Lopez-Bucio *et al*. (2003), in higher plants, the root system consists of main, lateral and adventitious root. Adventitious root is formed from the stem or hypocotyl and lateral root is formed from the main root. Only main root emerges on two treatments combination. According to Li *et al*. (2006), application of singly BAP inhibits lateral root formation. But due to auxin is abundant in young leaves, floral organs and developing fruits and seeds able to stimulate root formation (George *et al*., 2008). According to Cheng *et al*. (1992), the ability of root formation is not affected by explant source. The different biochemical or physiological content on each explant is triggers factor of root formation. As reported by Gomez *et al*. (1995) that, the peroxide activity in apical shoot where the activity is related to cell division and differentiation cambium are essential process of root formation.

The callus is developed at surface damaged by excision. According to Gill *et al*. (2004), callus formation occurs when plant tissue get injured. In that scar, absorb more nutrients that lead to cell division fastly then followed by callus formation. Auxin and cytokinin are the key factors to determine the embryogenic response due to their pervasive participation in the cell cycle regulation and cell division (Francis and Sorrell, 2001). The results from the present study, also shown that BAP is essential for inducing embryogenic callus from petioles and nodal segments of *J. Curcas* (Kumar *et al*., 2015). The nutrition components of medium also affect on callus induction. There are some nutrients only found in WPM medium while they are lacking in MS medium such as calcium nitrate, potassium sulfate and mangan sulfate (Saad and Elshahed, 2012). Those nutrients are likely to be the factor which influence the responses of explants towards the formation of callus (Osman *et al*., 2016). In the present study, the effects of using WPM medium, in bay leaf tree (*Cinnamomum tamala*) by Sharma and Nautiyal (2009) reported that almost all explants and plant growth regulator treatments produced compact callus and also by Avil´es *et al*. (2009) in Juglans regia L even in endosperm explant of *Barringtonia racemosa* L by Osman *et al*. (2016) Non embryogenic callus generally has lost regeneration capacity. But the present investigation reveals that non embryogenic callus can be turned into embryos and plantlets if cultured on appropriate medium. Bibi *et al.* (2011) reported that, *Centella asiatica* non-embryogenic callus can be converted into embryogenic and shoots when cultured on appropriate concentrations of BA and NAA in MS medium.

Conclusion

In this research, conclude that:

- All treatments able to induce shoot and leaf, but only several treatments able to induce root and callus formation.
- Lateral meristem is an appropriate explant to induce high number of leaves significantly
- Lateral meristem has the highest rate of multiplication 1.7 with application of BAP 2 ppm
- This result can be referenced to promote shoot multiplication in order to provide great quality seedling but still need further research

Acknowledgement

The authors are thankful to Mr. Sutiman in Kepuhsari Village, Manyaran, Wonogiri for providing sapodilla seedling.

Funding Information

The author's sincere thanks to PUPT DP_2M, Ministry of Research, Technology and Higher Education 2015-2016 as a single funder for this research.

Author's Contributions

All authors equally contributed in the preparation, development and publication of this manuscript.

Ethics

This article is original research paper. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved after the publication of this manuscript.

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