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Effect of BAP and 2,4-D on callus induction of *Jatropha curcas* in vitro

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Abstract. *Andaryani S, Samanhudi, Yunus A. 2019. Effect of BAP and 2,4-D on callus induction of* Jatropha curcas *in vitro. Cell Biol Dev 3: 56-65.* The aim of this study was to obtain appropriate concentrations of Benzyl Amino Purine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) to increase callus induction in vitro using shoot explants of *Jatropha curcas* L. The research was conducted at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia, from July to August 2010. The study used a completely randomized design (CRD) with two treatment factors and three replications. The first factor is the level of BAP concentration, namely: 0.5 ppm, 1 ppm, 1.5 ppm, and 2 ppm. The second factor is the concentration of 2,4-D, namely: 0 ppm, 0.25 ppm, 0.5 ppm, and 0.75 ppm. Observation variables included callus emergence time, callus color, callus texture, root emergence time, number of roots, shoot emergence time, number of shoots, leaf emergence time, number of leaves, and callus fresh weight. Qualitative data are presented descriptively. Quantitative data includes callus fresh weight data, which was analyzed for variance based on the 5% F test and continued with the DMRT test at the 5% level. The results showed that the combination of 2 ppm BAP treatment and 0.25 ppm 2,4-D was the fastest in inducing callus, 5.67 Days After Planting (DAP). All the resulting calluses have a crumb texture. The average callus color in all treatment combinations was yellowish-green. Only the treatment combination of 1 ppm BAP without 2,4-D could produce roots at 9 DAP. The combination of 0.5 ppm BAP treatment and 0.25 ppm 2,4-D was the fastest to produce shoots, 6 DAP. The fastest leaf emergence was obtained in the 1 ppm BAP treatment without 2,4-D, 11 DAP. The combination of 2 ppm BAP treatment and 0.5 ppm 2,4-D resulted in the largest callus fresh weight (2.56 g).

Keywords: Benzyl Amino Purine, BAP, callus, dichlorophenoxyacetic acid, 2,4-D, in vitro, *Jatropha curcas*

INTRODUCTION

The need for fuel in Indonesia has recently been increasing along with the increase in oil-fueled transportation and other machines that use oil fuel. Now, Indonesia is no longer an exporter of petroleum but only an importer, especially from Arabia (Sumanto 2005). For this reason, it is necessary to find alternative sources and Jatropha (*Jatropha curcas* L.) is one of the plants that have the potential as a source of biofuel.

As a biofuel, Jatropha has several advantages compared to other plants. First, this plant has only a few limited functions, so competition for its use is also limited. In addition to being environmentally friendly, jatropha oil is not an edible oil, so the price of raw materials is lower, and it is not considered food (Prastowo 2007).

According to Syah (2006), the main problem in developing biodiesel from jatropha oil is the availability of raw materials, which is still very low, considering that the plantations have just been developed. Therefore, it is necessary to accelerate the productive Jatropha cultivation business to meet the raw material needs of the national biodiesel industry.

Along with the increasing demand and need for Jatropha plant material, it is necessary to make efforts to propagate plants in large quantities and in a short time. The provision of superior seeds is one of the factors supporting the success of Jatropha development. However, conventional propagation of plants is still limited by the ability of plants to produce new seeds in large quantities, uniformly, and in a short time. Until now, Jatropha seedlings have been produced in two ways: seeds and cuttings. However, the jatropha propagation business using cuttings or seeds has problems. Using seeds for plant propagation in large quantities will reduce the number of seeds processed into oil. In addition, the propagation technique through cuttings requires many parent trees. At the same time, the availability of parent trees is very limited, and there are also concerns about damage to the parent trees (Lizawati et al., 2009).

Cultivating tissue culture (in vitro) is necessary to overcome the problem above. Plant tissue culture is a technique for growing plant parts, either in the form of cells, tissues, or organs, under aseptic culture conditions in vitro (Yusnita 2004). Therefore, propagation by tissue culture will offer a great opportunity to produce large numbers of seeds in a relatively short time. In addition, tissue culture can maintain superior parental characteristics and produce seeds free of fungi, bacteria, viruses, and pests (Prihandana and Hendroko 2006).

The principle of this tissue culture technique is that all plant parts, whether in the form of cells, tissues, or organs, could become newer plants if grown under aseptic conditions in a sterile manner. Therefore, Jatropha tissue culture techniques will work well if the conditions are met. These techniques include the selection of explants as planting material, using a suitable medium, aseptic conditions, and good air regulation (Hendaryono and Wijayani 1994).

One of the factors influencing the success or failure of procuring Jatropha seedlings through tissue culture is the presence of growth regulators (ZPT). However, the hormone content in plants must also be considered. Hormones in plants are also called phytohormones. According to Pierik (1987), phytohormones are compounds produced by higher plants endogenously. These compounds play a role in stimulating and increasing the growth and development of plant cells, tissues, and organs towards a certain direction of differentiation. Other compounds with the same characteristics as hormones but are produced exogenously are known as PGR. Wetter and Constable (1991) suggested that one of the most frequently used compounds to induce cell division is 2,4-dichlorophenoxyacetic acid (2,4-D). In in vitro cultivation, callus induction is an important step. Suppose the endosperm of dicot plants is used, and hormones from the auxin group, namely 2,4-D or IAA, are added to the medium. In that case, hormones from the cytokinin group, namely kinetin or Benzyl Amino Purine (BAP), must be added (Suryowinoto 1996).

This study aimed to obtain the appropriate concentrations of BAP and 2,4-D to induce callus of Jatropha in vitro.

MATERIALS AND METHODS

Research material

The plant material used as explants was shoots of *J. curcas,* derived from sterile germinated seeds.

Research design

This study used a completely randomized design (CRD) arranged in a factorial manner with two treatment factors. The first factor was the concentration of BAP consisting of 4 levels, namely: B1 = treatment with the addition of 0.5 ppm BAP, B2 = treatment with the addition of BAP 1 ppm, B3 = treatment with the addition of 1.5 ppm BAP, B4 = treatment with the addition of 2 ppm BAP. The second factor is the concentration of 2,4-D consisting of 4 levels, namely: D1 = treatment without the addition of 2,4-D (0 ppm), D2 = treatment with the addition of 2,4-D 0.25 ppm, D3 = treatment with the addition of 2,4-D 0.5 ppm, D4 = treatment with the addition of 2,4-D 0.75 ppm. Thus, 16 treatment combinations were obtained, each repeated three times.

Research procedure

Tool sterilization

The equipment for this research were culture bottles, scalpels, petridish, and tweezers. First, they were washed using laundry soap, rinsed, then dried. Next, the drying equipment was wrapped in newspaper (except for culture bottles). Finally, all types of equipment were sterilized by autoclave at a temperature of 121°C and a pressure of 1.5 Psi (kg/cm^2) for 45 minutes.

Stock solution preparation

The stock solutions were prepared by weighing the chemicals, macronutrients, micronutrients, and PGR according to the composition of MS media (Appendix 1). These materials were dissolved with sterile distilled water and then stirred until completely homogeneous using a magnetic stirrer. The materials were then put into bottles, labeled according to the treatment, and stored in the refrigerator.

The making of planting media

The planting media was made by taking and measuring each stock solution according to the treatment and the determined size and then putting it into a measuring flask. These materials were dissolved with aquadest until the volume of the solution reached 250 mL (¼ liter) and added with 7.5 g sugar. After that, the solution was put in a glass beaker and stirred using a magnetic stirrer. The solution was conditioned at pH 6.3 by adding NaOH (if the pH was too low or HCl (if the pH was too high). Then, the solution was added with 2 g of agar, stirred with a magnetic stirrer, and boiled with a hot plate. After boiling, the solution is poured into culture bottles ± 25 mL each. The bottle was closed with 0.3 mm PP plastic and tied with rubber. The media was sterilized by autoclave at a temperature of 121°C at a pressure of 1.5 kg/cm^3 for 45 minutes. After that, the bottles were placed on the culture shelves.

Seed germination

Seed germination was carried out in a Laminar Air Flow Cabinet (LAFC), which had been previously cleaned with denatured alcohol. Before germination, the seeds were washed with soapy water and rinsed thoroughly. Then, their seed coats were peeled off with pliers.

The planting begins by bringing the mouth of the culture bottle close to the Bunsen lamp. During the planting, the mouth of the bottle must be close to the Bunsen lamp to prevent contamination. Next, the seeds were sterilized with 100% chlorox solution for 1 minute. Afterward, the seeds were opened, and the embryos were taken using tweezers and a scalpel. Then, they were planted on MS media without ZPT. First, the bottles were covered with aluminum foil, and then they were covered again with 0.3 mm PP plastic. Finally, the bottle with the embryo in it was labeled according to the planting date.

Explants planting

The explants used were the shoots of 11-day-old plants that had been germinated sterile. The explants were planted by taking the plant from the bottle with tweezers and then placing it on a petridish. The shoots of the plant were ready to be cut using a scalpel. First, the mouth of the bottle was preheated with a Bunsen lamp to prevent contamination. Then the explants were planted on the treatment media with sterile tweezers. The scalpel and tweezers were always heated to maintain the sterilization of the tool. Before closing, the mouth of the bottle was heated again. After that, the bottles were covered with aluminum foil and covered again with 0.3 mm PP plastic. Bottles were labeled according to the treatment and date of planting.

Maintenance

Maintenance of culture bottles was done by placing them on the culture shelves and spraying them with denatured alcohol every two days to prevent contamination.

Research variable

Observations were done daily by counting the days of the first callus appearance, expressed in days after planting (DAP). The callus appearance was indicated by swelling or the appearance of greenish-white tissue on the surface of the explant.

Visual observation of callus color was carried out at the end of observation (30 DAP). A score determined the callus color, i.e., 0: white, 1: whitish-green, 2: yellowish-green, 3: green, 4: brownish-green.

Callus texture was observed at the end of the observation (30 DAP) by observing the formed callus texture, whether compact or crumbly.

The time of emergence of the first roots was calculated from the time of planting until the emergence of roots and expressed in DAP. It was characterized by the presence of yellowish-white protrusion (± 2 mm) on the lower part of the explant.

The number of roots was observed by counting the total roots in each growing explant. It was done at the end of the observation (30 DAT).

The first shoots' emergence time was calculated from planting until the emergence of shoots and expressed in DAP. The formation of shoots was indicated by the presence of a greenish-white protrusion (± 2 mm) on the upper part of the explant.

The number of shoots was observed at the end of the observation (30 DAP) by counting the number of shoots that emerged from the surface of the explants.

The observation at the time of leaf emergence was carried out using the same research method as Nofiyanti's in 2007, namely by counting the days from planting until the opening of the leaves completely. It was expressed in DAP.

The number of leaves was observed by counting the entire leaves in each growing explant. It was performed at the end of the observation (30 DAP).

Callus Fresh Weight was observed at the end of the observation (30 DAP). The fresh weight of callus was measured by weighing on an analytical balance the fresh weight of callus along with culture bottles complete with lids minus the weight of culture bottles (without callus) and lids.

$$WW = WW_t - WW_O$$

Note:
WW : fresh callus weight (g)
WWt: fresh callus weight+culture bottle+cover (g)
WWo: weight of culture bottle+cover (g)

Data analysis

The qualitative analysis included visual data. In addition, the data were analyzed using descriptive methods. Meanwhile, quantitative data were analyzed using analysis of variance based on the F test at a 5% level. If there was a significant difference, it was continued with the DMRT test at a 5% level.

RESULTS AND DISCUSSION

Callus appearance time

One indicator of the growth of in vitro culture is the appearance of callus on explants. According to Hendaryono and Wijayani (1994), callus is undifferentiated cells formed on one or all of the explant slices. In this study, the callus was first formed at the tip of the explant in contact with the media. Next, start with swelling of the explants, then callus appears on the base of the explants with a greenish color, according to Hartmann et al. (1990) cit. Dwiyono (2009), callus produced through in vitro propagation is formed due to tissue injury and hormone response. The average callus appearance of Jatropha explants at various concentrations of BAP and 2,4-D is presented in Table 1.

Table 1 shows that callus was induced in almost all treatments. Auxins are generally added to the nutrient media to induce callus from explants (George and Sherrington 1984). In this study, callus was not formed in explants that were not added with 2,4-D. The absence of callus was possible because endogenous auxin in Jatropha explants had not been able to induce callus. In other words, explants had low auxin content, so they still needed additional exogenous auxin in the culture medium. Pierik (1987) stated that auxin is a hormone capable of inducing callus. It is reinforced by Haensch's (2007) statement that the combination without 2,4-D does not show any somatic embryogenesis. Somatic embryogenesis is the process of the formation of embryos derived from somatic cells (not the result of the fusion of male and female gametes). Explants often die or do not change. However, some of the explants formed a little callus.

This study showed that the B4D2 treatment (BAP 2 ppm and 2,4-D 0.25 ppm) was the fastest to induce callus at 5.67 DAP. It is in line with Hanifah's research (2007), in the treatment of 0.5 ppm NAA and 1 ppm BAP and 0.5 ppm and 2 ppm NAA treatment gave the fastest callus appearance of 13.33 DAP. Although auxin is known as a hormone capable of inducing callus, cytokinins are often used as a combination ingredient for callus induction.

Gustian (2009) states that adding auxin at low concentrations will generally stimulate callus formation. On the contrary, if the ratio of auxin and cytokinin in the medium is higher, it will stimulate callus explants to regenerate to form organs. In addition, Table 1 shows that the longest callus induction was obtained in the B1D4 treatment (BAP 0.5 ppm and 2,4-D 0.75 ppm) at 7 DAP. It was possible because the concentration of 2,4-D given to the explants was high, thus inhibiting callus growth in the explants. At high levels, auxin inhibits rather than stimulates growth (Hendaryono and Wijayani 1994). The inhibition of callus growth was due to the culture mass being grown for too long in the same medium, which caused the loss of nutrients and water. Running out of nutrients and water can occur because, apart from being

sucked in for growth, the media evaporates water from time to time. In addition to running out of nutrients, the callus releases compounds resulting from metabolism, eventually inhibiting the callus's growth (Anonim 2010).

Callus color

Explant growth indicators of *in vitro* cultivation in the form of callus color describe the visual appearance of the callus so that it can be known whether a callus still has cells that are actively dividing or have died. Callus tissue produced from an explant usually shows different colors. Good quality callus has a green color. According to Fatmawati (2008), callus color indicates the presence of chlorophyll in the tissue. The greener color of the callus means more chlorophyll content. A light or white color can indicate that the callus condition is still quite good.

Figure 1 showed that the color in the callus of J. curcas ranged from white (indicated by a score of 0) to brownish-green (indicated by a score of 4). Based on Table 2, the color of the callus ranges from whitish-green, yellowish-green, and brownish-green. The difference in callus color indicated that the level of callus development was different. Almost all treatments showed a yellowish-green color on the formed callus. According to Hanifah (2007), adding cytokinins with increasing concentrations tends to show a green (bright) color on the callus to last longer. The green color of callus is due to the effect of cytokinins in the formation of chlorophyll. In contrast, the whitish-green callus color was shown in the BAP treatment of 1 ppm and 2,4-D 0.25 ppm.

Brownish green callus color was found in the treatment of 2,4-D 0.75 ppm, which was added with 0.5 ppm BAP and 1 ppm BAP. Callus color getting darker (becomes brown) means that callus growth is decreasing. In this study, callus with a brownish-green color was produced on media containing 2,4-D with a fairly high concentration. The results of research reinforced by Dwiyono (2009) that the increasing addition of 2,4-D can lead to an increase in the formation of callus with brown color on *mahkota dewa* (*Phaleria macrocarpa* (Scheff.) Boerl.).

The brown color of the callus is due to the metabolism of toxic phenolic compounds, often stimulated by the explant sterilization process, which inhibits growth or even causes tissue death (Yusnita 2004). Santoso and Nursandi (2004) stated that the browning event was a natural event and a process of adaptive changes in plant parts due to physical influences such as stripping and cutting. Browning symptoms were signs of physiological deterioration of explants. In addition to indicating the occurrence of synthesis of phenolic compounds, the brown color was caused by the increasing age of cells or callus tissue. Following Palupi et al. (2004) research, brown callus is a callus that undergoes the cell aging process (senescence) due to the absence of BA in the media. Thereby this condition accelerates the aging process (senescence) of cells. High concentrations of 2,4-D and BA (2,4-D 1.0 mg/l and BA 1.0 mg/l) can stimulate the aging process, inhibiting the callus growth process.

Callus texture

Callus texture is a marker used to assess a callus's quality. A good callus is assumed to have a crumbly texture (friable). Crumbly callus texture is considered good because it facilitates the separation into single cells in suspension culture and increases oxygen aeration between cells. Thus, efforts to propagate the number of callus through suspension culture are easier with this texture. Callus texture can be divided into three types, namely: compact (non-friable), intermediate and crumbly (friable) (Turhan 2004). Visually, the bonds between cells in the crumb callus formed on the explants of *J. curcas* looked loose and easily separated. If taken with tweezers, the callus was easily broken, and some stuck to the tweezers.

On the other hand, a compact callus has a texture that is difficult to separate and seems solid (Fitriani 2008). Meanwhile, the callus, partly compact and crumbly, is called the intermediate callus (Widiarso 2010). Callus textures formed on Jatropha explants at various concentrations of BAP and 2,4-D are presented in Table 3.

Table 1. Callus appearance time in Jatropha explants at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	6	6	7
1	~	6	6	6
1.5	~	6	6.33	6.33
2	~	5.67	6	6.33

Note: ~= no appearance of callus, DAP= days after planting, ppm = part per million (mg/L)

Table 2. Callus color of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	yellowish-green	yellowish-green	brownish-green
1	~	whitish-green	yellowish-green	brownish-green
1.5	~	yellowish-green	yellowish-green	yellowish-green
2	~	yellowish-green	yellowish-green	yellowish-green

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

Table 3. Callus texture of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP (ppm)	2,4-D (ppm)			
	0	**025**	**05**	**075**
05	~	crumbly	crumbly	crumbly
1	~	crumbly	crumbly	crumbly
15	~	crumbly	crumbly	crumbly
2	~	crumbly	crumbly	crumbly

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

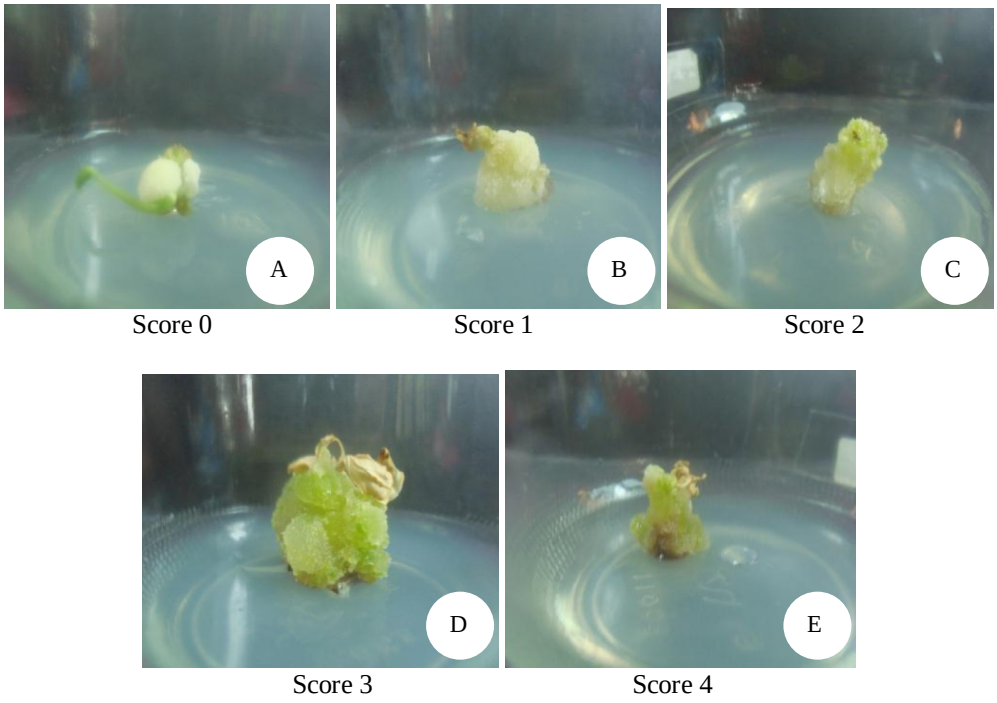

Figure 1. Callus color scoring category on Jatropha explants (A) white callus, (B) whitish-green callus, (C) yellowish-green callus, (D) green callus, (E) brownish-green callus

Pierik (1987) stated that the texture of callus could vary from compact to crumbly, depending on the type of plant, nutrient composition of the media, growth regulators, and environmental conditions of the culture. Table 3 showed that the combination of treatments formed callus with a crumbly texture, except for the treatment without 2,4-D. According to Fatmawati (2008), the callus that mostly had crumbly texture on leaf explants of *A. annua* was caused by 2,4-D in culture media. The same thing was also obtained in Ratnadewi's (1991) cit Fatmawati's (2008) research: Auxin 2,4-D combined with kinetin 1 mg/L was used to induce the formation and propagation of friable/crumbly callus in sugarcane plants. The formation of callus with a crumbly texture, according to Widyawati (2010), is triggered by the presence of endogenous auxin hormones produced internally by explants that have grown to form the callus.

Root appearance time

Roots for plant growth play a very important role because the roots are directly in contact with the planting medium where nutrients are stored. Zulkarnain (2009) said that roots function as a tool to absorb nutrients and nutrients and support the plant body. In addition, roots also function as transporters and food storage places such as carrots, sugar beets, and sweet potatoes. Therefore, the presence of roots is needed by plants, and in vegetative propagation, including tissue culture, various efforts are made to form roots. The emergence of roots in Jatropha explants at various concentrations of BAP and 2,4-D in vitro is presented in Table 4.

Table 4. The time of root appearance in Jatropha explants at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	~	~	~
1	9	~	~	~
1.5	~	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

Table 4 shows that the BAP treatment of 1 ppm without adding 2,4-D could produce roots. In this study, root formation only occurred in the combination treatment B2D1 (BAP 1 ppm, 2,4-D 0 ppm) at 9 DAP. Most of the explants were unable to grow roots. It is suspected that with the addition of 2,4-D to the culture medium, the explants focused more on callus induction than root emergence. In addition, this may occur because the concentration of auxin (2,4-D) given in the medium is too little. Judging from the combination of treatments in this study, the ratio of auxin and cytokinin concentrations was low, so the treatment could not produce roots. It is in line with the statement of Yunus et al. (2007), which states that if the ratio of the concentration of auxin to cytokinin is low, then the plants with this treatment will not be able to grow roots. Therefore, it can be concluded that the treatment has the roots grow with a high ratio of auxin and cytokinin, including IBA 0 ppm and BA 3 ppm, IBA 0.25 ppm and BA 1 ppm, and IBA 0.5 ppm and BA 1 ppm. This condition deviates from the opinion of Wetherell (1982) that for root formation, it is necessary to have a low ratio of auxin and cytokinin. This deviation was maybe because the endogenous growth substances (endogenous auxin)

contained in the explants in the treatment were sufficiently available.

In organogenesis, three possibilities can cause explants to fail. First, cells in explants lack totipotency, that is, the total genetic potential, which means that every living plant cell is equipped with a complete genetic and physiological device to grow into a whole plant under the right condition. Second, cells in explants could not differentiate and dedifferentiate due to a lack of essential induction stimulation of any type or inappropriate concentration of growth regulators (Tripepi 1997 cit. Prihatmanti and Mattjik 2004).

Number of roots

Many roots can optimize the absorption of nutrients in the culture media. Nickell (1982) cit. Rahmaniar (2007) stated that the active auxins used for root formation were Naphthalene acetic acid (NAA) and Indole Butyric Acid (IBA). Some other types that can be used are 2,4-D and 2,4,5-T. Both types form roots when used at low concentrations. The type of root system produced also depends on the growth regulator used. For example, the phenoxy acids at 2,4-D and 2,4,5-T produce a rich, thick, sturdy root system. In comparison, IBA produces a strong fibrous root system.

In this study, only the combination treatment without 2,4-D with the addition of 1 ppm BAP was able to grow roots. The root produced in this study was only 1 single root. In stem explants, roots do not always appear at the nodes (Rahmaniar 2007) but can also appear at the stem base in the media (Figure 2). Visually, the roots formed on the explants of Jatropha shoots were yellowish-white without root hairs, thin, and not sturdy, as Rahmaniar (2007) stated that in the treatment without 2,4-D, the roots formed were single roots that were thin and not sturdy.

The cells in the explants are thought to be able to produce their auxin to encourage cell metabolic processes. Therefore, adding auxin to the culture media will cause an unbalanced interaction with endogenous auxin and cannot produce a larger number of roots (Mujiyanto 2003 cit. Rahmaniar 2007).

Shoot appearance time

Shoots are plant parts obtained from vegetative propagation, which grow to carry out offspring on the plant. The formation of shoots indicates the success of the regeneration of explants inoculated on tissue culture media. Callus resulting from callus induction of Jatropha explants can differentiate to form shoots. However, in this study, not all callus formed could differentiate into shoots, so several shoots formed directly. The faster the shoots appear, the faster the material for plant propagation will be produced. The average shoots of Jatropha explants at various concentrations of BAP and 2,4-D are presented in Table 5.

Table 5 shows that the average fastest shoot appearance of Jatropha explants was in the treatment of 0.5 ppm BAP with the addition of 2,4-D 0.25 ppm, namely 6 DAP. This treatment produced bud shoots. From Table 5, it can be concluded that the effect of BAP and 2,4-D gave different responses to the appearance of shoots, as stated by George and Sherrington (1984), that the initiation of shoots and roots is determined by the concentration of cytokinins and auxins given to the medium and their interactions with cytokinins or endogenous auxins contained by explants.

In the combination treatment of 0.5 ppm BAP, and 2,4-D 0.75 ppm, the slowest effect in stimulating the appearance of shoots was at 22 DAP. Meanwhile, Jatropha explants at 1 ppm, 1.5 ppm, and 2 ppm BAP treatment added with 2,4-D 0.75 ppm did not produce shoots. It means that adding 2,4-D to the explants could not accelerate the appearance of shoots. However, as Hariyanti et al. (2004) stated, the increase in exogenous auxin and inhibition effect on the time of shoot formation is also increasing. Therefore, jatropha explants with BAP treatment of 1 ppm, 1.5 ppm, and 2 ppm without 2,4-D were able to produce shoots. This statement is supported by Nursetiadi (2008) that the endogenous auxin found in explants has been able to encourage the formation of shoots, so it only requires auxin that is not too high.

Adventitious shoots also appear on some explants, forming a callus. Adventitious shoots are derived from explant cells or tissues that previously did not have buds (Yusnita 2004). At the end of the observation, green protrusions (±2 mm) were seen on the formed callus (Figure 3A). These protrusions are adventitious shoots that will grow into new shoots. Adventitious shoots appeared in the combination treatment of 0.5 ppm BAP and 2,4-D 0.75 ppm, 2 ppm BAP and 2,4-D 0.25 ppm, 1 ppm BAP and 2,4-D 0.5 ppm, and BAP 2 ppm and 2,4-D 0,5 ppm. The formation of adventitious shoots is thought to influence the addition of cytokinins, in this case, BAP, into the culture medium. It follows the opinion of Yusnita (2004), which states that cytokinins can stimulate the formation of adventitious shoots. However, George and Sherrington (1984) stated that the interaction between auxins and cytokinins influenced the formation of adventitious shoots.

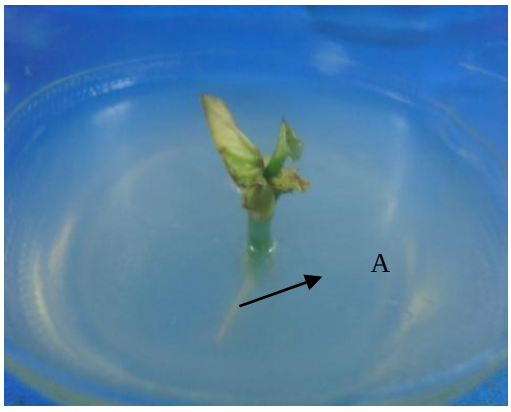

Figure 2. A root that was formed on jatropha explants in combination treatment B2D1 (BAP 1 ppm and 2,4-D 0 ppm)

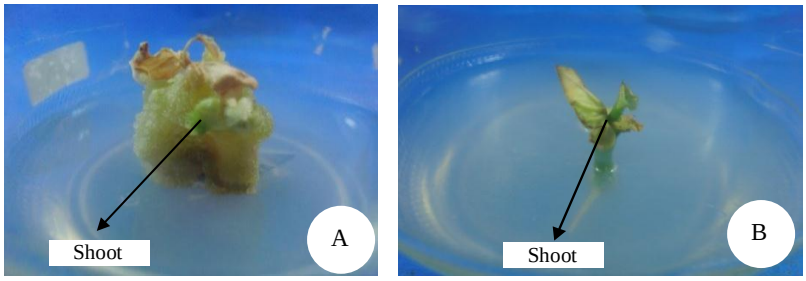

Figure 3. Types of shoots formed in Jatropha explants (A) adventitious shoots, (B) bud shoots

Hariyanti et al. (2004) reported that the higher the administration of exogenous auxin, the effect of its inhibition on the time of shoot formation also increased. However, in this study, with increasing concentration of 2,4-D, the effect of inhibition on the emergence of shoots was varied (Table 5); possibly, the explants contained endogenous auxin whose levels were not the same. Uniformity of size and method of taking explants is most likely not followed by the uniformity of endogenous plant hormones. Therefore, adding exogenous auxin into the culture media will cause a varied response.

Number of shoots

The number of shoots is the most important factor in plant multiplication in tissue culture. In tissue culture, the number of shoots can be indicated as success in multiplication. As many shoots are formed, multiplication of cultures to get more new shoots can be done. The number of shoots was calculated on all shoots that appeared on explants, both from elongation and adventitious shoots (not from shoots).

Figure 4 shows that the average shoot emergence in all treatments producing shoots was 1. The various concentrations of BAP and 2,4-D given in this study could not grow more than one shoot. The shoots formed came from the elongation of the shoots of the plant stems and shoots from the differentiation of callus tissue in explants. Therefore, it is in line with Nursetiadi's (2008) research which states that the types of media and the concentrations of BAP given in this study could not grow more than one shoot. Adding 1 to 3 ppm BAP concentrations tended to give the same results. Although the BAP concentration level had increased, there was no change; possibly that the administration of cytokinins with BAP concentrations of 3 ppm had not been able to stimulate shoot multiplication. According to George and Sherrington (1984), a higher cytokinin concentration than auxin concentration will stimulate shoot multiplication, which is reinforced by the statement of Haensch (2007) that the combination of 0 ppm 2,4-D with 4 ppm BAP causes shoot regeneration.

The role of auxin is to stimulate the division and enlargement of cells found in plant shoots and cause the growth of new shoots. Therefore, the addition of a larger amount of auxin, or the addition of a more stable auxin, such as 2,4-D acid, tends to cause callus growth from explants and inhibit plant shoot regeneration (Wetherell 1982).

Time of leaves appearance

Leaves are vegetative organs whose growth is influenced by the nitrogen content in the media. According to Pierik (1987), the source of organic N in tissue culture media is NH4+ and NO3-, and MS base media is the highest among other basic media. Therefore, using MS media can stimulate the growth of vegetative organs. However, almost all treatment combinations did not produce leaves. The average leaf appearance of Jatropha explants at various concentrations of BAP and 2,4-D is presented in Table 6.

Table 6 shows that leaves only appeared at 1 ppm BAP and 1.5 ppm BAP without adding 2,4-D and appeared at 11 DAP and 14 DAP, respectively. The factor that caused the absence of leaves in all treatment combinations was thought to be the explants dividing cells by dedifferentiation due to the addition of 2,4-D. As Yusnita (2004) stated, plant cells at the callus induction stage will experience dedifferentiation, namely the process of changing explant cells that were previously specialized to form plant organs such as roots and leaves or shoots to become no longer specialized. Under these conditions, the cells will return to being meristematic. Wetter and Constabel (1991) also said that cells growing on callus would be meristematic and usually undifferentiated.

Number of leaves

Leaves are the center of photosynthesis, a source of food for plants. So the more leaves, the better the plant growth is. The number of leaves is affected by adding growth regulators into the media. In this study, the average number of leaves that grew in the combination treatment without 2,4-D with 1 ppm BAP and 1.5 ppm BAP were 2 leaves and 1 leaf, respectively (Table 7).

Cytokinin hormones also influence leaf formation in tissue culture. In line with the statement of Yelnitis (1996) cit. Purwanto (2008) that adding BAP group cytokinins at a higher cytokinin ratio than auxin in the media can encourage an increase in the number of leaves. Widyawati's research (2010) also showed that only one treatment could form leaves, namely the 0.5 ppm BAP treatment without NAA. However, as in Hanifah's research (2007), treatment without NAA by giving 1 ppm BAP can produce the most leaves, namely 6 leaves. Therefore, it is suspected that adding cytokinins (BAP) in the media can encourage meristem cells in explants to divide and influence other cells to develop into shoots and form leaves.

Table 5. The appearance of Jatropha explant shoots at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP	**2,4-D (ppm)**			
(ppm)	**0**	**0,25**	**0,5**	**0,75**
0,5	~	6	8	22
1	11	7	18	~
1,5	10	~ 14	~ 10,5	~
2	8			~

Note: ~ = no appearance of callus, ppm = part per million (mg/l)

Table 6. Time of emergence of Jatropha explant leaves at various concentrations of BAP and 2,4-D in vitro (HST)

BAP	2,4-D (ppm)			
(ppm)	0	0.25	0.5	0.75
0.5	~	~	~	~
1	11	~	~	~
1.5	14	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, DAP= day after planting, ppm = part per million (mg/l)

Table 7. Number of leaves of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP	2,4-D (ppm)			
(ppm)	0	0.25	0.5	0.75
0.5	~	~	~	~
1	2	~	~	~
1.5	1	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, DAP= day after planting, ppm = part per million (mg/l)

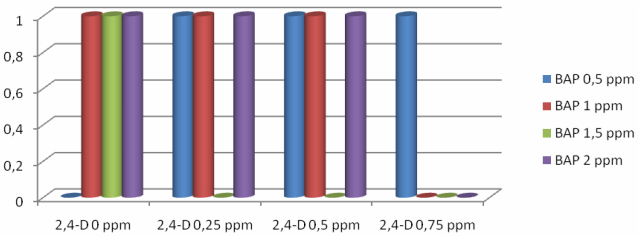

Figure 4. Histogram of the average number of *Jatropha* shoots with various concentrations of BAP and 2,4-D

In this study, some leaves changed color to brown, and some fell off; the leaves fell from the petiole base (Figure 5). It may be because the existing nutrients are not sufficient for the survival needs of the leaves until the explants can grow perfectly. It was suspected that the absorption was not optimal because there was no root formation in the explants; moreover, according to Supriyanto et al. (1992) cit. Triatminingsih et al. (1995), leaves that are formed experience loss due to chlorosis. Chlorosis is an event that decreases or decreases chlorophyll due to the addition of auxin. A combination of endogenous and exogenous auxin occurs in the tissue, then synthesizes ethylene which will cause leaf aging.

Callus fresh weight

An irreversible increase in weight characterizes growth. Therefore, the fresh weight measurement of the callus can represent the callus growth variable derived from shoots of Jatropha plant explants. According to Ruswaningsih (2007), physiologically fresh weight consists of two ingredients: water and carbohydrates.

The analysis of variance in the 5% F test showed that BAP and the interaction between BAP and 2,4-D had no significant effect. Meanwhile, the administration of 2,4-D in the media significantly affected the fresh callus weight (Appendix 16). The 5% DMRT test explained that the three treatments of 2,4-D (0.25, 0.5, and 0.75 ppm) had a different effect from the control (0 ppm 2,4-D) in increasing callus fresh weight (Table 8). In this study, the treatment without 2,4-D could not form a callus. It may occur because the endogenous auxin content in the explants was not sufficient to form a callus, so it still required exogenous growth regulators to form a callus. According to Santoso and Nursandi (2004), the direction of culture development is determined by the interaction and balance between growth regulators produced by plant cells endogenously because, in the explants, there are endogenous growth regulators. Still, in plant growth and development internally, the growth and development of plants are internal; therefore, in in-vitro exogenous growth, regulators are still being added. The addition of 2,4-D acid was carried out because 2,4-D acid played a role in promoting callus morphogenesis and callus induction and could affect the genetic stability of plant cells.

Based on the histogram (Figure 6), the highest callus fresh weight was 2.56 grams, obtained in the B4D3 treatment (BAP 2 ppm; 2,4-D 0.5 ppm) which was not much different from the fresh callus weight produced by B4D2 treatment (BAP 2). ppm; 2,4-D 0.25 ppm) of 2.48 grams. The callus formed in these two treatments was influenced by the presence of both endogenous and exogenous auxin with the addition of 2,4-D.

Rahayu et al. (2003) stated that the large fresh weight of callus was due to its high water content. The resulting wet weight is highly dependent on the speed at which these cells divide, multiply and continue with the enlargement of the callus. In addition, Pierik (1987) added that callus growth in one plant species might differ depending on the plant's original explant position and growing conditions. Growth and morphogenesis in in-vitro are influenced by interactions and balances between growth regulators added to the medium and growth hormones produced endogenously by cultured cells (George and Sherrington 1984). Therefore, BAP and 2,4-D treatments had no significant effect (ns) on fresh callus weight (Fatmawati 2008). Therefore, it could be concluded that the administration of 2,4-D 0.5 ppm was the best for fresh callus weight gain. However, giving 2,4-D 0.25 ppm was more beneficial because, besides having a very significant effect, adding a small concentration of it could increase the fresh weight of Jatropha callus.

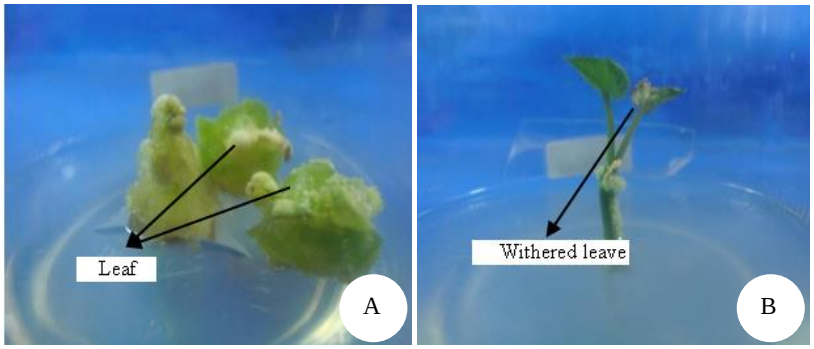

Figure 5. Jatropha shoots explants (A) Greenish yellow leaves fall off, which form a callus (B) Leaves turn brown (wither)

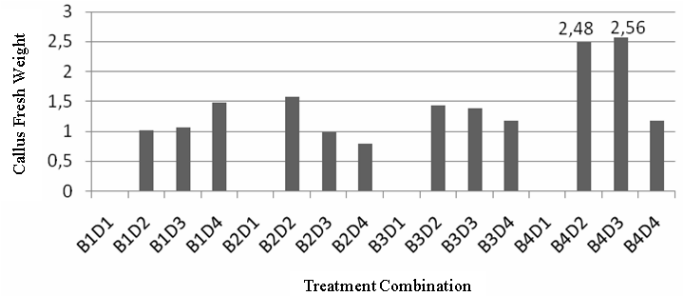

Figure 6. Effect of BAP and 2,4-D treatment on fresh weight of Jatropha callus in vitro. Note: B1: BAP 0.5 ppm; B2: BAP 1 ppm; B3: BAP 1.5 ppm; B4: BAP 2 ppm; D1: 2,4-D 0 ppm; D2: 2,4-D 0.25 ppm; D3: 2,4-D 0.5 ppm; D4:2,4-D 0.75 ppm

Table 8. Average fresh weight of Jatropha callus at various concentrations of 2,4-D in vitro

2,4-D (ppm)	Average callus weight
0	0.00 a
0.25	1.62 b
0.5	1.50 b
0.75	1.15 b

Note: Numbers followed by the same letter show no significant difference in the 5% DMRT test

In conclusion, concentrations of BAP 2 ppm and 2,4-D 0.25 ppm were the most optimal to induce the callus of *J. curcas* plant in vitro. The callus produced is generally yellowish-green in color and has a crumb texture. Adding 2 ppm BAP and 0.5 ppm 2,4-D produced the highest callus fresh weight of 2.56 grams. The combination of 1 ppm BAP treatment without 2,4-D produced roots at 9 DAP with 1 single root. Administration of 0.5 ppm BAP with the addition of 2,4-D 0.25 ppm gave the fastest shoot emergence, 6 DAP. The average of the fastest leaf emergence in BAP treatment was 1 ppm without 2,4-D, was 11 DAP with 2 leaves.

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