Development of banana in vitro from male bud culture supplemented with some concentration of sucrose and benzyladenine

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Abstract. Conventional techniques of propagating banana plants with corms require a relatively long time (10-18 months) and the amount produced is limited due to deforestation and industrialization. So production and supply of quality products are becoming a great challenge. In addition, there is a need to develop climate-resilient crop to face the consequences of global warming in the near future. Plant tissue culture is a proven technique for producing banana seeds in large quantities, uniformly and in a short time to support good quality banana seeds. The banana flower meristem can be a potential explant. The banana flower meristem offers the opportunity to regenerate plants with agronomic characteristics and results that can be controlled. This study aimed to regenerate banana flowers in vitro with different sucrose and BA (Benzyladenine) concentrations. The study used a Completely Randomized Design (CRD), two factorials sucrose concentration with 4 levels (20 g/L, 25 g/L, 30 g/L, and 35 g/L) and BA concentration with 4 levels (2 ppm, 4 ppm, 6 ppm and 8 ppm). The results showed that the combination of BA and sucrose concentration had not directly induced organogenesis in banana flower explants. Growth and development of banana flower explants maximally form Cauliflower-Like Bodies.

1. Introduction
Banana (Musa paradisiaca L.) are plants that are resistant to climate change characterized by the ability of the stem to store water then the banana plants can survive in drought conditions. In addition, there is a need to develop climate-resilient crop to face the consequences of global warming in the near future. Plant tissue culture is a proven technique for banana plants that can survive in drought conditions. Leaf size is wide, long and in a large number. Banana leaf is also a provider of oxygen through photosynthesis.

Bananas are the most widely produced and consumed as fruit commodities in Indonesia because they are readily available, affordable and can be enjoyed throughout the year. Bananas contain complete nutrients, including carbohydrates, proteins, vitamins (A, C and B complex) and minerals [1]. Until 2016, bananas ranked first for national fruit production. Bananas are the main contributor to national superior fruit production of 7.01 million tons, compared to oranges 2.01 million tons, mangoes 1.81 million tons, pineapple 1.4 million tons and papaya 0.9 million tons within one year of production [2]. Statistics Indonesia [3] shows that the development of bananas' national production began to decline from 7.29 million tons in 2015, 7 million tons in 2016, to 7.16 million tons in 2017.
Tissue culture techniques or propagation of banana seeds in vitro until they become whole plants that can be planted in the field require ± 5-8 months depending on the plant's vigor in maintaining its life [4]. Flower meristems can be potential explants. This method is cost-effective, has the advantage of not having latent contamination than shoots planted in the soil, and offers the opportunity to regenerate plants with agronomic characteristics and desired yields [5]. Cytokinin growth regulator (GR) is a critical factor in vitro propagation of plants. Benzyladenine (BA) is reported to be effective in stimulating the multiplication of side shoots. Increased sucrose concentrations have also been reported to cause increased multiplication of shoots [6]. However, this treatment is not necessarily effective for different explants. This study aimed to regenerate banana flowers in vitro to form plantlets by giving sucrose and BA concentrations.

2. Materials and methods
This research had been carried out at the Plant Physiology and Biotechnology Laboratory of the Faculty of Agriculture, Sebelas Maret University, Surakarta. The study used a completely randomized design (CRD) with 2 factors. Sucrose concentration with 4 levels (20 g/L, 25 g/L, 30 g/L, and 35 g/L) and BA concentration with 4 levels (2 ppm, 4 ppm, 6 ppm, and 8 ppm). Materials in this study included explants of Raja banana flowers, Murashige and Skoog (MS) media, agarose, 70% ethanol, Benzyladenin (BA), Indole Acetic Acid (IAA), deionized water, sucrose, sodium hypochlorite, pyroxylic spirit. Research activities included stock solutions preparation, media preparation, sterilization, initiating banana flowers, maintenance and observation. Variables included changes in the color of explants of banana flowers, when the enlargement of the explants of banana flowers appeared, the size and color of enlarged explants of banana flowers, browning on explants of banana flowers, and vigor of banana flowers. Data were analyzed descriptively due to the normality of the data set. Data could not be analyzed using variance analysis.

3. Results and discussion
Growth is interpreted as an irreversible process of increasing volume and occurs because of the increase and enlargement of cells. According to Solikin [7], the growth process is accompanied by changes in the shape, volume, number of cells, and protoplasm. The stages in growth and development include 3 phases, namely cell division, cell enlargement and cell differentiation. Cell division occurs which causes the number of cells to increase and the organelles in it. According to Kumianjani et al [8], growth and development are related to the increase in cell volume and the increase in cell numbers. Increasing the number of cells depends on the speed of cells to divide, one of which is influenced by growth media and GR. Figure color was 4% pale white, 13% yellow, 15% greenish-yellow, 21% green and 44% brown from all samples observed. Sucrose with 35 g/L at all BA levels (2 ppm, 4 ppm, 6 ppm, and 8 ppm) shows the average green color. Benzyladenine 4 ppm at all levels of sucrose (20 g/L, 25 g/L, 30 g/L, and 35 g/L) shows the average of green-yellow to green explants (Figure 1). The green color in the explants is formed due to the influence of the media's content and cytokinins added. According to Rainiyati et al [9], explants that are cultured under conditions of light underwent chlorophyll development due to light stimulation and photosynthesis. The photosynthesis process is also accompanied by absorption of nutrients and water from the planting medium, so enlargement and swelling. Nisa and Rodinah [10] stated that cytokinins and auxin provide an interaction effect on tissue differentiation in tissue growth. The presence of nitrogen in the media also influences the formation of chlorophyll. Cytokines can encourage the formation of chlorophyll. The greatest need for nitrogen is used to arrange nucleic acids, proteins, as coenzymes, or other compounds containing nitrogen such as chlorophyll, alkaloids, purine and pyrimidine derivatives and some endogenous hormones [11].
All explants showed a greenish color at the tip that spreads to the base, then expanded and turned completely green in 40-50 DAP (Day after Planting) after formation on MS basal media equipped with different concentrations and combinations of plant growth regulators [12]. Explant growth and morphogenesis are regulated by the interaction and balance of growth regulators on the media with endogenous hormones contained in explants (Figure 2). Swelling or enlargement of explants in the media can be caused by endogenous hormones and nutrients reserves [9]. Most of the food reserves in the form of carbohydrates and protein will accumulate in the wound tissue, resulting in swollen explants [13].

Figure 2 shows the fastest enlargement time to appear in the sample with 8 ppm BA and 35 g/L (B4S4) sucrose, 11.7 days after subculture in the treatment media. The longest enlargement of explants at 2 ppm BA and sucrose 20 g/L (B1S1) was 35.3 days after subculture in the treatment media. An increase in sucrose 30 g/L to 90 g/L without cytokinins, experiencing the fastest callus growth of 3 weeks after planting. The growth rate of banana explant tissue in regeneration is influenced by sucrose supply in the growing medium. Supply of endogenous auxins and cytokines present in the tissues is still sufficient to stimulate explant regeneration. A swollen explant shows that the cells have divided and enlarged [13]. Visually, living and dividing cells were characterized as green, firm, and getting bigger. The resulting enlargement colors were 13% pale white, 6% yellow, and 48% green from all samples observed. The average enlargement color of the explants was green in all sucrose treatment combinations at BA 2 ppm. Sucrose concentration 20-25 g/L affected on the enlarged explant color that was average green. In vitro response shown by wild banana zygotic embryos in germination is by changing the embryo’s color from milky white to yellowish white and subsequently will turn green followed by shoot formation [14]. The changing response in explant flowers after culturing can be said to be quite fast. Explant changed from yellowish white to brown on the former cutting and became greenish on the part that did not experience injury [10]. Two weeks, explants begin to break or open the deepest layers of bractea then swell to the wound, turned to greenish on the part that did not experience an injury.
Figure 2. Enlargement of banana flower explants on several sucrose and BA concentration (B1=BA 2 ppm; B2=4 ppm; B3=6 ppm; B4=8 ppm; S1=sucrose 20 g/L; S2=25 g/L sucrose; S3=30 g/L sucrose; S4=sucrose 35 g/L.

Figure 3. Enlargement of small white explants (A), enlargement of medium yellow explants (B), and enlargement of large green explants (C)

Figure 3 shows that the size of explant enlargement was 21% small, 29% medium size, 17% large, and 33% had no enlargement. Increasing the sucrose concentration with all BA levels (2 ppm, 4 ppm, 6 ppm, and 8 ppm) showed an average size of explant enlargement that was more or less than 1 cm. Benzyladenine 2 ppm with all levels of sucrose (20 g/L, 25 g/L, 30 g/L, and 35 g/L) showed the results of enlargement of explants with an average size of 0.5-1 cm. In vitro culture of cv. Grand Naine banana plant by Morfeine [15] found that an increase in sucrose concentration of 15-30 g/L also caused an increase in the number of shoots. However, a further increase to 45-75 g/L results in a decreased number of shoots. An increased number of shoots on banana in vitro culture at a sucrose concentration of 10-30 g/L [6]. Benzyladenine, is a type of synthetic cytokinin that has a strong activity, more effective than kinetin because BA has a benzyl group [16]. The use of BA on the formation of banana shoots with a
range of 2-7 ppm gives the best results for the emergence of shoots and the highest number of shoots [17]. Extreme color changes in explants can occur two weeks after culture. This relates to the response of plant adaptation with the given media. The extreme change in color of explants is known as browning and/or blackening by secondary metabolites. The latest observation results that explants experienced browning, namely 81%, blackening 15% and 4% contamination of all samples observed. Discoloration to brown (browning) in tissue culture occurs due to the accumulation of polyphenol oxidase released or synthesized tissue under oxidized conditions when cells are injured [18]. The isolated tissue became brown and/or blackish and failed to grow. Treatment of 25 g/L sucrose at 4 ppm and 6 ppm BA levels had blackening and explants did not develop (Figure 4). This browning event began to be seen within 1 (one) week after initiation and continued the following week. Browning occurred in all explants, but most explants were still able to live and develop. The presence of genomes B influences the level of phenol content and polyphenol oxidase activity, which the more genome B numbers, the higher the activity of the enzyme polyphenol oxidase [10]. When BA concentrations increase to more than 100mM, explants do not show any response and are instead covered by a thick black layer [19]. Phenolic compounds are also responsible for the high death rate (lethal blackening) initiated by blackening the surface of plant tissue, resulting in the formation of quinones that are very reactive and toxic to plant tissue [20]. According to Phenolic constituents in bananas are dopamine, catechin, chlorogenic acid, cinnamic acid, hydroxyl benzoic, resorcinol, pyrogallic acid, salicylic acid, ferulic acid, vanillin and coumaric acid and phenol [21]. According to Chikezie [22], this constituent is oxidized during tissue damage to prevent invasion of pathogen.

Figure 4. Explant browning but remains fresh (A) and explants undergo browning and then blackening (B)

Several attempts have been made to reduce the risk of browning in explants at the sterilization stage before the explants were planted. Treatment of flowed with water for 15 minutes to remove phenolic compounds reduces the risk of browning problems [13]. Explants of several species can avoid browning if initially cultured in a medium without growth regulators and transfer explants to new media. However, an increase in the number of subcultures often results in the accumulation of cell mutations and causes the loss of cells effective for forming embryogenesis [18]. Explant growth affects the ability to grow and develop to form new individuals. Explants that have low growth potential tend to experience slow growth or stunting. The results showed that the explant growing up to 12 MST experienced regeneration but was not accompanied by organ development. High sucrose and BA appeared to affect explant growth potential. Growth and development explant qualitatively was 23% vigor (very bad), 17% vigor (bad), 8% vigor (sufficient), 17% vigor (good) and 31% vigor (very good) for all observed samples. Sucrose 35 g/L for all BA levels showed a good average vigor. This growth was characterized by swelling with a large size and green color. Despite experiencing symptoms of browning, explants are still able to live in a less than optimal state. Treatment of 8 ppm BA in all sucrose combinations showed good average vigor. The combination of sucrose and BA had given an average explant growing and developing.
The induction of Curup banana callus using BAP and 2,4-D, the percentage of explant life is very high (100%) but has not followed by maximum explant growth. Growth of explants during the culture period requires a relatively long time forming callus and in some parts of the explant shows symptoms of browning. Browning symptoms in culture, if left too long it will cause explant death [13]. Organogenesis of explants in vitro occurs in two different ways, namely directly and indirectly [20]. This research suggested that BA and sucrose's addition would regenerate explants through organogenesis directly, inducing bud formation, but explants could only grow to form cauliflower-like bodies (CLB). Some explants that swell form CLB (Figure 5A), some explants form nodules with white nodules (Figure 5BC), and others did not develop. Nodules are initiated from the side adjacent to the former attaching bractea known as meristematic part [23]. The nodules formed in this study were white, similar to cauliflower. These organs are referred to as cauliflower-like bodies [5]. The organ turned green but had not been able to form buds. CLB formed in this study was 15% of all samples observed. Concentration of 4 mg/L BA does not induce male flowers and 98% of male flower hands turn brown in a month [24]. Comparable results have been reported by Hernandez and Garcia [25] for varieties cultivated by Musa AAA, Cavendish inflorescences that have not shown organogenesis or proliferation responses in basal media MS cytokinin deficiency. All cells or plants may not be manipulated in vitro due to differences in the ability to grow or regenerate from each cell type and plant genotype. Each type of explant or cell and plant genotype requires different media compositions [26].

4. Conclusion
A combination of BA and sucrose affected regenerating explants of banana flowers but had not yet been able to form organs. Growth and development of banana flower explants maximally form Cauliflower-Like Bodies resembling cauliflower. The banana plants can survive in drought conditions. There is a need to develop climate-resilient crops to face global warming in the near future.

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Figure 5. Explants undergo rupture to form CLB (A) explants undergo swelling (B) explants forming callus with small green size (C)
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