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Original research article

Induction Shoots of Black Pomegranate (*Punica granatum* L.) Seed Explant Using Thidiazuron And Kinetin Hormone *In Vitro*

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Abstract

Pomegranate is a fruit that has medicinal properties, it is proven by antibacterial, anti-inflammatory and anticancer activity. The aim of this study was to determine the best concentration of cytokinins in inducing shoots of black pomegranate seed explants (Punica granatum L.) in vitro. This study used one factorial, namely the addition of various concentrations of TDZ and kinetin. TDZ factor and kinetin with 6 treatment levels, namely 0 mg / L, 0.1 mg / L, 0.2 mg / L, 0.3 mg / L, 0.4 mg / L, and 0.5 mg / L, each treatment was repeated 4 times, for a total of 24 experimental units. The parameters observed included the day when the shoots appeared, the number of leaves, the length of the shoots and the day the leaves were fully open. Observation data were analyzed using Kruskal Wallis. The data showed that the cytokinin hormone, both kinetin and TDZ, was not able to significantly induce shoots on black pomegranate seed explants (Punica granatum L.). The hormone kinetin was able to induce shoots on explants of black pomegranate seeds (Punica granatum L.) with the best concentration of 0.4 ppm but the results were not significant. The optimal TDZ concentration in inducing callus appearance was 0.4 ppm with a callus appearance time of 24 DAP.

1. INTRODUCTION

Pomegranate is a plant that has medicinal properties, the fruit in the form of buni fruit which has many seeds with a coating of juicy and edible seeds. Pomegranate has three varieties, namely red, white and black pomegranate [1]. Pomegranate has anti-inflammatory and antibacterial activity [2]. In addition, the oil from pomegranate seeds has an inhibitory effect on the growth of skin and breast cancer cells. Pomegranate peel contains antidiabetic, antioxidant and anti-inflammatory activity [3].

Pomegranate plants can be propagated generative or vegetative. Generative propagation carried out in plant breeding to produce new varieties that have superior properties through cross-pollination and not recommended for mass production. In addition,

generative propagation also constrained by the texture of the pomegranate seeds which very hard so that germination takes a long time [4] The hard seed structure becomes a barrier to water entry into the seed. This event make difficult for the embryo to come out and germinate. This event cause a delay in the germination process and the seeds experience a period dormancy.

This has been proven in a study conducted stated, it took 71 days to grow pomegranate germination by 8% [5]. The seed will experience a period of dormancy when in an environment that not accordance with the physiological conditions of the seed which support the germination process so that the germination process will be delayed. Seed dormancy is a condition seeds will not experience germination even though the germination requirements have been met. The very hard seed coat structure inhibits the germination process, so it difficult for H2O, O2 and roots to penetrate the seed coat [6].

Plant propagation techniques using seeds are considered less effective because require a long time. So that tissue culture techniques are used which are based on the cell totipotency theory. Cell totipotency is the ability possessed by each cell to regenerate completely becomes new plants. The propagation method using plant tissue culture techniques is a type of vegetative propagation of plants by taking one of the plant organs that is placed in a medium containing nutrients in a sterile state which is carried out in the laboratory to produce complete plants with the same genetic characteristics as the parent in large quantities and a short time [7].

Treatment techniques in tissue culture must consider the appropriate environment for the physiological conditions of the plant. One of these determinants is to present the correct amount and measure of nutrients to the culture media. There are various kinds of media used in tissue culture, which are adjusted to the type of plant, tastes, goals and calculations of each researcher [8]. Hormone often used in tissue culture research is cytokinin and auxin hormones. The hormone that play an important role in the formation of shoots is auxin and cytokinin groups with a lower auxin concentration level than cytokinin. Cytokinin hormone include BAP or BA, kinetin and zeatin [9].

Thidiazuron is able to induce adventitious shoots and axillary shoots. TDZ can stimulate the formation of adventitious shoots in some plants because it can induce rapid cell division in a collection of meristem cells so that shoot primordia will be formed [10]. Kinetin is a cytokinin class hormone that first discovered [11] and a natural cytokinin produced in active growing tissues, especially in roots, embryos and fruit. Functions of kinetin to regulate cell division and morphogenesis [12].

TDZ hormone was able to induce shoot multiplication in jatropha with a concentration of 0.4 ppm while for shoot extension using 5 ppm kinetin with DKW media [13]. Induction of Jabon shoots at MS 1.10-1 mg/L 2,4-D + 3.10-1 mg/L kinetin within 2 days resulted in an average optimal shoot height of 1.28 cm [14].

Shoot induction through epicotyl using the hormone BA 0.5 mg / L and TDZ 3.0 mg / L can produce shoots of 9.6 with a height of 14.6 mm and number of leaves was 17 in each culture with regular shoots [15]. Effect of TDZ and IAA hormones on shoot induction of *Jatropa curcas* L. Showed that TDZ 0.5 ppm + IAA 1.5 ppm and TDZ treatment 0.5 ppm + IAA 2 ppm were able to form shoots [16].

Based on the description above, Then a study was conducted to determine the best concentration of cytokinins in inducing shoots of black pomegranate seed explants (*Punica granatum* L.) *in vitro.*

2. MATERIALS AND METHODS

The research was carried out at the Plant Tissue Culture Laboratory, Department of Biology, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University Malang, from September - December 2019.

The materials used in this study were black pomegranate seeds (*Punica granatum* L). obtained from Batu city. The chemicals used in this study were 70% alcohol, bactericide, fungicide, chlorox, detergent, 96% alcohol, methylated spirits, distilled water, petromax plastic, plastic wrap, MS (Murrasige and Skoog) media, agar, rubber, label paper, tissue, thidiazuron and kinetin hormone, sugar.

The tools used in tool sterilization include ovens, autoclaves, sponges, brushes, and plastics. The tools used for manufacture of media include analytical scales, micropipettes, stirrers, hotplate stirrers, beakers, petri dishes, pH meters, elenmeyer tubes, and measuring flasks. The tool used for media sterilization is an autoclave. Planting room sterilization (LAF) requires tools, including a hand sprayer and tissue. The initiation stage requires tools including tweezers, scapel, blade, bunsen, LAF, hand sprayer, petri dish, culture bottle and a lighter.

Research design

This study used one factorial, namely the addition of various concentrations of TDZ and kinetin. TDZ and kinetin factor with 6 treatment levels, namely 0 mg / L, 0.1 mg / L, 0.2 mg / L, 0.3 mg / L, 0.4 mg / L, and 0.5 mg / L, each treatment was repeated 4 times, so there were a total of 24 experimental units.

Sterilization of seeds

Explan sterilization, namely the seeds are cleaned with a tissue then flowed with water for 60 minutes. The seeds were put into a beaker glass, added with water and detergent as much as 3 ml, homogenized for 30 minutes. The seeds were rinsed with water until any traces of detergent are removed, then addited water and 2 grams of fungicide. Seeds were homogenized for 30 minutes. The seeds were rinsed with water, then addited water and 2 grams of bactericide. Seeds were homogenized for 30 minutes. The seeds were rinsed with water until clean and then put into LAF. In deep sterilization, the seeds were soaked with 30% chlorox solution for 15 minutes, then the seeds were rinsed with sterile distilled water for 15 minutes. The seeds were soaked with 10% chlorox solution for 5 minutes, then the seeds were rinsed with sterile distilled water for 15 minutes. Seeds were soaked with 70% alcohol for 2 minutes, then seeds were rinsed with sterile distilled water for 15 minutes. Treat 30% chlorox to 70% alcohol of the seeds by unidirectional shaking the bottle so that the seeds are sterile.

Initiation of the seeds

The work step at the initiation of explants is sterile pomegranate seeds are placed in a petri dish which has been added a little betadin. The seeds are split into two parts. Then the seeds are planted in the treatment medium. The bottle is covered with plastic and rubberised, and given a label paper. The initiation process is carried out at LAF

Data analysis

Parameter observed included shoots appearing day, leaf number, shoot length. The observations in this study were carried out without removing explants from the culture bottles. Observation data were analyzed using Kruskal Wallis.

3. RESULTS and DISCUSSION

A. Effect addition of Kinetin

Pomegranate seed is cut into 2, the tip of seeds (contain hilum,there is a black dot) and the second part is the base of the seeds. The two parts are planted separately but with the same hormone concentration. The kinetin given in this study were with concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 ppm. ZPT begins with the concept of hormones, namely plant organic compounds in low concentrations

affect physiological processes, especially differentiation and development plant. However, in the seeds sometimes the amount is limited. Then exogenous ZPT can be given as a treatment, especially in germination [17].

Table 1. Results of treatment with kinetin

Treatment	Percentage live of explants (%)		Result	
	Tip of the seed (hilum)	Base of the seed	Tip of the seed (hilum)	Base of the seed
Control	100	100	Callus	Shoots and sprouts
Kinetin 0,1 ppm	100	100	Callus	Sprouts
Kinetin 0,2 ppm	100	100	Callus	Sprouts
Kinetin 0,3 ppm	100	100	Callus	Sprouts
Kinetin 0,4 ppm	100	100	Callus	Shoots and sprouts
Kinetin 0,5 ppm	100	100	Calus	Shoots and sprouts

The treatments succeeded in inducing pomegranate shoots were only 3 treatments, namely explants at base of the seeds with kinetin concentrations of 0, 0.4 and 0.5 ppm. The treatment with fastest concentration of shoots appeared in treatment with kinetin concentration 0 (control), 27 days is the effective day to grow black pomegranate shoots.

This can be due to the fact that pomegranate seeds contain endogenous hormones that can stimulate shoot growth. The different plants can respond to hormones (auxin and cytokinin) in differently too. This is caused by differences in endogenous hormones in each plant [18]. So, the growth rate that occurs in the explants is due to the right interaction between the endogenous hormones of the explants and the addition of exogenous hormones which results in the physiological processes in the explants (Figure 1).

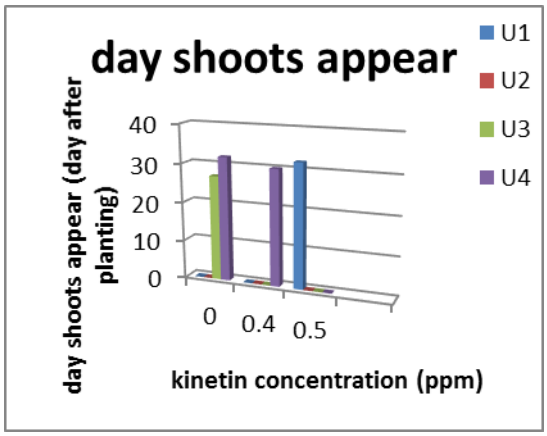


Figure 1. Days shoots appeared on kinetin treatment

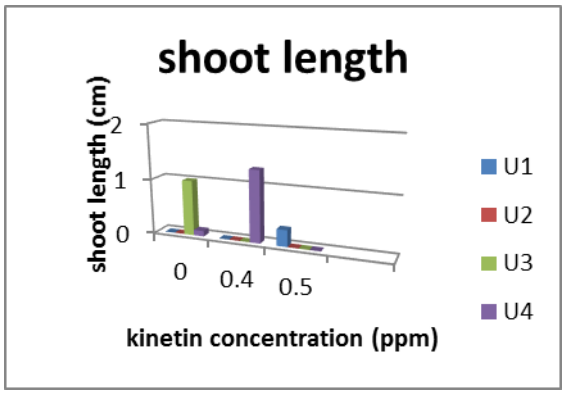


Figure 2. Shoot length on kinetin treatment

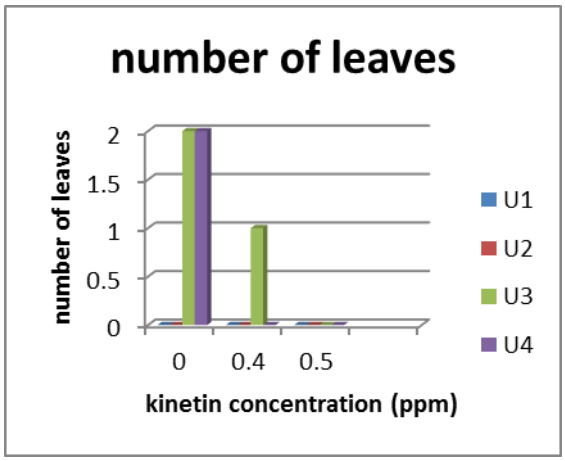


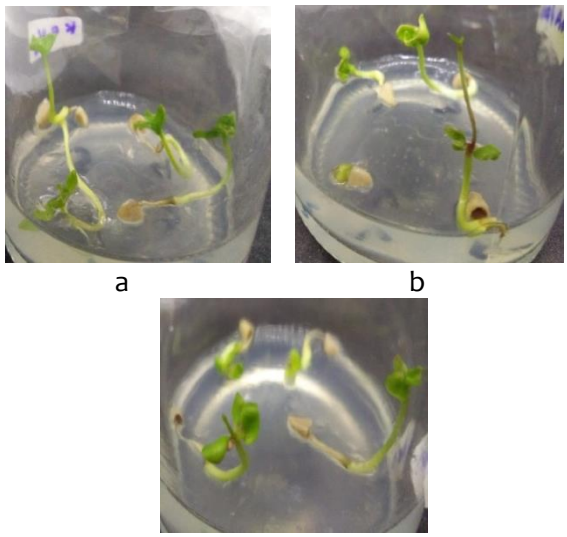
Figure 3. Number of shoots on kinetin treatment

The longest shoots found in kinetin treatment with a concentration of 0.4 ppm in replication 4, namely 1.3 cm, while the shortest shoots found in the 0 ppm kinetin treatment in replication 4, namely 0.1 cm. The highest number of leaves found in the kinetin concentration of 0 ppm in replications 3 and 4, namely 2 leaves, while in the 0.5 ppm kinetin treatment, 1 leaf was not formed completely. Leaves open perfectly, there is only 0 ppm kinetin treatment in repeat 3. From these results, it can be seen that if each plant growth has its own optimum concentration of hormones, although hormones in plants have a very important to stimulate growth, if it given in too high an amount will be inhibit the growth of the plant itself. Auxin and cytokinin growth regulators at low concentrations is able to stimulate the growth and development of explants and maintain the viability of explant tissue, but at high concentrations growth regulators can inhibit the development of explant morphogenesis [19].

Table 2. Result of Kruskall Wallis test

Observation variable	Asymp results
Day shoots appear	**0.766**
Shoot length	**0.810**
Number of leaves	**2.390**

The data were carried out by the Kruskall Wallis test. Asymp results. Significant in the Kruskall Wallis test greater than 0.05, which means that H0 is accepted. From these results it is known that there is no effect of adding kinetin on the induction of black pomegranate shoots.



a b c

Figure 4. Explant of the base of the seed. a. Kinetin 0 ppm, b. Kinetin 0.4 ppm, c. Kinetin 0.5 ppm

Treatment of seed base explants that had not yet grown shoots, namely the kinetin concentrations of 0.1, 0.2, 0.3 were still in the form of sprouts. The slow growth in this treatment can be due to several factors, namely the lack of lighting in the culture storage area so that cell metabolism is slow and the morphogenesis process is also slow. The presence of auxins and cytokinins in the medium can stimulate parenchymal tissue cells to divide [20]. Cytokinins are known to play an important role in almost all aspects of growth and development plant, including cell division, initiation and growth shoot, and the development of photomorphogenesis. Photomorphogenesis is a change in morphology due to the influence of light on tissue culture techniques.

Part of the seeds that contained hilum given some of the same kinetin concentrations as previous to produce callus at kinetin concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ppm. This shows that there is the influence of endogenous hormones in the seeds. The growth hormones produced plants are called endogenous hormones. These endogenous hormones are synthesized in meristematic tissues including leaves, branch primordium,

growing roots and seeds, while exogenous hormones are growth regulators synthesized outside the plant [21]. Based on theory, callus will be formed if the concentration of auxin and cytokinins is balanced. so, the callus is formed due to the presence of high auxin which can balance the concentration of the hormone kinetin given to the treatment.

Auxin increases cell elongation, cell division, and adventitious root formation [22]. Auxin has the effect of inhibiting adventitious and axillary shoot formation too, but its presence in tissue culture medium is required to increase adventitious root formation. The presence of auxin at high concentrations will stimulate callus formation and suppress morphogenesis. The balance between cytokinins and auxins increased the growth of root, shoot and callus *in vitro* culture [23]. The results of the callus formed in the treatment with a compact texture and a green color. The callus that had the largest size was found in the kinetin treatment with a concentration of 0.4 ppm.

Figure 5. Callus on 0.4 ppm kinetin treatment

B. Effect addition of TDZ

Based on the result of the study, life percentage of black pomegranate seed explant on MS medium with addition of TDZ with various consentration presented in table 3.

Table 3. Life percentage of black pomegranate seed explants on MS medium with addition of TDZ at 4 weeks after planting.

Treatment	Percentage live of explants (%)		Time of callus appears (DAP)	
	Base of the seed	Tip of the seed (hilum)	Base of the seed	Tip of the seed (hilum)
Control	0	0	-	-
TDZ 0,1 ppm	75	100	28-32	28-32
TDZ 0,2 ppm	100	100	30-32	27-30
TDZ 0,3 ppm	100	100	25-30	26-32
TDZ 0,4 ppm	100	100	25-29	25-31
TDZ 0,5 ppm	100	100	24-28	25-27

Note : (-) Callus does not appear

The formation of callus when addition TDZ hormone began to appear on average 25 DAP. Callus in the control treatment was not formed but form of sprouts. Other treatments except control resulted in 100% percentage of explants forming callus. This shows that the addition of cytokinins has an effect on increasing the percentage of callus growth because it can increase the concentration of endogenous ZPT in explants, so that it can trigger callus growth.

The fastest callus formation in this study was the concentration of the TDZ hormone (tip of the seed) 0.5 ppm, the time when the callus appeared on day 25 DAP and the addition of the TDZ hormone (base of seeds) was the most optimal with a concentration of 0.4 ppm, when the callus appeared on day 24 DAP. Giving TDZ with a low concentration is faster to induce callus that works at high concentrations.

The longest callus appearance was obtained by treatment with TDZ hormone (seed tip) 0.1 ppm, callus appearance time was on the 28th day DAP and the longest addition of TDZ hormone (seed base) with a

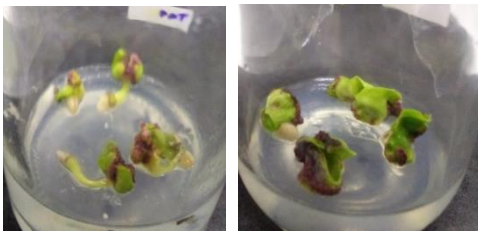
concentration of 0.2 ppm, callus appeared time was on the 30th day DAP.

Table 4. Callus texture of black pomegranate seed explants on MS medium with additionTDZ at 4 weeks after planting

Treatment	Texture of callus	
	Base of the seed	Tip of the seed (hilum)
Control	-	-
TDZ 0,1 ppm	Compact	Compact
TDZ 0,2 ppm	Compact	Compact
TDZ 0,3 ppm	Compact	Compact
TDZ 0,4 ppm	Compact	Compact
TDZ 0,5 ppm	Compact	Compact

Note : (-) Callus does not appear

Callus on black pomegranate seeds with TDZ hormone treatment was formed non-embryogenic callus. Callus form is compact with brownish yellow color. This is in accordance with the literature [24] that embryogenic callus is yellowish green, has a crumbly and dry texture, while non-embryogenic callus has a compact, wet and clear brownish texture. Callus with a compact texture is characterized by a dense, hard callus and the cells are difficult to separate because it is very tight. The texture of the callus depends on the type of plant used, the nutrient composition of the media, growth regulators and environmental conditions of the culture.



a b

Figure 6. Callus at 0.5 ppm kinetin, a. the base of the seeds, b. tip of the seed

The callus texture is a marker to assess the growth of a callus [25]. A good callus for use as a secondary metabolite producer is a compact texture (non-friable). The compact callus texture seems good because it can accumulate more secondary metabolites. Brownish color of callus was found in almost all treatments formed by callus. Brownish color in callus due to the presence of excessive phenolic compounds, which they are often aroused due to the explant sterilization process. The browning event is a natural event and a process of adaptive change in plant parts due to physical influences such as stripping and cutting. Symptoms of browning are signs of explant physiological deterioration.

4. CONCLUSION

The conclusion of this research is cytokinin hormones, both kinetin and TDZ, have not been able to significantly induce shoots on black pomegranate seed explants (*Punica granatum* L.). The optimal TDZ concentration in inducing callus was 0.4 ppm on the day 24 DAP. The hormone kinetin was able to induce shoots on explants of black pomegranate seeds (*Punica granatum* L.) with the best concentration of 0.4 ppm but the results were not significant.

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