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IN VITRO SHOOT REGENERATION OF *Citrus nobilis* Lour. FROM INTACT SEED AND COTYLEDON EXPLANTS

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SUMMARY

Siam orange (*Citrus nobilis* Lour.) is a fruit from Kampar Riau, Indonesia, which has a sweet flavor, is fragrant, and thin-skinned. However, plantation of siam orange is decreasing due to diseases. Preservation is required to prevent loss of siam orange by planting citrus on a large area. Plant propagation through tissue culture (*in vitro* culture) is an alternative approach to obtain seedlings in large quantities and potential for production of pathogen-free healthy plants. The effects of plant growth regulators of *6-benzylaminopurine* (BAP) on shoot regeneration of *Citrus nobilis* Lour. From intact seed and cotyledon explants were studied. The explants were cultured on Murashige and Skoog medium (MS) containing 30 g/L sucrose and 8 g/L agar supplemented with treatment of plant growth regulator (PGRs) (0, 1, 2, 3, 4, and 5 mg/L of BAP). The results showed that BAP treatment increased the number of shoots from cotyledon explants, but no increase the number of shoots from intact seed explants. The difference in BAP concentration (1, 2, 3, 4, and 5 mg/L) indicates the number of shoots was not significantly different (3.6 to 4 shoots) from cotyledon explants. The growth of shoots on MS medium without supplementation with BAP produced shoots that are longer, thinner, and have lower number of leaves. The growth of shoots on MS medium containing BAP produces shorter and thicker shoots and higher number of leaves. Shoots *in vitro* were successfully acclimatized and established in sand, soil, and compost (1:1:1) mixture covered with transparent plastic in each polybag with a survival percentage of 93.3%.

Key words: *Citrus Nobilis* Lour., cotyledon explants, *in vitro,* shoot, intact seed explants

Key findings: BAP treatment increased the number of shoots from cotyledon explants, but no increase the number of shoots from intact seed explants. The growth of shoots on MS medium containing BAP produces shorter and thicker shoots and higher number of leaves than shoots in MS medium without BAP.

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INTRODUCTION

Siam orange (*Citrus nobilis* Lour.) is a Kampar native fruit from Riau province of Indonesia, which has a sweet flavor, fragrant, thin-skinned, and high water content (Wahibah *et al*., 2011). Siam orange is utilized as fresh fruit, however, it also has the potential to be utilized in medication. In the field of medicine, citrus can protect humans from cancer, it can also help prevent kidney diseases, lower cholesterol level, is rich in potassium, boost heart health, and fights against virus infections. Citrus fruits contain a variety of vitamins (ascorbic acid, thiamin, niacin, and vitamin B6), minerals (potassium, calcium, phosphorus, magnesium, and copper), fiber, and phytochemicals such as carotenoids, flavonoids, and limonoids, which appear to have biological activities and health benefits (Clay, 1998; Turner and Betty, 2013). Siam orange plantation in Kampar, Riau decreases due to diseases. This resulted in a change of land of orange groves into oil palm plantations. Preservation is required to prevent loss of Siam citrus by planting on a large area. Citrus cultivation in large scale requires a lot of citrus seedlings. Plant propagation through tissue culture (*in vitro* culture) is an alternative method to obtain seedlings in large quantities. Tissue culture also allows fast selection for crop improvement, among others, for production of pathogen-free healthy plants.

Plant regeneration *in vitro* is influenced by the source of explants and the addition of growth regulators in the culture medium (Smith, 2009). Citrus seeds can be used as explants for shoot induction. The citrus seed is polyembrionic, with multiple embryos can be found in an individual seed. Generative seedlings are derived from fertilization (zygote), while the vegetative seedlings come from nucellar embryos. Nucellar embryos give rise to seedlings that are of the same genotype as the female parent (Koltunow *et al*., 1996; Setiono and Supriyanto, 2005; Chanana and Gill, 2008). Cotyledons are part of seeds containing nucellar embryos (Koltunow *et al*., 1996; Jajoo, 2010). Results of citrus propagation *in vitro* using cotyledon explants were tested with markers random amplification of polymorphic DNA (RAPD) which showed the same properties as its parent (Ramkrishna *et al*., 2005). Planting intact seeds or cotyledons through *in vitro* culture allows nucellar embryos to grow more, so that the number of seedlings will increase.

Shoot regeneration was enhanced through supplementation of growth regulators in the culture medium. The growth regulator class of cytokinin for example BAP (*6-Benzylaminopurine*), kinetin, either alone or in combination with auxin, for example NAA (*1-Naphthaleneacetic acid*) or IBA (*Indole-3-butyric acid).* Multiple shoots were obtained from seeds of lemon (*Citrus Jambiri* Lush.) when cultured on Murashige and Skoog (MS) medium, supplemented with 1.5 mg/L BAP (Altaf *et al*., 2008). *In vitro* culture of seed explants *Citrus limonia* Osbeck. on MS medium with the addition of BAP 2.22 mM was able to induce shoots with the highest number (18.26 shoots), while the addition of NAA which decreased the number of shoots were formed (Jajoo, 2010). The highest number of shoots was observed in MS medium containing 5 mg/L BAP from the cotyledonary explants of *Citrus reticula* L. (Sarma *et al*., 2011).

This study aims to investigate the effect of plant growth regulators of *6-benzylaminopurine* (BAP) on shoot regeneration of *Citrus nobilis* Lour. From intact seed and cotyledon explants.

MATERIALS AND METHODS

Sterilization and preparation of explant

Seeds were harvested from mature fruits of *Citrus nobilis* from a plantation in Kuok, Kampar Riau, Indonesia. The seeds were washed with running tap water and then treated with 10% detergent for 2 minutes. Thereafter, the seeds were rinsed in water. The surface sterilization of explants was done with 2 g/L fungicide (Dithane *M-45*) for 5 minutes followed by 2 g/L bactericide (Agarept) for 5 minutes and rinsed in water. Seeds were then soaked in 70% ethanol for 30 seconds followed by soaking in 20% sodium hypochlorite **(**Bayclin) for 20 minutes and then rinsed 2 times with sterile distilled water. There were two types of explant, including intact seeds and cotyledons. Seed explants were isolated through the release of the seed coat. Intact seed explants were cotyledon and embryonic axis. Cotyledon explants have two cotyledons, whereas zygotic embryos were removed.

Shoot regeneration

Explants were cultured in Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with 0.8% agar and supplemented with 30 g/L sucrose and different concentrations of *6-benzylaminopurine* (BAP): 6 concentration, namely 0, 1, 2, 3, 4, and 5 mg/L. Each culture bottle was filled with one citrus seed (for intact seed explants) and two cotyledons (for cotyledon explants) derived from a single seed. All experiments were conducted in a completely randomized block design (CRD) with five replications per treatment. Cultures were maintained at 22 ºC under 24 h photoperiod that was provided by cool white fluorescent light with 860 Lux. Data were collected after five weeks of culture. Analysis of variance (ANOVA) and Tukey test (honestly significance difference/HSD) were used to analyse the data.

Hardening and polybag establishment of shoots

In vitro shoots were removed from culture bottle and residual agar at the root side was removed with tap water. Plantlets were dipped in fungicide (2% Dithane solution), washed in tap water, then soaked in a commercial plant growth regulator (Growtone) (containing NAA) 2 ml/L for 10 minutes. *In vitro* shoots were transferred to polybag filled with a mixture of soil, sand, and compost (1:1:1). The study consisted of two treatments: the treatment of sachet technique and treatment of non sachet technique. Each treatment consisted of 15 replicates or polybags, and each polybag was filled with one seedling. In the treatment of Sachet technique, each polybag was covered with a transparent plastic bag and shoots were not watered. However, in the treatment of non sachet technique, polybags were not covered with a transparent plastic bag and shoots were watered every two days. Plants were established in the home screen for four weeks. The plastic bag was removed after 8 weeks and watered regularly. The percentage of survival of the seedlings was observed after 8 weeks of acclimatization.

RESULTS

In vitro morphogenesis from intact seed and cotyledon explants of *Citrus nobilis* Lour.

BAP treatment had a significant effect on the percentage of shoot regeneration and the percentage of root regeneration from cotyledon explants, but no significant effect on the percentage of live explants, shoot and root morphogenesis, and callus initiation. BAP treatment had no significant effect on the percentage of live explants, shoot and root morphogenesis, and callus initiation from intact seed explants of *Citrus nobilis*. The percentage of live explants and morphogenesis potential from intact seeds and cotyledon explants of *Citrus nobilis* after 49 days of culture was presented in Table 1. The percentage of live explants from cotyledon explants for all treatments was up to 100%. All explants were alive because of sterilization of explants. The explants used were cotyledons of citrus seeds that are meristematic because it contained nucellar embryos that will grow into shoots. Sterilization methods using a detergent with a soaking time of 30 minutes, 2 g/L fungicide for 10 minutes, 2 g/L bactericide for 10 minutes, 20% Na-hypochlorite for 7 minutes, and 70% alcohol for 3 minutes did not cause tissue damage, hence there was no tissue damage and death of explants.

The percentage of shoot regeneration from intact seed explants for all treatments reached 100%. The percentage of shoot regeneration from cotyledon explants on MS medium without growth regulator (control treatment), and with 1, 2, and 3 mg/L BAP treatment reached 100%. However, the percentage of shoot regeneration decreased (80%) in cotyledon explants treated with high concentration of BAP (4 and 5 mg/L BAP).

Intact seeds or cotyledon explants were capable of forming roots with a fairly high percentage (100%) in MS medium without growth regulators. BAP treatment on intact seed explants did not inhibit root formation. Only the highest concentration of BAP treatment (5 mg/L) inhibited root regeneration percentage (60%). The lowest percentage of root regeneration from cotyledon explants occurred on MS medium with the addition of 5 mg/L BAP (60%).

Percentage of callus formation is still low (0 to 20%). Callus was only formed from intact seed and cotyledon explants with BAP treatment (20%), while intact seeds and cotyledons grown on MS medium without BAP did not produce callus.

Table 1. Effect of BAP on the percentage of live explants, shoot and root morphogenesis, callus initiation from intact seeds and cotyledon explants of *Citrus nobilis* Lour. Kampar.

Growth regulators BAP (mg/L)	Explant life (%)		Shoot regeneration (%)		root regeneration (%)		callus initiation (%)	
	I	C	I	C	I	C	I	C
0	100	100	100	100b	100	100b	0	0
1	100	100	100	100b	100	100b	20	20
2	100	100	100	100b	100	100b	20	20
3	100	100	100	100b	100	100b	20	20
4	100	100	100	80a	100	100b	20	20
5	100	100	100	80a	100	60a	20	20

The numbers followed by different letters in the same column show significantly different at the 5% test level based on the Tukey test, I: Intact seeds explants; C: Cotyledon explants.

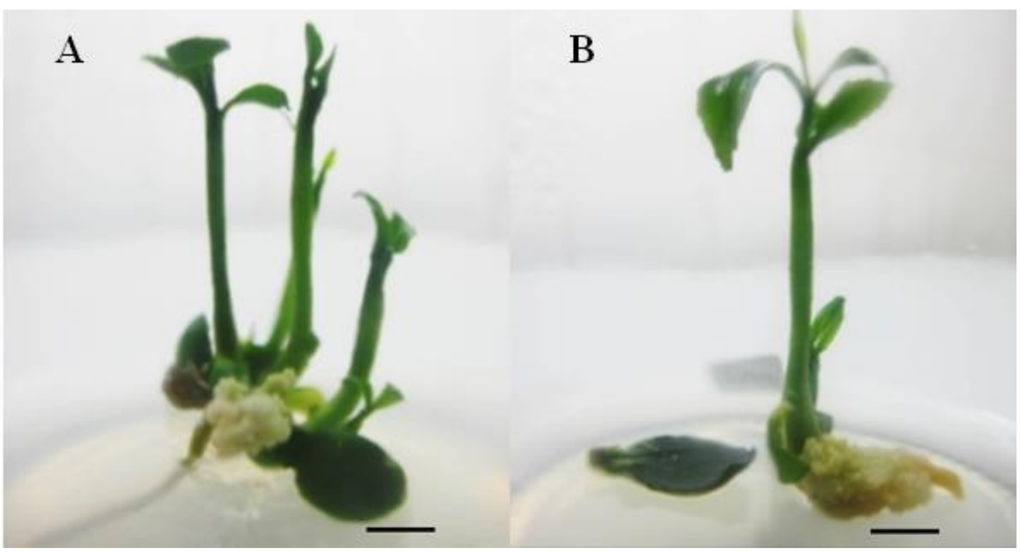

Figure 1. Callus formation from intact seed (A) and cotyledon (B) explants (Scale bar 5 mm).

Shooting response of intact seeds and cotyledon explants of *Citrus nobilis* Lour. Kampar

BAP treatment had significant effect on shoot length and the number of leaves from intact seeds. Effect of BAP on the shooting from intact seeds and cotyledon explants of *Citrus nobilis* Lour. Kampar was presented in Table 2. Shooting response from intact seeds and cotyledon explants on MS medium with the addition of BAP was shown in Figures 2 and 3.

Intact seeds were cultured on MS medium containing BAP which was able to form shoots after 16 to 20 days of culture, while it took 10 to 19 days for cotyledons. The fastest shoot regeneration was obtained from intact seed and cotyledon explants on MS medium without growth regulator (respectively 16 and 10 days of culture). The longest day to shooting from intact seeds and cotyledons found in the treatment of 4 mg/L BAP (respectively 19 and 20 days of culture). BAP treatment increases the number of days to shooting.

Table 2. Effect of BAP on the shooting from intact seeds and cotyledon explants of *Citrus nobilis* Lour. Kampar.

Growth regulators	Days to shoot		Number of shoots		Shoot length (cm)		Leaf number	
BAP (mg/L)	I	C	I	C	I	C	I	C
0	16	10	4,60	2.0a	6.00 ^{d}	4.60 ^{c}	2.40^{a}	3.20
1	19	14	4,00	4.0b	3.00 ^{c}	3.00 ^{b}	4.20^{b}	4.80
2	19	15	4,60	3.8b	2.80 ^{b}	2.40 ^{b}	5.20 ^{b}	4.40
3	16	12	4,00	3.6b	2.80 ^{b}	2.20 ^{ab}	4.20 ^{b}	5.40
4	20	19	4,60	4.0b	2.80 ^{b}	1.80 ^{a}	4.40 ^{b}	5.40
5	18	14	4,80	4.0b	2.00 ^{a}	1.60 ^{a}	4.60 ^{b}	4.00

The numbers followed by different letters in the same column show significantly different at the 5% test level based on the Tukey test, I : Intact seeds explants; C: Cotyledon explants.

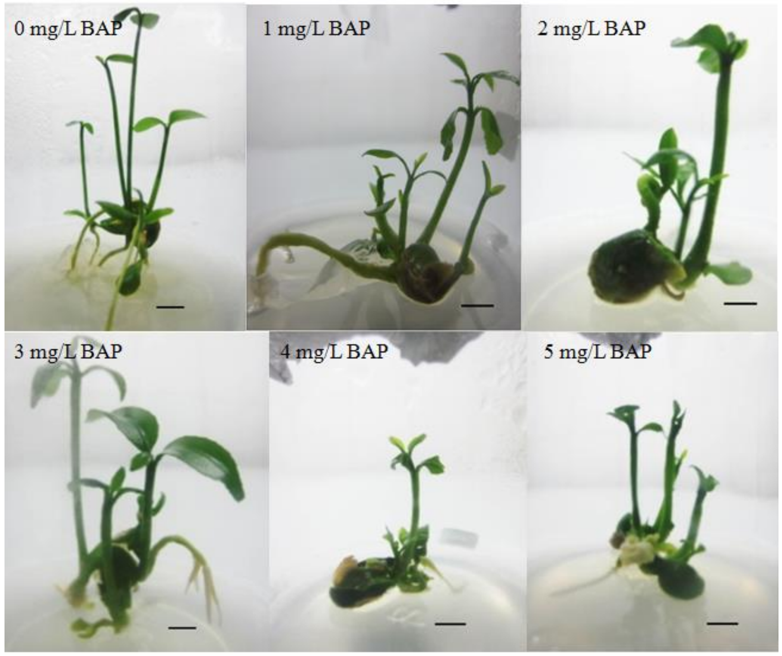

Figure 2. Shooting Response from intact seed explants of *Citrus nobilis* on MS medium supplemented with BAP at different concentrations after 49 days of culture (Scale bar 5 mm).

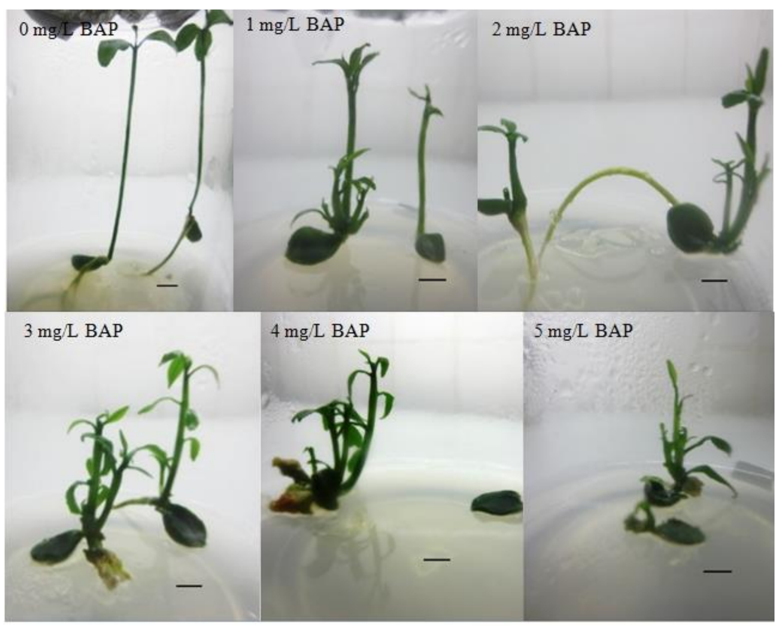

Figure 3. Shooting Response from cotyledon explants of *Citrus nobilis* on MS medium supplemented with BAP at different concentrations after 49 days of culture (Scale bar 5 mm).

Treatment of BAP had no significant effect on number of shoots from intact seeds explant. However, it had a significant effect on the number of shoots from cotyledon explants. Almost all of the BAP treatments on intact seed explants showed that the number of shoots were almost the same as that of treatment without growth regulators (4 – 4.8 shoots). BAP treatment of growth regulators tended to increase the number of shoots from cotyledon explants (3.6 - 4 shoots) compared to the control treatment (2 shoots).

Shoot growth was further shown by the length of shoots and number of leaves. There was a difference between mean length of shoot among BAP concentration treatments on whole seed explants and cotyledon explants. The highest length of shoots was obtained on MS medium without being supplemented with BAP. Treatment of 1 to 5 mg/L BAP showed a lower shoot length, and significantly different from control (without BAP treatment). The lowest shoot length was obtained at 5 mg/L BAP treatment, i.e. 2 cm (intact seed) and 1 cm (cotyledon). BAP treatment inhibited the elongation of shoots. Plant growth regulator that plays a role in the elongation of shoots is auxin, while cytokines can inhibit the extension of shoots. Morphology shoots formed from whole seeds or cotyledon explants showed shorter shoot length and larger stem diameter, while the control treatments showed higher and thinner stem (Figures 2 and 3). Treatment of exogenous cytokinin caused the stem tissue to became thicker because the elongation of cells occurred to the lateral side which was subsequently used to process initiation and growth of leaf (Gardner *et al*., 2008).

BAP treatment had significant effect on the number of leaf from intact seed explants, but no significant effect on cotyledon explants. Treatment of BAP could increase the number of leaves compared to control treatment from intact seed explants, but among different BAP concentrations showed no significant differences. The average number of leaves from the cotyledon explants was not significantly different between treatments, about 3.2 to 5.4 strands. This was because the number of leaves from cotyledon explants without treatment of BAP growth regulator were able to produce leaves of more than 2 strands.

Effect of sachet technique on survival of seedlings

The maximum survival of shoot *in vitro* (93.33%) was observed using a closed transparent plastic (Sachet technique) in polybag filled with a mixture of sand, soil, and compost (1:1:1). Whereas, shoot *in vitro* in polybag without sachet technique showed lower survival (46.67%) after four weeks. Citrus seedlings from sachet technique resulted in healthier, lighter leaf color (Figure 4). The main problem during the acclimatization of plantlets or *in vitro* shoots in *ex vitro* conditions was an increase in air temperature and high dehydration of *in vitro* shoots because of the thin cuticle layer of the leaves. Transparent plastic closure on shoots in polybag serves to prevent evaporation and maintain the humidity of soil on growth medium.

Figure 4. Citrus seedling is growing in polybag : without sachet technique (left), and with sachet technique (right).

DISCUSSION

Our results show that the treatment of exogenous BAP does not improve shoot regeneration. High concentrations (4 and 5 mg/L) decrease shoot regeneration (80%) from cotyledon explants. Intact seeds contain zygotic embryos, and cotyledons contain nucellar embryos which are naturally able to form shoots. In addition, the possibility of endogenous cytokinin is optimal to stimulate regeneration of shoots, so the addition of exogenous cytokinin with high concentrations inhibits shoot regeneration.

Regeneration of shoots from the cotyledons explants of *Citrus nobilis* Lour. from Kampar Riau, Indonesia was higher (100%) than the percentage of the highest shoots from cotyledonary explants of *Citrus clementina* cultivar "Monreal", "SRA 63", and "SRA 64" respectively 50%, 33.33%, and 25.93% by treatment of BAP (Lombardo *et al*., 2011). Shoot regeneration from cotyledon explants of *Citrus nobilis* from Kampar is also higher than the shoot regeneration from cotyledonary explants that are excised from 12 day old seedlings of *Citrus reticulata* with 84% highest shoot regeneration on MS medium containing 5 mg/L BAP after 4 weeks of culture. However, the shoot regeneration from cotyledonary explants of *Citrus reticulata* does not occur on MS medium without growth regulators (Sarma *et al*., 2011). The percentages of shoot regeneration from embryo and cotyledonary segment explants (2 - 3 pieces) citrus Pummelo (*C. grandis* L. Osbeck.) respectively, were 88% and 75% on MS medium supplemented with 2 mg/L BAP. Shoot regeneration does not occur from cotyledonary segment explants on MS medium without plant growth regulator, but the explants formed callus (Ibrahim, 2012). The highest of shoot regeneration from leaf explants of citrus lemon (*Citrus limon* Burm L. cv. 'Primofiore') only reached 37% in MT medium (Murashige and Tucker 1969) containing 3.5 mg/L BAP (Kasprzyk *et al*., 2015). The requirement for exogenous plant growth regulators varies with the explant type, species, and apparently depends on level of the endogenous hormone.

Cotyledonary explants from seed which germinated for 12 day (Sarma *et al*., 2011) resulted in reduced meristematic tissue because the nucellar embryos was regenerated from shoots after germination, so the cotyledons grown on MS medium without plant growth regulator cytokinin were not regenerated into shoots. Cotyledonary segment explants on MS medium without plant growth regulator were not regenerated into shoots (Ibrahim, 2012) because the cotyledonary segment's size was smaller. The explant size had an effect on the response of the tissue. The size of explants shoot affects the *in vitro* proliferation of globe artichoke shoots (*Cynara cardunculus* var. *scolymus* L .; accession 'Art 21'). The explants with the size 1 - 1.5 cm exhibited 100 rate of shoots survival buds (7 shoots) and they give the highest number shoots (7 shoots), while the explants with a smaller size (less than 1 cm) indicates the rate of shoot survival is lower (78%) and lower number of shoots (1 bud) (El Boullani *et al*., 2017).

Intact seeds and cotyledons contain embryos that naturally has been able to form roots. The addition of exogenous cytokinin increased the ratio of cytokinin to auxin which inhibits root formation. Root regeneration occurs if the ratio auxin is higher than cytokinin (Van, 2009).

In our study, no callus on intact seeds or cotyledonary explants were recorded in MS medium without growth regulators. BAP treatments on intact seeds and cotyledons explants stimulated callus formation (20%). The ability of callus formation from their injuries cropped cuts both cotyledons and embryos from the cotyledons. Injuries to the explant stimulated callus formation. Callus induction in dicotyledone explants occured due to a balanced ratio between cytokinin and auxin (Van, 2009). Treatment of exogenous cytokinin (BAP) was used as a balance of the ratio of auxin to cytokinin thus stimulated callus formation.

Percentage of callus formation is still low (20%) since there was no treatment for callus induction which generally is in the form of auxin growth regulator 2.4-D *(2.4-Dichlorophenoxyacetic acid*) or NAA (*Naphthaleneacetic acid*) which balances auxin to cytokinin ratio. The percentage of high callus formation was obtained from leaf cutting explants (90%) and stem cutting explants (95%) from Mandarin kinnow citrus (*Citrus reticulata* Blanco) with treatment of 1 mg/L 2,4-D + 0,5 mg/L NAA + 0,5 mg/L BAP (Kamruzzaman *et al*., 2003).

Several callus were formed with white color and friable texture. Such callus has the potential to form somatic embryo, which will form plantlets. Somatic embryogenesis may be used as one of the methods to obtain plants that are disease resistant. Elimination of virus from some plants, among others *Saccharum officinarum*, *Theobroma cacao*, and three citrus species (common mandarin, sweet orange, and Dweet tangor) through *in vitro* culture by somatic embryogenesis (D'Onghiaa *et al*., 2001; Dewanti *et al*., 2016; Edward and Wetten, 2016).

Intact seed explants of whole seeds which are able to form more shoots because intact seeds contain zygotic embryos on the embryo axis and nucellar embryos on cotyledons. Cotyledons contain food reserves and embryo nucellar, however the enzymatic activity in the cotyledons is regulated by embryonic axis so that the metabolic processes of the cotyledons can be disrupted if they are separated from the embryo axis (Ramkrishna *et al*., 2005). Citrus seed is naturally able to form more than one shoot because it is polyembrionic. Treatment of BAP (1, 2, 3, 4, and 5 mg/L) in cotyledon explants can increase the number of shoots formed (3.6 - 4 shoots). Treatment of 1 - 5 mg/L BAP increases the level of cytokinin that stimulates the cell division and cell differentiation of the meristematic cotyledon explants. The treatment of 1 - 5 mg/L BAP does not stimulate the formation of shoots from intact seed explants. This is likely because intact seeds have zygotic embryos and nucellar embryos which already contains endogenous cytokinin that do not require exogenous cytokinin. This result showed that supplement of 1 mg/L BAP was enough to trigger shoot regeneration, and it did not need to be increased up to 5 mg/L since it did not increase the number of shoots significantly.

The number of shoots formed from intact seed and cotyledon explants in this citrus (*Citrus nobilis* Lour.) from Kampar Riau is higher (each 4.8 and 4 shoots) compared to shoots formed from other explants. Shoot regeneration from *Citrus nobilis* Lour. orange nodes explant from Tawangmangu, produced the highest number of shoots (3.93 shoots) obtained on MS medium with supplement of 0.5 mg/L BAP (Samanhudi *et al*., 2010). Shoot regeneration from *Citrus cinensis* epicotyl explant produced 3.2 shoots with supplement of 2 mg/L BAP, *C. Limonia* epicotyl explant produced 2.4 shoots with supplement of 1.5 mg/L BAP, *C. volkameriana* epicotyl explants produced 2.9 shoots with supplement of 0.5 mg/L BAP, and *C. aurantium* epicotyl explant produced 1.4 shoots without any supplement of growth regulator (Schinor *et al*., 2011). Shoot regeneration from cotyledon explant from *Citrus nobilis* seed that has been germinated showed lower number of shoots (1.33 shoots) which were grown on MS medium with supplement of 0.1 mg/L IAA and 0.4 mg/L BAP (Harliana *et al*., 2012). Results from other studies used cotyledon explant from *C. reticulata* seed which was germinated for 12 days showed a higher number of shoots (6.5 shoots) which were grown on MS medium with supplement of 5 mg/L BAP (Sarma *et al*., 2011). Pummelo local orange (*Citrus grandis* (L.) OSBECK.) shoot induction results produced a higher number of shoots (9.33 shoots) grown on MS medium with supplement of 2 mg/L BAP, while cotyledon cut explants produced 3.33 shoots grown on MS medium with supplement of 1 mg/L BAP (Ibrahim, 2012). The difference in such number of shoots showed that shoot regeneration *in vitro* is affected by the type of explant and growth regulator used. The type of explant is related to the tissue meristematic level and endogenous hormone which affects cell division and cell differentiation to form shoots. Nucellar embryos from cotyledon explants from germinated seeds probably has grown and its endogenous hormone decreases. Therefore, the absence of nucellar embryos or a little embryo causes lower number of shoots. Such several studies showed that embryo explant produces the highest number of shoots. Possibly this is due to higher number of merismeratic tissue and level of embryo merismeratic tissue, as well as higher endogenous cytokinin. Such studies showed that shoot regeneration *in vitro* is affected by the interaction between endogenous hormone and exogenous hormone applied, as well as the merismeratic level of explant tissue.

The results of this study showed that BAP treatment increased the number of shoots from cotyledon explants, but no increase the number of shoots from intact seed explants. The difference in BAP concentration (1, 2, 3, 4, and 5 mg/L) indicates the number of shoots was not significantly different (3.6 to 4 shoots) from cotyledon explants. The growth of shoots on MS medium without being supplemented with BAP produces shoots that are longer, thinner, and have lower number of leaves. The growth of shoots on MS medium containing BAP produces shorter and thicker shoots and higher number of leaves.

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