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Effect of plant growth regulators on *in vitro* culture of pineapple (*Ananas comosus* L. Merr*)* MD2 variety

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Abstract

Pineapples (*Ananas comosus* L. Merr) are fruits that belong to the Bromeliaceae family. Pineapple variety MD2 is one of the varieties that has gained a place in the market among pineapple farmers due to its high value and quality. However**,** it is difficult to meet the demand for planting materials using conventional propagation techniques. Hence, plant tissue culture technology is one of the methods that has been widely used in the agriculture industry that boosts up the production of pineapple planting materials within a short period and is cost-efficient. The objective of this study was to determine the effect of plant growth regulator concentration to *in vitro* culture of MD2 variety pineapple. In this study, the various concentrations of 6-Benzylaminopurine (BAP) and α-Naphthalene Acetic Acid (NAA) for *in vitro* culture of MD2 pineapple were studied. The plantlets were effectively initiated from MD2 pineapple crown on Murashige and Skoog (MS) basal salt containing 30.0 g/L sucrose, and 2.0 mg/L BAP in two months of culture. Next, the pineapple plantlet was subculture on shooting medium containing full strength solid Murashige and Skoog (MS) medium supplemented with vitamins with various concentration BAP (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 mg/L) and NAA (0, 1.0, and 2.0 mg/L). The result obtained showed that the solid MS medium added with 30.0 g/L sucrose, without any BAP and NAA (T1) had the highest *in vitro* shoot regeneration. Meanwhile, the solid MS medium with 30.0 g/L sucrose with 1.0 mg/L NAA (T1) recorded the highest plantlet height (cm). The mean value for *in vitro* shoot regeneration in T1 and plantlet height (cm) in T1 were 2.80 (±0.5) and 4.40 (±0.3). To conclude, less amount plant hormone regulator required to obtain the mass quantity of *in vitro* clonal pineapple that can help solve the problem of lack of plant material in the pineapple crop industry.

1. Introduction

Ananas comosus L. Merr or Pineapple is a very popular tropical fruit in Malaysia because of its sweetness and attractive yellow colour. Pineapple is categorized as a commodity plant because of its great potential to generate income for farmers and contributes to a country's economy. It is available in the market throughout the year regardless of the season. It has high fibre content and vitamins especially A, B, and C. In addition, Malaysia is ranked 23rd place in the world among the world's pineapple producing countries with a production of 340 thousand metric tons of pineapple (MPIB, 2018).

There are many pineapple varieties such as Moris, N36, Josapine, Maspine, Sarawak, Gandul, and Yankee. Nonetheless, MD2 variety has been selected to lead Malaysia's ambitions in the pineapple exporting industry. MD2 variety has consistency in size and ripeness. Moreover, MD2 variety is high in vitamin C and contains four times the vitamin source (vitamins A, B6, E and K). It has lower acidity, thinner skin and longer shelf life of 30 days compared to the usual 21 days for other varieties which makes it is better for long-distance shipping (Amar *et al.,* 2015). It has also shown the highest sweetness and lowest astringency index (Chiet *et al*., 2014). Besides that, MD2 variety had the highest content of bioactive compounds, antioxidant

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capacities and bromelain activity with respect to biochemical properties compared to other cultivars (Chiet *et al*., 2014). Its price is also three times higher compared to other pineapple varieties and has returns in selling up to 200% which is a good sign for the economic impact in Malaysia (Amar *et al*., 2015)

However, the conventional way of producing healthy and high-quality pineapple is time-consuming. Conventional methods of obtaining materials from the sucker, crown, and slips of the pineapple take up to 16 to 18 months after the fruit is harvested. Furthermore, imported plant material is very costly (Hepton, 2003). In addition to that, the multiplication rate of pineapple is low when using conventional propagation method. This causes suppliers to be unable to meet the high demand for pineapple MD2 variety planting materials. Nevertheless, these constraints can be overcome through plant tissue culture technique where the mass quantity of *in vitro* clonal pineapple MD2 variety can be produced within a short period. Tissue cultured pineapples have similar physical and chemical properties when compared to conventionally grown plants (Jackson *et al.,* 2016).

In plant tissue culture innovation, plant tissue culture media is generally capable of *in vitro* development and plant tissue morphogenesis. Plant growth regulators are vital in plant tissue culture since they play an imperative part in stem elongation and apical dominance. The success rate of plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in cultured media (Saad and Elshahed, 2012). Plant growth regulators are the basic media parts in deciding the formative pathway of plant cells. Auxins and cytokinin are the most widely used plant growth regulators in plant tissue culture and are usually used together. The ratio of the auxin to the cytokinin determining the type of culture established or regenerated. Auxins promote both cell division and cell growth while cytokinin promotes cell division. A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Additionally, tissues from diverse parts of plants may have different requirements for satisfactory growth (Saad and Elshahed, 2012). The study of MD2 variety pineapple has been done by Danso *et al*. (2008) through direct *in vitro* micropropagation which displayed optimum result for *in vitro* proliferation when the concentration of NAA in the medium was 2.0 mg/L and the BAP 5.0 mg/L. A study by Hamid *et al*. (2013), found that in both direct and indirect regeneration of MD2 variety pineapple in various combination of plant growth regulator shows the highest number of shoot multiplication was in MS medium supplemented with BAP 3.0 mg/L, NAA 1.0 mg/L 3N1 (15±0.1). Therefore, the increased multiplication rate of MD2 variety *in vitro* will serve as an alternate source of planting materials for both subsistence and large-scale farmers in the pineapple industry. It is important to find the most suitable PGRs that can produce higher yield as well as reduce the cost of sustaining MD2 pineapple plant materials.

2. Materials and methods

This experiment was conducted in the postgraduate laboratory, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM), Branch Selangor, Campus Puncak Alam, Malaysia.

2.1 Plant material and surface sterilization method

The crown of MD2 variety pineapple was used as explants. The crown was obtained from KOSAS Berhad (Koperasi Serbaguna Anak-Anak Selangor Berhad). The crown was sterilized using a method established by Zuraida *et al.* (2011). The crown was cleaned with detergent and tap water before immersed in fungicide for one hour prior to the sterilization process inside the laminar airflow cabinet. Then, crown explant was sterilized in 50% (v/v) Clorox for 50 mins. Subsequently, the crown explant was rinsed three times with sterile distilled water and then again using 20% (v/v) Clorox for 10 mins. Finally, the crown explant was rinsed three times using sterile distilled water. The crown was cut into 1 cm^3 before culture in the shooting medium.

2.2 Plant tissue culture media preparation for initiation stage

Full strength plant tissue culture media was prepared by measuring 4.40 g/L MS basal salt (Murashige and Skoog, 1967), 30.0 g/L sucrose, and 8.0 g/L solidifying agar for initiation stage and supplementing it with 2.0 mg/L BAP. The culture media were adjusted to pH 5.70 using 1 M NaOH or 1 M HCl prior to autoclaving at 121˚C for 20 minutes. The cultures were incubated in the growth chamber at 25°C±2°C temperature and maintained with 16 hours' light photoperiod under fluorescent lights (40 $\mu molm^{-2}$ s^{-1}). Subculture was done for each plantlet in a monthly interval.

2.3 Plant tissue culture media preparation for multiplication stage

After two months in the initiation media with monthly interval subculture, the pineapple plantlets were

transferred into MS media containing 30.0 g/L (w/v) sucrose and supplemented with vitamins which were Glycine (2.0 mg/L), Myo-Inositol (100.0 mg/L), Nicotinic acid (0.50 mg/L), Pyridoxine HCl (0.50 mg/L), and Thiamine HCl (0.10 mg/L). Various concentration of BAP and NAA as per treatment table for multiplication stage (Table 1). All treatment media were adjusted to pH 5.70 using 1 M NaOH or 1 M HCl prior to autoclaving at 121˚C for 20 minutes. MS media containing 30.0 g/L (w/v) sucrose without any PGRs was used as control. Each treatment was carried out for each experiment with three replicates.

Table 1. Treatments for various combination of BAP and NAA

Treatment	BAP (mg/L)	NAA (mg/L)
T0 Control	0	0
T1	0	1
T2	0	2
T3	1	0
T4	1	1
T5	1	2
T6	2	0
T7	2	1
T8	2	2
T9	3	0
T10	3	1
T11	3	2
T12	4	0
T13	4	1
T14	4	2
T15	5	0
T16	5	1
T17	5	2
T18	6	0
T19	6	1
T20	6	2
T21	7	0
T22	7	1
T23	7	2
T24	8	0
T25	8	1
T26	8	2

2.4 Statistical analysis

The *in vitro* cultures were cultured for 6 weeks and subcultured at monthly interval. The *in vitro* cultures were maintained in a culture room at temperature 25±2 ˚C under 16 hours' light photoperiod. The pineapple plantlets growth was observed and data was collected on its number of shoots and height. The collected data were statically analysed using one-way analysis of variance (ANOVA) by SPSS program at the 5% level of significance and Microsoft Excel.

3. Results and discussion

There are two types of plant growth regulators used in this study. 6-Benzylaminopurine (BAP) is a member of the class of 6-aminopurines that is adenine in which one of the hydrogens of the amino group is replaced by a benzyl group. It has a role as a plant metabolite and a cytokinin (National Center for Biotechnology Information, 2020). The utilization of cytokines can be endogenously added to *in vitro* growth media. 1-Naphthaleneacetic acid (NAA) a synthetic plant hormone in the auxin family (Sauer *et al*., 2013). It is used to help induce root formation in various plant types, mostly in plant propagation.

Table 2 shows the result of the mean value for *in vitro* shoot regeneration and plantlet height (cm) in treatments applied. The plantlet of *Ananas Comosus* L. Merr MD2 variety well responds to the T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T15 and T18 treatments. MS medium without any plant growth regulator or control produced the highest shoot formation as compared to other treatment. The mean value for *in vitro* shoot regeneration in control was 2.9 (±0.5). Meanwhile, among the treatments, T1 and T6 showed the highest regenerated *in vitro* shoot. The mean value for *in vitro* shoot regeneration in T1 and T6 were 2.8 (±0.5) and 2.5 (±0.6). This result is much higher than in previous experiments. Results from Zuraida *et al*. (2011) recorded optimum *in vitro* shoot regeneration for Maspine variety was 1.0. In addition, finding from Bhatia *et al*. (2002) also recorded optimum *in vitro* shoot regeneration for Yeppon Gold variety was 1.3 (±0.33). Both from the previous experiment used 0.0 mg/L BAP and 0.0 mg/L NAA which similar concentration used in T1. This concludes plant tissue culture for pineapple is cost-effective because less amount of plant hormone regulator is required to obtain a mass quantity of *in vitro* clonal pineapple.

Next observation was pineapple plantlets height (cm). The result showed that T1 produced the highest pineapple plantlets (cm) compared to the control. The height of pineapple plantlets (cm) in T1 and control recorded a mean value of 4.4 (±0.3) and 3.4 (±0.4), respectively. However, this finding was lower from the previous study by Zuraida *et al.* (2011) which has reported that *in vitro* Maspine variety plantlets show satisfactory plantlets height of 5.0-7.0 cm when culture on MS media supplemented with BAP 1.0 mg/L and 0.0 mg/mL NAA.

Plant tissue culture medium that contains BAP and NAA has been studied in several species of the *Ananas Comosus* L Merr. Danso *et al*. (2008) studied various combinations of N6 -benzylaminopurine (BAP) and

Table 2. Effect of different PGRs concentration mean number of shoots and height produced per explant.

Treatment	Average height (cm)	Average shoot numbers
T0 Control	3.4±0.4^{b}	2.9±0.5^{a}
T1	4.4±0.3^{a}	2.8±0.5^{a}
T2	2.0±0.5^{c}	0.7±0.3^{ef}
T3	2.0±0.5^{c}	1.5±0.2^{bcde}
T4	1.0±0.3d^{e}	1.6±0.3^{bcd}
T5	1.4±0.3^{cd}	1.4±0.4^{bcde}
T6	2.9±0.3^{b}	1.8±0.6^{bcd}
T7	1.9±0.2^{c}	2.5±0.6^{b}
T8	1.3±0.3^{cd}	1.1±0.4^{cde}
T9	2.1±0.3^{c}	1.4±0.2^{bcde}
T10	0.8±0.1d^{ef}	0.1±0.1^{f}
T11	1.5±0.2^{cd}	0.1±0.1^{f}
T12	0.6±0.1^{ef}	No growth
T13	No growth	No growth
T14	No growth	No growth
T15	0.2±0.1^{f}	0.2±0.2^{ab}
T16	No growth	No growth
T17	No growth	No growth
T18	0.3±0.1^{ef}	0.1±0.2de^{f}
T19	No growth	No growth
T20	No growth	No growth
T21	No growth	No growth
T22	No growth	No growth
T23	No growth	No growth
T24	No growth	No growth
T25	No growth	No growth
T26	No growth	No growth

Values are expressed as mean±SE of 10 replications for the incubation period of 6 weeks. Values with the same superscript are not significantly different at $P \leq 0.05$ of Duncan multiple range test.

naphthalene acetic acid (NAA) in solid or liquid cultures. They reported that liquid cultures required 5.0 mg/L BAP to increase the multiplication rate. On the other hand, for solid MS medium supplemented with NAA or IBA alone or in combination resulted in root production in MD2 variety. Firoozabody and Gutterson (2003) used a combination of 1.5 mg/L BA and 0.5 mg/L NAA to produce the highest rate of shoot multiplication. Generally, when auxin concentration was higher than cytokinin, there will be root formation. On the other hand, when auxin concentration was lower than cytokinin there will be shoot regeneration. Reversed results for *in vitro* shoot regeneration and plantlets height (cm) was obtained from high BAP concentration of T13, T14, T16, T17, T19, T20, T21, T22, T23, T24, T25 and T26. It was found that pineapple plantlets exhibit retarded growth or no growth when supplemented with high BAP concentration. There are no new shoots formed as shown in Figure 1. Physical observation has shown the plantlets did not respond with the highest concentration of BAP and NAA. This might be because the concentration used was too high and might have cause shoot bud retardation resulting in zero shoot multiplication. This concludes, higher BAP concentration has made the explant to form buds, without any new shoot formation, explant became stunted and also formed buds and clumps. The ratio of hormones are not suitable as BAP concentration is too high and made the explant retarded. A higher concentration of BAP can cause shoot bud retardation because the excess hormone can become toxic to the plant (Azizan, 2017). Auxin and cytokinin ratio during *in vitro* tissue culture can play a critical role to induce the morphogenic response in higher plants (García *et al.,* 2008)

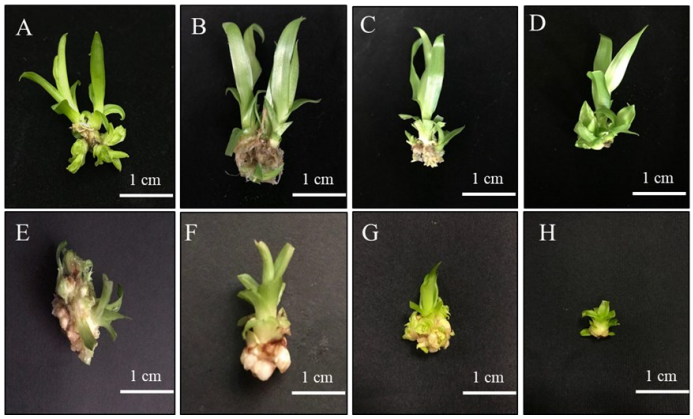

Figure 1. *In vitro* culture of MD2 variety pineapple in different plant growth regulators after 6 weeks of culture: A) T0 Control (B) T1 (C) T7 (D)T13 (E) T15 (F) T17 (G) T24 (H) T26

In general, explants need cytokinin to grow and auxin to form roots. Auxins and cytokinin are widely used in micropropagation work and important for the regulation of growth and promotion of callus growth. Among the factors that contribute to the success of *in vitro* mass production of healthy pineapple, plantlets are the addition of Benzylaminopurine (BAP) and Naphtalanaecetic acid (NAA) to the culture medium. This has been reported by Zuraida *et al.* (2011) that it is possible to produce 6,575 plantlets on a BAP supplemented medium. Therefore, MD2 variety of pineapple explant can grow and multiply well even without any PGR (Table 2). MS media without any supplemented BAP and NAA formed highest shoot numbers and only 1.0 mg/L NAA formed the highest height after 6 weeks of culture. The best PGR combination for this study is T1, MS medium with BAP 0.0 mg/L and NAA 0.0mg/L.

4. Conclusion

From the experimental study, it can be concluded that MS medium without any BAP combined with NAA at 1.0 mg/L (T1) is the best treatment in triggering shoot multiplication with a mean of 2.8(±0.5) *in vitro* shoots formed per explant and 4.4 cm height. In comparison, the *in vitro* shoot of *Ananas comosus* L. Merr (MD2 variety) did not respond to the BAP at higher concentration. It may be because there is a level of concentration that plants could tolerate the substance before it could become toxic.

Conflict of interest

The authors declare no conflict of interest.

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