In vitro responses of Areca catechu immature embryos on medium containing plant growth regulators

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Abstract. Betel (Pinang) nuts are used as herbs, traditional medicine, and in the beverage industry. Planting materials for Areca nut cultivation are seeds. However, the presence of 2 – 3 months dormancy hampers germination of Areca nut seeds. Therefore, developing alternative strategies to overcome such problems is necessary. This study evaluates Areca nut immature embryo in vitro responses on MS medium having various plant growth regulators. The experiments were set up using a single factor in a complete randomized design. The embryos were planted either on Murashige and Skoog (MS) medium (MS0), MS with a combination of BAP (1 ppm) and NAA (0.1 ppm), or MS medium supplemented with GA3 (3 ppm). Areca nut immature embryos planted on a medium containing BAP (1 ppm) and NAA (0.1 ppm) than that on a medium containing GA3 (3 ppm). The germinated embryos were best on medium supplemented with a combination of BAP (1 ppm) and NAA (0.1 ppm). Immature Areca nut embryos planted on MS0 medium were unable to grow. In conclusion, Areca nut immature embryos may be used as explants for this palm tree's micropropagation, and the embryos germinated well on a medium containing BAP and NAA.

1. Introduction

Areca nut (betel nut, Pinang) is a member of the Palmae family, and the fruits of this plant have many medicinal properties. Areca fruits are green when young and turn orange, yellow, or red when ripe. The community uses the brown seeds for Areca nut, gum strengthening drug, anti-intestinal worms, anti-diarrhea, wound medicine, uterine shrinkage, red dye, tanneries, and candy. Pinang seeds are used as industrial raw material and pharmacy [1]. Suhatri et al. [2] showed the efficacy of pinang seed extract to stimulant white mice's central nerve. Young Pinang fruits are used to make juice for health. According to Karina et al. [3], Cahyanto [4], Hidayah et al. [5], useful compounds produced from Areca plants include antioxidants (polyphenols, tannins and alkaloids).

Indonesians also use Areca trunks for traditional climbing competitions, as tools' making materials and as gutters and water channels. The Areca leaves are used in food packaging, while the roots of certain Areca varieties are poison sources.

Areca nuts are of tropical origin and distributed along the equator from South Asia to the Pacific Islands. In Indonesia, the Areca nuts are found worldwide from Aceh in the western region to Papua in the eastern-most regions. Areca nuts are monocot plants, with straight but small trunk diameter (20 - 40 cm). The stem height is varied according to the varieties. Miftahorrahman's research [6] indicated high
phenotype diversity among Areca nuts from Sumatra and Gorontalo [7], including plant height, fruit size, fruit yield, and fruit color. Five Indonesian Areca nut varieties have been released by the Ministry of Agriculture, including Betara from Jambi province and Golden Areca from North Sulawesi [8].

Seeds are used for planting materials in the Areca nut cultivation. However, the seeds are also the commercial products of these plants. Since Areca nuts are potentially important exported products, keeping a continuous supply of Areca nuts is necessary. The market share is increasing over the years, especially as export material to China and some European countries, which uses Areca seeds as ingredients in the medicinal and cosmetic industries [9]. The unavailability of high yielding betel nuts as the sources of planting materials will hamper seed productions.

As planting materials, Areca seeds are dormant and require 2 – 3 months of dormancy treatments to germinate the seeds. The presence of seed dormancy becomes a problem in the production of Areca nut seedlings. Therefore, alternative seedling production technologies are needed to provide Areca nut seeds, and plant tissue culture may be used as the alternative technology. Rapid propagation of Areca nut seedlings through in vitro technology may become the best seedling propagation technology to provide continuous seedlings for Areca nut cultivation.

Research using Areca nut in vitro is still very limited. In the experiment conducted by Bastaman et al. [10], an average of 48 percent of the Areca nut embryos germinated on the medium supplemented with only NAA at 4 - 8 ppm. The percentage of rooted cultures was 77 percent, and the highest callus culture was 19 percent. In kopyor coconut embryo rescue, a medium supplemented with 5 ppm BAP stimulates zygotic embryos' germination [11]. Therefore, it is necessary to evaluate the suitable growth regulators needed for germination, growth, and multiplication of Areca zygotic embryos' shoots. Based on kopyor coconut [12] and sugar palm embryo rescue [13], the cultured embryos can grow on in vitro media. Moon orchid germination in vitro was affected by gibberellin and coconut water [14]. The cytokinin's compositions, auxin or gibberellins in the medium, stimulate cell division and accelerate rescued embryos' germination. This experiment aims to study young Areca nut zygotic embryos' responses grown on a medium supplemented with plant growth regulators.

2. Methods

The experiment was carried out in Plant Tissue Culture Laboratory, Department of Agronomy, Faculty of Agriculture, IPB University from March to June 2020. The plant materials use young Areca zygotic embryos originated from Lhokseumawe district, Aceh Province, Indonesia. The medium used consisted of Murashige and Skoog (MS) basal medium, BAP, NAA, GA3, coconut water, activated charcoal, sugar, and aquadest. The KOH and HCl were added to stabilize medium pH into approximately 6.0.

The experimental design used a completely randomized design with one factor. The treatments consisted of three plant growth regulator (PGR) combinations, consisted of control MS medium without addition of PGR (MS0), MS medium supplemented with a combination of 1 ppm BAP and 0.1 ppm NAA, and MS medium supplemented with 3 ppm GA3. Each treatment was repeated three times, and there were a total of nine experimental units. Each experimental unit consisted of four young embryos (36 zygotic embryos were cultured in the whole experiment).

Observations were conducted three months after initial culture by observing surviving zygotic embryos, germinating embryos, rooted embryos, and plantlet regeneration. The number of shoots, the number of roots, and the number of leaves were also recorded. The collected data were statistically analysed using simple statistics.

The treatment medium was made by pipetting a volume of prepared MS stock solutions. Growth regulators are added according to the treatment combination. Furthermore, the media was supplemented with 30 g/L of sucrose. In the media containing BAP and NAA, coconut water was added at 100 mL/L. Finally, aquadest was added up to 1 liter. The medium pH was measured using a pH meter and adjusted to 5.7 by adding either HCl or KOH. Subsequently, 7 g / l of agar is added to the medium, and the agar was dissolved by heating. To the medium supplemented with BAP and NAA, two g/L of activated charcoal was also added. The media was then poured into sterile vials, covered with heat-resistant plastic and secured with rubber bands. Each bottle is labeled according to the appropriate treatment. The media was then autoclaved for 15 minutes at 121 °C and 17.5 psi (pounds per square per inch).
Young-green fruits (Figure 1.a.) with a fruit length of 7 – 10 cm are washed under running water, and the skin is cleansed with soaps. Subsequently, the fruits are soaked in 50% Bayclin, with an active sodium hypochlorite ingredient for 12 hours. The zygotic embryos were subsequently retrieved from the Areca nuts, and the seeds were vertically cut (1.b.) with the embryo containing section separated (Figure 1c). Part of the seeds containing embryos was brought to a laminar air-flow cabinet (LAFC) and subsequently soaked in 20% Bayclin solution for 15 minutes. The sterilized planting materials were rinsed thrice with sterile aquadest.

The sterilized embryos were isolated (Figure 1d) and placed in sterile Petri dishes. The isolated and intact embryos were then immersed in aquadest, containing five antiseptic Betadine drops for 5 minutes. The sterilized embryos were rinsed with sterile aquadest before planting on the treatment medium. The culture vials containing zygotic embryos were then placed on culture racks under white light, with 24 hours light exposure, and the culture room temperature was 23 ± 2 °C.

3. Results and discussion

3.1. Percentage of living, browning, germinating, and rooted embryos
Embryos that were isolated after two weeks of age grew with increasing embryo size. Areca nut embryos are shaped like a semicircular ball (Figure 1d), unlike coconut and oil palm embryos, which are tubular. The white embryo's color with the embryo's size originating from one bunch is not the same. The embryo's size is different from one bunch, presumably because the fertilization time is not the same between the fruit at the base and the tip and between the outer and inner inflorescences. Also found fruit without an embryo. The percentage of sterile embryos free from contaminants is very high, reaching 100 percent. The sterilization method is very good because the embryo is not in direct contact with air in the early stages until it is isolated in LAFC. Embryos that have been isolated and in intact or
undamaged conditions are planted in the treatment medium. Some small embryos showed very slow growth; some did not develop and remained white (Figure 2a), some browned and died (Figure 2b). The percentage of live embryos (sprouting) is not too high, only reaching 50 percent (Table 1), not too different from the results of the study by Bastaman et al. [10]. In this experiment, the low live embryos are thought to be due to the non-uniform embryo size and age. It is necessary to evaluate the correct embryo size or fruitage for higher germination.

The composition of PGR in the media affects the growth and development of Areca embryos. Based on the experiments conducted, Areca nut requires PGR to accelerate germination in vitro. Based on the data in Table 1, there are no live Areca nut embryos on media without PGR, and on media, with a plant growth regulator, the percentage of browning embryos is less than 20 percent. Young Areca embryos grown on the three media for development do not release phenolic compounds into the media. It is known that Areca seeds produce quite high phenolic compounds, but in this experiment, the isolated and grown embryos did not release phenolic compounds into the media. It is suspected that this happened because no isolated embryos were injured and during isolation did not include the endosperm tissue around the embryo. The dark brown color that occurs in embryos in the media without PGR may be due to the young Areca nut requiring PGR for cell division, and the absence of PGR makes the process of division and development of embryonic cells inhibited and causes embryo death. The presence of activated charcoal in BAP + NAA media is thought to have contributed to suppressing browning in embryos. Meanwhile, the cause of browning embryos on media containing PGR occurred in embryos that were too small in size, or there was damage during isolation.

Table 1. Percentage of some Areca zygotic embryo development at three months after culture

| Treatment media | Browning | Dormant | Germinated | Shoots | Roots | Plantlets |
|-----------------|----------|---------|------------|--------|-------|-----------|
| MS0             | 100      | 0       | 0          | 0      | 0     | 0         |
| MS with BAP and NAA | 17      | 33      | 50         | 83     | 100   | 75        |
| MS with GA3 (3 ppm) | 17      | 3       | 50         | 67     | 8     | 8         |

The composition of PGR in the media affects the growth and development of Areca embryos. Based on the experiments conducted, Areca nut requires PGR to accelerate germination in vitro. Based on the data in Table 1, there are no surviving embryos on media without plant growth regulators (PGR), and on media supplemented with PGR - the percentage of embryo browning was less than 20 percent. Young Areca embryos grown on the three media for development do not release phenolic compounds into the media. It is known that Areca seeds produce quite high phenolic compounds, but in this experiment, the isolated and grown embryos did not release phenolic compounds into the media. It is suspected that this happened because no isolated embryos were injured and during isolation did not include the endosperm tissue around the embryo. The dark brown color that occurs in embryos in the media without PGR may be due to the young Areca nut requiring PGR for cell division, and the absence of PGR makes the process of division and development of embryonic cells inhibited and causes embryo death. The presence of activated charcoal in BAP + NAA media is thought to have contributed to suppressing browning in embryos. Meanwhile, the cause of browning embryos on media containing PGR occurred in embryos that were too small in size, or there was damage during isolation.

The enlarged embryo will then produce a shoot from its widest part and turn green (Figure 2c). The base of the sprouts bulges and turns brownish. Embryos that are large enough show faster shoot growth than small embryos. The fastest embryos gave rise to shoots after one month of planting in the media. Small embryos less than 1 mm in size do not develop to form shoots or roots until three months after treatment (MAT) and, some embryos germinate after three MAT.
Areca nut embryos grow well and form shoots first before roots are formed. The embryos with the fastest response to shooting formation occurred in media supplemented with BAP 1 ppm + 0.1 ppm NAA. Shoot growth is faster, and shoot size is bigger than shoots that grow on media containing gibberellins. The presence of BAP and NAA and the addition of coconut water in the media are thought to encourage better Areca nut cell division according to the function of cytokinins and auxins in cell division. The gibberellins in this experiment had less effect on the growth of young Areca embryos. Maybe because the Areca nut is still young, the endogenous gibberellin content is still low, so the addition of gibberellin is not sufficient to accelerate the growth of embryo shoots, or it is suspected that the concentration of gibberelin given is too high. The results of research by Mukminin et al. [14] giving GA3 to 3 ppm reduced germination of moon orchid seeds in vitro. Gibberellins in chrysanthemum shoots also did not increase root formation [15].

The time required for embryos grown in media with gibberellin to germinate is longer than for embryos grown in media with BAP and NAA. Shoots that grew on media containing gibberellins looked slimmer than shoots formed on BAP and NAA media. The shape of the slender's shoots is due to the cell elongation because of the gibberellin's function.

The formation of shoots that occurred after one month of planting in culture media showed that young Areca nut embryos had a good germination response. According to research results, Miftuahorrahman and Iqbal [16] physiologically ripe Areca nuts take 2-3 months to germinate in the field. This experiment's results give hope that Areca nut germination can be accelerated in vitro for seed supply.

The germinated zygotic embryos were generally also formed roots at the base of the shoots. Roots formed on the media supplemented with BAP and NAA appeared faster, the root diameter was larger, and there were secondary roots (Figure 2d) than that in the other medium. Zygotic embryos cultured on medium supplemented with GA3 generated slender and short roots (Figure 2e). In this experiment, GA3 seems to inhibit root elongation. The percentages of zygotic embryos forming roots on medium containing BAP and NAA were higher than those on medium with GA3 (Table 1). The percentage of zygotic embryo cultures generating plantlets (shoots and roots) on medium containing BAP and NAA was higher than those of medium containing GA3 (Table 1). However, only 25 percent of the zygotic embryos generated plantlets with shoots and roots, while the other 75% were only generated shoots but without roots. This lack of root growth is presumably because either the auxin concentration is not sufficient to induce root formation or differences in the physiological development of the embryo may be the reasons. The small zygotic embryos generally slowly develop shoots and roots. Therefore, to generate plantlets ready for acclimatization, root induction from unrooted shoots on a medium supplemented with auxin to stimulate roots is necessary.

Not all zygotic embryos can generate shoots on a medium supplemented with either BAP and NAA or GA3 (Table 2). However, the highest number of shoots per explant were obtained on media supplemented with BAP and NAA. Shoot replication occurred on media with BAP and NAA (Figure 2f). The multiple shoots are formed at the base of the shoot in the form of adventitious shoots. The growth of the multiple shoots is almost the same as those growing only a single shoot. Therefore, the developed methods can shoot multiplication of the Areca nut to increase planting materials.

The average number of leaves and number of roots developing from the germinated embryos on media supplemented with BAP (1 ppm) and NAA (0.1 ppm) were higher than those on media with the addition of GA3 (3 ppm, Table 2). The results of Baidowi and Wiendi [17] studies showed that the addition of GA3 up to 2.60 mg/l reduced root formation from Tagetes shoots in vitro. The average number of roots and the percentage of rooted shoots were quite high in the media supplemented with BAP and NAA. In addition to the presence of auxin, it was also attributed to the addition of activated charcoal. The addition of activated charcoal to orchid media increases root formation from Dendrobium shoots. Activated charcoal plays an important role in root induction [18]. Moreover, the addition of activated charcoal simulated the dark effect at the base of the shoot. Thereby inhibiting the formation of IAA oxidase, which increases the concentration of IAA at the base of the shoots.

In this experiment, Areca nut plantlets were obtained (Figure 2g). Plantlets are complete plants with roots and shoots obtained from in vitro culture of young Areca embryo culture. The plantlets were also successfully acclimatized on a compost and husk charcoal (Figure 2h).
Table 2. The average number of shoot, leaf and root formation from zygotic embryos of Areca nut

| Treatment medium                  | Number of shoot/embryo | Number of leaves/shoot | Number of roots/shoot | Total number of shoot |
|-----------------------------------|------------------------|------------------------|-----------------------|-----------------------|
| MS0                               | 0                      | 0                      | 0                     | 0                     |
| MS with 1 ppm BAP and 0.1 ppm NAA | 0.83                   | 1.25                   | 11                    | 10 (8/12)*            |
| MS with 3 ppm GA3                 | 0.67                   | 0.08                   | 0.08                  | 6 (6/12)              |

Information: (x/y) x=embyo with shoot, y=total explant embryo

Figure 2. In vitro young Areca nut embryo culture. (a) Dormant zygotic embryos, (b) Browning embryos, (c) germinating embryos, (d) Primary and secondary root development on MS medium supplemented with BAP (1 ppm) and NAA (0.1 ppm), (e) developing roots on MS medium supplemented with GA3, (f) comparison of embryo culture on the three tested medium (left: MS0, middle MS + BAP and NAA, and right: MS + GA3), (g) Areca nut plantlets are ready for acclimatization. (h) acclimatization of Areca nut plantlets.
There is no callus formation in all Areca zygotic embryo cultures, presumably because the added plant growth regulators did not increase the endogenous auxin concentration. Media supplemented with 1 ppm BAP and 0.1 ppm NAA could accelerate shoot growth and development without developing callus at the base of the embryos.

4. Conclusion
Areca nut immature embryos planted on MS medium supplemented with a combination of BAP (1 ppm) and NAA (0.1 ppm) yielded a better percentage of germination and explant growth and development than that on medium supplemented with gibberellin. The immature embryos of Areca nuts planted on MS0 medium were unable to grow.

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