**MICROPROPAGATION VIA embryoGENESIS techniques UNDER COMMERCIAL SCALE of Vulnerable agarwood**

**(Aquilaria crassna Pierre ex Lecomte)**

**ABSTRACT**

*Introduction*: Agarwood is an important tree having valuable in medicine, pharmaceutical and cosmetic. Completely exploited by humans and driven to extinction. Reforestation of this precious tree species is necessary via seedlings production and supply to enlarge growing regions

*Aim:* Micropropagation via somatic embryogenesis of Golden Teak sourced from Myanma for regeneration and develoment of valuable tree species

*Method:* Using method cultivation of somatic embryos technique

*Result:* Leaf explants were the well samples for somatic embryogenesis cultivation. Embryogenic cell were inducted on MS medium + BA (3 mg/l) + K (5 mg/l) + IBA (0.5 mg/l) + vit B5 (10 mg/l); mass propagation on semi-solid MS medium + BA (1 mg/l) + K (1 mg/l) + NAA (0.5 mg/l) + vit B5 (5 mg/l); mass propagation on liquid MS medium + BA (3 mg/l) + K (5 mg/l) + IBA (0.5 mg/l); and layer on the semi-solid MS medium supplemented with BA (1 mg/l) + K (5 mg/l) + NAA (0.5 mg/l) + vit B5 (5 mg/l). Embryogenic cell were regenerated on MS medium + TDZ (0,1 mg/l) + NAA (0.1 mg/l) after 120 days of cultivation. Single shoot was used as materials for far micropropagation and/or other studies in agarwood improvement.

*Conclusion:* The report on agarwood regeneration for far micropropagation was set up

*Key world:* Agarwood, somatic embryo, regeneration, reforestation, induction, layering

**1. INTRODUCTION**

Agarwood is a specialty tree in the tropics (Cheng Seng Tan, *et al.,* 2019). This species is fast growing, hardy and can be harvested within a short rotation period of around 5-8 years (Ahmad Zuhaidi, 2016). Agarwood containing resin currently used as raw material for the perfume industry, cosmetics, incense, and preservatives types assesori, as essences, soap and shampoo.( Ella Yusnita, *et al.,* 2017). *Aquilaria* is a tree which is able to produce agarwood resins is the main product when attacked by bacteria or wounded (Turjaman *et. al.,* 2016; Faizal *et. al.,* 2017; Khairumddin Abdul Rahim, 2021). *Aquilaria* spp. belongs to the family Thymelaceaceae with more than 15 known species (Blanchette *et al.,* 2009; Chipa *et al.,* 2017). The formation process and mechanism of agarwood production are still a mystery (Rozi Mohamed, 2016; Chen and Rao, 2022; Clara Zaremski *et. al.,* 2022; Chen‑Chen Fu *et. al.,* 2024). With high economic value, agarwood is currently facing the risk of extinction (Red List, 2000).

*Aquilaria* spp. is considered ‘Vulnerable’ according to the current IUCN Red List Categories, and has been included in The World List of Threatened Trees (IUCN, 2010). There are very few agarwood trees in the primary forests today (Mohd Fauzi Elias *et. al.,* 2017, Arlene lopez-Sampson and Tony Page 2018). Restoration and cloning of agarwood by somatic embryo culture technique is very necessary (Pierik, 1987).

There are research results on techniques of embryogenesis cultures following steps of selection of explants (Nurul Hazwani Daud *et. al.,* 2012, Moitreyee Saikia and Karuna Shrivastava 2015, Mohd Syafik Mohamad Hamdan et. al., Melati *et. al.,* 2021, Albert A. Piñon and Tomas D. Reyes, Jr., 2021, Maulana Tamyiz *et. al.,* 2022),

Shoot multiplication (HE Meng-ling *et. al.,* 2005, Nor Hasnida Hassan *et. al.,* 2011, Zul Helmey Bin Mohamad Sabdin, 2016, , Chandrarathne, *et. al.,* 2023, Li *et. al.,* 2024).

Cultures for induction of callus (Moitreyee Saikia *et. al.,* 2012, Moitreyee Saikia *et. al.,* 2013, Jayaraman *et. al.,* 2014, Sara, 2014, Zul Helmey Bin Mohamad Sabdin, 2016, Baiq Erna Listiana *et. al.,* 2018, Ding Hou Nursaadiah Salam *et. al.,* 2019, Mohd Syafik Mohamad Hamdan *et. al.,* 2020, Benni Satria *et. al.,* 2021, Suklang Kharnaior and Shiny Chakkiath Thomas, 2021).

Process of embryogenesis (Rugkhla *et. al.,* 1998, Shashita Jayaraman and Rozi Mohamed, 2015, Nursaadiah Salam *et. al.,* 2019, Mohd Syafik Mohamad Hamdan *et. al.,* 2020, Ibrahim *et. al.,* 2022).

Application of embryogeneic cells on micropropagation and improvement of *Aquilaria crassna* have not yet record. With somatic embryo culture technique, agarwood trees are restored and return to a young state quickly (Thorpe, 1980). In a young state, agarwood trees are a source of raw materials for the development of upstream seed gardens, providing seedlings for the development of specialized agarwood export growing areas.

**2. MATERIALS AND METHODS**

**2.1 Materials**

*Culture sample:* Agarwood tree, ND clone (collected in the southern of Phu Quoc island). The selected *in vitro* seedlings are growing well and have full stems, leaves and roots. Young leaves and roots are used as explants in somatic embryogenesis culture. Leaves are cut into 1 cm2 pieces.

*Culture conditions:* The medium is sterilized at 121 oC and 1at for 25 minutes. Culture room temperature is 28+1oC. Illumination intensity is 34.2 μmol/m2/s. Photoperiod 8 hours/day

*Culture medium:* Basic mineral nutrient medium MS (Murashige-Skoog, 1962) and WPM (Lloyd and McCown, 1981), supplemented with BA (6-benzyl aminopurine), IBA (β-indole butyric acid), NAA (β-naphthalene acetic acid), glycine, vitamin B5, glutamin, and coconut water (CW)

**2.2 Experimental designs**

*Callus cell induction culture:* Samples are young leaves and roots of in vitro plantlets of ND clone (Southern of Phu Quoc Island). Basic media culture A: MS + CW (10 %) + sucrose (30 g/l) + glutamin (100 mg/l) with formuls are (mg/l): A1, A2, A3, A4, A5 to induce callus cell

|  |
| --- |
| A1: A + BA (1) + NAA (0.5) + K (1) + vit B5 (5)  A2: A + BA (3) + IBA (0.5) + K (5) + vit B5 (10)  A3: A + 2.4D (1)  A4: A + 2.4D (2)  A5: A + 2.4D (3) |

*Proliferation of pro-embryogenic**callus cell on agar medium:*Callus cell derived from roots were cultured on media A: MS + CW (10 %) + sucrose (30 g/l) + glutamin (100 mg/l) with formuls are (mg/l): B1, B2, B3 to induce pro-embryogenic callus cell

|  |
| --- |
| B1: A + BA (1) + NAA (0,5) + K (1) + vit B5 (5)  B2: A + BA (3) + IBA (0,5) + K (5) + vit B5 (10)  B3: A + 2.4D (1) |

*Proliferation of embryogeneic callus cell suspension in liquid medium:*Pro-Embryogenic callus cells were cultured on media A: MS + CW (10%) + sucrose (30 g/l) + glutamin (100 mg/l) and media B: MS + CW (10%) + vit B5 (10 mg/l) + glycin (5 mg/l) + sucrose (30 g/l) + glutamin (100 mg/l) with formuls are (mg/): C1, C2, C3, C4, C5, C6, C7, C8 to induce embryogenic callus cell

|  |
| --- |
| C1: A + 2.4D (0.5)  C2: A + 2.4D (1.0)  C3: A + 2.4D (1.5)  C4: A + 2.4D (2.0)  C5: B + BA (3) + K (5) + IBA (0.5)  C6: B + BA (3) + IBA (0.5)  C7: B + K (5) + IBA (0.5)  C8: B + BA (3) + K (5)  *Layering embryogenic cell suspension on semi-solid medium:* Pro-Embryogenic callus cells were cultured on media A: MS + CW (10%) + sucrose (30 g/l) + glutamin (100 mg/l) with formuls are (mg/l): D1, D2, D3 to layer embryogenic callus cell  D1: A + BA (1) + NAA (0,5) + K (1) + vit B5 (5)  D2: A + BA (3) + IBA (0,5) + K (5) + vit B5 (10)  D3: A + 2.4D (1) |

*Embryogenic cell regeneration:*Embryogenic cells from treatment D1 were used in regeneration studies. Pro-Embryogenic callus cells were cultured on media C: MS + CW (10%) + vit B5 (5 mg/l) + glycin (5 mg/l) + sucrose (30 g/l) + glutamin (100 mg/l) with formuls are (mg/l): E1, E2, E3 to regenerate of embryogenic cell

|  |
| --- |
| E1: C + TDZ (0,1) + NAA (0.1 mg/l)  E2: C + BA (0,5) + K (0,2)  E3: C + K (0,2) |

*Rapid propagation of Agarwood in vitro:* Single shoots were cultured into rapid propagation on media WPM + BA (0.1 mg/l) + NAA (0.1 mg/l) + CW (10%) to regenerate multiple-shoot clusters

**2.3 Data analysis**

The experiment was arranged according to RCBD (1 factor), 4 replicates, each replicate cultured 5 300 ml triangular flasks, each triangular flask contained 65 ml of experimental medium and was cultured with 5 samples. The collected data were statistically analyzed using MSTAT-C software (P=0.05). Somatic cell proliferation rate (%), cell biomass after 15, 30 days of culture (g), biomass increase (g), cell density (x 104 /ml)

**3. RESULTS AND DISCUSSION**

**3.1 Callus cell induction culture:**

the leaves are the parts used as culture samples. The culture samples are placed on the agar surface, with cuts in contact with the culture medium. The basic MS medium is used, supplemented with BA, Kinetin (K), NAA, IBA, 2.4D, and vitamin B5. The research results show that the rate of somatic cell generation is high with the culture sample being the leaves (ND clone in southern Phu Quoc island). On the culture medium supplemented with 2.4D, somatic cells arise quickly, with a clear white color; the medium supplemented with BA + NAA + Kinetin (K) + vitamin B5 gives somatic cells of male milkweed, viewed under the microscope, the cells are spherical in shape and transformed into somatic embryo cells (Table 1). With the ND root culture sample, treatment A1 gives lower somatic cell generation than treatment A2; but the ability of somatic cells to directly transform into somatic embryos is high (Figure 1, 2). Somatic embryos generated on A1 experimental medium were used in subsequent studies (Melati *et. al.,* 2021, Albert A. Piñon and Tomas D. Reyes, Jr., 2021, Maulana Tamyiz *et. al.,* 2022),

Table 1. Callus cell induction culture after 30 days

|  |  |  |  |
| --- | --- | --- | --- |
| **A** | Formula (mg/l) | Somatic cell induction rate (%) | |
| ND Leaf | ND Root |
| 1 | A + BA (1) + NAA (0.5) + K (1) + vit B5 (5) | 73.5 ab | 53.5 b |
| 2 | A + BA (3) + IBA (0.5) + K (5) + vit B5 (10) | 60.2 b | 94.2 ab |
| 3 | A + 2.4D (1) | 80.9 ab | 100.0 a |
| 4 | A + 2.4D (2) | 93.4 a | 93.3 ab |
| 5 | A + 2.4D (3) | 100.0 a | 100.0 a |
| CV (%) | | 21.1 | 29.9 |

A: MS + CW (10 %) + sucrose (30 g/l) + glutamin (100 mg/l)

ND: Samples are young leaves and roots of in vitro plantlets of ND clone (Southern of Phu Quoc Island)

**2**

**1**

Figure 1: Callus formation on leaves samples from the media (mg/l) A + BA (1) + NAA (0.5) + K (1) + vit B5 (5)

Figure 2: Callus formation on roots samples from the media (mg/l) A + BA (3) + IBA (0.5) + K (5) + vit B5 (10)

**3.2 Proliferation of pro-embryogenic callus cell on semi-solid medium:**

Callus cell derived from leaves and roots were cultured on MS semi-solid medium supplemented with BA, Kinetin (K), NAA, IBA, 2.4D, vitamin B5. The results showed that cells proliferated rapidly on the B1 treatment. Under the microscope, the pre-embryo cells had dense cytoplasm, small vacuoles, and rapidly produce secondary embryogenesis (Table 2) (Figure 3) (Benni Satria *et. al.,* 2021, Suklang Kharnaior and Shiny Chakkiath Thomas, 2021).

Table 2. Proliferation of pro-embryogeniccallus cell on semi-solid medium after 30 days

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **B** | Formula (mg/l) | Initial biomass  (g) | Biomass after 15 days (g) | Increased biomass  (g) |
| 1 | A + BA (1) + NAA (0,5) + K (1) + vit B5 (5) | 0.8 | 1.7 | 0.9 a |
| 2 | A + BA (3) + IBA (0,5) + K (5) + vit B5 (10) | 0.5 | 0.9 | 0.4 b |
| 3 | A + 2.4D (1) | 0.1 | 0.3 | 0.2 c |
|  | CV(%) |  |  | 35.6 |



**3**

Figure 3: Proliferation of pro-embryogeniccallus cell on media (mg/l) A + BA (1) + NAA (0,5) + K (1) + vit B5 (5)

**3.3 Proliferation of embryogenic callus cell suspension in liquid medium:**

Pro-embryogenic cells derived from leaves and roots were cultured in liquid medium, with an initial culture biomass of 1g / 65ml. The results showed that the highest proliferation density was in the MS basic medium supplemented with 2.4D (treatment C2) or BA + Kinetin (K) + IBA (treatment C5). However, in the medium supplemented with 2.4D, cells proliferated rapidly and tended to de-differentiate into somatic cells; on the contrary, in the medium supplemented with BA + Kinetin + IBA, somatic embryo cells entered the process of division and differentiation (Table 3) (Figure 4) (Nursaadiah Salam *et. al.,* 2019, Mohd Syafik Mohamad Hamdan *et. al.,* 2020, Ibrahim *et. al.,* 2022)

Table 3. Prolieration of embryogenic callus cell suspension in liquid medium after 21 days

|  |  |  |
| --- | --- | --- |
| **C** | Formula (mg/l) | Cell density after 21 days (x 104 /ml) |
| 1 | A + 2.4D (0.5) | 5150 a |
| 2 | A + 2.4D (1.0) | 7468 a |
| 3 | A + 2.4D (1.5) | 3650 b |
| 4 | A + 2.4D (2.0) | 3780 b |
| 5 | B + BA (3) + K (5) + IBA (0.5) | 7550 a |
| 6 | B + BA (3) + IBA (0.5) | 5220 a |
| 7 | B + K (5) + IBA (0.5) | 5825 a |
| 8 | B + BA (3) + K (5) | 5330 a |
|  | CV(%) | 41.90 |

A: MS + CW (10%) + sucrose (30 g/l) + glutamin (100 mg/l)

B: MS + CW (10%) + vit B5 (10 mg/l) + glycin (5 mg/l) + sucrose (30 g/l) + glutamin (100 mg/l)

The cultured sample is callus sourced from in vitro leaves and roots of ND clone (Southern Phu Quoc Island)



**4**

Figure 4: Prolieration of embryogenic callus cell suspension in liquid media (mg/l) B + BA (3) + K (5) + IBA (0.5)

**3.4 Layering embryogenic cell suspension on semi-solid medium**

The volume of 10ml of somatic embryo cell suspension on C5 treatment was taken and spread on agar culture medium. The culture medium was MS supplemented with BA, 2.4D, NAA, IBA, Kinetin, vitamin B5. The research results showed that after 15 and 30 days of culture, treatments D1 and D2 (Table 4) had higher biomass growth than treatment D3. Comparison between treatments D1 and D2 showed that in treatment D1, somatic embryos differentiated strongly, changing from milky white to light yellow, the transition to regeneration. Somatic embryos on treatment D1 were used in regeneration research (Figure 5, 6) (Ding Hou Nursaadiah Salam *et. al.,* 2019, Mohd Syafik Mohamad Hamdan *et. al.,* 2020)

Table 4. Layering embryogenic cell suspension on agar medium after 15 and 30 days

|  |  |  |  |
| --- | --- | --- | --- |
| **D** | Formula (mg/l) | Biomass after 15 days (g) | Biomass after 30 days (g) |
| 1 | A + BA (1) + NAA (0,5) + K (1) + vit B5 (5) | 8.85 a | 16.85 a |
| 2 | A + BA (3) + IBA (0,5) + K (5) + vit B5 (10) | 8.45 a | 16.30 a |
| 3 | A + 2.4D (1) | 2.25 b | 5.95 b |
|  | CV(%) | 12.32 | 13.52 |



**5**

**6**

Figure 5: Layering embryogenic cell suspension after 15 days on

semi-solid media (mg/l) A + BA (1) + NAA (0,5) + K (1) + vit B5 (5)

Figure 6: Layering embryogenic cell suspension after 30 days on

semi-solid media (mg/l) A + BA (1) + NAA (0,5) + K (1) + vit B5 (5)

**3.5 Embryogenic cell regeneration**

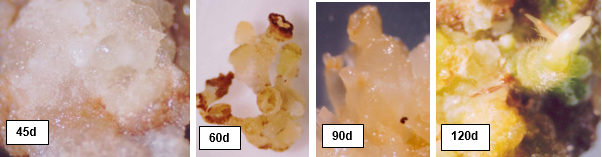
Embryogenic cells from treatment D1 were used in regeneration studies. With treatment E1 medium, Embryogenic cells underwent development of spherical (after 45 days), heart-shaped (after 60 days), torpedo-shaped (after 90 days) and regeneration as single shoots with primary leaves after 120 days (Table 5) (Figure 7) (Van Minh, 2020)

Table 5. Embryogenic cell regeneration

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **E** | Formula (mg/l) | Embryo differentiation | | | |
| Globula  45 DAP | Heart  60 | Torpedo  90 | Primary leaves  120 |
| 1 | C + TDZ (0,1) + NAA (0.1) | + | + | + | + |
| 2 | C + BA (0,5) + K (0,2) | + | + | + | - |
| 3 | C + K (0,2) | + | - | - | - |

C: MS + CW (10%) + vit B5 (5 mg/l) + glycin (5 mg/l) + sucrose (30 g/l) + glutamin (100 mg/l)

DAP: day after plating



**7**

Figure 7: Embryogenic cell regeneration on media C + TDZ (0,1) + NAA (0.1)

**3.6 Rapid propagation of Agarwood in vitro:**

Single shoots were introduced into rapid propagation on WPM + BA (0.1 mg/l) + NAA (0.1 mg/l) + CW (10%) (Chanh *et. al.,* 2025) to generate multiple-shoot clusters. The shoot clusters multiplied rapidly after several subcultures (Figure 8) (Chanh *et. al.,* 2025). The shoot clusters were used as a protocol for far rapid propagation in vitro (Figure 9) (Van Minh, 2020)

**9**

**8**

Figure 8: Agarwood cluster (after 60 days) on media (mg/l) WPM + BA (0.1 mg/l) + NAA (0.1 mg/l) + CW (10%)

Figure 9: Embryo-plantlets 5-months prepared to plantation

**CONCLUSION**

Root explants were the well samples for somatic embryogenesis cultivation. Embryogenic cell were initiated on MS medium + BA (3 mg/l) + K (5 mg/l) + IBA (0.5 mg/l) + vit B5 (10 mg/l); mass propagation on semi-solid MS medium + BA (1 mg/l) + K (1 mg/l) + NAA (0.5 mg/l) + vit B5 (5 mg/l); mass propagation on liquid MS medium + BA (3 mg/l) + K (5 mg/l) + IBA (0.5 mg/l); and layer on the semi-solid MS medium supplemented with BA (1 mg/l) + K (5 mg/l) + NAA (0.5 mg/l) + vit B5 (5 mg/l). Embryogenic cell were regenerated on MS + TDZ (0,1 mg/l) + NAA (0.1 mg/l) after 120 days of cultivation. Single shoot was used as materials for far micropropagation on media WPM + BA (0.1 mg/l) + NAA (0.1 mg/l) + CW (10%) or other studies in agarwood improvement. The report on agarwood regeneration for far micropropagation was set up

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