**MICROPROPAGATION UNDER PILOT-SCALE FOR REFORETATION**

**OF ENDANGERED SPECIAL AGARWOOD SPECIES**

**(AQUILARIA CRASSNA PIERRE EX LECOMTE)**

**ABSTRACT**

*Introduction:* Agarwood is a valuable and rare and valuable wood species. Planting Aquilaria to harvest Agarwood and Ky Nam.

*Method:* It is successfully in micropropagation.

*Result:* WPM is a basic media, supplemented with BA (0.1 mg/l), NAA (0.1 mg/l), coconut water (10 %), high light intensity (34,2 μmol/m2/s) and enhance air exchange improve multiple rate in vitro. It grows well and induce healthy roots in in vitro and acclimatisation. Circle cultivation was confirmed in 45 days.

*Conclusion:* A flush out system under pilot scale of agarwood micropropagation was developed.

*Keywords:* Agarwood, rare wood species, micropropagation, light intensity, air exchange

**1. INTRODUCTION**

The history of agarwood (Putra Desa Azren etal., 2019 [20]) is known through agarwood and has been known for centuries by Asian, Egyptian and Middle Eastern civilizations as a rare and precious product. Agarwood is an incense material originating from Southeast Asia and India. Agarwood is the most precious wood ranked in ancient civilizations.

*Aquilaria crassna* Pierre ex Lecomte is also known as agarwood or Ky Nam tree (Saikia and Khan, 2013 [23]), belongs to the genus Aquilaria, family Thymelaceae (agarwood), order Myrtales. Aquilaria has 15 different species including *Aquilaria crassna* Pieere ex Lecomte (agarwood), *Aquilaria agallocha* Roxb, *Aquilaria malaccensis* Lam are commonly grown (Lee and Mohamed 2016 [13]) due to their ability to accumulate agarwood.

Aquilaria is distributed throughout Asia, the Near East and the Middle East. Of these, there are three main species that are widely distributed: *Aquilaria malaccensis* Roxb. is mainly distributed in countries such as India (Arunachal, Assam, Meghalaya, Manipura, Tripura, West Bengal, Mizoram, Nagaland, Sikkim), Indonesia (Kalimantan, Sumatra), Bangladesh, Bhutan, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand (Elias, 2017 [8]). *Aquilaria agallocha* Lam. is mainly distributed in India, Egypt, the Near East, Laos. *Aquilaria crassna* Pierre ex Lecomte is mainly distributed in Vietnam, Cambodia

Aquilaria is a tree with evergreen leaves found in primary and secondary forests, distributed up to an altitude of 1000m above sea level. Agarwood wood is white or pale yellow, sometimes due to fungi on the wood grain causing black resin condensation, with a fragrant smell (Liu etal., 2017 [14]). Planting Aquilaria to get Agarwood and Ky Nam (Sen etal., 2017). Agarwood is a precious natural medicinal material (Kamonwannasit etal., 2013 [12]). The properties of Agarwood are spicy, bitter and warm. This property is applied in medicine and perfume. (Lee and Mohamed, 2016 [13]). Agarwood has strong antibodies (Kamonwannasit etal., 2013 [12]) and does not emit fragrance when not burned.

Agarwood oleoresin (Sergio Gutiérrez etal., 2024 [25]) has many different colors and will have different scents. Agarwood will contain high levels of oleoresin when seeing green or dark green clusters. The most valuable product of the agarwood tree, called Ky Nam in Vietnam, Kanam in Korea, Qie-Nan-Xiang in China, and Kanankoh in Japan. Ky Nam, also a type of agarwood, has medicinal value and has a delicate scent. Used to distill essential oils, it is used as an important fixative and is widely used in the high-end cosmetics industry. The most obvious difference between Ky Nam and Agarwood is that Ky Nam's oleoresin is sticky like glue (glutinous) while Agarwood is firm and hard; moreover, a little Ky Nam can also be rolled into balls.

*A. agarllocha* Roxb is extinct or near extinction in most of its countries of origin. Before the Ming Dynasty, *A. agallocha* Roxb was found on Hai-nang Island, southern China (Yin etal., 2016 [26]). Currently, Vietnam is the only country in the world that is still able to supply the best quality agarwood, better than *A. agallocha* Roxb, with high oleoresin content, from the related species *A. crassna* Pierre ex Lecomte. However, Vietnam currently prohibits agarwood exploitation and strictly manages it because *A. crassna* Pierre ex Lecomte is extinct (Harvey-Brown, 2018 [11]).

Plan for conservation and development of Agarwood gene resources in Vietnam (Red List, 2007 [21]): (1) Due to the situation of agarwood exploitation being too strong and uncontrollable, the competent authorities need to have strict regulations on agarwood exploitation (prohibiting agarwood exploitation in trees with a diameter of <30cm) (2) Building an effective seed system to serve natural regeneration, restoring traditional agarwood growing areas. However, agarwood seeds quickly lose their germination ability and have a low germination rate and are not yet able to multiply quickly by cuttings from seedlings (3) Due to the increasingly depleted seed source, it is necessary to quickly invest in surveying parent trees and building conservation populations, seed forests in key planning areas. (4) Proactively build an effective seedling production system and expand the agarwood growing area in the distribution area to proactively and rapidly increase the future agarwood source

However, understanding of the characteristics of this *A. crassna* Pierre ex Lecomte tree species is still too little, agarwood is a precious forest tree species (Rozi Mohamed, 2016 [22]), although widely distributed in Vietnam but is threatened with extinction (Red List, 2007 [21]). People have exploited agarwood indiscriminately, cutting down even small trees or trees that can be planted without effective measures to protect, maintain and propagate, causing agarwood trees to be at risk of extinction. People traditionally grow agarwood trees from seeds; however, not all trees from seeds produce agarwood (Clara Zaremski etal., 2020 [4]). Therefore, it is necessary to detect agarwood trees that are producing agarwood and apply modern technology to agarwood breeding work with the aim of preserving, rapidly propagating and developing agarwood-producing lines using the technique of shoot tip culture (Dodds, 1083 [7]; Esyanti etal., 2019 [9]; Benni Satria etal., 2021 [1]; Chandrarathne etal., 2023 [2]).

**2. MATERIALS AND RESEARCH METHODS**

**2.1 Materials:**

*Collecting culture samples:* Agarwood trees were surveyed on Phu Quoc island (Kien Giang). Four lines producing agarwood were discovered, labeled BD1, BD2 (agarwood clone 1, 2 of Northern of Phu Quoc Island), and ND1, ND2 (agarwood clone 1,2 of Southern of Phu Quoc Island). The samples were trimmed into 30-40cm long branches, cold-treated, and transferred to laboratory by airway in the day.

*Culture conditions:* The medium is sterilized at 121oC and 1at for 25 minutes. Culture room temperature is 28+1oC. Light intensity is 34.2 μmol/m2/s. Lighting time is 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), IAA (β-indole acetic acid), IBA (β-indole butyric acid), NAA (naphthalene acetic acid) and coconut water (CW)

**2.2 Experimental designs:**

*Sterilization of culture samples:* Media cultivation is WPM with none plant growth regulator. Samples are shoots and internode. Using HgCl2 (0.1 – 0.5 - 1 %) and Hypochlorite-Ca (1 – 5 %) for sterilisation in 7 minutes. Explants are trimmed into 3-5cm sections, washed with soap and sterilized with HgCl2 (0.1 %) for 7 minutes. After sterilization, explants are cut into 10-15 mm sections containing the growing apical parenchyma region. Explants are apical buds or stem nodes.

*In vitro culture of agarwood shoot tips:* Media cultivation are MS and WPM supplemented with BA (0.1 mg/l) and coconut water (CW 10 %). Samples are shoot and internode

*Effect of plant growth regulators on the ability to perform agarwood shoot clusters in vitro*: Media cultivation is WPM supplemented with BA (01. – 0.2 mg/l) and NAA (0.1 – 0.2 mg/l)

*Effect of plant growth regulators on rapid propagation of agarwood shoot cluster in vitro:* Media cultivation is WPM supplemented with BA (0.1 – 0.2 mg/l) and NAA (0.1 – 0.2 mg/l)

*Effect of coconut water (CW) on rapid propagation of agarwood shoot cluster in vitro:* Media cultivation is WPM supplemented with BA (0.1 – 0.2 mg/l), NAA (0.1 mg/l) and CW (10 %)

*Effect of gas exchange on rapid propagation of agarwood shoot cluster in vitro:* Media cultivation is WPM supplemented with BA (0.1 mg/l), NAA (0.1 mg/l) and CW (10 %). Gas exchange by using rubber cap and paper lib cover triangle glass 300 ml.

*Effects of light intensity and gas exchange on rapid propagation of agarwood shoot cluster in vitro:* Media cultivation is WPM supplemented with BA (0.1 mg/l), NAA (0.1 mg/l) and CW (10 %). Light intensity are (20.52 - 34.20 and 54.72 μmol/m2/s). Gas exchange by using rubber cap and paper lib cover triangle glass 300 ml.

*The influence of genetic source on rapid propagation of agarwood shoot cluster in vitro:* Media cultivation is WPM supplemented with BA (0.1 mg/l), NAA (0.1 mg/l) and CW (10 %). Light intensity are 34.20 μmol/m2/s. Samples are BD1, BD2, ND1, ND2.

*Growth of agarwood shoot in vitro: :* Media cultivation is WPM supplemented with BA (0.1 mg/l) and CW (10 %). Light intensity are 34.20 μmol/m2/s.

*In vitro culture of agarwood rooting:* Media cultivation is WPM supplemented with NAA, IBA, IAA (0.1 – 0.3 mg/l) and CW (10 %). Light intensity are 34.20 μmol/m2/s.

*Acclimatization of tissue cultured agarwood plantlets:* Agarwood plantlets are acclimatized by mist and fiber as substrate in 10 days. After true roots developed, plantlets were transferred to compose of soil: coconut fiber: manure (1:1:1)

**2.3 Data analysis:**

The experiment was arranged according to RCBD (1 factor), 4 replications, each replication cultured 5 300ml triangular flasks, each triangular flask contained 65 ml of experimental medium and was inoculated with 5 samples. The collected data were statistically analyzed using MSTAT software according to M, CV% and LSD (0.05).

The monitoring indicators were the number of shoots/cluster, stem height, number of internodes (no), number of leaf (no), leaf length (mm), leaf width (mm), leaf development (+/-), callus (+/-), number of root (no), root length (mm)

**3. RESULTS AND DISCUSSION**

**3.1 In vitro culture of agarwood shoot tips**

***3.1.1 Sterilization of culture samples:*** Culture samples were collected from natural forests, so the level of sample contamination was high. HgCl2 was selected to sterilize the samples. With a concentration of 0.1 - 1 % HgCl2, the sterilization result was 38.5 - 14.7 %. A concentration of 0.1 % HgCl2 gave a high sterilization efficiency of 38.5 % (Table 1) (Figure 1, 2) (Daud etal., 2012 [5])

Table 1: Sterile agarwood samples cultured in vitro

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Disinfectant | Concentration  (%) | Sterilization time (min.) | Sterility rate  (%) | Shoot growth  (+/-) | Leaf development  (+/-) |
| Hypo-Na | 1.0 | 7 | 0.0 | - | - |
|  | 3.0 | 7 | 0.0 | - | - |
|  | 5.0 | 7 | 13.2 | + | + |
| HgCl2 | 0.1 | 7 | 38.5 | + | + |
|  | 0.5 | 7 | 24.2 | + | + |
|  | 1.0 | 7 | 14.7 | + | + |
| LSD (0.05) | |  | 5.8 |  |  |

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**1**

Figure 1: Agarwood survey team on Phu Quoc island

Figure 2: Agarwood samples using in vitro cultivation

***3.1.2 In vitro culture of agarwood shoot tips:*** Agarwood shoot tips were cultured on two basic media, MS and WPM, supplemented with BA and coconut water. The results showed that agarwood plants grew better on WPM than on MS. On WPM, the combination of BA (0.1 mg/l) + CW (10 %) regenerated agarwood shoot tips better than the medium supplemented with only BA (0.1mg/l). On MS, the combination of BA (0.1mg/l) + CW (10 %) regenerated agarwood shoot tips better than the medium supplemented with only BA (0.1 mg/l); however, the cultured rootstocks produced callus and the shoots developed abnormally in both treatments. The combination WPM + BA (0.1 mg/l) + CW (10 %) gave better results in regenerating meristems than MS + BA (0.1 mg/l) + CW (10 %) (Table 2). WPM medium is suitable for cultivating meristems to generate shoots, stems and leaves, and to grow and develop normally without forming callus at the corner of the cultured sample; and was used for subsequent experiments (Figure 3, 4) (Debnath etal., 2013 [6])

Table 2: In vitro culture of agarwood shoot tips

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Media | BA  (mg/l) | CW  (%) | Stem height (mm) | Inrternode  (no) | leaf  (no) | Leaf lenght (mm) | Leaf width (mm) | Callus appearance  (+/-) |
| MS | 0.1 | - | 35.0 | 3.2 | 4.2 | 22.0 | 8.0 | + |
|  | 0.1 | 10 | 42.0 | 7.0 | 8.0 | 21.5 | 11.2 | + |
| WPM | 0.1 | - | 35.0 | 6.2 | 7.2 | 18.5 | 9.0 | - |
|  | 0.1 | 10 | 48.2 | 6.2 | 7.2 | 36.5 | 12.7 | - |
| LSD (0.05) | | | 3.5 | 1.4 | 1.4 | 3.4 | 2.2 |  |

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**3**

Figure 3: Regeneration of internode samples were cultured in vitro after 15 days

Figure 4: Regeneration of internode samples were cultured in vitro after 45 days on WPM + BA (0.1 mg/l) + CW (10 %)

***3.1.3 Effect of plant growth regulators on the ability to perform agarwood shoot clusters in vitro:*** Experiments with added growth regulators all produced better shoot clusters than the control. The medium supplemented with the combination BA (0.1 mg/l) + NAA (0.1 mg/l) was more suitable for shoot cluster formation than BA (0.2 mg/l) + NAA (0.2 mg/l). Shoot clusters are the basic unit of propagation (Table 3) (Figure 5) (Debnath etal., 2013 [6])

Table 3: Effect of growth regulators on the ability to form agarwood shoot clusters in vitro

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| BA (mg/l) | NAA (mg/l) | Stem height  (mm) | Shoot  (no) | Internode  (no) | Leaf lenght  (mm) | Leaf width  (mm) |
| - | - | 8.5 | 1.0 | 1.7 | 11.0 | 2.2 |
| 0.1 | - | 50.5 | 1.5 | 8.0 | 40.0 | 1.,7 |
| 0.1 | 0.1 | 32.7 | 5.7 | 3.7 | 20.0 | 5.7 |
| 0.1 | 0.2 | 14.0 | 2.2 | 2.7 | 26.0 | 5.7 |
| 0.2 | 0.1 | 44.2 | 1.5 | 5.0 | 31.5 | 11.0 |
| 0.2 | 0.2 | 21.0 | 2.2 | 6.2 | 31.7 | 9.0 |
| LSD (0.05) | | 5.3 | 0.9 | 1.2 | 5.2 | 2.7 |



**5**

Figue 5: To perform agarwood shoot clusters in vitro after 45 days on WPM + BA (0.1 mg/l) + NAA (0.1 mg/l)

**3.2 Rapid propagation of agarwood in vitro**

***3.2.1 Effect of growth regulators on rapid propagation of agarwood shoot cluster in vitro:*** Experiments with added growth regulators all gave better shoot multiplication results than the control. The medium supplemented with BA (0.1 mg/l) + NAA (0.1 mg/l) was more suitable for shoot cluster multiplication than the combination BA (0.2 mg/l) + NAA (0.2 mg/l). (Table 4) (Figure 6) (Hapsari etal., 2021 [10]; Benni Satria etal., 2021 [1])

Table 4: Effect of growth regulators on rapid propagation of agarwood shoot clusters in vitro

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| BA (mg/l) | NAA (mg/l) | Stem height  (mm) | Shoot  (no) | Internode  (no) | Leaf length  (mm) | Leaf width  (mm) |
| - | - | 9.0 | 1.5 | 1.7 | 9.0 | 2.2 |
| 0.1 | - | 48.5 | 5.5 | 9.5 | 2.0 | 9.2 |
| 0.1 | 0.1 | 21.0 | 9.2 | 4.0 | 33.2 | 6.5 |
| 0.1 | 0.2 | 31.0 | 3.5 | 3.0 | 21.2 | 5.0 |
| 0.2 | 0.1 | 44.2 | 2.7 | 8.2 | 32.5 | 9.0 |
| 0.2 | 0.2 | 23.5 | 3.2 | 5.7 | 31.2 | 11.2 |
| LSD (0.05) | | 6.0 | 2.1 | 2.2 | 5.5 | 2.6 |



**6**

Figure 6: rapid propagation of agarwood shoot cluster in vitro after 45 days on WPM + BA (0.1 mg/l) + NAA (0.1 mg/l)

***3.2.2 Effect of coconut water (CW) on rapid propagation of agarwood shoot cluster in vitro:*** Experiments supplemented with growth regulators and CW both gave higher shoot multiplication results than the control. The culture medium supplemented with BA (0.1 mg/l) + CW (10 %) gave better shoot multiplication results than the one supplemented with BA (0.1 mg/l) alone. The combinations of BA + NAA and BA + NAA + CW both gave lower shoot multiplication efficiency than the combinations of BA and BA + CW. In the absence of CW, the combination of BA (0.1 mg/l) + NAA (0.1 mg/l) achieved the highest shoot multiplication efficiency; when using CW, NAA (0.1 mg/l) can be replaced by CW (10 %) (Table 5) (Van Minh, 2020 [27])

Table 5: Effect of coconut water (CW) on rapid propagation of agarwood shoot clusters in vitro

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| BA  (mg/l) | NAA  (mg/l) | CW  (%) | Stem height  (mm) | Shoot  (no) | Internode  (no) | Leaf length (mm) | Leaf width (mm) |
| - | - | - | 10.0 | 1.5 | 2.0 | 8.2 | 3.0 |
| 0.1 | - | - | 48.0 | 5.5 | 8.5 | 20.0 | 8.5 |
| 0.1 | - | 10 | 41.2 | 11.2 | 6.7 | 19.5 | 6.0 |
| 0.1 | 0.1 | - | 31.2 | 7.7 | 5.7 | 24.7 | 9.0 |
| 0.1 | 0.1 | 10 | 23.5 | 8.5 | 4.5 | 24.5 | 5.0 |
| 0.2 | 0.1 | - | 31.7 | 3.2 | 3.5 | 19.5 | 4.0 |
| 0.2 | 0.1 | 10 | 19.0 | 2.5 | 4.7 | 25.0 | 6.5 |
| LSD (0.05) | | | 6.1 | 2.1 | 2.3 | 4.3 | 1.9 |

***3.2.3 Effect of gas exchange on rapid propagation of agarwood shoot cluster in vitro:*** Agarwood grows better in a jar covered with a paper lid than in a jar covered with a rubber lid, the leaves are dark green compared to the light green leaves in previous experiments (Tables 1-5). The process of shoot formation and multiplication is positively affected by gas exchange as shown through the growth and development of stems and leaves (increased leaf length and leaf width) and leaf color (Table 6) (Van Minh, 2020 [27])

Table 6: Effect of gas exchange on rapid propagation of agarwood shoot clusters in vitro

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Parameter | Triangle glass 300 ml (rubber cap) | Triangle glass 300 ml  (paper lid) | M | CV (%) | LSD (0.05) |
| Shoot (no) | 8.2 | 10.5 | 9.4 | 18.0 | 2.2 |
| Internode (no) | 4.5 | 6.5 | 5.5 | 29.5 | 2.2 |
| Leaf (no) | 5.5 | 7.5 | 6.5 | 24.7 | 2.2 |
| Leaf length (mm) | 27.0 | 35.7 | 31.4 | 18.2 | 6.1 |
| Leaf width (mm) | 6.7 | 10.7 | 8.7 | 28.5 | 2.4 |
| Stem height (mm) | 34.2 | 58.7 | 46.5 | 29.1 | 6.4 |
| Leaf color | Light green | Dark green | - | - | - |
| Leaf development (+/-) | + | +++ | - | - | - |

***3.2.4 Effects of light intensity and gas exchange on rapid propagation of agarwood shoot cluster in vitro:*** The culture flask treatment covered with paper lids gave better shoot and growth indicators than the culture flask treatment covered with rubber lids. With the culture flask covered with a tight lid, the shoot and growth indicators at a light intensity of 54.72 μmol/m2/s were not significantly different from those at a light intensity of 34.20 μmol/m2/s, but were significant compared to those at a light intensity of 20.52 μmol/m2/s. With the culture flask covered with a paper lid, the shoot and growth indicators increased significantly with light intensity. At low light intensity thresholds of 20.52 μmol/m2/s and 34.20 μmol/m2/s, gas exchange did not affect shoot multiplication and growth; On the contrary, at high light intensity (54.72 μmol/m2/s) combined with increased gas exchange, the number of shoots, growth and leaf development improved (Table 7). Increased gas exchange combined with culturing under high light intensity is indispensable to improve the efficiency of rapid in vitro agarwood multiplication (Van Minh, 2020 [27])

Table 7: Effects of light intensity and gas exchange on rapid propagation of agarwood shoot clusters in vitro

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Light intensity  (μmol/m2/s) | Glass trianlge cover | Stem height (mm) | Shoot  (no) | Internode  (no) | Leaf length (mm) | Leaf width (mm) | Leaf color | Leaf dev. (+/-) |
| 20.52 | Rubber cap | 34.5 | 6.5 | 4.5 | 23.2 | 5.7 | XL | + |
|  | Paper lib | 35.5 | 6.5 | 5.0 | 25.5 | 6.7 | XD | + |
| 34.20 | Rubber cap | 34.0 | 9.2 | 7.0 | 28.0 | 7.0 | XL | + |
|  | Paper lib | 35.7 | 10.0 | 7.7 | 36.5 | 12.2 | XD | ++ |
| 54.72 | Rubber cap | 59.5 | 10.0 | 8.7 | 30.0 | 11.2 | XL | ++ |
|  | Paper lib | 72.7 | 12.0 | 10.2 | 44.5 | 13.7 | XD | +++ |
| LSD (0.05) | | 3.5 | 2.4 | 2.2 | 5.7 | 2.6 |  |  |

XL: Light green XD: Dark green

***3.2.5 The influence of genetic source on rapid propagation of agarwood shoot cluster in vitro:*** mother plants for culture samples originated from seeds, genetic diversity was different. The results showed that the indicators of shoot number and in vitro growth fluctuated greatly without following the rule. Lines BD1, BD2 and ND1 produced good shoots in culture. Line ND2 produced average shoots. Different genetic origins between lines led to fluctuations in shoot generation and shoot growth in in vitro culture (Table 8)

Table 8: Effect of genetic origin on rapid propagation of agarwood shoot clusters in vitro

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Genetic source | Stem height  (mm) | Shoot  (no) | Internode  (no) | Leaf length (mm) | Leaf width  (mm) |
| BD1 | 50.0 | 9,2 | 7.7 | 38.7 | 11.2 |
| BD2 | 58.7 | 10.5 | 11.0 | 26.2 | 8.2 |
| ND1 | 31.2 | 6.5 | 10.2 | 31.2 | 11.7 |
| ND2 | 53.7 | 9.0 | 14.0 | 25.0 | 5.7 |
| LSD (0.05) | 7.8 | 2.1 | 2.7 | 4.5 | 2.5 |

***3.2.6 Growth of agarwood shoot in vitro:*** the indicators of the number of shoots and growth fluctuate slowly in the period of 15-30 days after culture; increase rapidly in the period of 30-45 days after culture; and gradually slow down in the period of 45-60 days after culture. Adventitious shoots arising from internode culture samples are better than those from apical shoots. The culture cycle is 45 days (Table 9). It can be divided into 3 growth stages: stage 1 (0-30 days) shoot generation, need to increase gas exchange and high light intensity; stage 2 (30-45 days) shoot growth is strongest, at the end of this stage, it is necessary to transfer to maintain a high rate of shoot growth; stage 3 (45-60 days) is suitable for root growth culture (Van Minh, 2020 [27])

Table 9: Growth of agarwood shoot in vitro

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| parameter | Shoot | | | | internode | | | | LSD |
|  | 15 DAP | 30 | 45 | 60 | 15 DAP | 30 | 45 | 60 | (0.05) |
| Shoot (no) | 1.0 | 1.0 | 1.0 | 1.0 | 1.7 | 4.7 | 9.7 | 10.5 | 1.6 |
| Internode (no) | 1.2 | 2.7 | 5.2 | 9.7 | 1.5 | 4.2 | 7.5 | 9.7 | 0.8 |
| Leaf (no) | 2.7 | 3.7 | 6.2 | 10.7 | 2.5 | 5.2 | 8.5 | 10.7 | 0.8 |
| Leaf length (mm) | 4.5 | 14.0 | 16.7 | 25.7 | 8.2 | 14.0 | 18.2 | 27.0 | 19 |
| Leaf width (mm) | 1.7 | 4.2 | 6.2 | 8.5 | 1.7 | 3.7 | 6.5 | 8.5 | 0.8 |
| Stem height (mm) | 13.2 | 21.2 | 44.7 | 56.2 | 11.0 | 26.7 | 40.0 | 54.7 | 2.5 |
| Leaf color | XV | XV | XD | XD | XL | XL | XD | XD | - |
| Leaf dev. (+/-) | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - |

XV: Pale-green XL: Light green XD: Dark green

***3.2.7 In vitro culture of agarwood rooting:*** Auxin at low concentrations (0.1 mg/l) does not stimulate root formation. When increasing the auxin concentration to 0.3 mg/l, it stimulates root formation. Higher auxin concentrations inhibit root formation (Table 10) (Figure 7). In nature, agarwood is very difficult to propagate by cuttings. Through in vitro culture, agarwood is brought back to a young physiological state, so it is easy to culture for root formation (Maulana Tamyiz etal., 2022 [16]).

Table 10: In vitro culture of agarwood rooting

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Auxin | Concentration (mg/l) | Stem height  (mm) | Internode (no) | Leaf  (no) | Root  (no) | Root length (mm) |
| IAA | 0.3 | 56.2 | 8.2 | 9.2 | 1.0 | 38.2 |
| IBA | 0.3 | 51.7 | 7.2 | 8.2 | 1.0 | 24.2 |
| NAA | 0.3 | 44.0 | 6.2 | 7.2 | - | - |
| IAA | 0.1 | 41.2 | 4.0 | 5.0 | - | - |
| IBA | 0.1 | 42.7 | 6.0 | 7.0 | - | - |
| NAA | 0.1 | 43.7 | 4.2 | 5.2 | - | - |
| LSD (0.05) | | 6.4 | 1.5 | 1.5 | ns | 2.8 |

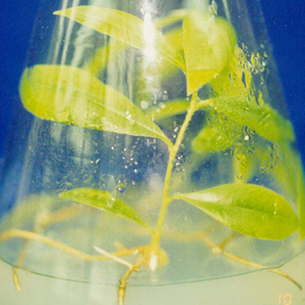


Figure 7: Agarwood plantlet rooting in vitro after after 45 days on WPM + IBA (0.3 mg/l)

**3.3 Acclimatization of tissue cultured agarwood plantlets**

Agarwood trees with a stem height of 40-50mm with developed stems, leaves and roots are put into acclimatization culture. The acclimatization mode is a misting chamber with a substrate of coconut fiber [mist + coconut fiber]. The misting time is 15 minutes and a rest period of 30 minutes, maintained for 10 days. Then the tissue cultured seedlings are cultured in soil-balls with a substrate of soil: coconut fiber: manure (1:1:1). New leaves appear on the 30th day after culture (Table 11) (Figure 8) (Page and Awarau, 2012 [19])

Table 11: Domestication of tissue cultured agarwood plantlets

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Date after culture  (ngày) | Stem height (mm) | Internode  (no) | Leaf  (no) | Leaf length  mm) | Leaf width (mm) | Survival  (%) |
| 0 | 42 | 4.4 | 5.4 | 20.5 | 7.8 | 100 |
| 15 | 55 | 5.2 | 6.2 | 28.2 | 9.5 | 85 |
| 30 | 68 | 6.5 | 7.5 | 35.6 | 12.4 | 85 |
| 45 | 72 | 7.1 | 8.1 | 45.8 | 18.9 | 85 |
| 60 | 91 | 9.5 | 1.5 | 62.1 | 23.5 | 85 |



**8**

Figure 8: Acclimatization in nursery after 3 months in soil-balls with a substrate of soil: coconut fiber: manure (1:1:1).

**4. CONCLUSION**

Micropropagation technology for agarwood trees has been established with WPM medium as the basic culture medium. Supplementing with BA, NAA, coconut water, enhancing the gas exchange process and high light intensity are factors that stimulate shoot growth. The concentration of 0.3 mg/l IBA is suitable for rooting culture. The in vitro culture cycle is 45 days. Application of micropropagation technology under pilot scale was set up for agarwood trees allows for the reforestation and development of traditional agarwood planting areas.

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