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In vitro propagation of *Aquilaria crassna* Pierre

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Abstract: *This study was focused on developing a micropropagation protocol for Aquilaria crassna. In experiment 01, four treatments (T1- 0.1% Captan, T2- 0.5% Captan, T3- 1.0% Captan, T4- 1.5% Captan together with 30% Clorox) were tested to identify the appropriate sterilization procedure using seeds on Woody Plant Medium. After 4 weeks, T3 performed the best (4.67±1.53). In experiment 02, four treatments (T1- 0.5% Carbendazim, T2- 1.0% Carbendazim, T3- 0.8% Mercuric Chloride, T4- 1.0% HgCl2 together with 30% Clorox) were tested to identify the best sterilization procedure for Aquilaria shoot tips. After 4 weeks, T3 performed the best (9.33±0.58). In experiment 03, different hormonal combinations (T1- 1.5 mg/L BA + 0.25 mg/L NAA, T2- 1.5 mg/L BA + 0.5 mg/L NAA, T3- 3.0 mg/L BA + 0.25 mg/L NAA, T4- 3.0 mg/L BA + 0.5 mg/L NAA) were tested for Aquilaria shoot multiplication. The highest shoot number (8.40±2.50) and leaves (10.3±3.15) were recorded in T3 after 12 weeks. The results revealed, T3 was the best treatment for Aquilaria in vitro multiplication. In experiment 04, different hormonal concentrations tested (T1- 20 mg/L NAA, T2- 40 mg/L NAA, T3- 20 mg/L IBA, T4- 40 mg/L IBA and T5- Control) revealed that T2 was the best treatment for Aquilaria crassna root induction.*

Keywords: *Aquilaria crassna, Explant sterilization, Micropropagation, Root induction media, Shoot multiplication media.*

I. Introduction

Aquilaria crassna Pierre is a tree-producing high-value Agarwood resin. This resin has been used as a medicine in China for centuries and also has high demand in other South East Asian and in Middle East countries. The resin has been utilized to produce medicine, perfumes, and incense [1]and [2]. *Aquilaria crassna* trees secrete secondary metabolites including terpenoids as a response to physical damages and microbial invasions which are later harvested as Agarwood resin[3]. The public attraction for *Aquilaria crassna* is now rising higher due to the high price of the Agarwood resin. According to [4] people are harvesting Agarwood from wild sources extensively. It has led the *Aquilaria crassna* to be categorized as a critically endangered species under IUCN red list classification [4]. At present commercial *Aquilaria crassna* plantations are established in different countries including Sri Lanka for Agarwood harvesting.
Aquilaria crassna Pierre belongs to the family Thymelaeaceae. There are 23 species belonging to the Thymelaeaceae family. Of 23 species belonging to the genus *Aquilaria* only 13 have been identified as Agarwood resin producers. *Aquilaria* species are mainly distributed in Southeast Asian countries [5]. Among 23 *Aquilaria* species, *Aquilaria crassna* is distributed in Vietnam, Cambodia, Lao PDR, and Thailand [4].

Agarwood can propagate through seeds and cuttings. But in commercial cultivation seeds are much more popular. However, as described by [6] and [1] the Agarwood seeds are recalcitrant and lose viability shortly. Propagation through cuttings is still under investigation. Micropropagation is an alternative method to overcome problems in seedling production. It is beneficial over the conventional method thus it can produce a large number of genetically uniform plants [7]. *In vitro* propagation of *Aquilaria crassna* was done using axillary buds [8]. In addition, shoots and leaf segments were used as explants to produce plants using the tissue culture technique [9]. Therefore, the present study was aimed to develop an *in vitro* propagation protocol for *Aquilaria crassna*Pierre.

II. Materials and Methods

Shoot tips and seeds (Fig.1) were collected from healthy *Aquilaria crassna* plants from the research field of the Central Research Station, in Matale, Sri Lanka to identify the best sterilization procedure. *In vitro* grown *Aquilaria crassna*shoots (Fig.1) available at the tissue culture laboratory in Central Research Station were also used. All the laboratory experiments were also conducted in the Central Research Station. Woody Plant Medium (WPM) was used as the basal medium with different hormonal combinations for all the experiments and sterilization treatments.

Figure 1. Agarwood (*Aquilaria crassna*) explants. (A) shoot tips, (B) seeds (C) *in vitro* developed shoots.

Experiment1: To find the best sterilization procedure for *Aquilaria crassna* P. shoot tips.

Three tofour cm long unopened shoot tips were collected from the research field and leaves were removed. The unopened shoot tips were cleaned well with a cotton bud soaked with water and Teepol(commercial detergent) solution. Subsequently, shoot tips were washed under running tap water with Teepol for 30 min and following treatments (Table 1) were applied to find out the best sterilization method for *Aquilaria crassna* Pierre shoot tips.

Table 1. Shoot tips sterilization treatments of *Aquilaria crassna*Pierre.

Treatment No.	Treatments
Treatment 01 (T1)	Carbendazim 0.5% (15 min) + Clorex 30% (15 min) + Ethanol 50% (5 min)
Treatment 02 (T2)	Carbendazim 1.0% (15 min) + Clorex 30% (15 min) + Ethanol 50% (5 min)
Treatment 03 (T3)	Mercuric Chloride 0.8% (15 min) + Clorex 30% (15 min) + Ethanol 50% (5 min)
Treatment 04 (T4)	Mercuric Chloride 1.0% (15 min) + Clorex 30% (15 min) + Ethanol 50% (5 min)

Single shoot tip was introduced into each test tube containing 10 ml of Woody Plant Medium. The cultures were kept at 22 ± 1ºC under 24 hours light (3000 lux) with 75% relative humidity. Bacterial contamination percentage (%), Fungal contamination percentage (%), Death percentage (%), and Success culture percentage (%) were recorded after one month of culture. Based on the data, the best sterilization procedure was selected.

Experiment 2.To find the best sterilization procedure for *Aquilaria crassna* P. seeds.

Seeds collected from the field were brought into the laboratory and seed tails were removed. All the seeds were kept in a container with Teepol and water and shaken well until the dirt in the seed coat was completely removed. Subsequently, the seeds were washed under running tap water with Teepol for 30 min. After cleaning, explants were subjected to the sterilization procedure under following sterilization treatments (Table 2).

Table 2. Seed sterilization treatments of *Aquilaria crassna*Pierre.

Treatment No.	Treatments
Treatment 01 (T1)	Captan 0.1% (30 min) + Clorex 30% (10 min)
Treatment 02 (T2)	Captan 0.5% (30 min) + Clorex 30% (10 min)
Treatment 03 (T3)	Captan 1.0% (30 min) + Clorex 30% (10 min)
Treatment 04 (T4)	Captan 1.5% (30 min) + Clorex 30% (10 min)

Different concentration levels of Captan (0.1%, 0.5%, 1.0%, 1.5%) solutions with 2 drops of Teepol were added separately to seeds and kept in the shaker for 30 min. Then seeds were washed with sterilized distilled water 3-4 times inside the laminar airflow cabinet. Subsequently, 30% Clorox solution was added to seeds with 2 drops of tween 20 and kept in a shaker for 10 min. Afterwards, seeds were washed 3 times with sterilized distilled water inside the laminar airflow cabinet and seeds were introduced to culture tubes. After sealing, tubes were kept in a culture room at 22+2ºC, under 24 hours light (3000 lux) with 75% relative humidity.

Bacterial contamination percentage (%), fungal contamination percentage (%), Death percentage (%), and germination percentage (%) were recorded after one month of culture. Based on the data, the best sterilization procedure was selected.

Experiment 3.To find the best plant growth regulator combination for *Aquilaria crassna* shoot multiplication using *in vitro* propagated shoots.

The following treatments were applied to find the best plant growth regulator combination for the shoot multiplication of *Aquilaria crassna* plants. *In vitro* propagated *Aquilaria crassna* plants were used as the explant for the experiment. Well-grown shoots were separated from the callus and introduced to the culture tubes with following treatments (Table 3). Then sealed culture tubes were kept in a culture room at 22+2ºC, under 16/8-hour light/dark (3000 lux) with 75% relative humidity. After twelve weeks of culture, number of new shoots, number of leaves, and length of longest shoot were recorded.

Table 3. Shoot multiplication treatments of *in vitro* propagated *Aquilaria crassna*Pierreshoots.

Treatment No.	Treatments
Treatment 01 (T1)	BA 1.5 mg/L + NAA 0.25 mg/L
Treatment 02 (T2)	BA 1.5 mg/L + NAA 0.5 mg/L
Treatment 03 (T3)	BA 3.0 mg/L + NAA 0.25 mg/L
Treatment 04 (T4)	BA 3.0 mg/L + NAA 0.5 mg/L

Experiment 4.To find the best plant growth regulator combination for *Aquilariacrassna*root induction using*in vitro* propagated shoots.

The following treatments were applied to find the best plant growth regulator concentration for root induction of *Aquilaria crassna* plants. *In vitro* propagated *Aquilaria crassna* shoots were used as the explant for the experiment. Two centimeters height shoots were selected and were put into liquid WPM medium containing different hormone combinations (Table 4) for 48 hours. Then shoots were introduced to a solid WPM medium containing 1 mg/L IBA. After one week, shoots treated with each hormone concentration were separated into two groups and transferred to solid WPM medium containing 1 mg/L IBA and with 2 g/LActivated Charcoal and solid WPM medium containing 1 mg/L IBA and without Activated Charcoal separately. Afterwards, sealed culture bottles were kept in the culture room at 22+2ºC, under 16/8-hour light/dark (3000 lux) with 75% relative humidity.The number of roots, length of the longest root, and rooting percentage were recorded during twelve weeks of culture.

Table 4. Root induction treatments of *in vitro* propagated shoots of *Aquilaria crassna*Pierre.

Treatment No.	Treatments
Treatment 01 (T1)	NAA 20 mg/L (48 hours)
Treatment 02 (T2)	NAA 40 mg/L (48 hours)
Treatment 03 (T3)	IBA 20 mg/L (48 hours)
Treatment 04 (T4)	IBA 40 mg/L (48 hours)
Treatment 05 (T5)	Control (48 hours)

2.1 Experimental design and data analysis.

Complete Randomized Design (CRD) was used as the experimental design. ANOVA was used to analyze the significant differences between treatments. Experiments were analyzed by using SAS OnDemand for academics online programme with a 95% confidence level. Mean separation was done by Tukey's test (TUKEY). Counts were transformed to square root values before being run through ANOVA. Percentages were transformed to arcsine values before running through ANOVA.

III. Results and Discussion

Experiment 1. To find the best sterilization procedure for *Aquilaria crassna* Pierre shoot tips.

The data revealed significant differences among treatments (Table5 and 6). Explants treated with 0.5% Carbendazim resulted in the highest percentage of fungal contamination (93.33%). The bacterial contamination percentage was zero in all four treatments. In T2, explants were treated with 1.0% Carbendazim, 30% Clorox, and 50% Ethanol resulted the highest rate of death, which is 23.33%. In preliminary studies, $HgCl_2$ was used in much lower concentrations and after four weeks, all samples were contaminated with fungi. However, in T4, the explants treated with 0.8% $HgCl_2$, 30% Clorox and 50% Ethanol resulted in the highest success percentage of 93.33. After 4 weeks of incubation period, few shoots exhibit development from leaf buds while other shoot tipsremain unchanged (Fig.2). [10] reported that increase number of success cultures resulted of*Aquilaria malaccensis*by pretreating shoot tips with Benomyl and later with a low concentration of $HgCl_2$.

Table 5. Fungal, bacterial contaminations, dead and success culture percentagesof sterilization of *Aquilaria crassna*Pierre shoot tips.

Treatment No.	Fungi (%)	Bacteria (%)	Dead (%)	Success (%)

Treatment No.	Contamination		Dead	Success
	Fungi	Bacteria		
Treatment 01 (T1)	9.33±1.15[a]	0.00	0.67±1.15[a]	0.00
Treatment 02 (T2)	6.6 ±1.53[b]	0.00	0.58±2.33[a]	1.00±1.00[b]
Treatment 03 (T3)	0.00	0.00	0.58±0.67[a]	9.33±0.58[a]

Treatment 01 (T1)	93.33	0	6.67	0
Treatment 02 (T2)	66.67	0	23.33	10
Treatment 03 (T3)	0	0	6.67	93.33
Treatment 04 (T4)	3.33	0	10	86.67

T1 had the highest mean number of fungal contaminations. There was no significant difference between treatments when considering the number of dead shoot tips (Table 6). T3 and T4 have the same mean number of success cultures. However, T3 and T4 had significant difference in success, when compared with T2. Based on the results, T3 (0.8% HgCl2 + 30% Clorox + 50% Ethanol) was selected as the best treatment for *Aquilaria crassna* shoot tips sterilization.In another study high success rate of surface sterilization of *Aquilaria malaccensis* shoot tips was achieved through a treatment combination of 5% Teepol solution and a 0.1% $HgCl_2$ solution [11].

Table 6. Average number of success culture, fungal, and bacterial contaminations and dead cultures±standard deviation of sterilization of *Aquilaria crassna* Pierre shoot tips.

Treatment 04 (T4)	0.33±0.58[c]	0.00	1.00±1.00[a]	8.67±1.53[a]

Means with the same letter (a,b,c) in a column are not significant at $p<0.05$.

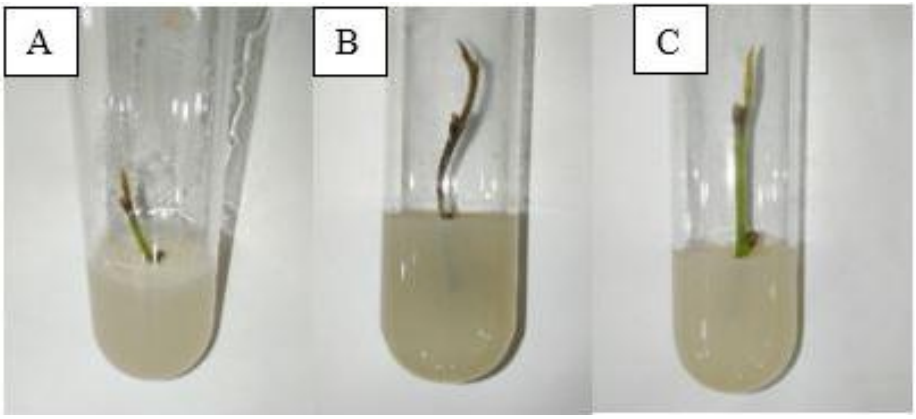

Figure 2. Performance of *Aquilaria crassna*shoot tips sterilization. (A) Fungal contamination (B) Dead culture (C) Success culture.

Experiment 2. Determination of the best sterilization method for *Aquilaria crassna* P. seeds.

Significant difference was observed between treatments used for seed sterilization. According to Table 7, in T3, explants were sterilized with 1.0% Captan and 30% Clorox resulted 46.67% success percentage, which is the highest success rate compared with the other three treatmentsand also the mean value for T3 success culture is significantly different from other treatments (Table 8). In T1, which the explants were sterilized with 0.1% Captan and 30% Clorox resulted very less success rate but resulted the highest (66.67%) fungal contamination percentage Also, in T2, explants result second highest fungi contamination rate, but the death rate is low when compare with the T1 and T4. In T4, explants were sterilized with 1.5% Captan and 30% Clorox resulted 23.33% success percentage but observed the highest rate of death (53.33%). According to the statistical analysis, in success culture values, T1 and T2 treatment pair and T3 and T4 treatment pair share means with same letters (Table 8), which indicate that there is no significant difference in success culture percentage between T1 and T2 and also between T3 and T4. But when comparingT1 and T2 with T3 and T4. there is a significant difference in success culture percentages.According to [10]pre-sterilization with a 0.2% Benomyl solution enhanced the number of*Aquilaria malaccensis* seeds that were both free of contaminations and viable, also in the same studythe highest number of contamination free and alive seeds were attained with 0.2% $HgCl_2$ treatment.

Table 7. Fungal contamination, bacterial contaminations, dead and success culture percentagesof sterilization of *Aquilaria crassna* P. seeds.

Treatment No.	Fungi (%)	Bacteria (%)	Dead (%)	Success (%)
Treatment 01 (T1)	66.67	0.00	30.00	3.33
Treatment 02 (T2)	60.00	0.00	26.67	13.33

Treatment 03 (T3)	23.33	3.33	26.67	46.67
Treatment 04 (T4)	23.33	0.00	53.33	23.33%

Table 8. Mean number of fungi, bacteria, death, and success cultures±standard deviation of sterilization of *Aquilaria crassna* P. seeds.

Treatment No.	Contamination		Dead	Success
	Fungi	Bacteria		
Treatment 01 (T1)	6.67 ± 0.58^{a}	0	3.00 ± 1.00^{ab}	0.33 ± 0.58^{b}
Treatment 02 (T2)	6.00 ± 2.00^{a}	0	2.67 ± 1.15^{b}	1.33 ± 1.15^{b}
Treatment 03 (T3)	2.33 ± 1.15^{b}	0.33 ± 0.58^{a}	2.67 ± 0.58^{b}	4.67 ± 1.53^{a}
Treatment 04 (T4)	2.33 ± 0.58^{b}	0	5.33 ± 1.15^{a}	2.33 ± 0.58^{ab}

Means with the same letter (a,b) in a column are not significant at $p<0.05$.

Experiment 3. Determining the best hormonal combination for *Aquilaria crassna* P. shoot multiplication.

*Aquilaria crassna*shoot multiplication was after twelve weeks of incubation period, WPM supplemented with 3.0 mg/L BA + 0.25 mg/LNAA resulted the highest number of shoots (8.0). The lowest number of shoots resulted from T4 - WPM supplemented with 3.0 mg/L BA + 0.5 mg/LNAA. [12] has reported 0.1 mg/L of BA and NAA supplemented with 10% coconut water resulted highest shoot multiplication in *Aquilaria crassna* subcultures. Also, [13] reported that Gamborg B5 medium supplemented with 0.5 mg/L BAP is good for shoot proliferation of *Aquilaria malaccensis*. [14] reported that in one of their studies which new shoots in *Aquilaria hirta*developed in both MS and WPM basal media. In contrast to the present study, they identified most effective medium for generating multiple shoots as the MS medium supplemented with 0.1 mg/L BAP, resulting in an average of 6.1 shoots per culture.According to Table 9, there is a significant difference among treatments on the number of shoots after 12 weeks of culture and T3 (Fig.3) is the best combination than other three treatments tested.

Table 9. Mean number of shoots after 12 weeks±standard deviation.

Treatment No.	Mean number of shoots
Treatment 01 (T1)	5.50 ± 2.63^{b}
Treatment 02 (T2)	5.11 ± 2.08^{b}
Treatment 03 (T3)	8.40 ± 2.50^{a}
Treatment 04 (T4)	4.95 ± 2.70^{b}

Means with the same letter (a,b) in a column are not significant at $p<0.05$.

The number of leaves was counted during the 12 weeks incubation period. According to Table 10, plants were treated with 3.0 mg/L BA and 0.25 mg/L NAA (T3) has resulted the highest number of leaves. The lowest number of leaves resulted by the plants treated with 0.5 mg/L NAA with 1.5 mg/L and 3.0 mg/L BA in T2 and T4 respectively. There was a significant difference between the treatments. T2 and T3 showed no significant difference between the mean, similarly T1 and T4. But T1 and T4 resulted a significant difference when compared with T2 and T4.

Table 10. Mean number of leaves after 12 weeks±standard deviation.

Treatment No.	Mean number of leaves
Treatment 01 (T1)	6.70±3.44[b]
Treatment 02 (T2)	9.11±2.88[a]
Treatment 03 (T3)	10.3±3.15[a]
Treatment 04 (T4)	6.95±2.66[b]

Means with the same letter (a,b) in a column are not significant at $p<0.05$.

The height of the shoots was measured after 12 weeks of incubation period. There was a significant difference in shoot height among treatments. According to Table 11, in T2, which the plants treated with 1.5 mg/LBA and 0.5 mg/L NAA resulted maximum height. However, the lowest shoot length was shown at a concentration of BAP at 1.5 mg/L + 0.25 mg/L NAA which was resulted 1.27 cm. [14]have reported that for *Aquilaria hirta*, highest length of new shoots was obtained in WPM containing 0.1 mg/L BAP.

Table 11: Mean shoot height after 12 weeks±standard deviation.

Treatment No.	Mean shoot height
Treatment 01 (T1)	1.27±0.58[b]
Treatment 02 (T2)	1.69±0.46[a]
Treatment 03 (T3)	1.49±0.41[ab]
Treatment 04 (T4)	1.31±0.47[b]

Means with the same letter (a,b) in a column are not significant at p<0.05.

Figure 3. Performance of shoot multiplication (*in vitro* developed *Aquilaria crassna* shoots in T3).

Experiment 4. Determine the best plant growth regulator concentration for root induction of *Aquilaria crassna* P.

In vitro propagated *Aquilaria crassna* plants were used for root induction. According to [13] NN medium without any hormones was suitable for the rooting of *Aquilaria malaccensis*. Also,[11] have reported root regeneration of *Aquilaria agallocha* was achieved by soaking the shoots in liquid half-MS medium supplemented with 40 mg/L IBA for 48 hours and transferred to solid half-MS medium supplemented with 2 mg/L IBA for one week then finally transferred to hormone-free medium supplemented with 0.2% Activated

Charcoal. According to [12], low percentage of rooting on *Aquilariacrassna* was resulted by using 0.3 mg/L of NAA or 0.3 mg/L of IBA.

The rooting percentage was calculated after 12 weeks incubation period. In T2, which plants were treated with 40 mg/L NAA then planted on WPM medium with Activated charcoal resulted the maximum rooting percentage (Table 12). The lowest rooting percentage was recorded in control treatment.

Table 12. Rooting percentage after 12 weeks in *In vitro* propagated *Aquilaria crassna.*

Treatment No.	Rooting percentage	
	With A. Charcoal (2 g/L)	Without A. Charcoal
Treatment 01 (T1)	50%	40%
Treatment 02 (T2)	80%	0%
Treatment 03 (T3)	25%	25%
Treatment 04 (T4)	50%	75%
Control	0%	0%

The number of roots were counted after the 12 weeks' incubation period. There was no significant difference in number of roots among treatments. But in treatment 01, which the plants treated with 20 mg/L NAA resulted the highest number of roots (09) after 12 weeks (Table 13).

Table 13. Number of roots after 12 weeks±standard deviation in *In vitro* propagated *Aquilaria crassna*.

Treatment No.	Number of roots	
	With A. Charcoal (2 g/L)	Without A. Charcoal
Treatment 01 (T1)	9 ± 0.97^{a}	2 ± 0.54^{a}
Treatment 02 (T2)	7 ± 1.14^{a}	0
Treatment 03 (T3)	3 ± 1.5^{a}	5 ± 0^{a}
Treatment 04 (T4)	4 ± 1.41^{a}	8 ± 1.82^{a}
Control	0	0

Means with the same letter (a,b) in a column are not significant at $p<0.05$.

The total length of roots was measured after 12 weeks of incubation period. There was no significant difference in root length among treatments as shown in Table 14. But in treatment 02, which the plants treated with 40 mg/L NAA resulted the highest length (1.6 cm) of roots after 12 weeks (Fig.4). Except T4, all other treatments with activated charcoal performed well, compared to the treatments without activated charcoal in root induction.However, [11] Debnath *et al.,* (2013)reported that the highest percentage of rootsof *Aquilaria*

*agallocha*was observed when using a half-strength MS medium supplemented with 1 mg/L of NAA. Also, they reported that roots were not developed in hormone-free MS medium.

Table 14. Number of roots after 12 weeks±standard deviation.

Treatment No.	Root length	
	With A. Charcoal (2 g/L)	Without A. Charcoal
Treatment 01 (T1)	1.6±0.37[a]	1.2±0.38[a]
Treatment 02 (T2)	3.8±0.25[a]	0
Treatment 03 (T3)	0.9±0[a]	3.1±0[a]
Treatment 04 (T4)	1.2±0.56[a]	2.6±0.92[a]
Control	0	0

Means with the same letter (a,b) in a column are not significant at $p<0.05$.

Figure 4. Performance of root induction *in vitro* developed *Aquilaria crassna*shoots in Treatment T2.

IV. Conclusion

*Aquilaria crassna*Pierre is a tree with high economic value. The study focused on investigating the best propagation method for producing good quality planting materials. Therefore, *in vitro* propagation method was selected to produce good planting material by experimenting with the best sterilization method, shoot multiplication and root induction. The best sterilization treatment for *Aquilaria crassna* Pierre seeds was observed in1.0% Captan (30 min) + 30% Clorox (15 min) (T3) during this study. The best sterilization treatment for *Aquilaria crassna* Pierre shoot tips was observed in 0.8% $HgCl_2$ (15 min) + 30% Clorox (15 min) + 50% Ethanol (5 min) (T3) during this study. WPM containing 3.0 mg/L BA with 0.25 mg/L NAA (T3) is the best hormonal combination for shoot multiplication of *Aquilaria crassna* Pierre. 40 mg/L NAA (T2)inWPM medium containing 1 mg/L IBA and 2g/L Activated Charcoal is the best treatment for root induction of *Aquilaria crassna* Pierre.

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