Plant regeneration of Amomum tsaoko Crevost & Lemarié in vitro

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Abstract. Cardamom (Amomum tsaoko Crevost & Lemarié) is a valuable medicinal herb in both traditional and western medicine. The pharmacological properties of the A. tsaoko seeds make it a useful cure for such ailments as abdominal pain, bloating, hiccups, vomiting, diarrhea, malaria, bad breath, and tooth decay. It has even further value for its use in food processing. Therefore, it is necessary to develop protocols that reduce the time required for the multiplication of cardamom using tissue culture techniques. The research was conducted to initially build up the process of in vitro propagation of cardamom from the rhizome buds. An 8 minutes treatment of HgCl₂, with a concentration of 0.1%, is the most efficient for the disinfection of the rhizome buds, resulting in the disease-free survival explants reaching 18.29%. MS medium supplemented with 1.0 mg/l BAP is the most suitable medium in the fast multiplication phase, with a multiplier of 4.54 shoots/explant, average shoot height of 5.45 cm, and good shoot quality after 6 weeks of culture. A suitable rooting medium for cardamom in vitro-shoots was MS medium supplemented with 0.5 mg/l IBA with a rooting rate of 100%, the average number of roots was 5.6 roots/shoot and the average root length was achieved 6.2 cm, after 8 weeks of culture.

1. Introduction
Amomum Roxb. (Family: Zingiberaceae Lindl.) as currently circumscribed is a plant genus of some 150 to 188 [1] species. In Vietnam, 21 species have been recorded of this genus [2]. The generic name was first used by Linnaeus (1753) but, as explained by Burtt & Smith (1968), none of the species Linnaeus included is now in Amomum. The name now used is Amomum Roxb. which is a conserved name. Roxburgh (1810) defined Amomum by its labellum, anther, and fruits. Amomum tsaoko is one of Amomum’s 188 species. It was established by Crevost & Lemarié (1917) and distributed in China, Laos, and Vietnam [1].

In Vietnam, Amomum tsaoko Crevost & Lemarié commonly known as “Cardamom” is a perennial herb and monocotyledon. It is one of the valuable non-timber forest products and is an important medicinal plant with excellent export potential in herbal drug trade. In traditional medicine, A. tsaoko seeds are used as a cure for abdominal pain, bloating, hiccups, vomiting, diarrhea, malaria, bad breath, and tooth decay [3-5]. In addition, they are widely used in many dishes as a spice [6].

According to Li Wei et al. (2011) [7], A. tsaoko essential oil has antimicrobial (Bacillus subtilis, Staphylococcus albus, and Escherichia coli) and antifungal (Aspergillus oryzae, Rhizopus sp., and
Penicillium sp.) activity. Besides, the dried fruit extract of A. tsaoko has also an inhibitory effect on NO production in RAW 264.7 macrophage cells, the protective effect on H2O2-induced apoptosis of PC-12 cells [8]; against cervical cancer cell Hela, HepG-2 and SMMC-7721 liver tumor cells, and A549 lung cancer cells [9]. These studies once again confirm the value of this plant.

For these reasons above, there is an urgent need to scale up this biotechnology for the agricultural sector. Currently, the large and long-standing cardamom growing areas of Vietnam, such as Ha Giang, Lao Cai, and Lai Chau, are mainly propagated by seeds and rhizome segments [10]. However, these methods have not brought about high efficiency and have not met the input needs of production. The propagation method using plant tissue culture technology promises to produce a large number of plants in a short time, with seedlings free of disease and uniform quality compared to traditional methods.

In the world, in vitro propagation has been carried out on some species of Amomum genus such as A. longiligulare [11], A. krekreva [12] and A. subulatum [13-15]. In Vietnam, plant tissue culture method has also been used to propagate some species of this genus such as A. longiligulare [16], Amomum sp. [17]. The research was conducted to initially build up the process of in vitro propagation of cardamom (A. tsaoko Crevost & Lemarié) from the rhizome buds.

2. Materials
Cardamom (A. tsaoko Crevost & Lemarié) was collected from Lai Chau province, Vietnam.

3. Methods

3.1. Sterilization and culture initiation
In the first step, rhizome segments of A. tsaoko Crevost & Lemarié were cut into 3-5 cm single nodal segments. In the second step, they were washed under running tap water for 15-20 min; outer scales of them were excised by a sharp blade. In the third step, they were soaked in a thin soap solution [1% (v/v)] for 10 minutes, washed directly under running tap water. In the fourth step, they were surface sterilized in 70% ethanol for 30 sec, followed by immersion in a 0.1% (w/v) aqueous mercuric chloride [0.05% or 0.1% (w/v)] or sodium hypochlorite [5% or 10%, (w/v)] for 4, 8 or 12 minutes. Lastly, after sterilizing with chemicals, they were removed from the damaged tissue at both ends after being rinsed 4-5 times with sterile distilled water and were placed on solid MS medium (Murashige and Skoog, 1962).

3.2. Shoot multiplication
Regenerated shoots 1.5-2.0 cm in length with 3-4 leaves from the initiation culture were used as explants for shoot multiplication. The shoot explants were placed on solid MS medium (Murashige and Skoog, 1962) supplemented individually with different concentrations of 6-Benzylaminopurine (BAP: 0, 0.5, 1.0, 1.5, and 2.0 mg/l) and kinetin (Kn: 0, 0.5, 1.0, 1.5, and 2.0 mg/l). Data were scored after 6 weeks of culture.

3.3. Root induction
Elongated healthy individual shoots 4-4.5 cm in length with 4-6 leaves obtained from the previous shoot proliferation experiments were transferred to solid MS medium supplemented individually with different concentrations of Indole-3-butyric acid (IBA: 0, 0.25, 0.50, 0.75, and 1.00 mg/l) and 1-Naphthaleneacetic acid (α-NAA: 0, 0.25, 0.50, 0.75, and 1.00 mg/l) for root induction. Data were scored after 8 weeks of culture.

3.4. Culture media and culture conditions
All solid media consisted of 0.8% (w/v) agar and 3% (w/v) sucrose, pH was adjusted to 5.6-5.8 by 1N NaOH before being autoclaved at 121ºC and at 1.1 atm for 20 min. The cultures were grown in a culture room at 25±2°C under a 16 h-photoperiod in cool white fluorescent light (2000-2500 lux).
3.5. Experimental design and statistical analysis

The experiments were arranged completely randomly and repeated three times with 28-45 explants per treatment. Analysis of variance (ANOVA) was performed using Sirichai Statistics 7.0 and means were compared using LSD at a 0.05 level of probability.

4. Results and discussion

4.1. Explants sterilization

Explants sterilization has an important role in an in vitro propagation process. Rhizome segments are in the ground. Therefore, they often contain many bacteria and fungi, making it difficult to sterilize explants. In this study, HgCl$_2$ or Ca(ClO)$_2$ at different concentrations were used to sterilize explants.

**Table 1.** Effect of chemical type and sterilization time on the effectiveness of explants sterilization.

| Chemical treatment / time | Disease rate (%) | Disease-free death rate (%) | Disease-free survival rate (%) |
|--------------------------|------------------|-----------------------------|-------------------------------|
| Control                  | 100              | 0                           | 0                             |
| 0.05 (w/v) HgCl$_2$/ 4 minutes | 80.36           | 14.07                       | 5.57                          |
| 0.05 (w/v) HgCl$_2$/ 8 minutes | 62.08           | 28.37                       | 9.55                          |
| 0.05 (w/v) HgCl$_2$/ 12 minutes | 57.30           | 34.90                       | 7.80                          |
| 0.10 (w/v) HgCl$_2$/ 4 minutes | 76.40           | 12.62                       | 10.98                         |
| 0.10 (w/v) HgCl$_2$/ 8 minutes | 54.06           | 27.65                       | 18.29                         |
| 0.10 (w/v) HgCl$_2$/ 12 minutes | 43.50           | 45.35                       | 11.15                         |
| 5 (w/v) Ca(ClO)$_2$/ 4 minutes | 96.35           | 1.85                        | 1.80                          |
| 5 (w/v) Ca(ClO)$_2$/ 8 minutes | 89.65           | 5.95                        | 4.40                          |
| 5 (w/v) Ca(ClO)$_2$/ 12 minutes | 85.20           | 10.35                       | 4.45                          |
| 10 (w/v) Ca(ClO)$_2$/ 4 minutes | 96.30           | 1.48                        | 2.22                          |
| 10 (w/v) Ca(ClO)$_2$/ 8 minutes | 84.40           | 10.38                       | 5.22                          |
| 10 (w/v) Ca(ClO)$_2$/ 12 minutes | 71.80           | 21.47                       | 6.73                          |

Research results show that, when using Ca(ClO)$_2$, increasing the concentration and sterilization time increases the sterilization efficiency of explants with the rate of survival and disease-free changes from 1.8% to 6.73%. The concentration of 10% Ca(ClO)$_2$ for better sterilization results, survival rate, and disease-free explant were 5.22% after 8 minutes and 6.73% after 12 minutes, respectively. However, this ratio is still lower than when sterilized with HgCl$_2$. Using HgCl$_2$, depending on the different concentrations and sterilization time, the survival and disease-free explant rate varied from 5.57% to 18.29% (etable 1). Thereby, it can be concluded, the sterilization of the rhizome buds with 0.1% HgCl$_2$ for 8 minutes gives the best results with 18.29% of the survival and disease-free explant. This result has been completely consistent with previous studies of Pradhan et al. (2014) [14], and Poudel et al. (2018) [15] on Large cardamom (A. subulatum Crevost & Lemarié).

4.2. Effect of cytokinin on the shoot regeneration

Among growth regulators of the cytokinin group, BAP and kinetin are commonly used in in vitro shoot multiplication in general as well as species of the genus *Amomum* [13-16]. This study evaluated the effects of kinetin and BAP separately on the ability to rapidly multiply in vitro cardamom shoots.

Research results show that by adding kinetin at a low concentration (0.5-1.0 mg/l) to the culture medium, the number of shoots/explant increased compared to when cultured on a medium without kinetin supplementation. At a concentration of 1.0 mg/l kinetin, the number of shoots/explant was the highest, with 3.45 shoots/explant, and the quality of shoots was good with the shoots being fat and strong with thick leaves of a dark green color (figure 1). When the concentration of kinetin was increased to 1.5-2.0 mg/l, the number of shoots was reduced to 2.56-2.75 shoots/explant (etable 2). The shoots obtained had a small stem diameter with thin and light green leaves. The use of kinetin in vitro shoot
multiplication was also performed in some species of the genus *Amomum*. According to research by Pradhan et al. (2014) [14], on *A. subulatum* Crevost & Lemarié, the highest shoots/explant coefficient was 9.34 in the culture medium supplemented with 3.0 mg/l kinetin. The medium suitable for rapid in vitro multiplication of *A. longiligulare* shoots is the medium with 1.0 mg/l kinetin with a multiplier of 5.67 [16].

On the other hand, when adding BAP, the shoot multiplier factor increased markedly, the quality of the shoot was good, the shoots were fat and the leaves were thick and of dark green color (figure 1). At the concentration of 1.0 mg/l BAP, the shoot multiplier was the highest with 4.54 shoots/explant. When the concentration of BAP was increased from 1.5-2.0 mg/l, the shoot multiplier decreased to 3.48-3.76 shoots/explant (table 2). This shoot multiplier was also higher which was the highest achieved using the kinetin in the above experiment. Furthermore, shoot height in the experiment using BAP also improved significantly compared to the experiment using kinetin. This result has been completely consistent with previous studies on *A. subulatum* Roxb. [13-15] and *A. longiligulare* T.L.Wu. [16].

**Table 2. Effect of Kn and BAP on the shoot regeneration after 6 weeks of culture.**

| Plant growth regulator, mg/l | No. of shoots per explant | Shoot length (cm) | Shoot quality |
|-----------------------------|---------------------------|------------------|---------------|
| MS                          | 1.85<sup>a</sup>          | 3.15<sup>a</sup>  | small shoot, thin and light green leaves |
| MS + 0.5 Kn                 | 2.01<sup>a</sup>          | 3.85<sup>c</sup>  | medium shoot, thin and light green leaves |
| MS + 1.0 Kn                 | 3.45<sup>c</sup>          | 3.12<sup>a</sup>  | fat shoot, thick and green leaves |
| MS + 1.5 Kn                 | 2.75<sup>b</sup>          | 3.56<sup>b</sup>  | medium shoot, thin and light green leaves |
| MS + 2.0 Kn                 | 2.56<sup>b</sup>          | 3.55<sup>b</sup>  | medium shoot, thin and light green leaves |
| MS + 0.5 BAP                | 3.56<sup>c,d</sup>        | 4.54<sup>d</sup>  | medium shoot, thin and light green leaves |
| MS + 1.0 BAP                | **4.54<sup>e</sup>**      | **5.45<sup>e</sup>** | fat shoot, thick and green leaves |
| MS + 1.5 BAP                | 3.76<sup>d</sup>          | 5.85<sup>f</sup>  | medium shoot, thin and light green leaves |
| MS + 2.0 BAP                | 3.48<sup>c</sup>          | 6.45<sup>f</sup>  | medium shoot, thin and light green leaves |
| LSD<sub>0.05</sub>          | 0.26                      | 0.21             |               |

Note: Different small letters mean that they significantly differ from each other at P=0.05.

**Figure 1.** Effect of Kn and BAP on the shoot regeneration after 6 weeks of culture (A: 0.0 mg/l kinetin; B: 0.5 mg/l kinetin; C: 1.0 mg/l kinetin; D: 1.5 mg/l kinetin; E: 2.0 mg/l kinetin; F: 0.0 mg/l BAP; G: 0.5 mg/l BAP; H: 1.0 mg/l BAP; I: 1.5 mg/l BAP; K: 2.0 mg/l BAP).
4.3. Effect of auxin on rooting of in vitro-propagated shoots

The in vitro rooting stage is an important stage to prepare the tree to absorb water and photosynthesis in the natural environment. IBA and α-NAA that are two phytohormones in the auxin group were used in the in vitro rooting stage of *Amomum* genus [14, 16, 17]. In this experiment, IBA and α-NAA were also used to stimulate in vitro rooting from *A. tsao ko* shoots.

The addition of IBA to the culture medium stimulates the in vitro rooting process, increasing the rooting rate, the number of roots/shoot, as well as the length of the root, compared to the medium without IBA supplementation (figure 2). When IBA was added, rooting shoots were 100% and explants did not form calluses. The highest number of roots obtained 5.6 roots/shoot on medium supplemented with 0.50 mg/l IBA. In this environment, the roots are of good quality, fat, and many hairs. However, when the IBA concentration increased to 0.75-1.00 mg/l, the rooting rate remained at 100%, but the roots formed were thinner and less hairy, and the number of roots reduced to only 4.2-4.4 roots/shoot (table 3). This result has been completely consistent with the previous study of Dang Ngoc Phuc et al. (2011) [16] on *A. longiligulare* T.L.Wu. According to Poudel et al. (2018) [15], the appropriate IBA concentration for rooting for Large cardamom shoots (*A. subulatum* Roxb.) was 1.0 mg/l; the highest number of roots at 4.8 roots/shoot.

**Table 3.** Effect of IBA and α-NAA on rooting of in vitro-propagated shoots after 8 weeks of culture.

| Plant growth regulator, mg/l | Rooting rate, % | No. of roots per shoot | Root length (cm) | Root quality |
|-----------------------------|-----------------|------------------------|------------------|-------------|
| MS                          | 75              | 2.1<sup>a</sup>        | 2.3<sup>a</sup>  | thin, few root hairs |
| MS + 0.25 IBA               | 100             | 4.5<sup>b</sup>        | 5.2<sup>c</sup>  | thin, a few root hairs |
| MS + 0.50 IBA               | 100             | 5.6<sup>c</sup>        | 6.2<sup>c</sup>  | medium or fat, many root hairs |
| MS + 0.75 IBA               | 100             | 4.2<sup>c,d</sup>      | 5.5<sup>c,d</sup>| thin, a few root hairs |
| MS + 1.00 IBA               | 100             | 4.4<sup>d</sup>        | 5.9<sup>d,e</sup>| thin, a few root hairs |
| MS + 0.25 α-NAA             | 100             | 3.5<sup>b,c</sup>      | 3.8<sup>b</sup>  | thin, a few root hairs |
| MS + 0.50 α-NAA             | 100             | 3.8<sup>b,c,d</sup>    | 4.1<sup>b</sup>  | medium or fat, many root hairs |
| MS + 0.75 α-NAA             | 100             | 3.5<sup>b,c</sup>      | 3.7<sup>b</sup>  | thin, a few root hairs |
| MS + 1.00 α-NAA             | 100             | 3.2<sup>b</sup>        | 2.5<sup>a</sup>  | thin, a few root hairs |
| LSD<sub>0.05</sub>          | 0.73            | 0.48                   |                  |             |

Note: Different small letters mean that they significantly differ from each other at P=0.05.

**Figure 2.** Effect of IBA and α-NAA on rooting of in vitro-propagated shoots after 8 weeks of culture (A: 0.00 mg/l IBA; B: 0.25 mg/l IBA; C: 0.50 mg/l IBA; D: 0.75 mg/l IBA; E: 1.00 mg/l IBA; F: 0.00 mg/l α-NAA; G: 0.25 mg/l α-NAA; H: 0.50 mg/l α-NAA; I: 0.75 mg/l α-NAA; K: 1.00 mg/l α-NAA).
Similar to the IBA, the addition of α-NAA also stimulated rooting with a 100% rate of root shoots. The number of roots/shoot also increased, ranging from 3.2 to 3.8 roots/shoot compared to 2.1 roots/shoot on the medium without α-NAA supplementation. The number of roots as well as root length was highest in the medium supplemented with 0.50 mg/l α-NAA with 3.8 roots/shoot and a mean root length of 4.1 cm (table 3). On this medium, the roots are of good quality, fat, and many hairs (figure 2). When the concentration of α-NAA increases to 0.75 - 1.00 mg/l, the roots become thinner, the number of roots decreases to 3.2 - 3.5 roots/shoot with less absorbent hair of a light yellow color. This result has been completely consistent with the previous study of Poudel et al. (2018) [15] on Large cardamom (A. subulatum Roxb.). In contrast, according to studies of Truong Thi Bich Phuong et al. (2017) [17] on Amomum sp. showed that MS medium supplemented with α-NAA was more effective in the induction of number of roots and their length than MS medium supplemented with IBA.

5. Conclusion

We have reported the initial results in the development of in vitro propagation of cardamom from the rhizome buds. An 8 minutes treatment of HgCl₂, with a concentration of 0.1%, is the most efficient for the disinfection of the rhizome buds, resulting in the rate of the disease-free survival explants reaching 18.29%. MS medium supplemented with 1.0 mg/l BAP is the most suitable medium in the fast multiplication phase, with a multiplier of 4.54 shoots/explant, average shoot height of 5.45 cm, and good shoot quality after 6 weeks of culture. A suitable rooting medium for cardamom invitro-shoots was MS medium supplemented with 0.5 mg/l IBA with a rooting rate of 100%, the average number of roots was 5.6 roots/shoot and the average root length was achieved 6.2 cm, after 8 weeks of culture.

Conflicts of interests

None of the authors have any competing interests to declare.

Authors’ contributions

This study was performed in collaboration between all authors. The two first authors, Khuat Van Quyet and Nguyen Thanh Hai, were the main investigators of the study. Other authors participated in the collection of materials, the preparation of experiments, and data collection. Kalashnikova E.A. designed the study, wrote the manuscript, and is the corresponding author of this research.

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