In vitro germination of amomum tsao-ko crevost & lemarié seeds

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Abstract. Cardamom (Amomum tsao-ko Crevost & Lemarié) belongs to the genus Amomum Roxb. (Family: Zingiberaceae), is widely used as a flavoring and spicy ingredient in many traditional Vietnamese dishes; furthermore, it is also used in many traditional Vietnamese remedies to treat digestive ailments, malaria, bad breath, tooth decay, etc. Due to the presence of hard seed coat, cardamom has low seed germination potential. Therefore the aims of the present investigation were undertaken to study the effect of different sterilization agents, mechanical scarification, and various culture media on germination parameters of cardamom seeds. From there, an efficient in vitro cardamom seed germination protocol allows for a high germination rate and provides efficient production of high-quality sterile seedlings was described. Intact seeds were removed from aril and were soaked in warm water for 8 hours before sterilization; in the next step, they were disinfected in 0.1% mercuric chloride for 10 minutes and rinsed 4-5 times with sterile distilled water; then, they were scarified manually by cutting 1-1.5 mm of the seed coat at the opposite site of hilum by a sterile scalpel; lastly, they were inoculated onto MS medium diluted to 1/16 concentration at 25±2ºC under a 16 h-photoperiod in cool white fluorescent light (2000-2500 lux). With this procedure, on average more than 33.33% of the seeds germinated after 90 days of culture, germination mean time (GMT) is 43.98 days, and germination rate index (GRI) is 0.1917.

1. Introduction
Amomum tsao-ko Crevost & Lemarié, a zingiberaceous plant called “Cardamom” or “Do-ho” is widely distributed and cultivated in Ha Giang, Lao Cai, and Lai Chau provinces of Vietnam [1]. Because of having a very characteristic aroma and taste, cardamom is widely used as a flavoring and spicy ingredient in many traditional Vietnamese dishes. Furthermore, cardamom seeds of Vietnamese origin rich in essential oil with an oil content of 1-1.4% of dry weight. The major components present in cardamom essential oil are 1,8-cineole (30.6%), 2-decenal (17.3%), geranial (10.6%), and neral (7.0%) [2]. Studies on the effects of 1,8-cineole, the predominant component in cardamom oil, in the treatment of respiratory ailments, myalgia, neurosis, rheumatism, and kidney stones have been reported [3].
In fact, cardamom has been used in many traditional Vietnamese remedies to treat digestive ailments, malaria, bad breath, tooth decay, etc [4].

In Vietnam, dried cardamom is often purchased by Chinese traders at prices ranging from 110,000-130,000 VND per kg. Due to the high economic value of cardamom, the cultivated area of this plant increases rapidly over the years, but mainly in the direction of spontaneity, low yield, and quality of cardamom. Cardamom is mainly propagated by seeds and rhizome segments. According to the experience of local people, plants grow from seeds have better yield, fruit quality, and longevity than from rhizome segments. Therefore, at present, local people mainly use seedlings grown from seeds. However, because of having a hard seed coat, cardamom has low seed germination potential; the onset of fruit for plants grow from seeds is 1-2 years longer than those grow from rhizomes. Thus, these methods have limitations and have not met the input needs of production. This problem can be solved by growing elite cultivars produced by in vitro clonal propagation.

In vitro germination of seeds has been reported in many crops. In most cases, agar-solidified basal salt media are used for germinating seeds for many species, such as Sophora toromiro [5], Swainsonia salsula [6], and cotton [7]. However, in others, the low-salt media are used and stated best for in vitro germination of seeds Dianthus zeyheri [8], Dalzellia zeylanica [9], and Bulbine canescense [10]. On the other hand, seeds of many species are reported to have the highest germination rates when sown in water agar or distilled water (DW), such as Annona muricata [11], Hypericum perforatum. cv. anthos [12], and Annona cherimola [13]. The above difference is explained because the mineral demand of seed germination is different in different species, depends on the amount of food reserves in the seed [13].

Srinivasa Rao et al. (1982) [14] successfully germinated cardamom (Elettaria cardamomum) seeds on White’s (1963) nutrient agar, which is also a low salt medium. Seed coat dormancy is caused by a hard and impermeable seed coat that prevents imbibition and sometimes gaseous exchange [15]. It may be the cause of the low germination rate of cardamom seed. The mechanical scarification can be used to break the dormancy of hard-coated cardamom seeds thereby improving seed germination. Improvement of seed germination through in vitro culture has been reported in banana [16] and papaya [17].

Seeds collected from the cardamom forest are often contaminated with exogenous and endogenous microbial contaminants that include fungi and bacteria. Hence, efficient seed sterilization is a prerequisite for successfully germinated cardamom seeds. Ethanol, calcium hypochlorite, and mercuric chloride have been commonly used for surface sterilization of plant and seed material of various species.

The aims of the present investigation were undertaken to study the effect of different sterilization agents, mechanical scarification, and various culture media on germination parameters of cardamom (Amomum tsao-ko Crevost & Lemarié) seeds. From there, we describe an efficient in vitro cardamom seed germination protocol that allows for a high germination rate and provides efficient production of high-quality sterile seedlings.

2. Materials and methods

2.1. Plant material
Mature seed capsules of cardamom (A. tsao-ko) were collected from cardamom forest at the Sin Cau village, Giang Ma commune, Tam Duong district, Lai Chau province, North-western region, Vietnam, in October 2020. Seeds were removed from dehisced capsules, aril and dried in the open sun for about three days. Then the seeds were transported to the Laboratory of Biotechnology at Russian State Agrarian University - MTAA, Russia, in a plastic bag that has been dehumidified. Here, they were stored at room temperature (27-28°C) in a desiccator for 24h.

2.2. Seed sterilization
The cardamom seeds were soaked in warm water for 8 hours before sterilization. In the first step, the seeds were kept under running water at room temperature for 1 hour. In the second step, they were washed in liquid soap for 10 minutes, then washed directly under running tap water. In the third step,
the seeds were transferred to a laminar cabinet, where they were surface sterilized in 70% ethanol for 30 sec, followed by immersion in a 0.1% (w/v) aqueous mercuric chloride or calcium hypochlorite [5% or 10%, (w/v)] for 5, 10 or 15 minutes. Lastly, after sterilizing with chemicals, they were rinsed 4-5 times with sterile distilled water and were inoculated onto basal MS medium [18].

2.3. In vitro seed germination

The best sterilization formula obtained from the above experiment was used to perform seed sterilization to study the effect of mechanical scarification and various culture media on germination parameters of cardamom seeds.

After sterilization, seeds were treated as follows: (1) mechanical scarification: seeds were scarified manually by cutting 1-1.5 mm of the seed coat at the opposite site of hilum by a sterile scalpel; (2) seeds were not treated. Then, they were transferred to the five germination media, including MS basal medium [18], modified MS media with macronutrients diluted to 1/2, 1/4, and 1/16 strength, as well as distilled water, as a control. The pH of four media 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±2ºC under a 16 h-photoperiod in cool white fluorescent light (2000-2500 lux). To avert the risk of losing cultures due to contamination, only one seed was sowed per bottle. Each experiment was performed in triplicate with 25 seeds per treatment per replicate.

2.4. Data collected

Seeds were considered germinated when the healthy, white radical had emerged through the integument. Data were scored from 30 to 90 days after culture. Counts were made every 10 days till the 90th day.

The following germination parameters were determined:

1. Germination percentage (GP); the number of germinated seeds as a percentage of the total number of tested seeds is given as;

\[ GP = \left( \frac{\text{germinated seeds}}{\text{total tested seeds}} \right) \times 100 \]

2. Germination mean time (GMT), is given according to Scott et al. (1984) [19] as;

\[ \text{GMT days} = \frac{\sum T_k N_k}{S} \]

where \( T_k \) is the number of days from the beginning of the experiment, \( N_k \) the number of seeds germinated per day and \( S \) is the total number of seeds germinated.

3. Germination rate index (GRI): it was calculated for each treatment using the following Equation:

\[ \text{GRI} = \left( \frac{G_1}{1} \right) + \left( \frac{G_2}{2} \right) + \ldots + \left( \frac{G_i}{i} \right) \]

where \( G \) is the germination day 1, 2,..., and \( i \) represents the corresponding day of germination (Esechie, 1994) [20].

Analysis of variance (ANOVA) was performed using Sirichai Statistics 7.0 and means were compared using LSD at a 0.05 level of probability.

3. Results and discussion

3.1. Seed sterilization

The seed surface sterilization effects of calcium hypochlorite and mercuric chloride are summarized in table 1 and figure 1.

Research results showed that simple treatment with 70% ethanol was ineffective, as there was 100% contamination. When using a combination of 70% ethanol with 0.1% mercuric chloride for 10 minutes, the optimal results were recorded, with 51.39% contamination-free seeds. Calcium hypochlorite showed lower sterilization efficiency than mercuric chloride, with the best results achieved at 10% concentration. Two main reasons can be given to account for such low sterilization efficiency: (1) the cardamom seed surface has striate that provides a haven for microorganisms; (2) the aril is difficult to
remove completely from the seed, its existence may be the cause of seed contamination. In addition, the effect of the contact time on the seed surface sterilization effects was also recorded. For calcium hypochlorite, as the contact time increased, the sterilization efficiency increased. In the first 2 weeks of observation, the disinfecting efficiency of 0.1% mercuric chloride within 15 minutes was the best; however, in the following weeks of observation, fungi appeared on the surface of many seeds in this treatment. This can be explained by the fact that long mercuric chloride exposure stimulates microorganisms to form spores that make them resistant to mercuric chloride. On the other hand, the experimental results also showed that the sterilizing agents did not affect the germination rate of cardamom seed on MS medium. Very low germination rates ranged from 0-6.94% between treatments.

### Table 1. Effect of various sterilizing agents on percentage of contamination-free in cardamom (A. tsao-ko) seeds 90 days after sowing*

| Sterilizing agent | Concentration (%, w/v) | Time of exposure (minutes) | Contamination-free seeds (% mean ± SE)b | Contamination seeds (% mean ± SE)b | Germinated seeds (% mean ± SE)b |
|-------------------|------------------------|---------------------------|----------------------------------------|------------------------------------|-------------------------------|
| T1 Ethanol        | 70 (%, v/v)            | 1                         | 0.00±0.00g                             | 100.00±0.00a                        | 0.00±0.00b                    |
| T2 Ca(ClO)2       | 5 (%, w/v)             | 5                         | 2.78±1.39f,g                           | 97.22±1.39b                        | 0.00±0.00b                    |
| T3 Ca(ClO)2       | 5 (%, w/v)             | 10                        | 5.56±1.38e-f                           | 94.44±1.38c                      | 0.00±0.00b                    |
| T4 Ca(ClO)2       | 5 (%, w/v)             | 15                        | 11.11±3.67d,e                          | 88.89±3.67c,d                    | 4.17±0.00a                    |
| T5 Ca(ClO)2       | 10 (%, w/v)            | 5                         | 6.94±1.38e-f                           | 93.06±1.38b                      | 2.78±1.39a                    |
| T6 Ca(ClO)2       | 10 (%, w/v)            | 10                        | 15.28±1.39c,d                          | 84.72±1.39d,e                    | 4.17±2.40a                    |
| T7 Ca(ClO)2       | 10 (%, w/v)            | 15                        | 19.44±3.67c                           | 80.56±3.67d,e                    | 2.78±1.39a                    |
| T8 HgCl2          | 0.1 (%, w/v)           | 5                         | 20.83±2.40c                            | 79.17±2.40c                      | 4.17±0.00a                    |
| T9 HgCl2          | 0.1 (%, w/v)           | 10                        | 51.39±5.01a                           | 48.61±5.01g                      | 6.94±1.39a                    |
| T10 HgCl2         | 0.1(% , w/v)           | 15                        | 37.50±2.40b                           | 62.50±2.40f                      | 5.56±1.39a                    |
| LSD0.05           |                        |                           |                                        |                                    | 6.23                          |

*In each column, different small letters mean that they significantly differ from each other at P=0.05.

bTransformed using the arcsine transformation before analysis.

Srinivasa Rao et al. (1982) [14] also used 0.1% mercuric chloride to clean samples for their in vitro propagation studies on Elettaria cardamomum Maton of Zingiberaceae. However, the author did not specify the time of seed sterilization in this article.

### 3.2. In vitro seed germination

The cardamom seeds were sterilized according to the T9 formula that had the best seed surface sterilization efficiency in the above experiment. After sterilization, seeds were mechanically treated or not, then were sowed in the five germination media. Results are tracked for 90 days. Effect of mechanical scarification and various culture media on germination parameters of cardamom seeds are shown in table 2 and figure 2, 3.

Results indicated that the composition of the basal medium influenced in vitro germination of cardamom seeds. Basal medium with full strength MS salts resulted in the least germination percentage (GP), longest mean days to germination (GMT) and the least germination rate index (GRI). The germination parameters of seeds were best recorded on MS medium diluted to 1/16 concentrations, giving about 21.33% untreated seeds germination and 33.33% treated seeds germination, as compared to the control (distilled water), which resulted in around 20% germination. In addition, seeds cultured on MS medium diluted to 1/16 concentration took a shorter time period for germination (47.11 days for untreated seeds and 43.98 days for treated seeds) and recorded the highest germination rate index (0.1161 for untreated seeds and 0.1917 for treated seeds). In general, the results from this study indicated media with low salt content to be the best for in vitro germination of cardamom seeds.
Figure 1. Effect of various sterilizing agents on percentage of contamination-free in cardamom (A. tsao-ko) seeds 90 days after sowing.

According to George (1993) [21], the hydrolysis of lipids stored in the endosperm is mainly governed by the amount of water infiltrating the seeds. On the other hand, the rate of water imbibition during seed germination completely depends on the osmotic potential of the internal and external environment of the seed. Therefore, culture media containing high concentrations of organic salts are characterized by high osmotic potential. This resulted in low germination of cardamom seeds on MS medium compared to dilute MS media.

Table 2. In vitro germination of cardamom (A. tsao-ko) seeds 90 days after sowing on five media formulation.

| Medium     | Seed                | GP (% mean ± SE)\(^b\) | GMT (days mean ± SE)\(^c\) | GRI (mean ± SE)\(^d\) |
|------------|---------------------|------------------------|-----------------------------|-----------------------|
| MS         | untreated           | 5.33 ±1.33\(^e\)       | 86.67 ±3.33\(^a\)          | 0.0153 ±0.0035\(^e\)  |
|            | mechanical scarification | 6.67 ±1.33\(^d,e\)    | 75.00 ±2.89\(^b\)          | 0.0226 ±0.0050\(^e\)  |
| 1/2-MS     | untreated           | 9.33 ±1.33\(^c,d\)     | 65.56 ±0.55\(^c\)          | 0.0357 ±0.0048\(^d,e\) |
|            | mechanical scarification | 12.00 ±2.31\(^c\)     | 58.33 ±1.67\(^d\)          | 0.0525 ±0.0116\(^d\)  |
| 1/4-MS     | untreated           | 13.33 ±1.33\(^c\)     | 60.00 ±1.92\(^d\)          | 0.0562 ±0.0059\(^d\)  |
|            | mechanical scarification | 18.67 ±1.33\(^b\)     | 57.17 ±0.60\(^d\)          | 0.0825 ±0.0063\(^c\)  |
| 1/16-MS    | untreated           | 21.33 ±1.33\(^b\)     | 47.11 ±2.56\(^c\)          | 0.1161 ±0.0124\(^b\)  |
|            | mechanical scarification | 33.33 ±1.33\(^a\)     | 43.98 ±0.23\(^d\)          | 0.1917 ±0.0067\(^a\)  |
| DW         | untreated           | 20.00 ±2.31\(^b\)     | 46.72 ±0.43\(^e\)          | 0.1089 ±0.0131\(^b\)  |

\(^a\) In each column, different small letters mean that they significantly differ from each other at 𝑃=0.05; MS = Murashige and Skoog (1962); 1/2-MS, 1/4-MS, and 1/16-MS = MS basal medium diluted to 1/2, 1/4, and 1/16 strength; DW = distilled water (control).

\(^b\) GP (%) = Germination percentage; transformed using the arcsine transformation before analysis.

\(^c\) GMT (days) = Germination mean time.

\(^d\) GRI = Germination rate index.
Figure 2. Effect of mechanical scarification and various culture media on germination percentage (GP) of cardamom (A. tsao-ko) seeds 90 days after sowing.

Figure 3. Effect of mechanical scarification and various culture media on germination mean time (GMT) and germination rate index (GRI) of cardamom (A. tsao-ko) seeds 90 days after sowing.

These results were in agreement with that of Srinivasa Rao et al. (1982) [14], who successfully germinated cardamom (Elettaria cardamomum) seeds on White’s (1963) nutrient agar, and Ergete Tefera W (2004) [22], who successfully germinated korarima (Aframomum corrorima) seeds on diluted MS media. However, it was in contrast to the findings of Dahanayake N (2015) [23], who stated MS medium to be the best for the in vitro germination of cardamom (Elettaria cardamomum) seeds.

On the other hand, the study results also showed that the untreated seeds had lower germination parameters compared to the treated seeds when cultured in the same medium. Overall, mechanical scarification significantly increased the germination rate of the seed and helped shorten the germination time of the seed.
4. Conclusion

The final protocol established for cardamom (A. tsao-ko) seed in vitro germination was: seeds were removed from aril and were soaked in warm water for 8 hours before sterilization; in the next step, they were disinfected in 0.1% mercuric chloride for 10 minutes and rinsed 4-5 times with sterile distilled water; then, they were scarified manually by cutting 1-1.5 mm of the seed coat at the opposite site of hilum by a sterile scalpel; lastly, they were inoculated onto MS medium diluted to 1/16 concentration at 25±2ºC under a 16 h-photoperiod in cool white fluorescent light (2000-2500 lux) (figure 4). Using this protocol that allows for a high germination rate and provides efficient production of high-quality sterile seedlings.

![Figure 4](image)

**Figure 4.** Protocol for cardamom (A. tsao-ko) seed in vitro germination: a- dried seeds with aril membranous; b- dried seeds have been completely removed from aril; c- the seed was inoculated onto MS medium diluted to 1/16 concentration after being disinfected and scarified; d- the seed germinated after 50 days of inoculation; e- the seedling observed after 70 days of seed inoculation; f- the seedling observed after 90 days of seed inoculation; scale bars = 1 cm.

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