In-vitro propagation: application of gibberellic acid (GA3) in enhancing germination in ‘Jernang’ or ‘Dragon Blood’ (Daemonorops sp.)

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Abstract. ‘Dragon blood’ (Daemonorops sp.) is widely known as one of the indigenous Non-Forest Timber Products (NFTP) with high economical value and grows mostly in the deep forests of Indonesian and Malaysian Peninsula. The name referred to red resinous exudations from four distinct plant species that are endemic to various eco-geographical regions. It has been highly valued in Chinese traditional medicines or painting industries since the 14th century. Its resin is strongly related with active bio-compound with strong curing activities for stomach ulcers or intestines’ related cancer. However, jernang’s number of population in its natural habitats has been decreasing over the past three decades as a result of unsustainable harvesting. Thus, this has dramatically reduced its population and lower genetic diversity. Another challenge is that the period of seed germination is relatively long; namely up to 12 weeks as the seeds are usually covered with thick coat. Thus, immersion in water is prerequisite in order to support the germination. The best performance was shown with 1*24 h water imbibed seeds with GA3 (W= 2 mg l⁻¹). For in-vitro, various organs from 3-4 months old of young seedlings were employed as explants. However, current mixture of media did not promote an accelerated growth of explants. Therefore, applying another mixture of growth hormone is highly recommended in order to deliver better experimental result. This paper can be concluded as a basic pioneer in ex-situ conservation of ‘jernang’.

1. Introduction
‘Dragon blood’ (Daemonorops sp.) is widely known as one of the indigenous Non-Forest Timber Products (NFTP) with high economical value and grows mostly in the deep forests of Indonesian and Malaysian Peninsula. ‘Dragon blood’ itself is a non-specific name for red resinous exudations from four distinct plant species that are endemic to various eco-geographical regions. They are: i) Croton (Euphorbiaceae), ii) Dracaena (Dracaenaceae), iii) Daemonorops spp. (Palmaceae), and iv) Pterocarpus (Fabaceae). Pterocarpus and Croton grow mostly in South America, while the Dracaena and Daemonorops are endemic in Africa and South-East Asia, respectively [1, 2].
There are various techniques in order to tap the resin depending on the species of the host trees. The dragon blood’s resin is containing in the host trees and can be obtained by performing the tapping technique on the stem part of main bark of the species of Dracaena cinnabari, Croton and Pterocarpus. Regardless where the resin comes from, the deep red resin has been highly valued since in the ancient time, e.g. in the Chinese medicinal uses and used as drug ingredients, antidiarrheal, antimicrobials, antioxidant, antiinflammation, anti-haemostatic, wound healing particularly in promoting wound recovery by the intestines and stomach ulcers [3]. It is also widely used as a colouring agent for varnishes, ceramics, marbles, woods, rattan, paintings, including in the reverse painting glasses decorating the ancient European churches in the 14th – 15th centuries [2,3]. In Indonesia, Daemonorops or in bahasa known as ‘jernang’ is still closely related with the rattan family (Calamus sp.).

They inhabit the deep Sumatran rain forests; ranging from the provinces of Aceh, on the ‘Bukit Tigapuluh National Park’ situated at the border between Jambi and Riau, until Lampung and on the western parts of Kalimantan. The resin is obtained from the secretion of tear-drops’ shaped fruits, with outer skin called as ‘scaly pericarp’ (figure 1). Up to 115 species exist, however, only 12 of them have been recognized as dragon blood-palm and produce resin. Out of 12, only five are recognized in terms of their high economic value, they are: Daemonorops draco Blume, D. didymophylla Becc., D. mattanensis Becc., D. micranthus Becc., D. micracanthus Becc. [3]. Those of D. draco contain bioactive compounds such as dracoresinotannol, dracorubin, dracorhodin, and abietic acid. In regard to the quality, meanwhile, the content of ‘dracorhodin’ is accounted as determining parameter [3].

Figure 1. Fruits of ‘dragon blood’ (Daemonorops sp.) that still contain resin at the outer skin.

Several indigenous tribes e.g. Suku Kubu, Anak Dalam, Talang Mamak, Melayu Tua, who live surrounding the area of the ‘Bukit Tigapuluh National Park’ and Suku Dayak (in Kalimantan) have been depending on ‘jernang’ harvesting as a source of livelihood [4]. There are two types of resin produced via traditional processing techniques, namely: the pure- or the mixed-one. By the mixed one, other lower-quality resin known in bahasa as ‘damar’ has been also added. Regardless of the type, both products should be immediately preserved in plastics as they tend to harden easily [5]. The final products are mostly prepared in granules, powders, lumps or sticks (figure 2).

The plants generally live in a cluster in the deep jungle and with at least three clusters exist per hectare. Each cluster, usually, has five main plants meaning up to 15 plants (3 *5) per hectare; with only nine female plants bearing fruits or equal to 60%. In one plant, there are up to five to six branches exist, however, only 50% are mature in the same period. Thus, only 27 branches are mature and ready to harvest per hectare. The harvesting period is maximum two times per year that would make 54 branches. In order to calculate the harvest potential and benefit of planting ‘jernang’, a much detailed calculation had been developed based as a representative sampling from Jambi [5].

Long term harvesting from nature is not accompanied with conservation efforts or no sustainable harvest has been employed among the traditional tribes. As an example is the Jambi province, where there were approximately 15 tonnes of harvested ‘jernang’ recorded in 1995. In However, there has been no exact record of harvested ‘jernang’ in its natural habitats within fifteen years later or since 2010. Thus, there has been no record since 2010. A similar tendency might also be applied in its other natural habitat such as in Aceh. Such phenomenon could be traced back due to some external factors and traditional practices or habits applied in the local tribes or community, such as: a) massive habitat destruction due to deforestation on Sumatra for oil palm and rubber plantations has led to a dramatical
population reduction; b) no conservation efforts; particularly natural breeding process leading to an optimum population number; c) a strong tendency to cut down male individual plants as they are considered as useless or bearing no fruits; d) local tribes tend to harvesting by cutting the plants completely; e) a strong belief that the plants can be propagated only in the nature, while no possibility to propagate artificially or in-vitro. All of these have led finally to lower population number in the nature as well as strong reduction of the genetic-diversity [4].

![Figure 2. Red resin obtained from ‘dragon blood’ A= in solid shape or clumps; B= powder (depicted from [3]).](image)

In-vitro technique or ‘plant tissue culture’ would be an appealing method to propagate plants in a rapid way. This can be also incorporated in various breeding programs e.g.: i) for generating clonal planting stocks employed in conservation program, ii) for propagating rare or elite germplasm, iii) for overcoming breeding barriers Therefore, the application of biotechnology and especially tissue culture provides an important tool to propagate the selected genotypes. Plants are generally regenerated under aseptic and controlled environment or Lab., where the name of ‘micropropagation’ is also referred for this kind of breeding method. The term ‘micro’ means that very small pieces of plant organs are used as initial vegetative tissue. In this case, small plantlets such derived from zygotic embryos, young seedlings, apical meristems, axillary shoots, and buds are mainly applied [6]. So far, this technique has been applied successfully in various plants; not only in Euphorbiaceae, Zingiberaceae, Curcubitaceae, Musaceae [7], but also in some hard or forest crops such as Acacia (family Fabaceae) [8], Scots Pine (Pinus sylvestris) [9], and Patchouli oil aromatic plant (Pogostemon cablin Benth.) [10].

So far, there has been no report available by the in-vitro attempts for ‘jernang’. However, few technical reports have highlighted the attempts to propagate in-vitro in rattan species especially, the commercial one (Calamus manan) [11], [12]. This can be applied as an initial reference to conduct similar work in ‘jernang’. Therefore, we would like to employ and pioneer a conservation effort via in-vitro propagation of the local ‘jernang’ resources. Before the main in-vitro technique experiment, we firstly tried to germinate the seeds collected from several local farmers in west Aceh and would like to emphasize the incorporation of plant growth hormone such as Gibberellic Acid (GA₃) in the germination process. The application of GA₃ in promoting the growth or stem elongation has been highlighted in many literatures.

2. Materials and Methods
2.1. Materials
2.1.1. For Germination. A kilogram of mature seeds equal to approximately 80 hard seeds with a diameter 3-5 cm; were prepared for the experiment. Soil medium consisting of sand, rice husk and cow dung (50%, 40%, 10%) was prepared for the out-door experiment. For the germination under lab. condition, all the seeds were initially sterilized by using fungicide, bactericide added with distilled water. All seeds were then put on wetted cotton placed on petri dishes. The first batch had been immersed initially with Gibberellic Acid or GA₃ (Phytogel, USA, W= 1 mg l⁻¹) per one liter water.
Meanwhile, the second batch was only immersed in distilled water with no GA$_3$ (Figure 3). Plastic container with a size of 25 * 35 cm (N=3 pieces), wet sterile cotton, distilled water, water container (N = 1 pieces), petri dishes (d= 10 cm, N= 3-5 pieces) and plastic wrap were applied.

2.1.2. For the In-Vitro Propagation. Diverse chemicals such as: Agar-agar (Caisson, USA), distilled water, Murashige and Skoog Medium with vitamin (Caisson, USA) the amount applied was according to the written instruction (W= 7-9 g l$^{-1}$), sucrose (Himedia), growth hormone belongs to auxine: 2,4-dichlorophenoxyacetic acid (2,4-D) (Phygenera, Germany, W= 2 mg l$^{-1}$), antiseptics such as: i) alcohol (70 % v/v), ii) dish liquid detergent (V= 200 ml), iii) ‘Dettol’ (V= 100 ml, but only few drops), and iv) Sodium hypochloride ‘Bayclin” (V= 250 ml, with concentration varied from 5; 10; 50% v/v) were purchased and prepared.

For the utensils, e.g. Autoclave (Thommy, Japan, V$_{capacity}$ = 250 l), middle size Laminar Air Flow (LAF), lab. beaker (V= 250 ml), glass cylinders, glass jars (V= 250 ml), Bunsen Brenner (V = 250 ml), scalpels {length (l =14 cm), forceps (l =14, 28 cm)}, knife tip (GEA, Germany), marking pens (water-proof Artline Snowman), paper label, thermometer, timer from Handphone (MI note 5, China), glass pipets, parafilm plastics, plastic wrap foils, sterile cotton buds, sterile masks, sterile oven (if necessary, T= 180–220 °C), nitrile gloves with allergen free were applied in lab. All working utensils were rinsed overnight in ‘bayclin’ solution, rinsed properly, washed and sterilized in an autoclave prior to single usage.

![Figure 3. The three types of germination process of ‘jernang’ (slightly modified from [13] Winarni 2017), and source of explants employed in the research.](image)

2.2. Methods
This work has been done in the Plant Tissue Culture laboratory belongs to the Provincial Bureau of Agriculture and Plantation (Dinas Pertanian dan Perkebunan Provinsi Aceh) at Lampineug, Banda Aceh, Indonesia. The work has been started in June 2019.
2.2.1. Germination Process. Seeds (N= 81) were employed. First, fruits were rinsed with tap water as some dirt (soils, sands) were available as well as to remove some pathogene that may hinder the germination process. The scaly pericarp and the fleshy sarcotesta of mature fruits or flesh fruits were removed by hand or knife. Then, these were rinsed several times with flowing tap water. The seeds were then placed in plastic containers and immersed in fresh water for 3*24 hours (h) at room temperature (T= 25-26 °C). Subsequently, seeds were air dried for 24 h.

| No. | Treatment | Media                          |
|-----|-----------|--------------------------------|
| 1.  | A         | Sand, husk, manure (70%, 20%, 10%) |
| 2.  | B         | water imbibed seeds            |
| 3.  | C         | water imbibed seeds with GA₃    |

2.2.1. In-vitro Propagation. The methodology was referring to [12]. After 4-12 weeks, the seeds would germinate, obviously marked with single yellowish-sprout emerged from the sprout eye. In short, there were three plant organ parts from young seedlings (height= 60-70 cm) were applied as explants: 1) young seedlings aged three months old. Then the shoot tip from leaf bases, leaves, root tips were obtained, 2) sprouted seeds, in which the seed-embryo excised from the collar region was derived from, 3) young shoot tips which were obtained from the apical meristem of the flower (figure 3). Each explant size was about 1-2 cm was taken with prior elimination at the tip region. These all were prepared and cut under sterile conditions inside the Laminar Air Flow (LAF) and subsequently planted on the already prepared agar medium. By the medium, two kinds of combination were applied (table 2). All working steps were referring to previous method and properly documented. Plantlets were then put on the metal shelves and lighting with cylinder form bulb (Philips) and constant air conditioned room (T= 24 °C) for 24 h.

|                | Agar-agar | Sucrose | Murashige-Skoog (MS) | 2,4-D |
|----------------|-----------|---------|----------------------|-------|
| Blank          | v*        | v       | v                    | x     |
| Added with auxine | v        | v       | v                    | v     |

*v= applied, x= no

2.3. Data Analysis
The data was compound based only on real counting and produced in Table (Windows Excel, Microsoft). The reported data was mostly based on qualitative descriptive analysis.

3. Results and Discussion
The germination process requires a very simple method by only immersing it in water (H₂O) or water imbibed, however, this is very crucial in determining the next growth stage in one single plant. Therefore, conducting a proper germination both has ecological and economic importance. This can be inferred that ‘jernang’ required up to 6-8 weeks to germinate and prior to that, seeds were water imbibed 1-3 days. The germination using sand-husk mixture did not result positively, as there was no germinated seeds (figure 5.A). The highest number of germinated seeds were resulted from the initiation with GA₃ (figure 4; figure 5.C), then followed with water only (figure 4; figure 5.B).

Before germination, most of the seeds experience the so called dormancy, which is an innate seed property that defines the environmental conditions. After overcoming the dormancy, seeds start to
germinate, and this could be inferred as natural sign or ‘inside-readiness’ from one plant or in another way around the external abiotic signals (light, temperature, moisture) from the environment has been responded well enough, so that kind of interactive mutual signs between the plant and environment have taken place in order to guarantee further development of one single seed. Plants are sessile organisms, that are essentially required to alter their development and growth responses in order to enable them to survive an ever-changing environment. From the plant developmental study point of view, seed dormancy is accounted as an adaptation that prevents premature germination in environments that are subjected to seasonal changes in growth conditions. Certainly, temperature; at first priority, then the light, and moisture. These are accounted as the essential ‘cocktails’ representing environmental factors that have considerable influence throughout the plant developmental processes. Thus, the three makes a major role in controlling the degree of seed dormancy [13].

Previous results reported that ‘jernang’ seeds performed dormancy ranging from 7 to 97 days after sowing (d.a.s) with temperature requirement 28 – 32 ºC. In order to break the dormancy, it is highly advisable to immerse them firstly in the water at least for a period of 24 h in order to accelerate the germination (figure 5.B). A period of 1*24 h of seed immersion in water showed the best result. It seems that such a period was sufficed to allow water to penetrate the relatively thick and hard seed cover as well as to moisture the seeds. Water impermeability of the seed’s coat is often assumed as a natural endogenous barrier in some species e.g. Fabaceae and Lupinus spp. [14]. Contrastingly, an immersion of 48 hours had affected some seeds to be degraded and then unable to germinate. In this study, we were able to confirm that water imbibed seeds could applied as one of common methods to break dormancy [13].

Figure 4. Three types of germination process of ‘jernang’ (slightly modified from Winarni et al. 2017 [15]).
The highest number of germinated seeds was promoted due to GA$_3$ (figure 5.C). This is one of the known endogenous plant hormones involved in signal transduction. The other known one is abscisic acid (ABA). Many of the temperature-controlled responses are mediated via the manipulation of plant hormone levels and/or signal transduction. Both GA$_3$ and ABA have been playing deliberately role in temperature-controlled elongation responses. Mostly, the auxin level increases as ambient temperature rises, thus, auxin effect is widely known when it is related to elongation growth. In short, GA$_3$ affects stem elongation when plants are exposed under different temperatures between day and night [14]. Thus, our result also confirmed strongly previous result [14], that seed dormancy in many angiosperm plants can only be either broken by a long period of after-ripening or water imbibed seeds.

We confirmed that in-vitro propagation can be applied as potential method to conserve ‘jernang’ as this was already previously reported in rattan species [12]. Such method was applicable in order to speed up propagation of some elite cultivars and to ensure conservation of relatively rare rattan species threatened with extinction. Moreover, some organs could serve as explants, e.g. the root tips, basal stem, shoot and leaf tips (figure 6). The pictures being shown were the ones free from fungi and bacteria contamination. At the very beginning, contamination was the major threat in this micropropagation as > 50% of prepared explants were contaminated.

In addition to that, the growth ratio was very low, which is only < 2% (0.169%) over almost eight weeks. Only three explants were free from contamination and formed tiny root sprouts. Meanwhile, one single explant on MS supplemented with 2,4-D media formed very tiny sprouts obtained from our results. These were tendentially too low if compared with the previous result [12]. There, it was reported that cultured embryos of _C. trachycoleus_ on similar media or with NAA (5 mg l$^{-1}$) had already an amorphous swelling of the embryo after two weeks. We proposed that the amount of growth hormone was too low, and this was the reason why the growth was too slow.

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**Figure 5.** Results from the three germination process of ‘jernang’; A= using sand-husk medium, B= with water, C= with additional GA$_3$ with sprouted shoots (pictures were developed in 2019).

**Figure 6.** Explants obtained from several organs belong to ‘jernang’; A= root tip, B= basal stem from young seedlings (3-4 months old), C= shoot tips, D= leaf tip (pictures were developed in 2019).
4. Conclusion
This ex-situ conservation effort of ‘dragon blood’ (*Daemonorops* sp.) has just begun as the plants are getting rare in Aceh, although, they have brought additional extra income to some local tribes however, there has been no conservation efforts being started. In our work, we found out that the germination was relatively long in ‘jernang’. It required 6-12 weeks until young whitish sprout was emerged from the sprout eye. Moreover, the application of growth hormone e.g. GA3 was obviously enhancing the germination process. Seeds were firstly water imbibed for 1*24 with an addition of 1 mg GA3 l⁻¹. Some organs could be applied as explant source, however, several improvements are highly urged in the future for a better process & results especially in minimizing contamination. Furthermore, we are going to conduct in our subsequent work the application of two different growth hormones: NAA and BAP in order to see the differences compared to the initial application of 2.4-D. As the in-vitro work has not been fully finished, and still some steps need to be conducted. Thus, we are also planning to publish the final results, elsewhere.

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