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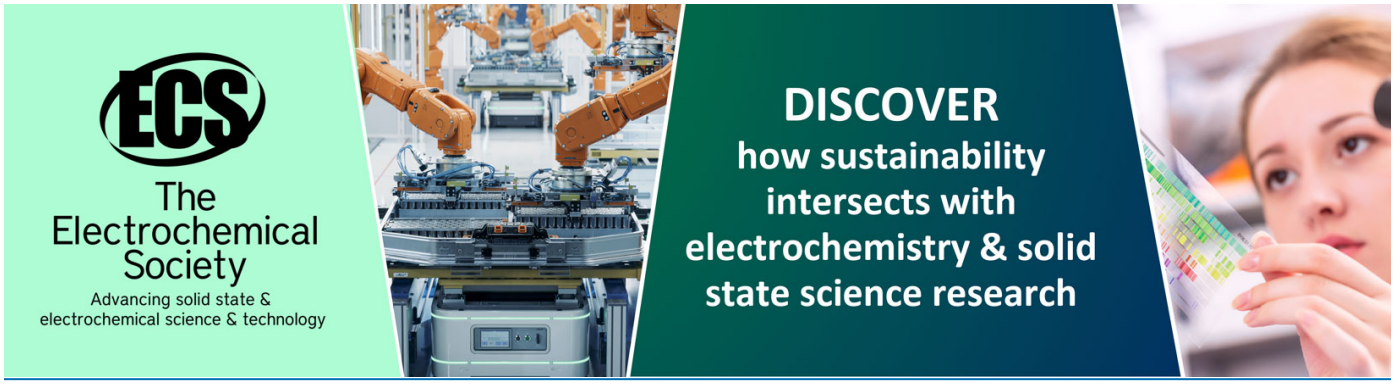
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Seed characteristics of *Curculigo latifolia* and its prospect to *in vitro* propagation

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Abstract. The propagation of *Curculigo latifolia* only occurs naturally in the wild and it is difficult to be domesticated via seed. It is suspected that the seeds are recalcitrant and require a special medium and care. In this study, we evaluated the structure of seed of *C. latifolia* using a stereo microscope, SEM and tetrazolium for seed viability test. The characteristics of the seeds observed in this study were the shape and color of the seeds, viability of the seeds with tetrazolim test. Seeds from the fresh fruit were cultured on MS0 medium to determine the ability of the seeds to germinate. This study showed that seeds aged 8-9 weeks after flowering were viable seeds with a red color indicated on all seed surfaces through the tetrazolium test.The structure of *C. latifolia* embryo is classified as an axial embryo with hypogeal germination type. Seeds of *C. latifolia* have potential to be used as explants for *in vitro* culture

1. Introduction

Curculigo latifolia is locally known as Lemba or marasi is an herb from the family of Hypoxidaceae that so far only naturally grows in the wild. Besides in Kalimantan, the plant can also be found in Sumatra, Bangka, Papua, and Java. Lemba is a Kalimantan's landrace plant having high potential for cultivation because of its high economic value and commercial prospect owing to its diverse purposes. Fibers of leaves of *C. latifolia* are utilized for making fishing net and rope. In Eastern Kalimantan especially among *Dayak* tribe, the fibers of leaves of *C. latifolia* are used for traditional cloth (*Ulap Doyo*) [1]. The plant is famous for its unique sweet proteins, namely, curculin and neoculin that have been proven to be 500 to 9000 times sweeter than sucrose by weight [2], and can be used as a low-calories sweetener for diabetic and obese people. *C. latifolia* has potential as an alternative sweetener to replace sugar cane.

C. latifolia is a monocotyledonous plant, growing erect, clumped with a height of less than 2 m. In the wild, multiplication in this plant use rhizome and could not domestification via seed. It is suspected that the seed are recalsitrant. There are many seeds in a fruit. The seed has hard and thick coat. The seeds coat has many important functions, one of them is to save the seeds from bad environmental condition. The germination of some species depends on the coat. Nyman punctured the seed coat of *P. Sylvestris* [3]. Seeds of different species have evolved to vary enormously in their

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structural and anatomical complexity and size (the weight of a seed varies from 0.003 mg for orchids to over 20 kg for the double coconut palm (*Lodoicea maldvica*) [4]. So far, there is no report that describes the detail structure of seeds of *C. latifolia*. The aim in this study is to describe the characteristics of *C. latifolia* seeds and its prospect to germinate in in vitro condition

2. Material and Method

2.1. Samples of seeds

The seeds were obtained from the fresh fruit of *C. latifolia* which grew in Pangandaran, West Java. The fruit was selected by the characteristics of fresh white color, the color of the seeds visible from the outside with the age of the fruit 8-9 weeks after flowering. Fruits size were measured using a digital caliper. Seeds were removed from the fruit by hand. Seeds were cleaned from fruit flesh and rinsed with water.

2.2. Observation of seed structure

Size of seed were measured using a digital caliper. Whole seeds were observed with a stereo microscope with a 40x magnification. Observations included seed coat color and seed shape. The seeds were observed for the structure of the cotyledon using SEM with 100x magnification to determine the seed diameter, seed coat thickness and cotyledon diameter.

2.3. Observation of seed viability with tetrazolium

35 seeds of *C. latifolia* without seed coat were thoroughly soaked in 0.1% 2,3,5- trifenil tetrazolium klorida (w/v) and incubated at 35^{O}C for 4 hours. Furthermore, the seeds were observed under a stereo microscope with 40x magnification. Observations included seed color which indicated the viability of seeds and determined endosperm and embryo staining.

2.4. In vitro propagtion

The seeds were planted on MS basal medium without plant growth regulator (MS0), 6 g/L agar and 3% sucrose. The medium pH was adjusted at 5.8. The cultures was incubated on white fluorescence lamp on 16 h/8 h light/dark photoperiod. The cultures were incubated for 4-5 weeks under temperature of 25 ± 2 °C.

3. Result and Discussion

In flowering plants, a fruit is a ripened ovary and seeds is a result of ovule development after fertilization. The inflorescences of *C. latifolia* are bunches, up to 5 cm long, the flowers emerge from the rhizome and look as if they are growing from the ground. The color of the flowers is bright yellow, and small. A flower in an inflorescence is composed of one pistil with one stigma, six stamina, and 6 petals (Figure 1). Fruit of *C. latifolia* was oval with the length of 2-3 cm approximately. The surface color of the fruit that was harvested 2-4 weeks after flowering appeared whitish; the fruit appearance turned greenish after 4–7 weeks of flowering and white or pale yellow after 8-10 weeks. Fruit contains many of seeds, approximately 35-45 seeds.



Figure 1. Morphology of *C. latifolia*. A). Whole plant; B). Inflorescence; C) and D). Fruits

A seed may be defined as the unit of reproduction which has the embryo and commonly food-laden endosperm enveloped by a seed coat derived from the integument of the ovule. Based on observations, it is clearly found that the shape of seed is ellipsoid, 2 - 4 mm long and with diameter of 1,5 -2,2 mm globose, with black seed coat, glossy, rough with wavy lines, beak knob-like,curved inside and hard (Figure 2). According to previous study [5], the seed coats of the fruits were pink or soft brown from 2 to 4 weeks and black from 6 to 8 weeks of flowering. The seeds and the seed coats were soft even 8 weeks after flowering, suggesting that the seeds were immature. After 10 weeks, the fruits matured, and seed coats hardened and black adequately to allow crushing by hand. In this study, it is showed that seeds having age 8-9 weeks was hard seed coat.

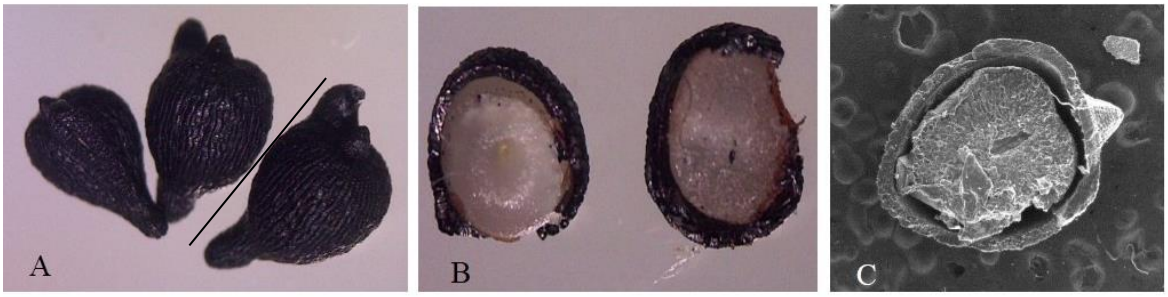


Figure 2. Morphology of *C. latifolia* seed. A). Whole seed; B). Endosperm; C). Longitudinal section of seed showed coat seed thickness. (A and B showed atenuation of 40x with stereo microscopy and C 100x with SEM) (Bar: 2 mm)

From Figure 2, it is found that the endosperm of *C. latifolia* has a white and shiny color. Observation of endosperm diameter can be known by using SEM. From the measurements, it can be seen that the diameter of the cotyledon is 1.55 – 1.55 mm and the thickness of the seed coat is 0.11 – 0.15 mm. *C latifolia* is monocotiledone plant. They contain one cotyledon.

The viability of the seed is a measure of how many seeds are alive and could develop into plants which will reproduce themselves, given at the appropriate conditions. Based on tetrazolium test, it is shown that the seeds had viable properties which were indicated by a red color on the entire surface of

the endosperm and pink or lighter color on the embryo (Figure 3). The percentage of germination of *C. latifolia* was low (41.440%). This phenomena was effected by dormancy. One of the method to break the dormancy was puctured the seed coat.

Tetrazolium tests have been carried out on several of seeds, for example, barley (*Hordeum vulgare*), *Cattleya labiata* and *C. tigrina*, gherkin (*Cucumis anguria*), papaya (*Carica papaya*), tambor or guapuruvu (*Schizolobium parahyba*) [6-10], and on several cereal crops [11].



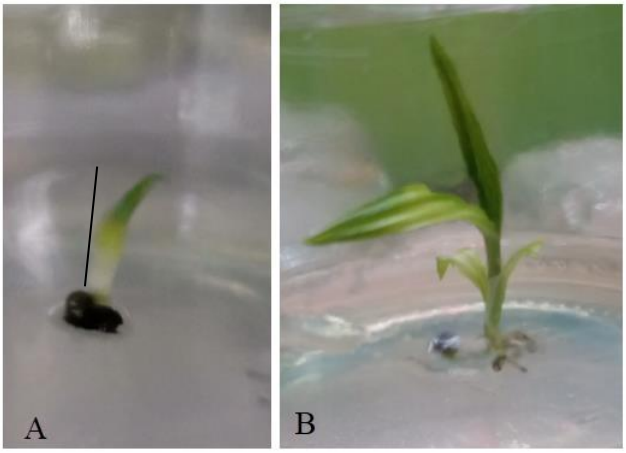
Figure 3. A. Transversal section of *C. latifolia* seed; B. Seed with tetrazolium test; c). Endosperm with peripheral embryo (Bar: 1,5 mm)

The tetrazolium (TZ) viability test uses a colorless solution of 2,3,5-triphenyl tetrazolium chloride, which is used as an indicator of the reduction process that occurs in living cells. This indicator will be imbibited by the seed, then interact with the reduction process in living cells and receive hydrogen from the dehydrogenase process. Through the hydrogenase of 2,3,5-triphenyl tetrazolium chloride, triphenyl formazan is formed which is red, stable and is an insoluble substance produced in living cells. This is what makes the difference between the red color of the live seed and the colorless part of the dead seed. The evaluation of the TZ test was carried out based on the staining pattern on the seed tissue, which would be categorized into viable and non-viable seeds. Viable seeds are seeds that show staining throughout the seed tissue required for normal germination development, while non-viable seeds show deficiencies and/or imperfect staining patterns, so that it can indicate the development of seeds into abnormal sprouts.The mature angiosperm embryo consists of an embryonic axis with a single cotyledon (monocots) [12]. Based on observations from the endosperm, it can be seen that the embryo of *C. latifolia* is classified as an axial embryo. This result is supported by Black et al. [12], it states that axial embryos range from small to occupying the entire seed interior, and may be central and straight, curved, coiled, bent or folded. The characteristics of seeds are summarized in Table 1.

Seeds with the above characteristics can be used as explants for in vitro propagation. Previous study [13] reported that the seeds of *C. latifolia* did not response on medium MS. In this study, the seeds were succesfully cultured on MS medium with free plant growth regulator and showed that the seeds could grow normally. Seed germination pattern showed the same hypogeal pattern with other monocot seeds. Hypogeal Germination is a type of seed germination that the cotyledons remain in the soil due to the epicotyl's length, which allows for faster growth and development. Seeds take 30-40 days to germinate. The average height of sprouts is 0.3-0.4 cm with 1 number of leaves. Furthermore, the stem will elongate and branch to form many petioles and leaves (Figure 4).

Table 1. Characteristic of seed *C. latifolia*

Fruit and seed	Characteristcs
Fruit shape	Oval
Fruit color	White glossy
Number of seeds on fruit	35-45 seeds
Seed shape	Ellipsoid, globose, black, glossy, rough with wavy lines, beak knob-like, curved inside.
Seed size	2-4 mm long
Seed coat color	Black and hard (mature)
Seed coat thick	110,7 - 151,5 um
Cotyledon diameter	1,45 mm
Endosperm color	White
Type of embrio	Axial embryo
Type of germination	Hypogeal
Germination time	30-40 days after planted
Percentage of seed germination	41,40%
Height of sprouts	0.3-0.4 cm



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Figure 4. Propagation of seed of *C. latifolia* on in vitro condition. A). Hypogeal type on in vitro germination ; B). Plantlet of *C. latifolia* (Bar: 0,4 cm)

Micropropagation of *C. latifolia* has been reported by Babaei et al [14], who used shoot tips of Malaysian Lemba as explants on MS basal medium that was supplemented with different concentrations and combinations of thidiazuron (TDZ: 0, 0.5, 1, 1.5, and 2 mg/L) and indole-3-butyric acid (IBA: 0, 0.25, and 0.5 mg/L) for shoot regeneration and scalp induction. Furthemore, Farzinebrahimi et al. [13], successfully induced callus from several explants of *C. latifolia* from Malaysia. They used MS basal medium containing 3% sucrose that was solidified with 2.5 g/L Gelrite and supplemented with various concentrations of IBA either alone or in combination with BAP (0.5–4 mg /L). In addition, the previous study [15] succeeded in inducing callus and its regeneration from leaf tissues for plant multiplication. This study succesfull to grow seeds in MS0 medium showed that viable seeds in large numbers had the potential to be used as explants for in vitro propagation. The results will contribute to *C. latifolia* propagation for green stock.

4. Conclusion

C. latifolia seeds at the age of 8-9 weeks had good characteristics and were viable based on the tetrazolium test. *C. latifolia* seeds with the characteristics discussed above have the potential to be used as explants in in-vtro propagation.

Acknowledgements

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