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THE PECULIAR PETIOLE CALLUSES GROWTH OF *Amorphophallus titanum* (Becc.) Becc. ex Arcang AND ITS IMPLICATIONS FOR EX SITU CONSERVATION EFFORTS

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ABSTRACT

Amorphophallus titanum is a plant species endemic to Sumatera. Land degradation and illegal capture of hornbills (a distributor of *A. titanum* seeds) is leading to potential extinction of *A. titanum* in the wild. In order to conserve the species and save it from extinction, there is an urgent need to develop methods to propagate it both *in situ* and *ex situ*. The aim of this research was to discover environmental factors triggering callus growth from petiole cuttings of *A. titanum* in its natural habitat in Sumatera and to determine the viability of callus pieces as a propagation material. A completely randomized design with a single factor, i.e., callus size, was employed on five callus replicates. Each replicate consisted of four callus samples. The treatments consisted of three callus piece sizes i.e. 0.5 x.0.5 cm^2, 1 x 1 cm^2, 2 x 2 cm^2. For each replicate, the following parameters of growth were assessed: the time of appearance of shoots; the shoot height when the first leaf fully opened; the petiole diameter; the diameter of the leaf lamina; and the number of young shoots. The results of our field observations showed that environmental factors such as temperature, humidity, and soil influence the formation of the callus in the wild. The size of the callus affected the shoot and root growth. The best result was obtained from callus 2 cm^2, which could produce 2–3 shoots with an average height of 18.8 cm at the time of first fully opened leaf. The collection of petiole calluses of *A. titanum* that formed in its natural habitat are recommended instead of carrying the tuber.This ex vitro calluses can be used as propagation materials and then it could be planted in the botanic gardens as one of *ex situ* conservation effort.

Keywords: *Amorphophallus titanum, ex vitro* propagation, *ex situ* conservation, petiole callus, Sumatera

INTRODUCTION

The genus *Amorphophallus* belongs to the Araceae family which comprises more than 220 species, ranging from the subtropical eastern Himalayas through subtropical and tropical Asia into the tropical western Pacific and northeastern Australia (Boyce *et al.* 2012; Yuzammi *et al.* 2017). Currently, 26 species (11.81 % of all *Amorphophallus* species) are found in Indonesia, of which 17 species (65.38 % of all Indonesian *Amorphophallus* species) are endemic (Hetterscheid & Ittenbach 1996; Yuzammi *et al.* 2014; Yuzammi *et al.* 2017).

One of the Indonesian endemic species of *Amorphophallus* is *A. titanum*. It is only found in Sumatera, the home of the largest inflorescence in the world. This endemic species is distributed throughout all Sumateran rainforests. Unfortunately, the conservation status of *A. titanum* as a vulnerable species is excluded from both the International Union for Conservation of Nature (IUCN) and World Conservation Monitoring Centre (WCMC) due to inadequate comprehensive population data on it.

The population of *A. titanum* in the wild decreased significantly in recent times. Therefore, population studies of *A. titanum* in its natural habitat are urgently needed. Several factors such as illegal logging, land degradation, illegal capture of hornbills (a distributor of *A. titanum* seeds) and destruction of the plant due to belief of local people based on a myth are leading to potential extinction of the species in the wild. Local people in Kapahiang Regency believe that within the petiole of *A. titanum,* there is a 'sacred stone' that can cure many kinds of diseases and even counter act effects of poisoning which encourages them

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to harvest the plant in the wild. Moreover, there is an additional belief by local people that the species is a predator of human beings due to the petiole pattern of *A. titanum* resembles a 'snake pattern', which leads them to destroy the species when they find it growing in their fields.

Based on several fields observation, *A. titanum* is likely to go extinct in the wild unless action is taken on many fronts. Increasing public awareness and properly educating local people about *A. titanum* will play an important role in conservation efforts. Another approach to conserving the species and saving it from extinction is through improvement in techniques for propagating the species.

Several propagation techniques of *A. titanum* have been applied such as from seeds, tuber and cutting. Other propagation technique could involve the of petiole calluses which are naturally formed in the wild. This peculiar petiole growth phenomenon is one of the plant's natural attempts to regenerate. The main objectives of this study were to investigate whether environment factors that trigger callus growth in its natural habitat and to determine the viability of callus pieces after growing them out as in *ex situ* or in botanic gardens.

MATERIALS AND METHODS

Field Study

Several field studies have been carried out over the past decade i.e., in April 2006, January 2013 and September 2015. The field studies were located in Bukit Daun Protection Forest and in selected farmers' fields in Tebat Monok Village, Kapahiang Regency, Bengkulu Province, Indonesia, where the material for *ex situ* research was taken. The Kepahiang Regency is about 40 km to northeast from Bengkulu city (Fig.1).

Ex situ Propagation

The research experiment was conducted in the nursery of the Centre for Plant Conservation, Bogor Botanic Gardens –Indonesian Institute of Sciences (LIPI), Indonesia from January to August 2013. Research materials were obtained from calluses which emerged on the chopped petiole of natural growing *A. titanum*. A completely randomized design with a single factor, i.e. callus size, was employed on five callus replicates. Each replicate consisted of four samples. The treatments consisted of three callus piece sizes i.e. 0.5 x.0.5 cm, 1 x 1 cm, 2 x 2 cm. Calluses raised from a chopped petiole were cut up according to the appropriate treatment size. However, the calluses tended to be irregular in shape therefore the callus-pieces were generally not perfect cubes. The callus pieces were planted on a mixed growing medium of 1:1 mix of chopped fern root with charcoaled rice husks.

The following parameters were assessed: time to appearance of shoots; shoot height when the first leaf fully opened; diameter of the emergent petiole; diameter of the lamina; and number of young shoots. All the data were analized using

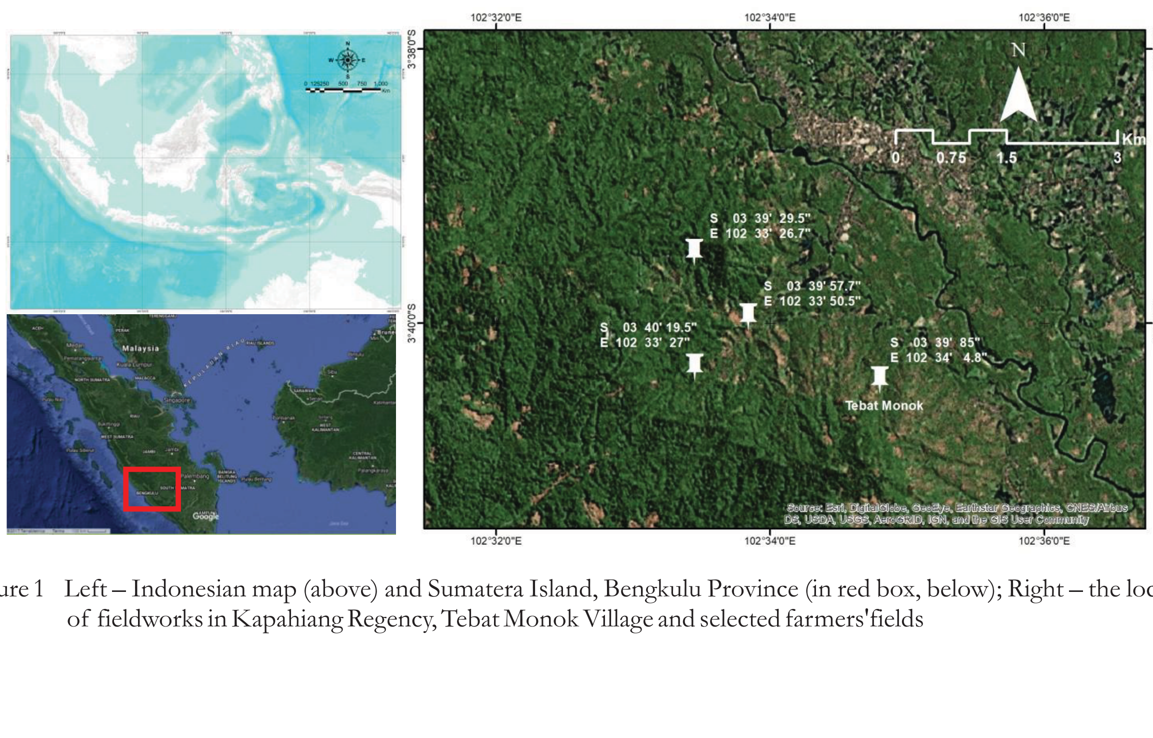

Figure 1 Left – Indonesian map (above) and Sumatera Island, Bengkulu Province (in red box, below); Right – the location of fieldworks in Kapahiang Regency, Tebat Monok Village and selected farmers'fields

SAS software. When several roots appeared from the new young shoot, it was separated from the callus immediately. This induced a new shoot from the callus.

A cross section of a fresh petiole from the seedling of *A. titanum* is made to demonstrate that the calluses are generated from epidermis tissue using a light microscope with 40 X magnification.

RESULTS AND DISCUSSION

Growth of *A. titanum* in Natural Habitat (*in situ*)

Based on our field study in Sumatera, *A. titanum* is often found in coffee and cocoa plantations, sometimes growing in extreme habitats such as on limestone outcrops and in places of heavy soil erosion. Most specimens of the species are found growing on steep areas with slope gradient ranging from 30% to 60% and mostly close to flowing water such as streams or rivers. The sites where *A. titanum* usually thrives have a temperature of 25°–27°C, atmospheric humidity of 85% – 95% and soil moisture of 65% – 90% which is relatively high and appears to be optimal for the growth of the species. The soil type is a dull yellowish-brown to dark brown forest soil, based on color charts (Oyama & Takehara 1976) and the soil pH is 5.1–6.4. In addition, Hetterscheid and Ittenbach (1996) have mentioned that the species is also found in semi-open spaces in young secondary forest from 0–1200 m altitude. The species has high tolerance of, and quickly adapts to, surrounding environments. However, the species prefers to have good soil drainage and balance of soil nutrients for better growth.

The presence of *A. titanum* in its natural habitat is not only determined by climate and soil nutrients but also by the integrity of all aspects of the biotic components of the habitat, including the support of the local human community and of government regulation. In addition, international conservation agencies are expected to support the preservation of the species in the wild.

Hidayat and Yuzammi (2008) reported that the distribution pattern for *A. titanum* tends to be clustered, which may increase the risk from threats in the wild. Moreover, unregulated trading by underpaid locals who collect large amounts of the species from the wild for transport to Japan and Korea (Hetterscheid & Ittenbach 1996) may lead to extinction in the future. In addition, land degradation, disappearance of the giant Hornbill Birds as seed distributors and the prevalence of destructive myths are all contributing to the species becoming vulnerable.

Nevertheless, *A. titanum* has its own ability to regenerate from its petioles in the wild. This peculiar growth from the petiole of *A. titanum* occurs when it is chopped and left naturally (Fig. 2c, 2d, 2e). Usually, the chopped petiole is best left in a standing position for calluses to form, the petiole being attached to the ground directly. It should not be far from a river for this to occur. If chopped and left in a prostrate position, the petioles did not form calluses during our observations in the field. It is assumed that after cutting of the petiole, the temperature and the environment humidity must be right for the petiole not to decay. Water and nutrients must continue to flow to the leaves in order that the leaves can still carry out photosynthesis. According to Mr. Holidin (pers. com.), one conservation-minded local community member, it takes more than six months to develop a new individual plant from a piece of chopped petiole in the forest (Fig. 2f, 2g).

Hejnowicz and Barthlott (2005) stated that the petiole of *A. titanum* is composed of a single layer of epidermis, a thin parenchymatous cortex, a thick layer of collenchyma strand embedded in compact pharenchyma and a core of aerenchyma (Fig. 3). This petiole produces exudates when cut, initially appears in epidermis layer (Fig. 2a, 2b) and covered the damage zone. It is presumed that this exudate stimulates the formation of callus in the damage zone. Moreover, Ikeuchi *et al.* (2013) mentioned that the callus form is associated with wounding. This callus can be produced from a single differentiated cell, many cells are totipotent, and those are able to regenerate.

Plant development is usually induced by many kinds of hormone growth, notably cytokinin and auxin. Mostly cytokinin is produced within the

Figure 2 Petiole cross section (a); Exudate from the petiole (b); A part of a petiole forming callus naturally (c); Callus appears with roots (d); Part of a rachis forming a callus (e); Seedling growth from a piece of planted petiole in the forest (f); A chopped section of petiole planted in the forest by a conservation-minded local community member (g)

roots naturally (Taiz & Zeiger 2010). Sometimes the plant can also produce both auxin and cytokinin in the leaves as well in the roots (Srivastava 2002). Therefore, when the petiole is cut, the transportation of cytokinin from the roots to the shoots is suddently stopped but the auxin and cytokinin are still delivered from the leaves to the damage zone of the petiole, polarly. Subsequently, the ratio of auxin and cytokinin may change in the damaged area of the petiole and needs to be appropriate for the formation of callus. The callus then is stimulated to grow until

Figure 3 Cross section of petiole from the seedling of *Amorphophallus titanum* (40 X) using light microscope; the emergence of the callus is probably initiated from the epidermis of the damage petiole zone

covering the entire damage petiole surface irregularly. The appearance of the callus is probably initiated from the epidermis of the damage petiole zone (Fig. 3).

The ratio of auxin to cytokinin will influence callus and root formation. Root tip elongation requires a high ratio of auxin to cytokinin while a somewhat lower ratio of auxin to cytokinin will induce callus formation (Gaba 2005). The callus develops a clump and then turns to producing small tubers. At this stage, the small tubers still adhere to each other and some are produced roots. A tuber starts to enlarge and young shoots emerge before separating eventually. In the juvenile stage, one tuber can generate two or three young shoots.

Production of *A. titanum* outside the Natural Habitat (*ex situ*)

The study explored improving a propagation technique for *A. titanum* to help address its extinction problem in its natural habitat. Petiole calluses of *A. titanum* were brought to the Bogor Botanic Gardens to examine their *ex situ* propagation potential (Fig. 4b).

The petiole of *A. titanum* resembles a tree trunk and its anatomy is typical of monocot stems (Fig. 4a). In appropriate condition, chopped petiole of *A. titanum* developed ex vitro calluses naturally, which can be used as propagation material. This is supported by totipotency theory that one cell can grow and develop to be a new

Figure 4 The petiole of *A. titanum* resembling a tree trunk in its natural habitat (a), cross section of a part of a petiole forming calluses initiated from the epidermis (b)

plant (Gahan 2007). These calluses then will emerge into a new plant in appropriate environment. The appearance of young shoot and roots depend on the ratio of auxin and cytokinin hormones. The inducement of shoot growth as a result of cytokinin content is higher than auxin. Meanwhile, the emergence of roots is caused the auxin content which is higher than cytokinin (Gaba 2005).

During the propagation experiment, all petiole calluses were able to produce young shoots, however the callus size significantly affected all measured parameters (Table 1). Cytokinin is known to be involved in shoot initiation (Davies 2004; Srivastava 2002) and our data showed that the induction of shoot growth may be proportional to the cytokinin content of a callus piece. In calluses of 0.5 cm² size, the first young shoot emerged 46.8 ± 2.9 days after planting, while for calluses of size 1 cm² and 2 cm², emergence appeared 52.4 ± 3 and 52.3 ± 3 days after planting, respectively. Furthermore, the number of shoots that emerged was also influenced by callus size. The callus 0.5 x 0.5 cm² size only produced one shoot with a plant height of 5.2 ± 0.2 cm (at the time of the first fully opened leaf). On the other hand, the calluses 1 x 1 cm² and 2 x 2 cm² in size generated one, two or three shoots, and plant height at the time of the first fully opened leaf was 11.4 ± 0.3 cm and 18.8 ± 0.3 cm, respectively (Table 1).

This finding suggests that bigger calluses retain greater amount of stored energy that is required for shoot and root formation. Lower energy available for shoot formation will produce a smaller plant. Smaller calluses are therefore expected to take a longer time to reach the same size in the young shoot compared to calluses 2 x 2 cm² in size. Since the young shoot which emerged from the callus had several roots, the new plant was separated quickly from the callus. The purpose of separating the new shoot from the callus was to stimulate the formation of more new shoots in the callus (Fig. 5). According to Gahan and George (2008), when a shoot emerges then it is likely that auxin production will inhibit further shoot formation. This means that all the stored energy of the callus will be used just for

Table 1 The effect of *A. titanum* callus size on shoot emergence, shoot height, leaf diameter, petiole diameter and number of shoots

Callus size (cm²)	Shoot emergence (days)	Shoot height (cm)	Leaf Diameter (cm)	Petiole diameter (mm)	Number of shoots
0.5 x 0.5	46.8 ± 2.9 b	5.2 ± 0.2 c	6.8 ± 0.5 c	2.41 ± 0.13 c	1.0 ± 0.0 b
1 x 1	52.4 ± 3.1 a	11.4 ± 0.3 b	10.1 ± 0.6 b	3.64 ± 0.21 b	1.2 ± 0.2 b
2 x 2	52.3 ± 3.2 a	18.8 ± 0.3 a	14.4 ± 0.1 a	5.69 ± 0.34 a	2.4 ± 0.7 a

Note: The shoot height, leaf and petiole diameters are measured when first leaf is fully opened (values for a particular parameter followed by the same letter are not significantly different at $P<0.05$)

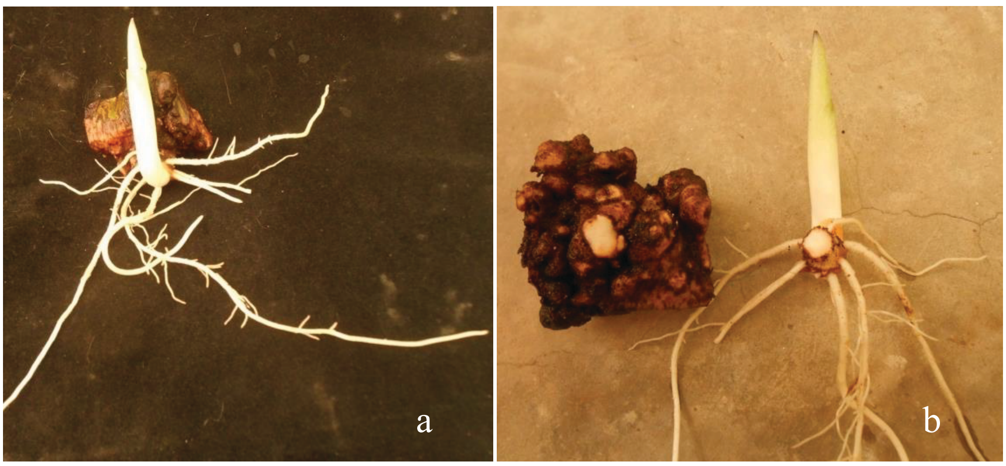

Figure 5 The *A. titanum* shoot emerging from the callus (a) and the shoot separated from the callus (b)

growing this one shoot. This experiment showed that a callus with 0.5x0.5 cm² size appeared to be totally depleted of its stored energy in forming a single shoot with its roots. Therefore, not more than one shoot was produced. For calluses of 2x2 cm² size, separation of an emergent shoot with its roots from the callus stimulated new shoot formation, because the inhibiting factor (auxin presumably) had been removed. This callus can then produce another one or even two shoots.

This research shows that natural calluses of *A. titanum* can be used as propagation material without plant growth regulator and provides a great potential to ensure its conservation in nature by local people. Besides, the result can also be applied by botanic gardens, as an ex situ conservation, to imply collecting the material of *A. titanum* from the fields. Using natural calluses as propagation material has several advantages namely maintains the population of *A. titanum* is in its habitat, provides more plant materials, easy to carry, less damage compared to carry the tuber, easy to grow, requires less space and will produce plenty of seedlings in short time.

CONCLUSIONS

Calluses that arise naturally on erect, chopped petioles of *A. titanum* attached directly to the ground are able to form new shoots. The appearance of these calluses in its natural habitat is supported by environmental factors such as water supply, good soil drainage and balance of soil nutrients for better growth. Ex vitro callus development also needs a day temperature of 25°–27°C, atmospheric humidity of 85% – 95%, soil moisture content of 65% – 90%, and soil pH of 5.1 – 6.4. When a callus is grown *ex situ* on an appropriate medium, the number and size of the shoots formed are influenced by the size of the callus. The optimum callus size for propagation is 2x2 cm², which can produce 2-3 shoots and 18.8 cm high at the time of first fully opened leaf. The difficulty to find *A. titanum* in its natural habitat was due to the over exploitation and destruction of this species. Nevertheless, the occurrence of natural calluses on its petiole cutting ensures its in situ and ex situ conservation. Ex vitro calluses of *A. titanum* is recommended as one of propagation materials in the future, notably for *ex situ* conservation e.g. in botanic gardens.

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