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IN VITRO PRESERVATION OF RODENT TUBER (TYPHONIUM FLAGELLIFORME LODD.) PEKALONGAN ACCESSION WITH PACLOBUTRAZOL

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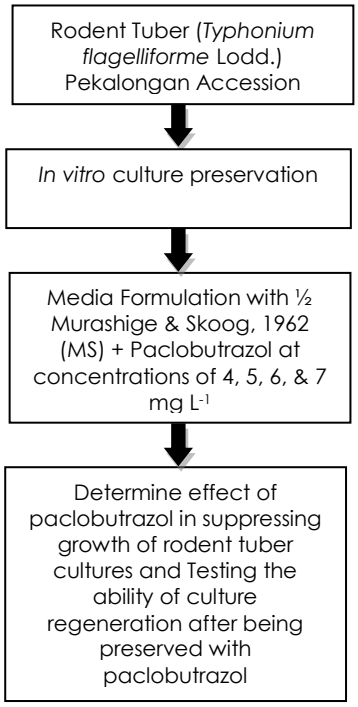
Graphical abstract



Abstract

Rodent tuber plant (*Typhonium flagelliforme* Lodd.), as one of the most potent medicinal plants, has to be developed as an active ingredient of degenerative drugs, including cancer drugs. However, this enormous potential must be supported by sustainable cultivation of the plant. The conventional preservation of rodent tuber can be done by planting various accessions in the field, but it will need land availability and intensive plant maintenance. Preservation through *in vitro* culture is an alternative method that can be used. The aim of this study was to determine the effect of paclobutrazol in suppressing the growth of rodent tuber cultures, and test the ability of culture regeneration after being preserved with paclobutrazol. The media formulation used was Murashige and Skoog (1962) (MS) with the addition of paclobutrazol at concentrations of 4, 5, 6, and 7 mg L^{-1}. The results showed that using paclobutrazol at 5 mg L^{-1} was the best concentration that can inhibit the elongation of buds, seedling formation, leaf formation, and root elongation until 5 months. Cultures of paclobutrazol treatment at 5 mg L^{-1} had shoot heights, number of shoots, number of leaves, and root lengths of 0.49, 2.33, 7.23, and 0.3 cm, respectively. Paclobutrazol could inhibit the growth of *in vitro* culture of rodent tuber and prolong the shelf life of culture up to 5 months. The culture of rodent tuber from paclobutrazol treatment had normal growth and regenerative ability after transfer to the medium regeneration.

Keywords: *Typhonium flagelliforme* (Lodd.), *in vitro*, paclobutrazol, normal shoot regeneration

1.0 INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a medicinal plant that lives in a humid environment not exposed directly to sunlight. Rodent tubers are native plants of Indonesia found in many islands of Java, and live at an altitude of 1-300 m above sea level [4]. The extract of rodent tuber is useful to treat cancer, such as breast cancer [2], lung cancer [10], leukemia [16], and has toxicity to *Artemia salina* [24]. Rodent tuber has chemicals, which include sterols [11], cerebrosides, glycosides [8], saponins [27], terpenoids [13], and alkaloids and flavonoids [18].

Rodent tuber has many properties that need to be developed as a raw material of medicine. According to Essai [4] there are 40 accessions of rodent tuber benefits which have not been much explored. Propagation of rodent tuber was done vegetatively with tubers. A technique that can multiply plants rapidly in large quantities and retain the same properties as its mother plant has been developed to anticipate increased exploitation of plants from nature. *In vitro* preservation is primarily performed on plants with short seed viability and with vegetatively propagated plants [31]. Rodent tuber Pekalongan accession has been successfully propagated through *in vitro* culture, and there are several collections of rodent tuber mutant clones that need to be maintained and stored [20]. Storage and collection of plant material in the field often failed because of pests and diseases, and other environmental stressors. Short-lived plants require repeated renewal efforts that resulted in increasing energy and cost [12]. *In vitro* cultures can be used as an alternative technology to solve these problems [12].

In vitro preservation has several advantages. It can preserve endangered plants that are almost extinct, save plants that do not produce seeds, prevent plants from pests and diseases caused by nature, and can store plants in a disease-free condition, as well as can be done in a room that is relatively small [30]. One method of plant preservation with *in vitro* cultures that can be used to conserve germplasm is called minimal storage. Minimized nutrients can also be applied using a growth regulator that has low activity, such as kinetin, or using growth inhibitors or retardant [28]. Plant conservation through minimal growth can be done in various ways, including reduction of medium salt composition, low temperature, osmotic stress induction, and the use of growth inhibitors [26].

Minimum growth can be done by using one or a combination of several factors. Minimum growth can be conditioned by reducing light, temperature, carbon source, and minerals [15]. *In vitro* preservation of rodent tuber have minimal growth, which was successfully performed on a medium containing paclobutrazol for 5 months [28].

Paclobutrazol in the tissues is translocated acropettally through the xylem which can affect the shortening of plant height [1]. Paclobutrazol is a plant-inhibiting agent that inhibits gibberellin biosynthesis [3]. Paclobutrazol prevents kaurene oxidase, and blocks oxidative reactions *ent*-kaurene to *ent*-kaurenoic acid that synthesizes gibberellin [6, 30, 15]. The results of *in vitro* preservation that have been successfully studied, among others, were in daun dewa (*Gynura procumbens*) with the usage of paclobutrazol and ABA inhibitor [12], temulawak (*Curcuma xanthorrhiza*) with the usage of retardants paclobutrazol [26], pineapple with the usage of paclobutrazol and ABA [19], and purwoceng (*Pimpinella pruatjan*) with paclobutrazol [21], mannitol and sucrose [21].

Propagation of rodent tuber through tissue culture has been performed and it is known that the best basic medium is Murashige & Skoog, 1962 (MS) medium with additional of 1-Naphthalene Acetic Acid (NAA) 0.5 mg L^{-1} , and 6-benzylamniopurin (BAP) 0.5 mg L^{-1} [23]. The study used MS base medium with ½ concentration of macro salt and paclobutrazol to minimize the availability of nutrients, and suppress the growth of shoots.

This study was conducted to determine the effect of several treatments of paclobutrazol concentrations on the growth of *in vitro* culture of rodent tuber, and to test their regeneration ability after *in vitro* storage. From this research it will be known what media formulation is ideal to store rodent tuber *in vitro* in long shelf life, and which has a normal regeneration ability.

2.0 METHODOLOGY

Initiation of Explant

The explant materials were *in vitro* shoots from rodent tuber Pekalongan accession (*Typhonium flagelliforme* Lodd.) at 2.5 months old. The research activities were conducted in the laboratory of Biotechnology, Pelita Harapan University. The activities undertaken include multiplication of *in vitro* shoots as a source of explants, *in vitro* storage as well as regeneration of post-storage *in vitro* culture. Propagation multiplication was done by planting buds of rodent tuber in Murashige & Skoog, 1962 (MS) medium with additional of 1-Napthalene Acetic Acid (NAA) 0.5 mg L^{-1} and 6-benzylamniopurin (BAP) 0.5 mg L^{-1}. Culture removal (subculture) was done every 2.5 months to obtain sufficient plant material for storage activities.

Experimental Design

Plant materials or the explant used *in vitro* were shoots without leaves ± 0.5 cm in size. The media formulation used was ½MS agar medium without the addition of paclobutrazol, ½MS macro salt concentration with the addition of paclobutrazol (control, 4, 5, 6, and 7 mg L^{-1}). The explant was

planted on a culture bottle containing ½MS medium and paclobutrazol in various concentrations. The number of replicates for each treatment was 10. The incubation was carried out in a culture room with a temperature of 20 ± 2°C. The intensity of illumination was applied at 800-1000 lux and 16 hours of bright photoperiodicity.

Data collection variables were recorded on the shoot heights, number of shoots, number of leaves, and root lengths. The shoot height was observed from the top of the hump to the shoots. The number of shoots observed was the number of new plants growing from the stumps. The number of leaves observed was the number of leaves that were completely open. Recovery was done after 2.5 and 5 months preservation period. Post-recovery was done by subculturing *in vitro* on MS medium .

Shoots Regeneration After Minimum Storage

In vitro cultures of rodent tuber that had been preserved in minimal growth media with the addition of paclobutrazol were transferred to propagation medium (MS with the addition of NAA 0.5 mg L^{-1} and BAP 0.5 mg L^{-1}). Observations were made on plant heights, number of shoots, number of leaves, and root lengths.

Statistical Analysis

The experimental design of this research was a completely randomized design with one factor. Plant heights, shoot heights, number of shoots, number of leaves, and root lengths in the last week of observation were analysed with SPSS 19 programme using One Way Variance Analysis (ANOVA), and Duncan significance test at 5% level.

3.0 RESULTS AND DISCUSSION

Shoot Heights

The growth response of rodent tuber cultures to various storage treatments is shown in Figure 1. The results showed that MS or ½MS media treatment without the addition of paclobutrazol still supported the growth of culture until 2.5 months, but at 5 months, it decreased in culture height which indicated that the culture had died. Shoots in the control treatment (MS and ½MS) grew until 2.5 months old, then there was a decrease in shoot height at 5 months old.

In ½MS medium all shoots had died, whereas in MS medium only some shoots died. The results showed that the shoot height at all concentrations of paclobutrazol treatment with ½MS and MS medium was different for all treatments at 2.5 and 5 months (see Table 1). The usage of media with media concentration being ½ of the most macro reduction rates, especially NH_4^+ and NO_3^-. Nitrogen is a key factor for reducing growth [13]. Shoots on all paclobutrazol treatments had a higher shoot height. This suggests that the use of paclobutrazol may have inhibited the growth of plant height. The addition of paclobutrazol influenced the endogenous as an inhibitor of gibberellin synthesis pathway [14]. The usage of paclobutrazol caused the culture to be roset (short sections) [22], and visually short-sighted cultures [19]. There was a high rate of shortened shoots with a paclobutrazol treatment at all stages of treatment, because paclobutrazol has physiological effects as anti-gellers used in the process of dilution in apical meristems [21]. Paclobutrazol works by preventing chlamene oxidase and blocking the oxidative reaction of *ent*-kaurene to an *ent*-kaurenoic acid that synthesizes gibberellin [6, 31, 16].

Tabel 1 The shoot height of rodent tuber in various medium formulations for preservation

Media	Shoot height (cm)	
	Month 2.5	Month 5
MS	$1,46^{c} \pm 0,09$	$1,16^{c} \pm 0,18$
½MS	$1,14^{b} \pm 0,15$	$0,00^{a} \pm 0,00$
½MS + Paclobutrazol 4 mg L^{-1}	$0,41^{a} \pm 0,03$	$0,61^{b} \pm 0,05$
½MS + Paclobutrazol 5 mg L^{-1}	$0,38^{a} \pm 0,03$	$0,49^{b} \pm 0,04$
½MS + Paclobutrazol 6 mg L^{-1}	$0,44^{a} \pm 0,03$	$0,65^{b} \pm 0,08$
½MS +Paclobutrazol 7 mg L^{-1}	$0,40^{a} \pm 0,03$	$0,53^{b} \pm 0,07$

The mean ± SE values followed by the same superscript notation on each row show no significant differences based on the Duncan test at 5% level.

Paclobutrazol at 5 mg L^{-1} is the best treatment to inhibit plant growth towards elongation. According to Surachman [29], paclobutrazol at 5 mg L^{-1} produces shorter shoots. Paclobutrazol at 5 mg L^{-1} is the best concentration to prolong the shelf life of the culture and significantly reduces the cell elongation process in the *in vitro* conservation of *Curcuma zanthorriza* [27]. The treatment of paclobutrazol at 4 mg L^{-1} in *Gynura segetum* produces the shortest shoots at 3 months old [13].

Number of Shoots

Statistical analysis showed that the usage of MS medium at the age of 2.5 and 5 months significantly produced the largest amount of shoots compared to other medium (see Table 2 and Figure 1), but the number of shoots decreased within 5 months. The usage of ½MS media could not support the formation of shoots, which was produced in 2.5 months only slightly (1.67 cm), even at 5 months the plant was dead.

Tabel 2 The number of shoots of rodent tuber in preservation media

Media	Number of shoots	
	Month 2.5	Month 5
MS	$6,17^{c} \pm 0,40$	$4,67^{b} \pm 0,33$
½MS	$1,67^{a} \pm 0,42$	$0,00^{a} \pm 0,00$
½MS+Paclobutrazol 4 mg L^{-1}	$3,17^{ab} \pm 0,65$	$3,50^{b} \pm 0,67$
½MS+Paclobutrazol 5 mg L^{-1}	$1,33^{a} \pm 0,76$	$2,33^{b} \pm 1,02$
½MS+Paclobutrazol 6 mg L^{-1}	$1,67^{a} \pm 0,56$	$3,00^{b} \pm 0,93$
½MS+Paclobutrazol 7 mg L^{-1}	$4,50^{bc} \pm 0,67$	$7,17^{c} \pm 1,25$

The mean ± SE values followed by the same superscript notation on each row show no significant differences based on the Duncan test at 5% level.

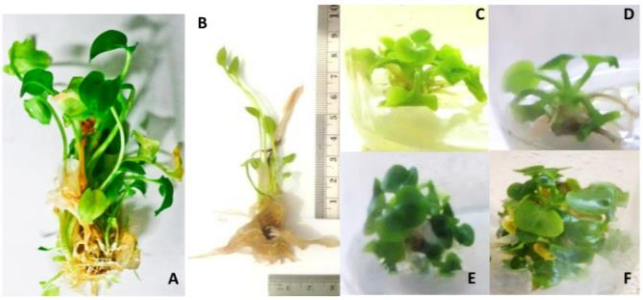


Figure 1 Effect of paclobutrazol treatment on the number of shoots *in vitro* of rodent tuber at 2.5 months old. (A) MS, (B) ½MS, (C) ½MS + paclobutrazol 4 mg L^{-1}, (D) ½MS + paclobutrazol 5 mg L^{-1}, (E) ½MS + paclobutrazol 6 mg L^{-1}, (F) ½MS + paclobutrazol 7 mg L^{-1}

The number of shoots with the usage of paclobutrazol at 4 to 6 mg L^{-1} did not differ significantly at 5 months old but were significantly different from the treatment of paclobutrazol 7 mg L^{-1}. Cultures on paclobutrazol treatment could produce shoots although no exogenous cytokines were added to the paclobutrazol treatment medium. The formation of shoots occurred because the paclobutrazol growth inhibitors could inhibit growth toward elongation but promoted the formation of multiple shoots [19]. Paclobutrazol affected plant growth regulators in plant tissues, which increased the content of cytokines [9] and ABA [25, 3]. Similar results were obtained from Syahid's research [27] where paclobutrazol 5 mg L^{-1} was able to suppress the number of shoots of *Curcuma zanthorriza* that was significantly different from the control. Paclobutrazol treatment at 4 mg L^{-1} produced the fewest shoots in daun dewa (*Gynura segetum)* [12].

Cultures in all paclobutrazol treatments were still increasing the number of shoots until 2.5 months old, which was suspected because of the high content of endogenous cytokines in the tissues. Syahid [27] suspected that the content of endogenous cytokines in temulawak tissue was high enough that the temulawak shoots continued to form until the 7 months old of all paclobutrazol treatments. Differences in the response of each plant to a given growth regulator depended on the content of cytokines and growth regulators such as GA in plant tissue and tissue physiology [12]. The increase in the number of shoots on the treatment of paclobutrazol 7 mg L^{-1} was caused by the formation of callus that regenerated to form buds.

The suppression of plant growth induced stress and allowed plants to expand other parts that were not inhibited by paclobutrazol. Inhibitors may have altered the ratio of plant growth regulators in plant tissues [5]. A higher concentration of paclobutrazol was thought to induce the formation of embryogenic callus, so in the treatment of paclobutrazol 7 mg L^{-1}, more shoots were produced.

Hamama *et al*. [14] said that paclobutrazol did not improve regeneration, and led to shoots of poor quality (hyperhydric) in *Pelargornium* sp. Paclobutrazol stimulated the *in vitro* morphogenesis process and allowed redifferation of *Centaurium. erythraea* root cells into embryogenic cells [26]. Paclobutrazol affected the ABA content [25, 3] that can promote callus growth and embryogenesis [8, 5].

Number of Leaves

The leaf colour of the shoot in ½MS medium turned brownish yellow, had a poor appearance, and the plant was weak, while most of the leaves on MS medium were yellow, and withered. Shoots in all paclobutrazol treatments had fresh green leaves and only a few leaves were white or yellow while the shoots looked tough. Leaf colour on the treatment of paclobutrazol 5 mg L^{-1} had fresh green leaves and a bit of pale leaves as shown in Figure 2.

At 5 months old, the least number of leaves were obtained from the treatment of paclobutrazol 5 mg L^{-1}, but this was not significantly different from the paclobutrazol 4, 6, and 7 mg L^{-1}. Shoots on paclobutrazol treatment 4 mg L^{-1} had the highest number of leaves at the age of 5 months, i.e., 8.72, whereas culture on MS had the lowest number of leaves, i.e. 0.86 (see Table 3). Nevertheless, the number of leaves increased in the treatment of paclobutrazol (see Figure 2). The treatment of paclobutrazol 7 mg L^{-1} had the lowest number of leaves compared to the other paclobutrazol treatments that had 7.09 although there were no significant differences (see Table 3). The results showed that paclobutrazol treatment had not been able to inhibit the formation of rodent tuber leaves significantly. This result differed from the results by Surachman [29] which showed that 5 mg L^{-1} of paclobutrazol could inhibit a number of leaves in rodent tuber and *Curcuma zanthorriza* [27]. The paclobutrazol treatment at 4 mg L^{-1} inhibited leaf formation in *Gynura segetum* [13].

Tabel 3 The number of leaves of rodent tuber in preservation media

Media	Number of leaves	
	Month 2.5	Month 5
MS	4,99^{ab} ± 0,18	0,86^{a} ± 0,07
½MS	8,07^{c} ± 1,08	0,00^{a} ± 0,00
Paclobutrazol 4 mg L^{-1}	4,02^{a} ± 0,39	8,72^{b} ± 0,89
Paclobutrazol 5 mg L^{-1}	5,25^{ab} ± 0,72	7,23^{b} ± 0,80
Paclobutrazol 6 mg L^{-1}	6,07^{b} ± 0,61	7,88^{b} ± 0,65
Paclobutrazol 7 mg L^{-1}	3,95^{a} ± 0,31	7,09^{b} ± 0,52

The mean ± SE values followed by the same superscript notation on each row show no significant differences based on the Duncan test at 5% level.

Root Length

The usage of paclobutrazol may have inhibited root elongation until 5 months old (see Table 4). The paclobutrazol treatment at 5 mg L^{-1} resulted in the shortest root (0.30 cm), significantly different from that of paclobutrazol 4, 6, and 7 mg L^{-1}, while in MS medium treatment the root grew elongated. The results showed that paclobutrazol might have inhibited root elongation (see Figure 2). Purnamaningsih *et al*. [19] stated that the treatment of paclobutrazol 0.1 mg L^{-1} in simadu pineapple had a more significant effect on root formation than without paclobutrazol. Shoots in the treatment of paclobutrazol appeared to be shorter, fatter, firmer, thicker, rounder, and greener (see Figure 2). Shorter buds occurred because paclobutrazol inhibited gibberellin synthesis, resulting in short stem segments [13, 23]. This could have occurred due to the influence of paclobutrazol which was very strong in suppressing the growth of plants during storage that still affected root growth at 2.5 months.

Tabel 4 The root length of rodent tuber in preservation media

Media	Roots length (cm)
	Month 5
MS	2,93^{c} ± 0,33
½MS	0,00^{a} ± 0,00
Paclobutrazol 4 mg L^{-1}	1,08^{b} ± 0,12
Paclobutrazol 5 mg L^{-1}	0,30^{a} ± 0,14
Paclobutrazol 6 mg L^{-1}	1,08^{b} ± 0,08
Paclobutrazol 7 mg L^{-1}	0,89^{b} ± 0,16

The mean ± SE values followed by the same superscript notation on each row show no significant differences based on the Duncan test at 5% level.

Growth retardants of paclobutrazol inhibited elongation but promoted the formation of multiple shoots [19] in all treatments of paclobutrazol. The shelf life under paclobutrazol treatment was longer because the retardants could have increased the content of grains in green leaves so that the photosynthesis process was better than without the additional paclobutrazol [27, 23). Paclobutrazol used in minimal growth media proved to be effective in prolonging shelf life and in maintaining the survival of rodent tuber *in vitro* preservation.

Figure 2 The appearance of rodent tuber *in vitro* culture in the control (½MS without paclobutrazol) and various treatments at 5 months paclobutrazol. (A) ½MS + paclobutrazol 5 mg L^{-1}, (B) ½MS + paclobutrazol 4 mg L^{-1}, (C) ½MS + paclobutrazol 5 mg L^{-1}, (D) ½MS + paclobutrazol 6 mg L^{-1}, (E) ½MS + paclobutrazol 7 mg L^{-1}.

In vitro Shoot Regeneration of Rodent Tuber after Minimum Storage

The regeneration test from the buds of rodent tuber after storage was performed using growth media. The results showed that buds from storage treatment with paclobutrazol could grow optimally after transfer to growth media (see Table 5). Plant height, number of shoots, number of leaves, and root length of all treatments with paclobutrazol were not significantly different from controls. The regeneration test showed that paclobutrazol used in storage medium did not cause a residual effect so that the shoots of all paclobutrazol treatments could be normal, and could reform shoots and leaves (see Figure 3).

Tabel 5 *In vitro* shoots growth from storage treatment with paclobutrazol in the regeneration medium

Paclobutrazol treatments	*In vitro* shoots growth (cm)			
	Plant height	Number of shoots	Number of leaves	Root length
0 mg L^{-1}	1.45^{a}	6.16^{a}	4.99^{a}	2.93^{a}
4 mg L^{-1}	1.39^{a}	6.16^{a}	5.21^{a}	3.30^{a}
5 mg L^{-1}	1.24^{a}	6.50^{a}	4.93^{a}	2.78^{a}
6 mg L^{-1}	1.24^{a}	6.83^{a}	5.52^{a}	1.63^{b}
7 mg L^{-1}	1.50^{a}	6.83^{a}	5.41^{a}	1.53^{b}

Description: Superscript of the same letter in the column shows no significant differences (p> 0.05)

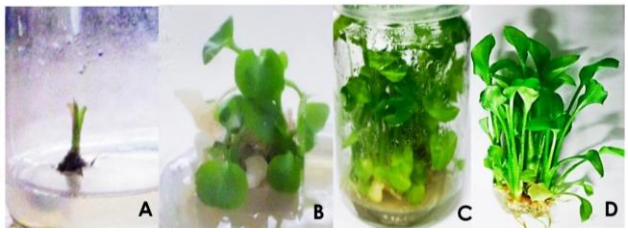


Figure 3 The growth and development of culture during and after storage time at the best paclobutrazol treatments. (A) new shoots in MS + 5 mg L^{-1} paclobutrazol in day 0, (B) shoots in ½MS + 5 mg L^{-1} paclobutrazol at 5 months old, (C) normal shoots regeneration of ½MS + 5 mg L^{-1} at 2.5 months, (D) shoots rooting of ½MS + 5 m L^{-1} paclobutrazol treatment

4.0 CONCLUSION

The usage of paclobutrazol at 5 mg L^{-1} is the best concentration that can inhibit buds, seedling formation, leaf formation, and root elongation. Cultures on the treatment of paclobutrazol at 5 mg L^{-1} had a shoot height, number of shoots, number of leaves, and root length of 0.49 cm, 2.33, 7.23 and 0.3 cm, respectively. Paclobutrazol can inhibit the growth of *in vitro* culture of rodent tuber and prolong the shelf life of culture up to 5 months. The explant of rodent tuber mutant clones from paclobutrazol treatment had normal growth and regenerative ability after transfer to regenerate medium up to 2.5 months.

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