

*Review Article*

## Micropropagation of Rodent Tuber Plant (*Typhonium flagelliforme* Lodd.) from Medan by Organogenesis

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### ABSTRACT

Rodent Tuber is an anticancer herbal plant from Araceae family which is very sensitive to environmental condition and has a low plantlet reproduction rate. This research was aimed to obtain effective method of micropropagation on Rodent Tuber plant with high rate multiplication factors. The source of explants used the mother plant originating from Medan (Indonesia). MS medium supplemented with the combination of 0.5 mg/L of BAP and various concentrations of NAA was used. Explants were successfully induced in medium containing 0.5 mg/L of BAP and 0.5 mg/L of NAA. Growing media for plant multiplication were ½ MS and MSO. In the treatment media, BAP was given in five different concentrations, i.e. 0.5, 1, 1.5, 2, and 2.5 mg/L. The result showed that, ½ MS medium added with 1.5 mg/L of BAP was effective in inducing the production of  $4.20 \pm 1.03$  plantlets. *In vitro* root induction of Rodent Tuber was achieved in MSO medium supplemented with 0.5, 1, 1.5, 2, and 2.5 mg/L of NAA. MSO medium supplemented with 1.5 mg/L of NAA could induce the formation of  $43.20 \pm 21.21$  new roots. Viability percentages of Rodent Tuber from Medan acclimatization was 100%. The usage of MS basal media enriched with BAP and NAA is able to effectively increase the production of new plants and roots of Rodent Tuber plant.

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## INTRODUCTION

Rodent Tuber (*Typhonium flagelliforme* Lodd.) is an Indonesian herbal plant that belongs to the Araceae family (Surachman, 2009). This plant can be found in several countries such as India (Mankaran et al., 2013), Indonesia (Essai, 1986), Malaysia (Lin, 2005), Sri Lanka and Australia (Sai et al., 2000). Rodent Tuber lives 1-300 m above sea level (Essai, 1986) in humid and shady areas (Nobakht et al., 2009).

Rodent Tuber is able to reach 26 cm in height. This plant has flowers that resemble a rat's tail. The flower of the rodent tuber plant is sterile i.e. it cannot reproduce sexually (Nobakht et al., 2009). Rodent Tuber is commonly propagated by the vegetative method i.e. by separating buds produced from the mother plant's tuber (Syahid & Kristina, 2007).

According to phytochemical analysis, this plant contains several bioactive compounds such as alkaloids, flavonoids (Mankaran et al., 2013), terpenoids, steroids (Nobakht et al., 2010), Ribosome Inactivating Proteins (RIP) (Indrayudha et al., 2006; 2011), antioxidants (Sukardi, 2011) and antibacterial compounds (Mohan et al., 2008). Besides that, Rodent Tuber extract has been found to be successful in inducing apoptosis of breast cancer cells T47D (Norrochmand et al., 2011) and MCF-7 (Putra et al., 2011). It was also found to inhibit the proliferation of human T4-lymphoblastoids (Mohan et al., 2008; Mohan et al., 2010) and NCI-H23 non-small cell lung carcinoma (Lai et al., 2008).

The ability of Rodent Tuber to inhibit and kill cancer cells has made this plant one of the plant commodities used as a raw material in complementary and alternative medicine (Mohan et al., 2011; Mankaran et al., 2013).

Rodent Tuber has a low reproduction rate and is very sensitive to environmental factors. Therefore, the number of Rodent Tubers in the plant's natural habitat is very low. However, demand for this plant is very high due to its biological activity. Vegetative propagation in *in vitro* culture is an appropriate method for overcoming this problem of low numbers because this method is able to produce many plants in a short period of time (Tiwari et al., 2011). The success of propagation in tissue culture is influenced by several factors i.e. genotype of plant, formulation media and physiological conditions of the mother plant. Same plants from different locations will show different results in the same formulation media. The influence of climate and conditions of the physical environment can also have an effect on the physiological conditions of the mother plant (George & Sherrington, 1984; Wattimena et al., 1992).

Plant Growth Regulators (PGR), if used in the right concentration, are able to optimise plant multiplication (Mustafa et al., 2012). PGR is instrumental in regulating the physiological activity of a plant such as growth, development and organogenesis (George & Sherrington, 1984). One of the PGRs that is usually used

to induce the production of plants in *in vitro* culture is 6-benzylamniopurin (BAP). BAP is a cytokinin-type PGR that is effective in inducing the production and propagation of buds from bud eyes (Gunawan, 1987). The supplementation of BAP in a Murashige-Skoog (MS) medium could induce the production of buds from plants such as *Amygdalus communis* L.cv. Yaltsinki (Akbas et al., 2009), *Psoralea corylifolia* Linn (Pandey et al., 2013), *Vinca rosea* L. (Haq et al., 2013) and *Potulaca grandiflora* Hook (Jain et al., 2010).

In Indonesia, Rodent Tuber has been found in several regions such as Bogor, Pekalongan and Medan. Laurent et al., (2013) identified the genetic differences between Rodent Tubers from three different regions by analysing their RAPD molecular marker profiles. Rodent Tuber from Bogor was multiplied in MS medium to which was added 1 mg/L of NAA and 0.5 mg/L of BAP (Sianipar et al., 2011). Besides that, Rodent Tuber from Pekalongan has also been propagated *in vitro* in MS medium supplemented with 0.5 mg/L of NAA and 0.5 mg/L of BAP (Sianipar et al., 2015). Rodent Tuber from Medan is one of the plant accessions in Indonesia. The regeneration of this plant in *in vitro* culture can be done in two ways i.e. by somatic embryogenesis and organogenesis. In our previous research (Sianipar et al., 2011), the somatic embryogenesis micropropagation method was performed on Rodent Tuber from Bogor. However, plants from different accessions usually have different optimal micropropagation

conditions as well. Therefore, this research aimed to formulate a new and efficient micropropagation method for propagating Rodent Tuber plants from Medan by applying different concentrations of BAP as PGR.

## MATERIALS AND METHOD

### Sterilisation and Initiation of Explant

This research used bud eyes and tubers of Rodent Tuber from Medan as the initial plant materials/explant source. The explants were washed in running tap water to remove any soil particles attached to them. The explants were submerged in 200 ml solution containing 0.25 g of bactericide and fungicide for 30 min, and 2.25 mg/ml of rifampicin for 4 h. The explants were then submerged in 2% *Clorox*<sup>®</sup> bleach for 15 min, 1.5% *Clorox*<sup>®</sup> bleach for 15 min, 1% *Clorox*<sup>®</sup> bleach for 10 min and 0.1% HgCl<sub>2</sub> for 10 min. The explants were washed twice with sterile water and then cultured in growth media for initiating buds. There were three types of growth media for initiating buds, each with a different PGR composition. The media used were all Murashige-Skoog (MS) with 30 g sucrose and 100 mL coconut water added for a one-Litre media. The first medium formulation contained 0.5 mg/L *Benzylaminopurine* (BAP) and NAA in three different concentrations i.e. 0.5, 1.0 and 1.5 mg/L. The explants were stored in an incubation room with light intensity 2000 lux for 16 h with a room temperature of about 24 ± 1°C for shoot and root multiplication.

### Shoot Multiplication

The shoot multiplication media were  $\frac{1}{2}$  MS and MSO, and to each was added BAP in five different concentrations i.e. 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. Each treatment was replicated 10 times. The variables observed were the number of shoots in the 10<sup>th</sup> week. The explants were then subcultured in the optimal medium.

### Root Induction

The growth medium for root induction was an MSO basal medium supplemented with NAA in five different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). The experiment was repeated 10 times. The variables observed were the length and number of roots in the 10<sup>th</sup> week.

### Acclimatisation

Plantlets which had many roots were acclimated after the 10<sup>th</sup> week. The plantlets, especially their roots, were washed with water and submerged in 1 g/L of Agrimycin bactericide and 0.25 g Benlate fungicide solutions for 3 min each. The plantlets were then acclimated in a medium composed of husk and compost in the ratio of 1:1 in plastic bottles in a greenhouse. Watering was done twice a day to maintain humidity. The variable observed was the viability percentage of plantlets during four weeks of pre-acclimatisation. Plants which could withstand the pre-acclimatisation period

were planted in media containing soil and compost in the ratio of 1:1. These plantlets were maintained in a greenhouse. The parameters observed were the plantlet's viability percentages for a period of four weeks.

### Research Design and Statistical Analysis

The experimental design of this experiment was a completely random design with eight replications. The statistical analysis of the average number of shoots was done at the 10<sup>th</sup> week. The statistical analyses used were the normality test Kolmogorov-Smirnov, followed by an analysis of variance (ANOVA) test for normally distributed data.

## RESULTS AND DISCUSSION

### Induction and Initiation of Explant

Rodent Tuber explants from Medan, Indonesia were cultured in MS medium supplemented with BAP and NAA. The part of explant used for initiating buds formation was the bud on the mother plant's tuber. Buds were used as explants for micropropagation because they contain meristematic somatic cells. According to Nobakht et al. (2009), the bud eye of Rodent Tuber is a potential explant for micropropagating Rodent Tuber in *in vitro* culture.

An *in vitro* culture medium of Rodent Tuber was MS combined with 0.5 mg/L of BAP and three different concentrations

of NAA i.e. 0.5, 1.0 and 1.5 mg/L. The percentages of sterile and viable explants in different media were supplemented with 0.5 mg/L BAP and NAA and 0.5 mg/L BAP and 1.5 mg/L NAA, both at 100% (Table 1).

Table 1  
*Percentage of sterile and viable explants in initiation media*

The composition of growth medium (mg/L)		The percentage of sterile and viable explants*
BAP (mg/L)	NAA (mg/L)	
0.5	0.5	100%
0.5	1.0	66.6%
0.5	1.5	100%

\* The number of explants initiated for each treatment was three explants

Sterile and viable explants were obtained from all of the three types of media during the initiation stage. The percentage of sterile explants in medium containing 1.0 mg/L NAA was quite low i.e. 66.6% because the explants were contaminated with fungi.

At the bud induction and initiation stages, the percentage of sterile and viable explants was determined by the type, concentration and time of sterilisation. Organic and inorganic components of the medium were the determining factors of differentiation and de-differentiation (for example, formation of meristem-interfascicular cambium and cork cambium from fully differentiated parenchyma cells) processes. PGRs largely contributed to the plant's morphogenesis (George & Sherrington, 1984). In this research, the MS medium to which had been added sucrose and coconut water was able to fulfil the explant's requirements of macro- and micronutrients. The addition of BAP

is important for mitosis cell division and inducing bud formation, while NAA is for cell division and root induction. Roots will be induced if the ratio of NAA to BAP is higher than one (George & Sherrington, 1984). According to Nobakht et al. (2009), the application of BAP as cytokinin, either alone or combined with NAA as auxin, is effective in increasing the proliferation rate of buds and the fresh weight of Rodent Tuber explant (Syahid & Kristina, 2007).

### **Multiplication**

Rodent Tuber from Medan was propagated in two different types of growth media i.e.  $\frac{1}{2}$ MS and MS. The  $\frac{1}{2}$ MS medium contained half of the macro- and micronutrients of basal MS medium. Explants that were cultured in the  $\frac{1}{2}$ MS medium produced a higher number of shoots. The highest increase in the number of shoots was achieved in the medium supplemented with 1.5 mg/L of BAP (Figure 1).

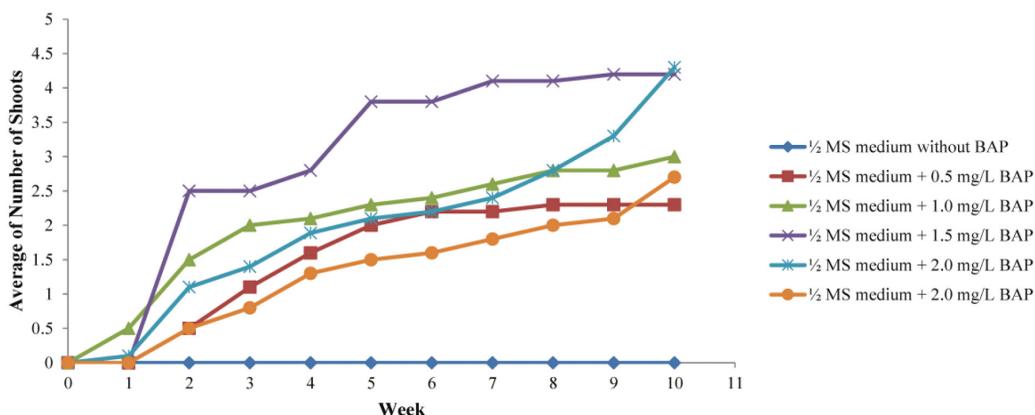


Figure 1. The increase in the number of shoots in various 1/2MS media supplemented with BAP

The highest number of shoots i.e. 4.20 was achieved on 1/2MS medium supplemented with 1.5 mg/L of BAP. A Duncan test at the accuracy level of 5% showed that the number of shoots in 1/2MS medium supplemented with 1.5 mg/L BAP was significantly different from that produced by the control, 1/2MS with 0.5

mg/L BAP, 1/2MS with 1.0 mg/L BAP and 1/2MS with 2.5 mg/L BAP. Meanwhile, the average number of shoots in the 1/2MS medium supplemented with 1.5 mg/L of BAP was not significantly different from that produced by the 1/2MS medium supplemented with 2.0 mg/L of BAP (Table 2 and Figure 4(c)).

Table 2  
Multiplication of Rodent Tuber from Medan in 1/2MSO medium

Media	Average number of shoots (mean ± SD)*
T0=1/2 MS + 0 mg/L BAP	0.00±0.00 <sup>a</sup>
T1=1/2 MS + 0.5 mg/L BAP	2.30±1.49 <sup>b</sup>
T2=1/2 MS + 1.0 mg/L BAP	3.00±.15 <sup>bc</sup>
T3=1/2 MS + 1.5 mg/L BAP	4.20±1.03 <sup>d</sup>
T4=1/2 MS + 2.0 mg/L BAP	4.10±0.88 <sup>cd</sup>
T5=1/2 MS + 2.5 mg/L BAP	2.70±1.95 <sup>b</sup>

\* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

The increasing concentration of BAP up to 2.5 mg/L lowered the number of shoots. This showed that a relatively low concentration of PGR will greatly affect the differentiation process. Like the 1/2MS treatment, the MSO medium supplemented

with BAP was also able to induce the production of new shoots every week. The MSO medium supplemented with 2.5 mg/L BAP was the optimum medium for propagating plants *in vitro* (Figure 2).

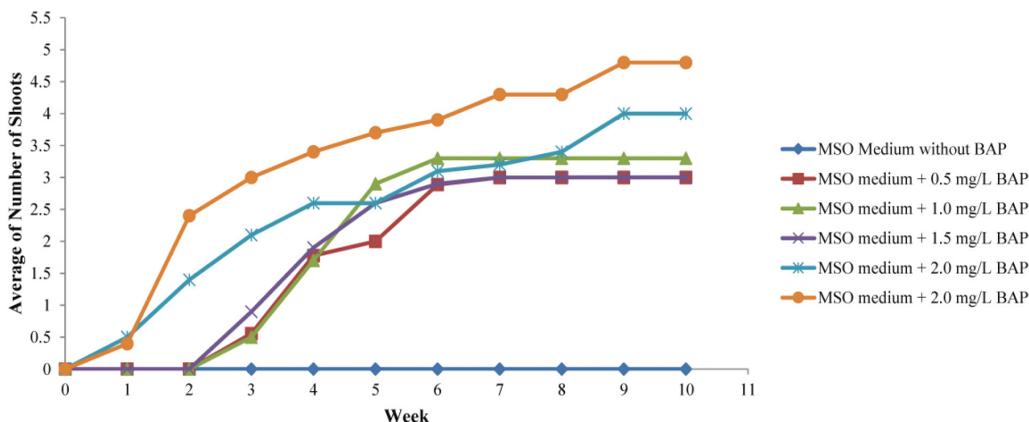


Figure 2. The number of shoots of Rodent Tuber from Medan in MS medium supplemented with BAP

Among the MS medium treatments, the highest average number of shoots was obtained by the MS medium supplemented with 2.5 mg/L of BAP. The average number of shoots in that medium reached 4.8. A Duncan test with an accuracy level of 5% showed that there were significant differences in the number of shoots between the MS medium to which had been added

BAP 2.5 mg/L and the control medium, as well as between the MS medium to which had been added 0.5 mg/L of BAP and the MS medium to which had been added 1.5 mg/L of BAP. The average number of shoots between the MS medium supplemented with 1.0 mg/L of BAP and the MS medium supplemented with 2.0 mg/L of BAP was not significantly different.

Table 3  
Multiplication of Rodent Tuber from Medan in MS medium

Media	Average number of shoots (mean ± SD)*
T0=MS+0 mg/L BAP	0.00±0.00 <sup>a</sup>
T1=MS+0.5 mg/L BAP	3.00±2.16 <sup>b</sup>
T2=MS+1.0 mg/L BAP	3.30±2.26 <sup>bc</sup>
T3=MS+1.5 mg/L BAP	3.00±1.41 <sup>b</sup>
T4=MS+2.0 mg/L BAP	4.00±1.76 <sup>bc</sup>
T5=MS+2.5 mg/L BAP	4.80±1.23 <sup>c</sup>

\* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

The ½MS and MSO basal media were able to support the growth of the plant cultures based on the observation of the number of healthy shoots produced. However, the application of the ½MS and MS without the addition of BAP was not able to increase the number of shoots. BAP is important for inducing shoot multiplication (Wattimena et al., 1992) and it can induce cell division and differentiation to produce new buds, either directly or indirectly. The same concentration of BAP can also induce shoot production of *Musa acuminata* cv. Berangan plant (Jafari et al., 2011). The application of PGR outside the safe concentration range will destroy plant tissue and inhibit bud production and cell enlargement, so plant growth will also be inhibited (George & Sherrington, 1984; Sharman et al., 2012). The same applied to *Melissa officinalis* (Tavares et al., 1996) and *Hedeoma multifolium* (Koroch, 1997). The ½MS medium to which had been added 1.5 mg/L BAP produced the highest number of shoots compared to the

other treatment with ½MS, while the MSO medium to which had been added 2.5 mg/L BAP was the optimum treatment compared to the other treatments with MSO.

### Root Induction

Root induction of *in vitro* shoots was achieved in the MS medium supplemented with five different concentrations of NAA i.e. 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L and 2.5 mg/L. Root length and average number of roots were observed in the 10th week. Root length was not increased significantly in the medium supplemented with NAA. The longest root was obtained in the MSO medium i.e. 7.79 cm (Table 4). NAA was important for root induction, according to the analysis of the average number of roots (Table 5). The highest average number roots was obtained by the culture in the MS medium supplemented with 1.5 mg/L NAA i.e. 43.20 (Figure 4D). The Duncan test with an accuracy level of 5% showed that there were significant differences between the treatments.

Table 4  
Average root length of Rodent Tuber from Medan

Media	Average length (cm) (mean±SD)*
T0=MS+0 mg/L NAA	7.79±2.97 <sup>b</sup>
T1=MS+0.5 mg/L NAA	5.66±0.57 <sup>a</sup>
T2=MS+1.0 mg/L NAA	6.11±1.39 <sup>a</sup>
T3=MS+1.5 mg/L NAA	6.14±1.56 <sup>a</sup>
T4=MS+2.0 mg/L NAA	5.14±0.92 <sup>a</sup>
T5=MS+2.5 mg/L NAA	5.36±0.58 <sup>a</sup>

\* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value ≤0.05 according to the Duncan analysis.

Table 5  
The average number of roots of Rodent Tuber from Medan

Media	Average number of roots (mean $\pm$ SD)*
T0=MS + 0 mg/L NAA	19.20 $\pm$ 10.67 <sup>ab</sup>
T1=MS + 0.5 mg/L NAA	12.50 $\pm$ 4.03 <sup>a</sup>
T2=MS + 1.0 mg/L NAA	26.20 $\pm$ 17.42 <sup>ab</sup>
T3=MS + 1.5 mg/L NAA	43.20 $\pm$ 21.21 <sup>c</sup>
T4=MS + 2.0 mg/L NAA	26.40 $\pm$ 11.92 <sup>ab</sup>
T5=MS + 2.5 mg/L NAA	28.20 $\pm$ 17.69 <sup>b</sup>

\* Each treatment was replicated 10 times. Average numbers with the same letters indicated that there were no significant differences at p-value  $\leq$ 0.05 according to the Duncan analysis.

The addition of NAA in the MS medium affected the production of the roots of the Rodent Tuber plant from Medan but did not significantly affect the elongation of roots. NAA is a synthetic auxin that is able to stimulate cell growth, cell division and the formation of fruit and roots (Wattimena et al., 1992). Auxin that has been absorbed will be metabolised through the auxin transport method and is important for stimulating the elongation of roots, formation of adventive shoots and root hairs and determining the roots' growth direction (Teale et al., 2004). The application of exogenous auxin in the culture medium was essential for lateral root formation (Thimann, 1936).

The application of exogenous auxin in a culture medium is important for initiating lateral root formation (Chhun et al., 2003). NAA in plant tissue culture medium was able to induce the formation of lateral roots of red betel plant (Sianipar et al., 2016), rice mutant *Lrt 1* (Chhun et al., 2003) and *Mellissa officinalis* (Sevik & Guney, 2013). This research generated the micropropagation method of Rodent Tuber

from Medan, Nort Sumatra (Indonesia) up to the phase of acclimatisation. Although Nobakht et al. (2009) conducted micropropagation research on the same plant with a mother plant originating from Malaysia, the different climatic conditions and the physical environment had a huge effect on the physiological conditions of the mother plant as a source of the explants. This phenomenon is a problem that is common with tissue culture even in the same location as a different isolation of plants (dry/wet climate) will affect the success of micropropagation (totipotency cells) (George & Sherrington, 1984; Wattimena et al., 1992).

### Acclimatisation

The plantlets were acclimated in media composed of husk and compost in the ratio of 1:1 in a greenhouse. After four weeks, the viability percentages of the Rodent Tuber plantlets during pre-acclimatisation and post-acclimatisation were observed (Figure 3 and 4E).

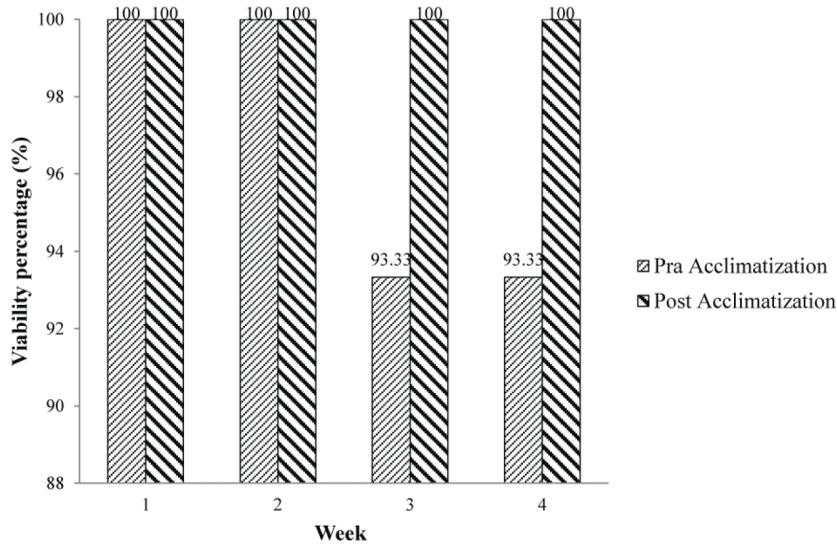


Figure 3. Acclimatisation of the plantlets of Rodent Tuber from Medan by plant tissue culture method

The viability percentage of the plantlets during pre-acclimatisation was very high i.e. reaching 93.33%. This has also been observed by Sianipar et al (2015), who showed a viability percentage of 100% from Rodent Tuber from Pekalongan. The viability percentage of Rodent Tuber from Malaysia documented by Nobakht et al. (2009) reached 90%. During post-acclimatisation, the viability percentage of Rodent Tuber from Medan even reached 100%. This was even higher than the viability percentage of Rodent Tuber from Pekalongan, which was only 58%. This result showed that different accessions/genotypes

of plants have different adaptability to environmental conditions. The role of roots in acclimatisation is crucial because roots determine the effectiveness of the absorption of nutrition and water from the medium. The ability of Rodent Tuber from Medan to withstand environmental conditions during pre-acclimatisation and post-acclimatisation is largely increased if plants have a good root system during the *in vitro* culture period. The application of auxin in an appropriate concentration is vital for supporting the growth and developmental physiological response of the root meristem (Figure 4F).



Figure 4. Micropropagation of Rodent Tuber from Medan

Note: A- Rodent Tuber from Medan used as the explant mother plant; B- Initiation in MS medium to which had been added 0.5 mg/L BAP and 0.5 mg/L NAA; C: Multiplication of shoots in the 10<sup>th</sup> week in 1/2MS medium to which had been added 1.5 mg/L BAP; D: Root induction in MS medium to which had been added 1.5 mg/L NAA; E: Pre-acclimatisation of plantlets; and F: Post-acclimatisation of the plant that had been propagated in *in vitro* culture previously.

## CONCLUSION

Rodent Tuber from Medan was able to multiply and produce a high number of shoots i.e.  $4.20 \pm 1.03$  shoots in a 1/2MS medium supplemented with 1.5 mg/L BAP in the 10<sup>th</sup> week. Rodent Tuber from Medan is unique because it can grow optimally in a 1/2MSO medium. The highest number of shoots i.e. 4.80 was obtained from the MSO medium supplemented with 2.5 mg/L BAP. The highest number of roots i.e. 43.20 lateral roots was achieved from the MSO medium supplemented with 1.5 mg/L NAA. Rodent Tuber from Medan has good adaptability to environmental conditions, with a viability percentage of 93.33% during pre-acclimatisation and 100% during acclimatisation.

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