



Shoot Generation and Callus Induction of *Dioscorea hispida* Dennst by Different Plant Growth Hormones and Basal Media

Nor Hasima Mahmod*, Zakiah Mustapha, Ahmad Hilman Ariffin Rusni, Nurul Anisah Ishak, Hafsa Jaafar

Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, Malaysia.

***Corresponding author: norhasima@unisza.edu.my**

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ABSTRACT

Dioscorea hispida Dennst produces tuber which possess valuable medicinal properties but unsustainable harvesting has led to its reduction. The plant propagates slowly because of its low tuber sprouting rate. In average, *Dioscorea hispida* Dennst tubers took approximately 60 d to break dormancy and sprout. Hence, callus culture is proposed as a possible efficient type of culture for manipulation of this species. In the present study, calli were induced from stem segments to evaluate callus culture potential of *Dioscorea hispida* Dennst. Results indicate that the combination of 1 mgL⁻¹ naphthaleneacetic acid (NAA), 1 mgL⁻¹ 6-benzylaminopurine (BAP) and 0.5 mgL⁻¹ 2,4-dichlorophenoxyacetic acid (2, 4-D) in Gamborg (B5) medium improved callus multiplication and differentiation in the stem culture as opposed to those in Murashige and Skoog (MS) medium. The findings from the present study provide the basis of callus culture protocol for stem explant of *Dioscorea hispida* Dennst with B5 being the more effective basal medium.

Keywords: *Dioscorea*, shoot generation, callus, tissue culture, plant growth hormone

INTRODUCTION

Dioscorea hispida, also known as ubi gadong in Malaysia is an intoxicating yam species from Dioscoreaceae family. This rare and exotic plant is particularly grown in the East Coast region of Malaysia and consumed especially during the rainy season (Nashriyah *et al.*, 2011). *D. hispida* is normally obtained from the wild but can be vegetatively planted using either root tuber pieces or as a whole. Depends on the tuber maturity during harvest, storage and growth conditions, *Dioscorea* spp. generally takes a relatively longer period than other tuber plants to produce tubers and to mature as seed tubers (Ondo *et al.*, 2015). Regeneration of plantlets from this species has been achieved through direct organogenesis using nodal explants (Behera *et al.*, 2008) but there is no report on callogenesis on this species.

The presence of alkaloid in its tubers, called dioscorine, combined with high level of oxalate and cyanogens are responsible for its toxic properties (Bhandari & Kawabata, 2005). If consumed untreated, the tubers can cause serious health complications such as paralysis of nervous system and diarrhea. It is the toxicity of the

tubers that have hampered its value as a commercial crop but recent studies have proven that it also possesses numerous medicinal properties. For example, it has been traditionally used to relieve rheumatism symptoms (Wagh & Jain, 2014) as well as deworming alternatives (Nashriyah *et al.*, 2012). Congruently, scientific evidence on antioxidant, anti-inflammatory, anticancer and analgesic activities have been demonstrated in *D. hispida* leaf and tuber extract (Lim, 2012). *D. hispida* was shown to be promising in preventing liver fibrosis in rats administered orally with dioscin, an active compound abundant in *D. hispida* tuber extract (Zhang *et al.*, 2015). However, the tuber extract is lethal in pregnant rats if the dose exceeded 2000 mg/kg body weight while a lower dose of 250 mg/kg body weight caused activation of hepatotoxicity-related genes (Lokman *et al.*, 2017).

Callus is a mass of cells formed from various abiotic and biotic stimuli but *in vitro*, it requires an exogenous supply of auxin and cytokinin at a certain ratio. It is generally established that a higher ratio of cytokinin to auxin promotes shoot formation, a higher ratio of auxin to cytokinin promotes root formation while high levels of both results in formation of callus. Despite (2, 4-Dichlorophenoxyacetic acid) (2,4-D) being pointed out as an ultimate callus inducer (George *et al.*, 2008), there is no golden rule for effective callus formation as the type of cytokinins or auxins greatly vary between species. Callus culture is capable of producing the same types of secondary metabolites as *in vivo* but in variable amount. For example, callus derived from shoot and root of *Moringa oleifera* Lam (Shank *et al.*, 2013) possessed significantly higher peroxidase activities than those of *in vivo*. On the other hand, in the case of *Senecio candicans* the reverse was observed as antioxidant activity of *in vitro* leaf was found lower than that of *in vivo* leaf sample (Hariprasath *et al.*, 2015). However, through callogenesis, not only the process can be shortened because the whole plant does not need to be regenerated and the number of labor works can be substantially reduced.

The potentially high medicinal benefits offered by *D. hispida* is hindered by its slow propagation rate and toxicity of the tubers it produced. Therefore, a viable and reproducible callus formation protocol for *D. hispida* is desirable as an alternative for this species manipulation. In this study, different basal media augmented along with several combinations of plant hormones were evaluated on callus induction of stem explants. The callus formed would be useful for further manipulation including callus suspension culture for recovery of secondary metabolites.

MATERIALS AND METHODS

Plant materials

A total of 304 mature tubers from Besut (N 05° 31-46', E 102° 29') and Setiu area, Terengganu, Malaysia were collected and planted as whole in polybags containing mineral soil, sand, compost (3:2:1) in a greenhouse. Misting was occasionally done to induce green sprouting. Earlier sprouted tubers were harvested for stem explant and node segments (5 mm) from 40 days old plants were taken from lateral branches.

Sterilization of Explants

Surface sterilization method was modified from Daud *et al.* (2012). In this method, explants were thoroughly washed under running tap water to remove adhering soil before being soaked in tap water for 30 min. The explants were then soaked in 0.05% (v/v) Teepol solution for another 30 min and rinsed five times with distilled water. The explants were transferred into a conical flask containing 2% (w/v) Benomyl (fungicide) solution and the flask was shaken for 30 min. The explants were rinsed five times with distilled water and immediately transferred into a laminar air flow. In the laminar air flow, the explants were submerged in 70% ethanol for 1 min, rinsed once with sterile distilled water and then soaked in 20% (v/v) commercial bleach (Clorox®: sodium hypochlorite, 5.25%) solution for 20 min. Finally, the explants were thoroughly rinsed with sterile distilled water.

Induction of Callus Formation

Stem segments were cultured either on solidified Murashige & Skoog (MS) or Gamborg (B5) medium supplemented with 0.5 mgL⁻¹ 2, 4-D and nine combinations of treatments of 1 mgL⁻¹ auxins and 1 mgL⁻¹ cytokinins (Table 1).

Table 1. Experimental matrix for induction of callus. Treatments are named by alphabets A-I. Abbreviation; BAP = 6- benzylaminopurine, KIN = kinetin, ZEA = zeatin, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid, NAA = 1-naphthaleneacetic acid.

Cytokinin (1 mgL ⁻¹)	Auxin (1 mgL ⁻¹)		
	IAA	IBA	NAA
BAP	A	B	C
KIN	D	E	F
ZEA	G	H	I

For each treatment, nine replicates consisting of six stem segments per replicate were inoculated for callus induction and they were incubated at 25 °C in complete dark. Photographs were taken after 30 d of inoculation, the morphology of callus was recorded and the mean of callusing percentage was calculated. The experiments were performed according to completely randomized design (CRD).

Data Analysis

Observations were performed twice a week and data were recorded for percentage of callus formation. The callus morphology was evaluated based on its color and texture. The rate of callus growth was graded by cross comparison and represented as (+) symbol. The (+) symbol represents poor callusing, (++) represents moderate callusing and (+++) represents profuse callusing. Data were analyzed using one-way Analysis of Variance (ANOVA) from SPSS version 12 software. Multiple comparisons were done using Duncan's Multiple Range Test (DMRT). Data were expressed as mean $\pm$ SD. Student t-test was performed at $p=0.05$, two-tailed level of significance to test if there was difference in the application of different basal medium.

RESULTS AND DISCUSSION

Low tuber sprouting rate suggests the needs for tissue culture intervention

Tubers collected were of various sizes, weighing from 10 to over 1500 g but a vast majority ranged from 100 - 499 g or medium size. The lack of uniformity in the size of the tubers had a major influence on the success of sprouting (Fig. 1). Out of 304 mature tubers planted, only 26 or 8.5% sprouted with the highest percentage in tubers of weight less than 100 g. The greater size and weight of tubers are strongly related to increased maturity which normally is attributed to decreased duration of dormancy (Otrosky & Struik, 2008) but this was not observed in *D. bispida* as none of the tubers over 1500 g germinated. However, it is erroneous to claim that the frequency of sprouting to be weight-dependent as only 4% of larger group size (100-499 g) sprouted despite being the group with the highest number of tubers collected.

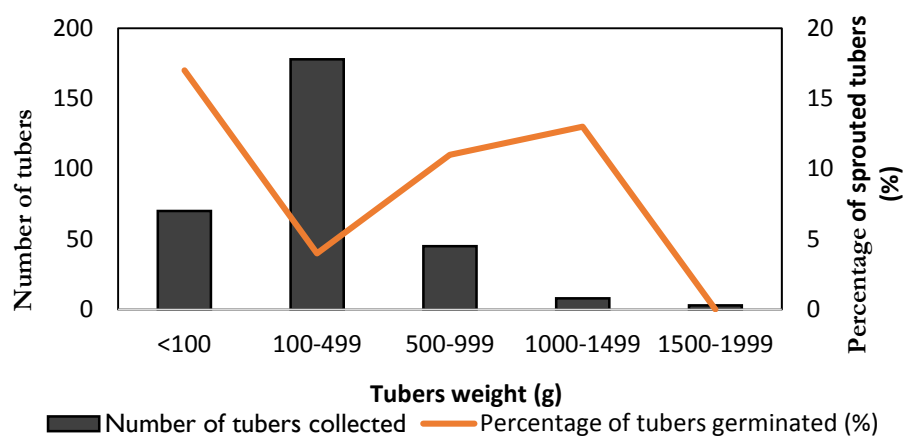


Fig. 1. Germination pattern of *D. hispida* according to tubers weight (g)

Other factors that may influence variation in germination of tubers planted in a controlled environment such as in greenhouse include the number of eyes (germination point) and wounding of the tubers that have possibly occurred during handling. Cutting the tubers before planting is a normal practice to accelerate sprouting but it may increase the risk of fungal infection. For example, the miniset technique which has been practiced in the multiplication of *D. alata* was proved to be independent of sizes but still require proper treatments with fungicides (Ayankanmi *et al.*, 2005).

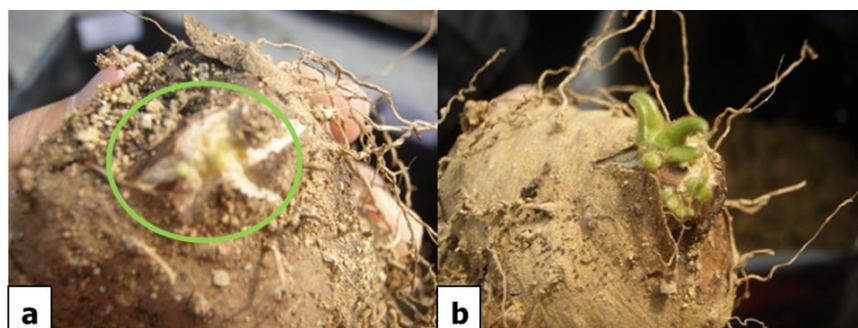


Fig. 2. Protuberance of shoots from sprouting locus (circled). The time taken between photograph a and b is 23 d

In average, *D. hispida* tubers took approximately 60 d to break its dormancy and sprout (Fig. 2). This is about four times longer than important tuber crop like potato but is rather similar with other *Dioscorea* species like *D. rotundata* (white yam) (Ile *et al.*, 2006) and *D. alata* (water yam) (Shiwachi *et al.*, 2013). Unlike potatoes, yam, in general does not possess any internal or external apical shoot buds but only a layer of meristematic cells below the surface of the tuber and this explains the extended dormancy. Essentially, prolonged dormancy accounts for economic losses as crop production cycle per year becomes limited and supply becomes restricted. Therefore, shortening dormancy has become a priority for breeders but the control of dormancy is still poorly understood (Olivier *et al.*, 2012).

B5 is more suitable basal media for callus induction

Overall, all treatments were capable of inducing callus in which the calli formed shortly 9 d after incubation. The highest mean percentage for callus formation was shown in treatment A/B5 (1 mgL⁻¹ IAA + 1 mgL⁻¹ BAP) and D/MS (1 mgL⁻¹ IAA + 1 mgL⁻¹ KIN) with 89% callus formation but a more profuse callus growth was observed in the former. The lowest mean percentage was shown by treatment I/MS medium (1 mgL⁻¹

NAA + 1 mgL⁻¹ ZEA) with only 41% callus formation. Together with treatment E/MS, they were statistically low in terms of callus percentage formation (Table 1). In both types of basal media, auxin and cytokinin combination as in treatment B and C tended to produce a higher callus growth than the other treatments. Both B and C contain BAP suggesting it as an indispensable key regulator to promote its formation. Behera *et al.* (2008) also used hormone combination as in treatment C but managed to achieve direct shoot organogenesis mainly because their treatment was devoid of 2,4-D which is an efficient callus-inducing hormone in many plant species (Tahir *et al.*, 2011). Also, the concentration of both hormones were slightly different as 2.0 mgL⁻¹ BAP + 0.5 mgL⁻¹ NAA were added instead of 1.0 mgL⁻¹ of each hormone. On the other hand, G, H and I combination showed the least favorable condition for callus induction and this is most likely caused by the inhibitory effect of ZEA. Similar to other cytokinins, ZEA which is a naturally occurring plant hormone isolated from genus *Zea* functions to promote cytokinesis or cell division but not in this case. Likewise, ZEA has also been shown to decrease callus formation in *Labisia pumila* stem explant at a higher concentration (Ling *et al.*, 2013).

Regardless of auxin/cytokinin combinations, all calli in MS medium were clear and soft in appearance (Table 2, Fig. 3). On the other hand, treatment A, B, D and F using B5 as basal medium were slightly white with a soft and spongy texture. However, none of the treatments produced calli with distinct embryo-like or organ-like structures observable on their surface (Table 2, Fig. 4). Except for some treatments, calli formed were prone to phenolic browning which tended to appear 15 d after incubation. Exudation of phenolics can lead to retardation of cell division and ultimately death of cell and tissues. Some species are prone to browning than the others but the common way to resolve this problem is to incorporate antioxidants like ascorbic acid (Ndakidemi *et al.*, 2014) and activated charcoal (López-Pérez *et al.*, 2015) in the media preparation.

Table 2. Callusing rate and characteristics of callus produced according to treatments using MS and B5 as basal media

Treatment	Percentage of callus formation (%) (SD)	Callus color / Texture	Degree of callus growth
MS			
A	76 ± 25 ^b	Clear, soft	++
B	87 ± 20 ^b	Clear, soft	+++
C	84 ± 20 ^b	Clear, soft	+++
D	89 ± 8 ^b	Clear, soft	++
E	54 ± 23 ^a	Clear, soft	+
F	76 ± 17 ^b	Clear, soft	+
G	85 ± 13 ^b	Clear, soft	+
H	75 ± 12 ^b	Clear, soft	+
I	41 ± 18 ^a	Clear, soft	+
B5			
A	89 ± 12 ^a	White, soft	+++
B	78 ± 20 ^a	White, soft	+++
C	72 ± 17 ^a	Clear, soft	+++
D	69 ± 13 ^a	White, soft	+++
E	72 ± 22 ^a	Clear, soft	+++
F	72 ± 19 ^a	White, soft	++
G	85 ± 18 ^a	Clear, soft	++
H	84 ± 21 ^a	Clear, soft	++
I	70 ± 24 ^a	Clear, soft	+

Means denoted with identical letters are not significantly different at $P \leq 0.05$ Duncan's multiple comparison, n=9. Callus growth rating value= (+) poor, (++) moderate, (+++) profuse.

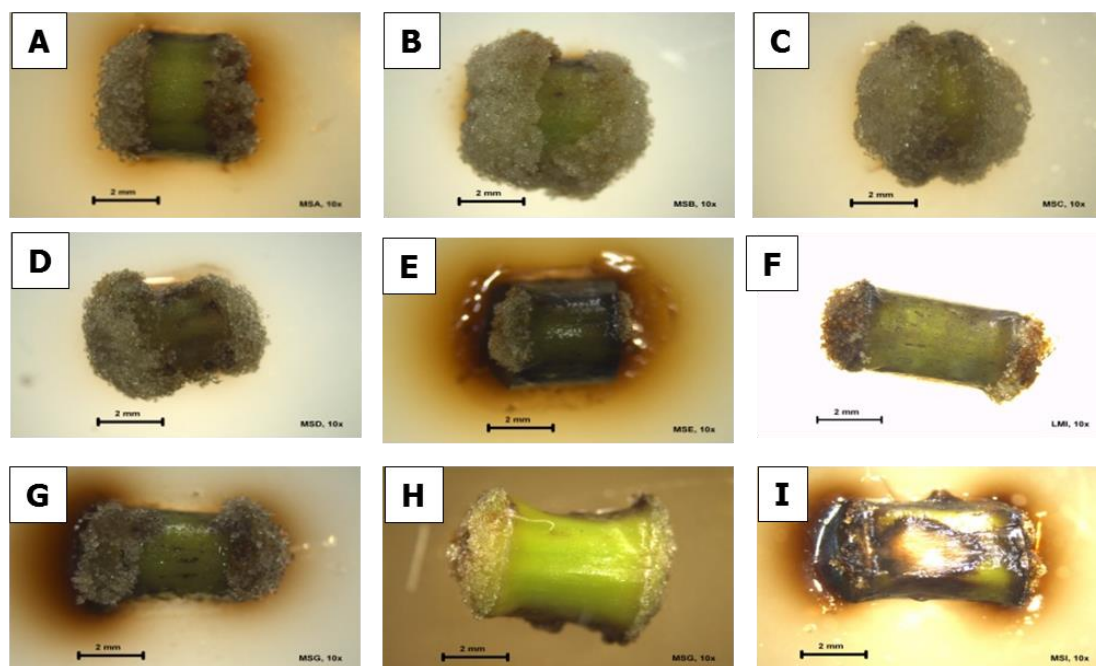


Fig. 3. Photographs of calli induced in MS medium using various combinations of auxins and cytokinin at 30 d after induction (Table 1). All images were captured under a light microscope at 10X magnification. Scale bar = 2 mm.

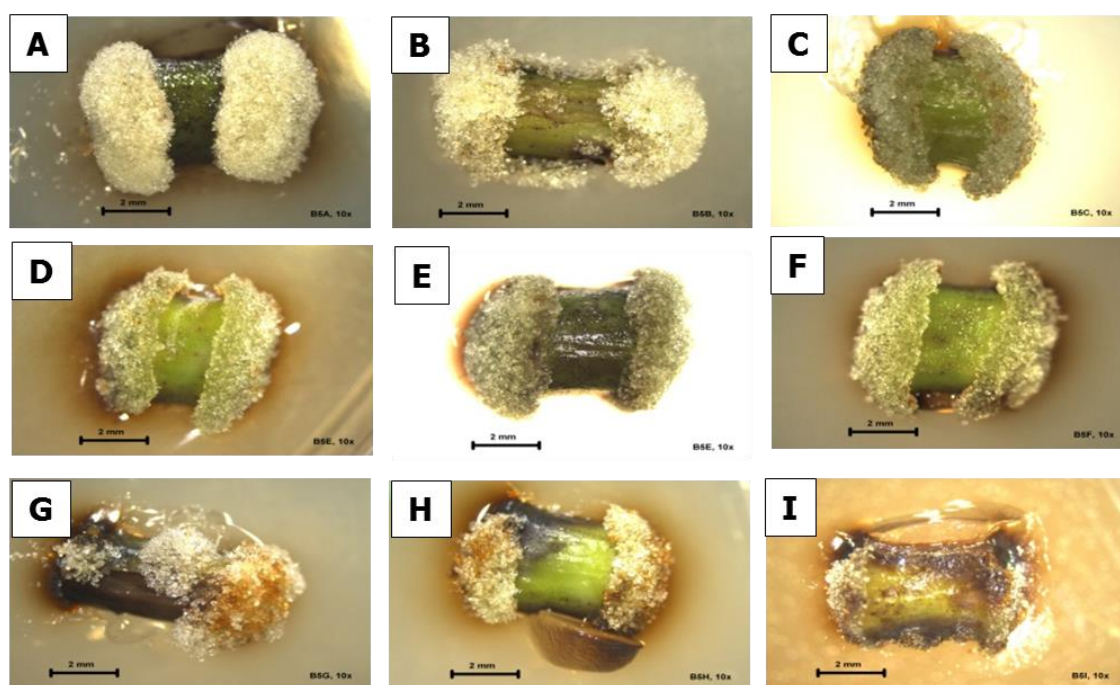


Fig. 4. Photographs of calli induced in a B5 medium using various combinations of auxins and cytokinin at 30 d after induction (Table 1). All images were captured under a light microscope at 10X magnification. Scale bar = 2 mm.

Morphological observation and degree of callus formation suggests that callus growth was greater in most treatments using B5 as a basal medium, as compared to those using MS medium. Although, there is no statistically significant difference between the two media (Student t-test, $p > 0.05$) there were two treatments in the MS experiment which showed statistically low callus formation. The findings is consistent with Belarmino and Gonzales (2008) who also found MS as an incompatible medium for their nodal and shoot tip culture.

CONCLUSION

The arbitrary pattern of sprouting suggests that callus culture offers an alternative to exploit *D.hispida*. Based on callus morphology, color and growth in stem culture, B5 is more compatible as a basal medium compared to MS. Essentially, incorporation of antioxidants is may be desirable to prevent phenolic browning in the cultures.

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REFERENCES

- Ayankanmi, T., Shiwachi, H., & Asiedu, R. (2005). Sprouting and yield of yam (*Dioscorea* spp.) minisetts in relation to sett size, soil moisture and agro-ecology. *Tropical Science*, 45(1), 23-27.
- Behera, K. K., Sahoo, S., & Prusti, A. B. (2008). Effect of plant growth regulator on *in vitro* micropropagation of 'bitter yam' (*Dioscorea hispida* Dennst.). *International Journal of Integrative Biology*, 4(1), 50-54.
- Belarmino, M. M., & Gonzales, J. R. (2008). Somatic embryogenesis and plant regeneration in purple food yam (*Dioscorea alata* L.). *Annals of Tropical Research*, 30(2), 22-33.
- Bhandari, M. R., & Kawabata, J. (2005). Bitterness and toxicity in wild yam (*Dioscorea* spp.) tubers of Nepal. *Plant Foods for Human Nutrition*, 60(3), 129-135.
- Daud, N. H., Jayaraman, S., & Mohamed, R. (2012). Methods Paper: An improved surface sterilization technique for introducing leaf, nodal and seed explants of *Aquilaria malaccensis* from field sources into tissue culture. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 2, 55-58.
- George, E. F., Hall, M. A., & De Klerk, G. J. (2008). *Plant propagation by tissue culture 3rd Edition*. The Netherland: The Back Ground Springer.
- Hariprasath, L., Jegadeesh, R., Arjun, P., & Raaman, N. (2015). *In vitro* propagation of *Senecio candicans* DC and comparative antioxidant properties of aqueous extracts of the in vivo plant and in vitro-derived callus. *South African Journal of Botany*, 98, 134-141.
- Ile, E. I., Craufurd, P. Q., Battey, N. H., & Asiedu, R. (2006). Phases of dormancy in yam tubers (*Dioscorea rotundata*). *Annals of Botany*, 97(4), 497-504.
- Lim, T. K. (2012). *Dioscorea hispida*. In *Edible Medicinal and Non-Medicinal Plants*, Springer, Netherlands. 659 pp.

- Ling, A. P., Tan, K. P., & Hussein, S. (2013). Comparative effects of plant growth regulators on leaf and stem explants of *Labisia pumila* var. *alata*. *Journal of Zhejiang University. Science. B*, 14(7), 621–631.
- Lokman, E.F, Muhammad, H., Awang, N., Hasyima Omar, M., Mansor, F., & Saparuddin, F. (2017). Gene expression profiling associated with hepatotoxicity in pregnant rats treated with ubi gadong (*Dioscorea hispida*) extract. *International Journal of Biomedical Science*, 13(1), 26–34.
- López-Pérez, A. J., Carreño, J., Martínez-Cutillas, A., & Dabauza, M. (2015). High embryogenic ability and plant regeneration of table grapevine cultivars (*Vitis vinifera* L.) induced by activated charcoal. *VITIS-Journal of Grapevine Research*, 44(2), 79.
- Ma, Y., Sawhney, V. K., & Steeves, T. A. (1993). Staining of paraffin-embedded plant material in safranin and fast green without prior removal of the paraffin. *Canadian Journal of Botany*, 71(7), 996-999.
- Mendoza, A. B., Hattori, K., Nishimura, T., & Futsuhara, Y. (1993). Histological and scanning electron microscopic observations on plant regeneration in mungbean cotyledon (*Vigna radiata* (L.) Wilczek) cultured *in vitro*. *Plant Cell Tissue Organ Culture*, 32(2), 137-143.
- Nashriyah, M., Nur Athiqah, M. Y., Syahril Amin, H., Norhayati, N., Mohamad Azhar, A. W., & Khairil, M. (2011). Ethnobotany and distribution of wild edible tubers in Pulau Redang and nearby islands of Terengganu, Malaysia. *World Academy of Science, Engineering and Technology*, 60, 1832-1835.
- Nashriyah M., Salmah T., Nur Atiqah M.Y., Siti Nor Indah O., Muhamad Azhar A.W., Munirah S., Nornasuha Y., Abdul Manaf A. (2012) Ethnobotany and distribution of *Dioscorea hispida* Dennst. (Dioscoreaceae) in Besut, Marang and Setiu Districts of Terengganu, Peninsular Malaysia. *World Academy of Science, Engineering and Technology*, 72, 240–243
- Ndakidemi, C. F., Mneney, E., & Ndakidemi, P. A. (2014). Effects of ascorbic acid in controlling lethal browning in *in vitro* culture of *Brachylaena huillensis* using nodal segments. *American Journal of Plant Sciences*, 5(1), 187.
- Olivier K. A., Konan K. N., Anike F. N., Agbo G. N., & Dodo H. W. (2012). *In vitro* induction of minitubers in yam (*Dioscorea cayenensis*-*D. rotundata* complex), *Plant Cell Tissue Organ Culture*, 109(1), 179-189.
- Ondo Ovono, P., Kevers, C., & Dommes, J. (2015). Behavior of various types of seeds of two species of yams tuber (*Dioscorea cayenensis* Lam. and *Dioscorea rotundata* Poir.) in Gabon. *International Research Journal of Agricultural Science and soil Science*, 5, 58-66.
- Otroshy, M., & Struik, P. C. (2008). Effects of size of normal seed tubers and growth regulator application on dormancy, sprout behaviour, growth vigour and quality of normal seed tubers of different potato cultivars. *Research Journal of Seed Science*, 1(1), 41-50.
- Shank, L. P., Riyathong, T., Lee, V. S., & Dheeranupattana, S. (2013). Peroxidase activity in native and callus culture of *Moringa oleifera* Lam. *Journal of Medical and Bioengineering*, 2(3), 163-167.
- Tahir, S. M., Victor, K., & Abdulkadir, S. (2011). The effect of 2, 4-Dichlorophenoxy acetic acid (2, 4-D) concentration on callus induction in sugarcane (*Saccharum officinarum*). *Nigerian Journal of Basic and Applied Sciences*, 19(2), 213-217.
- Shiwachi, H., Chen, S., Matsumoto, R., Sanada, A., & Toyohara, H. (2013). Anatomical observation on release of tuber dormancy in water yam (*Dioscorea alata* L.). *African Journal of Agricultural Research*, 8(16), 1365-1369.

Wagh V. V. & Jain A. K. (2014) Ethnomedicinal uses of underground plants parts in Jhabua district, Madhya Pradesh, India. *Advances in Biological Research*, 8(4), 151-156.

Zhang, X., Han, X., Yin, L., Xu, L., Qi, Y., Xu, Y., & Peng, J. (2015). Potent effects of dioscin against liver fibrosis. *Scientific reports*, 5, 9713.

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