*BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA*, March 2018. Vol. **15**(1), p. 51-58

# Optimization of Culture Media Formulations for Micropropagation of *Lepisanthes fruticosa*

**Zuraida Ab Rahman[1], Mohd Shukri Mat Ali[2], Mohd Norfaizal Ghazalli[2], Khadijah Awang[2] and Ayu Nazreena Othman[1]**

[1]Biotechnology & Nanotechnology, Research Centre, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia.
[2]Genebank and seed centre, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia.

http://dx.doi.org/10.13005/bbra/2607

(Received: 11 February 2018; accepted: 23 February 2018)

**Tissue culture provides an avenue for the production of high quality clonal plants in large numbers within a short time. Here, we describe the development of protocols for reproducible *in vitro* micropropagation of *Lepisanthes fruticosa via* direct organogenesis. Shoots were initiated from two types of explants, nodes and young shoots, to establish *in vitro* cultures on Murashige and Skoog's (MS) medium or Woody Plant Medium (WPM) supplemented with different concentrations of benzylaminopurine (BAP). Semi-solid WPM media containing 1 mg/L BAP was most effective in shoot initiation in both node and young shoot explants, giving 40% and 20% shoot induction, respectively. The highest rate of shoot proliferation from young shoot explants was obtained using BAP at 3.0 mg/L in combination with NAA at 1.0 mg/L in WPM culture medium. This combination of growth regulators in the medium was also suited to root initiation.**

**Keywords:** BAP, *Lepisanthes fruticosa*, micropropagation, sterilization, shoot initiation.

Malaysia possesses a rich diversity of tropical fruit trees. Besides those yielding commercial fruits, there are many other species that produce fruits that are not so popular or well-known with consumers. Many of the latter species have great potential for commercialization, but they remain under-exploited. Such fruits may, in fact, be locally abundant but they are globally rare, and scientific information or knowledge about them is limited (Gruere *et al.*, 2009). These fruits, usually consumed locally, have a broad range of flavours and colours, and more importantly, have potential health benefits (Ikram *et al.*, 2009). Some of these lesser-known fruits are sold at night markets, '*pekan sari*', or along the roadside. *Lepisanthes fruticose*, known as *Ceri Terengganu* in Malaysia, is a non-seasonal fruit. The fruits are borne in bunches or clusters, with about 20 fruits per bunch. Each fruit contains 1 – 3 seeds and its flesh is soft and sweet. The tree has a spreading canopy and grows to a height of about 2 m. Young leaves that are purplish in colour add to the attractiveness of the tree (FRIM, 2012). *Lepisanthes fruticosa* is an under-utilised tree which has potential to be

*Corresponding author E-mail: azuraida@mardi.gov.my

exploited for commercial production of its fruits, especially in view of its potential pharmacological properties.

Before this fruit tree can be commercialized, a reliable source of planting material has to be readily available. The micropropagation technique offers a method of propagation which can provide an adequate supply of planting materials. Thus, the aim of this study was to establish an efficient micropropagation protocol for plant tissue culture of *Lepisanthes fruticosa* which can be used for the mass production of planting materials.

## MATERIALS AND METHODS

Healthy plants of *Lepisanthes fruticose*, 5 - 6 months old, were maintained in a glass house for a few weeks prior to the excision of explants for establishment *in vitro*. After defoliation, the aerial parts of the plants were harvested and washed in running tap water for 1 h. These explants were cut into 2-3 cm pieces of young shoots (Figure 3a) or stem segments bearing nodes (Figure 3b).

The main sequences of explant sterilization are shown in Table 1. The plant segments were kept in a conical flask and thoroughly rinsed under running water for 1 hour Following this, the plantlets were treated with the fungicide solution (5% Bavestin) for 30 min or 1 hour before rinsing with distilled water. The explants were transferred to laminar air flow chamber where they were surface sterilized with 50% of Clorox® containing several drops of Tween-20 for 15 or 30 min on a rotary shaker. Two further cycles of sterilization using 30% or 5% Chlorox were performed. After rinsing with sterile water, the sterilized explants (Figure 3c) were cultured onto Murashige and Skoog's (MS) (1962) basal medium or Woody Plant Medium (WPM) (1981).

In experiments to examine the use of MS medium in shoot initiation, the culture medium was prepared with 3% sucrose and 0, 1 or 3 mg/L of the cytokinin 6-benzylaminopurine (BAP) supplemented. Gelrite agar 3% (solid) and 1.5% (semi solid) was added for gelling. The pH of the medium was adjusted to 5.8 prior to autoclaving (15 min, 121$^{o}$C). The cultures were incubated in a culture room maintained at 25±2°C, under white fluorescent light at an intensity of 3000 lux, with a photoperiod of 12 hours light and 12 hours darkness. Cultures were checked regularly for contamination; those showing signs of infection were immediately discarded after recording. Data were expressed as percent of surviving cultures, shoot initiation, and appearance of browning after 30 days of culture.

In another set of experiments to examine the use of WPM in shoot initiation, the culture medium was prepared with 0.3% (solid) or 0.15% (semi-solid) Gelrite agar for gelling, and supplemented with 3% sucrose. BAP was added in concentrations of 0, 0.5, 1, 3 and 5 mg/L) to induce shoot formation. Sterilization was performed by autoclaving at 121$^{o}$C for 15 min. The pH was adjusted to 5.7-5.8 before agar was added. Explants were inoculated into 150 ml flask containing 40 mL of the selected medium. Ten replicate flasks were used for each medium treatment. Each flask contained 3 explants which were in contact with the medium surface. The cultures were incubated in a plant growth room at a temperature of 25 $^{o}$C±1 and under a 16 hour light: 8 hour darkness photoperiod. Lighting was supplied by cool-white fluorescent lamps (1000–2000 lux). Results were expressed as percent of shoot induction, number of shoots per explants and mean shoot length after 45 days of culture.

Besides studies on shoot initiation, separate experiments were conducted to optimize shoot multiplication. Cultures were established on basal WPM medium supplemented with either BAP (0, 1.0, 3.0 and 5.0 g/L), NAA (0 or 1.0 mg/L) or combinations of both growth regulators. Ten replicated flasks were used in each treatment and observations were recorded after 45 days of culture. The results were expressed as percent explants showing shoot proliferation, the number of shoots per culture, and number of roots per explant. Complete plantlets produced *in vitro* were removed from the culture medium and the roots were washed to remove the agar. The plantlets were then transferred into polybag containing organic soil mixed with garden soil (1:1) and placed in the net house under controlled conditions, with 75% shading and temperatures between 28ÚC to 32ÚC. To maintain humidity, the plants were watered twice a day. Observations were recorded on the percent survival of rooted and acclimatized plants. Means and standard errors of the means were calculated for the degree of response.

## RESULTS AND DISCUSSION

### Explant sterilization

Contamination and low multiplication rates are the limiting factors in generating *in vitro* plantlets. Hence, optimization of the techniques to minimize contamination for the survival of explants was carried out (Table 1). Contamination of the explants was evaluated for each treatment 4 weeks after inoculation. Browning is an indication of over-sterilization that injures the explant. Over-sterilization also leads to the leaching of phenolic compounds during explant excision. When the phenolics oxidize, they turn phytotoxic and cause necrosis and death (Preece & Compton, 1991). This development was evident in all six treatments but the incidence of contamination varied.

In the sterilization treatments of nodal segment explants, Treatment 4 showed the best balance between the incidence of contamination and the incidence of tissue browning (Table 1). With this treatment, 75% of the explants were free from contamination while the incidence of browning remained low, at 10% (Figure 1a). The contamination rates in Treatments 1, 3, and 5 were even lower, but the plants nevertheless displayed higher than 70% tissue browning.

In the sterilization of explants from young shoots, Treatment 2 was the most effective with 80% of the explants remaining uncontaminated and only 10% showing symptoms of browning (Figure 1b). Uncontaminated explants were removed from the flask to prevent further contamination by those already infected. The remaining non-contaminated explants were further observed for 4 weeks for shoot induction.

### Shoot induction

In developing a simple and effective protocol for the *in vitro* micropropagation of *Lepisanthes fruticose*, we investigated the effect of the cytokinin BAP on shoot propagation. Explants were cultured on solid or semi-solid MS and WPM media supplemented with different concentration of BAP (1.0 and 3.0 mg/L) to evaluate their effect on shoot multiplication. Over the duration of 30 days, shoots developed directly from explants (nodal segments and young shoots). In general, semi-solid WPM medium supplemented with 1.0 mg/LBAP was more effective in promoting shoot than solid medium (Table 2, Figure 3 d,e,f). No shoot was induced from cultures on the solid MS medium from both types of explants used, whether node or young shoots.

Cultures of young shoots on WPM semi-solid medium with 1.0 mg/L BAP, showed on average 20% shoot induction (Table 3). Treatment with a higher concentration (3.0 mg/L) BAP decreased the percentage of shoot induction. At the same time, nodal segment cultures on semi-solid WPM medium containing 1.0 mg/L BAP gave the highest success at shoot induction (40% of explants with average shoot length of 3.4 cm), whereas solid medium showed only 5% in shoot induction at best (Table 3).

The use of BAP in tissue culture media has been found to be useful in shoot initiation. As reported in previous studies on *in vitro* proliferation of the f G xN15 (hybrid of almond x peach) vegetative rootstock, MS medium supplemented with 1 mg/L BAP resulted in the production of 8.5 new micro shoots/explant (Arab *et al.*, 2014). Many studies have been undertaken

**Table 1.** Sterilization steps of *Lepisanthes fruticosa* explants (Nodes and young shoots)

| Treat. No. | Step-1 Fungicide (5%) | Step-2 Clorox (50%)+ Tween 20 | Step-3 Clorox(30%) +Tween 20 | Step-4 Clorox (5%) + Tween 20 | Step-5 Distilled water |
|---|---|---|---|---|---|
| 1 | 30 mins | 30 mins | 0 | 10 mins | Rinse 3X with distilled water |
| 2 | 30 mins | 15 mins | 15 mins | | |
| 3 | 1 hour | 30 mins | 0 | | |
| 4 | 1 hour | 15 mins | 15 min | | |
| 5 | 1 hour | 30 mins | 15 mins | | |
| 6 | 1 hour | 15 mins | 5 mins | | |

to determine the optimal culture medium and the concentration of cytokinin for *in vitro* proliferation in woody plants (Ruzic & Vujovic, 2008). While MS is the most commonly used basal medium for micropropagation, WPM has been shown to be useful in many cases (Radmann *et al.*, 2009). In the present study, WPM has been found to be superior to MS medium in the *in vitro* proliferation of *Lepisanthes alata.* Our results are in agreement with those of Sadeghi *et al.* (2015) on Prunus empyrean 3 that showed WPM containing 0.4 mg/L BAP enhanced induced shoot number and length. In contrast, Seema and Vijaya (2015) reported that in the medicinal plant *Clerodendrum Serratum*, MS medium (supplemented with 0.5 mg/L BAP) was more effective than WPM (supplemented with 2.0 mg/L BAP) in the initiation of shoots and their subsequent growth. Different *in vitro* cultured plants can thrive on solid, semi-solid or liquid media. In the present study, the explants displayed the best shoot initiation when cultured in semi-solid medium. Working with *Cadaminelyrata*, Sakularat *et al.* (2015) observed that liquid MS medium supplemented with 0.5 mg/l NAA and 2 mg/l BA gave the highest average number of shoots (68.80 shoots/explant), followed by semi-solid MS medium (41.71 shoots/explant) and solid MS medium (28.60 shoots/explant).

**Shoot proliferation and rooting**

Culture medium containing BAP and NAA initiated shoot proliferation in all treatments tested. Various concentrations of these two growth

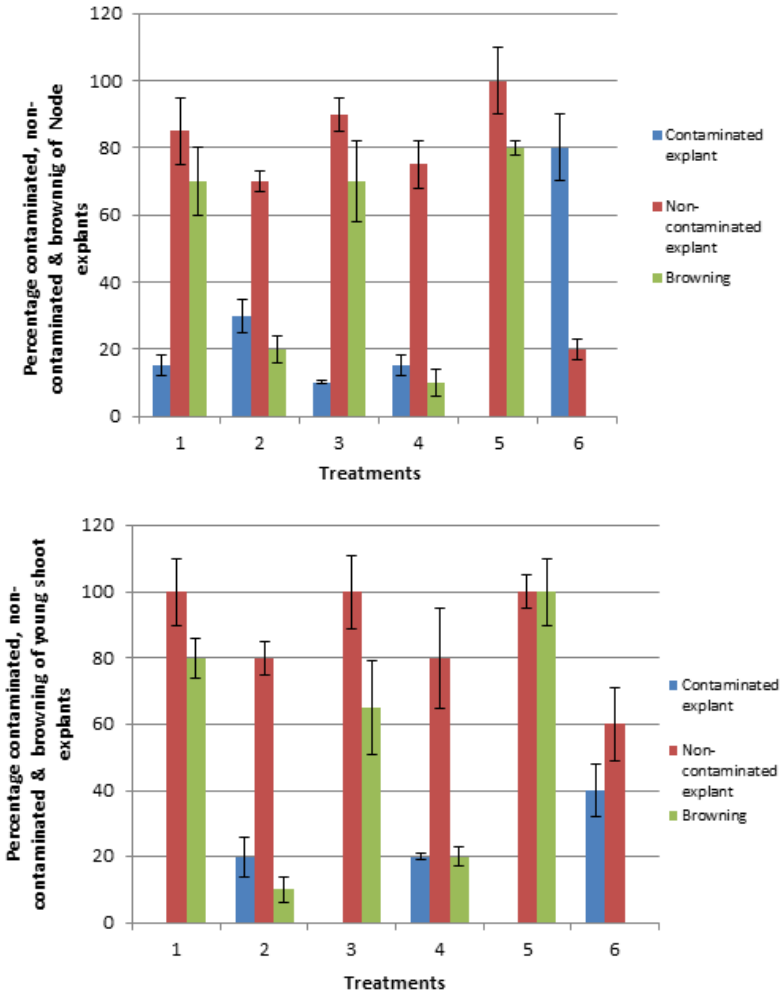


**Fig. 1.** Effect of various sterilization treatments of explants (a: nodal segments, b:young shoots) on incidence of contamination and tissue browning

regulators had an effect on the number of shoots and roots produced. The best shoot proliferation rate (occurring in 80% of the explants, 5.4 shoots/explant) was obtained on WPM containing 3 mg/L BAP+1 mg/L NAA (Table 4). This was followed by supplementation with 5 mg/L BAP + 1 mg/L NAA (shoot proliferation in 70% of the explants, 4.5 shoots/explant). Poor results were obtained with all concentrations of BAP in the absence of NAA (shoot proliferation in 40% of the explants).

**Table 2.** Effect of MS medium containing different concentrations of BAP on shoot initiation from nodal segments and young shoots (after 30 days of culture)

| Explants | Culture medium | BAPconc. mg/L | Shoot induction (%) | No. of shoot/ Explants | Mean shoot length (cm) |
|---|---|---|---|---|---|
| Node | Solid | 0 | 0 | 0 | 0 |
| | | 1 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 |
| | Semi solid | 0 | 0 | 1 | 0 |
| | | 1 | 20 | 1 | 2.4±0.5 |
| | | 3 | 10 | 1 | 2.9±0.4 |
| Youngshoots | Solid | 0 | 0 | 0 | 0 |
| | | 1 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 |
| | Semi solid | 0 | 0 | 0 | 0 |
| | | 1 | 20 | 1 | 2.1±0.2 |
| | | 3 | 0 | 0 | 0 |

**Table 3.** Effect of WPM medium containing different concentrations of BAP on shoot initiation from nodal segments and young shoots (after 45 days of culture)

| Explants | Culture medium | BAPconc. mg/L | Shoot induction (%) | No. of shoot/ Explants | Mean shoot length (cm) |
|---|---|---|---|---|---|
| Node | Solid | 0 | 0 | 0 | 0 |
| | | 0.5 | 0 | 0 | 0 |
| | | 1 | 5 | 1 | 2.1±0.1 |
| | | 3 | 5 | 1 | 1.9±0.5 |
| | | 5 | 0 | 0 | 0 |
| | Semi solid | 0 | 20 | 1 | 3.1±0.09 |
| | | 0.5 | 35 | 1 | 2.4±0.2 |
| | | 1 | 40 | 2 | 3.4±0.3 |
| | | 3 | 30 | 1 | 3.1±0.1 |
| | | 5 | 0 | 0 | 0 |
| Young shoots | Solid | 0 | 0 | 0 | 0 |
| | | 0.5 | 0 | 0 | 0 |
| | | 1 | 0 | 0 | 0 |
| | | 3 | 5 | 1 | 2.4±0.3 |
| | | 5 | 0 | 0 | 0 |
| | Semi solid | 0 | 0 | 0 | 0 |
| | | 0.5 | 5 | 1 | 2.2±0.05 |
| | | 1 | 20 | 2 | 2.3±0.10 |
| | | 3 | 0 | 0 | 0 |
| | | 5 | 0 | 0 | 0 |

**Table 4.** Effect of plant growth regulators (BAP and NAA) on microshoot propagation (% of shoot explants forming microshoots), number of microshoots per explants, and mean number of roots produced after 45 days of culture on WPM medium

| BAP (mg/L) | NAA (mg/L) | Shoot explants showing shoot proliferation (%) | Mean number of shoots/ explant | No. of roots |
|---|---|---|---|---|
| 0 | 0 | 10 | 1.2±0.23 | 3.2± |
| 1 | 0 | 40 | 1.3±0.24 | 3.1± |
| 3 | 0 | 40 | 2.5±0.91 | 3.9± |
| 5 | 0 | 40 | 2.4±0.71 | 2.2± |
| 1 | 1 | 60 | 3.3±0.41 | 4.7± |
| 3 | 1 | 80 | 5.4±0.52 | 4.1± |
| 5 | 1 | 70 | 4.5±1.25 | 3.2± |

Data are means ± SD of results from 10 replicates

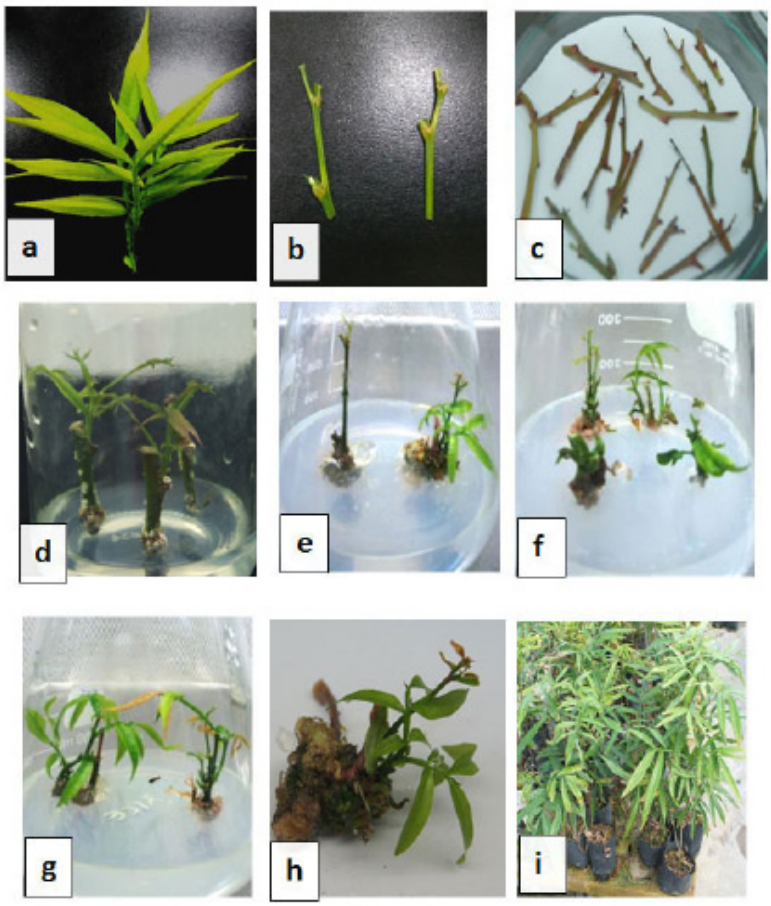


**Fig. 2.** *In vitro* micropropagation of *Lepisanthes fruticosa. The explant, young shoots (a), nodal segments (b), nodal segments after sterilization (c,), S*hoot initiation from nodal segments cultured on semi solid WPM medium with 1.0 mg/1 BAP (d,e,f). Plantlets ready for transfer into polybags in the glasshouse (g,h,i)

The number of shoots produced per explant was similarly poor (1.3 to 2.4 shoots per explant) (Table 4). Supplementation of the culture medium with 1 mg/L NAA improved root formation regardless of the BAP added (Table 4).

In plant micropropagation, a balance between two groups of growth regulators, auxins and cytokinins, controls cell division and elongation (Soledad *et al.*, 2012). The combination of BAP and NAA in tissue culture media is most effective for shoot formation. As reported in previous studies, the used of both BAP (0.5 – 1.0 mg/L) and NAA (0.02 mg/L) promoted shoot proliferation in *Sequoia sempervirens* (Boulay,1989). According to Hossain *et al*. (2016), the highest shoot regeneration was obtained using 5.0 mg/L BAP in exotic banana.Root formation was most successful on MS medium supplemented with 4.0 mg/L NAA. Findings by Zuraida *et al*. (2013) showed that nodal segments of *V. planifolia* cultured on a medium containing 1 mg/L BAP produced the highest number of shoots and the greatest lengths of shoots.

**Plant hardening and transplantation**

The *in vitro* grown complete plantlets were transferred from the culture room to polybags where they underwent successive phases of acclimatization (Figure 3g,h,i).·A survival rate 85% was achieved during acclimatization.

## CONCLUSION

In this study on the *in vitro* micropropagation of the underutilized fruit tree *Lepisanthes fruticosa*, semi solid (0.15% agar) WPM culture medium containing 1.0 mg/L BAP gave the best rate of shoot induction using explants derived from nodal segments or young shoots. The highest rate of shoot proliferation was obtained using BAP at 3.0 mg/L in combination with NAA at 1.0 mg/L. In addition, this treatment was also suited to root initiation. The plantlets obtained survived and grew normally in the greenhouse. This procedure can be recommended for rapid *in vitro* shoot micropropagation of *Lepisanthes fruticosa*.

## REFERENCES


1. Arab.Mohammad M., Abbas Yadollahi, Abdolali Shojaeiyan, Saber Shokri, Shoresh Maleki Ghojah. Effects of nutrient media, different cytokinin types and their concentrations on in vitro multiplication of G ·N15 (hybrid of almond ·peach) vegetative rootstock. *Journal of Genetic Engineering and Biotechnology* . 2014. **2**(2): 81-87.
2. Dj.V. Ruzic, T.I. Vujovic, The effects of cytokinin types and their concentration on in vitro multiplication of sweet cherry cv. Lapins (Prunus avium L.). *Horticultural Science.* 2008; **35**(1):12-21
3. Sadeghi F, A. Yadollahi b, M. Jafarkhani Kermani , M. Eftekhari. Optimizing culture media for in vitro proliferation. and rooting of Tetra (Prunus empyrean 3) rootstock. *Journal of Genetic Engineering and Biotechnology.* 2015; **13** (1): 19-23
4. Seema Upadhyay, Vijaya Koche. Comparison of Different Medium and Establishment of an Efficient Micropropagation Technique of Clerodendrum Serratum L. An Endangered Medicinal Plant. *Journal of Environmental Science, Toxicology and Food Technology,* 2015. **1**(1): 27-35
5. Sakularat Sanputawong1, Tiwa Raknim and Sorapong Benchasri. Influence of different type of Culture Media and activated charcoal on Callus Induction and ShootMultiplication of Cadaminelyrata. *Journal of Agricultural Technology* 2015; **11**(8): 1697-1704.
6. FRIM (2012). Buah ceri terengganu yang masak ranum. Retrieved on 25 Sept. 2012.from http://www.frim.gov.my/?page_id=6531&lang=ms
7. MS RM1_RM1 Rare Fruits of Malaysia Serie III. http://stamps-my.club/28-03-2006-rare-fruits-series-iii-buah-buahan-nadir/
8. Gruere, G.L., Nagarajan, O. and King, E.D.I. The role of collective action in the marketing of underutilised plant species: Lessons from a case study on minor millets in South India. 2009. *Food Policy* **34**: 39 – 45
9. Ikram, E.H.K., Eng, K.H., Jalil, A.M.M., Ismail, A., Idris, S., Azlan, A., Nazri, H.S.M., Diton, N.A.M. and Mokhtar, R.A.M. Ora antioxidant capacity and total phenolic content of Malaysian underutilised fruits. *J. Food Comp. Anal*. 2009; **22**: 388 – 393
10. M. Boulay, "4 Redwood (*Sequoia sempervirens*)," *Bio- technology in Agriculture and Forestry.* 1989. **5**: 549-573. doi:10.1007/978-3-642-61535-1_29
11. M. Soledad Diaz, P. Lorena, C. F. Ana and E. G. Marta, "*In Vitro* Propagation of Muña-Muña (*Clinopodium odo- rum* (Griseb.) Harley)," Biotechnology Research International, Vol. 2:1-6. doi:10.1155/2012/196583
12. Preece, J.E. and M.E. Compton. 1991. Problems

with explant exudation in micropropagation. In: Biotechnology in Agriculture and Forestry. (Ed): Y.P.S. Bajaj, Springer, Berlin, 168-189.

13. Hossaina Md. Anwar , Mehede Hassan Rubelb , Khondoker M. Nasiruddinc and Fatematuz Zuhura Evamonia . Influence of BAP and NAA on in vitro plantlet regeneration of local and exotic banana cultivars . *Journal of Bioscience and Agriculture Research.* 2016. **06**(02): 553-564
14. Zuraida Ab Rahman, Kamarulzaman Hassan Fatin Liyana Izzati1, Othman Ayu Nazreena1, Wan Sembok Wan Zaliha, Che Mohd Zain Che Radziah, Zainal Zamri, Subramaniam Sreeramanan. A Simple and Efficient Protocol for the Mass Propagation of *Vanilla planifolia. American Journal of Plant Sciences,* 2013, **4**: 1685-1692
15. Radmann E.B, V.J. Bianch, J.C. Fachinello, R.P. Oliveira, Sci. Agrar. 2009.10: 95–101.
16. McCown B.H. and G. Lloyd. 1981. Woody plant medium (WPM) – a mineral nutrient formulation for microculture of woody plant species. HortScience. **16**: 453-453.
17. Murashige T. and F. Skoog. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 1962; **15**: 473-497.