การประชุมเสนอผลงานวิจัยระดับชาติ มหาวิทยาลัยสุโขทัยธรรมาธิราช ครั้งที่ 6
The 6th STOU National Research Conference

In Vitro Embryo Culture in Pineapple Hybrids

Somruethai Wongwirattana[1] Varaporn laksanalamai[2] Tharathorn Teerakathiti[3]
Oranuch Leelapon[4]

Abstract

The objective of this study is to focus on germination and development of embryo culture in 4 pineapple hybrids; Smooth cayenne x Phuchawa, Phuchawa x Smooth cayenne, MD2 x Phuchawa and Phuchawa x MD2. It was found that the Smooth cayenne x Phuchawa hybrid showed the highest seed production (181 seeds). Embryogenic shoots were initiated after 7 days of culture on Murashige and Skoog (MS medium). The Phuchawa x MD2 hybrid had the highest germination rate of 25. 34%. Patterns of embryogenic development could be unambiguously categorized into 3 types; individual embryos, clusters and embryogenic calluses. All cross combination had individual embryo and cluster types. Furthermore, cluster type was the highest. Phuchawa x Smooth cayenne and MD2 x Phuchawa showed the same highest numbers of cluster type at 60%. Likewise, Phuchawa and MD2 showed the highest number of individual embryo type at 41.18%. However, embryogenic callus type was obtained only from the hybrids of Smooth cayenne x Phuchawa, and Phuchawa x MD2.

Keywords: Pineapple hybrids, Embryo culture

[1] Stady of master degree Biotechnology in Rangsit University wongwirattana.s@gmail.com

[2] Assistant Professor; Ph.D, Dean of food technology in Rangsit University varaporn@rsu.ac.th

[3] Ph.D, Researcher in National for Genetic Engineering and Biotechnology tharathorn@biotec.or.th

[4] Ph.D, Researcher in National for Genetic Engineering and Biotechnology oranuch@biotec.or.th

Introduction

Pineapple is the most economically important plant in the family Bromeliaceae. It is one of the world's most popular tropical fruits and is consumed worldwide. Fresh fruit is good source of manganese and contains significant amounts to vitamin A, B and C (Akbar, et al., 2003). Value of exports is up to 625 million tons per year, representing 20,000 million baht (Office of Agricultural Economics (OAE), 2014). Hence, pineapple is an important industrial crop of Thailand (Klakhai & Boonrod, 1992).

Pineapple originated in South America. In Thailand there are 3 main groups of pineapple varieties are grown; Spanish, Queen and Smooth cayenne. Spanish is a native variety, resistant to pests and diseases more than other varieties. Queen is locally cultivated for fresh fruit market. Smooth cayenne or Pattawia is grown for both pineapple canning industry and fresh consumption (Thongtham, 1983). At present, MD2 variety, hybrid of Smooth cayenne developed by Del Monte scientists in Hawaii, is popular in Thailand for fresh fruit consumption. It is sweeter, less fiber and contains four times more vitamin C than regular varieties, but less acidity. It satisfies the market with exceptional sweetness, uniformity and consistency in size and ripeness (Ruangaiam, 2011). In addition, Phuchawa, the hybrid variety Smooth cayenne x Phuket (Queen) from breeding programs in Thailand, is introduced in fresh consumption with light yellow flesh and sweetness (Siriphat, 2011).

Current breeding has been carried out to increase the diversity of species and to meet the needs of consumers. Generally, seed production is rare because most varieties of pineapple possess reduced fertility combined with self-incompatibility. Germination rate is low because seed coat is hard (Verawudh, 1998). Conventional breeding is tedious and time consuming. Moreover, seeds occurring from this method mostly have low germination rate as well as slow embryo development. To solve the problem, embryo culture has been used to promote the development of embryo and then shorten the period of breeding (Zhang, et al., 2003; Cisneros & Tel-Zur, 2010).

Embryo culture is the aseptic isolation and culture on the medium. Many authors have reported that embryo culture is as the tool for producing plants from hybridization in which failure of endosperm development leading to embryo abortion (Liu, et al., 2007; Salamma & Rao, 2013), and for overcoming seed dormancy (Mohan, et al., 2011). The success of this method depends on media, embryo age and culture (Reed, 2005). Plantlet develops directly from the embryo or first callus formation (Burun & Poyrazolu, 2002). Finally, the plantlet would be multiplied for mass propagation within a short time period (Phongsupasamit, 2004; Thawaro & Te-

chato, 2010). Embryo culture was the proper method for mass propagation of pineapple within a short time period (Almeida, et al., 2002; Zuraida, et al., 2011; Usman, et al., 2013; Akin-Idowu, et al., 2014). The objective of this experiment is to study growth pattern of plantlets developing from the embryos of various pineapple hybrids.

Objectives

Study on germination and development of embryo culture in pineapple hybrids

Materials and methods

The characteristics of 4 groups of pineapple hybrid fruits after fertilization for 45 day (breeder; Sarawut ruangaiam, Amornchan farm) Smooth cayenne x Phuchawa, Phuchawa x Smooth cayenne, MD2 x Phuchawa and Phuchawa x MD2 were recorded. Each pineapple fruit was peeled and washed with detergent in water. Surface sterilization was done by the immersion of the fruit in 50% of Clorox (5.25% sodium hypochlorite) for 20 minute, followed by 3 rinses in sterile distilled water. Thereafter, seeds were separated form sterile fruit and counted. Then, the embryos were excised from disinfected seeds and cultured on MS medium (Murashige & Skoog, 1962) supplemented with 30 g/l sucrose and 2.5 g/l kelcogel. The medium pH was adjusted to 5.7 using NaOH and HCl and dispensed into 8 ounce-glass bottle. The bottle was closed with plastic lid and sterilized at 121 °C and 1.5 kg/cm^2 for 30 min. The culture condition was 25 °C and photoperiod of 16 hr of light (30 μmol m^2 s^1) provided by cool white inflorescence lamps. The embryos were maintained and subcultured on the same media every 4 weeks. The germination rate was recorded by comparing geminating embryo with total embryo. Growth pattern of plantlets developing from the embryo of various pineapple hybrids were compared.

Results and Discussion

Character of pineapple hybrid fruits

The character of pineapple hybrid fruits after fertilization for 45 days was observed. The hybrid of Smooth cayenne x Phuchawa has rather big crown, wide and shallow eyes. However, the hybrid of Phuchawa x Smooth cayenne has cylindrical shape with bottleneck, small crown and big eyes like Smooth cayenne. The hybrid of MD2 x Phuchawa has rather circle shape, small crown and wide eyes like Phuchawa. The hybrid of Phuchawa x MD2 has cylindrical shape with

small bottleneck, big eyes and crown bigger than the hybrid of Phuchawa x Smooth cayenne. (Table 1)

Table 1 Character of fruit after fertilization for 45 days**;** Smooth cayenne x Phuchawa, Phuchawa x Smooth cayenne, MD2 x Phuchawa and Phuchawa x MD2

Cross combination (mother x father)	Mother plant	Father plant	F1 hybrid
Smooth cayenne x Phuchawa			
Phuchawa x Smooth cayenne			
MD2 x Phuchawa			
Phuchawa x MD2			

Character of hybrid seeds

The seed of pineapple hybrids after fertilization were found in three carpels (locules). To excise the disinfected seeds were done by carefully piercing a pointed forceps around the eye and separate seed form fruit (Figure 1). Young seed was white and turned to dark brown with hard seed coat when it became old. The white embryo inside young seed connected with endosperm (Figure 2). Seed size of the hybrid of Smooth cayenne x Phuchawa was approximately 2-3 millimeter. However, the hybrids of Phuchawa x Smooth cayenne, MD2 x Phuchawa and Phuchawa x MD2 were aprroximately 3-5 millimeter (Figure 3). Seed production of the hybrid of Smooth cayenne x Phuchawa was highest at 181 seeds, while the hybrids of MD2 x Phuchawa, Phuchawa x MD2 and Phuchawa x Smooth cayenne had 153,134 and 81 seeds respectively.

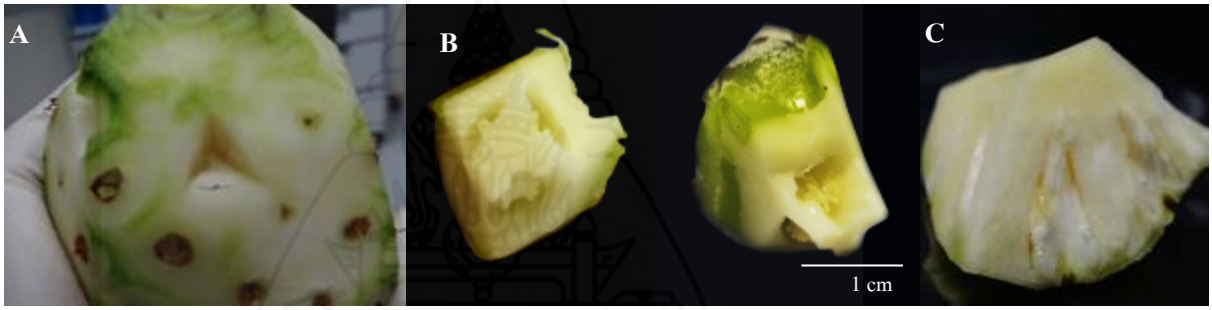

Figure 1 (A) Piercing the pineapple eyes to excise seeds from the fruit. (B) Pineapple ovary (C) Seed in ovary (locules)

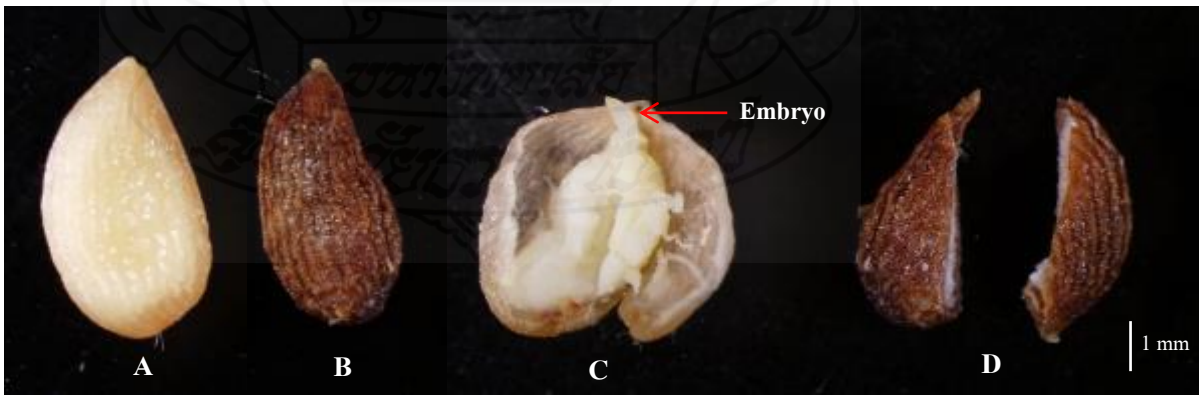

Figure 2 Pineapple seeds (A) young seed (B) old seed (C) inside young seed (D) inside old seed

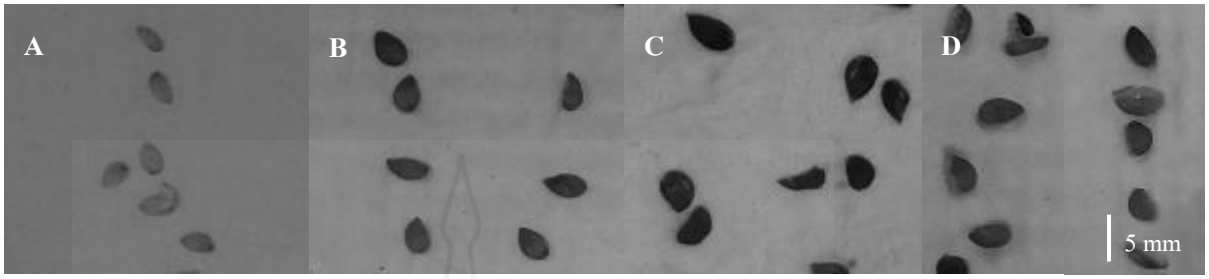

Figure 3 Pineapple hybrid seeds

(A) Smooth cayenne x Phuchawa (B) Phuchawa x Smooth cayenne

(C) MD2 x Phuchawa and (D) Phuchawa x MD2

Embryo culture in pineapple hybrids

Embryo of pineapple hybrids were excised aseptically from the seed and cultured on MS basal medium with no hormone. The germination and growth pattern of embryo were observed every 7 days for 56 days. The germination of embryo occurred obviously at 14 days after cultured on MS medium, as well as cultured embryos of pinus the embryo occurred in 1 day and the germination rate of 80% within 14 days (Stojicic, et al., 2012). Phuchawa x MD2 showed the highest germination rate at 25.37% . Smooth cayenne x Phuchawa, MD2 x Phuchawa, and Phuchawa x Smooth cayenne had the germination rate at 14.81% , 6.54% , and 5.52 % , respectively (Table 2). Pattern of embryogenic development could be divided into three types; 1) Individual embryo that developed into one plantlet. Root initiated from the radicles at 7 days after cultured. Then, shoot developed further into one plantlet within 21 days. 2) Cluster that embryo developed from the clump of shoot buds into more individual plantlets. Embryos swell up at 14 days and etiolated into clump of shoot buds within 28 days. Finally, the clump had fully developed into 3-5 plantlets in one week later. 3) Embryogenic callus. White callus formation initiated from the radicles at 7 days and eventually enveloped the entire embryo within 35 days. Embryogenic callus had been established from primary callus and differentiated into cluster of shoot buds at 49 days. Then the cluster grew and developed into plantlets within 56 days. On average, 5–10 fully developed plantlets were obtained from one hybrid embryo (Figure 4), as well as embryo culture in hybrids derived from a cross between *Brasica juncea* with *Brasica napus* that found younger embryos had a higher tendency to proliferate and form callus. However, the percentage of plants regenerated from younger embryos was lower than those from relatively older embryos. Calluses are produced, it is rather difficult to germinate and regenerate directly into plants. The authors suggest subculturing would be necessary to induce

the germination of callus to produce shoots but Time consuming and labour intensive (Zhang, et al., 2003). Developmental pattern in all cross combination had the numbers of individual embryo and cluster (Table 2). The percentage of cluster type was higher than any other types. Phuchawa x Smooth cayenne and MD2 x Phuchawa showed the same highest numbers of cluster at 60%. Smooth cayenne x Phuchawa, and Phuchawa x MD2 had the numbers of cluster at 58.33% and 52.94% , respectively. In the other hand, Phuchawa and MD2 showed the highest number of individual embryo at 41.18%. Phuchawa x Smooth cayenne and MD2 x Phuchawa showed the same numbers of individual embryo at 40%. Smooth cayenne x Phuchawa had the lowest of individual embryo at 16.67%. However, embryogenic callus type was obtained only from the cross combination of Smooth cayenne x Phuchawa, and Phuchawa x MD2.

Table 2 Germination rate and pattern of embryogenic development of four hybrids, Smooth cayenne x Phuchawa, Phuchawa x Smooth cayenne, MD2 x Phuchawa, and Phuchawa x MD2

Cross combination	Germination rate (%)	Pattern of embryogenic development (%)		
		Individual embryo	Cluster	Embryogenic callus
Smooth cayenne x Phuchawa	14.81	16.67	58.33	25.00
Phuchawa x Smooth cayenne	5.52	40.00	60.00	00.00
MD2 x Phuchawa	6.54	40.00	60.00	00.00
Phuchawa x MD2	25.37	41.18	52.94	5.88

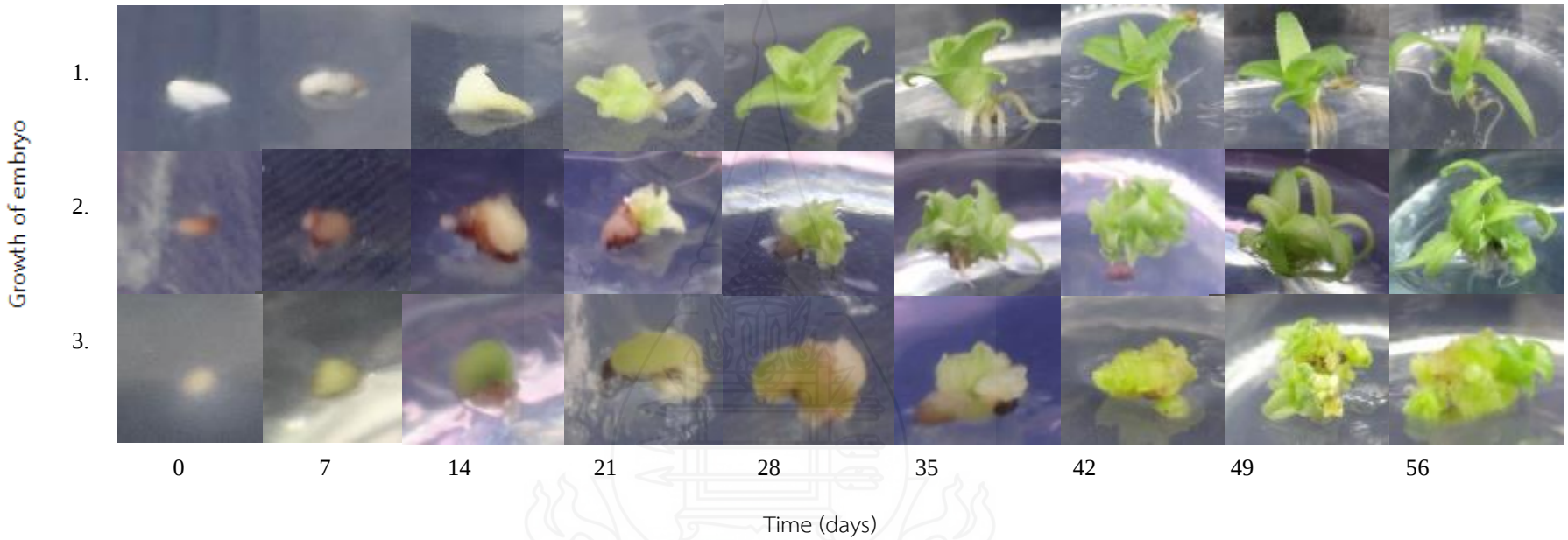

Figure 4 Embryogenic development of Smooth cayenne x Phuchawa, when cultured on MS medium for 56 days.
1. Individual embryo 2. Cluster 3. Embryogenic callus.

Conclusion

Embryo of pineapple hybrids were excised aseptically from the seed and cultured on MS medium for 56 days. The germination rate including growth and development pattern of embryo were observed every 7 days. The embryo germinated obviously within 14 days. The hybrid of Phuchawa x MD2 showed the highest germination rate at 25.37% . Pattern of embryogenic development was divided into three types; 1) Individual embryo that developed into one plant. 2) Cluster that one embryo developed from the clump of shoot buds into plantlets. 3) Embryogenic callus that established from primary callus into cluster of shoot buds, then developed into plantlets. Developmental pattern of embryo in all cross combination had the numbers of individual embryo and cluster, However, embryogenic callus type was obtained only from the cross combination of Smooth cayenne x Phuchawa and Phuchawa x MD2. The percentage of cluster type was the highest of all types. Phuchawa x Smooth cayenne and MD2 x Phuchawa showed the same highest numbers of cluster type at 60%. In the other hand, Phuchawa and MD2 showed the highest number of individual embryo type at 41.18% . Smooth cayenne x Phuchawa showed the highest number of embryogenic callus type at 25.00%.

Acknowledgements

The authors are grateful to Sarawut ruangaiam for pineapple hybrids used in this work. The authors would specifically like to thank my advisor Asst.Prof.Dr.Varaporn Laksanalamai, Dr.Tharathorn Teerakathiti and Dr.Oranuch Leelaporn, for guidance and encouragement throughout my research. The authors would also like to acknowledge Research Assistant and lab members for help and encouragement. The last the authors are grateful to Laboratory of Molecular Genetics and Plant Biotechnology, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for support this research.

References

Akbar, M.A., Karmakar, B.K., & Roy, S.K. (2003). Callus induction and high-frequency plant regeneration of pineapple (*Ananas comosus* (L.) Merr.). *Plant Tissue Cult*, *13*(2), 109-116.

Akin-Idowu, P.E., Akinyemi, S.O.S., & Ibitoye, D.O. (2014). Influence of medium type and growth regulators on *in vitro* micropropagation of pineapple (*Ananas comosus* (L.), var. Smooth Cayenne). *Afri.J.Pl.Sci*, *8*(9), 450-456.

Almeida, W.A.B.D., Santana, G.S., Rodriguez, A.P.M., & Carvalho Costa, A.P.D. (2002). Optimization of a protocol for the micropropagation of pineapple. *Rev.Bras.Frutic*, *2*(24), 296-300.

Burun, B. & PoyrazOlu E.Ç. (2002). Embryo culture in barley (*Hordeum vulgare* L.). *Turk J Biol*, *26*, 175-180.

Crisneros, A., & Tel-Zur, N. (2010). Embryo rescue and plant regeneration following interspecific crosses in the genus *Hylocereus* (Cactaceae). *Euphytica*, *174*, 73-82.

Klakhai, M. & Boonrod, S. (1992). *Pineapple*. n.p. (In Thai).

Liu, W., Chen, X., Liu, G., Liang, Q., He, T., & Feng, J. (2007). Interspecific hybridization of *Prunus persica* with *P. armeniaca* and *P. salicina* using embryo rescue. *Pl. Cell Tiss Organ Cult*, *88*, 289-299.

Murashige, T & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol plant*, *15*, 473-479.

Office of Agricultural Economics. (2014). Pineapple exports in 2014. Retrieved may 29, 2015, from http://www.oae.go.th/oae_report/export_import/export.php.

Phongsupasamit, S. (2004). *Crop Improvement*. 1st ed. Chiangmai : Nopburi Printing. (In Thai).

Reed, S.M. (2005). Embryo rescue. *Plant Development and Biotechnology, 18*, 235-239.

Ruangaiam, S. MD2 Pineapple, Pineapple Breeding New Varieties. *Dailynews*, September 23, 2011.

Salamma, S. & Rao, B.R.P. (2013). *In vitro* embryo culture of *Croton scabiosus* Bedd. (Euphorbiaceae), an endemic plant of Southern Andhra Pradesh. *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, *5*(2), 108-114.

Siriphat, S. (1975). Phucawa Pineapple Varities. The Announcement of the Department of Agriculture.

Stojicic, D., Janosevic, D., Uzelac, B., Cokesa, V., and Budimir, S. "*In vitro* zygotic embryo culture of *Pinus peuce* Gris.Optimization of culture condition affecting germination and early seeding growth." Arch. Biol. Sci. 64(2) (2012): 503-509.

Thawaro, S. & Te-chato. S. (2010). Eeffect of culture medium and genotype on germination of hybrid oil plam zygotic embryos. *ScienceAsia*, 36, 26-32.

Thongtham, C. (1983). *The Pineapple Industry in Thailand*. Bangkok: Aksorn Publishing House. (In Thai).

Usman, I.S, Abdulmallk, M.M., Sani, L.A., & Muhammed, A.N. (2013). Development of an efficienct protocol for micropropagation of pineapple (*Ananas comosus* L. var. Smooth cayenne). *African Journal of Agricultural, 8*(18), 2053-2056.

Verawudh, J. (1998). *The Pineapple Physiological Growth.* 1st ed. Bangkok: Kasetsart University. (In Thai).

Zhang, G.Q., Zhou, W.J., Gu, H.H., Song W.J., and Momoh, E.J.J. "Plant regeneration from the hybridization of *Brassica juncea* and *B. napus* through embryo culture." *J. Agronomy and Crop Science*. 189(2003) : 347-350.

Zuraida, A.R., Nurul Shahnadz, A.H., Harteeai, A., Roowi, S., Che Radziah, C.M.Z., & Sreeramanan, S. (2011). A novel approach for rapid microprapagation of maspine pineapple (*Ananas comosus* L.) shoots using liquid shake culture system. *African Journal of Biotechnology*, 10(19), 3859-3866