THE EFFECT OF THIDIAZURON CONCENTRATIONS ON THE INDUCTION OF COCOA SOMATIC EMBRYOS

Norasekin, T.*, Lea, J. and Rosmin, K.
Center for Cocoa Biotechnology Research, Malaysian Cocoa Board, Norowot Road, Commercial Zone 1, KKIP, 88450 Kota Kinabalu, Sabah, Malaysia
**Corresponding author: norasekin@koko.gov.my*

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ABSTRACT – *Somatic embryogenesis provides an effective method of in vitro clonal propagation making it possible to rapidly propagate elite cocoa (*Theobroma cacao *L.) clones for research and production. Adjustment on the concentration of nutrients and plant growth regulator in the culture medium is necessary to increase the efficiency of embryogenic response. This study was conducted to determine the effect of thidiazuron (TDZ) on the induction of primary somatic embryos (SEs) using five recommended clones. Staminodes were excised and cultured on induction medium supplemented with different concentrations of TDZ (3.125 µL/L, 6.25 µL/L, 12.5 µL/L, 25 µL/L, and 50 µL/L). Data obtained suggest that the size of callus produced corresponds with the volume of TDZ added. The percentage of primary SEs observed after eight weeks range from 0% to 80% on each media. Too much TDZ appeared to be toxic to some genotypes of cacao.*

***Key words*:** Thidiazuron, induction, primary somatic embryos, *Theobroma cacao* (cocoa)

INTRODUCTION

Theobroma cacao is a tropical tree originating from the Amazon basin, which presently is grown all over the tropics to fulfil the global demand for cocoa (Maximova *et al.*, 2002). Generally, the chocolate tree is propagated by seeds. As a consequence, a wide variety of genetic variance exists among seed-derived plants, leading to a low yield of single desired traits of plantation (Irizarry & Rivera, 1999). Therefore, a system that is fast and highly efficient needs to be developed for vegetative propagation of new variance of cacao in selected breeding methods and novel wild germplasm (Traore *et al.*, 2003).

Advances in biotechnology have offered a way to boost the strategy to increase yield through genetic engineering including breeding methods with desired traits and faster production of crops using tissue culture (Timmis, 1998). This is because through genetic engineering, plants can be generated from asexual propagation with uniform traits, for example, in cacao, somatic embryogenesis provides a system for clonal production of orthotropic plants with normal dimorphic architecture and taproot formation (Maximova *et al.*, 2002). Besides, through tissue culture, the disease-free crops and germplasm conservation can be tested via cryo-preservation that is important for cacao improvement, preservation and distribution (Maximova *et al.*, 2002).

Cacao tissue culture mainly centred on somatic embryogenesis (Maximova *et al.*, 2002). Many laboratories have developed culture systems from somatic tissues such as leaves, nucellus and floral explants including petals and staminodes (Figuera & Janick, 1993). However, previously these systems were only partially successful and applicable to certain species and genotypes as it shows a low rate of conversion (Maximova *et al.*, 2002). A method reported by Li *et al.* (1998), then has given new insight into the propagation of a wide variety of cacao genotypes. Thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic have been shown to produce primary SEs from floral explants. High yields of plants can be acclimated and grown until maturity by increasing the efficiency of the method by optimizing the media conversion (Maximova *et al.*, 2002).

Thidiazuron has unique characteristics of imitating auxin and cytokinin properties on explants developments and growth (Sankhla *et al.*, 2003). However, a small amount of TDZ treatment gives different morphogenetic responses whereby it could predispose tissue to receive other stimuli or work in combinations (Guo *et al.*, 2011). Alternatively, it still can provide the pathway for full regeneration even after it was removed from the system (Guo *et al.*, 2011). Both still account for observations, moreover, TDZ can initiate plants' basic survival mechanism to regenerate asexually (Guo *et al.*, 2011). In this paper, we will study the effects of TDZ concentrations on the induction of cocoa SEs using five recommended clones.

MATERIALS AND METHODS

Plant materials and explant preparation

Unopened cocoa (Theobroma cacao) two-to-three-week old (4 – 8 mm in length) flower buds were used as a source of explants. Samples from cocoa genotype PBC140, PBC154, MCBC5, KKM4, and BR25 were obtained from bud grafted and field-grown clonal plants at Centre for Cocoa Biotechnology Research, Kota Kinabalu Industrial Park, Sabah, Malaysia. These immature flower buds were collected in a clean falcon tube containing cold water, early in the morning (around 8 – 9 am). Samples were surface sterilized as described in Norasekin *et al.,* (2013).

Effect of TDZ concentration on somatic embryogenesis

Staminodes were extracted and cultured on 30 mL of induction medium in a plastic petri dish (90 mm diameter). The medium contained DKW basal salts, DKW vitamin solution (Thiamine-HCl, Nicotinic acid, Glycine and Tryptophan), 20 g/L glucose, 250 mg/L glutamine, 200 mg/L myo-inositol, 2.0 g Phytagel, 2,4-D and various concentration of TDZ. The petri dishes were sealed with Parafilm and maintained in the dark at 25±2 ^{o}C for 14 days. Various concentrations of TDZ used were 3.125 µL/L, 6.25 µL/L, 12.5 µL/L, 25 µL/L, and 50 µL/L (0.2mg/ml stock). This induction medium was used to assess the SE formation for the genotype used.

Samples were then subcultured onto a secondary callus growth medium containing McCown's salt, Gamborg's vitamin solution and 2.0 g Phytagel. The dishes were sealed and the cultures were maintained in the dark for another 14 days. Callus were then transferred to an expression medium containing DKW Basal Salts, DKW vitamin solution, sucrose and 2g Phytagel. The explants were then subcultured onto a fresh expression medium every 14 days until SEs reach maturity. At this stage, mature SEs with torpedo shapes were selected and cultured onto embryos expression development medium.

The experiment was carried out in triplicate. 20 explants (4 flower buds) were cultured per genotype per replicate. The earliest time taken to produce SEs and the percentage of SEs formation were calculated and the data were recorded.

RESULTS

The results from this study showed the significant effect of TDZ concentration on inducing callus and SEs of five Theobroma cacao. Staminodes that were cultured on an induction medium were grown to two-to-three times larger in size after 2 – 3 weeks. At the end of the secondary induction medium, several types of callus formation were generally observed, mainly white compact and yellow-brown friable callus. However, white compact callus is not able to produce SEs even though it grows robustly in size, unlike the dark brown friable callus.

The effect of TDZ on callus formation of all genotypes was determined by the number of callus formations after 4 weeks from culture initiation. All 5 genotypes of cocoa that were utilized in the experiment have produced callus at all concentrations of TDZ at various degrees of responses. Staminodes from MCBC5, KKM4 and BR25 cultured on induction medium supplemented with 3.125 µL/L of TDZ managed to grow little in size with least callus formation, whereas PBC140 and PBC154 show no response (Table 1).

On induction medium supplemented with 6.25 µL/L and 12.5 µL/L, explants from all genotypes were able to produce callus even at low frequencies. On an induction medium containing 25 µL/L TDZ, explants from all genotypes utilized in this study have produced approximately 70-90% yellow friable callus (Table 1) with fresh weight increment. However, the number of callus declines to 40-60% as TDZ concentration increases at 50 µL/L and explants turn black. This result shows that TDZ with 25 µL/L promoted the highest rate of callus induction.

The SE formation of BR25, MCBC5 and KKM4 were first observed after 3 to 4 weeks transferred to the expression medium. Meanwhile, SEs from PBC140 and PBC154 first emerged after 6 to 7 weeks on the expression medium. At this stage, clusters of pre-embryonic lumps were seen attached to the callus which later developed into globular-shaped embryos (Figure 1). After 5 weeks, heart-shaped SEs can be seen.

SEs formation was below 10% for explants cultured on medium supplemented with 3.125 µL/L of TDZ. The formation increases as TDZ concentration increases. The medium supplemented with 25 µL/L TDZ shows the highest percentage (80%) of embryo formation for MCBC5. However, in a medium supplemented with 50 µL/L TDZ, the frequencies of SEs emerged was decreased. It is seen that it has a direct influence on callus emergence at the early stage. Therefore, medium supplemented with 25 µL/L TDZ was determined as an optimum concentration for all genotypes tested in this study.

Table 1: Callus and embryogenic potential of cocoa genotypes on callus induction medium supplemented with various concentration of TDZ.

Induction medium supplemented with TDZ (0.2mg/ml stock)	Mean Callusing (%)					Mean Primary somatic embryos (%)				
	MCBC5	KKM4	PBC 140	PBC 154	BR25	MCBC5	KKM4	PBC 140	PBC 154	BR25
3.125 µL/L	32.5	25.0	0.0	0.0	20.0	10.0	7.5	0.0	0.0	0.0
6.25 µL/L	37.5	30.0	22.5	5.0	42.5	15.0	12.5	7.5	0.0	10.0
12.5 µL/L	60.0	37.5	22.5	7.5	52.5	15.0	17.5	15.0	5.0	22.5
25.0 µL/L	90.0	85.0	77.5	70	85.0	80.0	72.5	62.5	52.5	72.5
50 µL/L	62.5	60	47.5	52.5	50.0	45.0	37.5	27.5	32.5	30.0

Figure 1*: Clusters of pre-embryonic lumps and SEs attached to callus for MBC5*

DISCUSSIONS

Tan and Furtek (2002) reported that among the different physiological ages of the flowers used, unopened flower buds of 2-3 weeks old yielded the highest percentage of explants-producing embryos. Flower buds collected after 9 am often open during surface sterilization, leading to contamination of explants. As staminodes were the best explants for embryogenesis compared with anthers, staminodes and petals were extracted and cultured on induction media for callus initiation.

From this study, we can't deny that the use of Thidiazuron as cytokinin was critical, particularly for the onset of embryogenic callus and subsequent production of SEs in *Theobroma cacao*. In addition, TDZ has a unique property that can mimic both auxin and cytokinins' effect on the differentiation and growth of cultured explants.

In this study, all five (5) genotypes tested showed various degrees of responses in terms of callus and SEs formation on an induction medium supplemented with five (5) different concentrations of TDZ. It seems that mostly the size of callus produced corresponds with the concentration of TDZ added. The higher the concentration, the bigger the callus developed, which resembles cytokinin function that encourages cell division. However, the size and number of callus decreased as TDZ concentration increased at 50 µL/L TDZ and explants turned black. This is because a higher concentration of TDZ might be toxic to the explants and might lessen callus growth and embryo production.

Generally, SEs production starts 3-7 weeks on embryo expression medium for most genotypes tested. Percentage of embryo production

increased with culture time for genotypes demonstrating a sign of high embryogenesis instead of genotypes generating low frequencies of embryos, which is the SEs decreased with culture time. These observations conform to the observation of Li *et al*., (1998), Maximova *et al*., (2002) and Quainoo & Dwomo (2012) and show that the effect of these two growth regulators concentration on somatic embryogenesis is genotype-dependent.

The results show that medium supplemented with 25 µL/L of TDZ resulted in a great density of callus formation and SEs emergence. However, in this study, the callus induction medium was supplemented with TDZ and 2,4-D. A combination of these two growth regulators with suitable concentration seems to be suitable for all five genotypes tested. A high percentage of callusing, SEs formation and normal somatic embryo can be seen as compared to higher or lower TDZ concentrations. This finding is supported by the previous research done by other researchers on cocoa (Quainoo & Dwomo, 2012) and other plants such as *Falcataria moluccana* (Sunandar *et al.*, 2017).

CONCLUSIONS

The response of TDZ concentration on the induction of callus and somatic embryos of cocoas seem to be genotype-dependent. Generally, lower concentrations of TDZ are appropriate to produce normal somatic embryos thus increasing plantlets conversion.

REFERENCES

Figuera, A., & Janick, J. (1993). Development of nucellar somatic embryos of *Theobroma cacao*. *Acta Horticulture*, **336**: 231-238.

Guo, B., Abbasi, B. H., Zeb, A., Xu, L. L., & Wei, Y. H. (2011). Thidiazuron: A multi-dimensional plant growth regulator. *African Journal of Biotechnology*, **10(45)**: 8984-9000.

Irizarry, H., & Rivera, E. (1999). Early yield of five cacao families at three locations in Puerto Rico. *Journal of Agricuture of the University of Puerto Rico*, **82**: 167-176.

Li, Z., Traore, A., Maximova, S., & Guiltinan, M. J. (1998). Somatic embrygenesis and plant regeneration from floral explants of cacao (*Theobroma cacao L*.) using Thidiazuron. *In Vitro Cellular & Developmental Biology*, **34**: 293-299.

Maximova, S. N., Alemanno, L., Young, A., Ferriere, N., Traore, A., & Guiltinan, M. J. (2002). Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao L. In Vitro Cellular & Developmental Biology*, **38**: 252-259.

Norasekin, T., Siti Norhana, M.A., Nik Iryani, N.A and Azhar, M. (2013). Somatic embryogenesis of cocoa genotype PBC140. Poster presented at Malaysian International Cocoa Conference.

Quainoo, A.K and Dwomo, I.B. (2012). The effect of TDZ and 2,4-D concentrations on the induction of somatic embryo and embryogenesis in different cocoa genotypes. *Journal of Plant Studies*, **1(1)**: 72-78.

Sankhla, N., Mackay, W. A., & Davis, T. D. (2003). Reduction of flower abscission and leaf senescence in cut phlox inflorescences by Thidiazuron. *Acta Horticulture*, **628**: 837-841.

Sunandar, A., Dorly., Supena, E.D.J. (2017). Induction of somatic embryogenesis in Sengon (*Falcataria moluccana*) with Thidiazuron and light treatments. *HAYATI Journal of Biosciences*, **24(2)**: 105-108.

Tan, C.L. and Furtek, D.B. (2003). Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues. *Plant Sci.* **164**: 407-412.

Timmis, R. (1998). Bioprocessing for tree production in the forest industry: Conifer somatic embryogenesis. *Biotechnology Progress*, **14(1)**: 156-166.

Traore, A., Maximova, S. N., & Guiltinan, M. J. (2003). Micropropagation of *Theobroma cacao L.* using somatic embryo-derived plants. *In Vitro Cellular Developmental Biology*, *39*: 332-337.

Zhijian, L., Abdoulaye T., Siela, M. & Guiltinan, M. J. 1998. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using Thidiazuron. *In Vitro Cell. Dev. Biol – Plant*, **34**:293 – 299.