Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 41 (2) MAY 2018

PERTANIKA
JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

JTAS

Journal of Tropical Agricultural Science

AN INTERNATIONAL PEER-REVIEWED JOURNAL

EDITORIAL OFFICE

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Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
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43400 Serdang, Selangor Malaysia.
Gen Enq.: +603 8947 1622 | 1616
E-mail: executive_editor.pertanika@upm.my
URL: www.journals-jd.upm.edu.my

PUBLISHER

UPM PRESS
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia.
Tel: +603 8946 8855, 8946 8854
Fax: +603 8941 6172
E-mail: penerbit@upm.edu.my
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Pertanika Journal of Tropical Agricultural Science
Vol. 41 (2) May 2018

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Short Communication

Pertanika J. Trop. Agric. Sci. 41 (2): 855 – 864 (2018)

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Effect of Cytokinins on *In Vitro* Growth of Hypocotyl and Cotyledon of Tomato (*Lycopersicon esculentum*)

Wina Dian Savitri*, Popy Hartatie Hardjo, Leonardo Tejo Gunawan Putra Hardianto and Steven Sutanto

Department of Biology, Faculty of Biotechnology, University of Surabaya, Jawa Timur, 60292, Indonesia

ABSTRACT

Study of regeneration from different tissues or organs of plants is important as it gives information on how a piece of a plant can transform into its whole form. This process is even substantial when we talk about genetic engineering in plants, since no genetic engineering is valuable without knowing first the standard protocol for regenerating the transformed tissue or organs to become a whole plant. This experiment used hypocotyl and cotyledon of tomato cv. Tymoti as the explants was used to study how different concentrations (1.5-3 ppm) of cytokinins (Kinetin (Kin), 6-benzylaminopurine (BAP), thidiazuron (TDZ) and Zeatin (Zn)) affect its growth. As many as 16 explants were used for each treatment. The growth of both explants in the Murashige and Skoog (MS) media + vitamins showed that Zn and TDZ were superior among the other treatments in inducing calli and primordia organ.

Keywords: Cotyledon, cytokinins, hypocotyls, *in vitro* growth, tissue regeneration

INTRODUCTION

Cytokinins, theoretically, are plant growth regulators (PGRs) that trigger the differentiation of shoots. This PGR is primarily produced in root caps and then distributed into shoots (Aloni et al., 2005). Several kinds of cytokinins have been discovered, namely thidiazuron (TDZ), 6-benzylaminopurine (BAP), 6-γ-γ-dimethylaminopurine (2-ip), kinetin and zeatin. Among all the cytokinins that have been mentioned above, 2-ip and zeatin are naturally occurring, while the rest are derived synthetically (Razdan, 2002).

ARTICLE INFO

Article history:
Received: 18 September 2017
Accepted: 30 April 2018

E-mail addresses:
winasavitri@staff.ubaya.ac.id (Wina Dian Savitri)
poppy_hardjo@staff.ubaya.ac.id (Popy Hartatie Hardjo)
leonardo.tejogunawan@gmail.com
(Leonardo Tejo Gunawan Putra Hardianto)
ssutanto2@gmail.com (Steven Sutanto)
*Corresponding author

ISSN: 1511-3701

Yet, some plant species showed a different responses toward cytokinins. For example, less than 25% up to 50% of callus occurred on muskmelon's cotyledon explants cultured on MS media + vitamins incorporated with 1-2 ppm BAP, although 11.11% (1 out of 9 explants) and 44.44% (4 out of 9 explants) shoots were also produced from 1 ppm BAP and 2 ppm BAP respectively (Ishak, 2015). Our preliminary data on tomato cv. Tymoti showed that 0.5-3 ppm BAP applied on cotyledon and hypocotyls generated low to high callus structure on each explant. Savitri (2015) suggested that cotyledon explants of tomato cv. Tymoti cultured on MS medium + vitamins with the adding of 1-3 ppm BAP in combination with 0.1 ppm TDZ produced not only shoots but also calli that ranged from 18.75-56.25%. In addition, 0.5-2.5 ppm BAP or TDZ mixed with 0.1 ppm indole acetic acid (IAA) yielded 100% callus structure when applied to 10-week-old leaf discs of tomato cv. Tymoti cultured in dark condition (Savitri et al., 2016). Those findings represent that in relatively low concentration, cytokinins could also give rise to callus formation instead of shoot differentiation. Tomato cv. Tymoti is a hybrid that has already been sold commercially. This product is unique because it is suitable to be cultured on lowland, such as in Surabaya. Additionally, this product is resistant to Geminivirus and *Pseudomonas solanacearum*. This cultivar seems more promising than the others because it can be planted in lowland, so that the hybrid can be used in the experiment as a sample to learn about the tomato regeneration by in vitro culture.

The current experiment is aimed at studying the effect of four different cytokinins, i.e. TDZ, BAP, kinetin and zeatin on four concentrations; these are 1.5, 2, 2.5 and 3 ppm for each cytokinin. The results could be beneficial to give information about tomato regeneration through indirect pathway. The indirect pathway is very useful to produce a new traits, because the callus can divide very fast without certain direction. This can lead to cell mutation where some of the daughter cells are different from the parent cell. The ultimate aim of this research is to find a new trait from tomato cv. Tymoti (crop improvements), such as shorter reproduction cycle and greater yields.

MATERIALS AND METHODS

Plant Materials

The seeds of tomato cv. Tymoti were collected and surface sterilised by double dipping methods using sodium hypochlorite (NaOCl) solution, namely 2.63% (5 minutes) and 1.8% (15 minutes) respectively. These method were followed by rinsing it with sterile distilled water three times. The surface-sterile seeds (10-15) were cultured on ½ MS medium for 14 days. The hypocotyl and cotyledon were collected after that.

Culture Media

Half strength MS medium was prepared to culture the surface-sterile seeds. Each culture bottle contained 25 mL ½ MS medium. MS media + vitamins (Phytotech) were prepared for the treatments. Zeatin (Zn), Thidiazuron (TDZ), Benzylaminopurine (BAP), and Kinetin (Kin), at a concentration of 1.5, 2, 2.5 and 3 ppm respectively were added to the MS media + vitamins. Each bottle contained 25 mL MS medium + vitamins each enriched with cytokinin in a certain concentration. As much as 3% sucrose was added to the media. Before the adding of 1.2% agar, the pH was set at 5.6 for both media. Four cotyledon or hypocotyl were cultured on each culture bottle. Each treatment was repeated four times.

Incubator Condition

Incubator room was set at 25°C with 80-85% humidity, white fluorescent lamps were used to provide light, approximately equalling to 2000 lux. The photoperiod was regulated at 16 hours light/ 8 hours dark.

Data Analysis

Data was collected after eight weeks of culture. The callus and shoot formation data were derived from the number of explants that produced callus or shoot, compared with all the explants on each culture bottle and converted into a percentage. Because each treatment was repeated 4 times, percentage average was used. Data of every explant was noted from the average of callus score (Figure 1) for every 16 explants in each treatment. Data related to friable callus, compact callus, 'friable callus with nodule' and 'compact callus with nodule' were derived from number of callus matched with each type of callus compared with total number of explants that produced callus in each treatment. This data was converted into percentage. Data of 'number of shoots per explant' was calculated from the average number of shoots produced by every 16 explants in each treatment. The Kruskal-Wallis test (Minitab 17) was used to analyse data of 'callus score', 'number of shoots' and comparison between hypocotyl and cotyledon on both data. Correlation coefficients between callus formation (%) vs. callus score and vs. shoot formation (%) were performed using Microsoft Excel 2007 program.

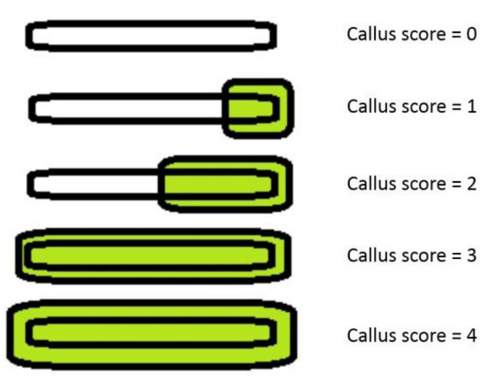

Figure 1. Illustration of callus score. 0, no callus formation; 1, quarter of explant formed callus; 2, half of explant formed callus; 3, entire explant formed callus; 4, callus size is twice of the initial explant Green indicates the growth of callus

RESULTS AND DISCUSSION

Effect of Cytokinins on Hypocotyls' Development

Based on Table 1, callus formation on hypocotyls, after being exposed to different kinds and concentrations of cytokinins, ranges from 43.75-100%. The lowest callus formation was produced by 3 ppm Kinetin, while the highest was produced by 1.5-2.5 ppm TDZ and 2.5 ppm Zeatin. This finding shows that TDZ and Zeatin are the best among the treatments. Even though 3 ppm TDZ, 1.5-2 ppm Zeatin, and 3 ppm Zeatin were not the highest, they are still higher among other treatments (93.75%). However, for Kinetin and BAP, callus formation varied between 43.75% and 62.5%.

The callus score is shown in Table 1 while the different letters show the significant differences among the treatments. Callus formation was the highest (93.75%-100%) when hypocotyls is exposed to 2.5 ppm TDZ and 1.5-3 ppm Zn. Given the situation, 2.5 ppm TDZ was chosen because it contributed to the highest shoot formation (31.25%), although the number of shoot per explant was low. This was probably because the explants were not sub-cultured in a new fresh media, as the explants' age was already 8 weeks old when data was collected. The longer the usage of culture medium, the lower the nutrients. There are not enough nutrients on the media to produce more shoots. Moreover, TDZ is much cheaper than Zn. Osman et al. (2010) reported that the 8-week-old hypocotyls and cotyledon tomato explants transferred to ½ MS + 1 ppm Indole acetic acid (IAA) produced plantlets with fine roots. The experiments also suggested that 0.5-3 ppm TDZ was suitable to produce 5-6 shoots from a cotyledon explant. Razdan (2002) proposed that a low concentration of auxins and cytokinins induce production of shoot and axillary buds while the high levels lead to callus and root formation. Yet in this experiment, a relatively low concentration of cytokinins (1.5-3 ppm) led to callus formation.

Table 1
Effect of cytokinins on *hypocotyl's development*

Treatment (ppm)	Callus Formation (%)	Callus Score[v]	Friable Callus (%)	Compact Callus (%)	Friable Callus with Nodule (%)	Compact Callus with Nodule (%)	Shoot Formation (%)	No. of Shoots
BAP 1.5	56.25	1 c[w]	22.22	33.33	0	33.33	25	1[x]
BAP 2	62.5	1 c	20	50	0	30	18.75	1
BAP 2.5	50	1 c	75	12.5	0	12.5	6.25	0
BAP 3	68.75	1 bc	9.09	36.36	0	54.55	37.5	1
Kin 1.5	43.75	1 c	28.57	57.14	0	14.29	6.25	0
Kin 2	56.25	1 c	0	55.56	11.11	33.33	25	1
Kin 2.5	50	1 c	37.5	50	0	12.5	6.25	0
Kin 3	43.75	1 c	0	28.57	0	71.43	6.25	0
TDZ 1.5	100	3 b	100	0	0	0	6.25	0
TDZ 2	100	4 a	81.25	0	18.75	0	18.75	1
TDZ 2.5	100	3 a	0	0	0	100	31.25	1
TDZ 3	93.75	3 a	0	86.67	0	13.33	18.75	1
Zn 1.5	93.75	2 bc	0	0	0	100	25	1
Zn 2	93.75	3 b	0	0	0	100	25	2
Zn 2.5	100	3 ab	0	0	0	100	25	1
Zn 3	93.75	2 b	0	0	0	100	31.25	1

Note. [v]Callus Score: 0, no callus formation; 1, quarter of explant formed callus; 2, half of explant formed callus; 3, entire explant formed callus; 4, callus size is twice of the initial explant; [w]Mean values with the same letter are not significantly different at $P \geq 0.05$; [x]Mean values are not significantly different at $P \geq 0.05$.

Callus scores were used to describe how much calli were formed from a single explant. The scores ranged from 0 to 4. Each score shows the size of callus descriptively from 'no callus formation' to 'the size of callus as twice the initial explant'. This descriptive data was then analysed using the Kruskal-Wallis test after being converted into scores. Figure 2 shows the callus formed from hypocotyls explants. The callus score 4, 3 and 1 are as shown on Figure 2A, 2B and 2C respectively. The nodules that occur on callus indicate the sign of organogenic callus, meaning that it will develop into organ primordia which usually are shoot buds rather than root. Later, the nodules or the organogenic calli will form calli with partial organ regeneration. Ikeuchi et al. (2013) categorised these calli as shooty, rooty and embryonic, based on the adventitious organ's type that regenerated from the callus. The nodules formed from compact callus are shown in Figure 3, while nodules formed from friable callus are described in Figure 4.

There is a positive correlation between callus formation (%) and its score in hypocotyl (Figure 7), and between callus formation (%) and shoot formation (%) (Figure 8). These data indicate that the higher the percentage of callus formation, the higher the callus score and shoot formation.

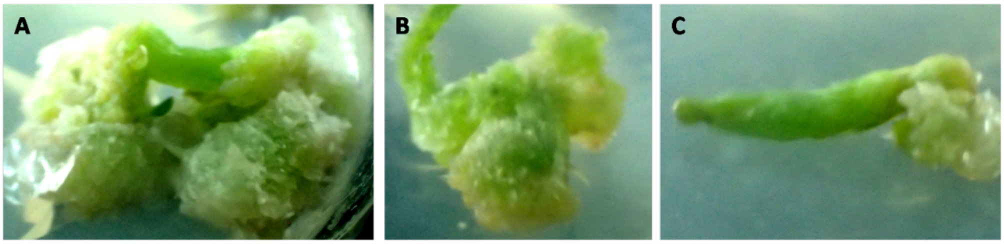

Figure 2. Callus formation on the hypocotyls explant as the effect of cytokinins after 8 weeks of culture on MS medium + vitamins. A, 2 ppm TDZ (callus score: 4); B, 3 ppm Zn (callus score: 3); C, 1.5 ppm Kin (callus score: 1)

Figure 3. Shoot formation on hypocotyl explant after 8 weeks of culture on MS medium + vitamins enriched with 2 ppm BAP. The arrows show the nodules that later will develop into shoot buds

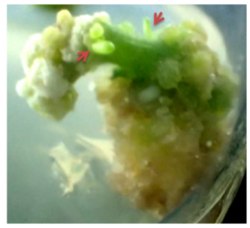

Figure 4. Friable callus with nodules formed on hypocotyl explant after 8 weeks of culture on MS medium + vitamins incorporated with 2 ppm TDZ. The arrows show the nodules that later will develop into shoot buds

Effect of cytokinins on cotyledon's development

The callus formation in all treatments achieved by cotyledon explants was relatively lower than those by hypocotyl (Table 2). The callus score per explant was also lower than hypocotyl. In terms of shoot growth, 1.5 ppm TDZ, 2.5 Zn and 2.5- 3 ppm BAP gave a higher number compared with hypocotyl by 2, 2 and 2-3 number of shoot, respectively. Yet, Table 3 shows there is no significant difference in the number of shoot per explant produced by hypocotyl and cotyledon. This finding is not supported by Wayase and Shitole (2014) on tomato cv. Dhanashri. They concluded that cotyledonary explants were better than hypocotyl in producing shoots. If the statistical data can be ignored, it is likely that 2.5 ppm BAP can be chosen because BAP is cheaper than TDZ and Zn. The BAP is the most commonly used cytokinin (Bhojwani & Dantu, 2013), and TDZ is the most active cytokinin (Huetteman & Preece, 1993). Zeatin is naturally occurring cytokinin in plants (Mok et al., 2002).

Table 2
Effect of *cytokinins* on *cotyledon's development*

Treatment (ppm)	Callus Formation (%)	Callus Score[v]	Friable Callus (%)	Compact Callus (%)	Friable Callus with Nodule (%)	Compact Callus with Nodule (%)	Shoot Formation (%)	No. of Shoots
BAP 1.5	25	1[x]	0	100	0	0	0	0[x]
BAP 2	56.25	1	11.11	88.89	0	0	0	0
BAP 2.5	31.25	0	40	40	0	20	6.25	2
BAP 3	56.25	1	22.22	33.33	0	44.44	25	3
Kin 1.5	12.5	0	0	50	0	50	12.5	1
Kin 2	12.5	0	0	50	0	50	6.25	2
Kin 2.5	18.75	0	33.33	66.67	0	0	0	0
Kin 3	25	0	0	50	0	50	12.5	2
TDZ 1.5	31.25	1	0	40	0	60	25	2
TDZ 2	37.5	1	0	83.33	0	16.67	0	0
TDZ 2.5	43.75	2	71.42	0	28.57	0	12.5	2
TDZ 3	56.25	1	0	0	0	100	6.25	1
Zn 1.5	31.25	0	0	0	0	100	0	0
Zn 2	6.25	0	0	0	100	0	0	0
Zn 2.5	62.5	1	0	0	20	80	25	2
Zn 3	43.75	1	0	0	14.29	85.71	0	0

Note. [v]Callus Score: 0, no callus formation; 1, quarter of explant formed callus; 2, half of explant formed callus; 3, entire explant formed callus; 4, callus size is twice of the initial explant; [w]Mean values with the same letter are not significantly different at $P \geq 0.05$; [x]Mean values are not significantly different at $P \geq 0.05$.

Just like the hypocotyl, in cotyledon, four callus types and 5 kinds of callus score (0, 1, 2, 3 and 4) were present. Figure 5A shows compact callus with nodules and Figure 5B shows friable callus with nodules. They both scored 1 and 3 respectively based on the callus size. Figure 5C shows 0 callus score (i.e. no callus is formed on the cotyledon explant). Figure 6A and 6B show the buds on this explant.

The correlation between callus formation (%) and shoot formation (%) was also analysed (Figure 7 and 8). As in the cotyledons, correlation between callus formation (%) and callus score is also clearly shown by coefficient correlation (r) 0.67. Furthermore, a lower positive correlation was shown by callus formation (%) versus shoot formation (%) (r = 0.35).

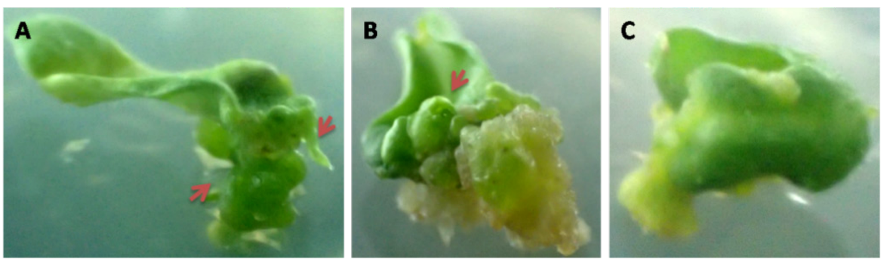

Figure 5. Callus formation on the cotyledon explants as the effect of cytokinins after 8 weeks of culture on MS medium + vitamins. A, 1.5 ppm Kin (callus score: 1); B, 3 ppm TDZ (callus score: 2); C, 1.5 ppm Zn (callus score: 0). The arrows show the nodules that later will develop into shoot buds

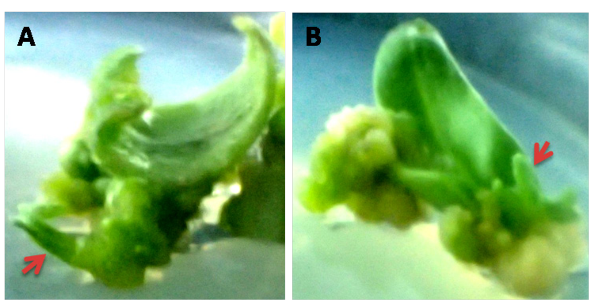

Figure 6. Cotyledon explants formed callus after 8 weeks of culture on MS medium + vitamins. A, Compact callus was produced after exposed to 1.5 ppm BAP; B, Friable callus was produced after being exposed to 2.5 ppm TDZ. The arrows show the shoot buds

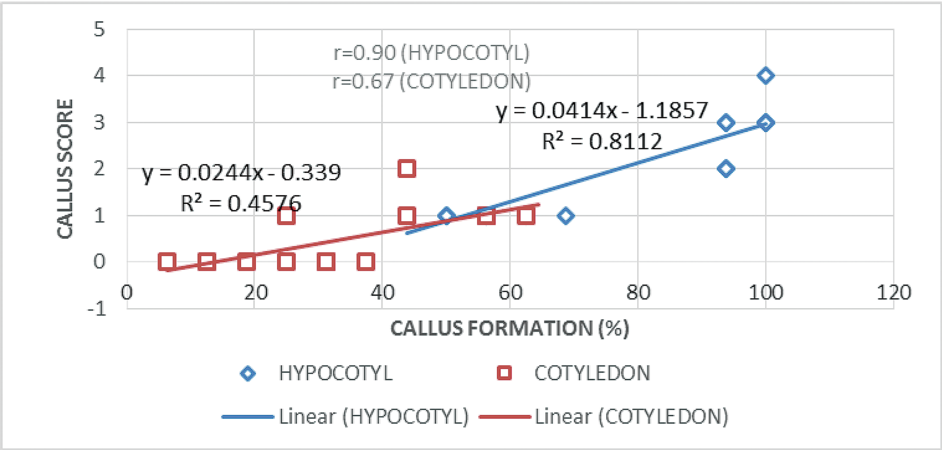

Figure 7. Positive correlation between callus formation (%) and callus score on hypocotyl and cotyledon

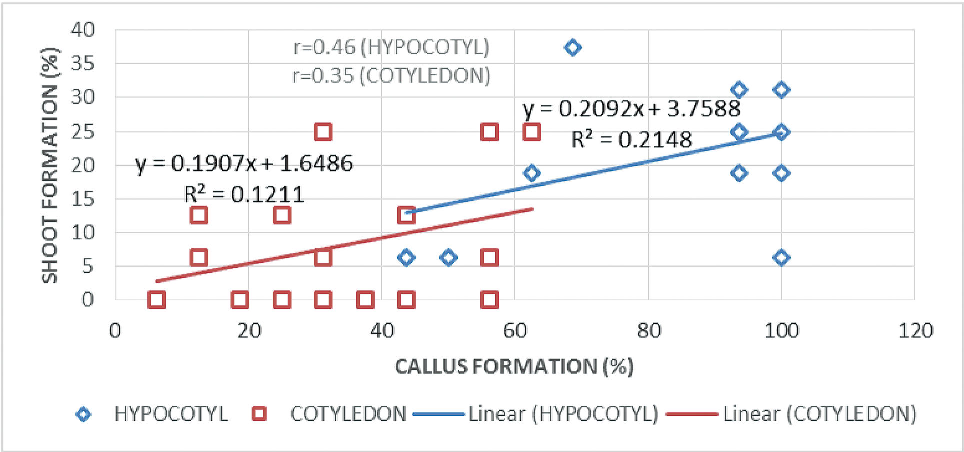

Figure 8. Positive correlation between callus formation (%) and shoot formation (%) on hypocotyl and on cotyledon

Comparing the two explants

In terms of shoot production from callus (indirect pathways), cotyledon explants showed better result. This is a common cultivar specific result. Genetic and environmental conditions are two major causes that effect regeneration. Moghaieb et al. (1999) reported the opposite finding, that hypocotyl explants in tomato cv. Pontaroza produced greater number of shoots compared with cotyledons.

Therefore, hypocotyl produces a higher callus formation and a higher callus score per explant. This finding was supported by correlation data between callus formation versus callus score and callus formation (%) versus shoot formation (%), that both showed a positive relationship. The comparison test performed by the Kruskal-Wallis showed no significant difference between two groups of data (Table 3). The additional experiment, such as sub-culturing the incubated explants into fresh medium, is needed to prove that cotyledon produces greater number of shoots than those that are not sub-cultured.

Table 3
Comparing hypocotyl and cotyledon explants in callus score and number of shoots per explant

Treatment	Callus Score per Explant*	Number of Shoot per Explant*
BAP 1.5	NS**	NS
BAP 2	NS	NS
BAP 2.5	NS	NS
BAP 3	NS	NS
Kin 1.5	NS	NS
Kin 2	Sig	NS
Kin 2.5	NS	NS
Kin 3	NS	NS
TDZ 1.5	Sig	NS
TDZ 2	Sig	NS
TDZ 2.5	NS	NS
TDZ 3	Sig	NS
Zn 1.5	Sig	NS
Zn 2	Sig	NS
Zn 2.5	Sig	NS
Zn 3	Sig	NS

Note. * Data of hypocotyl and cotyledon's comparisons were analysed using Kruskal-Wallis Test by significance level of 0.05; ** NS: not significantly different; ***Sig: significantly different.

CONCLUSION

BAP, TDZ, Kinetin and Zeatin induced the production of callus on hypocotyl and cotyledon of tomato cv. Tymoti. The shoots were also produced but in a very low percentage because the explants had not been sub-cultured in a new fresh MS medium. There was positive correlation between percentage of callus formation and callus score and shoot formation in both hypocotyl and cotyledon. In spite of the fact there was no significant difference between hypocotyl and cotyledon in producing shoots, using a hypocotyl explant and exposing it to 1.5-3 ppm TDZ or Zeatin may lead to a higher probability in producing callus.

ACKNOWLEDGEMENT

Authors would like to thank Lembaga Penelitian dan Pengabdian kepada Masyarakat (LPPM) Ubaya for funding this research, and the Faculty of Biotechnology Ubaya for providing the facilities to complete these experiments.

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Pertanika Journal of Tropical Agricultural Science

Country	Malaysia - SIR Ranking of Malaysia
Subject Area and Category	Agricultural and Biological Sciences Agronomy and Crop Science
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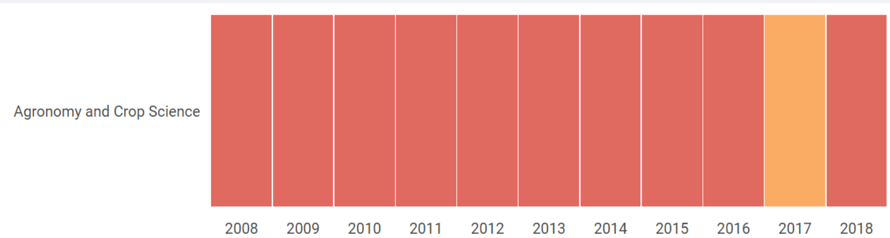

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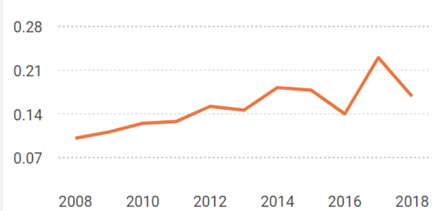

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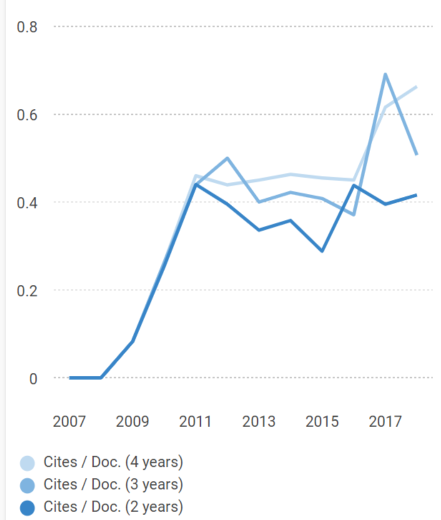

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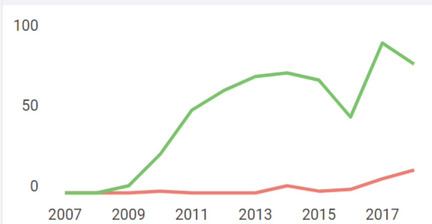

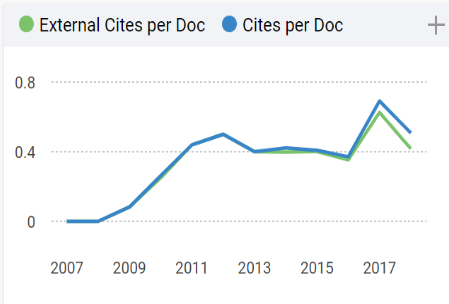
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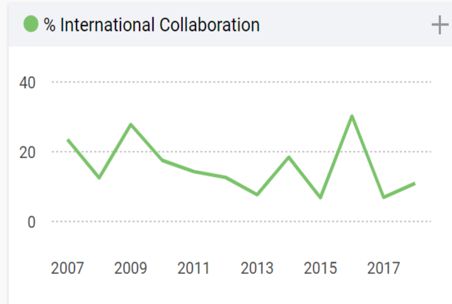
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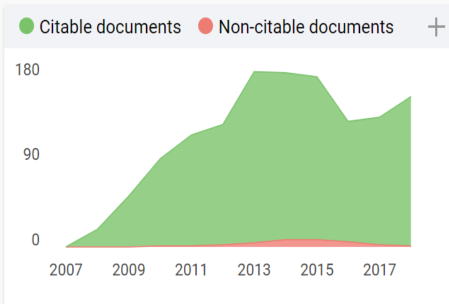
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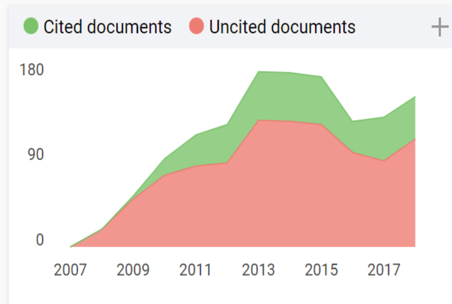
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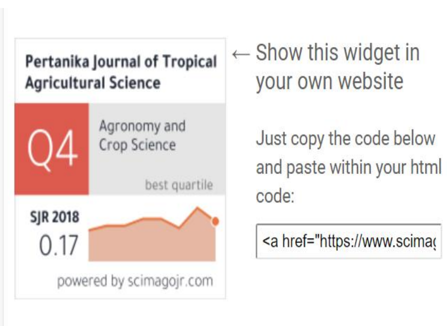
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