Journal of Applied Biology & Biotechnology Vol. 11(4), pp. 128-134, Jul-Aug, 2023
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2023.115801

Thidiazuron-mediated and genotype-independent regeneration system for tomato (*Solanum lycopersicum* L.)

Arshlyonel C. Velda[1], Sherry Anne G. Rodriguez[2], Michelle Lyka V. Descalsota[1], Olivia P. Damasco[1]*

[1]National Plant Genetic Resources Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, Philippines.
[2]Plant Cell and Tissue Culture Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, Philippines.

ARTICLE INFO

Article history:
Received on: January 20, 2023
Accepted on: March 20, 2023
Available online: June 04, 2023

Key words:
In vitro,
Benzylaminopurine,
Naphthalene acetic acid,
Recalcitrance,
Callus.

ABSTRACT

Solanum lycopersicum L. is one of the most studied crops, with numerous genetic improvement techniques employed to enhance its fruit quality. Recalcitrance of tomato to certain regeneration medium remains to be a limiting factor for a successful *in vitro* culture protocol. This study was conducted to establish an efficient regeneration system for the target tomato genotype Vb-15 for its use in gene editing using Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 vector system delivered by *Agrobacterium tumafaciens*. Cotyledons excised from 10 to 12-day-old *in vitro* germinated seedlings were cultured onto MS medium supplemented with BAP, TDZ, and NAA. Explants cultured on TDZ medium showed enlargement of the explant followed by callusing with multiple shoot development, while explants on BAP-medium showed early signs of callus formation and early development of shoot structures. The highest number of regenerants was obtained in MS with 0.5 mg^{-1} or 2.5 mg^{-1} TDZ yielding a mean of 4.45–4.64 shoots per explant. When transferred onto MS basal medium, regenerants from 0.5 mg/L TDZ or BAP showed maximum shoot elongation compared with other treatments. Rooted plantlets transferred to the greenhouse after acclimatization showed 78% survival. The optimum TDZ concentration gave significantly higher regeneration response in seven genotypes compared with Vb 15. Cluster analysis showed that at 0.6 average distances, the response of Vb-15 was unique showing its recalcitrance to *in vitro* culture.

1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an economically important agricultural commodity. It provides several nutrients including lycopene, beta carotene, flavonoids, Vitamin C, hydroxycinnamic derivatives and antioxidants that reduce risks in cancer, cardiovascular diseases, and Vitamin A deficiency [1]. In 2019, tomato was cultivated in a total of 16,360 ha of land yielding 223 thousand tons of fruit harvest, in the Philippines [2].

Due to increasing demand, tomato has been the subject of research focusing on improving fruit quality and developing disease resistance and stress tolerance through genetic transformation and other new breeding techniques. However, success in transformation relies on establishing an efficient regeneration system. Although there was significant progress in tomato transformation [3], successful transformation was confined to model cultivars such as Moneymaker [4], Micro-Tom [5,6], and Rio Grande [7].

Plant regeneration can be affected by several factors, including plant's genetic makeup, type of explant, components of the culture medium, concentration, and type of plant growth regulators, and their interaction [8]. In tomato, plant growth regulators specifically auxin and cytokinin source and concentration, play a significant role in mediating the morphogenic capacity of the explant [9]. The addition of 2.0 mg/L kinetin and 0.5 mg^{-1} indole acetic acid (IAA) on Chu (N6) medium was found to be effective in enhancing plant regeneration percentage and number of shoots in tomato [10]. High frequency of shoot regeneration was also observed on Murashige and Skoog (MS) medium supplemented with 0.1 mg^{-1} IAA, 1.0 mg^{-1} Zeatin and 2.0 mg^{-1} benzylamino purine (BAP) along with 8–10 mg^{-1} silver nitrate in tomato varieties Rio Grande, Roma, and Moneymaker [11]. Adventitious shoot regeneration from the hypocotyl and cotyledon explants of Micro-Tom tomato was observed on MS medium containing 1.0 mg^{-1} Zeatin and 0.1 mg/L IAA [12].

The recalcitrance of tomato in *in vitro* culture makes it difficult to standardize one protocol for plant regeneration that will work for various genotypes, particularly in identifying plant growth hormones. With the advent of synthetic plant growth hormones, overcoming the phenomenon of *in vitro* recalcitrance is made possible. Thidiazuron (TDZ) is a synthetic cytokinin that is highly active even at lower

*Corresponding Author:
Olivia P. Damasco,
Institute of Plant Breeding, College of Agriculture and Food Science,
University of the Philippines Los Baños, College, Laguna, Philippines, 4030.
E-mail: opdamasco @ up.edu.ph

concentration and is more resistant to the degrading enzymes in plants [13]. Due to its high activity, exposing an explant to TDZ can trigger various morphogenetic responses and will still be effective even after transferring to a medium devoid of TDZ, especially when inducing *in vitro* regeneration [14]. Specifically, it is known for its high potency for *in vitro* morphogenesis responses such as shoot proliferation and regeneration [15]. Its supplementation in the media applied whether at low or high concentrations can effectively induce *in vitro* shoot responses as observed in several crops such as date palm, bamboo, and apple [16-18].

An efficient regeneration system for tomato genotype Vb-15 is vital for its further genetic manipulation. This genotype is originally a Florida line (FLA-456) bred from *Solanum chilense* LA2779 and Royal Sluis tomato hybrid "Tyking" [19]. It has resistance to several begomoviruses, including the Tomato yellow leaf curl virus and has a high intensity of orange fruit color. Silencing of phytoene desaturase (PDS) gene involved in carotenoid biosynthesis in tomato was previously described in cv. Micro-Tom. This causes photobleaching mainly on fruit and leaves expressing that PDS gene has been knocked out [20]. Similarly, a clear manifestation of color change can be observed in Vb-15 to validate lycopene cyclase gene knockout using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated protein-9 (CRISPR/Cas9) vector system delivered by *Agrobacterium tumefaciens*. However, preliminary regeneration experiments showed that Vb-15 is recalcitrant in *in vitro* culture.

This study aimed to develop an efficient regeneration system for tomato genotype Vb-15. The objectives were to select the best medium for callus induction and shoot regeneration and determine differences in morphogenic potential of the cotyledon explant in the presence of TDZ, BAP, and naphthalene acetic acid (NAA). The study also aimed to evaluate the reproducibility of the best medium on other tomato genotypes and to assess if the regeneration protocol developed can be utilized as a general procedure for future transformation studies.

2. MATERIALS AND METHODS

2.1. Preparation of Plant Materials

Seeds of tomato genotypes used in the study were obtained from the collections of Institute of Plant Breeding - University of the Philippines Los Baños (14.1546° N, 121.2631° E). The tomato variety Vb-15 was obtained from the Genetics Laboratory, tomato hybrids (Apollo and Improved Apollo) from Plant Physiology Laboratory, open-pollinated varieties (Discovery, Rosanna, and Northern Red) from the National Seed Foundation; and open-pollinated tomato breeding lines (Tm 14-1-1-5, Tm 15-26-0-10, Tm 163147, and Tm 12-18-0-8) from the Vegetables Section. Seeds were surface disinfected with 10% sodium hypochlorite (NaOCl, 5.25% a.i.) for 30 min and rinsed with sterile distilled water. These were soaked overnight followed by the same disinfection procedure before inoculating on *in vitro* germination medium (refer to 2.4).

2.2. Selection of Best Plant Growth Hormones for Callus Induction and Shoot Regeneration

Cotyledon explants were excised from the *in vitro* germinated seeds after 10–12 days. The explants were cut in half with approximately 0.5 cm in length and cultured on MS medium supplemented with BAP, TDZ, and NAA treatments with the abaxial side down.

2.3. Assessment of Shoot Elongation, Rooting and Greenhouse Establishment after Prolonged Cytokinin Treatment

Regenerated shoots through direct and from callus were excised after 12 weeks and were then transferred to MS basal medium for shoot elongation and rooting. After 1 week of acclimatization, well-rooted plantlets were cultured in sterilize soil mixture (garden soil: coir dust, 1:1, v/v) and maintained in the greenhouse until flowering.

2.4. Culture Condition and Maintenance

The culture medium for all experiments was based on Murashige and Skoog [21] salts and vitamins with 3% sucrose and solidified with 0.8% plant agar. The *in vitro* germination medium contained MS basal medium alone. For the selection of best plant growth hormones, the following were added (in mgl^{-1}: T1- 0.5 BAP, T2 - 2.5 BAP, T3 - 0.5 TDZ, T4 - 2.5 TDZ, T5 - 0.5 NAA, T6 - 2.5 NAA, T7 - 0.5 BAP + 0.5 TDZ, T8 - 2.5 BAP + 0.5 TDZ, T9 - 0.5 BAP + 2.5 TDZ, T10 - 2.5 BAP + 2.5 TDZ, T11 - 0.5 BAP + 0.5 TDZ + 0.5 NAA, T12 - 2.5 BAP + 0.5 TDZ + 0.5 NAA. Moreover, the medium used to screen the *in vitro* response of ten tomato genotypes was MS supplemented with 0.5 mg^{-1} TDZ. The culture medium was adjusted to pH 5.6 and autoclaved at 15 psi for 20 min. Cultures were transferred to fresh medium with the same composition as the initial medium for two subculture cycles and were transferred to MS basal medium for shoot elongation and rooting. All cultures were maintained under 16-h photoperiod using cool white light emitting diode emitting a light intensity of approximately 30–50 μmol/(m^{2} s).

2.5. Evaluation of the *In Vitro* Response and Statistical Analysis

A total of 252 cotyledons with seven replicates having three samples were studied for the 12 treatments tested in Complete Randomized Design with subsampling. In callus induction and regeneration experiments, percent callus formation, callusing index (CI), percent shoot formation, and number of shoots were collected after 4 weeks of incubation. For the CI, the scale was: 0 - no tissue growth to swelling of the cotyledon with no callus formation; 1 - callus formation on one cut end of the cotyledon; 2 - callus formation on both cut ends of the cotyledon; and 3 - calli growth has completely covered the cotyledon explant and with at least two-fold increase in mass. For the experiment on the assessment of the reproducibility of the optimized treatment combination, 25 cotyledons per genotype with five replications were studied and laid out in a Complete Randomized Design. *In vitro* response diversity on the ten genotypes was analyzed using hierarchical cluster analysis based on Euclidean distance matrix. In addition, shoot characteristics were also classified into single – those with complete shoot structure and distinct apical meristem; clump – those with leafy shoot structure without distinct meristem. For all experiments, differences on the mean values were assessed at 95% confidence level ($P \leq 0.05$). Means for each treatment and the standard deviation were calculated. Data analysis was performed using the analysis of variance and Least Significant Difference Test for CI and number of regenerants. Regeneration efficiency of responsive explants, callus, and shoot were obtained by quantifying the responsive cultures for each stage, and a binary logistic regression analysis was performed to analyze the data obtained. A student t-test was also performed to compare means per genotype. All analysis was processed in Statistical Analysis Software (version 3.8).

3. RESULTS

3.1. Callus Induction

The Vb-15 tomato seeds germinated with a relatively high efficiency (>85%). Cotyledon segments were obtained when they reached approximately 1 cm in length. During the first 5 days of incubation on the modified media, cotyledons started to swell followed by formation of cell clusters at the margin. Callus induction was effective in all treatments except for medium containing NAA alone which only produced adventitious roots. However, combining BAP, TDZ, and NAA at relatively low concentration induced rapid callus proliferation.

The ability of plant growth hormones in inducing callus growth was measured using CI where a significant variation (P = 0.0232) was observed. Maximum response was attained in MS + 0.5 mg^{-1} BAP + 0.5 TDZ mg^{-1} + 0.5 mg^{-1} NAA, with the highest CI of 2.94 [Table 1]. Although, there was a similar efficiency when BAP alone, TDZ alone, and their combination was used, explants cultured on MS + 0.5 mg^{-1} BAP + 0.5 mg^{-1} TDZ + 0.5 mg^{-1} NAA have more callus graded as 3 (mean CI difference = 1.26) compared to MS + 0.5 mg^{-1} BAP + 0.5 mg^{-1} TDZ. This only showed that adding NAA promotes rapid callus proliferation.

3.2. Shoot Regeneration

Shoot differentiation started when the callus showed protruding clusters of green structures [Figure 1a] which later developed into torpedo structure [Figure 1b] and eventually showed early cotyledonary stage of shoots [Figure 1c]. On the other hand, some cultures induced adventitious shoot formation from the cotyledon explant under TDZ-treated medium during the early incubation period [Figure 1d]. These structures were stunted and developed large leafy structures. Regeneration was only observed in cultures inoculated on a medium devoid of NAA, showing that shoot formation was highly cytokinin-dependent [Table 1].

This study demonstrated the significant role of cytokinin on shoot formation. Based on the results, there is a progressive increase in percent shoot formation in the presence of TDZ or when combined with BAP [Table 1]. The highest shoot percentage (58.82%) was obtained in high concentrations of TDZ and BAP both at 2.5 mg^{-1}. In addition, the regeneration frequency, measured by counting the number of shoots formed per callus and shoot formation, was highly dependent on the concentration and type of plant growth hormones present in the media (P = 0.0439). The highest number of regenerated shoots was observed in MS medium fortified with 2.5 mg^{-1} TDZ with 4.64 shoots per callus. Although it has a similar efficacy (P = 0.8428), the use of 0.5 mg^{-1} TDZ has a higher percentage of shoot formation (55.6%). Regenerants from this treatment have distinct apical meristem and can grow into a complete shoot structure as compared with regenerants from 2.5 mg^{-1} TDZ that has a leafy structure of blinded shoots (without distinct meristem) and are usually stunted. A significantly higher frequency of shoots was obtained on TDZ medium than on BAP medium. Statistical analysis showed that explants cultured on TDZ would yield approximately two to three more shoots than on BAP. However, a combination of BAP and TDZ in the medium reduced the number of regenerated shoots manifesting the negative effect of BAP in multiple shoot organogenesis.

3.3. Shoot Elongation, Rooting, and Potting Out

All regenerated shoots were transferred to MS basal medium for shoot elongation and rooting [Figure 2]. After 4 weeks of incubation, maximum height in cultures was obtained on shoots previously treated with 0.5 mg^{-1} BAP and 0.5 mg^{-1} TDZ [Table 1]. However, regenerants from other treatments continue to produce multiple shoots showing that apical dominance was inhibited. Further incubation on the basal medium induced root formation (100% of the cultures). Before transferring to sterilized soil mixture, the plantlets were acclimatized for 1 week when they reached approximately 5 cm in height. Relatively high survival rate (75–78%) was obtained on well-developed plantlets after 14 days in the greenhouse.

3.4. *In vitro* Response of Different Tomato Genotypes on MS + 0.5 mg^{-1} TDZ

The callus induction and plant regeneration system developed [Figure 2] was evaluated on other tomato genotypes including Vb-15 as the reference genotype (control). Culturing cotyledons onto MS + 0.5 mg^{-1} TDZ from the ten genotypes tested resulted to 92–100% responding explants with signs of positive callus cell growth and rapid longitudinal expansion of the explant [Table 2]. Callus formation was observed only after 6 days of culture in Discovery, a commercially

Table 1: Quantitative growth response of Vb-15 relative to different concentrations and combination of plant growth regulators.

Treatment (in mgl^{-1})	% RE	% Callus formation per RE	Mean callusing index	% Shoot formation per RE	No. of regenerated shoot per RE	Highest shoot length (cm)**
0.5 BAP	94.44	100	1.94±1.01^{ab}	41.18^{a}	2.45±0.59^{b}	3.91±0.83^{a}
2.5 BAP	100	100	2.61±0.50^{ab}	27.78^{a}	2.18±0.55^{b}	2.64±0.78^{b}
0.5 TDZ	90	100	1.90±0.85^{ab}	55.6^{a}	4.45±0.92^{a}	3.22±0.35^{ab}
2.5 TDZ	100	100	2.45±0.76^{ab}	55^{a}	4.64±0.68^{a}	1.74±0.42^{c}
0.5 NAA	0	0	0^{c}	0^{a}	0^{c}	NA
2.5 NAA	27.78	40	0.11±0.32^{c}	0^{a}	0^{c}	NA
0.5 BAP+0.5 TDZ	89.47	94.12	1.68±1.11^{b}	35.29^{a}	2.27±0.54^{b}	1.17±0.15^{d}
2.5 BAP+0.5 TDZ	89.47	100	2.16±1.01^{ab}	35.29^{a}	2.91±0.78^{ab}	1.60±0.15^{c}
0.5 BAP+2.5 TDZ	89.47	100	2.11±0.88^{ab}	41.18^{a}	3.00±0.57^{ab}	1.61±0.13^{c}
2.5 BAP+2.5 TDZ	94.44	100	2.33±0.77^{ab}	58.82^{a}	2.63±0.39^{b}	0.95±0.07^{e}
0.5 BAP+0.5 TDZ+0.5 NAA	88.89	100	2.94±1.16^{a}	0^{a}	0^{c}	NA
2.5 BAP+0.5 TDZ+0.5 NAA	94.44	100	2.33±0.77^{ab}	0^{a}	0^{c}	NA

*Means±the standard error with the same letter are not significantly different using least significant difference. **Measured from regenerated plantlets transferred to MS basal medium after 4 weeks, RE: Responsive explants.

propagated tomato genotype. In most genotypes, dedifferentiation of callus was found at the embryo axis attachment to the hypocotyl, and at the wounded margins of the cotyledon. Although a high percentage of callus formation was observed (96–100%), there is a relatively slow callus growth in terms of CI where all genotypes formed callus only at one cut end or at both cut ends of the cotyledons. The medium optimized for Vb-15 has also stimulated shoot formation on other genotypes following different pathways. In tomato varieties Rosanna and Discovery, shoot has emerged directly without callus formation in 7–8 days. The two hybrid tomatoes have significantly different responses in terms of number of days and percent shoot formation. Shoot formation in Improved Apollo was observed in 21 days while in Apollo it was only 8 days. All varieties, except for Apollo, produced more shoots compared to Vb-15. For all Tm breeding lines tested, shoot formation was observed after 10–12 days. These Tm breeding lines were also superior to Vb-15 in terms of shoot formation. In general, all open-pollinated tomato genotypes have superior response in *in vitro* culture compared with hybrid tomatoes. From these results, the optimized medium for Vb-15 is reproducible and can be used for other genotypes. Moreover, the recalcitrance of Vb-15 in *in vitro* culture was also evident since the other genotypes produced more shoots with relatively higher responding explants per culture.

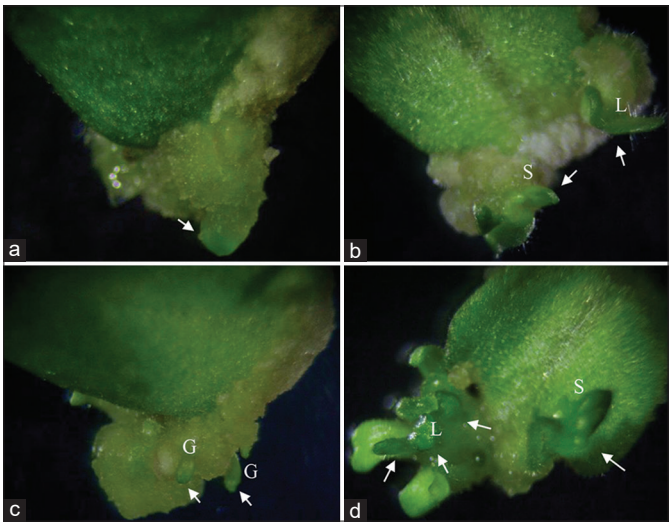

Figure 1: Different morphological structures observed after 17 days of culture from the cotyledon explants of Vb-15. (a) Callus formation and green color of friable callus, (b) dedifferentiation of callus into leaf (L) and shoot (S) structures, (c) formation of globular (G) structures from friable callus and (d) direct shoot regeneration (S) and leaf organogenesis (L). Arrow indicates structures which developed into shoots at different stages from callus initiation to shoot formation.

Two different characteristics of shoot (clump, single) were observed in the regenerated cultures. Single shoots have a complete shoot structure with a distinct apical meristem and normal leaf development [Figure 3a]. Clumped shoots appeared as a cluster of leafy, stunted, and shoot-like structures joined together in a single axis [Figure 3b]. For Discovery and Rosanna, the number of clumped shoots was higher than single shoots while Northern Red produced more single shoots [Figure 4]. The differences in shoot appearance can be attributed to the initial response of the cotyledon explant to TDZ. It was found that more clumped shoots arose when shoot development followed the direct

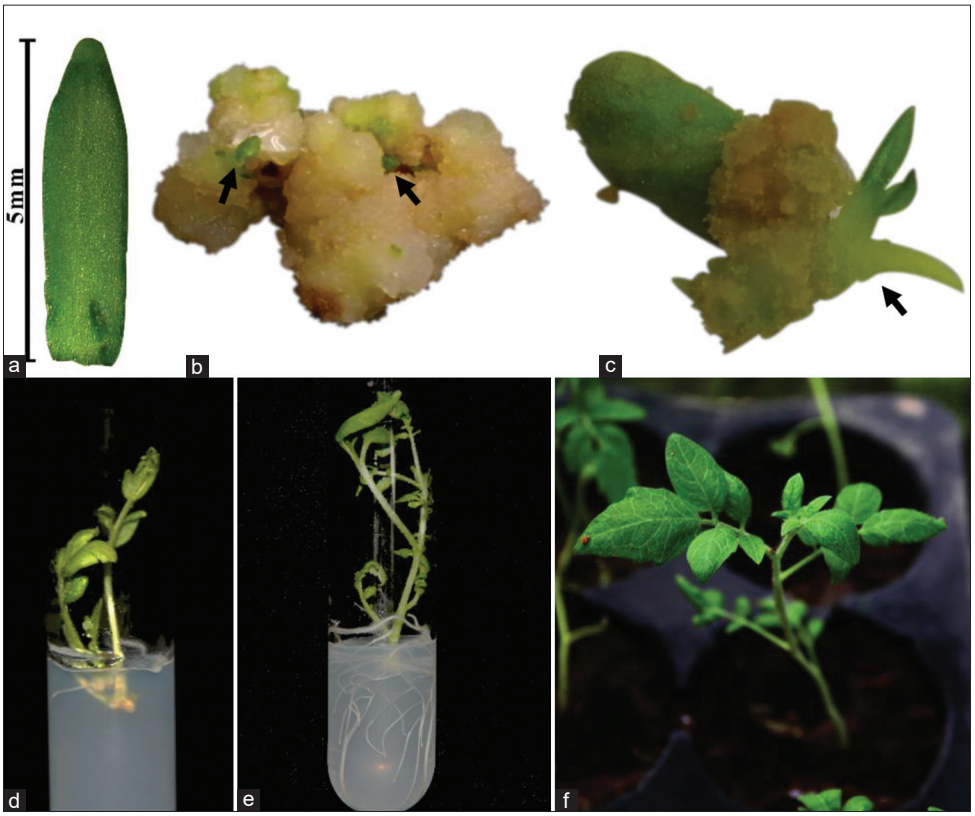

Figure 2: Callus induction and plant regeneration in Vb-15. (a) 10-day-old cotyledon explant excised from *in vitro* germinated seed. (b) Friable callus with few differentiating shoots indicated by arrow. (c) Regenerating shoot indicated by arrow. (d) 8-week-old regenerants at the elongation stage. (e) Rooted plantlets. (f) 2-week-old potted out plantlet.

Table 2: Mean response of different tomato genotypes in terms of callus and shoot formation after 6 weeks of culture on MS+0.5 mg/L TDZ.

Genotype	Responding explant (RE) (%)*	Callus formation/ RE (%)*	Shoot formation/ RE (%)*	No. of days to callus formation**	Mean callusing Index**	No. of days to shoot formation**
Vb-15	92	100	56.52	10.2 ± 0.20^{c}	1.2 ± 0.12^{b}	10.64 ± 0.13^{cd}
Improved Apollo	92^{ns}	100^{ns}	78.26^{s}	10 ± 0.0^{c}	1.32 ± 0.13^{ab}	21 ± 0.0^{f}
Apollo	100^{ns}	100^{ns}	44^{ns}	10 ± 0.0^{c}	1.48 ± 0.10^{ab}	11.18 ± 0.35^{d}
Discovery	100^{ns}	100^{ns}	100^{s}	6.6 ± 0.92^{a}	1.76 ± 0.09^{a}	8.6 ± 0.16^{b}
Rosanna	100^{ns}	96^{ns}	100^{s}	9 ± 0.0^{b}	1.64 ± 0.11^{ab}	7.8 ± 0.08^{a}
N. Red	100^{ns}	100^{ns}	80^{s}	10 ± 0.0^{c}	1.48 ± 0.10^{ab}	7.75 ± 0.10^{a}
Tm 163147	100^{ns}	100^{ns}	100^{s}	10.4 ± 0.40^{c}	1.64 ± 0.10^{ab}	10.2 ± 0.08^{c}
Tm 12-18-0-8	100^{ns}	100^{ns}	80^{s}	10 ± 0.0^{c}	1.48 ± 0.10^{ab}	12 ± 0.0^{e}
Tm 14-1-1-5	92^{ns}	100^{ns}	65.22^{ns}	10 ± 0.0^{c}	1.44 ± 0.13^{ab}	12 ± 0.0^{e}
Tm 15-26-0-10	96^{ns}	100^{ns}	79.17^{s}	10 ± 0.0^{c}	1.28 ± 0.11^{ab}	12 ± 0.0^{e}

*Significant (s) or not significant (ns) in comparison with the reference genotype Vb 15 using binary logistic regression analysis. **Means±standard error with the same letter is not significantly different using least significance difference, RE: Responsive explants.

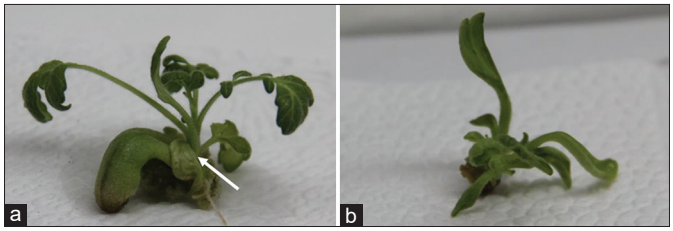

Figure 3: Morphological characteristics of the regenerated shoots from tomato genotypes cultured on MS medium supplemented with 0.5 mg/L TDZ. (a) Complete structure with single shoot and distinct apical meristem; (b) clump and leafy shoot structures without distinct apical meristem. (Arrow indicates the distinct apical meristem).

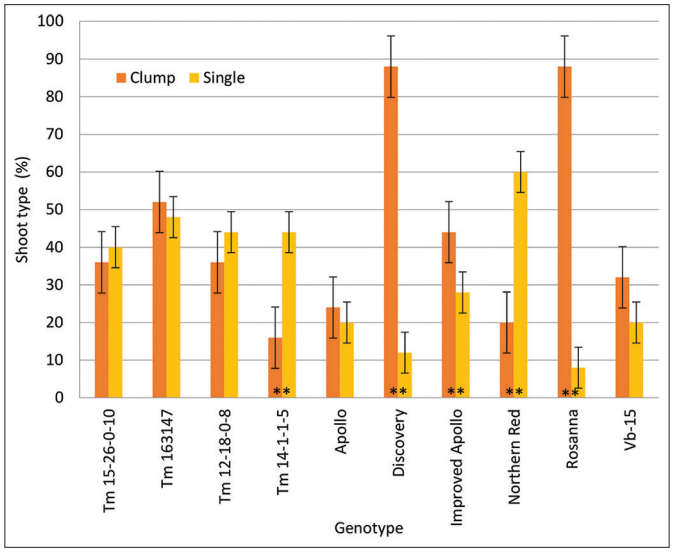

Figure 4: Comparison of the type of shoot regeneration (clump vs. single) observed on different tomato genotypes after 6 weeks of culture on MS + 0.5 mgl^{-1} TDZ. Values are mean shoot type ± standard error of the mean. Per genotype, means with ** shows significant difference in the type of shoot regeneration using t-test at 95% confidence interval.

organogenesis pathway. In contrast, single shoots most likely developed from callus following an indirect organogenesis pathway as observed in Northern Red, Tm 15-26-0-10, and Tm 12-18-0-8 [Figure 4].

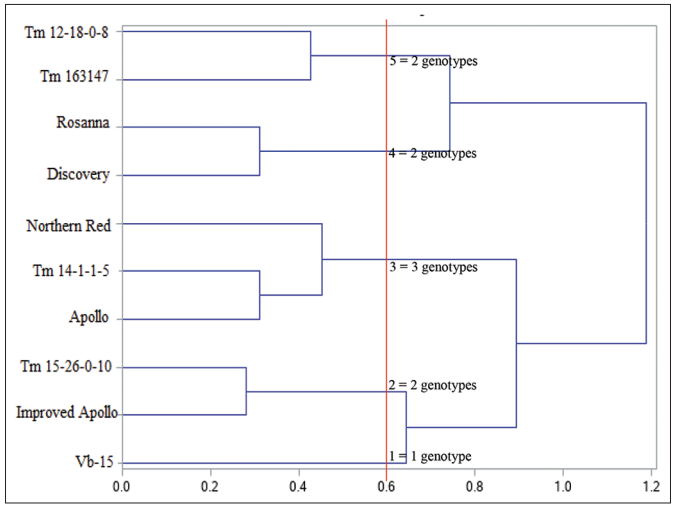

Figure 5: Dendrogram showing relationship of ten tomato genotypes based on tissue culture parameters (number of days to callus formation, percent callus formation, mean callusing index, percent shoot formation, and mean number of shoots per explant) generated by the Average Linkage Clustering method using Euclidean Dissimilarity Distance. Five clusters were generated when truncated at 0.6 distance coefficient as represented by the vertical line.

3.5. Cluster Analysis based on the *In Vitro* Growth Response of Ten Tomato Genotypes

A dendrogram was constructed based on Euclidean dissimilarity measure of the *in vitro* response standardized with maximum absolute value for ratio variables and standard deviation for ordinal variables (number of days to callus formation, percent callus and shoot formation, mean CI, mean number of shoots per explant, callus appearance, CI, and shoot characteristics) [Figure 5]. The Euclidean distance ranged from 0.28 to 1.19. At 0.6 average distance, five clusters were generated. The mean response of Vb-15 was separated at 0.64 distance from Tm 15-26-0-10 and Improved Apollo. Both Apollo and Tm 14-1-1-5 were unique from Northern Red at 0.45 average distance. Rosanna and Discovery showed a relatively low variation and were separated at a small distance of only 0.31. Although it was demonstrated that Vb-15 was unique, the optimized medium developed for this genotype was also applicable for other genotypes and regeneration response of most of the genotypes were superior to Vb-15.

4. DISCUSSION

This study developed a tissue culture regeneration system for tomato genotype Vb-15 and other genotypes after a thorough evaluation of factors affecting the regeneration capacity. The *in vitro* morphogenesis of plant can be mediated by the levels of auxin and cytokinin in the medium as well as the levels of endogenous growth hormones in the tissue explants [22]. In general, a balanced ratio of auxin to cytokinin induces callus formation while high levels of cytokinin stimulate shoot formation [23]. On the contrary, using cotyledon explant requires high cytokinin and a relatively low auxin level for regeneration [24,25].

In this study, callus induction and plant regeneration in tomato was found to be cytokinin-dependent. It was observed that callus was initiated at the margins of the cotyledons obtained proximal to the hypocotyl, following the patterns of cell growth when cytokinin interacts with high levels of endogenous auxin. It was demonstrated in Arabidopsis that auxin distribution in the cotyledon flows from the petiole toward the ground tissue and accumulates along the margins and the apex [26]. Following this auxin transport pattern in dicots, it can be noted that upon excision, free auxins accumulated in the cotyledons of tomato balances the effect of exogenous cytokinin in the medium, thus, resulting to the formation of callus. In contrast, in tomato cv. ArkaAbha, a high concentration of cytokinin (2 mg^{-1} BAP) and low level of auxin (0.2 mg/L) enhanced shoot formation and proliferation [27].

The two most important growth hormones in tissue culture are auxin and cytokinin. Cytokinin functions in controlling shoot apical meristem proliferation and in promoting cell division. For younger plant tissues, cytokinin is involved in the formation of shoot meristems through a transition process from undifferentiated cells to differentiated tissues [28]. Cytokinins can be classified into adenine-type, naturally occurring in plants, and phenylurea-type, synthetic compounds. BAP is an example of adenine-type cytokinin recognized by its aromatic ring [29] The phenylurea TDZ, however, is a glucosyl derivative which is highly active [30]. These two cytokinins have different modes of action in plant cells. TDZ was found to be effective in enhancing regeneration with the highest percent shoot formation and number of regenerants in Vb-15 compared with BAP. Similarly, in tomato cv. Omdurman, highest number of shoot regenerants from cotyledon explants was obtained on medium containing TDZ at 3 mg^{-1} [31]. In contrast to Vb-15, 0.5 mg^{-1} of TDZ gave the highest number of regenerants which has the same efficacy when a high TDZ concentration at 2.5 mg^{-1} was supplied in the medium. An important result of this work showed that exogenous application of TDZ indirectly affects *in vitro* morphogenesis by changing the endogenous level of phytohormones in plant cells and tissues. TDZ enhances cytokinin activity by removing a phosphate group from naturally occurring cytokinin ribonucleotide and making them biologically active with more free bases present in plant tissues [32]. This synthetic hormone also triggers *de novo* production of purine-based cytokinins such as BAP [30]. In peanut, the application of TDZ decreases the levels of 6-(γ,γ-Dimethylallylamino) purine (2iP) but stimulates adenine and adenosine synthesis in the cotyledon [33]. The fluctuating level of 2iP is due to its conversion to adenosine which is brought by the physiological activity of TDZ. Moreover, TDZ can also increase the levels of tryptamine, a precursor of IAA, indicating that *de novo* synthesis of this naturally occurring auxin was also stimulated [34]. As observed in Vb-15, *in vitro* competence of the cotyledon explants was enhanced following the direct regeneration from the cotyledon and regeneration after minimal callusing as triggered by TDZ by increasing the levels of free auxins and the metabolic activity of endogenous cytokinin.

Combined application of TDZ and BAP seemed to show an antagonistic effect on the morphogenic competence of tomato as shown by low level of regeneration compared to TDZ alone. It was observed that when both BAP and TDZ were supplied in the medium, there was a significant decrease in the number of regenerants in Vb-15 as compared with single application of BAP and TDZ in the medium. As proposed in a study on *Miscanthus* X *ogiformis* Honda [35], cytokinin can bind to cytokinin-binding protein receptor that has two different binding sites specific for adenine-type and phenylurea-type cytokinin. Exogenous application of both TDZ and BAP will initiate a competition with the endogenous adenine-type cytokinin for their active binding site. The excess free molecules of TDZ and BAP was said to trigger a conformational change in the adenine-type binding site, thus, inhibiting cytokinin activity. Although TDZ is efficient in producing multiple shoots, continuous exposure to this hormone suppressed elongation of the regenerated shoots in tomato. Similarly, strawberry shoots sub-cultured on TDZ-containing medium showed signs of stunted growth and maximum height of shoots was attained only when the cultures were transferred to medium with a low concentration of zeatin [36].

5. CONCLUSION

The results in this study demonstrated that the morphogenic competence of tomato in *in vitro* culture was influenced by the interaction between the explant and plant growth regulators. The addition of TDZ in the medium induced either direct shoot formation from the cotyledon explant or regeneration with minimal callus formation. The most efficient medium combination for inducing shoots with complete structure was MS medium added with 0.5 mg^{-1} TDZ. Transferring regenerated shoots onto MS basal medium enhanced root formation and further growth of the shoot. This two-step regeneration protocol was also effective on other tomato genotypes that are either open-pollinated or hybrid. Moreover, this plant regeneration using cotyledon explant of tomato line Vb-15 can be used for *Agrobacterium*-mediated transformation.

6. ACKNOWLEDGMENT

The authors would like to thank all the support staff in the *in vitro* Unit of National Plant Genetic Resources Laboratory and the Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (PCAARRD) - Department of Science and Technology (DOST) for funding this research.

7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. FUNDING

This research study was part of the project funded by the Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development (PCAARRD) – Department of Science and Technology (DOST).

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

12. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

REFERENCES

1. Rodriguez-Concepcion M, Avalos J, Bonet ML, Boronat A, Gomez-Gomez L, Hornero-Mendez D, *et al*. A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. Prog Lipid Res 2018;70:62-93.
2. Food and Agriculture Organization of the United Nations. FAOSTAT Database. Rome, Italy: Food and Agriculture Organization. Available from https://www.fao.org/faostat/en/#data/QC [Last accessed on 2021 Sep 09].
3. McBride KE, Summerfelt KR. Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 1990;14:269-76.
4. Saker MM, Salama HS, Salama M, El-Banna A, Ghany NM. Production of transgenic tomato plants expressing cry 2Ab gene for the control of some lepidopterous insects endemic in Egypt. J Genet Eng Biotechnol 2011;9:149-55.
5. Yasmeen A. An improved protocol for the regeneration and transformation of tomato (cv Rio Grande). Acta Physiologiae Plantarum 2009;31:1271-7.
6. Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar micro-tom. Plant Cell Rep 2013;32:239-47.
7. Arshad W, Haq I, Waheed MT, Mysore KS, Mirza B. *Agrobacterium*-mediated transformation of tomato with rolB gene results in enhancement of fruit quality and foliar resistance against fungal pathogens. PLoS One 2014;9:e96979.
8. Bolibok H, Rakoczy-Trojanowska M. Genetic mapping of QTLs for tissue-culture response in plants. Euphytica 2006;149:73-83.
9. Schaller GE, Bishopp A, Kieber JJ. The yin-yang of hormones: Cytokinin and auxin interactions in plant development. Plant Cell 2015;27:44-63.
10. Sheeja TE, Mondal AB, Rathore RK. Efficient plantlet regeneration in tomato (*Lycopersicon esculentum* mill.). Plant Tissue Cult 2004;14:45-53.
11. Shah SH, Ali S, Jan SA, Din JU, Ali GM. Assessment of silver nitrate on callus induction and *in vitro* shoot regeneration in tomato (*Solanum lycopersicum* mill.). Pak J Bot 2014;46:2163-72.
12. Lee MH, Lee J, Jie EY, Choi SH, Jiang L, Ahn WS, *et al*. Temporal and spatial expression analysis of shoot-regeneration regulatory genes during the adventitious shoot formation in hypocotyl and cotyledon explants of tomato (CV. micro-tom). Int J Mol Sci 2020;21:5309.
13. Huetteman CA, Preece JE. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss Organ Cult 1993;33:105-19.
14. Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH. Thidiazuron: A multi-dimensional plant growth regulator. Afr J Biotechnol 2011;10:8984-9000.
15. Lu CY. The use of thidiazuron in tissue culture. *In Vitro* Cell Dev Biol Plant 1993;29:92-6.
16. Al-Mahayi AM. Thidiazuron induced *in vitro* bud organogenesis of the date palm (*Phoenix dactylifera* L.) cv. Hillawi. Afr J Biotechnol 2014;13:3581-90.
17. Lin C, Kalpana K, Chang WC, Lin NS. Improving multiple shoot proliferation in bamboo mosaic virus-free *Bambusa oldhamii* munro propagation by liquid culture. HortScience 2007;42:1243-6.
18. Geng F, Moran R, Day M, Halteman W, Zhang, D. Increasing *in vitro* shoot elongation and proliferation of 'G.30' and 'G.41' apple by chilling explants and plant growth regulators. HortScience 2016;51:899-904.
19. Kadirvel P, de la Peña R, Schafleitner R, Huang S, Geethanjali S, Kenyon L, *et al*. Mapping of QTLs in tomato line FLA456 associated with resistance to a virus causing tomato yellow leaf curl disease. Euphytica 2013;190:297-308.
20. Fernandez AI, Viron N, Alhagdow M, Karimi M, Jones M, Amsellem Z, *et al*. Flexible tools for gene expression and silencing in tomato. Plant Physiol 2009;151:1729-40.
21. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
22. Su YH, Liu YB, Zhang XS. Auxin-cytokinin interaction regulates meristem development. Mol Plant 2011;4:616-25.
23. Mastuti R, Munawarti A, Firdiana ER. The Combination Effect of Auxin and Cytokinin on *in vitro* Callus Formation of *Physalis angulata* L.-a Medicinal Plant. Vol. 2017. In: AIP Conference Proceedings; 2017. p. 040007.
24. Costa MG, Nogueira FT, Figueira ML, Otoni WC, Brommonschenkel SH, Cecon PR. Influence of the antibiotic timentin on plant regeneration of tomato (*Lycopersicon esculentum* mill.) cultivars. Plant Cell Rep 2000;19:327-32.
25. Otroshy M, Khalili Z, Ebrahimi MA, Nekoui MK, Moradi K. Effect of growth regulators and explant on plant regeneration of *Solanum lycopersicum* L. var. cerasiforme. Russ Agric Sci 2013;39:226-35.
26. Lewis DR, Wu G, Ljung K, Spalding EP. Auxin transport into cotyledons and cotyledon growth depend similarly on the ABCB19 multidrug resistance-like transporter. Plant J 2009;60:91-101.
27. Arulananthu G, Bhat SG, Ramesh N. Callus induction *in vitro* regeneration of tomato (*Lycopersicon esculentum* mill.). Res J Life Sci 2019;5:491-503.
28. Werner T, Motyka V, Strnad M, Schmülling, T. Regulation of plant growth by cytokinin. Proc Natl Acad Sci U S A 2001;98:10487-92.
29. Nandi SK, Letham DS, Palni LM, Wong OC, Summons RE. 6-benzylaminopurine and its glycosides as naturally occurring cytokinins. Plant Sci 1989;61:189-96.
30. Thomas JC, Katterman FR. Cytokinin activity induced by thidiazuron. Plant Physiol 1986;81:681-3.
31. Osman M, Elhadi E, Khalafalla M. Callus formation and organogenesis of tomato (*Lycopersicon esculentum* mill, CV Omdurman) induced by thidiazuron. Afr J Biotechnol 2010;9:4407-13.
32. Capelle SC, Mok DW, Kirchner SC, Mok MC. Effects of thidiazuron on cytokinin autonomy and the metabolism of n-(delta-isopentenyl) [8-c]adenosine in callus tissues of *Phaseolus lunatus* l. Plant Physiol 1983;73:796-802.
33. Murthy BN, Murch SJ, Saxena PK. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): Endogenous growth regulator levels and significance of cotyledons. Physiol Plant 1995;94:268-76.
34. Hutchinson MJ, Krishnaraj S, Saxena PK. Morphological and physiological changes during thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* x *hortorum* bailey) hypocotyl cultures. Int J Plant Sci 1996;157:440-6.
35. Nielsen JM, Hansen J, Brandt K. Synergism of thidiazuron and benzyladenine in axillary shoot formation depends on sequence of application in *Miscanthus* X *ogiformis Giganteus*. Plant Cell Tiss Organ Cult 1995;41:165-70.
36. Debnath CS. Zeatin overcomes thidiazuron-induced inhibition of shoot elongation and promotes rooting in strawberry culture *in vitro*. J Hortic Sci Biotechnol 2006;81:349-54.

How to cite this article:
Velda AC, Rodriguez SA, Descalsota ML, Damasco OP.
Thidiazuron-mediated and genotype-independent regeneration system for tomato (*Solanum lycopersicum* L.). J App Biol Biotech. 2023;11(4):128-134.
DOI: 10.7324/JABB.2023.115801