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Original Article

In vitro Callus Induction of Tomato and Evaluation of Antioxidant Activity of Aqueous Extracts and Enzymatic Activities in Callus Cultures

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ABSTRACT

Background: Tomato (*Solanum lycopersicum* L.) is an important plant rich in many vitamins and antioxidant enzymes.
Methods: Tomato leaves of two cultivars ('Peto 86' and 'Strain B') were used as explant sources for callus induction. The antioxidant activity of the calli ethanol (ET) and methanol (ME) extracts were determined. The enzymatic activities were evaluated in callus cultures. Callus induced from leaf explants of tomato cultivars on the Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of cytokinins and auxins such as BAP (6-benzylaminopurine), NAA (1-naphthalene acetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (indole-3-acetic acid) and Kin (Kinetin) for rapid induction of callus and biomass growth.
Results: The medium (M2) containing 3 mg L^{-1} BAP with 1 mg L^{-1} IAA produced the highest percentage of callus induction (PCI) (100%) in two cultivars. The relative fresh weight growth (RFWG) was reported by the fresh callus weighed after four weeks of culture and again weighed after one month of sub-culture. In both cultivars cultured on M2 medium the RFWG was (1.60) in 'Peto 86' and (1.47) in 'Strain B'. The results showed that PCI and RFWG differed with the cultivars tested. The scavenging DPPH free radical activity in callus (ET) extracts exhibited a significant increase in ($P < 0.05$) than the activity in callus (ME) extract. The peroxidase and polyphenol oxidase activities were found in calli of both tomato cultivars. The enzymatic activities were higher in callus of 'Peto 86' cultivar than in callus of 'Strain B' cultivar.
Conclusions: Calli had antioxidant and enzyme activities that can be beneficial for extracting important components or for plant regeneration.

Key words: Callus induction, DPPH, Enzyme activity, Plant growth regulators, Tomato

1. Introduction

Tomato (*Solanum lycopersicum* L.) belongs to Solanaceae family, and is an essential crop of vegetables. Tomato plays a vital role in keeping people safe and vigorous. Tomatoes are an important source of vitamin C, B, and an excellent source of β-carotene [1]. Furthermore, tomato is considered to be one of the most important vegetable crops for genetic engineering because it was used as a model plant to introduce important agronomic genes into

dicotyledonous crop plants [2]. Tomato is commonly known as a sensitive plant to biotic and abiotic stresses. Salinity, high temperature, drought, and insect pests are causing damage in all the plant parts. Tomato cultivars 'Peto 86' and 'Strain B' are determinate, open pollinated, 'Strain B' is a relatively small plant and resistant to high temperature also 'Peto 86' is cultivated in large areas with high temperature [3]. 'Peto 86' and 'Strain B' are TYLCV-susceptible cultivars and attacked by several pathogens [4].

The pathogenic organisms such as fungi, viruses and nematodes cause a number of diseases in tomato cultivation. Tissue culture has been used to improve crops productively through the rapid production of superior planting material so that it considered as an important tool of biotechnology [5]. The plant tissue culture techniques are an appropriate tool for improving many crops and vegetables [6] and also it is considered as an important tool for modern plant improvement programs to develop suitable cultivars in minimum time [7].

Callus formation was the effective technique of tissue culture by which embryogenesis, organogenesis and cell suspension are formed when callus is transferred to suitable media [8]. Callus is vacuolated cells contain regions of unorganized small meristematic cells cluster, that are induced by plant growth regulators (PGRs) from a cut region of the explants [9]. The effect of different PGRs has been investigated on callus induction, *in vitro* shoot regeneration and hairy root formation in tomato by [10, 11]. Some workers have previously reported callus induction in many crops such as tomato [12] and rice [13] depending on PGRs in culture media.

Callus formation and tomato organogenesis rely on endogenous and exogenous growth regulators [14]. According to Coenen and Lomax [15], callus induction requires cytokinins and auxins which are essential for cell growth by promoting cell division and elongation. Cytokinins in combination with auxins or alone in media have enhanced callus induction for many tomato cultivars [16]. The nodal explants were grown on NAA and BAP induced callus but showed comparatively slow growth, so sub-cultures on media containing either 2,4-D or NAA+BAP combinations could enhance the callus growth of *Decalepis hamiltonii* without shoot regeneration [17].

Enzymes are considered as a major contributor to the physiological and biochemical processes of plant cells. Many studies are interested in the role of enzymes, such as peroxidase and polyphenol oxidase, in the biological processes of plant cells [18] and callus culture [19, 20].

The present study aimed at callus induction from tomato leaf explants by various growth regulators. Moreover, the objective was to evaluate the free radical scavenging activity of aqueous extracts coupled with peroxidase and polyphenol oxidase activities in callus cultures.

2. Material and Methods

2.1. Plant materials

The cultivars of tomato (*Solanum lycopersicum* L.) 'Peto 86' and 'Strain B' were used in this study. Seeds of two cultivars were obtained from Horticulture Research Institute, Agriculture Research Center, Giza, Egypt. Seeds were germinated in pots at the first of October in greenhouse of National Research Centre. After 30 days of germination, healthy leaves of seedlings were collected for use as explants. All experiments were performed in the Tissue Culture Laboratories, Department of Botany, National Research Centre, Giza, Egypt.

2.2. Callus induction

Leaf explants of tomato cultivars 'Peto 86' and 'Strain B' were washed with tap water then with sterilized distilled water. The explants were surface sterilized with 70%

ethanol for 1 min and rinsed four times in sterilized distilled water. The explants were disinfected with 30% solution of commercial bleach containing 5% of sodium hypochlorite with a few drops of Tween-20 for 10 min, subsequently washed with sterilized distilled water for five times. The leaves were cut into 0.5-1 cm^2 sized explants, and only those possessing central mid-rib were cultured on the Murashige and Skoog (MS) medium [21] supplemented with 30 g/l sucrose and 8 g/l agar (Sigma-Aldrich, USA).

Leaf explants were cultured on different culture media composed of MS medium with different types and concentrations of PGRs such as BAP, IAA, 2,4-D, NAA and Kinetin. The PGRs were added in varying combinations and concentrations shown in Table 1. The pH of the medium was maintained at 5.8 with 1.0 N NaOH or 1.0 N HCl before autoclaving at 121ºC for 20 min. The cultures were incubated at 25±2 ºC temperature, under 16 h photoperiod and photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1} provided by white fluorescent lights. For both cultivars 4 explants per jar and 5 repetitions for each cultivar and treatment were investigated.

Table 1. Culture media composition for callus induction in two tomato cultivars

Medium	Plant Growth regulators (PGR)
M0	PGR- Free (control)
M1	2 mg L^{-1} BAP + 0.5 mg L^{-1} IAA
M2	3 mg L^{-1} BAP + 1 mg L^{-1} IAA
M3	3 mg L^{-1} BAP + 2 mg L^{-1} IAA
M4	3 mg L^{-1} Kin + 2 mg L^{-1} NAA + 1 mg L^{-1} IAA
M5	2 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP

After 30 days of culture, the callus induction frequency was recorded for each treatment combination. The percentage of callus induction (PCI) was calculated using the following formula:

Callus induction (%) **=**

$$\frac{\text{Number of explants induced callus}}{\text{Total number of explants cultured}} \times 100$$

Calli obtained after 4 weeks of culture were used for callus growth study. Sub-cultures to media with the same composition were performed after 30 days. Calli were weighted before their transfer to the fresh callus induction medium (W1). They were weighted again after 4 weeks of sub-culture (W2). Relative fresh weight growth (RFWG) was calculated as (W2 – W1) / W1 [22].

2.3. Preparation of methanol and ethanol extracts

Over two days, fresh calli were dried at 40 ºC, and then dried calli were powdered in liquid nitrogen using a mortar and pestle. The extracts of dried calli were prepared by adding 10 g of powdered calli to 200 ml of 95% methanol or ethanol at room temperature and placing on shaker at 120 rpm for 24 h, then filter methanol and ethanol extracts. The filtrates were evaporated under reduced pressure and vacuum drier to get dried residue and stored at 4 ºC for the estimation of antioxidant activity [23].

2.4. Antioxidant activity (DPPH) assay

DPPH' assay was carried out according to [24] with some modification. The antioxidant activity of the extracts and standard was assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging. Added 10 μl of methanol or ethanol extracts or standard (from 0.0 1μg/ml) to 90 μl of a 100 μM methanolic solution of DPPH in a 96-well microliter plate (Sigma-Aldrich). After

incubation in the dark at 37 °C for 30 min, the decrease in absorbance of each solution was measured at 520 nm using an ELISA micro plate reader. The control was done as the same way by adding methanol and DPPH without extract sample. Every measurement was replicated three times. The percentage of scavenging inhibition was determined and compared with ascorbic acid as standard. The radical-scavenging capacity of the extracts was calculated with the following equation:

Antioxidant activity (%) = $(Abs_{control} - Abs_{sample}) / Abs_{control} *100$

Where $Abs_{control}$ is the absorption of the blank; Abs_{sample} is the absorption of the extract sample.

2.5. Enzyme extraction and enzyme assays

The enzymatic activities were evaluated in fresh calli. All steps of the enzyme extraction were performed under cooling at 4 ºC. Fresh calli (2 g) were homogenized in a mortar with 10 ml of (0.1 M) phosphate buffer (pH 7.5). Then centrifugation at 15000 g and 4 °C for 15 min was done, the supernatant was used for enzyme assays. Peroxidase (POD) and polyphenol oxidase (PPO) activities were determined by the method of Kar and Mishra [25], and then measured by recording the absorbance at 420 nm. The results were recorded in three replicates. One unit of enzyme activity (U) was defined as the amount of enzyme that changed the absorbance min^{-1}. The enzyme activity was presented as U min^{-1} mg^{-1} fresh weight.

2.6. Statistical analysis

All the experiments were analyzed by SPSS program for windows version 14.0, using one way analyses of variance (ANOVA) test. The results were presented as means of five replicates ± standard error (±SE) which compared using Duncan's multiple range test at $p < 0.05$.

3. Results and discussion

3.1. Callus induction

The culture media and concentrations of PGRs affected callus formation from the leaf explants of tomato (*Solanum lycopersicum*) (Table 2). The medium compositions are considered as an important factor of *in vitro* culture for callus induction. All culture media containing different concentrations of PGRs, callus could be initiated on the cut surfaces of the leaf explants within seven days of culture.

Medium (M0) was used as the control medium, without PGRs. It was also observed that the surfaces of leaf explants swelled without callus induced when the leaf explants were cultured on the control medium (M0), but after four weeks the roots were formed from the leaf explants of two cultivars (Figure 1a, g). Similarly, no callus growth was observed on leaf explants of *Artemisia scoparia* cultured on PGRs-free MS medium [26].

In general, calli formation were observed on all surfaces of the leaf explants cultured on media containing both auxins and cytokinins after four weeks. The induction percentages of calli ranged from 90.61% to 100% and 89% to 100% respectively for 'Peto 86' and 'Strain B' according to different concentrations of PGRs in media. Callus formation from tomato leaves depended on BAP, IAA, Kin and 2,4-D concentrations. With the combination of BAP and IAA in MS medium, high percentages of callus induction response were exhibited in two cultivars. The best medium for callus induction was M2 medium consisting of MS medium with 3 mg L^{-1} BAP and 1 mg L^{-1} IAA. The calli were friable and white-creamy or greenish in color, then turned into greenish yellow or green compact calli (Figure 1).

Table 2. Callus induction and the relative fresh weight of leaf explant of two tomato cultivars cultured on different media

Cultivars / Media	'Peto 86'		'Strain B'	
	PCI	RFWG	PCI	RFWG
M0	–	–	–	–
M1	96.11	1.05± 0.16 [b]	95.33	0.84± 0.33 [b]
M2	100.00	1.60± 0.67 [a]	100.00	1.47± 0.64 [a]
M3	98.00	0.70± 0.18 [bc]	97.00	0.50± 0.21 [bc]
M4	94.50	0.64± 0.20 [bc]	96.10	0.72± 0.29 [bc]
M5	90.61	0.48± 0.13 [d]	89.00	0.35± 0.17 [cd]
Mean	79.87	0.75 ± 0.11	79.57	0.65 ± 0.10

Means with the same letter(s) in the column are not significantly different according to Duncan's multiple range tests at $P < 0.05$.

The combination of auxins with cytokinins showed a significant increase especially in M2 medium at ($P < 0.05$) for both cultivars in callus induction and callus growth, RFWG were 1.60 ± 0.67 and 1.47 ± 0.64 for 'Peto 86' and 'Strain B' cultivars. Leaf explants of two cultivars 'Peto 86' and 'Strain B' cultured on M5 medium had shown minimum values of PIC were (90.61% and 89.00%) and RFWG were (0.48 ± 0.13 and 0.35 ± 0.17) (Table 2). The best callus formation from tomato leaf as explant on M2 medium with high percentage 100 % at two cultivars. Similarly, the highest frequency of callus induction recorded on MS medium was supplemented with 2 mg L^{-1} IAA + 2.5 mg L^{-1} BAP, unlike the MS medium added with 1 mg L^{-1} IAA + 1 mg L^{-1} NAA + 2 mg L^{-1} Kin and MS medium containing 0.5 mg L^{-1} IAA + 2 mg L^{-1} 2,4-D gave low callus induction frequencies from hypocotyl and leaf explants of *S. lycopersicum* [11]. MS medium supplemented with BAP 2 mg L^{-1} + NAA 3 mg L^{-1} and BAP 3 mg L^{-1} + NAA 2 mg L^{-1} were proved to be best for callus induction of *Citrullu scolocynthis* [27].

The results revealed that cytokinins especially BAP were suitable for efficient callus induction in tomato cultivars compared to other cytokinins such as Kin. Also, the combination of BAP at higher concentration than that of auxins gave the highest percentage of callus induction and promoted calli growth value by increasing callus fresh weight. Jatoi *et al*. [28] reported that an increasing of BAP concentration in media increased the formation of callus from tomato hybrids. According to Rzepka-Plevneš *et al*. [29], MS medium supplemented with 2 mg L^{-1} of IAA and 1 mg L^{-1} of BAP was the best medium for callus induction from leaves of tomato cv. 'Maskotka'. Sherkar and Chavan [30] also observed that the best formation of callus from internode segments of tomato explants on MS media containing 0.2 mg L^{-1} NAA and 2 mg L^{-1} BAP. The maximum callus induction from tomato leaf was obtained on MS media using high amount of 2,4-D with BAP [31].

The importance of BAP in combination with auxin (2, 4-D) in medium (M5) for induction of callus in tomato cultivars was evident in this research. Callus induction and growth affected by the combination of equal concentrations of cytokinin (BAP) and auxin (2, 4-D) in the culture media of *Artemisia scoparia* [26]; and also, *Glycyrrhiza glabra* calli were produced by media containing 2, 4-D with cytokinin [32]. In contrast, MS medium containing auxin alone in the form of 2, 4-D at concentrations 3.0 mg L^{-1} and 4.0 mg L^{-1} was not able to induce callus from leaf explants of tomato [11] or produced poor callus induction [10]. The present study demonstrated that tissue culture conditions causing the physiological and metabolic changes were related to the

fundamental role of cytokinins as BAP in regulation of tomato calli formation and growth. Cytokinin in tissue culture media had an important role in cell division [33]. Therefore, the media containing higher concentrations of cytokinin BA were induced calli proliferation [34].

3.2. Antioxidant activity of tomato calli extracts

Methanol (ME) and ethanol (ET) extracts prepared from calli of tomato cultivars were investigated to determine their antioxidant activity using DPPH assay. The results of calli extracts exhibited antioxidant activity (59.67 ± 0.13% and 55.80 ± 0.36%) for ET and ME extracts of callus induced from 'Peto 86' cultivar (Figure 2). Additionally, ET extract of callus from 'Strain B' exhibited antioxidant activity (56.21 ± 0.47%) and ME extract has weak antioxidant activity (49.08 ± 0.13%) in Table 3. The different cultivars have been shown to differ in the antioxidant activities that determined by DPPH assay [35].

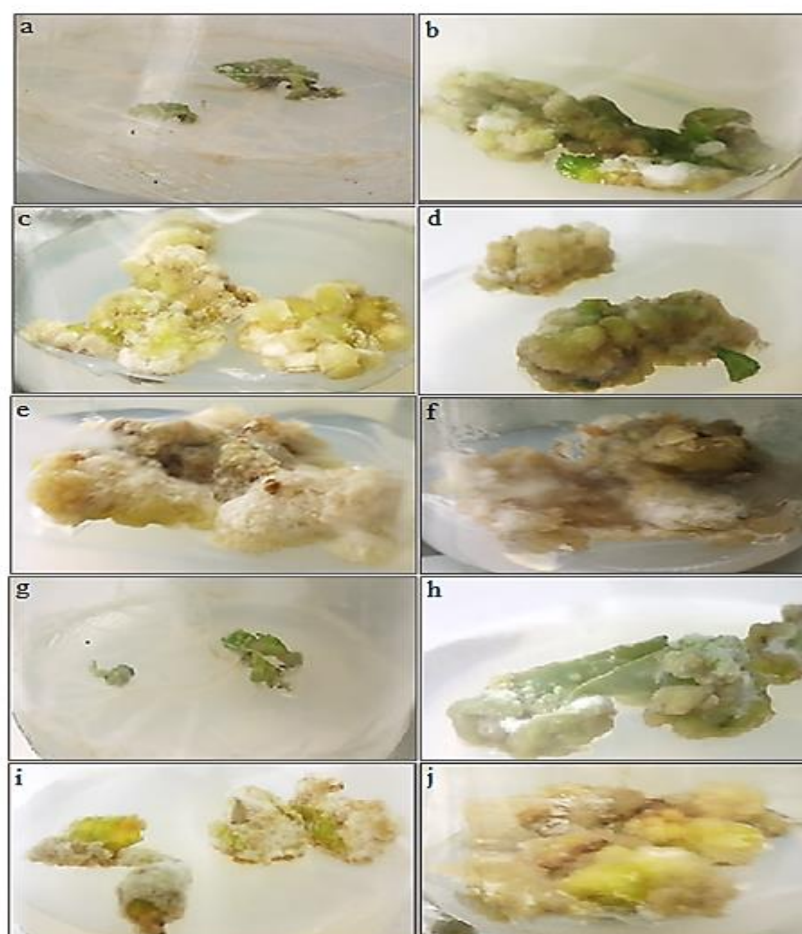

Figure 1. *In vitro* callus induction and growth from leaf explants of two tomato cultivars on MS medium. (a, b, c, d, e, f) Callus induction of 'Peto 86' cultivar. (g, h, i, j) Callus induction of 'Strain B' cultivar. (a, g) Leaf explants on PGR-free MS medium (M0) forming roots. (b, h) Callus initiated on the cut surfaces of the leaf explants. (c, d, i, j) Creamy-greenish granular and compact callus induced on M2 medium. (e, f) Brownish compact callus induction on M5 medium

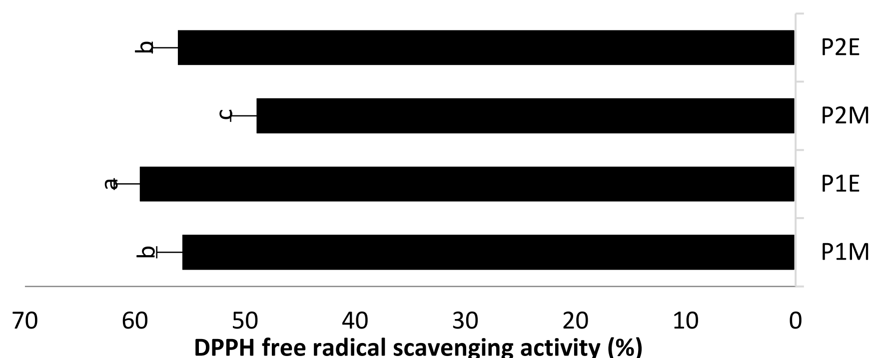

Figure 2. Antioxidant activity of call us methanol and ethanol extracts by DPPH assay. P1M, Callus methanol; P1E, Callus ethanol extracts of 'Peto 86'. P2M, Callus methanol; P2E, Callus ethanol extracts of 'Strain B'. Values are the mean of three replicating with ± standard error. Different letter (s) differs significantly at P < 0.05 according to Duncan's multiple range test

The scavenging DPPH free radicals in callus are performed as a result of the secondary metabolites. Similarly, previous studies referred to the presence of secondary metabolites in callus tissues may be the reason for performance of free radicals scavenging DPPH activity [36]. According to Abbasi *et al.* [37], the callus culture had antioxidant activity of that assessed by DPPH-free radical scavenging.

In general, the callus ET extract showed a significant increase (P < 0.05) in the antioxidant activity compared to the callus ME extracts activity in both cultivars. Ethanolic extracts have more effective to evaluate the antioxidant activity of tomato extracted [38].

Table 3. DPPH activity of callus extracts, peroxidase and polyphenol oxidase activity in callus culture of two tomato

Cultivars	DPPH		POD	PPO
	ME	ET	U min^{-1} mg^{-1} FW	U min^{-1} mg^{-1} FW
Peto 86	55.80 ± 0.36 ^{b}	59.67 ± 0.13^{a}	130 ± 0.53^{a}	20 ± 0.61^{c}
Strain B	49.08 ± 0.13^{c}	56.21 ± 0.47 ^{b}	80 ± 0.75 ^{b}	11 ± 0.27 ^{d}

Means with the same letter(s) in the column are not significantly different according to Duncan's multiple range test at P < 0.05.

3.3. Enzyme activity of tomato calli

The POD and PPO enzymes activities are evaluated in the calli of two tomato cultivars. There are differences between two tomato cultivars calli in their enzyme activity. Calli of two cultivars gave significantly specific activity of POD and PPO. The activities of POD and PPO were considered higher in 'Peto 86' cultivar than in 'Strain B' cultivar (Table 3). According to previous study, the enzyme activities during growth varied with genotype and conditions [8]. The activities of POD and PPO enzymes are present in all plant organs [40, 41]. The physiological changes in callus are related to increasing in the enzymes such as POD and PPO [42]. In addition, the growth regulators in culture media effected on the antioxidant activity that enhances callus growth. The peroxidase activity in callus varied according to different concentrations of growth regulators [43]. Khalid and Aftab [44] referred to the growth regulators able to maintain the endogenous hormonal levels and increase the antioxidant enzyme activities.

4. Conclusions

In conclusion, callus induction from leaf explants was formed in both tested tomato cultivars. The combination of auxins and cytokinins has been the most appropriate for induction of tomato calli, especially in a medium that containing 3 mg L^{-1} BAP combined with 1 mg L^{-1} IAA. In addition, plant growth regulators in callus induction media affect enzyme activity in callus culture. The formation of callus under specific conditions would enhance the antioxidant and enzyme activities. Therefore, calli had antioxidant and enzyme activities can be beneficial for extracting important components or for plant regeneration.

Conflict of interest

None.

Consent for publications

The author read and approved the final manuscript for publication.

Availability of data and material

All data generated and/or analyzed during the current study are embedded in this manuscript.

Authors' contributions

The author designed and performed the idea, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate:

Not applicable.

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