



Micropropagation of pokeweed (*Phytolacca americana* L.) and comparison of phenolic, flavonoid content, and antioxidant activity between pokeweed callus and other parts

Attachai Trunjaruen^{1,*}, Prathan Luecha^{2,*} and Worasitikulya Taratima¹

¹ Salt-Tolerant Rice Research Group, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

² Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Science, Khon Kaen University, Khon Kaen, Thailand

* These authors contributed equally to this work.

ABSTRACT

Background. Pokeweed (*Phytolacca americana* L.) is regarded as an invasive plant in many parts of the world but possesses therapeutic characteristics used for antitumor and rheumatism treatment. This study investigated the effects of auxins and four explants on pokeweed callus induction. The effects of cytokinins and combinations between cytokinins and NAA on shoot and root induction were also studied. TPC, TFC and antioxidant activity of calli were screened and compared with other pokeweed plant parts.

Methods. Four explants were used to induce callus using 2,4-D and IBA at 1, 2, 3 and 4 mg/l for each auxin. Direct shoot organogenesis from nodal explants was investigated using BAP, kinetin and TDZ (1, 2 and 4 mg/l for each cytokinin). Combined effects between cytokinins and NAA at 0.1, 0.2 and 0.3 mg/l were further simultaneously estimated with root induction. Calli derived from the leaves were compared with other plant parts for TPC, TFC and antioxidant activity using the Folin-Ciocalteu, AlCl₃ colorimetric assay and DPPH assays, respectively.

Results. Results showed that MS medium containing 2 mg/l 2,4-D induced callus formation on leaf explants that provided highest fresh and dry weights. Three types of synthetic cytokinins as kinetin, TDZ and BAP were used for direct shoot organogenesis from pokeweed nodes. MS medium containing 2 mg/l kinetin was effective in stimulating normal shoots, with the largest number of shoots and leaves and the longest shoots. The combination between cytokinins and NAA showed no positive effect on shoot and root induction from pokeweed nodal explants. For TPC and TFC determination, pokeweed seeds and leaves possessed the highest phenolic and flavonoid contents, respectively. Highest phenolic content of pokeweed seeds led to lowest IC₅₀ by DPPH assay. Phenolic content was higher than flavonoid content.

Conclusion. Results suggested promising conditions for callus induction. Leaf explants cultured on MS medium with 2 mg/l 2,4-D and nodal explants cultured on MS medium with 2 mg/l kinetin provided the largest number of normal shoots and leaves. NAA did not show positive effects on shoot and root induction when combined with cytokinins. Chemical constituent screening indicated that seeds and leaves provided

Submitted 20 September 2021

Accepted 16 January 2022

Published 7 February 2022

Corresponding author
Worasitikulya Taratima,
worasitikulya@gmail.com

Academic editor
Robert Winkler

Additional Information and
Declarations can be found on
page 15

DOI 10.7717/peerj.12892

© Copyright
2022 Trunjaruen et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

highest TPC and TFC, respectively, while pokeweed calli contained higher phenolic than flavonoid content. This is the first report describing chemical constituent screening and antioxidant activity of calli and other parts of the pokeweed plant. Results provided significant information to further enhance bioactive compound contents of pokeweed calli using elicitation methods.

Subjects Biochemistry, Biotechnology, Plant Science

Keywords Antioxidant, Callus, Chemical constituents, Micropropagation, Pokeweed

INTRODUCTION

Phytolacca americana L. or pokeweed (family Phytolaccaceae), is widely distributed as a native weed in North America and regarded as an invasive plant in many parts of the world (Balogh & Juhasz, 2008). Pokeweed seeds are phenolic-rich and possess interesting biological properties. Seed derivative americanin A shows antitumor activity for human colon cancer, while isoamericanin B and C inhibit tyrosinase activity (Jung et al., 2015; Petrillo et al., 2019). However, copious quantities of roots and seeds would be required for commercial purposes and plant tissue culture is now an effective technique to produce large numbers of plants and also metabolites that can be used for medicinal purposes.

Mass plant production within a limited time is the major advantage of the plant tissue culture technique (Bhoite & Palshikar, 2014). Callus can be induced from all parts of plants and used as materials for plant regeneration to stimulate production of bioactive compounds. Direct shoot organogenesis is a process whereby new shoots are directly produced from other vegetative parts (Bhatia & Bera, 2015). Callus induction and direct shoot organogenesis are different manipulation methods, and factors influencing both processes represent the balance between auxins and cytokinins. High ratios of auxins and cytokinins induce roots, while low ratios induce shoots from explants and equal ratios promote callus induction (Ikeuchi, Sugimoto & Iwase, 2013). However, optimal concentrations of auxins and cytokinins need to be estimated for plant species and specific types of explants. Therefore, it is necessary to select types of explants that are appropriate for micropropagation.

Most previous research about pokeweed micropropagation focused on direct shoot organogenesis (Zou et al., 2008 and El-Minisy et al., 2017) but research concerning callus induction of pokeweed is limited. Pokeweed callus induction was first established for cell suspension production. Sakuta, Hirano & Komamine (1991) induced calli from pokeweed stems and produced pokeweed cell suspension from the calli. Some phytochemicals, like betalains, can be produced from calli and cell suspension. Consequently, this study determined the effects of auxins and explant types on pokeweed callus induction to obtain calli that could be used for chemical constituents and antioxidant activity determination. The effects of PGRs and explants on direct shoot organogenesis were also evaluated for more efficient pokeweed micropropagation. Total phenolic and flavonoid contents and total antioxidant activity by DPPH assay were also evaluated to compare phytochemical contents between the callus and other parts of wild plants.

MATERIALS & METHODS

Plant materials

Pokeweed plants were cultivated, and seeds were obtained from ripened pokeweed fruits grown in Sakon Nakhon Province, Thailand (17°27'24.1"N 103°27'20.2"E). The plant was identified by Dr. Sakuntala Ninkaew, and the specimens were deposited at KKU herbarium (KKU No. 26576). For *in vitro* germination, pokeweed seeds were soaked in concentrated sulfuric acid for 10 min and then rinsed with running tap water. Seeds were then sterilized by agitation with 2% (v/v) sodium hypochlorite for 30 min and rinsed with sterilized distilled water for 5 min three times. The sterilized seeds were cultured on solid Murashige and Skoog (MS; 1962) medium supplemented with 30 g/l sucrose (pH 5.8). Cultures were incubated at 25 ± 2 °C with a 16/8 h (light/dark) cycle at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Media and equipment were sterilized by autoclaving at 121 °C for 20 min. Cotyledons, leaves, nodes and internodes were obtained from *in vitro* grown plants aged 2 months after germination. Cotyledons and leaf explants were cut into 1×1 cm squares, and 1 cm long nodal and internodal explants were used for callus induction and direct shoot organogenesis.

Callus induction

Cotyledons, leaves, nodes and internodes from *in vitro* grown pokeweed were used as initial explants. Solid MS medium fortified with 30 mg/l of sucrose (pH 5.8) was used as basal medium and supplemented with 2,4-D and IBA at 1, 2, 3 and 4 mg/l for both PGRs. MS medium without PGRs was considered as the control. Callus induction cultures were maintained under aseptic conditions at 25 ± 2 °C with a 16/8 h (light/dark) cycle at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity for four weeks before data collection.

Survival, response and callus formation percentages were calculated at week four after callus induction. Explants showing viable characteristics were considered as surviving. Response percentages were estimated from the surviving explants as changes in growth. Explants with callus formation were used to calculate callus induction percentage, fresh weight and dry weight. For dry weight, the calli were dried at 45 °C for 3 days.

Direct shoot organogenesis

Explants of cotyledons, leaves, nodes and internodes were used as plant materials. Solid MS medium with 30 g/l sucrose (pH 5.8) was supplemented respectively with three types of synthetic cytokinins as BAP, kinetin and TDZ applied for each cytokinin at 1, 2 and 4 mg/l. MS medium without PGRs was considered as the control. The cultures were maintained under aseptic conditions at 25 ± 2 °C with a 16/8 h (light/dark) cycle at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity for six weeks before data collection.

Survival percentages, response, callus formation, and root and shoot formation percentages were collected six weeks after culture for direct shoot organogenesis. Explants with shoots and roots were used to calculate shoot and root formation percentage. Number of shoots per explant, shoot length and number of leaves per shoot were evaluated simultaneously.

Combined effects of cytokinins and auxins

From four explant types, only nodal explants showed potential for shoot organogenesis. Therefore, nodes from *in vitro* grown pokeweeds were used as plant materials in this experiment. Solid MS media (pH 5.8) with three cytokinin conditions as 1 mg/l BAP, 2 mg/l kinetin and 1 mg/l TDZ were combined with four concentrations of NAA (0, 1, 2 and 3 mg/l). MS medium without PGRs was considered as the control. The cultures were maintained under aseptic conditions at 25 ± 2 °C with a 16/8 h (light/dark) cycle at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity for six weeks before data collection.

Percentages of survival, response, callus formation and root and shoot formation were collected at six weeks after culture for direct shoot organogenesis. Explants with shoots and roots were used to calculate shoot and root formation percentages. Number of shoots per explant, shoot length and number of leaves per shoot were evaluated simultaneously.

Chemical constituents and antioxidant activity

Calli induced from leaf explants cultured on MS medium added with 2 mg/l 2,4-D, leaves, roots and seeds were dried at 45 °C for 3 days. All samples were ground and then extracted with methanol. Briefly, 100 mg of powdered sample were extracted with 1 ml of methanol and sonicated for 30 min at 30 °C. The extraction was repeated three times and the extract solutions were assembled. Methanol was evaporated using a vacuum oven at 45 °C for 48 h. The extracts were used to determine chemical constituents and antioxidant activities.

Total phenolic content of the pokeweed callus extract was determined and compared with TPC of other plant parts using the Folin-Ciocalteu assay with some modifications following [Mwamatope et al. \(2020\)](#). Extracts at 20 μl were mixed with 100 μl of 10-fold diluted Folin-Ciocalteu solution and then added with 80 μl sodium carbonate (Na_2CO_3) in microplates. The microplates were incubated in darkness for 30 min. TPC was determined at 760 nm and calculated as mg gallic acid equivalent (GAE)/g dry weight extract.

Total flavonoid content of pokeweed callus extract was determined and compared with TFC of other plant parts based on the complex between flavonoid compounds and aluminum chloride (AlCl_3) ([Pekal & Pырzynska, 2014](#)). Extracts at 100 μl were mixed with 50 μl of 2% AlCl_3 and 50 μl of water in microplates. The reaction was carried out in the dark for 30 min, determined at 425 nm and calculated as mg quercetin equivalent (QE)/g dry weight extract.

DPPH assay was carried out following the method of [Mwamatope et al. \(2020\)](#). The solution was incubated with DPPH reagent for 30 min at room temperature under dark condition. After incubation, absorbance was measured at 517 nm using a spectrophotometer. Methanol and ascorbic acid were used as negative and positive controls, respectively. Free radical scavenging activity was calculated by the following equation.

$$\% \text{ free radical scavenging activity by DPPH} = [1 - (A_{\text{sample}} / A_{\text{control}})] \times 100$$

A sample means the absorbance of sample solution, while A control denotes the absorbance of negative control, with both measured at 517 nm.

Statistical analysis

All experiments were carried out with ten replications per treatment. Data were analyzed by one-way Analysis of Variance (ANOVA) and means were separated using Least Significant Difference (LSD; $p < 0.05$). Data were expressed as mean \pm standard error of mean (SE). Statistical analysis was performed with Statistix 10 software.

RESULTS

Effects of explant types and auxins on callus induction

Results showed that all types of explants survived and responded in all treatments without significant difference. Cotyledons and leaves only generated calli in media with 2,4-D, while roots were induced from explants by media supplemented respectively with IBA (Fig. 1A). Similar responses were also found in treatments of nodal and internodal explants cultured in the media with IBA (Fig. 1B). Interestingly, shoot formation was observed only in the treatments of nodal explants cultured on MS medium without PGRs and media with IBA, singly (Fig. 1C). This phenomenon showed the shoot organogenesis potential of pokeweed nodal explants.

This experiment identified conditions that were suitable for callus induction of pokeweed. Calli from all treatments showed similar morphology as friable with pink to purple color. Observation under a stereomicroscope revealed elongated parenchymal cells, mostly with accumulated green or purple pigment and some elongated and clear without pigment (Fig. 1D). Considering callus induction percentage, all explant types cultured on media containing 2,4-D produced calli leading to 100% callus induction percentage in those treatments (P -value = 0.00; Table 1). Callus production was also observed in treatments of internodes and nodes cultured on media with IBA, ranging 37.50–62.50% and 56.25–81.25%, respectively. However, callus percentages from IBA treatments were significantly lower than those from 2,4-D treatments (P -value = 0.00; Table 1). Significantly highest callus fresh weight was obtained from nodes cultured on medium containing 3 mg/l IBA (Fig. 1B) and leaves cultured on medium containing 2 mg/l 2,4-D (Fig. 1E) at 0.81 g and 0.80 g, respectively while these two treatments also provided the highest dry weights of calli at 0.05 g (P -value = 0.00; Table 1). Callus induction percentage of leaf explants cultured on 2 mg/l 2,4-D reached 100%, while nodes cultured on 3 mg/l IBA provided only 62.50% (Table 1). Fresh and dry weights of calli generated from leaf explants (0.69–0.80 g and 0.04–0.05 g, respectively) were higher than calli from other explants (Table 1). Therefore, culture of leaf explants on MS medium supplemented with 2 mg/l 2,4-D was the most appropriate for callus induction from pokeweed.

Effects of explant types and cytokinins on direct shoot organogenesis

Different types and concentrations of cytokinins were tested with cotyledon, leaf, internodal and nodal explants of pokeweed to induce direct shoot organogenesis. Six weeks after culture, percentages of root formation, shoot formation, number of shoots per explant, shoot length and number of leaves per shoot were recorded. Most treatments produced callus, while only nodal explants produced roots in the control treatment (Fig. 1F).

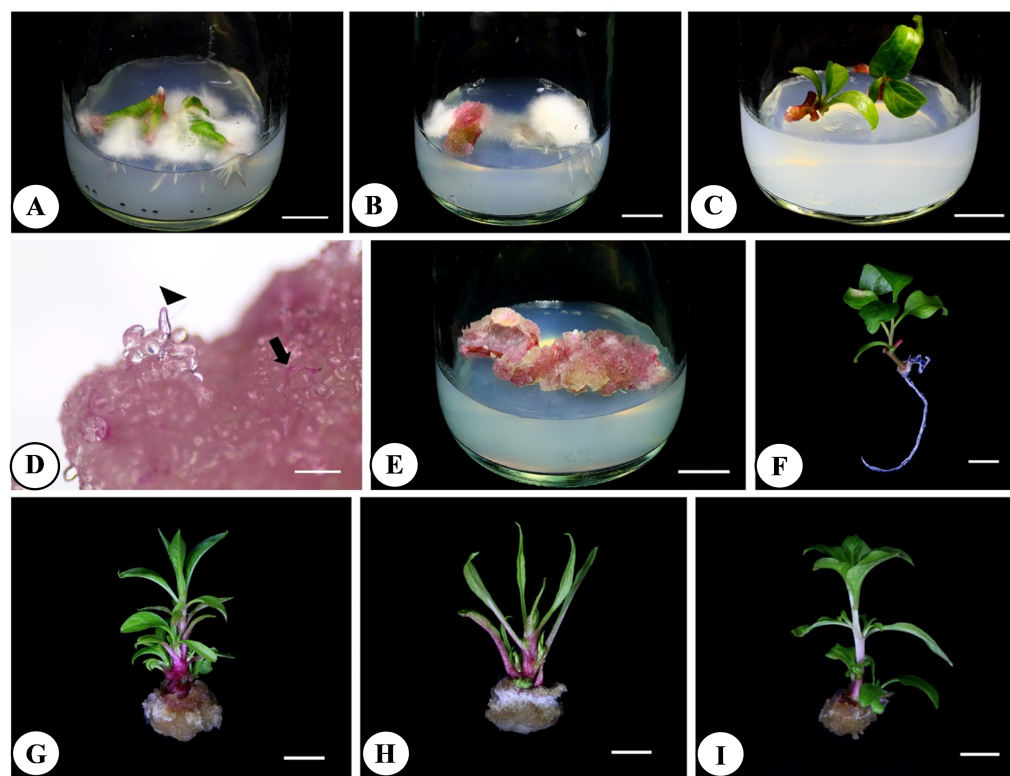


Figure 1 *In vitro* calli and plantlets grown on MS media supplemented with different PGRs. (A) Leaf explants cultured on MS medium with 4 mg/l IBA; (B) nodal explants cultured on MS medium with 3 mg/l IBA; (C) nodal explants cultured on MS medium at four weeks; (D) Callus with purple pigment inside the cells (arrow) and elongated clear cells (arrowhead); (E) leaf explants cultured on MS medium with 2 mg/l 2,4-D; (F) nodal explants cultured on MS medium at six weeks; (G) nodal explants cultured on MS medium with 1 mg/l BAP; (H) nodal explants cultured on MS medium with 1 mg/l TDZ; (I) nodal explants cultured on MS medium with 2 mg/l kinetin; scale = 1 cm (A–C, E–I); scale = 500 μ m (D).

Full-size [DOI: 10.7717/peerj.12892/fig-1](https://doi.org/10.7717/peerj.12892/fig-1)

Cotyledon explants turned from green to purple in treatments of control and kinetin without any other changes, while color change was also found when leaf explants were cultured on the control medium.

Shoot formation percentages were calculated to identify suitable conditions for direct shoot organogenesis. Results showed that only nodal explants produced novel shoots that emerged directly from the leaf axillary. All treatments provided 100% shoot formation percentage, except for the control that gave 37.50% and significantly lower than the others (P -value = 0.00; Table 2). All treatments provided new shoots from the explants, but some abnormalities were observed from these shoots and leaves. In the control treatment, generated shoots gave expanding leaves with slender stems, which were considered normal characteristics (Fig. 1F). Shoots from BAP and TDZ treatments showed aberrations such as curling at the leaf margin and fleshy thick stems (Figs. 1G and 1H), whereas more slender and less fleshy stems with normal leaves were observed in treatments of kinetin (Fig. 1I). The number of shoots per explant, shoot length and number of leaves per shoot were also recorded. Treatment of 1 mg/l TDZ provided significantly largest number of shoots

Table 1 Callus induction percentage of different pokeweed explants affected by various types and concentrations of auxins.

Explants	PGRs		Callus induction (%)	Fresh weight (mg)	Dry weight (mg)	
	Auxins	Conc. (mg/l)				
Cotyledon	control	0	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
		1	100.00 ± 0.0 ^a	0.44 ± 0.0 ^s	0.03 ± 0.0 ^c	
		2	100.00 ± 0.0 ^a	0.54 ± 0.0 ^{ef}	0.03 ± 0.0 ^c	
		3	100.00 ± 0.0 ^a	0.49 ± 0.0 ^g	0.03 ± 0.0 ^c	
	2,4-D	4	100.00 ± 0.0 ^a	0.53 ± 0.0 ^{ef}	0.03 ± 0.0 ^c	
		1	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
		2	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
		3	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
	IBA	4	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
		control	0	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f
		1	100.00 ± 0.0 ^a	0.70 ± 0.0 ^b	0.04 ± 0.0 ^b	
		2	100.00 ± 0.0 ^a	0.80 ± 0.0 ^a	0.05 ± 0.0 ^a	
Leaf	2,4-D	3	100.00 ± 0.0 ^a	0.69 ± 0.1 ^b	0.04 ± 0.0 ^b	
		4	100.00 ± 0.0 ^a	0.75 ± 0.0 ^{ab}	0.04 ± 0.0 ^b	
		1	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
		2	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
IBA	3	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f		
	4	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f		
	control	0	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ^f	
	1	100.00 ± 0.0 ^a	0.22 ± 0.0 ^j	0.01 ± 0.0 ^e		
Internode	2,4-D	2	100.00 ± 0.0 ^a	0.21 ± 0.0 ^j	0.01 ± 0.0 ^e	
		3	100.00 ± 0.0 ^a	0.24 ± 0.0 ^j	0.01 ± 0.0 ^e	
		4	100.00 ± 0.0 ^a	0.22 ± 0.0 ^j	0.01 ± 0.0 ^e	
		1	56.25 ± 14.7 ^c	0.30 ± 0.0 ⁱ	0.02 ± 0.0 ^d	
IBA	2	62.50 ± 12.5 ^c	0.35 ± 0.0 ^{hi}	0.03 ± 0.0 ^c		
	3	56.25 ± 11.3 ^c	0.35 ± 0.1 ^{hi}	0.03 ± 0.0 ^c		
	4	37.50 ± 12.5 ^d	0.38 ± 0.0 ^h	0.03 ± 0.0 ^c		
	control	0	0.00 ± 0.0 ^e	0.00 ± 0.0 ^k	0.00 ± 0.0 ⁱ	
Node	2,4-D	1	100.00 ± 0.0 ^a	0.61 ± 0.0 ^{cd}	0.04 ± 0.0 ^b	
		2	100.00 ± 0.0 ^a	0.56 ± 0.0 ^{d-f}	0.03 ± 0.0 ^c	
		3	100.00 ± 0.0 ^a	0.56 ± 0.0 ^{c-e}	0.03 ± 0.0 ^c	
		4	100.00 ± 0.0 ^a	0.58 ± 0.0 ^{c-e}	0.04 ± 0.0 ^b	
IBA	1	62.50 ± 8.1 ^c	0.62 ± 0.0 ^c	0.04 ± 0.0 ^b		
	2	81.25 ± 9.1 ^b	0.61 ± 0.0 ^{cd}	0.04 ± 0.0 ^b		
	3	62.50 ± 8.1 ^c	0.81 ± 0.0 ^a	0.05 ± 0.0 ^a		
	4	56.25 ± 6.2 ^c	0.59 ± 0.0 ^{c-e}	0.04 ± 0.0 ^b		

Notes.

Means ± SE followed by different letters are significantly different by ANOVA and least significant difference tests ($p < 0.05$). Different letters (a, b, c, \dots) represent significant differences in columns ($p < 0.05$).

Table 2 Direct shoot organogenesis from nodal explants of pokeweed influenced by various types and concentrations of cytokinins.

Cytokinins conc. (mg/l)	Root formation (%)	Shoot formation (%)	Number of shoots	Shoot length (cm)	Number of leaves
Control					
0	31.25 ± 9.1 ^a	37.50 ± 8.1 ^b	1.00 ± 0.0 ^c	4.41 ± 0.4 ^{ab}	6.06 ± 0.4 ^d
BAP					
1	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	2.18 ± 0.1 ^{ab}	4.18 ± 0.1 ^b	9.50 ± 0.3 ^{ab}
2	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	2.06 ± 0.1 ^{ab}	3.82 ± 0.1 ^{bc}	8.81 ± 0.5 ^{bc}
4	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	1.25 ± 0.1 ^c	2.49 ± 0.1 ^c	5.56 ± 0.6 ^d
Kinetin					
1	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	1.25 ± 0.1 ^c	4.93 ± 0.2 ^a	10.37 ± 0.5 ^{ab}
2	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	1.81 ± 0.1 ^b	5.04 ± 0.2 ^a	10.93 ± 0.7 ^a
4	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	2.12 ± 0.2 ^{ab}	3.82 ± 0.1 ^{bc}	9.50 ± 0.8 ^{ab}
TDZ					
1	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	2.43 ± 0.2 ^a	3.12 ± 0.2 ^{de}	7.18 ± 0.5 ^{cd}
2	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	2.18 ± 0.1 ^{ab}	2.93 ± 0.1 ^{de}	7.06 ± 0.6 ^d
4	0.00 ± 0.0 ^b	100.00 ± 0.0 ^a	1.81 ± 0.1 ^b	3.44 ± 0.2 ^{cd}	6.93 ± 0.5 ^d

Notes.

Means ± SE followed by different letters are significantly different by ANOVA and least significant difference tests ($p < 0.05$). Different letters (*a, b, c...*) represent significant differences in columns ($p < 0.05$).

per explant (2.43 shoots), while nodal explants cultured on medium with 2 mg/l kinetin gave significantly longest shoots and largest number of leaves per shoot as 5.04 cm and 10.93 leaves, respectively (P -value = 0.00; Table 2). Interestingly, all treatments except for the control generated callus at the basal part of nodal explants. In each type of explant, cytokinin concentrations at 1, 2 and 1 mg/l gave significantly highest number of shoots, shoot length and number of leaves for BAP, kinetin and TDZ, respectively. Therefore, these concentrations were used in the basal medium to investigate the combined effects of cytokinins and auxins on direct shoot organogenesis.

Combined effects of cytokinins and auxins on direct shoot organogenesis

According to the previous study, 1 mg/l BAP, 2 mg/l kinetin and 1 mg/l TDZ as suitable conditions for direct shoot organogenesis were combined with 0.1, 0.2 and 0.3 mg/l NAA to optimize conditions for direct shoot organogenesis and root induction simultaneously. The same parameters with the previous experiments were collected after culture for six weeks. Results showed that survival and response percentages were not affected by these combinations, leading to 100% in all treatments. However, shoots from the control have roots emerged from the base, while the media containing both cytokinin and NAA could not produce root from the explants (Table 3).

The control treatment gave 37.50% shoot formation, significantly lower than percentages from other treatments that all recorded 100% (P -value = 0.00; Table 3). Abnormal morphology increased when NAA concentration increased, for example curling at the leaf margin, non-expanding leaves and fleshy stems. Media supplemented with 2 mg/l kinetin

Table 3 Combined effects of cytokinins and NAA on direct shoot organogenesis from pokeweed nodal explants.

PGRs		Roots formation (%)	Shoots formation (%)	Shoots number	Shoot length	Leaves number
Cytokinins	NAA conc. (mg/l)					
Control		31.25 ± 9.15 ^a	37.50 ± 8.18 ^b	1.00 ± 0.00 ^e	4.41 ± 0.49 ^{ab}	6.06 ± 0.49 ^{cd}
1 mg/l BAP	0	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.18 ± 0.16 ^{a-c}	4.18 ± 0.13 ^b	9.50 ± 0.37 ^b
	0.1	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.06 ± 0.19 ^{a-d}	3.86 ± 0.11 ^b	7.25 ± 0.50 ^c
	0.2	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.68 ± 0.17 ^{cd}	3.80 ± 0.11 ^b	6.43 ± 0.34 ^{cd}
	0.3	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.43 ± 0.15 ^{de}	3.18 ± 0.18 ^{cd}	7.12 ± 0.40 ^c
2 mg/l KIN	0	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.81 ± 0.16 ^{b-d}	5.04 ± 0.22 ^a	10.93 ± 0.70 ^a
	0.1	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.50 ± 0.32 ^a	3.16 ± 0.19 ^{cd}	5.25 ± 0.44 ^d
	0.2	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.50 ± 0.30 ^a	2.98 ± 0.25 ^d	5.25 ± 0.49 ^d
	0.3	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.93 ± 0.21 ^{a-d}	2.79 ± 0.23 ^d	6.81 ± 0.66 ^c
1 mg/l TDZ	0	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.43 ± 0.25 ^{ab}	3.12 ± 0.21 ^d	7.18 ± 0.57 ^c
	0.1	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	2.37 ± 0.28 ^{ab}	3.21 ± 0.18 ^{cd}	7.31 ± 0.29 ^c
	0.2	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.73 ± 0.26 ^{cd}	2.60 ± 0.15 ^d	6.00 ± 0.34 ^{cd}
	0.3	0.00 ± 0.00 ^b	100.00 ± 0.00 ^a	1.68 ± 0.23 ^{cd}	2.96 ± 0.18 ^d	6.62 ± 0.42 ^c

Notes.

Means ± SE followed by different letters are significantly different according to ANOVA and Least Significant Different Test ($p < 0.05$). Different letters (a, b, c...) represent significant differences in columns ($p < 0.05$).

combined with 0.1 and 0.2 mg/l NAA showed significantly largest number of shoots per explant (2.5 shoots; P -value = 0.00). For shoot length and number of leaves per shoot, the highest parameters were obtained from single treatment of 2 mg/l kinetin as 5.04 cm and 10.93 leaves, respectively (P -value = 0.00; Table 3). Interestingly, these parameters tended to decrease when concentration of NAA combined with each type of cytokinin increased (Table 3). Therefore, parameters from media supplemented with only each cytokinin were higher than those from treatments of cytokinin-auxin combination.

Chemical constituents and antioxidant activity

Phenolic and flavonoid contents and IC_{50} values for DPPH assay of the four explants were determined as preliminary screening of some bioactive pokeweed compounds. Parameters of calli, leaves, roots and seeds of pokeweed were compared (Table 4). Results demonstrated that pokeweed seeds provided the highest phenolic content as 155.83 mg GAE/g dry weight extract, while calli, leaves and roots had significantly lower phenolic content than seeds ranging from 14.03 to 26.03 (P -value = 0.00). The flavonoid content of pokeweed leaves was significantly higher than other explants (57.31 mg QE/g dry weight extract). Pokeweed seeds and roots contained lower flavonoid content than leaves as 49.81 and 33.46 mg QE/g dry weight extract, respectively while pokeweed calli had the lowest flavonoid content (11.73 mg QE/g dry weight extract; P -value = 0.00).

Antioxidant activities of the four pokeweed explants were determined and compared with ascorbic acid as control using DPPH assay and expressed inhibitory concentration at 50% (IC_{50}). Pokeweed seeds and ascorbic acid had significantly lowest IC_{50} at 12.66 and 3.91 μ g/ml, respectively. Higher IC_{50} values were obtained from leaves and calli at 228.70 and 341.99 μ g/ml, respectively, while roots showed the highest IC_{50} at 11256.51 μ g/ml.

Table 4 TPC, TFC and IC₅₀ by DPPH assay of different types of pokeweed explants.

Explant types	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	IC ₅₀ by DPPH assay (μg/ml)
Callus	26.03 ± 0.6 ^b	11.73 ± 0.5 ^d	341.99 ± 15.3 ^b
Leaf	28.56 ± 0.4 ^b	57.31 ± 0.7 ^a	228.70 ± 4.0 ^c
Root	14.03 ± 0.3 ^b	33.46 ± 1.2 ^c	1125.51 ± 21.5 ^a
Seed	155.83 ± 8.6 ^a	49.81 ± 1.3 ^b	12.66 ± 0.6 ^d
Ascorbic acid	–	–	3.91 ± 0.0 ^d

Notes.

Means ± SE followed by different letters are significantly different by ANOVA and least significant different tests ($p < 0.05$). Different letters (*a, b, c, ...*) represent significant difference in columns ($p < 0.05$).

These results demonstrated that pokeweed calli from *in vitro* culture generated phenolic and flavonoid compounds that were responsible for antioxidant activity determined by DPPH assay.

DISCUSSION

Different responses of explants to auxins in pokeweed callus induction

Different types of pokeweed explants from aseptic plants were cultured on MS medium with 2,4-D and IBA at various concentrations to investigate the effects of explant types and auxins on callus induction efficiency. Survival and response percentage from all treatments were not affected but fresh and dry weight of calli were strongly influenced. Remarkably, callus from any part of pokeweed showed green to purple, resulting from the betacyanin pigments accumulated in callus cells (*Sakuta, Hirano & Komamine, 1991*).

Our results found that calli derived from leaf explants had larger fresh and dry weights than calli derived from other explants. The success of callus induction from leaf explants was also reported in *Vanda* sp. as callus induction from leaf segments (*Budisantoso, Amalia & Kamsinah, 2017*). Possible reasons for the success were high stomatal density and spongy mesophyll that facilitated nutrients and PGR uptake, therefore, leaf explants provided high callus efficiency (*Zhang et al., 2020*). These reasons were also valid for callus induction from cotyledons that have stomata and spongy mesophyll. Recent results showed that fresh and dry weight of cotyledon-derived calli were similar to calli from leaf explants. In lotus (*Nelumbo nucifera*), four explant types were tested for callus induction. Results showed that immature cotyledons were suitable for callus induction of lotus, with 20% callus formation after three weeks of culture (*Deng et al., 2020*). However, our results showed adverse results of nodal and internodal explants, while some previous studies demonstrated the positive effects of those explants. *Kumlay & Ercisli (2015)* recorded high performance for callus induction from nodal explants of potato (*Solanum tuberosum*). They explained that potato leaves have a large surface area with high rate of water loss, while lower surface area and stomata of nodal explants led to better efficiency of callus induction. Our results confirmed that differences in explant histology affected callus induction efficiency and response of explants to PGRs.

The balance of PGRs in growth media is a vital factor that affects plant callus induction. Equal ratios of auxins and cytokinins promoted callus induction (Ikeuchi, Sugimoto & Iwase, 2013) but appropriate types and concentrations of auxins and cytokinins must first be considered as PGRs for specific plant species. Most studies reported that exogenous auxins were necessary for callus induction. Therefore, here, we focused on the effects of auxin types and concentrations on callus induction of pokeweed. In this study, 2,4-D and IBA were applied with different pokeweed explants. Results showed that callus induction media containing 2,4-D induced calli from all types of explants, consistent with previous studies. Callus induction from bamboo (*Dendrocalamus hamiltonii*) was carried out by shoot tip culture on medium with 3 mg/l 2,4-D and 1 mg/l benzyladenine (Zang et al., 2016). Callus induction medium with 2 mg/l 2,4-D was identified as the optimal condition for landrace rice (*Oryza sativa*) from Thailand (Trunjaruen et al., 2020). Auxins, e.g., 2,4-D and NAA in callus induction medium were reported to promote expression of important genes like LBD family and ARF19 (Ikeuchi, Sugimoto & Iwase, 2013), resulting in callus formation. Supplementation of 2,4-D also induced several genes involving signal transmission and overexpression of transcription factors ZmBBM in maize (*Zea mays*) that induced callus formation from their scutella (Du et al., 2019). All these genes have been proved for involvement of auxins with callus formation under *in vitro* conditions.

Some studies have investigated pokeweed cell suspensions. Medium supplemented with 1 mg/l 2,4-D was used for callus induction from stem explants and cell suspension establishment from those calli (Sakuta, Takagi & Komamine, 1986; Sakuta, Hirano & Komamine, 1991). However, reports about the effects of explant types and other auxins on callus induction are limited. Therefore, this study determined improved and reliable conditions for pokeweed callus induction. The calli obtained from this study could be also extracted and determined phenolic, flavonoid content, and antioxidant activity.

Direct shoot organogenesis influenced by explant and cytokinin types

Callus induction is a vital step for plant micropropagation because callus can be applied as the initial materials for indirect organogenesis. However, our preliminary experiments demonstrated that indirect shoot organogenesis could not be induced from calli, therefore, direct shoot organogenesis was an alternative method to produce *in vitro* pokeweed plantlets. The plantlets can be used for pokeweed propagation, while leaves from the plantlets can also be used as explants for callus induction.

In a recent study, we investigated the effects of cytokinins on direct shoot organogenesis of pokeweed from different types of explants. Our results showed that new shoots were only generated from nodal explants and were induced in all treatments. Several studies reported the success of direct shoot organogenesis from nodal explants. Direct shoot organogenesis of maize was carried out with nodal explants cultured on medium supplemented with 1.8 mg/l benzyladenine (Mushke, Yarra & Bulle, 2016), while compared with three different nodal explants, *Tylophora indica* showed the highest efficiency of direct shoot organogenesis (Najar et al., 2018). New shoots emerged from the leaf axils of nodal explants, possibly influenced by apical dominance that promoted multiple shoots (Oliveira et al., 2013).

Our findings revealed that nodal explants from all treatments provided new shoots. The control treatment gave lowest shoot formation percentage, while other treatments reached 100% (Table 2). These results proved that applications of exogenous cytokinins had positive effects on direct shoot organogenesis, consistent with many previous studies. A concentration of 3 mg/l BAP was reported as the optimal condition over other cytokinins for direct shoot regeneration of potato tree (*Solanum erianthum*; Sarkar & Banerjee, 2020). In direct shoot organogenesis of *Cryptocoryne wendtii*, shoot tips in the control treatment gave lower shooting percentage than treatments of BAP and kinetin, while the appropriate cytokinin conditions for direct shoot organogenesis was 3 mg/l kinetin (Klaocheed et al., 2020). For direct shoot organogenesis of apple, appropriate types and concentrations of cytokinins mainly depended on genotypes and initial explants (Magyar-Tabori et al., 2010). The AHK4/WOL cytokinin receptor is the major component in shoot regeneration and induces many downstream genes. Overexpression of *ARR1* and *WUS*, which are normally upregulated by AHK4/WOL, promoted shoot regeneration in a cytokinin-free medium (Ikeuchi et al., 2019). This information proved that cytokinins promote shoot regeneration through the regulation of gene expression.

Results showed that 1 mg/l TDZ provided the largest number of shoots per explant, while shoots from treatments of TDZ showed abnormalities like thick and succulent stems and curling leaves. These characteristics were considered as hyperhydricity or vitrification, as phenomena that always occur in plants grown under *in vitro* conditions (Kevers et al., 2004). Micropropagated pear showed higher hyperhydricity in the shoots from TDZ treatments than benzyladenine and kinetin (Kadota & Niimi, 2003). Therefore, treatment of 2 mg/l kinetin was considered to be the most suitable conditions for pokeweed direct shoot organogenesis because novel shoots were not abnormal, with longest shoot length and highest number of leaves as 1.81 shoots per explant, 5.04 cm length and 10.94 leaves per shoot (Table 2).

Previous studies reported protocols for direct shoot organogenesis of pokeweed. MS medium with 2.0 mg/l benzyladenine and 0.2 NAA mg/l was appropriate for direct shoot induction from stem segments of pokeweed (Zou et al., 2008). MS medium supplemented with 2 mg/l BAP and 0.2 mg/l NAA provided 2.6 shoots generated from stem segments, higher than our results

(El-Minisy et al., 2017). However, micropropagated shoots reported by El-Minisy et al. (2017) were longer with greater numbers of leaves than previous studies. As well as direct shoot organogenesis, callus generation was induced from the base of micropropagated shoots in all treatments except for the control. This proved that the application of cytokinins stimulated callus formation through the cytokinin-mediated callus formation pathway (Ikeuchi, Sugimoto & Iwase, 2013).

Our results provided promising conditions for direct shoot organogenesis, which gave abundant pokeweed shoots that could be further propagated. The leaves from *in vitro* plantlets could also be used as explants for callus induction.

Combined effects between cytokinins and auxin

Three cytokinins as BAP, TDZ and kinetin at 1, 2 and 1 mg/l, respectively were selected from the highest efficiency of shoot organogenesis from the previous experiment. These treatments were combined with 0.1, 0.2 and 0.3 mg/l NAA. Results showed that kinetin treatments tended to increase the number of shoots, while other parameters decreased (Table 3). Application of NAA with cytokinins produced vitrified shoots with thick and fleshy stems and abnormal leaves.

Most previous studies reported positive effects of cytokinin-auxin combination on shoot organogenesis. Multiple shoots of *Ruta graveolens* were induced from shoot tip meristems by MS medium supplemented 2.25 mg/l benzyladenine and 0.47 mg/l NAA (Faisal et al., 2018). Positive effects of the combination were also reported in pokeweed shoot induction. Zou et al. (2008) induced new pokeweed shoots from stem segments using 2 mg/l BA and 0.2 mg/l NAA, while 1 mg/l BAP combined with 0.2 mg/l NAA was found to be suitable for shoot regeneration from stem cuttings (El-Minisy et al., 2017). However, our results showed that single cytokinins gave better results than cytokinin-auxin combination treatments. Similar results were also found in shoot multiplication of *Amygdalus communis* apical shoot tips. Shoot formation was not observed in treatments containing benzyladenine and kinetin combined with NAA (Akbaş et al., 2009). In this study, we hypothesized that the addition of NAA with cytokinins may induce shoots and roots, simultaneously. However, the results indicated that media fortified with cytokinins and NAA did not affect pokeweed root induction. A high ratio between cytokinins and auxins was suitable for shoot induction (Ikeuchi, Sugimoto & Iwase, 2013). We also found that micropropagated shoots showed some vitrified characters when NAA concentration increased. Hyperhydricity may result from excessive concentrations of PGRs (Karimpour et al., 2013). Therefore, we suggested that 2 mg/l kinetin without supplementation with NAA could induce multiple shoots of pokeweed from nodal explants.

Chemical constituents and antioxidant activity

Among all callus induction treatments, leaf explants cultured on MS medium added with 2 mg/l 2,4-D gave calli with the highest dry weight. Nazir et al. (2020) found that the highest biomass purple basil (*Ocimum basilicum* L. var *purpurascens*) calli provided the highest phenolic and flavonoid contents. Therefore, calli possessing the highest dry weight were used to determine phenolic and flavonoid contents and antioxidant activity and compared with other pokeweed plant parts.

Different parts of pokeweed possess diverse major secondary metabolites. Here, phenolic and flavonoid contents of various pokeweed explants were determined to preliminarily screen the chemical constituents, and the results showed that pokeweed seeds provided the highest phenolic content. Neo-lignans and phenolic compounds accumulated mainly in pokeweed seeds (Bailly, 2021). These phenolic compounds have many medicinal properties and may be responsible for high antioxidant activity, as shown by the lowest IC₅₀ value in DPPH assay. Other explants showed lower phenolic content, especially in roots. Results proved that phenolic compounds were not the major accumulated compounds in leaves and roots. Leaves accumulated the highest flavonoid content compared with other explants.

Flavonoid content was also higher than phenolic content in leaves and roots. The major secondary metabolites stored in leaves and roots were not phenolics and flavonoids. Research on chemical constituents of pokeweed leaves is limited but it has been reported that the leaves are green and their veins are always purple due to the accumulation of betacyanins (Jerz *et al.*, 2008), while there was strong evidence that triterpenes and phytosterols accumulated in pokeweed roots (Choe *et al.*, 2020; Jeong *et al.*, 2004).

Previous studies of pokeweed callus chemical constituents focused on betacyanin extraction. Callus induced from leaf explants also showed purple pigments of betacyanins (Kobayashi *et al.*, 1995; Fig. 1D), while betacyanins were also extracted from cell suspension maintained under aseptic conditions (Sakuta, Hirano & Komamine, 1991). Recent studies reported that low levels of phenolic and flavonoid contents accumulated in pokeweed calli resulted in low antioxidative potential. Similar results were found in most research. Calli of *Helicteres angustifolia* produced lower phenolic, flavonoid and saponin contents than the wild roots (Yang *et al.*, 2019). Phenolic and flavonoid contents of blueberry (*Vaccinium corymbosum*) calli were lower than field-grown and *in vitro* leaves (Kolarevic *et al.*, 2021). The phenomenon could be explained by the dedifferentiated property of the callus that resulted in lesser production and accumulation of complicated metabolites (Kolarevic *et al.*, 2021; Yang *et al.*, 2019). Lower metabolite contents in callus may result from limited metabolite production by *in vitro* explants but could be enhanced by callus elicitation to reduce cost and time to obtain pokeweed seeds and roots possessing major compounds of pokeweeds.

CONCLUSIONS

Pokeweed plants possess medicinal properties and may be applied in agricultural and medicinal fields. This study investigated the effects of explant types and auxins on pokeweed callus induction. Results showed that all types of explants generated callus by the application of exogenous 2,4-D. Suitable conditions for callus induction were culture of leaf explants on MS medium supplemented with 2 mg/l 2,4-D. This study proposed an improved and reliable system for pokeweed callus induction. In direct shoot organogenesis, pokeweed nodes were the only explant type for multiple shoot induction by cytokinins. Shoots with normal characteristics and largest number of shoots and leaves with the longest shoots were induced from MS medium fortified with 2 mg/l kinetin. We also found that inhibitory effects of combination with vitrified shoots increased when NAA concentration was increased. This study proposed an effective method for fast multiplication of pokeweed to utilize the plant in several fields. Pokeweed seeds showed the highest phenolic contents and lowest IC₅₀ by DPPH assay, while leaves contained the highest flavonoid contents. Pokeweed calli showed higher phenolic than flavonoid content. This is the first report describing chemical constituent screening and antioxidant activity of calli and other parts of the pokeweed plant.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was financially supported by the Science Achievement Scholarship of Thailand (SAST). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
The Science Achievement Scholarship of Thailand (SAST).

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Attachai Trunjaruen conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Prathan Luecha conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Worasitikulya Taratima conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, supervise this project, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:
The data is available in the [Supplementary Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.12892#supplemental-information>.

REFERENCES

- Akbaş F, Işikalan Ç, Namli S, Ak BE. 2009.** Effect of plant growth regulators on in vitro shoot multiplication of *Amygdalus communis* L. cv. Yaltsinki. *African Journal of Biotechnology* **8(22)**:6168–6174 DOI [10.5897/ajb09.782](https://doi.org/10.5897/ajb09.782).
- Bailly C. 2021.** Medicinal properties and anti-inflammatory components of *Phytolacca* (Shanglu). *Digital Chinese Medicine* **4**:159–169 DOI [10.1016/j.dcm.2021.09.001](https://doi.org/10.1016/j.dcm.2021.09.001).
- Balogh L, Juhasz M. 2008.** American and Chinese pokeweed (*Phytolacca americana* L. Ph. Esculenta Van Houtte). In: Botta-Dukat Z, Balogh L, eds. *The most important invasive plants in Hungary*. Budapest: AS Institute of Ecology and Botany, 35–46.
- Bhatia S, Bera T. 2015.** Somatic embryogenesis and Organogenesis. In: Bhatia S, ed. *Modern applications of plant biotechnology in pharmaceutical science*. Amsterdam: Elsevier Inc., 209–230.

- Bhoite HA, Palshikar GS. 2014.** Plant tissue culture: a review. *World Journal of Pharmaceutical Science* 2:565–572.
- Budisantoso I, Amalia N, Kamsinah . 2017.** *In vitro* callus induction from leaf explants of *Vanda* sp stimulated by 2, 4-D. *Biosaintifika* 9(3):492–497.
- Choe S, Jeong S, Jang M, Yeom H, Moon S, Kang M, Yang W, Kim S. 2020.** Identification of phytolaccosides in biological samples from pokeweed intoxication patients using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* 1149:122123 DOI 10.1016/j.jchromb.2020.122123.
- Deng X, Xiong Y, Li J, Liu J, Sun H, Song H, Wang Y, Ma J, Liu Y, Yang M. 2020.** The establishment of an efficient callus induction system for lotus (*Nelumbo nucifera*). *Plants* 9:Article 1436 DOI 10.3390/plants9111436.
- Du X, Fang T, Liu Y, Huang L, Zang M, Wang G, Liu Y, Fu J. 2019.** Transcriptome profiling predicts new genes to promote maize callus formation and transformation. *Frontiers in Plant Science* 10:Article 1633 DOI 10.3389/fpls.2019.01633.
- El-Minisy AM, El-Sawy A, El-Shabrawi HM, Soliman MH, El-Assal S, Mahfouze HA. 2017.** *In vitro* propagation and molecular analysis of pokeweeds (*Phytolacca americana*) plant. *Middle East Journal of Agriculture* 6:424–432.
- Faisal M, Ahmad N, Anis M, Alatar AA, Qahtan AA. 2018.** Auxin-cytokinin synergism in vitro for producing genetically stable plants of *Ruta graveolens* using shoot tip meristems. *Saudi Journal of Biological Science* 25:273–277 DOI 10.1016/j.sjbs.2017.09.009.
- Ikeuchi M, Favero DS, Sakamoto Y, Iwase A, Coleman D, Rymen B, Sugimoto K. 2019.** Molecular mechanisms of plant regeneration. *Annual Reviews of Plant Biology* 70:377–406 DOI 10.1146/annurev-arplant-050718-100434.
- Ikeuchi M, Sugimoto K, Iwase A. 2013.** Plant callus: mechanisms of induction and regression. *The Plant Cell* 25:3159–3173 DOI 10.1105/tpc.113.116053.
- Jeong SI, Kim KJ, Choi MK, Keum KS, Lee S, Ahn SH, Back SH, Song JH, Ju YS, Choi BK, Jung KY. 2004.** α -Spinosterol isolated from the root of *Phytolacca americana* and its pharmacological property on diabetic nephropathy. *Plant Medica* 70:736–739 DOI 10.1055/s-2004-827204.
- Jerz G, Skotzki T, Fiege K, Winterhalter P, Wybraniec S. 2008.** Separation of betalains from berries of *Phytolacca americana* by ion-pair high-speed counter-current chromatography. *Journal of Chromatography A* 1190:63–73 DOI 10.1016/j.chroma.2008.02.093.
- Jung C, Homg J, Bae SY, Kang SS, Park HJ, Lee SK. 2015.** Antitumor activity of americanin A Isolated from the seeds of *Phytolacca americana* by regulating the ATM/ATR signaling pathway and the Skp2 –p27 axis in human colon cancer cells. *Journal of Natural Products* 78:2983–2993 DOI 10.1021/acs.jnatprod.5b00743.
- Kadota M, Niimi Y. 2003.** Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. *Plant Cell, Tissue and Organ Culture* 72:261–265 DOI 10.1023/A:1022378511659.
- Karimpour S, Davarynejad GH, Tehranifar A, Bagheri A. 2013.** Comparative Effects of Some PGRs Combination on Proliferation and Hyperhydricity of Sebri Pear Cultivar. *International Journal of Farming and Allied Sciences* 2(9):202–205.

- Kevers C, Franck T, Strasser RJ, Dommes J, Gasper T. 2004.** Hyperhydricity of micro-propagated shoots: a typically stress-induced change of physiological state. *Plant Cell, Tissue and Organ Culture* 77:181–191 DOI [10.1023/B:TICU.0000016825.18930.e4](https://doi.org/10.1023/B:TICU.0000016825.18930.e4).
- Klaocheed S, Jehsu W, Choojun W, Thammasiri K, Prasertsongsun S, Rittirat S. 2020.** Induction of direct shoot organogenesis from shoot tip explants of an ornamental aquatic plant, *Cryptocoryne wendtii*. *Walailak Journal of Science and Technology* 17:293–302.
- Kobayashi A, Hagihara K, Kajiyama S, Kanzaki H, Kawazu K. 1995.** Antifungal compounds induced in the dual culture with *Phytolacca americana* callus and *Botrytis fabae*. *Zeitschrift fur Naturforschung C. Journal of Biosciences* 50:398–402.
- Kolarevic T, Milincic DD, Vujovic T, Gasic UM, Prokic L, Kostic AZ, Cerovic R, Stanojevic SP, Tesic ZL, Pesic MB. 2021.** Phenolic compounds and antioxidant properties of field-grown and *in vitro* leaves, and calluses in blackberry and blueberry. *Horticulturae* 7: Article 420.
- Kumlay AM, Ercisli S. 2015.** Callus induction, shoot proliferation and root regeneration of potato (*Solanum tuberosum* L.) stem node and leaf explants under long-day conditions. *Biotechnology & Biotechnological Equipment* 29(6):1075–1084 DOI [10.1080/13102818.2015.1077685](https://doi.org/10.1080/13102818.2015.1077685).
- Magyar-Tabori K, Dobranszki J, Silva JAT, Bulley SM, Hudak I. 2010.** The role of cytokinins in shoot organogenesis in apple. *Plant Cell, Tissue and Organ Culture* 101:251–267 DOI [10.1007/s11240-010-9696-6](https://doi.org/10.1007/s11240-010-9696-6).
- Mushke R, Yarra R, Bulle M. 2016.** Efficient *in vitro* direct shoot organogenesis from seedling derived split node explants of maize (*Zea mays* L.). *Journal of Genetic Engineering and Biotechnology* 14:49–53 DOI [10.1016/j.jgeb.2016.03.001](https://doi.org/10.1016/j.jgeb.2016.03.001).
- Mwamatope B, Tembo D, Chikowe I, Kampira E, Nyirenda C. 2020.** Total phenolic contents and antioxidant activity of *Senna singueana*, *Melia azedarach*, *Moringa oleifera* and *Lannea discolor* herbal plants. *Scientific African* 9:e00481 DOI [10.1016/j.sciaf.2020.e00481](https://doi.org/10.1016/j.sciaf.2020.e00481).
- Najar RA, Fayaz M, Bhat H, Bashir MH, Bashir M, Kumar A, Jain AK. 2018.** An efficient micropropagation protocol for direct organogenesis from nodal explants of medicinal climber, *Tylophora indica*. *Bioscience Biotechnology Research Communications* 11:144–153 DOI [10.21786/bbrc/11.1/20](https://doi.org/10.21786/bbrc/11.1/20).
- Nazir M, Ullah MA, Younas M, Siddiquah A, Shah M, Gigliloli-Guivarc'h N, Hano C, Abbasi BH. 2020.** Light-mediated biosynthesis of phenylpropanoid metabolites and antioxidant potential in callus cultures of purple basil (*Ocimum basilicum* L. var *purpurascens*). *Plant Cell, Tissue and Organ Culture (PCTOC)* 142:107–120 DOI [10.1007/s11240-020-01844-z](https://doi.org/10.1007/s11240-020-01844-z).
- Oliveira LF, Quoirin M, Koehler HS, Amano E, Higa AR, Ribas LLF. 2013.** Propagation from axillary buds and anatomical study of adventitious roots of *Pinus taeda* L. *African Journal of Biotechnology* 12:5413–5422 DOI [10.5897/AJB2012.2960](https://doi.org/10.5897/AJB2012.2960).
- Pekal A, Pyrzynska K. 2014.** Evaluation of aluminium complexation reaction for flavonoid content assay. *Food Analytical Methods* 7:1776–1782 DOI [10.1007/s12161-014-9814-x](https://doi.org/10.1007/s12161-014-9814-x).

- Petrillo AD, Gonzalez-Paramas AM, Rosa A, Ruggiero V, Boylon F, Kumar A, Pintus F, Santos-Buelga C, Fais A, Era B. 2019.** Chemical composition and enzyme inhibition of *Phytolacca dioica* L. seeds extracts. *Journal of Enzyme Inhibition and Medicinal Chemistry* **34**:519–527 DOI [10.1080/14756366.2018.1563077](https://doi.org/10.1080/14756366.2018.1563077).
- Sakuta M, Hirano H, Komamine A. 1991.** Stimulation by 2, 4-dichlorophenoxyacetic acid of betacyanin accumulation in suspension cultures of *Phytolacca americana*. *Physiologia Plantarum* **83**:154–158 DOI [10.1111/j.1399-3054.1991.tb01295.x](https://doi.org/10.1111/j.1399-3054.1991.tb01295.x).
- Sakuta M, Takagi T, Komamine A. 1986.** Growth related accumulation of betacyanin in suspension cultures of *Phytolacca americana* L. *Journal of Plant Physiology* **125**:337–343 DOI [10.1016/S0176-1617\(86\)80155-9](https://doi.org/10.1016/S0176-1617(86)80155-9).
- Sarkar J, Banerjee N. 2020.** Influence of different cytokinins on micropropagation of an important medicinal plant, *Solanum erianthum* D. Don, and assessment of the genetic fidelity of the regenerants. *In Vitro Cellular & Developmental Biology - Plant* **56**:480–490.
- Trunjaruen A, Raso S, Maneerattanaringroj P, Taratima W. 2020.** Effects of cultivation media on *In vitro* callus induction and regeneration capabilities of pakaumpuel rice (*Oryza sativa* L.) Thai Rice Landrace. *Walailak Journal of Science and Technology* **17**:37–46.
- Yang X, Lei Z, Yu Y, Xiao L, Cheng D, Zhang Z. 2019.** Phytochemical characteristics of callus suspension culture of *Helicteres angustifolia* L. and its *in vitro* antioxidant, antidiabetic and immunomodulatory activities. *South African Journal of Botany* **121**:178–185 DOI [10.1016/j.sajb.2018.11.006](https://doi.org/10.1016/j.sajb.2018.11.006).
- Zang Q, Zhou L, Zhuge F, Yang H, Wang X, Lin X. 2016.** Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. *SpringerPlus* **5**:Article 1799 DOI [10.1186/s40064-016-3520-7](https://doi.org/10.1186/s40064-016-3520-7).
- Zhang Y, Bozorov TA, Li DX, Zhou P, Wen XJ, Ding Y, Zhang DY. 2020.** An efficient *in vitro* regeneration system from different wild apple (*Malus sieversii*) explants. *Plant Methods* **16**:Article 56 DOI [10.1186/s13007-020-00599-0](https://doi.org/10.1186/s13007-020-00599-0).
- Zou L, Suzhi X, Hujim Y, Yua M, Wang X. 2008.** Tissue culture and rapid propagation of *Phytolacca americana*. *Journal of Chinese Medicinal Materials* **31**:1299–1301.