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Research Article

The effects of cytokinin and plating density on protoplast culture of sunflower

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Abstract Sunflower (*Helianthus annuus* L.) protoplasts were isolated from seven-day-old etiolated hypocotyls of 10 A line and four-week-old fully expanded young leaves of PI 441983 line *in vitro* seedlings using an enzymatic method. Purified protoplasts were collected by filtration and floatation in sucrose solution. Semi-solid protoplast culture was performed using the L4 regeneration protocol with various culture media and plating densities to achieve the highest efficiencies for protoplast culture of hypocotyl and mesophyll protoplasts of 10 A and PI 441983 lines, respectively. The concentrations in liquid L'4M medium and different plating densities were evaluated in two types of cytokinins, the adenine-type 6-benzyladenine (BA) and the phenylurea-type thidiazuron (TDZ). The highest colony formation was achieved in both sunflower lines when 0.5 mgL^{-1} BA and 0.5 mgL^{-1} TDZ were applied with a high plating density (3×10^5 protoplasts mL^{-1}). These conditions led to 38.45% and 39.40% colony formation for hypocotyl protoplasts of the 10 A line and mesophyll protoplasts of the PI 441983 line, respectively. Moreover, many hypocotyl protoplast-derived colonies developed into micro-calli. In addition, superior development of both sunflower protoplasts was observed with all plating densities when BA was used in combination with TDZ. This finding will be applicable to future sunflower hybrid production via somatic hybridization.

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Introduction

Generally, most sunflower (*Helianthus annuus* L.) cultivars used are F_1 hybrids. The transfer of normal cytoplasm trait into a cytoplasmic male sterile line (CMS; A-line) to generate a cytoplasmic fertile line (maintainer line; B-line) is required for hybrid seed production. Development of a B-line can be rapidly generated in a single step by using protoplast fusion. Protoplast is a naked plant cell and it can form new wall and dedifferentiate under *in vitro*, becoming totipotent (Costa et al. 2018). Protoplast fusion was developed and successfully used to transfer cytoplasmic traits in brassica (Liu et al. 1996), tobacco (Zubko et al. 2003), citrus (Bona et al. 2009; Xu et al. 2006), chicory (Varotto et al. 2001) and celeriac (Bruznican et al. 2021) etc. Development and regeneration *in vitro* of sunflower usually depend on genotypes, culture conditions and tissue sources (Cravero et al. 2012; Dagustu 2018; Davey et al. 2005; Jie et al. 2011; Kativat et al. 2021; Radonic et al. 2008; Rákosy-Tican et al. 2007). In previous reports different tissue sources of sunflower protoplasts were used such as hypocotyls (Burrus et al. 1991; Henn et al. 1998b; Keller et al. 1997; Krasnyanski and Menczel 1993), mesophylls (Henn et al. 1998a; Krasnyanski et al. 1992) and cotyledons (Fischer et al. 1992). The composition of enzyme solutions for protoplast isolation is crucial to obtain large quantity of viable protoplasts. Greater viability and larger amount of protoplasts were increasingly achieved when using appropriate combinations of enzymes i.e., cellulase, pectinase, macerozyme, hemicellulase, meicelase and driselase at optimum concentrations (Mastuti and Rosyidah 2018;

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da Silva Júnior et al. 2012; Wang et al. 2022). In sunflower, we previously found that the isolation solution containing 1% cellulase and 0.5% macerozyme was optimum for isolating protoplasts from hypocotyls of 10 A line, giving the highest number of viable protoplasts (4.24 $\times 10^6$ protoplasts/g fresh weight [FW]). However, for leaf explants of PI 441983 line, the highest numbers of viable protoplasts (8.81 $\times 10^6$ protoplasts/g FW) were achieved when incubated with 0.5% cellulase, 0.05% driselase, 0.02% macerozyme and 0.1% bovine serum albumin (BSA) (Kativat et al. 2012).

Various culture protocols were applied including L4 regeneration protocol described by Burrus et al. (1991), mKM regeneration protocol (Wingender et al. 1996) and VKM regeneration protocol (Krasnyanski and Menczel 1993), and culture conditions such as plating densities, plant growth regulators (PGRs), artificial oxygen carriers and antibiotics were varied to enhance the efficiency of protoplast culture (Davey et al. 2005; Rákosy-Tican et al. 2007). The major PGRs that are most necessary for growth and development of protoplasts (auxin and cytokinin) have been extensively studied. Generally, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are auxin types which are often used for protoplast culture while 6-benzyladenine (BA) and kinetin which are adenine type are mainly used as cytokinin (Beyl et al. 2015; Guo et al. 2011). Nevertheless, current findings show that the phenylurea type, especially thidiazuron (TDZ), is more effective for growth and regeneration of cells and tissues than the adenine type (Guo et al. 2011; Ricci et al. 2001; Tsuro et al. 1999; Visser et al. 1992). Therefore, this current investigation was to evaluate the effects of TDZ and BA in culture medium, and to determine the optimal plating density for protoplast culture of sunflower.

Materials and Methods

Plant materials

Two sunflower genotypes including a cytoplasmic male sterile line with high oil content, 10 A, developed in Thailand for hybrid production and a fertile cytoplasmic line, PI 441983, provided by the North Central Regional Plant Introduction Station, Iowa, USA were used.

Seed surface sterilization and seedling culture

Seeds of two sunflower lines were surface sterilized in 20% (v/v) clorox for 20 min and then were rinsed three times in sterile distilled water. The seeds were sterilized again in 20% (v/v) clorox for 30 min after pericarps were removed. After washing in sterile distilled water three times, seed coats were removed after soaking in 5% (v/v) hydrogen peroxide (H_2O_2) for 5 min. The seeds were germinated in Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar. The 10 A seeds were cultured on MS medium at 25°C for 7 d in the dark condition, while PI 441983 seeds were cultured on MS medium at 25°C under 2,000 lux of Gro-lux fluorescence at 16 hr duration for 14 d, and shoots were sub-cultured and grown in vermiculite supplemented with liquid MS medium and 2% (w/v) sucrose for 4 wk.

Protoplast isolation and purification

For protoplast sources, we used seven-day-old etiolated hypocotyls of 10 A line and four-week-old fully expanded young leaves of PI 441983 line of *in vitro* seedlings. Their small pieces after segmentation were incubated in enzyme solutions. The component of enzymes, 1% (w/v) cellulase onozuka R-10 (from *Trichoderma viride*; Yakult Honsha, Japan) and 0.5% (w/v) macerozyme R-10 (from *Rhizopus* sp.; Kinki Yakult MFG, Japan) in isolation solution (308 mM NaCl, 5.37 mM KCl, 41.7 mM, $CaCl_2 \cdot 2H_2O$ and 3.3 mM MES, pH 5.6-5.7 (Krasnyanski and Menczel 1995)) was applied for hypocotyl digestion. For mesophyll protoplasts isolation, 0.5% (w/v) cellulase, 0.02% (w/v) macerozyme R-10, 0.05% (w/v) driselase (from *Basidiomycetes* sp.; Sigma-Aldrich, Germany) and 0.1% (w/v) BSA in isolation solution (336 mM KCl, 13.6 mM $CaCl_2$ and 3.59 mM MES, pH 5.6-5.7 (Keller et al. 1997)) was used. Purified protoplasts were collected by filtration using nylon filters with 82, 62 and 40 μm pore diameters, respectively, and floatation in the sucrose solution (0.5 M sucrose, 14 mM $CaCl_2 \cdot 2H_2O$, 3 mM MES, pH 5.6 (Henn et al. 1998b)), described by Kativat et al. (2012). Fluorescein diacetate (FDA) staining (Henn et al. 1998b) and haemacytometer were used to observe yields and viability of protoplasts, respectively.

Protoplast culture

Protoplast culture was undertaken in 500-μL agarose-solidified droplets (8 droplets/100 × 15 mm Petri dish) of culture medium (Shillito et al. 1983) using the L4 regeneration protocol (Lénée and Chupeau 1986), according to Burrus

et al. (1991). An original liquid L’4M medium (1 mg L^{-1} BA) and three modified liquid L’4M media including L’4M 1 (0.5 mgL^{-1} BA and 0.25 mgL^{-1} TDZ), L’4M 2 (0.5 mgL^{-1} BA and 0.5 mgL^{-1} TDZ) and L’4M 3 (1 mgL^{-1} TDZ), and two final plating densities, 1×10^5 and 3×10^5 protoplasts mL^{-1} (viable protoplasts) were assessed. The mean plating efficiency which is defined as the percentage of cell division was observed under an inverted microscope every 7 d and was recorded at 14 d after culture for 3 wk, and the percentage of colony formation was recorded at 28 d after culture for 4 wk. The percentages of cell division and colony formation were calculated following the formulas:

$$\text{Cell division (\%)} = \frac{\text{Number of dividing protoplasts}}{\text{Number of all protoplasts}} \times 100$$

$$\text{Colony formation (\%)} = \frac{\text{Number of dividing protoplasts formed colony}}{\text{Number of all protoplasts}} \times 100$$

Statistical analysis

The research was established in a completely randomized factorial design (factorial in CRD) using 3 replications for each source of protoplasts, hypocotyl of 10 A and mesophyll of PI 441983 lines. Two factors including media and plating densities and interactions between factors were analyzed for cell division and colony formation. SPSS version 14.0 (Levesque and SPSS Inc. 2006) was used to conduct an analysis of variance (ANOVA) and compare the means through Duncan’s post hoc statistical tools.

Results

When the hypocotyl protoplasts were cultured, percentages of cell division and colony formation continuously increased during the period of culture and reached up to 47-56% and 25-38%, respectively, when cultured for 28 and 50 d, respectively (Table 1, Fig. A1-H2). The culture medium resulted in no effect on average cell division ($p > 0.05$) at 28 d, but significantly affected average colony formation ($p < 0.01$) at 50 d (Table 2A). Modified liquid L’4M media tended to give higher average cell division and significantly higher colony formation than liquid L’4M, especially L’4M 2 (1.1 and 1.4-fold higher average cell division and colony formation than L’4M, respectively) (Table 2A). Plating density was significantly responsible for both cell division and colony formation ($P < 0.01$) at 28 and 50 d, respectively. The culture at a high density of protoplasts (3×10^5 protoplasts mL^{-1}) promoted higher plating efficiencies than a lower density (1×10^5 protoplasts mL^{-1}) (Table 2B). However, the effects of interaction between culture medium and plating density were not detected on any parameters measured ($p > 0.05$). The highest average cell division was achieved when using liquid L’4M 1 with plating density 3×10^5 protoplasts mL^{-1} (56.20%), however no significant difference was observed when compared to those cultured on other media with any of the plating densities except when using liquid L’4M 3 and L’4M with 1×10^5 protoplasts mL^{-1} (48.83 and 47.14%, respectively) (Table 1). Nevertheless, at the end of the culture period using liquid L’4M 2 with high plating density (3×10^5 protoplasts mL^{-1}) was found to be the best combination to promote the development of divided protoplasts into colonies

Table 1 The effects of culture media and plating densities on cell division at 14, 21, and 28 d, and colony formation at 28, 35, 42, and 50 d of cultured hypocotyl protoplasts of sunflower line 10 A

Liquid media	Plating densities (protoplasts mL^{-1})	Cell division (%)			Colony formation (%)			
		14 d	21 d	28 d	28 d	35 d	42 d	50 d
L’4M	1×10^5	20.59 ± 0.59^{cd}	39.04 ± 0.92^{bc}	47.14 ± 0.60^{c}	2.16 ± 0.31^{e}	15.38 ± 5.93	18.21 ± 0.96^{c}	24.54 ± 1.58^{d}
	3×10^5	19.51 ± 2.86^{d}	36.07 ± 3.31^{c}	51.13 ± 1.49^{abc}	10.85 ± 0.99^{c}	17.78 ± 0.37	29.12 ± 1.78^{ab}	29.64 ± 0.44^{c}
L’4M 1	1×10^5	31.49 ± 1.45^{ab}	50.67 ± 1.59^{a}	50.68 ± 3.32^{abc}	3.66 ± 0.60^{de}	21.51 ± 7.83	28.45 ± 2.53^{ab}	33.99 ± 1.54^{b}
	3×10^5	26.37 ± 3.15^{bc}	40.93 ± 2.84^{bc}	56.20 ± 1.00^{a}	15.01 ± 0.23^{a}	22.23 ± 1.18	31.91 ± 2.04^{ab}	34.37 ± 0.90^{b}
L’4M 2	1×10^5	35.95 ± 1.85^{a}	52.03 ± 1.41^{a}	52.87 ± 0.78^{abc}	4.32 ± 0.42^{d}	22.16 ± 6.70	35.70 ± 3.59^{a}	36.44 ± 0.89^{ab}
	3×10^5	28.14 ± 2.02^{b}	43.76 ± 1.52^{b}	55.63 ± 2.95^{a}	12.45 ± 0.71^{bc}	25.47 ± 0.38	33.36 ± 1.39^{ab}	38.45 ± 0.85^{a}
L’4M 3	1×10^5	31.51 ± 1.75^{ab}	50.73 ± 3.00^{a}	48.83 ± 1.08^{bc}	2.86 ± 0.31^{de}	20.90 ± 6.77	27.51 ± 2.51^{b}	28.79 ± 0.92^{c}
	3×10^5	25.20 ± 1.58^{bcd}	41.84 ± 1.21^{bc}	53.51 ± 1.66^{ab}	14.31 ± 1.09^{ab}	22.45 ± 0.27	30.78 ± 2.32^{ab}	34.82 ± 1.07^{b}
F-test		**	**	*	**	ns	**	**

Means with different letters within the same column are significantly different ($p < 0.05$) based on Duncan’s multiple range test

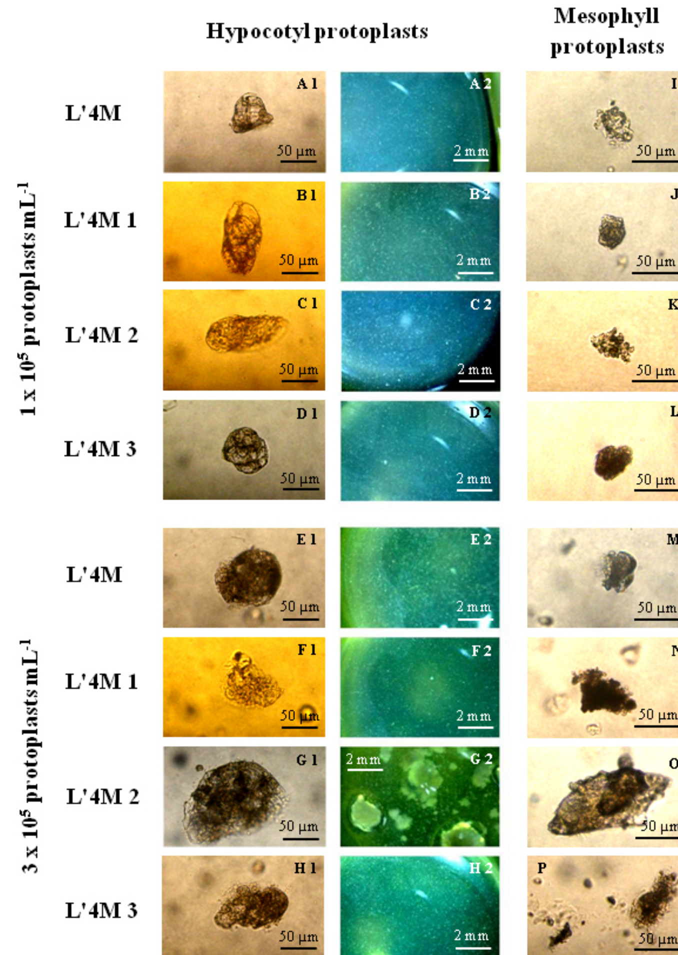

Fig. 1 The development of sunflower protoplasts at 50 d after culturing in various media (L'4M, L'4M 1, L'4M 2, and L'4M 3) and plating densities (1×10^5 and 3×10^5 protoplasts mL^{-1}). (A1-H1) Characteristics of colonies developed from hypocotyl protoplasts of 10 A line. (A2-H2) Visible colonies and microcalli in agarose droplets developed from hypocotyl protoplasts of 10 A line. (I-P) Characteristics of colonies developed from mesophyll protoplasts of PI 441983 line

Table 2 Average percentages of cell division and colony formation of hypocotyl protoplasts of 10 A line when cultured with different culture media and plating densities

A. Effects of culture media

Liquid media	Cell division (%)			Colony formation (%)			
	14 d	21 d	28 d	28 d	35 d	42 d	50 d
L'4M	20.05^{b}	37.56^{b}	49.13	6.51^{b}	16.58	23.66^{c}	27.09^{d}
L'4M 1	28.93^{a}	45.80^{a}	53.44	9.34^{a}	21.87	30.18^{ab}	34.18^{b}
L'4M 2	32.05^{a}	47.89^{a}	54.25	8.39^{a}	23.82	34.53^{a}	37.44^{a}
L'4M 3	28.36^{a}	46.28^{a}	51.17	8.58^{a}	21.67	29.15^{b}	31.81^{c}
F-test	**	**	ns	**	ns	**	**

B. Effects of plating densities

Plating densities (protoplasts mL^{-1})	Cell division (%)			Colony formation (%)			
	14 d	21 d	28 d	28 d	35 d	42 d	50 d
1×10^5	29.89^{a}	48.12^{a}	49.88^{b}	3.25^{b}	19.99	27.47^{b}	30.94^{b}
3×10^5	24.80^{b}	40.65^{b}	54.12^{a}	13.15^{a}	21.98	31.29^{a}	34.32^{a}
F-test	**	**	**	**	ns	*	**

Means with different letters within the same column are significantly different ($p < 0.05$) based on Duncan's multiple range test

Table 3 The effects of culture media and plating densities on cell division at 14, 21, and 28 d, and colony formation at 28, 35, 42, and 50 d of cultured hypocotyl protoplasts of sunflower line PI 441983

Liquid media	Plating densities (protoplasts mL^{-1})	Cell division (%)			Colony formation (%)			
		14 d	21 d	28 d	28 d	35 d	42 d	50 d
L'4M	1×10^5	19.42 ± 1.37^b	26.29 ± 1.76^b	37.74 ± 7.29	0.98 ± 0.50	17.10 ± 4.55	22.75 ± 2.95^c	27.30 ± 2.25^b
	3×10^5	21.36 ± 0.41^{ab}	30.63 ± 0.46^{ab}	42.22 ± 5.50	3.04 ± 1.65	20.54 ± 5.04	29.99 ± 0.75^b	32.91 ± 2.03^{ab}
L'4M 1	1×10^5	22.68 ± 1.04^a	34.18 ± 0.99^a	43.43 ± 7.45	2.28 ± 1.16	21.43 ± 4.08	32.26 ± 1.22^{ab}	35.21 ± 2.53^a
	3×10^5	22.43 ± 0.30^a	33.37 ± 1.94^a	47.72 ± 5.87	5.41 ± 2.76	25.29 ± 3.65	34.29 ± 0.81^{ab}	35.28 ± 1.57^a
L'4M 2	1×10^5	23.45 ± 0.78^a	35.16 ± 1.05^a	43.77 ± 8.06	5.31 ± 2.66	23.43 ± 3.11	32.36 ± 1.12^{ab}	36.30 ± 2.07^a
	3×10^5	22.76 ± 0.27^a	37.01 ± 3.02^a	47.09 ± 8.27	5.54 ± 2.95	26.79 ± 3.56	37.47 ± 2.48^a	39.40 ± 1.45^a
L'4M 3	1×10^5	21.93 ± 0.19^a	37.59 ± 2.21^a	41.13 ± 4.97	4.98 ± 2.64	21.74 ± 2.87	31.02 ± 0.66^b	35.30 ± 2.10^a
	3×10^5	21.89 ± 0.72^a	37.62 ± 3.39^a	48.45 ± 7.24	5.84 ± 2.94	23.93 ± 2.80	34.52 ± 2.06^{ab}	37.52 ± 2.56^a
F-test		*	*	ns	ns	ns	**	*

Means with different letters within the same column are significantly different ($p < 0.05$) based on Duncan's multiple range test

Table 4 Average percentages of cell division and colony formation of mesophyll protoplasts of PI 441983 line when cultured with different culture media and plating densities

A. Effects of culture media

Liquid media	Cell division (%)			Colony formation (%)			
	14 d	21 d	28 d	28 d	35 d	42 d	50 d
L'4M	20.39^b	28.46^b	39.98	2.01	18.82	26.37^b	30.11^b
L'4M 1	22.55^a	33.78^a	45.58	3.85	23.36	33.28^a	35.25^a
L'4M 2	23.11^a	36.09^a	45.43	5.43	25.11	34.91^a	37.85^a
L'4M 3	21.91^{ab}	37.61^a	44.79	5.41	22.84	32.77^a	36.41^a
F-test	*	**	ns	ns	ns	**	*

B. Effects of plating densities

Plating densities (protoplasts mL^{-1})	Cell division (%)			Colony formation (%)			
	14 d	21 d	28 d	28 d	35 d	42 d	50 d
1×10^5	21.87	33.31	41.52	3.39	20.92	29.60^b	33.53
3×10^5	22.11	34.66	46.37	4.96	24.14	34.07^a	36.28
F-test	ns	ns	ns	ns	ns	**	ns

Means with different letters within the same column are significantly different ($p < 0.05$) based on Duncan's multiple range test

(38.45%). No significant difference on colony formation was observed when using the same medium with low plating density (1×10^5 protoplasts mL^{-1}) (36.44%), however, this medium gave significantly higher percentage of colony formation than other media at both plating densities (Table 1). In addition, a large number of colonies obtained using liquid L'4M 2 with 3×10^5 protoplasts mL^{-1} also developed into micro-calli (Fig. 1G2).

Similarly, mesophyll protoplasts of sunflower PI 441983 line, which were found recalcitrant in culture, developed on the culture media. The highest cell division and colony formation were observed at 28 and 50 d of culture, respectively (Table 3, Fig. 1I-P). Cell division was not significantly influenced by culture medium ($p > 0.05$) at 28 d. However, colony formation was found to be significantly affected by the culture medium ($p < 0.05$) at 50 d (Table 4A). All modified liquid L'4M media still promoted higher average colony formation, especially L'4M 2 which gave 1.26-fold higher average colony formation than L'4M medium at 50 d (Table 4A). Plating density had no significant effect on any of parameters measured ($p > 0.05$) at the end of experiment. Although high plating density tended to promote higher levels of both cell division and colony formation than lower density,

which were 1.12 and 1.08-folds higher, respectively at 28 and 50 d, respectively. (Table 4B). In addition, interactions between culture medium and plating density were also not significant on either cell division or colony formation ($p > 0.05$). The highest average cell division was obtained when using liquid L'4M 3 with high plating density 3 × 10^5 protoplasts mL^{-1} (48.45%), but there was no significant difference with those obtained from other combinations (Table 3). However, when colony formation was evaluated, the highest average colony formation was achieved using a combination of liquid L'4M 2 and plating density of 3 × 10^5 protoplasts mL^{-1} (39.40%), which was not significantly different from those obtained using other media for all plating densities except for using liquid L'4M with low plating density which gave the lowest average colony formation (27.30%) (Table 3). Nevertheless, the development of colonies into micro-calli was not observed in all combinations.

Discussion

Culture medium affected only colony formation of both hypocotyl protoplasts of the 10 A line and mesophyll protoplasts of the PI 441983 line, whereas plating density influenced both cell division and colony formation of hypocotyl protoplasts of 10 A line, but was not responsible for either parameters in the mesophyll protoplasts of the PI 441983 line. The newly modified media with supplementation of TDZ in different concentrations with and without BA (L'4M 1, L'4M 2 and L'4M 3) tended to promote colony formation of protoplasts from both sources. L'4M 2 (0.5 mgL^{-1} each of BA and TDZ) was the best medium for promoting the highest colony formation on both kinds of protoplasts of the 10 A and PI 441983 lines. In particular, using L'4M 2 with high plating density (3 × 10^5 protoplasts mL^{-1}) resulted in the highest colony formation of protoplasts from both sources and induced micro-callus formation in hypocotyl protoplasts of the 10 A line. These results may be due to the effects of TDZ, which has been frequently reported to be more efficient for promoting cell division and growth than BA (Chupeau et al. 1993; Guo et al. 2011; Murthy et al. 1998; Wallin and Johansson 1990). Similarly, Chupeau et al. (1993) observed that when replacing BA with TDZ, it could induce a high yield of colonies and reduced the release of toxic exudates from protoplasts, resulting in a high production of micro-calli in the culture of hybrid poplar (*Populus tremula* × *P. alba*) protoplasts. The findings indicate the role of TDZ for sustaining the development of protoplast-derived colonies and micro-calli. However, the combining effects of adenine and phenylurea cytokinin types on protoplast culture have never been reported. Interestingly, we are the first to report the synergistic effects of BA and TDZ in protoplast culture. These results agree with various researchers who found that using BA together with TDZ can enhance the efficiency of cell development and regeneration of cells and tissues in other systems (Guo et al. 2011; Jonoubi et al. 2004; Khalafalla and Hattori 1999; Nielsen et al. 1995; Taha et al. 2021; Tantasawat et al. 2015).

Cell division and colony and callus formation also depended on the concentrations of PGRs, genotypes, and plating densities. For plating density, a high protoplast density (3 × 10^5 protoplasts mL^{-1}) was more beneficial for both cell division and colony formation than a lower density (1 × 10^5 protoplasts mL^{-1}) in both protoplast sources. Similarly, Schween et al. (2003) found that the culture of moss protoplasts (*Physcomitrella patens*) with high plating density at 3 × 10^5 protoplasts mL^{-1} sustained viability and promoted the development of cells better than with using a lower density. These results may be due to the fact that high plating density protoplasts are able to release a sufficient amount of the physiologically active compounds, including growth factors and/or amino acids, into the medium to stimulate and sustain mitotic division, normally known as the nurse-effect (Davey et al. 2005; Fu et al. 2009; Schween et al. 2003; Tomar and Dantu 2010; Xu and Xue 1999). In addition, Rákosy-Tican et al. (2007) also found that plating efficiency could be improved for all sunflower genotypes and culture protocols (L4 and VKM regeneration protocols) when a low plating density used at 5 × 10^4 protoplasts mL^{-1} was adjusted to 8 × 10^5 protoplasts mL^{-1}. However, optimal plating density usually depended on genotypes and plant tissues (Bajaj 1994).

The development of protoplasts from different sunflower genotypes and tissues varied. Only hypocotyl protoplasts of 10 A line could develop into micro-calli especially when using L'4M 2 medium with plating density of 3 × 10^5 protoplasts mL^{-1}. Whereas, the development of mesophyll protoplasts from PI 441983 line could reach only colony formation, indicating that donor plant genotypes and tissue sources may have specific requirement for growth and development in *in vitro* culture (Morrison et al. 1991; Wiszniewska and Pindel 2013). However, supplementation of TDZ in all modified L'4M media (L'4M 1, L'4M 2 and L'4M 3) could enhance the efficiency of protoplasts culture procedure especially when using with high plating density of protoplasts (3 × 10^5 protoplasts mL^{-1}). Further study is needed for future development and regeneration

of these protoplasts derived colonies and micro-calli into plantlets.

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