Pak. J. Bot., 49(5): 1903-1910, 2017.

PEG-MEDIATED SYMMETRIC AND ASYMMETRIC PROTOPLAST FUSION IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

CHITPAN KATIVAT[1,2,3] AND PIYADA ALISHA TANTASAWAT[1,2*]

[1]*School of Crop Production Technology, Suranaree University of Technology, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand*
[2]*Center of Excellence on Agricultural Biotechnology (Ag-BIO/PERDO-CHE), Bangkok 10900, Thailand*
[3]*National Research Council of Thailand (NRCT), 196 Paholyotin Rd., Chatuchak, Bangkok 10900, Thailand*
[*]*Correspondence author's email: piyada@sut.ac.th; Tel.: + 66 44224276; Fax: + 66 44224281*

Abstract

Symmetric and asymmetric protoplast fusions were evaluated with sunflower (*Helianthus annuus* L.) PI441983 and 10A lines. The optimal cytoplasmic inactivation procedure and conditions for induced fusion of protoplasts by using polyethylene glycol (PEG) were developed. The cell division activities of hypocotyl protoplasts of the 10A line with a cytoplasmic male sterile (CMS) trait were inhibited with different concentrations of iodoacetic acid (IOA; 0, 1.5, 3.0 and 4.5 mM) and incubation periods (15 and 20 min) to generate a recipient parent for a normal cytoplasm trait. The optimal inactivation was achieved with 20 min incubation in 1.5 mM IOA, which was the lowest concentration leading to low levels of both cell division (20.41%) and colony formation (3.70%). When various concentrations of PEG 8000 (0, 10, 20 and 30% (w/v)) and fusion periods (10, 15 and 20 min) were used to induce fusion between hypocotyl protoplasts of the 10A line and mesophyll protoplasts with a normal cytoplasm trait of the PI441983 line (donor parent), 20% (w/v) PEG 8000 for 15 min was found to be optimal for induced fusion, giving a high frequency of binary fusion (26.16%) and lowfrequency of multi fusion (12.96%). When both symmetric and asymmetric protoplast fusion procedures were performed, fusion products could develop, divide and form colonies in the culture medium, and also have a tendency to generate microcalli. However, the densities of protoplast-derived colonies in asymmetric fusion were lower than those in symmetric fusion. The efficient procedures developed in this study will be beneficial for future sunflower breeding programs for hybrid production.

Key words: Cytoplasmic inactivation, Iodoacetic acid, Normal cytoplasm, Polyethylene glycol, Recipient parent.

Introduction

Sunflower (*Helianthus annuus* L.) is an important oilseed crop. At present, sunflower hybrid cultivars are extensively used around the world, including Thailand, because they give superior yields and provide several important agronomic traits. Therefore, new hybrid cultivars are being continually developed and released. For commercial hybrid seed production, parental lines containing cytoplasmic male sterile (CMS; A-line) and cytoplasmic fertile (maintainer line; B-line) traits are essential. CMS is a maternally inherited trait that is controlled by genes in the cytoplasm which are usually found in the mitochondrial genome, resulting in plants that have no pollen or are unable to produce functional pollen (Eckardt, 2006). This trait is applied instead of the hand emasculation procedure in breeding programs, leading to the mass production of F_1 hybrid seeds that are easy to manage (Poehlman, 1994; Hou *et al*., 2016). Because the A-line is male sterile, a cytoplasmic fertile trait in the B-line is required for seed multiplication of the A-line. The transfer of cytoplasmic traits such as CMS and cytoplasmic fertile traits to generate A- and B-lines can be performed either by continued backcrossing for several generations (Dagustu *et al.*, 2012) or using protoplast fusion (Galun *et al.*, 1988). Nevertheless, to minimize the cost and time and to overcome sexual incompatibility barriers to generate these stable lines, protoplast fusion is frequently considered and used (Yousuf *et al*., 2015).

Protoplast fusion is an efficient method for genetic modification of both nuclear and cytoplasmic genomes by fusing two genetically different plant protoplasts together, especially between different plant species (Taski-Ajdukovic *et al.*, 2008; Scholze *et al.*, 2010; Rezazadeh *et al.*, 2011) or genera (Cheng & Xia, 2004;Vassilevska-Ivanova *et al.*, 2014; Tudses *et al.*, 2015) to generate a new hybrid. To induce protoplast fusion, chemical agents and an electrical field were used (Lakshmanan *et al.*, 2013). For chemical induced fusion, several fusogenic agents such as salt solutions (NaCl, KCl, KNO_3, $NaNO_3$ and $CaNO_3$), dextran sulfate, polyvinyl alcohol, lysolethicin and polyethylene glycol (PEG) were applied. However, PEG was most frequently utilized due to its ability to provide a high frequency of heterokaryon formation and high heterokaryon viability, preventing the protoplast from bursting, and because it is a less complicated procedure than others (Compton *et al.*, 2000; Navrátilová, 2004). Successful uses of PEG in protoplast fusion to generate new hybrids were found in several plant families such as Brassicaceae (Scholze *et al.*, 2010; Wang *et al.*, 2011), Musaceae (Xiao *et al.*, 2009), Poaceae (Xiang *et al.*, 2004) and Solanaceae (Sun*et al.*, 2005) etc. Protoplasts may be fused symmetrically and asymmetrically (donor-recipient). For symmetric fusion, whole genomes of both parental protoplasts were fused, causing complexity of genomes in hybrids and the negative effects from undesired chromosomes or genes were always found, especially for the fusion of protoplasts between cultivars and wild types, which leads to abnormal growth and regeneration of hybrids and low fertility of hybrids, etc. (Eeckhaut *et al.*, 2006). To overcome these limitations, asymmetric fusion was developed. In this method, partial genomes of parental protoplasts were fused for transfers of specific traits in the cytoplasm genome (mitochondria (mtDNA) and chloroplast (cpDNA)) or only a few genes and chromosomes in the nuclear genome. Before inducing fusion by using the asymmetric method, genomes of parental

protoplasts were fragmented to generate donor or recipient protoplasts with the desired traits. Nuclear genome fragmentation can be accomplished by several techniques, including irradiation (UV, X- and ϒ-rays) and microprotoplasts (MPPs), whereas metabolic inactivation of cytoplasm is achieved by using chemicals, such as iodoacetic acid (IOA) and rhodamine 6-G (Lakshmanan *et al.*, 2013). Asymmetric fusion was used successfully in several economic crops to transfer specific traits, including CMS, disease and pest resistance, and environmental tolerance e.g., common wheat (*Triticum aestivum* L.) (Xu *et al.*, 2003; Xia, 2009), maize (*Zea mays* L.) (Xu *et al.*, 2003), cotton (*Gossypium* spp.) (Fu *et al.*, 2009), citrus (*Citrus* spp.) (Xu *et al.*, 2006; Bona *et al.*, 2009) and sunflower (*Helianthus annuus* L.) (Varotto *et al.*, 2001; Taski-Ajdukovic *et al.*, 2008). This research was aimed at optimizing conditions for induced fusion of protoplasts using PEG, developing a cytoplasmic inactivation procedure to produce a recipient parent for the transfer of a normal cytoplasm trait of sunflower protoplasts, and generating cell hybrids of sunflower by using both symmetric and asymmetric fusions.

Materials and Methods

Plant materials and protoplast isolation: Two sunflower genotypes, a cytoplasmic male sterile line, 10A, which was developed in Thailand for the production of hybrids with high oil content (41.40%), and a fertile cytoplasmic line, PI441983, from the North Central Regional Plant Introduction Station, Iowa, USA, were used. The seeds of the sunflower genotypes were germinated and maintained *In vitro* on hormone-free MS (Murashige & Skoog, 1962) medium containing 2% (w/v) sucrose and 0.8% agar at pH 5.6-5.7. Seven-day-old etiolated hypocotyls of the 10A line and four-week-old fully expanded young leaves of the PI441983 line were used for protoplast isolation by using the mixed enzymatic method according to Kativat *et al.* (2012), and purified as described by Henn *et al.* (1998b). Yield and viability of protoplasts were determined by using haemacytometer and fluorescein diacetate (FDA) staining (Henn *et al.*, 1998b), respectively. In this study, hypocotyl protoplasts of the 10A line were used as a recipient parent to receive a normal cytoplasm trait from mesophyll protoplasts of the PI441983 line, a donor parent.

Cytoplasmic inactivation treatment: Optimal conditions to inactivate cytoplasm of hypocotyl protoplasts of the 10A line were examined. Four concentrations of IOA (0, 1.5, 3.0 and4.5 mM) in W5 solution (154 mM NaCl, 10.73 mM KCl, 125 mM $CaCl_2.2H_2O$, 5.55 mM glucose and 13.32 mM glycine, pH 5.8 (Menczel & Wolfe, 1984)) and two incubation periods (15 and 20 min) were used. The purified hypocotyl protoplasts were adjusted to a final density of 1×10^6 protoplasts mL^{-1} with an isolation solution (Kativat *et al.*, 2012). Hypocotyl protoplast suspension was gently mixed with IOA solution at the ratio of 1:5. IOA-treated hypocotyl protoplasts were washed to remove IOA from the protoplasts (Greplová *et al.*, 2011) by centrifuging for 5 min at 1,000 rpm, and protoplast pellets were washed twice in the isolation solution. The cleaned protoplast pellets were resuspended and adjusted to a final density of 5×10^5 protoplasts mL^{-1} with liquid L4 medium (Lenée & Chupeau, 1986). IOA-treated hypocotyl protoplasts were cultured in 1-mL agarose-solidified droplets (4 droplets/ 100 × 15 mm Petri dish) of culture medium using the L4 regeneration protocol (Lenée & Chupeau, 1986), described by Burrus *et al.* (1991). The frequencies of cell division at 10, 20, 30 and 40 d of culture and colony formation at 20, 30 and 40 d of culture were monitored to estimate the best conditions for inhibition of cytoplasmic activities, and calculated using the following formulas:

$$\text{Frequency of cell division (\%)} = \frac{\text{Number of dividing protoplasts}}{\text{Total number of protoplasts}} \times 100$$

$$\text{Frequency of colony formation (\%)} = \frac{\text{Number of dividing protoplast-formed colonies}}{\text{Total number of protoplasts}} \times 100$$

PEG-mediated protoplast fusion: Using optimized conditions for fusion induction by a chemical fusogen, PEG, was evaluated for hypocotyl and mesophyll protoplasts of 10A and PI441983, respectively. Various concentrations of PEG 8000 (0, 10, 20 and 30% (w/v)) in fusion solution (5% (v/v) DMSO, 90 mM mannitol, 60 mM $CaCl_2$ and25 mM glycine, pH 5.6-5.7 (Binsfeld *et al.*, 2000)) and fusion periods (10, 15 and 20 min) were evaluated. The purified protoplasts of each source were adjusted to a final density of 1×10^6 protoplasts mL^{-1} and mixed together at a ratio 1:1. An equal volume of mixed protoplast suspension and PEG solution were gently mixed together. The occurrence of binary (fusion of only two protoplasts) and multi fusion (fusion of more than two protoplasts) were recorded under an inverted microscope and the percentages of binary and multi fusion were calculated using the following formulas:

$$\text{Percentages of binary fusion (\%)} = \frac{\text{Number of fusions between only two protoplasts}}{\text{Total number of protoplasts}} \times 100$$

$$\text{Percentages of multi fusions (\%)} = \frac{\text{Number of fusions among more than two protoplasts}}{\text{Total number of protoplasts}} \times 100$$

Symmetric and asymmetric induced fusion and culture: The final density of both parental protoplasts was 1×10^6 protoplasts mL^1. An equal volume of mixed protoplast (1:1) suspension was instantly mixed with PEG solution for symmetrically induced fusion, whereas in asymmetric fusion, hypocotyl protoplasts were pretreated with IOA using two conditions - 1.5 mM IOA for 20 min and 3.0 mM IOA for 15 min - before being mixed with mesophyll protoplasts and then fusion was induced. Both methods of fusion (symmetric and asymmetric) were induced by using 20% (w/v) PEG 8000 for 15 min. Fusion products were cleaned to remove PEG by centrifugation and washed in the isolation solution, and cultured in 500-µL agarose-solidified droplets (8 droplets/ 100 × 15 mm Petri dish) of culture medium using the L4 regeneration protocol (Burrus *et al.*, 1991). Cell division, and colony and callus formations were observed.

Statistical analysis: The data were statistically analyzed by the analysis of variance (ANOVA) using a completely randomized factorial design (factorial in CRD) with 4 replications for both PEG-induced fusion and cytoplasmic inactivation experiments, and the means were compared

by Duncan's new multiple range test (DMRT). All statistical analyses were performed using SPSS version 14.0 (Levesque & SPSS Inc., 2006).

Results

Cytoplasmic inactivation: Freshly isolated hypocotyl protoplasts of 10A were treated with different concentrations of IOA and incubation periods to inactivate cytoplasm, resulting in disruption of cell division. The inhibition effect was observed when IOA-treated protoplasts were plated in L4 medium solidified with agarose and cultured under the L4 regeneration protocol. The percentages of cell division and colony formation were compared to untreated protoplasts and protoplasts treated with W5 solution (0 mM IOA). The results show that IOA concentrations significantly affected both cell division and colony formation ($p<0.01$) and increasing IOA concentrations resulted in continuously reduced development of both (Table 1). The lowest average percentage of cell division and colony formation at 40 d of culture were observed when 4.5 mM IOA was used, 4.48 and 3.30-fold lower than untreated protoplasts and protoplasts treated with 0 mM IOA, respectively for cell division, and 30.36 and 19.21-fold, respectively for colony formation. The incubation periods had no effect on average percentages of both cell division and colony formation ($p>0.05$); however, a better cytoplasmic inactivation tended to be achieved when using a longer period (20 min). In general, increasing the concentrations of IOA, especially together with using a longer incubation period (20 min), could promote a stronger inhibitory effect. At 40 d of culture, using 4.5 mM IOA for 15 min gave the lowest average percentage of cell division (10.65%), which was 5.17 and 3.80-fold lower than untreated protoplasts and protoplasts treated with W5 solution (0 mM IOA) ($p<0.05$), respectively, but it was not significantly different when compared with other combinations of IOA concentrations (1.5, 3.0 and 4.5 mM) and incubation periods (15 and 20 min) except when using 1.5 mM IOA for 15 min (31.04%). However, when colony formation was simultaneously observed, it was found that the lowest average percentage of divided cells that could develop into a colony was achieved when using 4.5 mM IOA for 20 min (0.64%). No significant difference of colony formation was found among any combinations of IOA concentrations (1.5, 3.0 and 4.5 mM) and incubation periods (15 and 20 min), leading to average percentages of colony formation ranging from 1.32-10.93%. Conversely, a significant colony formation difference was observed when compared with untreated protoplasts and protoplasts treated with W5 solution (0 mM IOA) (33.09 and 20.65%, respectively). For efficient cytoplasmic inactivation, the lowest concentration of IOA that results in the best inactivation is required. Our results suggest that using 1.5 mM IOA for 20 min and 3.0 mM IOA for 15 min were optimal and should be applied for asymmetric fusion.

PEG-mediated protoplast fusion: PEG 8000 was added to a mixture of equal volume of hypocotyl and mesophyll protoplasts of 10A and PI441983, respectively, to induce fusion (Fig. 1A). Two characteristics of fusion were observed, binary and multi fusions (Fig. 1B). It was found that the concentrations of PEG 8000 and fusion periods significantly affected the frequency of binary fusion ($p<0.01$). Increasing both PEG 8000 concentrations and fusion periods promoted a binary fusion event. The highest average percentage of binary fusion was observed when using 20 and 30% (w/v) PEG 8000 (25.10 and 25.09%, respectively), which were significantly higher than using 0 and 10% (w/v) PEG 8000 1.85 and 1.28-fold, respectively. For fusion periods, the highest average percentage of binary fusion (23.28%) was found at the longest period (20 min), and continuously decreased when 15 and 10 min were used (21.08 and 18.22%, respectively). However, the frequency of multi fusions was significantly influenced only by fusion periods ($p<0.01$), and the highest average percentage of multi fusions was achieved at 20 min (21.42%), which was 1.88-fold higher than that obtained at 10 min. The increase of PEG 8000 concentrations, especially together with fusion periods tended to enhance the frequencies of both binary and multi fusions. The highest fusion frequencies of both binary and multi fusions were achieved when 30% (w/v) PEG 8000 was used for 20 min (27.65 and 24.28%, respectively). However, no significant difference was observed when compared with using 20% (w/v) PEG 8000 for 20 min (26.15 and 22.26, respectively), 30% (w/v) PEG 8000 for 15 min (23.56 and 19.78, respectively) and 10% (w/v) PEG 8000 for 20 min (22.14 and 17.97, respectively). In contrast, using 0% (w/v) PEG 8000 for 10 min gave the lowest frequencies of both binary and multi fusions (9.03 and 9.32%, respectively). Because both high frequency of binary fusion but low frequency of multi fusions are usually required for efficiently induced fusions, using 20% (w/v) PEG 8000 for 15 min, which resulted in a high frequency of binary fusions (26.16%) and a low frequency of multi fusions (12.96%), was found to be the most appropriate condition (Table 2).

Symmetric and asymmetric fusions: Both symmetric and asymmetric fusions of hypocotyl protoplasts of 10A and mesophyll protoplasts of PI441983 were induced togenerate hybrid cells and evaluate their efficiency. Freshly prepared parental protoplasts wereimmediately fused after isolation and purification procedures for symmetric fusion, whereas in asymmetric fusion hypocotyl protoplasts were pretreated with IOA (1.5 mM IOA for 20 min and 3.0 mM IOA for 15 min) before fusion with mesophyll protoplasts. Protoplast fusion was induced with 20% (w/v) PEG 8000 for 15 min. Fusion products were plated in L4 medium solidified with agarose and cultured under the L4 regeneration protocol. It was found that the fusion products of both symmetric and asymmetric fusions continually developed - dividing, forming colonies and having a tendency to generate microcalli in the culture medium. Nevertheless, slower growth and development were observed when compared with the culture of each parental protoplast (hypocotyl and mesophyll), especially with the fusion products of asymmetric fusion. The reduced densities of protoplast-derived colonies and microcalli from asymmetric fusion may result from the inability of unfused hypocotyl protoplasts and fusion products among hypocotyl protoplasts to develop in the culture medium (Fig. 2).

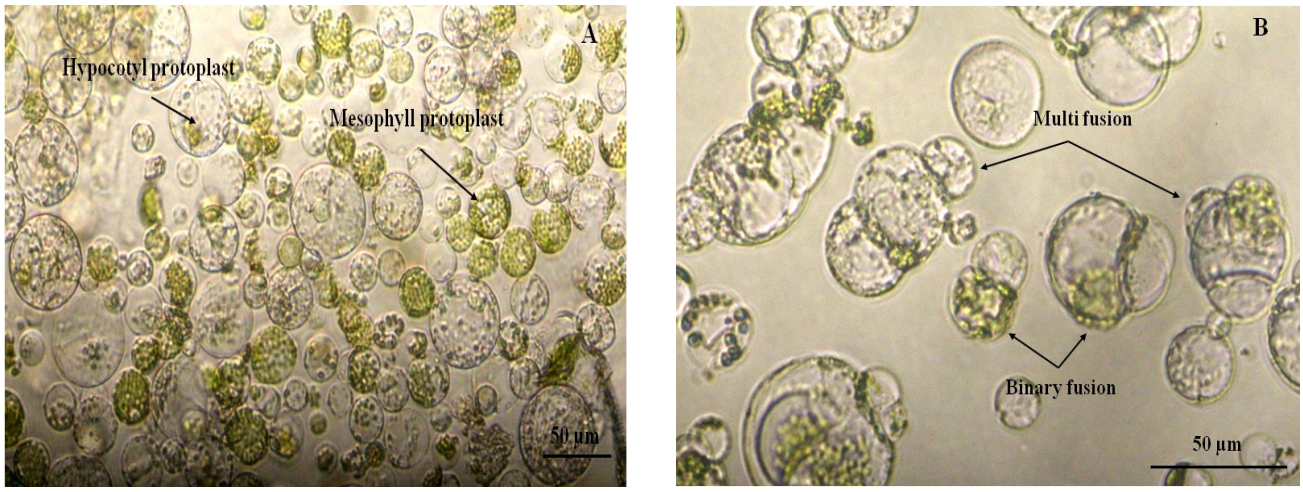

Fig. 1. Induced fusion of sunflower protoplasts by PEG 8000 between hypocotyl and mesophyll protoplasts of 10A and PI441984 lines, respectively. (A) Mixed protoplast suspension of hypocotyl and mesophyll protoplasts. (B) Characteristics of binary and multi fusions.

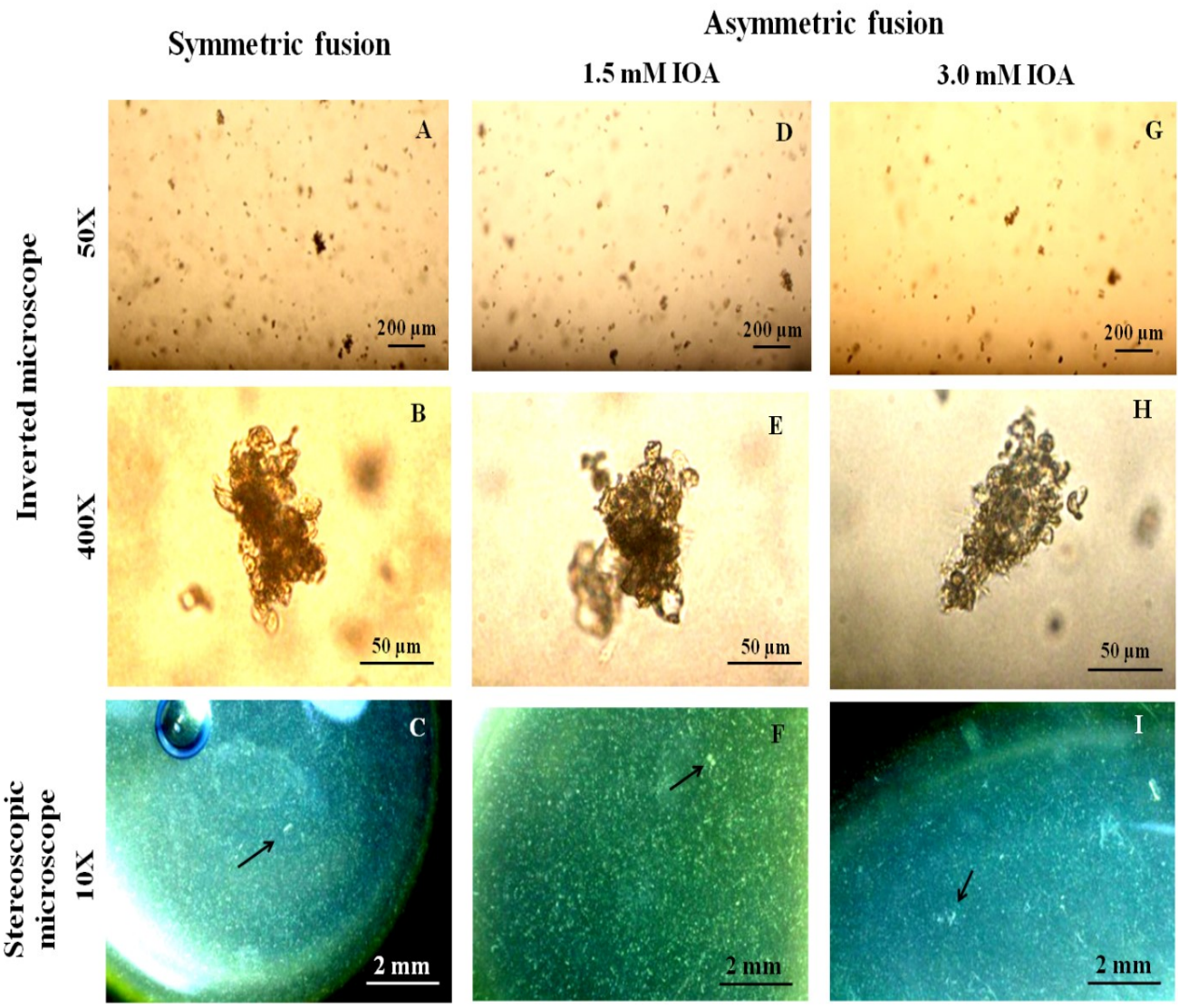

Fig. 2. The development of sunflower fusion products from symmetric and asymmetric induced fusions using PEG 8000. (A-C) Fusion products of symmetric fusion divided into colonies. (D-F) Fusion products of asymmetric fusion when hypocotyl protoplasts were treated with 1.5 mM IOA for 20 min and divided into colonies. (G-I) Fusion products of asymmetric fusion when hypocotyl protoplasts were treated with 3.0 mM IOA for 15 min and divided into colonies. (Arrows) colonies that could develop into microcalli. IOA = Iodoacetic acid

Table 1. The effects of iodoacetic acid concentrations and incubation periods on cell division and colony formation of hypocotyl protoplasts of 10A sunflower.

Iodoacetic acid (mM)	Incubation period (min)	Cell division (%)				Colony formation (%)		
		10 d	20 d	30 d	40 d	20 d	30 d	40 d
Untreated protoplasts	-	19.04 ± 4.68 a	33.12 ± 6.08 a	41.80 ± 3.92 a	55.03 ± 5.37 a	7.66 ± 1.55 a	19.23 ± 0.39 a	33.09 ± 4.83 a
0 (W5 solution)	15	13.99 ± 3.45 ab	34.78 ± 6.61 a	34.49 ± 6.09 ab	41.55 ± 5.73 ab	10.69 ± 2.73 a	15.62 ± 4.52 a	22.73 ± 4.82 ab
	20	13.81 ± 2.96 ab	32.95 ± 6.46 a	37.30 ± 8.46 ab	39.30 ± 6.55 ab	7.46 ± 1.54 a	15.84 ± 4.12 a	18.56 ± 4.87 bc
1.5	15	8.91 ± 3.29 bc	24.39 ± 6.06 ab	25.20 ± 3.10 bc	31.04 ± 9.77 bc	6.03 ± 1.96 a	5.86 ± 1.21 b	10.93 ± 2.91 dc
	20	6.68 ± 1.66 bc	17.95 ± 3.54 ab	22.69 ± 4.36 bc	20.41 ± 2.13 dc	1.12 ± 0.58 b	4.99 ± 0.90 b	3.70 ± 1.09 d
3.0	15	5.45 ± 1.61 bc	12.29 ± 3.62 b	14.74 ± 4.34 c	15.08 ± 2.50 dc	0.46 ± 0.23 b	1.80 ± 0.10 b	2.93 ± 0.88 d
	20	4.07 ± 0.66 c	11.69 ± 1.92 b	16.12 ± 3.34 c	13.89 ± 3.16 d	0.53 ± 0.23 b	1.27 ± 0.40 b	2.56 ± 1.70 d
4.5	15	3.29 ± 1.23 c	9.83 ± 1.40 b	9.37 ± 2.76 c	10.65 ± 1.70 d	0.74 ± 0.43 b	0.74 ± 0.48 b	1.32 ± 0.85 d
	20	3.02 ± 0.65 c	10.44 ± 4.42 b	10.69 ± 5.46 c	15.58 ± 1.52 dc	0.00 ± 0.00 b	1.50 ± 0.39 b	0.64 ± 0.64 d

Data are expressed as mean ± S.E. Values with different letters within the same column are significantly different at $p<0.05$ by Duncan's multiple range test

Table 2. Percentages of binary and multi fusion between hypocotyl protoplasts of the 10A line and mesophyll protoplasts of the PI441983 line of sunflower induced by PEG 8000.

PEG 8000 (% (w/v))	Fusion period (min)	Binary fusion (%)	Multi fusion (%)
0	10	9.03 ± 1.45 f	9.32 ± 3.87 c
	15	14.60 ± 2.64 e	18.28 ± 2.23 ab
	20	17.18 ± 3.80 cde	21.18 ± 6.11 a
10	10	16.80 ± 2.22 de	9.61 ± 2.20 c
	15	20.01 ± 2.78 bcd	14.15 ± 3.72 bc
	20	22.14 ± 3.14 abc	17.97 ± 3.21 ab
20	10	22.98 ± 2.20 ab	13.57 ± 4.86 bc
	15	26.16 ± 3.17 a	12.96 ± 4.50 bc
	20	26.15 ± 5.59 a	22.26 ± 4.27 a
30	10	24.06 ± 6.26 ab	13.05 ± 3.61 bc
	15	23.56 ± 0.82 ab	19.78 ± 7.61 ab
	20	27.65 ± 2.54 a	24.28 ± 2.83 a

Data are expressed as mean ± S.E. Values with different letters within the same column are significantly different at $p<0.05$ by Duncan's multiple range test

Discussion

IOA concentrations affected both cell division and colony formation of hypocotyl protoplasts of the 10A line, but incubation periods had no effect on these two parameters. Inhibition of cell division occurred when protoplasts were treated with IOA (1.5, 3.0 and 4.5 mM), because it is an irreversible metabolic inhibitor of cytoplasm that affected the division of cells by inhibiting the activity of the mitotic spindle at the prophase of mitosis, making cells unable to divide, and their spindle fibers deteriorate (Varotto *et al.*, 2001; Bona *et al.*, 2009; Lakshmanan *et al.*, 2013). Thus, the frequencies of both cell division and colony formation in our study continually decreased with increasing concentrations of IOA, in agreement with previous studies (Varotto *et al.*, 2001; Ge *et al.*, 2006; Fu *et al.*, 2009; Xiao *et al.*, 2009). In this study, using 4.5 mM IOA gave maximum inhibition; average percentages of cell division and colony formation at 40d of culture were 10.65-15.58% and 0.64-1.32%, respectively, which were 2.52-2.90 and 17.22-29.00-fold lower than protoplasts treated with W5 solution (0 mM IOA), respectively. However, when average percentages of both cell division and colony formation at lower IOA concentrations (1.5 and 3.0 mM) were considered, it was found that using 3.0 mM IOA resulted in only slightly higher average percentages of cell division and colony formation than 4.5 mM IOA, indicating that this concentration was also effective for cell division inhibition. Conversely, 1.5 mM IOA tended to give high frequencies of both developments, suggesting that this concentration was not sufficient to suppress cell division activities. Therefore, treated protoplasts were able to reorganize their cytoplasm and divide again (Varotto *et al.*, 2001). However, when 1.5 mM IOA was used with a longer incubation period (20 min), a substantial decrease in both cell division and colony formation was achieved, which were 1.52 and 2.96-fold lower than when using 15 min, respectively, indicating that longer periods could enhance the efficiencies of cytoplasmic inactivation. These results agree with those of Bona *et al.* (2009) who found that using 3.0 mM IOA for 20 min gave the best cytoplasmic inactivation of protoplasts from embryogenic suspension cells of *Citrus* (grapefruit; *Citrus paradisi* Macfad. and Murcott tangor; *C. reticulata* × *C. sinensis*) when compared with 10 or 15 min. Nevertheless, the frequencies of cell division and colony formation were often taken into account along with the toxicity effect of IOA to obtain optimal

conditions. High concentrations of IOA, especially with long periods of incubation, cause severe damage to protoplasts including loss of viability, shrinking or bursting of protoplasts, decreasing the regeneration ability of protoplasts or loss of the ability to generate complete plants (Liu & Deng, 1999; Liu & Deng, 2002; Ge *et al.*, 2006; Zhao *et al.*, 2008; Fu *et al.*, 2009; Lakshmanan *et al.*, 2013). Consequently, using 1.5 mM IOA for 20 min and 3.0 mM IOA for 15 min were selected and applied for asymmetric fusion.

For PEG-induced fusion between hypocotyl and mesophyll protoplasts of the 10A and PI441983 lines, respectively, concentrations of PEG 8000 and fusion periods affected the frequencies of binary fusion, while multi fusion was influenced only by fusion periods. However, increasing PEG 8000 concentrations, especially together with fusion periods, tended to promote the frequencies of both binary and multi fusions. These results agree with several reports on sugar beet (*Beta vulgaris* L.) (Badr-Elden *et al.*, 2010), ginger (*Zingiber officinale* Rosc.) (Guan *et al.*, 2010) and brassica (*B. carinata* Braun. and *B. rapa* L.) (Beránex *et al.*, 2007) which found that when PEG concentrations increased from 0 to 30% (w/v), continuous increases of both binary and multi fusion frequencies were obtained. In our study, using PEG 8000 at 30% (w/v) for 20 min led to the highest average percentages of binary and multi fusions (27.65 and24.28%, respectively). This might be due to the properties of PEG that help reduce the negative charges around the plasma membrane of isolated protoplasts, allowing them to fuse (Tomar & Dantu, 2010). Therefore, using high concentrations (30% (w/v)) was more effective in reducing the negative charges than lower concentrations (10 and 20% (w/v)) and the fusion event could also occur faster. Similarly, Beránex *et al.* (2007), Xiao *et al.* (2009), Badr-Elden *et al.* (2010) andGuan *et al.* (2010)found that induced fusion with 20-30% (w/v) PEG resulted in the highest frequencies of binary fusion. In addition, using higher concentrations of PEG than 30% (w/v) tended to continually increase multi fusion, while binary fusion continually decreased (Xiao *et al.*, 2009). Moreover, increasing fusion periods also enhanced the frequencies of both binary and multi fusions. In our study, the highest average percentages of binary and multi fusions were observed when the longest period (20 min) was applied, which agrees with Beránex *et al.* (2007), Badr-Elden *et al.* (2010) and Guan *et al.* (2010) who found that the optimal period that led to the highest frequency of binary fusion was 10-20 min. Furthermore, using 0% (w/v) PEG 8000 also resulted in high frequencies of both binary and multi fusions in our study, possibly due to the effects of DMSO in the PEG solution (5% (v/v)) that helps enhance fusion frequencies (Menczel & Wolfe, 1984; Henn *et al.*, 1998a). A high frequency of binary fusion and a low frequency of multi fusion are desirable for protoplast fusion. Therefore, 20% (w/v) PEG 8000 for 15 min was chosen for symmetric and asymmetric induced fusions.

To generate sunflower hybrids, symmetric and asymmetric protoplast fusions using PEG 8000 were evaluated. Hypocotyl protoplasts were pretreated with IOA to generate a recipient parent before the induced asymmetric fusion. When each fusion procedure was performed, and fusion products were cultured, it was found that all of them developed in the culture medium, divided and formed colonies and also had the potential to generate microcalli, indicating that the fusion condition (20% (w/v)PEG 8000 for15 min) used was suitable. Under this condition, PEG had no toxic effect that makes protoplasts burst, die or be unable to develop (Henn *et al.*,1998a; Navrátilová, 2004; Verma *et al.*, 2008). However, when the densities of protoplast-derived colonies and microcalli in the culture medium were examined, differential densities of fusion product-derived colonies of symmetric and asymmetric fusions were observed. The densities of protoplast-derived colonies of asymmetric fusion (hypocotyl protoplasts were treated with 1.5 mM IOA for 20 min and 3.0 mM IOA for 15 min) were fewer than those of the symmetric fusion, especially when hypocotyl protoplasts were treated with a high concentration of IOA (3.0 mM IOA for 15 min). These results might be due to the inhibitory effects of IOA on the cell division of hypocotyl protoplasts (Varotto *et al.*, 2001; Bona *et al.*, 2009), resulting in the selection of fusion products (Fu *et al.*, 2009; Lakshmanan *et al.*, 2013). Furthermore, high concentrations of IOA also give a strong inhibitory effect to the metabolism of protoplasts that results in slow growth and development of cells (cell division, colony and callus formation and plant regeneration), and a loss of viability in protoplasts (Liu & Deng, 1999; Varotto *et al.*, 2001; Liu & Deng 2002; Ge *et al.*, 2006; Fu *et al.*, 2009). Similarly, our results show that the lowest density of colony formation was observed in asymmetric fusion using 3.0 mM IOA for 15 min.

Conclusions

IOA concentrations affected both cell division and colony formation of sunflower hypocotyl protoplasts. We found that the best condition for cytoplasmic inactivation of 10A hypocotyl protoplasts was 1.5 mM IOA treatment for 20 min. PEG concentrations and fusion periods affected frequencies of both binary and multi protoplast fusion. Using 20% (w/v) PEG 8000 for 15 min, which resulted in high frequency of binary fusion (26.16%) and low frequency of multi fusion (12.96%), appears to be the most appropriate method. Generating sunflower hybrids by using asymmetric fusion could help reduce confusion in the selection of hybrids by eliminating the unfused hypocotyl protoplasts and fusion of protoplasts among hypocotyl protoplasts. Pretreatment with 1.5 mM IOA for 20 min was optimal for asymmetric fusion because it gave more colonies and the potential to generate microcalli, which are essential to the production of sunflower hybrids.

Acknowledgements

The authors would like to express their gratitude to Dr. Paisan Laosuwan and Dr. Thitiporn Machikowa for providing 10A and PI441983 seeds. We also thank Mr. Peter C. Bint and Mr. Chris Boyle for editing the manuscript. This work was supported by the National Research Council of Thailand (NRCT), Suranaree University of Technology (SUT), the Office of the Higher

Education Commission under the NRU project of Thailand, and the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE), Thailand.

References

Badr-Elden, A.M., A.A. Nower, M.I. Nasr and A.I. Ibrahim. 2010 Isolation and fusion of protoplasts in sugar beet (*Beta vulgaris* L.). *Sugar Tech.*, 12: 53-58.

Beránex, M., M. Bechynêm and M. Klíma. 2007. Protoplast isolation and fusion between *Brassica carinata* Braun. and *Brassica rapa* L. *Agric. Tropica et Subtrop.*,40: 1-6.

Binsfeld, P.C., R. Wingender and H. Schnabl. 2000. Characterization and molecular analysis of transgenic plants obtained by microprotoplast fusion in sunflower. *Theor. Appl. Genet.*, 101: 1250-1258.

Bona, C.M., J.H. Gould, J.C. Miller, D. Stelly and E.S. Louzada. 2009. Citrus asymmetric hybrids produced via fusion of gramma-irradiated and iodoacetamide treated protoplasts. *Pesq. Agropec. Bras.*, 44: 454-462.

Burrus, M., C. Chanabe, G. Alibert and D. Bidney. 1991. Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.). *Plant Cell Rep.*, 10: 161-166**.**

Cheng, A.X. and G.M. Xia. 2004. Somatic hybridization between common wheat and Italian ryegrass. *Plant Sci.*, 116: 1219-1226.

Compton, M.E., J.A. Saunders and R.E. Veilleux. 2000. Use of protoplast for plant improvement. In: *Plant Tissue Culture Concepts and Laboratory Exercise*. (Eds.): R.N. Trigiano and D.J. Gray CRC Press, New York, pp. 249-261.

Dagustu, N., G. Bayram, M. Sincik and M. Bayraktaroglu. 2012. The short breeding cycle protocol effective on diverse genotypes of sunflower (*Helianthus annuus* L.). *Turk. J. Field Crops*, 17: 124-128.

Eckardt, N.A. 2006. Cytoplasmic male sterility and fertility restoration. *Plant Cell*, 18: 515-517.

Eeckhaut, T., K. Van Laere, J. De Riek and J. Van Huylenbroeck. 2006. Overcoming interspecific barriers in ornamental plant breeding. In: *Floriculture, Ornamental and Plant Biotechnology: Advance and Topical Issues*. (Ed.): J.A. Teixeira da Silva. Global Science Books, UK, pp. 540-551.

Fu, L.L., X.Y. Yang, X.L. Zhang, Z.W. Wang, C.H. Feng, C.X. Liu, P.Y. Jiang and J.L. Zhang. 2009. Regeneration and identification of interspecific asymmetric somatic hybrids obtained by donor-recipient fusion in cotton. *Chinese Sci. Bull.*, 54: 3035-3044.

Galun, E., A. Perl and D. Aviv. 1988. Protoplast fusion-mediated transfer of male sterility and other plasmone-controlled traits. In: *Applications of Plant Cell and Tissue Culture.* (Eds.): G. Bock and J. Marsh. A Wiley-Interscience Publication, UK, pp. 97-112.

Ge, T.M., X.H. Lin, F.L. Qin, S.W. Yu and Y.J. Yu. 2006.Protoplast electrofusion between common wheat (*Triticum aestivum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) and regeneration of mature cybrids. *In Vitro Cell Dev. Biol. Plant*, 42: 179-187.

Greplová, M., E. Greplová, H. Polzerová and J. Domkářová. 2011. Procedure of asymmetric somatic hybridization (in *Solanum* genus). *Potato Research Institute.*

Guan, O., Y. Guo, Y. Wei, F. Meng and Z. Zhang. 2010. Regeneration of somatic hybrids of ginger via chemical protoplast fusion. *Plant Cell Tiss. Organ Cult.*, 102: 279-284.

Henn, H.J., R. Wingender and H. Schnabl. 1998a. Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L. and wild *Helianthus* species. *Plant Cell Rep.*, 18: 220-224.

Henn, H.J., R. Wingender and H. Schnabl. 1998b. Regeneration of fertile plants from *Helianthus nuttallii* T&G and *Helianthus giganteus* L. mesophyll protoplasts. *Plant Cell Rep.*, 18: 288-291.

Hou, Z., D. Tang and N. Tang. 2016. Isolation and analysis of differentially expressed genes between male fertile and male sterile flower buds of marigold (*Tagetes erecta* L.). *Pak. J. Bot*., 48: 2423-2431.

Kativat, C., O. Poolsawat and P.A. Tantasawat. 2012. Optimization of factors for efficient isolation of protoplasts in sunflower (*Helianthus annuus* L.). *Aust. J. Crop Sci.*, 6: 1004-1010.

Lakshmanan, P.S., T. Eeckhaut, D. Deryckere, E. Van Bockstaele and J. Van Huylenbroeck. 2013. Asymmetric somatic plant hybridization: Status and applications. *AJPS.*, 4: 1-10.

Lenée, P. and Y. Chupeau. 1986. Isolation and culture of sunflower protoplasts (*Helianthus annuus* L.): Factors influencing the viability of cell colonies derived from protoplasts. *Plant Sci.*, 43: 69-75.

Levesque, R. and SPSS Inc. 2006. *SPSS programming and data management*, 3rd edn. SPSS Inc., Chicago, IL.

Liu, J.H. and X.X. Deng. 1999. Production of hybrid calluses via donor-recipient fusion between *Microcitrus papuana* and *Citrus sinensis*. *Plant Cell Tiss. Organ Cult.*, 59: 81-87.

Liu, J.H. and X.X. Deng. 2002. Regeneration and analysis of citrus interspecific mixoploid hybrid plants from asymmetric somatic hybridization. *Euphytica,*125: 13-20.

Menczel, L. and K. Wolfe. 1984. High frequency of fusion induced in freely suspended protoplasm mixtures by polyethylene glycol and dimethylsulfoxide at high pH. *Plant Cell Rep.*, 3: 196-198.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*., 15: 473-497.

Navrátilová, B. 2004. Protoplast cultures and protoplast fusion focused on Brassicaceae - a review. *Hort. Sci. (PRAGUE)*, 31: 140-157.

Poehlman, J.M. 1994. *Breeding Field Crops.* (3rd Ed)Van Nostrand Reinhold, New York.

Rezazadeh, R., D.K. Harrison and R.R. Williams. 2011. Intraspecific somatic hybridization of mango (*Mangifera indica* L.) through protoplast fusion. *J. Appl. Hort.*, 13: 101-107.

Scholze, P., R. Kramer, U. Ryschka, E. Klocke and G. Schumann. 2010. Somatic hybrids of vegetable brassica as source for new resistances to fungal and virus diseases. *Euphytica*, 176: 1-14.

Sun, Y.H., Q.Z. Xue, C.M. Ding, X.Y. Zhang, L.L. Zhang, W.F. Wang and S. Ali. 2005. Somatic cybridization between *Nicotiana tabacum* and *N. repanda* based on a single inactivation procedure of nuclear donor parental protoplasts. *Plant Sci.*, 168: 303-308.

Taski-Ajdukovic, K., N. Nagl and D. Miladinovic. 2008. Somatic hybridization for diseases resistance breeding in sunflower. *In:* Teixeira da Silva, J.A. (Ed.), *Floriculture, Ornamental and Plant Biotechnology Advances and Topical Issues Volume V*. Global Science Books Ltd, Japan, pp. 130-137.

Tomar, U.K. and P.K. Dantu. 2010. Protoplast culture and somatic hybridization. In:*Cellular and Biochemical Sciences*. (Ed.): G. Tripathi, I.K. International Publishing House Pvt Ltd, India, pp. 876-891.

Tudses, N., S. Premjit and D. Premjit. 2015. Establishment of method for protoplast fusion with PEG-mediated between *Jatropha curcas* L. and *Ricinus communis* L. *Int. J. Life Sci. Biotech. Pharm. Res.*, 4: 172-178.

Varotto, S., E. Nenz, M. Lucchin and P. Parrini. 2001. Production of asymmetric somatic hybrid plants between *Cichorium intybus* L. and *Helianthus annuus* L. *Thero. Appl. Genet.*,102: 950-956.

Vassilevska-Ivanova, R., B. Kraptchev, I. Stancheva, M. Geneva, I. Iliev and G. Georgiev. 2014. Utilization of related wild species (*Echinacea purpurea*) for genetic enhancement of cultivated sunflower (*Helianthus annuus* L.). *Turk. J. Agric. For.*, 38: 15-22.

Verma, N., M.C. Bansal and V. Kumar. 2008. Protoplast fusion technology and its biotechnological applications.*Chem. Eng. Trans.*, 14: 113-120.

Wang, G.X., Y. Tang, H. Yan, X.G. Sheng, W.W. Hao, L. Zhang, K. Lu and F. Liu. 2011. Production and characterization of interspecific somatic hybrids between *Brassica oleracea* var. *botrytis* and *B. nigra*and their progenies for the selection of advanced pre-breeding materials. *Plant Cell Rep.*, 30: 1811-1821.

Xia, G. 2009. Progress of chromosome engineering mediated by asymmetric somatic hybridization. *J. Genet. Genomics*, 36: 547-556.

Xiang, F., G. Xia, D. Zhi, J. Wang, H. Nie and H. Chen. 2004. Regeneration of somatic hybrids in relation to the nuclear and cytoplasmic genomes of wheat and *Setaria italica*. *Genome*, 47: 680-688.

Xiao, W., X. Huang, Q. Gong, X.M. Dai, J.T. Zhao, Y.R. Wei and X.L. Huang. 2009. Somatic hybrids obtained by asymmetric protoplast fusion between *Musa* Silk cv. Guoshanxiang (AAB) and *Musa acuminate* cv. Mas (AA). *Plant Cell Tiss. Organ Cult.*, 97: 313-321.

Xu, C., G. Xia, D. Zhi, F. Xiang and H. Chen. 2003. Integration of maize nuclear and mitochondrial DNA into the wheat genome through somatic hybridization. *Plant Sci.*, 165: 1001-1008.

Xu, X.Y., J.H. Liu and X.X. Deng. 2006. Isolation of cytoplasts from Satsuma mandarin (*Citrus unshiu* Marc.) and production of alloplasmic hybrid calluses via cytoplast-protoplast fusion. *Plant Cell Rep.*, 25: 533- 539.

Yousuf, S., F. Ashraf, S.K. Kazmi, S. Khan and H.A. Kayani. 2015. A study on the isolation of protoplasts from the callus of *Lilium longiflorum* Overig. *Pak. J. Bot.*, 47: 2391-2396.

Zhao, Z.G., T.T. Hu, X.H. Ge, X.Z. Du, L. Ding and X.H. Li. 2008. Production and characterization of intergeneric somatic hybrids between *Brassica napus* and *Orychophragmus violaceus* and their backcrossing progenies. *Plant Cell Rep.*, 27: 1611-1621.

(Received for publication 2September 2016)