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Research Note

Optimization of factors for efficient isolation of protoplasts in sunflower (*Helianthus annuus* L.)

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Abstract

Various factors influencing the efficiency of sunflower protoplast isolation including genotypes (10A and PI 441983), tissue types (young leaves and hypocotyls), isolation methods (M1-M5) and cellulase concentrations (0.1, 0.5, 1.0 and 1.5%) were evaluated to obtain optimum protoplast isolation procedures. Young leaves were preferable to hypocotyls in production of viable protoplasts. Genotypes and isolation methods significantly affected both yields and viability of protoplasts from both tissues. Moreover, protoplast yield from leaf tissues was also influenced by cellulase concentrations. Using hypocotyls as explants, 10A line gave the highest number of viable protoplasts (4.24×10^6 protoplasts/ g fresh weight [FW]) when incubated at 25°C for 16 h in the optimal isolation solution containing 1% cellulase and 0.5% macerozyme. Higher number of viable protoplasts observed in this genotype, compared to PI 441983, may result from differences in hypocotyl morphology between the two genotypes. By contrast, for leaf explants the highest numbers of viable protoplasts, 6.13×10^6 protoplasts/ g FW for 10A line and 8.81×10^6 protoplasts/ g FW for PI 441983 line, were achieved when incubated with 0.1 and 0.5% cellulase, respectively, in the optimal isolation solution containing 0.05% driselase, 0.02% macerozyme and 0.1% bovine serum albumin (BSA) at 25°C for 16 h. These results suggest that suitable sunflower protoplast isolation procedures varied according to genotypes and tissue types, and need to be individually optimized.

Keywords: Cellulase concentration**,** Sunflower genotype, Protoplast isolation method, Protoplast yield and viability, Tissue type, Viable protoplast.
Abbreviations: BSA, Bovine serum albumin**;** FW, Fresh weight.

Introduction

Sunflower is one of the major economic oil crops of the world. Most sunflower cultivars used intensively in Thailand are F_1 hybrids whose production requires three parental lines (a cytoplasmic male sterile line [CMS; A-line], a maintainer line [cytoplasmic fertile line; B-line] and a restorer line [R-line]). Generally, an A-line is crossed with an R-line to produce F_1 hybrid seeds, while a B-line, which is similar genetically to an A-line but differs only in the traits of cytoplasm is used as a male parent to cross with an A-line for seed multiplication of the CMS line. Development of a B-line can be accomplished either by continued backcrossing for several generations, or rapidly produced by using protoplast fusion to transfer the fertile cytoplasm trait to the A-line in a single step. A nucleus from a protoplast derived from a CMS line can be combined with the cytoplasm derived from a protoplast of a male fertile line via protoplast fusion to create a male fertile cybrid that regenerates into a male fertile maintainer line (Aviv and Galun, 1986). Protoplast fusion has been used successfully to transfer the cytoplasmic traits in various plants such as perennial ryegrass, rice, brassica, tobacco, carrot and citrus (Creemers-Molenaar et al., 1992; Bhattacharjee et al., 1999; Arumugam et al., 2000; Davey et al., 2005; Cai et al., 2007). Furthermore, protoplasts have been used in somatic hybridization to form interspecific or intergeneric hybrids, in the generation of somaclonal variants, as well as in several modern biotechnological techniques such as genetic transformation, genomics, proteomics and metabolomics (Davey et al., 2005). In the case of *Helianthus* species, the application of protoplast fusion has previously been reported for the transfer of *Sclerotinia sclerotiorum* resistance genes from wild *Helianthus* species to cultivated sunflowers to produce resistance sunflower hybrids (Henn et al., 1998a; Taski-Ajdukovic et al., 2006). Nevertheless, successful development of protoplast-to-plant systems was still limited in many plants because it usually depends on various factors especially genotypes (Krasnyanski et al., 1992; Krasnyanski and Menczel, 1993; Schum et al., 2001; Davey et al., 2005). The effects of genotypes on plant development have been demonstrated in several *in vitro* systems including protoplast isolation and regeneration (Rákosy-Tican et al., 2007; Meyer et al., 2009; Taski-Ajdukovic et al., 2009). Isolation of protoplasts was generally performed by enzymatic digestion of various types of plant tissues. Among the commonly used enzymes are cellulases, hemicellulases and pectinases. Efficiency of protoplast isolation has been reported to be influenced by plant species, plant genotypes, tissue types, developmental stages, mixture of enzymes, isolation solutions, temperature, plasmolysis and

duration of enzyme incubation (Davey et al., 2005). Although a number of procedures for protoplast isolation of *Helianthus* species have been documented for various tissues including hypocotyls, cotyledons, leaves and roots (Bohorova et al. 1986; Krasnyanski and Menczel, 1995; Keller et al., 1997; Henn et al., 1998b; Binsfeld et al., 2000; Özdemir et al., 2002), the appropriate procedures will normally vary depending on the plant genotypes, as well as the plant tissues being used, suggesting that specific conditions are probably required for each genotype or tissue being processed (Uchimiya and Murashige, 1974; Davey et al., 2005). Studies on the isolation of sunflower protoplasts from varieties developed in Thailand have never been reported before. Therefore, this study was conducted to establish optimum protoplast isolation procedures in order to produce high quality protoplasts with maximum yields for future use in protoplast fusion to ultimately transfer the fertile/sterile cytoplasmic trait for sunflower F_1 hybrid production in Thailand.

Results

Protoplast isolation from hypocotyls and young leaves

Relatively pure protoplast fractions were recovered from most isolation procedures after floatation in the sucrose gradient. However, the efficiency of protoplast isolation (determined by protoplast yield and viability) from different tissue types appears to depend on the genotypes, isolation methods and cellulase concentrations. Because of the distinct tissue characteristics associated with hypocotyls and young leaves, different isolation methods were used for these tissue types. Although this prevents direct comparison between different tissue types on isolation efficiency, our results suggest that on average young leaves give a 1.7-fold higher protoplast yield, but a 1.3-fold lower protoplast viability, resulting in a 1.3-fold higher number of viable protoplasts than hypocotyls.

Factors influencing protoplast isolation from hypocotyl tissues

Freshly isolated hypocotyl protoplasts from all genotypes, isolation methods and cellulase concentrations were generally spherical, colorless and varied in size between 12.2 and 171.7 µm with an average of 36.4 µm (Fig. 1A, C). Genotypes significantly affected yields, viability and numbers of viable protoplasts ($P < 0.01$). 10A gave 1.9-fold higher average protoplast yield and number of viable protoplasts than PI 441983. Protoplast yields and viability were also significantly influenced by isolation methods. M2 gave an average protoplast yield 1.2-fold higher than M1 ($P < 0.05$), but protoplast isolated by M2 had lower average viability than those isolated by M1 ($P < 0.01$). However, no effect of isolation methods was observed on the numbers of viable protoplasts. In addition, cellulase concentrations had no significant effect on all parameters measured. Because protoplast yields, viability and numbers of viable protoplasts were significantly affected by interactions between genotypes and isolation methods ($P < 0.01$), it was found that an appropriate isolation method for each genotype was different. Protoplasts of 10A could be released with the highest average protoplast yield and number of viable protoplasts when using M2 (4.06 and 3.71 × 10^6 protoplasts/ g FW, respectively). However, the highest average protoplast yield and number of viable protoplasts of PI 441983 were achieved using M1 (1.95 and 1.81 × 10^6 protoplasts/ g FW, respectively). Nevertheless, protoplasts isolated by M1 had the highest average viability in both 10A and PI 441983 (92.19 and 91.06%, respectively). No effect of other interactions among factors was found on all parameters.

When all combinations of factors were considered (Table 1), it was found that the highest number of viable protoplasts was obtained from the hypocotyl tissues of 10A using M2 with 1.0% cellulase (4.24 × 10^6 protoplasts/ g FW), although there was no significant difference as compared to those obtained using M2 with 0.5 and 1.5% cellulase. The same isolation method (M2) and cellulase concentration (1.0%) released significantly fewer numbers of viable protoplasts with PI 441983 (1.53 × 10^6 protoplasts/ g FW). By contrast, the highest number of viable protoplasts of PI 441983 (1.96 × 10^6 protoplasts/ g FW) was obtained using M1 with 1.0% cellulase. In this genotype using M1 with 1.5% cellulase also gave similarly high numbers of viable protoplasts (Table 1).

Factors influencing protoplast isolation from young leaf tissues

Most freshly isolated mesophyll protoplasts from leaf tissues were spherical, green in color with a large number of chloroplasts, and heterogeneous in size between 15.6 and 108.6 µm with an average of 36.3 µm (Fig. 1B, D). It was found that genotypes significantly affected protoplast yields, viability and numbers of viable protoplasts. PI 441983 gave average protoplast yield and number of viable protoplasts 1.4- and 1.6-fold higher than 10A, respectively ($P < 0.01$). Similarly, the average viability of PI 441983 protoplasts was 1.2-fold higher than that of 10A ($P < 0.05$). In addition, isolation methods were found to significantly affect all parameters. M3 gave the highest average protoplast yield (6.25 × 10^6 protoplasts/ g FW) which was 9.9-fold higher than M5 ($P < 0.01$), whereas no significant difference was observed between M3 and M4. However, M3 gave the lowest average viability of protoplasts (48.77%) which was 1.6-fold lower than M4 and M5 ($P < 0.01$). Therefore, when numbers of viable protoplasts were calculated from both yields and viability, it was found that M4 gave the highest average number of viable protoplasts (5.18 × 10^6 protoplasts/ g FW). The levels of cellulase concentrations significantly affected protoplast yields and numbers of viable protoplasts, but had no effect on viability. The average protoplast yields and numbers of viable protoplasts generally declined with increasing cellulase concentrations. Low cellulase concentrations of 0.1 and 0.5% released similar average protoplast yield and number of viable protoplasts, however, increasing cellulase concentrations to 1.5% significantly reduced both parameters by up to 2.9-fold ($P < 0.01$). The highest (4.31 × 10^6 protoplasts/ g FW) and lowest (1.5 × 10^6 protoplasts/ g FW) values of average numbers of viable protoplasts were obtained with 0.1% and 1.5% cellulase, respectively. Protoplast yield was also significantly affected by an interaction between genotypes and isolation methods, an interaction between isolation methods and cellulase concentrations, and an interaction between genotypes and cellulase concentrations ($P < 0.05$). 10A can be released with the maximum average protoplast yield when using M3 (5.67 × 10^6 protoplasts/ g FW), whereas M4 was most favorable for PI 44198 (6.92 × 10^6 protoplasts/ g FW). By contrast, M5 had the lowest protoplast releasing ability in both genotypes. Interactions among all factors were not significant on protoplast viability and numbers of viable protoplasts. When all combinations of factors were considered (Table 2), the highest numbers of viable leaf mesophyll protoplasts of 10A (6.13 × 10^6 protoplasts/ g FW) and PI 441983 (8.81 × 10^6 protoplasts/ g FW) were achieved using M4 with 0.1 and 0.5% cellulase, respectively. It should be noted that these maximum

Table 1. Yield and viability of protoplasts, and number of viable protoplasts isolated from hypocotyl tissues of *H. annuus* L. with various factors

Genotypes	Isolation methods	Cellulase concentrations (%)	Yield ($\times 10^6$ protoplasts/ g FW)	Viability (%)	No. of viable protoplasts ($\times 10^6$ protoplasts/ g FW)
10A	M1	0.5	2.34 ± 0.60[a] cde	92.66 ± 0.65 a	2.17 ± 0.09 cde
		1.0	2.88 ± 0.09 cde	92.70 ± 0.57 a	2.67 ± 0.09 bcd
		1.5	3.19 ± 0.31 bc	91.21 ± 1.10 a	2.90 ± 0.26 bc
	M2	0.5	3.70 ± 0.31 ab	91.76 ± 0.39 a	3.40 ± 0.29 ab
		1.0	4.67 ± 0.30 a	90.86 ± 0.92 a	4.24 ± 0.26 a
		1.5	3.80 ± 0.77 ab	91.84 ± 0.73 a	3.48 ± 0.70 ab
PI 441983	M1	0.5	1.63 ± 0.27 de	91.78 ± 0.77 a	1.52 ± 0.33 de
		1.0	2.15 ± 0.32 cde	91.02 ± 0.95 a	1.96 ± 0.30 cde
		1.5	2.07 ± 0.37 cde	90.52 ± 1.94 a	1.88 ± 0.34 cde
	M2	0.5	1.94 ± 0.56 cde	87.42 ± 0.51 b	1.70 ± 0.50 de
		1.0	1.80 ± 0.60 de	86.34 ± 1.38 b	1.53 ± 0.48 de
		1.5	1.38 ± 0.25 e	86.26 ± 0.95 b	1.19 ± 0.23 e

[a] Data are presented as means ± SE. Data not followed by the same letter in a column are significantly different ($P < 0.05$).

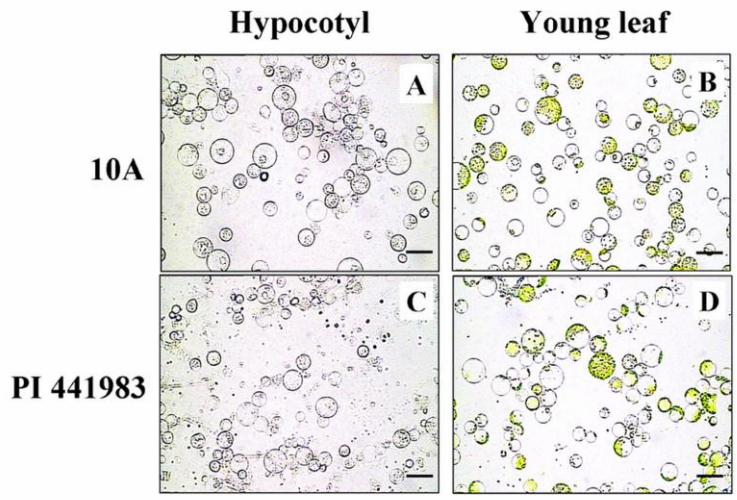

Fig 1. Isolated protoplasts from hypocotyl (A, C) and young leaf (B, D) tissues of 10A (A, B) and PI 441983 (C, D). Bar = 50 μm

numbers are not significantly different from those obtained from other combinations of M3 and M4 with various cellulase concentrations (Table 2).

Discussion

Tissue types, genotypes, isolation methods and cellulase concentrations affected both yields and viability of protoplasts which finally determined the number of viable protoplasts, a criterion useful for choosing the most favorable isolation procedures. Genotypes and types of tissues are often considered as important plant material factors that govern the release of viable protoplasts. The differential responses in yields and viability may result from the differences in the extent of cell wall thickening and the physiological status of plant materials (Davey et al., 2005; Raikar et al., 2008; Lord and Gunawardena, 2010). In our study, on average, leaf tissues allowed a higher release of protoplast yields than hypocotyls which is in agreement with previous research (Boonrumpun et al., 1997; Taski-Ajdukovic et al., 2006). Genotypes also played a critical role in the protoplast releasing ability. 10A could release 1.9-fold higher number of viable protoplasts than PI 441983 when hypocotyl tissues were used. Conversely, PI 441983 gave 1.6-fold higher number of viable protoplasts than 10A when young leaves were used. There are several reasons for this occurrence.

Firstly, genotypic characteristics of most etiolated hypocotyl tissues of PI 441983 were shorter and hyperhydrated, resulting in lower numbers of initial cells (per 1 g tissue) than 10A. Moreover, the protoplasts isolated from this genotype lost their viability more easily. Secondly, the thickness of young leaf tissues of 10A was less than that of PI 441983, which may make them more prone to damage occurring during the isolation process (Lord and Gunawardena, 2010). These results confirm the previous findings that plant genotypes and types of tissues influenced the efficiency of protoplast isolation in various plants (Guangyu et al., 1997; Chabane et al., 2007; Raikar et al., 2008; Badr-Elden et al., 2010). Moreover, age as well as environmental and seasonal conditions of plant materials also affected the success of protoplast isolation (Lenee and Chupeau, 1986; Krasnyanski et al., 1992; Henn et al., 1998b; Davey et al., 2005; Pongchawee et al., 2006; Raikar et al., 2008). Several factors associated with the enzymatic digestion of plant cell walls, including a mixture of cellulase, hemicellulase and pectinase enzymes, osmotic potential and pH of the isolation solution, temperature, plasmolysis and duration of enzyme incubation, have previously been examined (Lenee and Chupeau, 1986; Krasnyanski et al., 1992; Sinha et al., 2003; Davey et al., 2005; Badr-Elden et al., 2010). In this study, selected levels of cellulase concentrations and isolation methods (pectinase concentrations, incubation time, osmoticum and pH of the

Table 2. Yield and viability of protoplasts, and number of viable protoplasts isolated from young leaf tissues of *H. annuus* L. with various factors.

Genotypes	Isolation methods	Cellulase concentrations (%)	Yield ($\times 10^6$ protoplasts/ g FW)	Viability (%)	No. of viable protoplasts ($\times 10^6$ protoplasts/ g FW)
10A	M3	0.1	6.94 ± 0.75[a] bcd	48.10 ± 16.50 abc	2.86 ± 0.75 bcde
		0.5	7.03 ± 1.18 bcd	44.22 ± 17.17 abc	2.63 ± 0.84 bcde
		1.0	4.90 ± 0.52 cde	52.15 ± 13.17 abc	2.40 ± 0.32 bcde
		1.5	3.83 ± 0.78 def	38.62 ± 25.28 bc	1.31 ± 0.54 cde
	M4	0.1	6.30 ± 1.99 bcd	86.15 ± 3.19 ab	6.13 ± 2.09 ab
		0.5	4.51 ± 1.14 def	86.23 ± 4.24 ab	4.16 ± 1.09 bcde
		1.0	3.49 ± 1.36 def	80.60 ± 6.84 abc	3.51 ± 1.64 bcde
		1.5	1.01 ± 0.13 ef	65.05 ± 18.28 abc	0.50 ± 0.05 e
	M5	0.1	0.22 ± 0.11 f	68.48 ± 15.93 abc	0.20 ± 0.13 e
		0.5	0.46 ± 0.27 f	63.72 ± 13.86 abc	0.46 ± 0.31 e
		1.0	0.22 ± 0.01 f	NA	NA
		1.5	0.18 ± 0.06 f	58.87 ± 19.34 abc	0.12 ± 0.09 e
PI 441983	M3	0.1	9.01 ± 2.04 abc	60.67 ± 10.28 abc	5.88 ± 1.13 abc
		0.5	12.03 ± 1.47 a	48.83 ± 38.72 abc	6.54 ± 5.28 ab
		1.0	1.74 ± 0.29 ef	NA	NA
		1.5	1.86 ± 1.43 ef	NA	NA
	M4	0.1	7.22 ± 1.58 bcd	79.95 ± 6.37 abc	5.52 ± 1.07 abcd
		0.5	9.48 ± 1.84 ab	82.53 ± 1.59 abc	8.81 ± 1.25 a
		1.0	6.37 ± 1.26 bcd	79.38 ± 4.21 abc	5.98 ± 0.16 abc
		1.5	4.52 ± 1.19 def	63.74 ± 13.75 abc	3.89 ± 1.67 bcde
	M5	0.1	NA	NA	NA
		0.5	0.77 ± 0.33 ef	90.44 ± 0.92 a	1.00 ± 0.41 de
		1.0	0.72 ± 0.13 ef	90.62 ± 2.78 a	0.71 ± 0.11 e
		1.5	1.34 ± 0.43 ef	81.68 ± 3.63 abc	1.09 ± 0.34 de

[a] Data are presented as means ± SE. Data not followed by the same letter in a column are significantly different ($P < 0.05$).
NA, data not available due to low numbers of protoplasts obtained.

isolation solution) reported to be highly efficient for sunflower protoplast isolation were evaluated. For hypocotyl protoplast isolation, both selected isolation methods (M1 and M2) were able to release sufficient numbers of viable protoplasts. On average, protoplast yield isolated by M2 was significantly higher than M1, but the difference was only 1.2-fold. However, an interaction between genotypes and isolation methods was found, and the most favorable isolation methods for the two genotypes differed, M2 for 10A and M1 for PI 441983. The pectinase concentrations also appeared to be crucial for leaf mesophyll protoplast isolation. When protoplasts were isolated from young leaf tissues, M5 released the lowest protoplast yield from both 10A and PI 441983. It should be noted that M5 used the lowest concentration of pectinase (0.05% pectolyase) and was given less incubation time (6 h) compared to those of M3 (16 h) and M4 (16 h), which may not have been sufficient to release a large number of protoplasts. However, the protoplasts derived from M5 were more viable than those from M3. When M3 and M4 (with equal incubation time) were compared, the average protoplast yields of both methods were not significantly different even if M3 contained ca. 40-fold higher pectinase concentration than M4, suggesting that the levels of pectinase in M3 might be excessive. The lowest viability of protoplasts obtained by this method (M3) further implicated the toxicity of excessive pectinase which is known to be the cause of cytoplasm acidification, membrane depolarization, oxidative burst and K^+ leakage (Raikar et al., 2008). Furthermore, the level of cellulase concentrations was considered as an important factor influencing the release of leaf protoplasts. High levels of cellulase concentrations decreased protoplast yield and tended to decrease the viability of protoplasts, possibly from the influence of cellulase on the integrity of membrane and the reduction of physical activities of protoplasts (Zhu et al., 2005; Raikar et al., 2008). Therefore, when high levels of both cellulase and pectinase enzymes were used, shorter incubation time should be considered to minimize the damage of protoplasts from toxicity of enzymes (Uchimiya and Murashige, 1974; Zhu et al., 2005; Pongchawee et al., 2006; Ling et al., 2010). The optimization of various factors in this study has led to improved protoplast yields compared to previous results on *Helianthus* species. It should be noted that with the most appropriate isolation procedure, the maximum leaf mesophyll protoplast yield achieved in our study (12.03×10^6 protoplasts/ g FW) was substantially higher than those previously reported in *H. maximiliani* ($1.5\text{-}5 \times 10^6$ protoplasts/ g FW), *H. giganteus* (3×10^6 protoplasts/ g FW) and *H. nuttallii* (2×10^6 protoplasts/ g FW [Henn et al., 1998b; Dragana et al., 2001; Taski-Ajdukovic et al., 2006]). Similarly, we were able to obtain the highest protoplast yield from hypocotyls (4.67×10^6 protoplasts/ g FW) at a higher level than those previously reported in other cultivated varieties ($4\text{-}5 \times 10^5$ to $2\text{-}3 \times 10^6$ protoplasts/ g FW [Krasnyanski and Menczel, 1993; Taski-Ajdukovic et al., 2006]). The regenerability of protoplasts isolated from these optimized procedures is currently being investigated. In summary, optimized sunflower protoplast isolation procedures that give both high yields and viability, resulting in maximum numbers of viable protoplasts for each genotype and tissue type were obtained from this study. M2 with 1.0% cellulase was the most favorable for protoplast isolation from hypocotyl tissues of 10A, whereas M1 with 1.0% cellulase released the highest number of viable protoplasts from PI 441983 hypocotyls. For young leaf tissues, where no interaction between factors was found, M4 with 0.1 and 0.5% cellulase were the most favorable isolation procedures for 10A and PI 441983, respectively. These results suggest that the most suitable

Table 3. Various factors (genotypes, isolation methods and levels of cellulase concentrations) used for protoplast isolation from hypocotyl and young leaf tissues of *H. annuus* L.

Tissue sources	Isolation methods	Enzymes and proteins	Isolation solutions	pH	Temperature (°C)	Incubation periods (h)	References
Hypocotyl	M1	1% macerozyme[a], 1% BSA, and 0.5, 1.0 or 1.5% cellulase[b]	336 mM KCl, 16 mM $CaCl_2$ and 3 mM MES	5.7	25	4	Binsfeld et al. (2000)
	M2	0.5% macerozyme, and 0.5, 1.0 or 1.5% cellulase	308 mM NaCl, 5.37 mM KCl, 41.7 mM $CaCl_2.2H_2O$ and 3.3 mM MES	5.6	25	16	Krasnyanski and Menczel (1995)
Young leaf	M3	0.05% pectolyase[c], 0.75% macerozyme, 0.005% driselase[d], 1% BSA, and 0.1, 0.5, 1.0 or 1.5% cellulase	340 mM KCl, 1.4 mM $CaCl_2.2H_2O$ and 3 mM MES	5.6	25	16	Henn et al. (1998b)
	M4	0.05% driselase, 0.02% macerozyme, 0.1% BSA, and 0.1, 0.5, 1.0 or 1.5% cellulase	336 mM KCl, 13.6 mM $CaCl_2$ and 3.59 mM MES	5.7	25	16	Keller et al. (1997)
	M5	0.05% pectolyase, and 0.1, 0.5, 1.0 or 1.5% cellulase	0.5 M mannitol and 20 mM MES	5.6	25	6	Özdemir et al. (2002)

[a] Macerozyme R-10 (from *Rhizopus* sp.), Kinki Yakult MFG, Japan, catalogue no. 202047
[b] Cellulase Onozuka R-10 (from *Trichoderma viride*), Yakult Honsha, Japan, catalogue no. 216012
[c] Pectolyase (from *Aspergillus japonicus*), Sigma-Aldrich, Germany, catalogue no. P5936
[d] Driselase (from *Basidiomycetes* sp.), Sigma-Aldrich, Germany, catalogue no. D8037

isolation methods, cellulase concentrations and tissue types vary according to sunflower genotypes and need to be determined individually. These isolation procedures will be further used for future protoplast isolation to transfer the fertile/sterile cytoplasmic trait for the production of sunflower F_1 hybrids via protoplast fusion.

Materials and methods

Plant materials

A sunflower genotype with fertile cytoplasm, PI 441983, from the North Central Regional Plant Introduction Station, Iowa, USA and a cytoplasmic male sterile line, 10A, developed in Thailand for production of hybrids with high oil content, were used. Seven-day-old etiolated hypocotyls and 4-week-old fully expanded young leaves of *in vitro* germinated seedlings were used for protoplast isolation.

Protoplast isolation and purification

One gram of cut etiolated hypocotyls and leaf pieces were incubated in 15 and 25 ml of enzyme solutions, and shaken at 70 and 40 rpm, respectively, in the dark (Krasnyanski et al., 1992; Krasnyanski and Menczel, 1993). Two isolation methods (M1 and M2) and three cellulase concentrations (0.5, 1.0 and 1.5%) were evaluated for hypocotyl tissues, while in leaf tissues, three isolation methods (M3, M4 and M5) and four cellulase concentrations (0.1, 0.5, 1.0 and 1.5%) were used (Table 3). Digested materials were filtrated through nylon filters with 82, 62 and 40 µm pore diameters to remove undigested cell mass and debris. The filtrates were centrifuged for 5 min at 1,000 rpm, and the protoplast pellets were washed twice in the isolation solutions. The protoplasts were purified by floatation in the sucrose gradient using sucrose solution (0.5 M sucrose, 14 mM $CaCl_2.2H_2O$, 3 mM MES, pH 5.6 [Henn et al., 1998b]), washed and resuspended with the isolation solutions. Yields and viability of protoplasts were determined by using a haemacytometer and fluorescein diacetate (FDA) staining (Henn et al., 1998b), respectively. The number of viable protoplasts was calculated following the formula: Number of viable protoplasts = Yield × Viability/100. The data were statistically analyzed by the analysis of variance (ANOVA) using randomized complete block design (RCBD) with 5 replications, and the means were compared by Duncan's new multiple range test (DMRT). All statistical analyses were performed using SPSS version 14.0 (Levesque and SPSS Inc., 2006).

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