Pak. J. Bot., 46(2): 673-677, 2014.

INFLUENCES OF EXPLANT TYPE AND ENZYME INCUBATION ON ISOLATED PROTOPLAST DENSITY AND VIABILITY IN TWO GARLIC CULTIVARS

E.I. METWALLY[1], M.E. EL-DENARY[2] AND Y.H. DEWIR[1*]

[1]*Horticulture Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt*
[2]*Horticulture Research Institute, Giza, Egypt*
[*]*Corresponding author e-mail: ydewir@hotmail.com*

Abstract

The present study reports on optimizing protoplast isolation and fusion in two garlic cultivars 'Balady' and 'Seds 40'. Protoplast density and viability were investigated in four different explants (etiolated and green parts of the pseudostem and lower and upper parts of the leaves) under enzyme incubation for 1, 2, 3 and 4 h. Among different explants, used for protoplast isolation in Balady cultivar, the upper and lower parts of the leaves produced the highest number of total protoplasts (70 and 66 pps/0.1 ml) at 4 and 3 h enzyme incubation, respectively. However, the etiolated part of pseudostem produced the highest number of viable protoplast in which 52.5 pps/0.1 ml were obtained at 3 h enzyme incubation. For protoplast isolation in Seds 40 cultivar, the highest number of total protoplasts (125 and 107.5 pps/0.1 ml) as well as viable protoplasts (105 and 107.5 pps/0.1 ml) was obtained from the etiolated and the green parts of pseudostem, respectively. The cultivar 'Seds 40' yielded higher total and viable protoplasts than Balady cultivar. Isolated protoplasts of 'Seds 40' and 'Balady' were fused successfully at a protoplast density of 1×10^5 using either physical and/or electrical method. Optimization of the source of plant material as well as protoplast isolation conditions for garlic is a crucial step towards a successful protoplast fusion and subsequent colony formation.

Introduction

Garlic (*Allium sativum* L., Liliaceae) is an important and widely cultivated crop which is known for its culinary and medicinal use. Garlic has been cultivated vegetatively because of its sexual sterility. Vegetative Propagation of garlic is achieved through division of the ground bulbs and/or aerial bulbs. Therefore, the multiplication rate is fairly low. Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and they suffer from insect pests (Davies, 1994; Verbeek *et al*., 1995). Improvement of garlic through breeding programs is limited due to difficulties of inducing flowering and the absence of sexual reproduction which impoverishes garlic's genetic variability (Fellner, 1995; Barandiaran *et al*., 1999). Garlic regeneration from protoplasts has been considered as a prerequisite step to overcome the problems involved in sexual reproduction. Plants of Allium genus are fastidious for culture *In vitro*. Intensive studies on the protoplast culture of the genus Allium have been reported (Wang *et al*., 1986; Fellner & Havranek, 1992; Buiteveld & Creemers-Molenaar, 1994; Schum *et al*., 1994; Ayabe *et al*., 1995; Karim & Adachi, 1997; Hasegawa *et al*., 2002; Song *et al*., 2009). However, for garlic only a few reports have been successful (Ayabe *et al*., 1995; Hasegawa *et al*., 2002).

Tissue physiology influences the release of viable protoplasts, necessitating strict attention to source material (Davey *et al*., 2005). The starting plant materials for the isolation of protoplast, isolation procedure and protoplast density are important factors influencing colony formation. Therefore, the objective of the present study was to determine the suitable explants and the duration of enzyme incubation for protoplast isolation and fusion in two garlic cultivars 'Balady' and 'Seds 40'.

Materials and Methods

Plant material: Cloves of two garlic cultivars (Balady and Seds 40-Chinese type) were surface sterilized in a gyratory shaker at 100 rpm using 70% (*v/v*) ethanol for 30 sec. followed by 50% (*v/v*) commercial bleach clorox (5.25% sodium hypochlorite) with a few drops of Tween 20 as a wetting agent for 20 min. The sterilized cloves were rinsed five times with sterile distilled water and cultured onto 375 ml jars containing half strength basal salts of MS (Murashige & Skoog, 1962) medium. The cultures were incubated in a growth chamber at 25 ± 1°C with a 16 h photoperiod at 40 µ mol m^{-2} s^{-1}. Regenerated Garlic plants at 3-4 leaves stage were incubated in the dark for 24 h at 26°C before protoplast isolation.

Enzyme preparation, cell wall digestion and protoplast isolation: The enzyme solution was prepared by dissolving 1% (*w/v*) Onozuka RS (Yakult Honsha Co., Tokyo, Japan) and 0.1% (*w/v*) pectolyase Y23 (Seishin Pharm. Co., Tokyo, Japan) in the pre-enzyme solution (0.6 M mannitol solution and l0 mM $CaCl_2$). The pH of the solution was adjusted to 5.8 and sterilized by filtration through 0.45 µM pore size filter. One gram of each explant was cut into small pieces and treated with ten ml of the enzyme solution in small flasks. The flasks were incubated at 28°C on a gyratory shaker at 60 rpm under dark conditions for 1, 2, 3 and 4 h. After digestion, the obtained protoplasts were filtered through 40 µm nylon mesh and centrifuged at 800 rpm for 5 min. Then, the precipitant pellet was washed thrice with washing solution, containing 0.6 M mannitol and l0 mM $CaC1_2$. Then, they were purified by 20% sucrose using l000 rpm centrifugation for l0 min.

Estimation of protoplasts density and viability: The protoplasts density (number of total protoplasts per 0.1 ml) was estimated for each treatment using a double chamber haemocytometer (Barakat, 1999). The protoplast viability in terms of viability percentage and the number of viable protoplasts per 0.1 ml were determined using a fluorescein diacetate (Wildholm, 1972; Barakat, 1999) and a fluorescent research microscope (Reichert Microstar IV).

Protoplast fusion: The isolated protoplasts from the best treatment of each cultivar were used for protoplast fusion. Physical and electrical methods were used for the fusion. For physical fusion, 2 ml of each protoplast cultivar with a density of 1×10^5 protoplast/ml were mixed and centrifuged at 1000 rpm for 10 min. (Barakat, 1999). Electrical fusion was conducted using Jasco CET-200 Continuous Electro Manipulator according to Ayabe *et al*., 1995 and Barakat, 1999 in a two-step process:

a. Di-electrophoresis: The membranes of neighboring protoplasts were brought into contact with an AC field. Protoplasts formed transient dipoles with one side of the cell surface negatively charged relative to the other side. The induced charges on the cell surface caused the cells to be drawn to regions of higher electric field strength. As the protoplasts approached the electrodes, they tended to form chains of protoplasts because the electrical conductivity of the cells is greater than the conductivity of the medium leading to locally higher electric fields at the cell poles. This pearl chain formation brings the plasma membranes of neighboring cells into close contact. The optimal conditions for the induction of fusion in the first step were as follows: AC fields, 1.0 MHz, 125 V/cm, 60 sec.

b. Cell fusion: At the points of contact between protoplasts, the plasma membranes were broken down electrically with a short DC pulse. Membrane reorganization, following the DC (square-wave) pulse, resulted in cell fusion. The optimal conditions for the induction of fusion in the second step were as follows: DC fields, 1,250 V/cm square-wave 50 µsec. in duration, 5 times at 1.0 sec intervals.1.0 MHz, 125 V/cm, 60 sec.

Experimental design and data analysis: The experiment included 32 treatments which were combined of three factors: two garlic cultivars (Balady cv. and Seds 40), four different explants (the etiolated part of pseudostem, the green part of pseudostem, the lower part of leaves and the upper part of leaves) and four enzyme exposure at 1, 2, 3 and 4 h. Data were subjected to Duncan's multiple range test using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

Results and Discussion

Number of total protoplasts and viable protoplasts: The number of total protoplasts and viable protoplasts have been greatly influenced by enzyme incubation as well as a source of plant material in terms of explants type and cultivar. Among different explants used for protoplast isolation in Balady cultivar, the upper and lower parts of the leaves produced the highest number of total protoplasts (70 and 66) at 4 and 3 h enzyme incubation, respectively (Fig. 1A). However, the etiolated part of pseudostem produced the highest number of viable protoplast in which 52.5 protoplasts were obtained at 3 h enzyme incubation. The lowest number of viable protoplasts was obtained from the upper part of the leaves (Fig. 1B). For protoplast isolation in Seds 40 cultivar, the highest number of total protoplasts (125 and 107.5) as well as viable protoplasts (105 and 107.5) was obtained from the etiolated and the green parts of pseudostem, respectively. Leaf explants produced the lowest number of total as well as viable protoplasts (Fig. 1C, D). In general, Seds 40 cultivar produced higher number of total protoplast and viable protoplasts than that of Balady cultivar. The duration of enzyme incubation has been reported to stimulate protoplast release (Sinha *et al*., 2003). In the present study, the number of total protoplasts and viable protoplasts was increased with increasing enzyme incubation up to 3 h and then decreased at 4 h incubation (Fig. 1).

Previous reports have indicated that protoplast viability is influenced by source of plant material. For garlic, protoplasts have been isolated from tissue cultured shoot primordia (Ayabe *et al*., 1995), long term shoot primordia-derived calluses (Hasegawa *et al*., 2002) and callus derived from the bulbils (Song *et al*., 2009). It has been reported that cotyledons from *In vitro*-grown seedlings of white lupin gave higher yields compared to leaves, hypocotyls and roots (Sinha *et al*., 2003). Embryogenic cell suspensions have been the preferred source of viable protoplasts in cereals, particularly rice (Tang *et al*., 2001), rye (Ma *et al*., 2003), and banana (Assani *et al*., 2002). Mesophyll cells were used as a source of protoplasts in sugarcane (Aftab *et al*., 2002). Guard cells are a unique source of totipotent protoplasts in sugarbeet (Hall *et al*., 1996) and *A. thaliana* (Pandey *et al*., 2002).

Percentage of protoplast viability: Enzyme incubation influenced the percentages of protoplast viability (Table 1). For Seds 40 cultivar, enzyme incubation from 1 to 3 h produced 100% protoplast viability from all different explants used. However, 4 h enzyme incubation significantly decreased the percentages of protoplast viability. In Balady cultivar, enzyme incubation from 1 to 3 h produced the highest percentages of protoplast viability from etiolated parts of the pseudostem. Also, incubation of the upper part of the leaves for 1 h resulted in a 100% protoplast viability. The optimal duration of enzyme incubation required for isolation of garlic protoplasts has been reported to vary from 4 to 6 h depending on explants type and cultivar used. Enzyme incubation for 4 and 5 h was optimal for garlic cultivars 'Jejujaerae' and 'Namdo', respectively (Song *et al*., 2009). Garlic protoplasts have been isolated from leaf primordia after 5 - 6 h enzyme incubation (Ayabe *et al*., 1995), callus derived from the pedicel and leaf tissue after 5 and 4 h, respectively (Suh & Park, 1995). Also, 4 - 5 h incubation was optimal for protoplast isolation from long term shoot primordia-derived calluses (Hasegawa *et al*., 2002). Protoplast isolation is a stress-inducing procedure (Papadakis & Roubelakis-Angelakis, 2002), particularly during enzymatic isolation, leading to the accumulation of peroxides and other reactive oxygen species. Additionally, Commun *et al*., (2003) reported the production of stilbene phytoalexins in protoplasts of *Vitis spp*., accompanied by a decrease in viability. Other workers confirmed that leaf protoplasts of *Brassica napus* and *Petunia hybrida* experience stress during isolation (Watanabe *et al*., 2002). The effects of stress during isolation may be long-term and may be associated with the subsequent recalcitrance of protoplasts during culture (Davey *et al*., 2005).

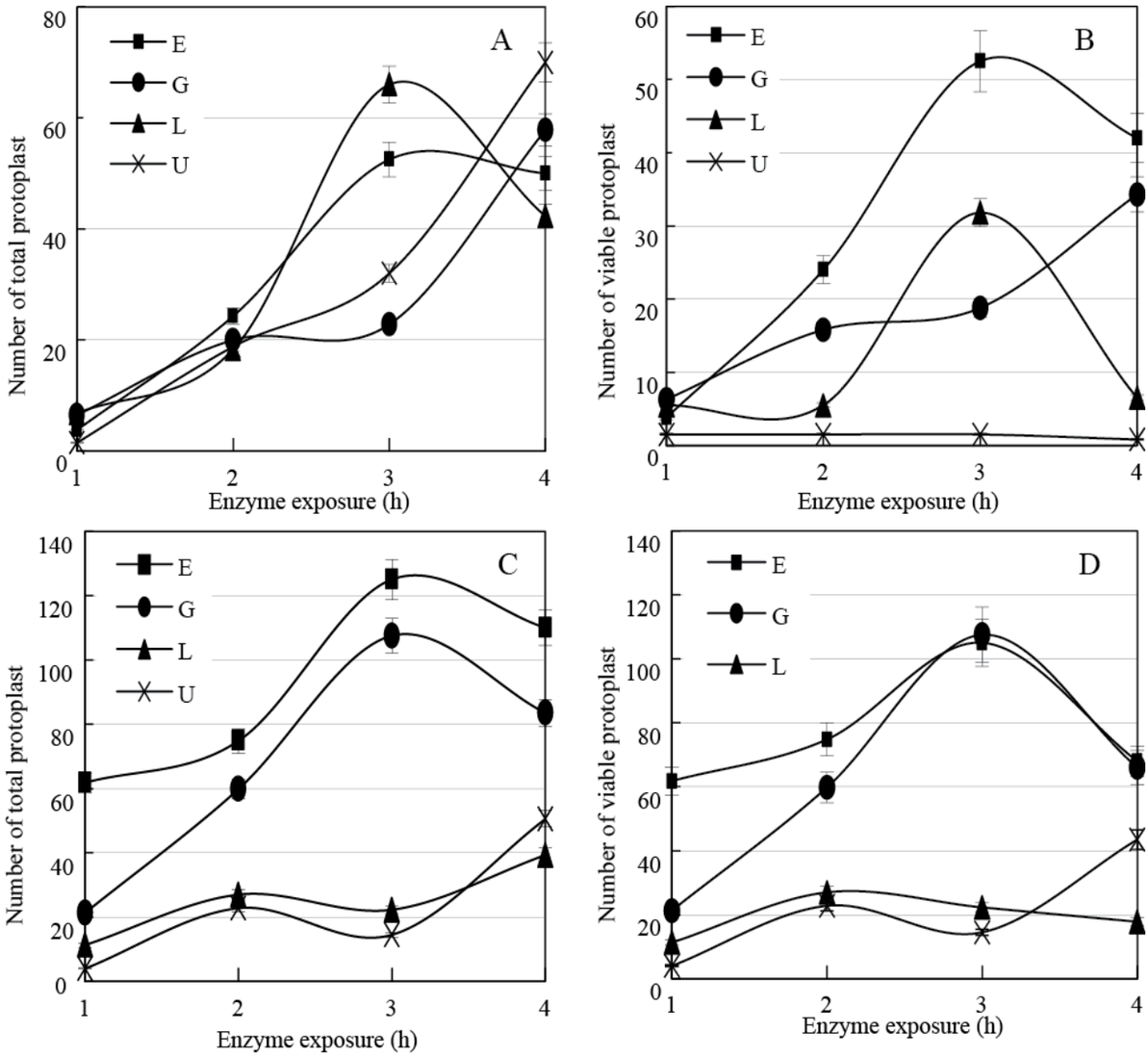

Fig. 1. Number of total protoplasts and number of viable protoplasts as affected by enzyme incubation and explant type in *Allium sativum* Balady cv. (A, B) and Seds 40 cv. (C, D). Legend abbreviations (E = etiolated part of the pseudostem; G = green part of the pseudostem; L = lower part of the leaves; U = upper part of the leaves).

Table 1. Effects of explant type and enzyme incubation on percentage of protoplast viability in Balady and Seds 40 garlic cultivars.

Enzyme incubation (h)	Etiolated part of the pseudostem	Green part of the pseudostem	Lower part of the leaves	Upper part of the leaves
		Balady cv.		
1	100.0 a[Z]	96.9 ab	83.5 c	100.0 a
2	99.1 a	80.1 c	30.4 g	9.4 hi
3	100.0 a	82.2 c	48.1 ef	4.4 i
4	84.4 c	59.0 de	15.9 h	1.1 i
		Seds 40 cv.		
1	100.0 a	100.0 a	100.0 a	100.0 a
2	100.0 a	100.0 a	100.0 a	100.0 a
3	83.9 c	100.0 a	100.0 a	100.0 a
4	62.5 d	78.7 c	45.0 f	85.9 bc

[Z] Mean separation within each column by Duncan's multiple range test at 5% level

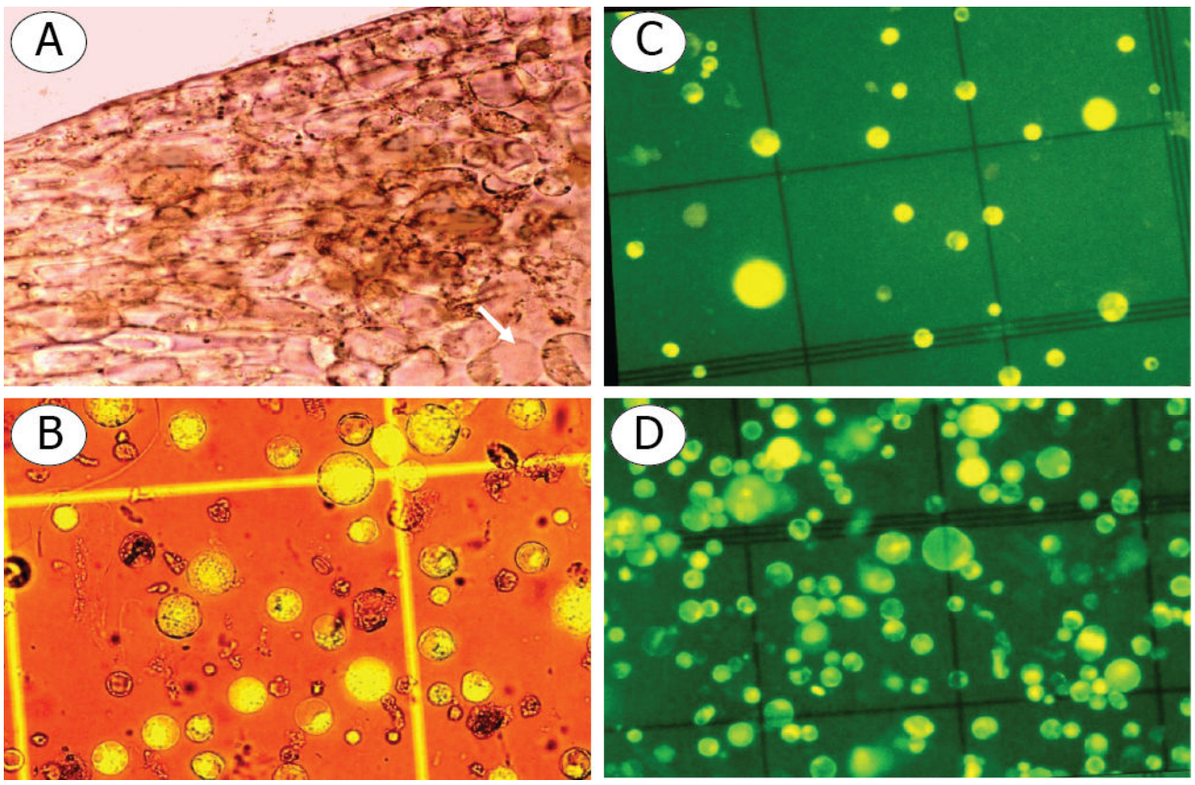

Fig. 2. Protoplast isolation in garlic A) Releasing of protoplasts from leaf tissue using the enzymatic solution B) Low viability with low density of protoplasts during protoplast isolation C) High viability with low density of protoplasts during protoplast isolation D) High viability with high density of protoplasts during protoplast isolation.

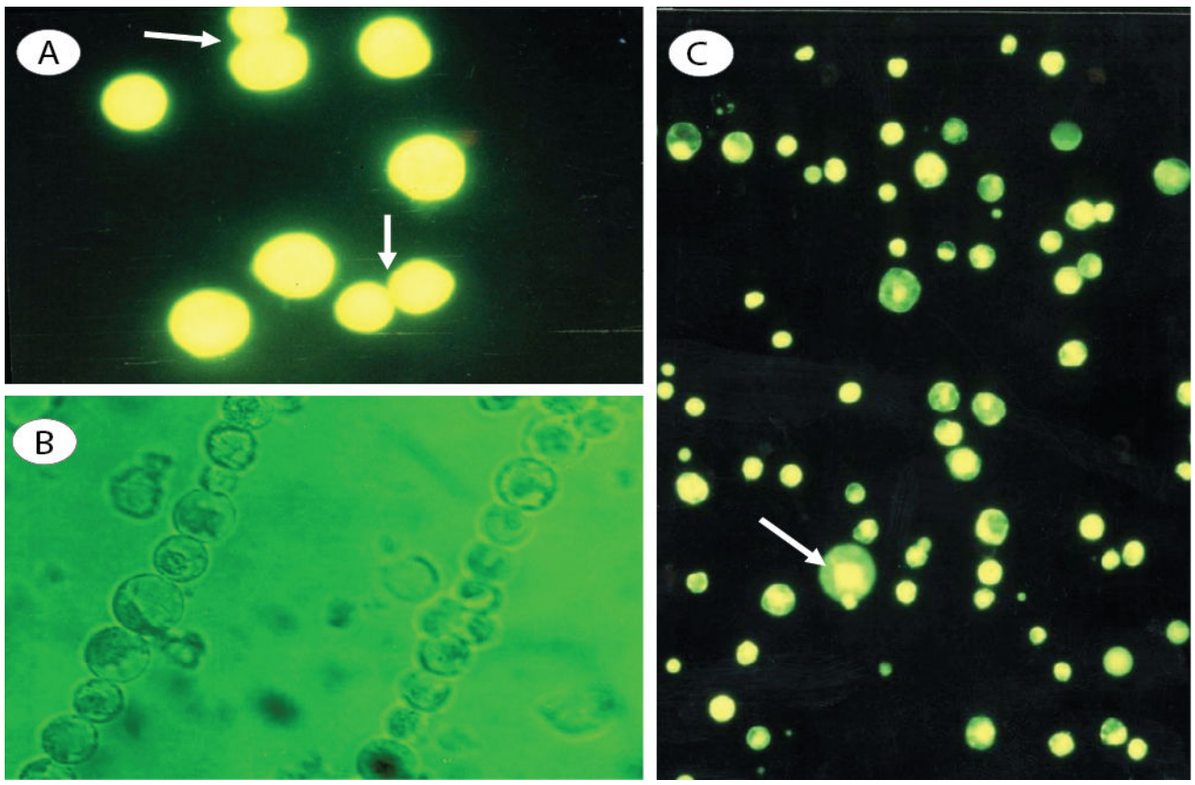

Fig. 3. Protoplast fusion in garlic A) start of a protoplast fusion B) Chain formation and protoplasts fusion during DC stage of the electric fusion C) Hybrid and single protoplasts.

Protoplast density and protoplast fusion: The density of protoplasts in the culture medium is crucial for their sustained mitotic division. Consequently, protoplasts fail to grow when cultured below a minimum density. In general, the optimum plating density is 5×10^2 to 1×10^6 protoplasts per ml, depending on the species. In the present study, a protoplast density of 1×10^5 protoplast per ml was optimal for the protoplast fusion of 'Balady' and 'Seds 40' cultivars. Protoplast isolation, with different density and viability, is shown in Fig. 2. However for garlic, the optimal protoplast density depends on the source of plant material and cultivars. A density of 1×10^4 protoplasts per ml was used for protoplasts derived from tissue-cultured shoot primordia (Ayabe *et al*., 1995) and protoplasts derived calluses of garlic cv. 'Howaito roppen' (Hasegawa *et al*., 2002); 1.8×10^4 and 1.1×10^4 for protoplasts isolated from callus derived from the bulbils of garlic cultivars 'Jejujaerae' and 'Namdo', respectively (Song *et al*., 2009). The isolated protoplasts from the two cultivars 'Balady' and 'Seds 40' were fused successfully through physical and/or electrical methods (Fig. 3).

Conclusion

In the present study, it has been found that etiolated and/or green parts of the pseudostem rather than the upper or lower parts of the leaves produced the highest viable protoplasts under enzyme incubation for 3 h. The cultivar 'Seds 40' yielded higher total and viable protoplasts than Balady cultivar. Isolated protoplasts of 'Seds 40' and 'Balady' were fused successfully at a protoplast density of 1×10^5 using either physical or electrical methods. Optimization of the source of plant material, as well as protoplast isolation conditions for garlic, is a crucial step towards a successful protoplast fusion and subsequent colony formation.

References

Aftab, F., Y. Zafar and J. Iqbal. 2002. Optimization of conditions for electrofusion in sugarcane protoplasts. *Pak. J. Bot.,* 34: 297-301.

Assani, A., R. Haicour, G. Wenzel, B. Foroughi-Wehr, F. Bakry, F.X. Cote, G. Ducreux, A. Ambroise and A. Grapin. 2002. Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (*Musa* spp.). *Plant Sci.,* 162: 355-362.

Ayabe, M., K. Taniguchi and S. Sumi. 1995. Regeneration of whole plants from protoplasts isolated from tissue-cultured shoot primordia of garlic (*Allium sativum* L.). *Plant Cell Rep.*, 15: 17-21.

Barakat, M.N. 1999. Protoplast fusion, somaclonal and gametoclonal variation technology. The Central Lab. for Agric. Biotech. and Molecular Biology, A course on biotechnology and genetic engineering in industrial fungi and higher plants, Fac. Agric., Alex. Univ., Egypt, pp. 37-67.

Barandiaran, X., N. Martin, M. Rodrfguez, A. Di Pietro and J. Martin. 1999. Genetic variability in the callogenesis and regeneration of garlic. *Plant Cell Rep.*, 18: 434-437.

Buiteveld, J. and J. Creemers-Molenaar. 1994. Plant regeneration from protoplasts isolated from suspension cultures of leek (*Allium ampeloprasum* L.). *Plant Sci.*, 100: 203-210.

Commun, K., M.C. Mauro, Y. Chupeau, M. Boulay, M. Burrus and P. Jeandet. 2003. Phytoalexin production in grapevine protoplasts during isolation and culture. *Plant Physiol. Biochem.,* 41: 317-323.

Davey, M.R., P. Anthony, J.B. Power and K. Lowe. 2005. SIVB congress symposium proceedings "thinking outside the cell": Plant protoplast technology: status and applications. *In vitro Cell. Dev. Biol.-Plant*, 41: 202-212.

Davies, J.L.M. 1994. Chemical control of Allium white rot: a review. In: *Proceedings of the Fifth International Workshop of Allium white rot. Cordoba, Spain*: (Eds.): A.R. Entwistle and J.M. Melero-Vara. Instituto de Agricultura Sostenible, pp. 7-8.

Fellner, M. 1995. Influence of antibiotic ciprofloxacin on culture of *Allium longicuspis* callus-derived protoplasts. *Ann. Bot.,* 76: 219-223.

Fellner, M. and P. Havranek. 1992. Isolation of *Allium* pollen protoplasts. *Plant Cell Tiss. Org. Cult.*, 29: 275-279.

Hall, R.D., T. Riksen-Bruinsma, G.J. Weyens, I.J. Rosquin, P.N. Denys, I.J. Evans, J.E. Lathouwers, M.P. Lefebvre, J.M. Dunwell, A. van Tunen and F.A. Krens. 1996. A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. *Nature Biotechnol.,* 14: 1133-1138.

Hasegawa, H., M. Sato and M. Suzuki. 2002. Efficient plant regeneration from protoplasts isolated from long term, shoot primordia derived calluses of garlic (*Allium sativum*). *J. Plant Physiol.*, 159: 449-452.

Karim, M.A. and T. Adachi. 1997. Cell suspension, isolation and culture of protoplasts of *Allium cepa*. *Plant Cell, Tiss. Org. Cult.*, 51: 43-47.

Ma, R., Y.D. Guo and S. Pulli. 2003. Somatic embryogenesis and fertile green plant regeneration from suspension cell-derived protoplasts of rye (*Secale cereale* L.). *Plant Cell Rep.*, 22: 320-327.

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

Pandey, S., X.Q. Wang, S.A. Coursol and S.M. Assmann. 2002. Preparation and applications of Arabidopsis thaliana guard cell protoplasts. *New Phytol.,* 153: 517-526.

Papadakis, A.K. and K.A. Roubelakis-Angelakis. 2002. Oxidative stress could be responsible for the recalcitrance of plant protoplasts. *Plant Physiol. Biochem.,* 40: 549-559.

Schum, A., H. Junge and L. Mattiesch. 1994. Plant regeneration from protoplasts of *Allium porrum* L. *Gartenbauwissenschafr,* 59: 26-30.

Sinha, A., A.C. Wetten and P.D.S. Caligari. 2003. Optimisation of protoplast production in white lupin. *Biol. Plant.,* 47: 21-5.

Song, S.Y., C.H. Kim, K.C. Seong, K.H. Kang and I.J. Kim. 2009. Protoplast isolation, fusion and colony formation from two garlic (*Allium sativum* L.) cultivars, 'Namdo' and 'Jejujaerae'. *Kor. J. Hort. Sci. Technol.*, 27: 470-475.

Suh, S.K. and H.G. Park. 1995. Protoplast isolation, fusion, and culture in garlic (*Allium sativum* L.). *J. Kor. Soc. Hort. Sci.,* 36: 614-619.

Tang, T., X. Sun, D. An, J.B. Power, E.C. Cocking and M.R.A. Davey. 2001. Simple and rapid procedure to establish embryogenic cell suspensions as a source of protoplasts for efficient plant regeneration from two Chinese commercial rice cultivars. *Plant Cell Tiss. Org. Cult.,* 66: 149-153.

Verbeek, M., P. Van Dijk and E.M.A. Van Well. 1995. Efficiency of eradication of four viruses from garlic *(Allium sativum* L.) by meristem-tip culture. *Eur. J. Plant. Pathol.*, 101: 231-239.

Wang, G.Y., Z.A. Xia and L.F. Wang. 1986. Regenerated plantlets from cultured mesophyll protoplasts of onion (*Allium cepa* L.). *Acta Biol. Exp. Sinica*, 19: 409-413.

Watanabe, M., K. Suzuki, H. Kawasaki and Y. Watanabe. 2002. Differential responses of *Brassica napus* and Petunia hybrida to leaf protoplast isolation stress. *Physiol. Plant*., 114: 645-651.

Wildholm, J.M. 1972. The use of fluorescein diacetate and phenosufranine for determining viability of cultured plant cells. *Stain Tech.,* 47: 189-194.

(Received for publication 16 September 2012)