In vitro regeneration of onion through repetitive somatic embryogenesis

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Abstract

A reliable protocol for the regeneration of onion through repetitive somatic embryogenesis was established. Embryogenic callus was derived from mature seeds on Murashige and Skoog (MS) medium supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D). Somatic embryos aroused on the surface of calli cultures and formed plantlets after the removal of 2,4-D or its substitution with 1 mg dm^{-3} kinetin (Kin). Reculturing the somatic embryos on 2,4-D containing medium led to secondary embryos formation. The embryogenic cultures which were preserved for five months on maintenance medium containing 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin have retained their ability for regeneration, while those kept on 2,4-D only, failed to form plantlets. Electrophoretic analysis of total soluble proteins revealed that the competence for successful conversion of somatic embryos into plantlets is associated with the expression of new set of proteins (112, 58 and 30 kD). The regenerated plants were successfully transferred to the soil.

Additional key words: *Allium cepa*, proteins, tissue culture.

Introduction

Although onion is the fourth largest vegetable crop throughout the world, there are still limits for its introduction into tissue culture (Reynolds 1986). Proliferation of callus from different explants of onion and subsequent plant regeneration *via* organogenesis have been investigated by many workers, *e.g.* Dunstan and Short (1979), Hussey and Falavigna (1980) and Phillips and Luteyn (1983). Abo-El-Nil is the first who reported somatic embryogenesis for *A. sativum* (garlic) in 1977 and Dunstan and Short (1978) have reported somatic embryogenesis for onion. Recently, Van der Valk *et al.* (1992) described a protocol for regeneration of onion through

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Abbreviation: 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; Kin - kinetin; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; SDS-PAGE - sodium dedocyl sulfate polyacrylamide gel electrophoresis.

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somatic embryogenesis from zygotic embryos explant and mentioned that significant differences in regeneration potential from callus tissue existed among genotypes. This means that the regenerative capacity of each genotypes should be individually investigated. Although, Schavemaker and Jacobsen (1995) developed a system for cyclic somatic embryogenesis regeneration system for leek, there are no reports dealing with repetitive somatic embryogenesis in onion.

Thus, in order to ensure effective regeneration system for Egyptian onion, the aim was to develop and optimize a method for regeneration of onion *via* repetitive somatic embryogenesis. Also, we attempted to characterize the regenerative capacities of embryogenic cultures preserved *in vitro* using SDS-PAGE protein profiles. This system has been already used to identify embryogenic cultures before morphogenesis (Roberts *et al.* 1989, Feirer and Simon 1991).

Materials and methods

Primary somatic embryogenesis: Seeds of *Allium cepa* L. cv. Giza 20 were obtained from Horticulture Research Institute, Ministry of Agriculture, Egypt. Seeds were surface sterilized in 70 % ethanol for 1 min followed by 50 % *Clorox* (5.25 % NaOCl) for 20 min and thoroughly washed with sterilized distilled water. Seeds were placed onto different embryogenic media. These contained MS salts, B5 vitamins (Gamborg *et al.* 1968), 0.7 % agar, 3 % sucrose and increasing levels of 2,4-D (from 0.5 to 3.5 mg dm^{-3}). pH was adjusted to 5.7 before autoclaving 20 min at 121 °C and 1.2 kg cm^{-2}. Five seeds were transferred to glass jar containing 25 cm^3 of embryogenic media. Ten replicates were employed for each treatment. Cultures were incubated in 18-h photoperiod, using cool white fluorescent lamps (irradiance of 25 $\mu mol\ m^{-2}\ s^{-1}$) and media were refreshed at three weeks intervals. After 8 weeks of cultivation, percentage of explants (seeds) proliferated calli, fresh mass yield of the proliferated calli and frequency of embryogenic calli among total proliferated calli were recorded.

Germination of somatic embryos: Somatic embryos formed on the surface of embryogenic cultures were separated and planted onto conversion medium *i.e.* MS medium containing 1 mg dm^{-3} Kin alone or in combination with 0.5 mg dm^{-3} of IAA, IBA, NAA and GA_3. After 6 weeks, the percentage of germination and shoot length were recorded.

Repetitive somatic embryogenesis: Callus pieces of 100 mg fresh mass containing about five primary somatic embryos were recultured onto MS medium either with 2 mg dm^{-3} 2,4-D or 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin (maintenance media). Five replicates were employed for each medium. Growth rate (mg fresh mass per day) and number of secondary embryos proliferated per piece of callus (explant) were monthly recorded. For testing the regenerative capacity of embryogenic cultures preserved on the two maintenance media, each month one replication was separated and

transferred to outgrowth medium which was previously optimized and the percentage of conversion of secondary embryos into plantlets was recorded.

Electrophoresis: For SDS-PAGE analysis, protein of 1 g fresh callus tissue, taken from the embryogenic cultures which were maintained for five months on either 2,4-D or 2,4-D + Kin was extracted in Na-phosphate buffer, pH 6.8. Calli samples used for protein analysis were morphologically similar. Electrophoresis was performed according to Laemmli (1970) using 10 % acrylamide in the separating gel and 3 % in the stacking gel. Protein samples (20 - 30 μg) in a total volume of 0.016 cm^3 (0.008 cm^3 of sample in extraction buffer + 0.008 cm^3 denaturing buffer) were denatured for 3 min in boiling water bath; cooled; centrifuged and 0.015 cm^3 was applied. Electrophoretic separation was carried out using EC mini gel unit at 60 V for 4 h. After electrophoresis, gel was stained with Coomassie brilliant blue (R-250) and destained with high methanol solution. Molecular masses of polypeptide bands were calculated from a calibration curve of low molecular mass marker kit of *Pharmacia* (Uppsala, Sweden).

Results

Induction of somatic embryogenesis: The percentage of callus proliferation varied between 5 % and 90 %, when the 2,4-D was added to the culture media in the range from 0.5 to 3.5 mg dm^{-3}. The highest percentage of callus proliferation and frequency of embryogenic callus was recorded on MS with 2 mg dm^{-3} 2,4-D. However, the number of somatic embryos per seed as well as the average fresh mass yield was the highest on 3 mg dm^{-3} 2,4-D (Table 1).

Table 1. Effect of various concentrations of 2,4-D on callus proliferation and induction of somatic embryogenesis from seed explants of onion. Mean ± SE; $n = 10$.

2,4-D [mg dm^{-3}]	Callus formation [%]	Embryogenic callus [%]	Number of somatic embryos per seed	Fresh mass yield [g]
0.5	5	4	4 ± 0.08	0.5 ± 0.10
1.0	20	10	5 ± 0.05	0.7 ± 0.30
1.5	50	40	8 ± 0.11	1.0 ± 0.13
2.0	80	60	10 ± 0.04	1.4 ± 0.20
2.5	90	55	10 ± 0.11	1.5 ± 0.11
3.0	90	50	14 ± 0.15	1.6 ± 0.40
3.5	77	55	15 ± 0.09	1.3 ± 0.45

Conversion of somatic embryos: After cultivation of isolated somatic embryos on different conversion media for six weeks, the highest percentage of somatic embryos converted into plantlets (50 %) occurred in somatic embryos cultured on MS with 1 mg dm^{-3} Kin and this percentage decreased to (40 %), when 0.5 mg dm^{-3} IAA is added. The percentage of conversion depended on the type of auxin (IAA, NAA,

IBA) or GA_3 added (Table 2). The highest shoot length (6 cm) was observed on basal MS medium. Addition of 0.5 mg dm^{-3} GA_3 in combination with 1 mg dm^{-3} Kin induced relatively high percentage of conversion (45) and shoot length (5 cm).

Table 2. Conversion of somatic embryos into plantlets, after six weeks of cultivation on different conversion media. Mean ± SE; $n = 10$.

Conversion Media	Germination [%]	Shoot length [cm]
Basal MS medium	20	6.0 ± 0.10
MS + 0.5 mg dm^{-3} Kin	30	3.0 ± 0.05
MS + 1.0 mg dm^{-3} Kin	50	3.5 ± 0.60
MS + 1.0 mg dm^{-3} Kin + 0.5 mg dm^{-3} IAA	40	4.0 ± 0.20
MS + 1.0 mg dm^{-3} Kin + 0.5 mg dm^{-3} NAA	35	3.2 ± 0.12
MS + 1.0 mg dm^{-3} Kin + 0.5 mg dm^{-3} IBA	45	4.5 ± 0.30
MS + 1.0 mg dm^{-3} Kin + 0.5 mg dm^{-3} GA_3	45	5.0 ± 0.22

***In vitro* maintenance of embryogenic cultures:** Primary embryos proliferated from seed callus (embryogenic cultures) on 2 mg dm^{-3} 2,4-D containing medium (Fig. 1) were subcultured on MS medium containing either 2 mg dm^{-3} 2,4-D or 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin. The growth rate of cultures on medium with 2,4-D was higher than that of cultures on medium with 2,4-D + Kin (Table 3, Fig. 1). Similarly, cultures on 2,4-D alone gave the highest number of secondary somatic embryos throughout the five months of cultivation. However, the highest percentage of somatic embryos converted into plantlets was observed in embryonic cultures maintained on 2,4-D + Kin. The most striking observation is the failure of all secondary somatic embryos on 2,4-D to convert into plantlets, after five months of preservation on maintenance medium. On the other hand, 50 % of embryos preserved for five months on 2,4-D + Kin were converted into plantlets. Generally, the percentage of germination of somatic embryos was decreased as the period of cultivation on maintaince medium increased.

Table 3. Effect of two maintenance media (MS with 2 mg dm^{-3} 2,4-D or MS with 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin) on the regeneration capacity of embryogenic onion cultures preserved for five months *in vitro* (explant - primary embryos isolated from the embryogenic cultures) and growth rate of secondary embryos. Mean ± SE; $n = 10$.

Media Number of subculturing	MS + 2,4-D second. embryos [number explant^{-1}]	conversion [%]	growth rate [mg(f.m.) d^{-1}]	MS + 2,4-D + Kin second. embryos [number explant^{-1}]	conversion [%]	growth rate [mg(f.m.) d^{-1}]
1	8 ± 0.09	50	1.0	7 ± 0.14	70	0.73
2	15 ± 1.00	40	1.3	10 ± 0.11	70	1.50
3	30 ± 0.10	30	2.7	20 ± 0.30	65	2.22
4	38 ± 0.20	10	2.6	27 ± 0.20	60	2.25
5	40 ± 0.10	0	2.3	34 ± 0.90	50	2.20

SDS-PAGE protein patterns of embryogenic cultures: The results of maintenance of embryogenic cultures on either 2 mg dm^{-3} 2,4-D or 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin demonstrated that only somatic embryos maintained on Kin containing medium were successfully developed into plantlets through normal outgrowth. The use of SDS-PAGE protein patterns as a molecular marker to distinguish between the two embryogenic cultures, illustrated that when the embryogenic culture was maintained on MS + 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin, three polypeptide bands (112, 58 and 30 kDa) were newly expressed and their expression was confined to cultures

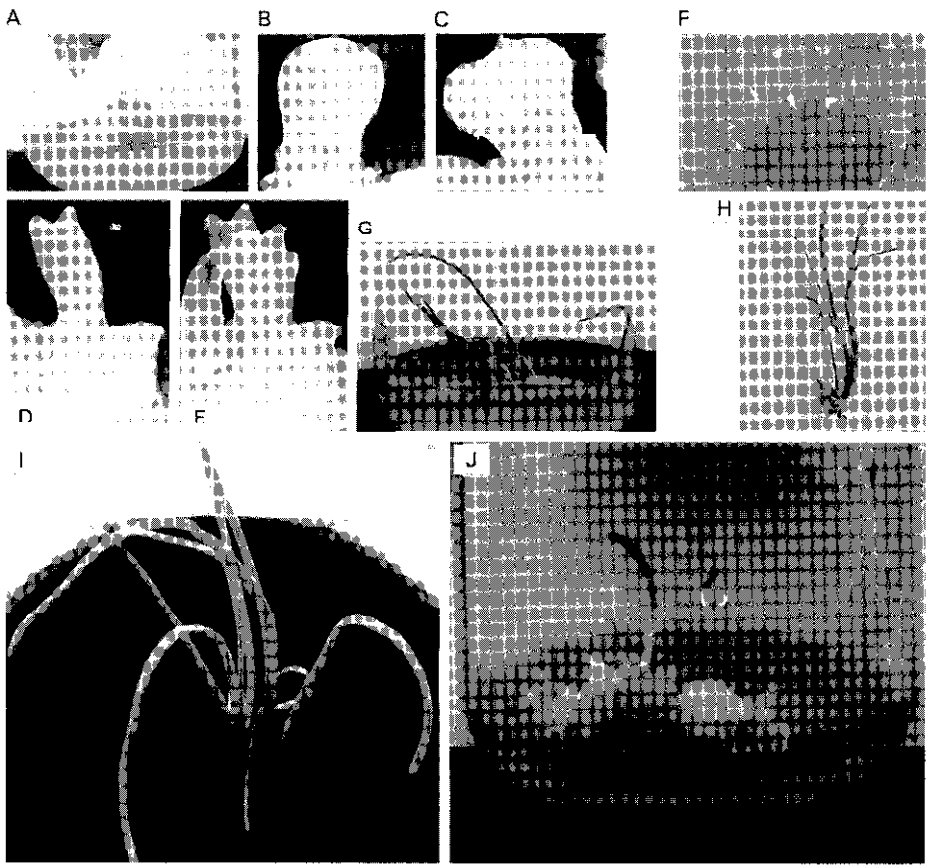

Fig. 1. Different stages of regeneration of onion plants *via* somatic embryogenesis: *A*) Proliferation of embryogenic callus on MS medium + 2 mg dm^{-3} 2,4-D; *B,C,D,E*) Somatic embryos at different stages of development, *i.e.*, globular (heart), torpedo, cotyledonary and multiple somatic embryos, respectively; *F*) Isolated somatic embryos; *G,H*) Germination of somatic embryos; *I*) Onion plant; *J*) Embryogenic culture maintained for five months on MS medium + 2,4-D + 0.5 Kin. Some embryos were germinated on the maintenance medium.

maintained on Kin containing medium (Fig. 2). Although the calli were similar in appearance, the newly expressed proteins were not detected in cultures preserved on 2,4-D containing medium.

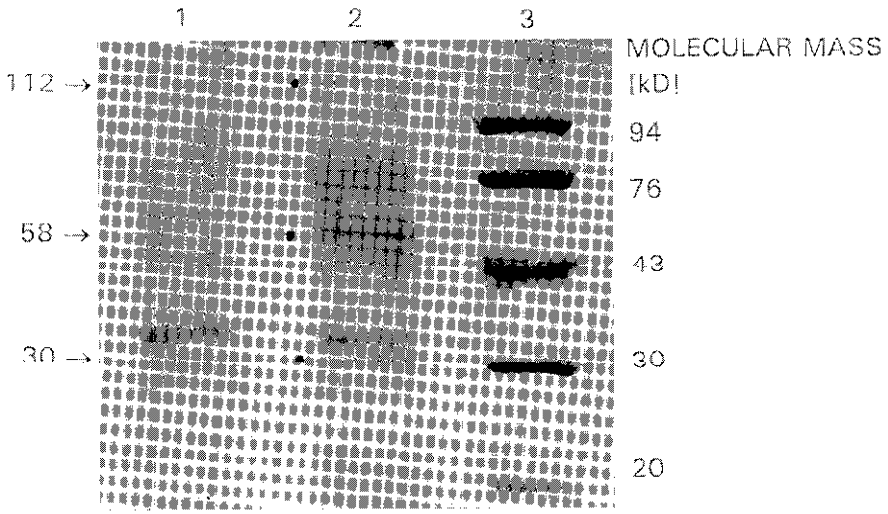

Fig. 2. Protein profiles of onion embryogenic cultures preserved for 5 months onto maintenance medium containing either 2 mg dm^{-3} 2,4-D (lane 1) or 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin (lane 2) and low molecular mass standard marker (lane 3). The black dots between lanes identify bands discussed in the text.

Discussion

Previous reports dealing with regeneration in onion have been confined to either organogenesis or direct somatic embryogenesis (Dunstan and Short 1978, Van der Valk *et al.* 1992, Yassen *et al.* 1994, Pinto and Rodrigues 1995). In the present study, we have described a method for repetitive somatic embryogenesis in onion. 2,4-D at concentration 2 mg dm^{-3} was found to be the optimum for proliferation of embryogenic cultures, meanwhile cultures preserved on maintenance medium contained 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} Kin showed the highest percentage of secondary somatic embryos converted into plantlets. An obvious limitation to plant transformation through direct somatic embryogenesis is the multicellular origin of somatic embryos arising directly from explant (Williams and Maheswaran 1986). On the other hand, embryos formed during repetitive embryogensis can originate from single cell and allows the propagation of transformed cells (Polito *et al.* 1989). The successful role of Kin in recovering of plantlets from somatic embryos, observed here, is in agreement with the results of Saxena and King (1987) who found that the frequency of somatic embryos formation is apparently enhanced by the presence of cytokinins in callus induction medium.

Some efforts have been made to investigate the relationship between the biochemical status of *in vitro* cultured cells and tissues and their ability for regeneration (Roberts *et al.* 1989, Feirer and Simon 1991, Győrgyey *et al.* 1991, Egertsdotter *et al.* 1993). Most of previously published results identify some categories of proteins associated with the regeneration capacity, However, it was also stated that SDS-PAGE protein patterns did not discriminate between embryo forming and proliferating calli (Feirer and Simon 1991). In the present study, variations in SDS-PAGE protein patterns revealed the novel expression of high molecular mass (112 kDa) and middle molecular mass (58 and 30 kDa) proteins by cultures on Kin containing medium and their absence in cultures on 2,4-D containing medium. This may be linked with successful development of somatic embryos into plantlets. Kin may be considered the inducer of these set of proteins and or *in vitro* cultivation for long time on 2,4-D may be the repressor of it. It may be concluded that the successful germination of secondary somatic embryos preserved on Kin containing medium is associated with the expression of new set of proteins.

Some worker observed the expression of new proteins during different embryogenic stages. Győrgyey *et al.* (1991) isolated novel 18 kDa protein during embryogenic stages in alfalfa. Recently, Hendriks and Varies (1995) detected a group of proteins (54, 47, 38, 32 and 10 kDa) in embryogenic cultures of carrot and Saker (1995) has detected 110 and 85 kDa proteins in somatic embryos of pea produced from immature cotyledon explants on 2 mg dm^{-3} NAA, However, somatic embryos produced on 1 mg dm^{-3} 2,4-D failed to synthesize these two proteins and failed to develop into planlets.

References

Abo-El-Nil, M.M.: Organogenesis and embryogenesis in callus cultures of garlic (*Allium sativum L.*). - Plant Sci. Lett. 9: 259-264, 1977.

Dunstan, D.I., Short, K.C.: Shoot production from onion tissue cultures. - Sci. Hort. **9**: 99-100, 1978.

Dunstan, D.I., Short, K.C.: Shoot production from cultured *Allium porrum* tissues. - Sci. Hort. **11**: 37-43, 1979.

Eggertsdotter, U., Mo, L.H., von Arnold, S.: Extracellular proteins in embryogenic suspension cultures of Norway spruce (*Picea abies*). - Physiol. Plant. **88**: 315-321, 1993.

Feirer, P.R., Simon, W.P.: Biochemical differences between carrot inbreds differing in plant regeneration potential. - Plant Cell Rep. **10**. 152-155, 1991.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspensions cultures of soybean root cells. - Exp. Cell Res. **50**: 148-151, 1968.

Győrgyey, J., Gartner, A., Németh, K., Magyar, Z., Hirt, H., Heberle-Bors, E., Dudits, D.: Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. - Plant mol. Biol. **16**: 999-1007, 1991.

Hendriks, J., Varies, S.: The role of secreated proteins in carrot somatic embryogenesis. - In: Terzi, M., Cella, R., Falavigna, A. (ed.): Current Issues in Plant Molecular and Cellular Biology. Pp.359-368. Kluwer Academic Publishers, Dordrecht 1995.

Hussey, G., Falavigna, A.: Origin and production of *in vitro* adventitious shoots in the onion, *A. cepa* L. - J. exp. Bot. **31**: 1675-1686, 1980.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head bacteriophage T4. - Nature **224**: 680-685, 1970.

Phillips, G.C., Luteyn, K.J.: Effect of picloram and other auxins on onion tissue cultures. - J. amer. Soc. hort. Sci. **108**: 948-953, 1983.

Pinto, P., Rodrigues, M.: Micropropagation of onion through *in vitro* bulblet formation. - *In vitro* **31**: 3, 1995.

Polito, V.S., McGranahan, G., Pinney, K., Leslile, C.: Origin of somatic embryo from repetitively embryogenic cultures of walnut (*Juglans regia* L.): Implication for *Agrobacterium*-mediated transformation. - Plant Cell Rep. **8**: 219-221, 1989.

Reynolds, F.J.: Regeneration in vegetable species. - In: Vasil, K.I. (ed): Cell Culture and Somatic Cell Genetics of Plants. Pp. 152-177. Academic Press, New York 1986.

Roberts, R.D., Flinn, S.B., Webb, T.D., Webster, B.F., Sutton, S.C.: Characterization of immature embryos of interior spruce by SDS-PAGE and microscopy in relation to their competence for somatic embryogenesis. - Plant Cell Rep. **8**: 285-288, 1989.

Saker, M.M.: Studies on tissue cultures of some legumes. - Ph. D. Thesis, Faculty of Science, Tanta Unversity, Tanta 1995.

Saxena, P.K., King, J.: Morphogenesis in lentil: Plant regeneration from callus cultures of *Lens culinaris* Med. *via* somatic embryogenesis. - Plant Sci. **52**: 223-227, 1987.

Schavemaker, M.C., Jacobsen, E.: Development of cyclic embryogenesis regneration system for leek (*Allium ampeloprasum L.*) using zygotic embryos. - Plant Cell Rep. **14**: 227-231, 1995.

Van der Valk, Scholten, O.E., Verstappen, F., Jansen, R.C., Dons, J.M., Van der Valk, P.: High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species. - Plant Cell Tissue Organ Cult. **30**: 181-191, 1992.

Williams, E.G., Maheswaran, G.: Somatic embryogenesis: Factors influencing coordinated behaviour of cells as an embryogenic group. - Ann. Bot. **57**: 443-462, 1986.

Yasseen, M.Y., Splittstoesser, W.E., Litz, R.E.: *In vitro* shoot proliferation and production of sets from garlic and shallot. - Plant Cell Tissue Organ Cult. **36**: 243-247, 1994.