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INDUCTION OF SOMATIC EMBRYOGENESIS IN ONION USING IMMATURE FLOWER BUDS

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ABSTRACT

A novel method of direct organogenesis in onion resulting in the formation of somatic embryos and subsequent plantlets induced on immature flower buds is described. Immature flower bud explants were *in vitro* cultured on medium contained various combinations of growth regulators. Nodular cultures and somatic embryos were proliferated in high frequency using MS medium supplemented with 0.5 mg /l kin +1mg/l 2,4-D.The highest percentage of embryo germenation as well as fresh and dry mass of proliferated cultures were registered when medium contained 0.5 mg /l kin + 0.5 mg /l 2,4-D was used . Among three concentrations of each of IAA and IBA used for plantlets elongation and improvement of root formation, IBA at a concentration of 2 mg/l gave the best results of shoot height and root length. However, the highest number of root per plantlet was observed when 2 mg /l IAA was added to culture medium. The highest percentage of survival in free-living conditions was obtained when plantlets were transplanted into pots contained peatmoss and perlite (1:1). The results of SDS-PAGE protein patterns showed identification of *in vitro* regenerated plants to that grown *in vivo*.

Key words: Onion, Somatic embryogenesis, SDS-PAGE protein analysis

INTRODUCTION

Onion is one of the most important vegetable species worldwide and is produced in almost all climatic regions. It is considered of great economic importance in Egypt and is one of the main exported vegetables. For decades there have been well established onion breeding and seed production programs. The development of many cultivars of onion has proceeded by mass or individual selection at the deploid level. There are several methods for producing improved onion cultivars i.e. hybridization, mass pollination within cultivars and /or selfing to produce inbred lines for hybrid production. Recently, the discovery of male sterility enabled the commerical production of hybrid seeds. Genetic-cytoplasmic male sterile lines utilized for the production of hybrid onion seeds are maintained and increased using genetically similar lines which are genetic sterile cytoplasmic fertile

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.However, male sterile plants often occur in onion progenies within breeding lines and open-pollinated cultivars, but cannot be utilized in seed production because of the lack of a genetically similar female maintainer . Development of tissue culture system will be useful to solve this problem. Moreover *in vitro* proliferation of onion plants through somatic embryogenesis could be great advantage not only for multiplication of specific genotypes, but also for offering the high regeneration ability needed for use in genetic transformation studies. In this respect, different organogenesis responses have been studied in several onion *in vitro* culture systems. In general, two types of tissues have been used for induction of shoot cultures: (1) inoculation of scale bases excised from the basal parts of bulbes or onion sets **(Hussey, 1978; Fujieda *et al* 1979; Hussey and Falavigna, 1980; Kahane *et al* 1992)** and (2) inoculation of mature flower parts such as receptacles **(Matsubara and Hihara, 1978)**. Poor regenerative capacity and subsequently low number of shoot formation have been achieved when immature flower buds were used **(Pike and Yoo, 1990)**.

The objective of this work is to recognize an effective regeneration system through somatic embryogenesis of onion using immature flower buds and to characterize the regenerated cultures using SDS-PAGE protein profiles.

MATERIAL AND METHODS

Immature flower buds of onion (*Allium cepa*) cv. Giza 6 were used as plant material (Fig. 1-A). Flower buds were immersed in 70 % ethanol for 1 min followed by commercial Clorox (contained 5.25 % sodium hypochlorite) for 15 min and then rinsed three times with sterilized distilled water. Flower buds inside the closed spathe are usually free from contamination. Sterilized flower heads were then cut longitudinally into halves and cultured on MS **(Murashige and Skoog, 1962)** medium in addition of combinations of benzyleadenine (BA), kinetin (Kin), naphthalenacetic acid (NAA) and dichlorophenoxyacetic acid (2,4-D). Nodular cultures and somatic embryogenesis frequencies and the number of embryo per culture were registered after six weeks of culturing. To investigate the effect of growth regulators on conversion of somatic embryos to plantlets, the proliferated embryos were recultured on MS medium supplemented with various combinations of BA, NAA, Kin, and 2,4-D. After six weeks, germination frequency and fresh and dry mass of the developed plantlets were recorded. For elongation of plantlets and improvement of root formation, the developed plantlets (2.5 cm length) were cultured on medium contained three concentrations (1,2 and 3 mg/l) of each of indoleacetic acid (IAA) and indolebutyric acid (IBA). Plant height, number of root per plantlet and root length were measured after five weeks of culturing. For acclimatization of *in vitro* derived cultures to free-living conditions, plants with good root system were taken and washed with tap water and then disinfected by immersion in benlate solution (1 mg/l) for 20 min. Plants were then transplanted in pots contained peatmoss alone and in combination with perlite (2:1, 1:1, and 1:2). The pots were covered with clear polyethylene bags and they were sprayed with water to maintain a high relative humidity. The percentage of survival were recorded after four weeks of transplanting.

Culture media were adjusted to pH5.8 before autoclaving at 126°C and 1.5 Ib/M² for 25 min and cultures were incubated at 25 ±2 °C under 16 hr light and 8 hr dark. All experiments were designed in completely randomized design and obtained data were statistically analyzed using standard error (SE) according to the method described by **Snedecor and Cochran (1967)**.

For protein analysis using SDS-PAGE, 1 g fresh samples of *in vitro* proliferated shoots, *ex vitro* adapted plantlets and *in vivo* grown plants were taken and protein extraction was achieved according to **Laemmli (1970)**. Each sample was homogenized in 1 ml sodium phosphate buffer (pH 6.8). Samples were centrifuged for 15 min at 6000 rpm that contained 4 ml water soluble extraction buffer (1.0 M Tris pH 8.6 and 0.25 M EDTA). Supernatant containing water soluble proteins were used for SDS-PAGE analysis. Electrophoresis was performed using 10 % acrylamide in separting gel and 3 % in stacking gel. Samples (50 µl) were denatured by heating at 100°C for 5 min in 1 % SDS containing 2-mercaptoethanol. Protein patterns of the different types of cultures were compared using low molecular weight standard (M.W. range from 14 to 94 KD Pharmacia, Motrea l).

RESULTS AND DISCUSSION

1. Effect of growth regulators on somatic embryogenesis

The influence of different combinations of phytohormones on induction of nodular cultures and somatic embryogenesis of onion from immature flower buds was investigated. Within three weeks of culturing, flower bud explants formed the visual structures. At this stage, regenerated structures had globular embryogenic apparance and became completely white after five weeks (Fig. 1-B). Concerning the differentation parameters, data of Table (1) reveal that the best results of nodular culture formation and somatic embryo proliferation were obtained when combinations of kin and 2,4-D were used. Also the results showed significant differences among the culture media for the paramater number of proliferated embryos per culture. The highest percentage of nodular cultures (86 %) was observed on medium contained 0.5 mg/l kin + 2 mg/l 2,4-D. However, the highest frequency of somtatic embryogenesis (80%) as well as the number of embryo per culture (14.00) were noticed when 0.5 mg/l kin + 1 mg/l 2,4-D added to culture medium. The obtained results have demonstrated that immature onion flowers could be induced to produce multiple high frequency of organogenic structures via direct regeneration. These results are in line with those obtained by **Luthar and Bohance (1999)** in their study on onion. They reported that direct organogenesis structures induced on mature flower buds or ovaries when cultured on medium containing 2mg/l 2,4-D. Moreover, **Zheng *et al* (1998)** reported that 2,4-D is the most important determining factor for callus production and later regeneration in onion. In the same direction, **Saker (1998)** used medium contained 2 mg/l 2,4-D for embryogenic callus formation of onion from seeds. However, **Pike and Yoo (1990)** used medium contained 05 mg/l NAA + 5 mg/l BA for *in vitro* proliferation of adventitious shoots from immature flower buds of onion.

Table 1. Effect of different combinations of growth regulators on somatic embryogenesis of onion from immature flower buds

Culture media	Nodular cultures (%)	Somatic embryogenesis frequency (%)	No. of embryo per culture
MS + 0.5 mg/l BA + 0.5 mg/l NAA	13	33	8.00 ± 0.20
MS + 0.5 mg/l BA + 1 mg/l NAA	20	20	5.00 ± 0.25
MS + 0.5 mg/l BA + 2 mg/l NAA	26	-	-
MS + 0.5 mg/l Kin + 0.5 mg/l 2,4-D	53	73	10.00 ± 0.40
MS + 0.5 mg/l Kin + 1 mg/l 2,4-D	73	80	14.00 ± 0.33
MS + 0.5 mg/l Kin + 2 mg/l 2,4-D	86	66	12.00 ± 0.20

Each treatment is the average of 15 replicates. ± SE = Standard error

2. Conversion of somatic embryos to plantlets

To investigate the effect of plant growth regulators on *in vitro* conversion of onion somatic embryos into plantlets, the proliferated embryos were isolated and cultured on medium contained different combinations of BA and NAA or Kin and 2,4-D (Table, 2). The results generally show that the combinations of Kin and 2,4-D were more effective on conversion of somatic embryos to plantlets compared with the combinations of BA and NAA. Also, results obviously indicated that reducing of 2,4-D in culture medium gave high frequency of conversion since the highest percentage of germination (93 %) and the highest values of fresh and dry mass were registered with medium contained 0.5 mg/l Kin + 0.5 mg/l 2,4-D. The balance of Kin to 2,4-D in recovering of plantlets from somatic embryos of onion observed in present study is in agreement with the results of **Saker (1998)** . Who found that the embryogenic cultures of onion which were preserved for five months on medium containing 2,4-D and Kin had retained their ability for regeneration, while those kept on 2,4-D only failed to form plantlets. Also, the results are in line with those obtained by **Bekheet (2000)** who reported that the highest percentage of embryos germination of asparagus was obtained when medium contained 0.5 mg/l 2,4-D was used. In this connection, **Luthar and Bohance (1999)** mentioned that, the auxin 2,4-D was superior to NAA or picloram, which partially or completely inhibited *in vitro* regeneration of onion cultures.

Table 2. Conversion of somatic embryos to plantlets on medium contained various combinations of growth regulators

Culture media	Germination frequency (%)	Fresh mass (g)	Dry mass (mg)
MS basal medium	33	0.85 ± 0.11	77.27 ± 1.50
MS+0.5 mg/l BA + 0.5 mg/l NAA	46	1.20 ± 0.20	120.00 ± 2.30
MS + 1 mg/l BA + 1 mg/l NAA	40	1.10 ± 0.33	104.76 ± 2.10
MS+0.5 mg/l Kin + 0.5 mg/l 2,4-D	93	1.80 ± 0.25	180.00 ±2.50
MS + 1 mg/l Kin + 1 mg/l 2,4-D	80	1.65 ± 0.10	166.60 ± 3.00

-Each treatment is the average of 15 replicates ± SE = Standard error

3. Elongation of converted plantlets and improvement of root formation

Root development can be performed *in vitro* in most species using auxins such as NAA or IBA (0.1-1.0 mg/l). Otherwise, to save manual labour, instead of transplanting the shoots to fresh medium, liquid media can be added to established cultures (Double layer technique). In the present work, the developed plantlets were cultured on medium contained three concentrations of each of IBA and IAA (Table, 3). The obtained data generally revealed that addition of IBA was more effective for plantlets elongation compared with IAA. The best height of shoots and root length were observed with medium contained 2 mg/l IBA (Fig. 1-C). However, the highest number of root per plantlet was noticed when medium contained 2 mg/l IAA was used.In this respect, several researchers found that it is necessary to transfer the shoots of onion grown *in vitro* to the medium with a low auxin concentration for promoting root formation **(Dunstan and Short, 1979; Bohojwani, 1980; Novak *et al* 986)**. Otherwise, the regenrants of onion may develop root directly on the regeneration medium **(Hussey, 1978; Dunstan and Short, 1977)**. In this respect, **Pike and Yoo (1990)** reported that *in vitro* grown shoots (2-3 cm) of onion were transferred to sterilized culture tubes containing vermiculite moistened with agar-free medium. About two weeks were required to form roots, which developed on almost 100% of plants.

4. Acclimatization of plantlets to free-living conditions

Several environmental conditions are essential in the initial period after transplanting into free –living conditions.

Table 3. Elongation and rooting improvement of converted plantlets of onion on medium contained different types and concentrations of auxins

Culture media	Means of plant height (cm)	No of root per plantlet	Means of root length (cm)
MS + 1 mg/l IBA	4.50 ± 0.15	3.00 ± 0.15	2.50 ± 0.13
MS + 2 mg/l IBA	6.00 ± 0.20	3.50 ± 0.12	3.00 ± 0.11
MS + 3 mg/l IBA	4.00 ± 0.10	3.20 ± 0.19	2.60 ± 0.05
MS + 1 mg/l IAA	3.50 ± 0.25	3.40 ± 0.15	1.50 ± 0.09
MS + 2 mg/l IAA	3.10 ± 0.30	4.00 ± 0.20	1.90 ± 0.08
MS + 3 mg/l IAA	2.80 ± 0.33	3.80 ± 0.25	1.80 ± 0.10

Each treatment is the average of 15 replicates ± SE = Standard error

One is maintenance of high relative humidity for two to three weeks to protect the plant from desiccation and enable it to initiate new roots. The second requirement is a loose, aerated, well-drained transplanting medium, which allows new roots to develop quickly. This part of study aimed to investigate the effect of transplantig media on survival of *ex vitro* adapted plants of onion. Data of Table (4) generally indicate that using mixture of peatmoss and perlite gave good results of survival compared with using peatmoss alone. The highest percentage of survival (90%) was recorded with peatmoss: perlite (1:1) followed by 1peatmoss: 2 perlite (Table, 4 and Fig. 1-D). The successful transplanting may be also due to the strong and healthy root system of plantlets. In this respect, **Barringer *et al* (1996)** in their study on onion reported that, *in vitro* grown shoots with roots over 30 mm were successfully established in potting mix and grown in glasshouse. **Pike and Yoo (1990)** obtained 80 % of survival when onion plants were transplanted into trays filled with Fison`s Sushie Mix, Blend No.1.

Table 4. Effect of different types of transplanting media on acclimatization of *in vitro* derived plantlets of onion

Transplantig media	Survival (%)
Peatmoss	50
Peatmoss + perlite (2: 1)	70
Peatmoss + perlite (1: 1)	90
Peatmoss + perlite (1: 2)	80

Each treatment is the average of 10 replicates

5- Protein analysis

The results of SDS-PAGE protein patterns presented in Fig. (2) demonstrated that somatic embryos of onion developed into normal plantlets. Since the

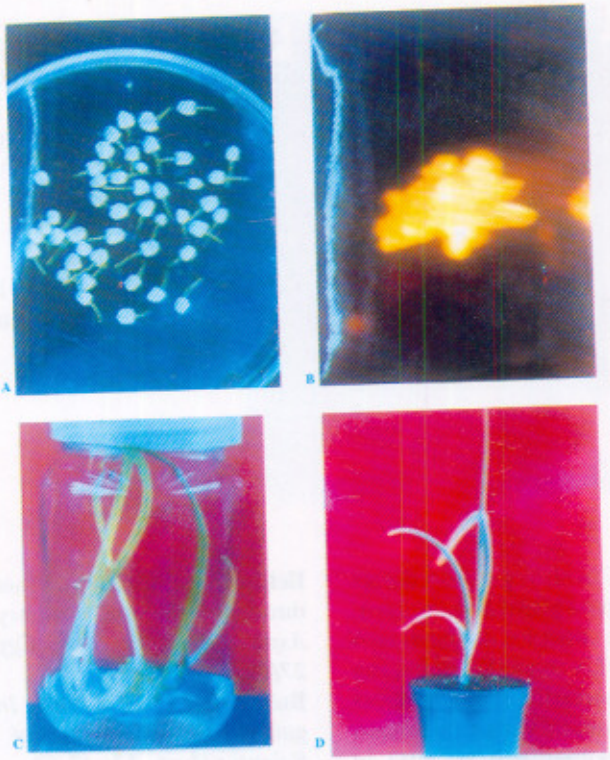

Fig. 1. A- Immature flower buds of onion cv. Giza 6.
B- Globular structures of onion proliferated from flower bud explants cultured on MS+0.5 mg/l Kin + 1 mg/l 2,4-D.
C- Elongation of onion plantlets using medium contained 2 mg/l IBA.
D- Adapted plants of onion transplanted into pots contained peatmoss and perlite (1:1).

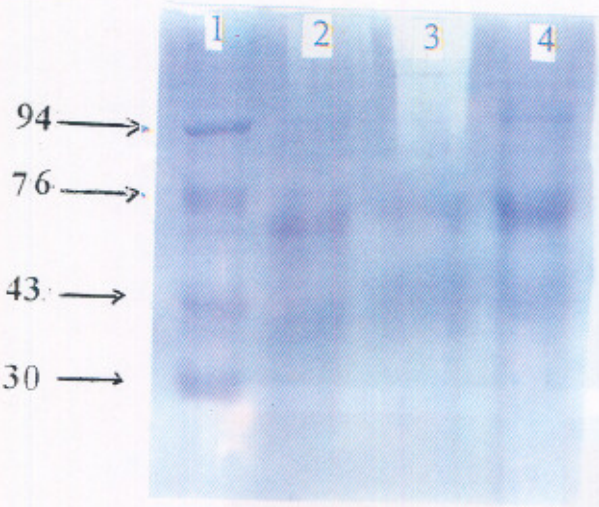

Fig. 2. Protein patterns of various types of onion cultures : low molecular weight protein marker (Lane 1), *in vitro* proliferated cultures (Lane 2), *ex vitro* adapted plants (Lane 3) and *in vivo* grown plants (Lane 4).

protein profiles of *in vitro* growing shoots and *ex vitro* adapted plantlets were identity and they were similar with the *in vivo* growing plants. This show that onion can be grown commercially through direct somatic embryogenesis without variation and this methodology can be used in breeding programs. In this connection, SDS-PAGE protein patterns was used to proof the identity of regenerated plants *in vitro* to their intact plants (**El-Kazzaz and Taha, 2002**) as well as to assess the variations which could occur in tissue cultures (**Ultrika *et al* 1993**).

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تخليق الأجنة الجسدية في البصل باستخدام البراعم الزهرية الغير مكتملة النمو

[١٦]

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يصف هذا البحث طريقة جديدة للتكشف المباشر للاعضاء في البصل معمليا متمثلا في الأجنة الجسدية و بالتالى تكوين النباتات من البراعم الزهرية الغير مكتملة النمو. حيث زرعت أجزاء هذه البراعم الزهرية تحت الظروف المتحكم فيها على بيئة مغذية صناعية (بيئة موراشيج وسكوج) تحتوى على توافيق مختلفة من منظمات النمو . قد لوحظ أفضل تكشف للنموات الجنينية والأجنة الجسدية على بيئة موراشيج وسكوج مضافا اليها ٠,٥ ملجم/لتر كينيتين + ١ ملجم/لتر ٤,٢- داى كلوروفينوكسى حمض الخليك . و قد سجلت أعلى نسبة لانبات الأجنة الجسدية المتكونة و كذلك أعلى قيم للوزن الطازج والجاف عند استخدام بيئة تحتوى على ٠,٥ ملجم/لتر كينيتين + ٠,٥ ملجم/لتر ٤,٢- داى كلوروفينوكسى حمض الخليك . ومن بين ثلاث تركيزات من كل من أندول حمض الخليك و أندول حمض البيوتريك استخدمت لأستطالة النموات وتحسين الجذير فقد سجل استخدام ٢ ملجم/لتر أندول حمض البيوتريك أحسن النتائج بالنسبة لارتفاع السيقان وأطوال الجذور . بينما لوحظ أعلى قيم لعدد الجذور عند إضافة ٢ ملجم/لتر أندول حمض الخليك إلى بيئة الزراعة . و كانت أعلى نسبة لبقاء النباتات حية بعد الأقلمة للظروف البيئية الطبيعية قد تم الحصول عليها عند زراعة النباتات فى أصص تحتوى على البيت موس والبرليت بنسبة ١:١ . وقد أشارت نتائج تحليل البروتين إلى تطابق النباتات المتحصل عليها من الأنابيب مع تلك النامية في الظروف الحرة .

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