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In vitro propagation of garlic (*Allium sativum* L.) through adventitious shoot organogenesis

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The present study investigates *in vitro* regeneration of three garlic cultivars; that is, Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type through adventitious shoot organogenesis. Shoot and root apices were subjected to eight callus induction treatments. A combination of 1 mg L^{-1} 2,4-D + 5 mg L^{-1} BA + 5 mg L^{-1} NAA produced a 100% of callus induction from root apices for all garlic cultivars tested. Shoot apices showed higher frequency of callus induction than root apices. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. Shoot apices had higher callus fresh weight than root apices in Balady and Sids 40 cultivars as well as wild type. The cultivar Balady had the highest callus fresh weight whereas the wild type had the lowest values. There were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. The wild type failed to regenerate shoots. The highest shoot number per root induced callus (48.8) was obtained from C7 callus line cultured into B5 medium supplemented with 10 mg L^{-1} Kin and 2 mg L^{-1} IAA. The cultivar Sids 40 showed the lowest regeneration efficiency among other cultivars with 1.5 shoots per explant. For callus induced from shoot apices, the eight callus lines for both Sids 40 cultivar and the wild type failed to regenerate shoots and the cultivar VFG 180 (3 -1) showed very low regeneration efficiency. The Balady cultivar showed the highest regeneration efficiency with 39 shoots per explant. The regenerated garlic shoots were *in vitro* rooted and acclimatized in greenhouse prior to their cultivation in open field. Garlic plantlets derived through tissue culture required three vegetative generations to produce bulbs of commercial size.

Key words: Acclimatization, garlic, organogenesis, tissue culture.

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 (Etoh, 1985). Vegetative

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **Kin,** kinetin; **IAA,** indole-3-acetic acid; **BA,** 6-benzyladenine; NAA**,** naphthalene acetic acid.

Table 1. Main characters for the investigated garlic cultivars (Balady, Sids 40 and VFG 180 (3-1)) and an Egyptian wild type.

Characters / Source	*Allium sativum* L.			Egyptian wild type
	Balady	Sids 40	VFG 180 (3-1)	
	Egypt	Egypt	Taiwan	North region of Egypt
Plant height (cm)	104.4±2.09a[Z]	65.5±1.64d	85.2±1.70c	96.5±2.51b
Pseudostem length (cm)	41.3±1.65a	28.0±0.56c	34.4±0.71b	17.5±0.53d
No. of leaves/plant	13.0±0.57a	10.3±0.31b	8.8±0.30b	9.5±0.35b
5th leaf length (cm)	62.0±1.24a	39.3±0.98b	58.0±1.16a	33.5±0.87c
5th leaf width (cm)	1.6±0.05c	3.7±0.13a	4.1±0.16a	2.5±0.10b
Total yield (ton/fed.)	5.3±0.23b	5.6±0.17b	8.3±0.28a	1.2±0.04c
Bulb weight (g)	76.4±2.29b	73.1±1.97b	117.4±2.35a	18.0±0.37c
Bulb diameter (cm)	5.8±0.12b	5.1±0.15b	7.7±0.21a	2.5±0.06c
No. of cloves / bulb	55.2±1.13a	17.0±0.51b	16.5±0.33b	2.0±0.03c
Clove weight (g)	1.38±0.05d	4.3±0.12c	7.2±0.15b	8.5±0.22a
Bulb skin color	White	Purple	Purple	Cream
Bulblets (helmet-shaped)	-	-	-	Many small with stolon from stem disk
Bulbils	Few small	Few small to medium	Few medium	-
Number of chromosomes*	16	16	16	32

*The cytological study was carried out according to Darlington and La Cour (1976) technique. [Z]Data presented are means ± standard error. Mean separation within rows by Duncan's multiple range tests at 5% level.

propagation of garlic is achieved through division of the ground bulbs and/or aerial bulbs therefore, the multiplication rate is fairly low. Also, due to difficulties of inducing flowering, improvement of this crop through breeding programs is limited (Barandiaran et al., 1999a; Metwally et al., 2014). Many of the elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect pests (Verbeek et al., 1995). Virus infection was shown to reduce the bulb yield by 20 to 60%, and up to 80% in case of mixed infection, depending on cultivar and stage of infection (Lot et al., 1998). The virus infection is inevitable for vegetative propagation of garlic. Therefore, *in vitro* propagation would be one of the key technologies for sustainable supply of this important plant source

The low propagation rate and the continuous accumulation of deleterious viruses produced in the field have promoted the development of *in vitro* propagation of garlic (Nagakubo et al., 1993; Seabrook, 1994; Koch et al., 1995; Mohamed-Yassen et al., 1995; Haque et al., 1997, 2003; Ayabe and Sumi, 1998; Myers and Simon, 1998, 1999; Robledo-Paz et al., 2000; Kim et al., 2003; Luciani et al., 2006; Keller and Senula, 2013). According to these reports, the physiological condition of the explant, the genotype and the growth regulator combinations used in the culture medium were the most important factors affecting plant regeneration. However, several months up to one year are required for plant regeneration; therefore, the efficiency of plant regeneration has not been reproducibly high. In continuation to improve *in vitro* culture and propagation for garlic cultivars, in the present study, a detailed investigation was carried out in order to establish *in vitro* culture protocol for three garlic cultivars; that is Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type.

MATERIALS AND METHODS

Plant material and culture establishment

The present study was conducted on three cultivars of garlic (*A. sativum* L.); that is Balady, Sids 40 and VFG 180 (3 - 1) as well as an Egyptian wild type collected in 2009 from El-Arish city at the North region of Sinia peninsula. The main vegetative characteristics as well as chromosomal number for these garlic genotypes are presented in Table 1 and Figure 1. Garlic cloves were separated from the compound bulb and pealed manually, washed and air dried. Small and injured cloves were excluded. The cloves were rinsed in 70% (v/v) ethanol for 30 s followed by 3.5% sodium hypochlorite containing one drop of Tween 80 for 20 min under a constant hand agitation. Cloves were then washed thrice with sterile distilled water for 5 min each. The cloves were inoculated in sterile glass jars of 100 ml each containing 20 ml of half strength MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). Root and shoot tips of the sprouting plantlets were used as plant material for callus induction. Root tips were 0.8 to 1.2 cm in length whereas shoot tips were 1.0 to 1.5 mm in length.

Culture conditions

All media were supplemented with 3% (w/v) sucrose and 0.8% (w/v) Sigma agar-agar. The pH of the medium was adjusted to 5.8 before

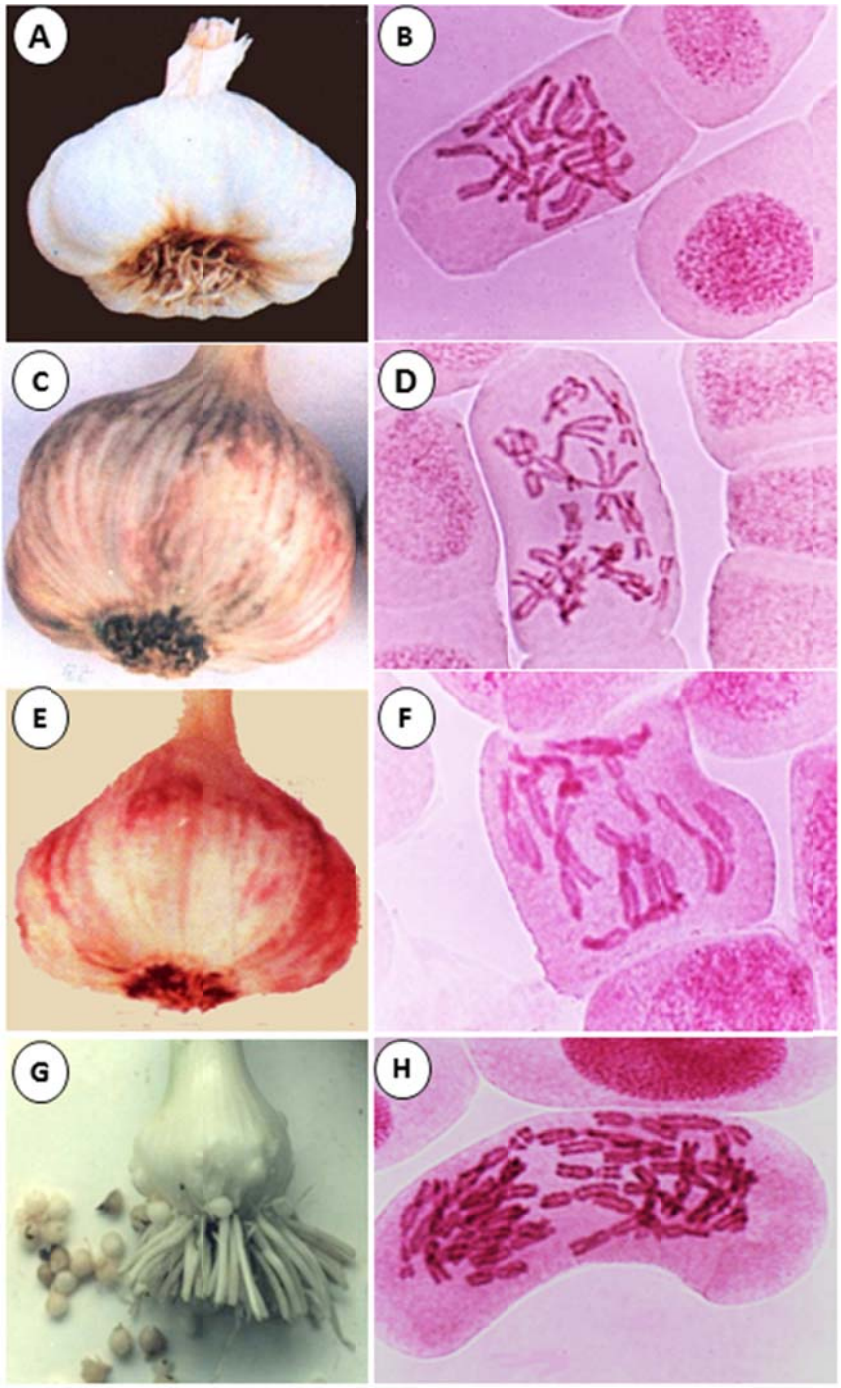

Figure 1. Morphological characteristics and chromosomal number in garlic cultivars. **A, B)** Balady; **C, D)** Sids 40; **E, F)** VFG 180 (3 - 1) and **G, H)** wild type.

autoclaving for 20 min at 121°C. The cultures were incubated for three days under dark conditions at 25 ± 2°C then under 16 h light at 35 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by cool white fluorescent tubes.

Callus induction

Root and shoot apices were cultured on either MS medium supplemented with different PGRs concentration and combinations [1.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) + 2.0 mg L^{-1} Kinetin (Kin) + 1.7 mg L^{-1} indole-3-acetic acid (IAA); 2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} 6-benzyladenine (BA); 5 mg L^{-1} BA + 5.0 mg L^{-1} 1-naphthalene acetic acid (NAA); 1 mg L^{-1} NAA; 3 mg L^{-1} 2,4-D; 1 mg L^{-1} 2,4-D; 1 mg L^{-1} 2,4-D + 5 mg L^{-1} BA + 5 mg L^{-1} NAA] or B5 medium (Gamborg et al., 1968) supplemented with 3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} 2ip. Culture conditions are as described above. There were four explants per replicate and four replicates per treatment

Table 2. Frequency of shoot and root apices induced callus of three garlic cultivars {Balady, Sids 40 and VFG 180 (3 - 1)} as well as wild type after 8 weeks of culture.

Medium code[Y]	Balady	Sids 40	VFG 180 (3 - 1)	Wild type
Root apex				
C1	100a[Z]	90.6b	100a	100a
C2	100a	100a	85c	68d
C3	100a	20g	100a	100a
C4	0h	0h	100a	0h
C5	100a	100a	25.8g	76d
C6	100a	100a	42.9f	100a
C7	100a	100a	100a	100a
C8	100a	30g	100a	100a
Shoot apex				
C1	100a	91.7b	100a	100a
C2	100a	66.7d	68.8d	100a
C3	100a	50.0f	100a	100a
C4	94.5b	91.7b	100a	90b
C5	100a	66.7d	62.5e	70d
C6	100a	58.3e	93.8b	100a
C7	100a	41.7f	87.5c	70d
C8	100a	50.0f	100a	100a

[Y]Callus induction media were supplemented with various PGRs as follows: C1 (1.1 mg l^{-1} 2,4-D + 2.0 mg l^{-1} Kin + 1.7 mg l^{-1} IAA); C2 (2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA); C3 (5 mg l^{-1} BA + 5.0 mg l^{-1} NAA); C4 (1 mg l^{-1} NAA); C5 (3 mg l^{-1} 2,4-D); C6 (1 mg l^{-1} 2,4-D); C7 (1 mg l^{-1} 2,4-D + 5 mg l^{-1} BA + 5 mg l^{-1} NAA); C8 (3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2ip). [Z]Mean separation within columns by Duncan's multiple range test at 5% level.

rendering a group of 16 explants per treatment. The percentage of callus formation and callus fresh weight were recorded after eight weeks of culture.

Adventitious shoot regeneration

All calluses were transferred onto either MS medium supplemented with different concentrations and combinations of BA and NAA as follows: BA (1.0 mg L^{-1}) + NAA (0.5 mg L^{-1}); BA (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}); BA (2.0 mg L^{-1}) + NAA (1.0 mg L^{-1}) or B5 medium supplemented with Kin (10.0 mg L^{-1}) + IAA (2.0 mg L^{-1}). There were five explants per Petri dish and four replicates per treatment. Culture conditions are as described above. The number of proliferated shoots per callus was recorded after eight weeks of culture for Balady cultivar while after 12 months for Sids 40 and VFG 180 (3 - 1) cultivars.

Root formation and plant acclimatization

Proliferated shoots obtained through organogenic calli were individually separated and inoculated to cylindrical culture jars (375 ml capacity) containing 35 ml MS medium without PGRs. The medium was supplemented with 3% (*w/v*) sucrose and solidified with 0.8% (*w/v*) agar. The pH of the medium was adjusted to 5.8 before autoclaving (at 121°C and 1.2 kg cm^{-2} pressure for 15 min). All the culture jars were maintained for four weeks at the same culture conditions described above.

For pre-acclimatization of garlic plantlets, the leaves were trimmed and the plantlets were sub-cultured for four weeks. The jars were subjected to a light intensity of 35 µ mol m^{-2} s^{-1} PPF for 2 weeks followed by 50 µmol m^{-2} s^{-1} PPF for two weeks. The plantlets were carefully cleaned from the medium and washed with tap water. They were then transplanted into sterilized clay pots (20 cm diameter) containing a mixture of peat moss: silt: sand (1:1:1, v/v/v). The leaves were trimmed into half size and the pots were incubated in a growth chamber. The environment in the growth chamber was adjusted to 25 ± 1°C air temperature, 50 µmol m^{-2} s^{-1} PPF with a 16 h photoperiod provided by cool white fluorescent tubes and 40 to 50% relative humidity. The pots were covered with a clear polyethylene bags for the first week, gradually removed and grown for three weeks. The acclimatized plants were then transferred to a greenhouse for 6 weeks before their planting in an open field.

Experimental design and data analysis

Experiments were set up in a completely randomized design. Data were subjected to Duncan's multiple range tests using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

RESULTS AND DISCUSSION

Callus induction

Both shoot and root apices excised from *in vitro* plantlets of three garlic cultivars as well as wild type were subjected to eight callus induction treatments in the first series of experiments (Table 2). Shoot apices formed callus in all eight treatments and in all garlic cultivars

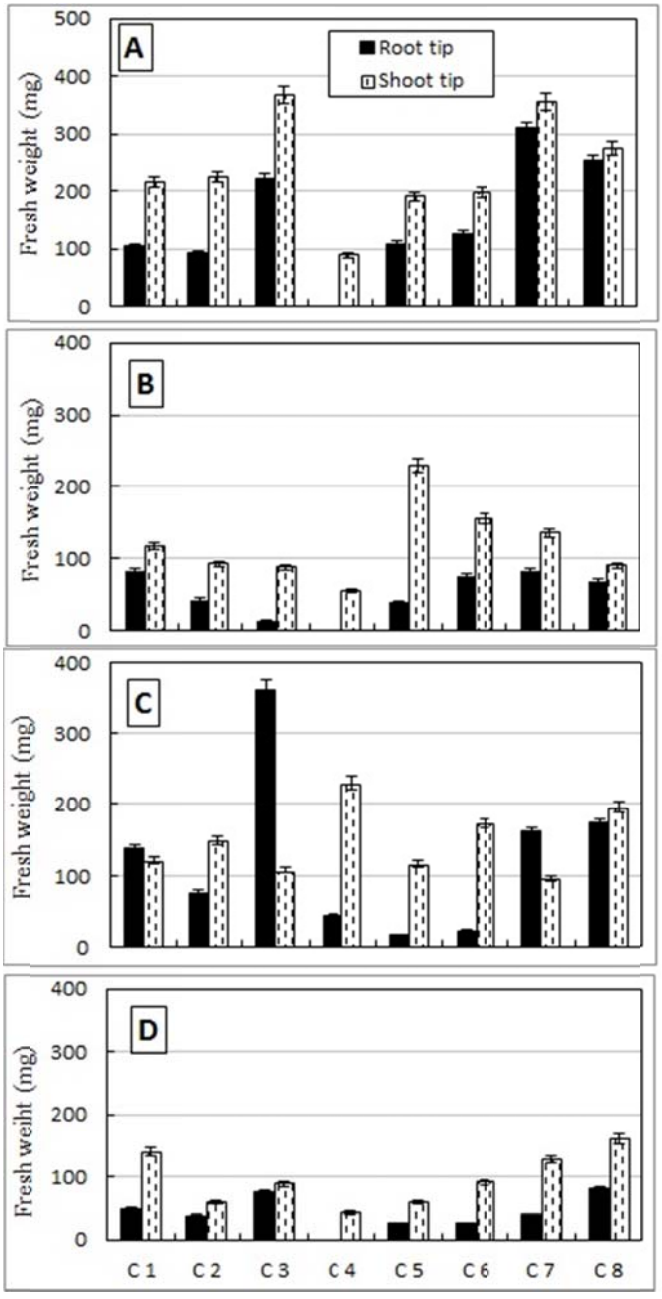

Figure 2. Fresh weight of root/shoot tips induced callus of Balady (A), Sids 40 (B), VFG 180 (3 - 1) (C) and wild type (D) after eight weeks on callus induction media supplemented with various PGRs: C1 (1.1 mg l^{-1} 2,4-D + 2.0 mg l^{-1} Kin + 1.7 mg l^{-1} IAA); C2 (2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA); C3 (5 mg l^{-1} BA + 5.0 mg l^{-1} NAA); C4 (1 mg l^{-1} NAA); C5 (3 mg l^{-1} 2,4-D); C6 (1 mg l^{-1} 2,4-D); C7 (1 mg l^{-1} 2,4-D + 5 mg l^{-1} BA + 5 mg l^{-1} NAA); C8 (3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2ip).

tested. Root apices also formed callus in all treatments except for C4 treatment which did not induce callus in Balady and Sids 40 cultivars as well as wild type. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. Shoot apices showed higher frequency of callus induction than root apices. There were differences among auxin/cytokinin callus induction treatments for the percentage of root and shoot apices that induced callus. A combination of 2,4-D, BA and NAA (C7 treatment) produced a 100% of callus induction from root apices for all garlic tested. For shoot apices, a combination of 2,4-D, Kin and IAA (C1 treatment) produced the highest frequency of callus induction at 100% for Balady, VFG 180 (3 -1) and wild type and 91.7% for Sids 40. In general, a high frequency of callus induction was obtained when auxin was combined with cytokinins.

The root and shoot apices of VFG 180 (3 -1) cultivar, contrary to the other garlic cultivars, produced a 100% of callus induction when NAA was employed alone without cytokinins. Garlic cultivars and different explants also showed variations for their callus fresh weight (Figure 2). Shoot apices had higher callus fresh weight than root apices in Balady and Sids 40 cultivars as well as wild type (Figure 2A, B and D). In VFG 180 (3 -1) cultivar, shoot apices also had higher callus fresh weight than root apices except for 3 callus induction treatments (C1, C3 and C7) (Figure 2C). In general, the cultivar Balady had the highest callus fresh weight whereas the wild type had the lowest values. Among the eight PGRs treatments, C3 (BA and NAA) produced the highest callus fresh from shoot apex in Balady cultivar and from root apex in VFG 180 (3 -1) cultivar. A combination of 2,4-D with BA and NAA (C7) or 2,4-D with 2ip (C8) also proved effective for callus fresh weight in Balady and VFG 180 (3 -1) cultivars as well as the wild type. Using auxins as the sole PGR in the medium such as 3 mg l^{-1} 2,4-D (C5) or 1 mg l^{-1} NAA (C4) produced the highest callus fresh weight from shoot apices in Sids 40 and VFG 180 (3 - 1), respectively. Previous reports demonstrated that callus differentiation in different garlic cultivars are determined by PGRs in the culture medium; 2,4-D was most effective (Myers and Simon, 1999; Robledo-Paz et al., 2000; Khar et al., 2005; Luciani et al., 2006).

In the present study, calluses were formed only on end of the root explant (Figure 3A). All calluses were compact and exhibited a nodular-like structure (Figure 3B). Previous reports demonstrated callus formation on the apical part of the root (Haque et al., 1998; Barandiaran et al., 1999b; Robledo-Paz et al., 2000). In contrary, it has been observed that calluses could be induced not only on the apical part of the root but also on the non-apical parts (Zheng et al., 2003).

Adventitious shoot regeneration from root apices induced callus

After callus induction, eight callus lines induced from root apices were transferred into four regeneration media rendering a group of 32 treatments for each cultivar as well as the wild type (Figure 4). In Balady cultivar, 25 out of 32 treatments produced shoots (Figure 4A). The

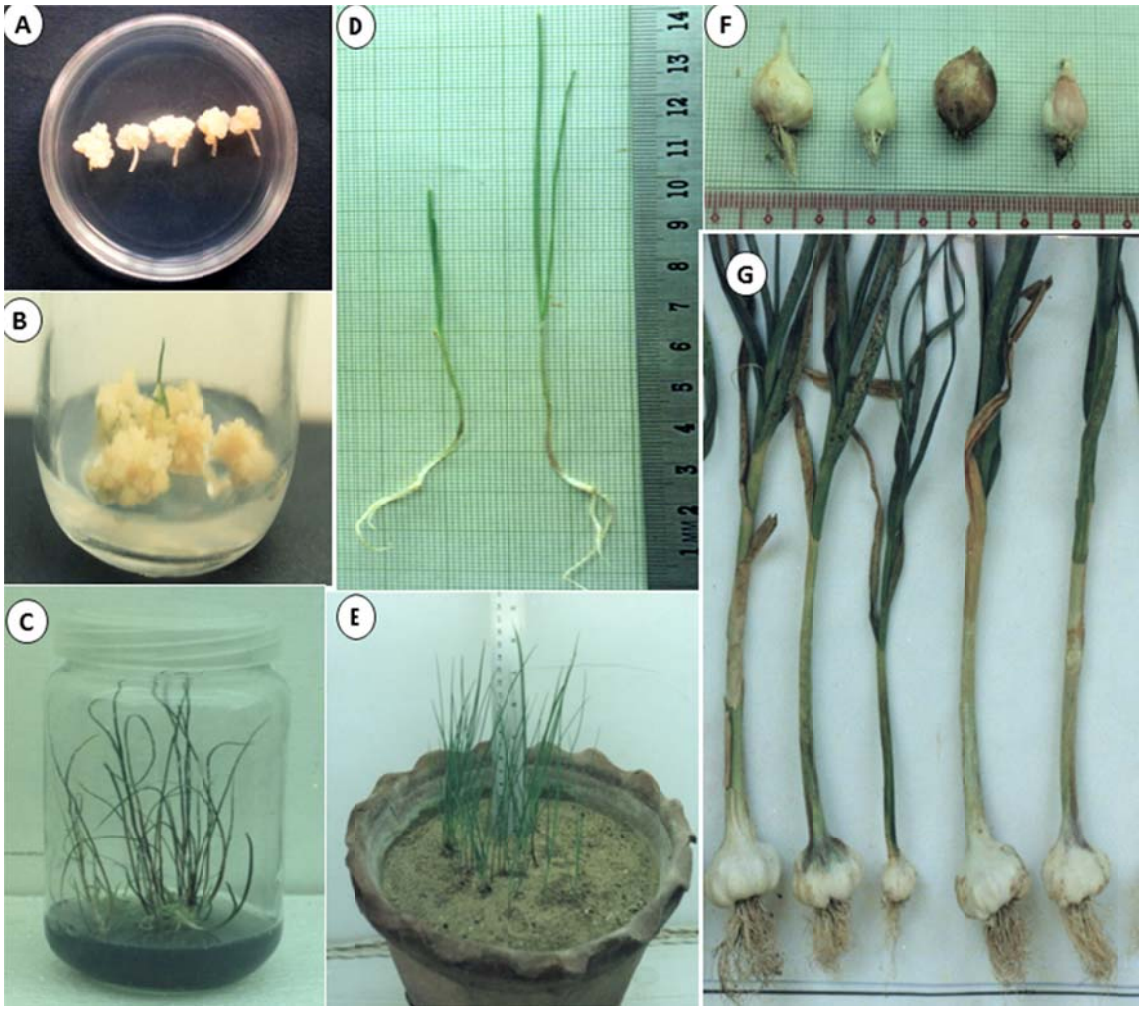

Figure 3. Indirect shoot organogenesis using root tip and development of garlic bulbs during three successive generations in Balady cultivar. **A)** Calluses formed on end of the root explants. **B)** Nodular meristematic callus. **C)** Regenerated garlic shoots. **D)** *In vitro* rooted plantlets. **E)** Acclimatized plantlets. **F)** Non-divided bulbs derived from the first vegetative generation in pots. **G)** Bulbs derived from the third vegetative generation in the field.

highest shoot number per explant (48.8) was obtained from C7 callus line cultured into R4 regeneration medium (B5 + 10 mg L^{-1} Kin + 2 mg L^{-1} IAA). It is also noted that the C7 callus line produced low number of shoots per explant (1.8 - 11.3) when cultured into other regeneration media supplemented with BA and NAA. However, C8 callus line produced 44 shoots per explants when cultured into R3 regeneration medium. For the cultivar VFG 180 (3 -1), only 5 out of the 32 treatments were able to produce shoots (Figure 4B). The highest number of shoots per explant (51.3) was obtained from C6 callus line cultured into R4 medium while the lowest (6.5) was obtained from C3 callus line cultured into R3 medium. The cultivar Sids 40 showed the lowest regeneration efficiency among other cultivars. Only 1 treatment (C1 callus line cultured into R1 medium) produced 1.5 shoots per explant. All of the 8 callus lines for the wild type failed to regenerate shoots at all four regeneration media. The obtained results indicate that there were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. Such variations have been previously reported for garlic callus lines (Myers and Simon, 1998; Zheng et al., 2003). For example, garlic callus grown in medium with either auxins or cytokinins alone produced no shoots (Myers and Simon, 1999). Therefore, a combination of auxin and cytokinin is necessary for regeneration of garlic callus (Haque et al., 1997; Zheng et al., 2003; Khar et al., 2005), however, the type and concentration of PGRs were cultivar-dependent.

Adventitious shoot regeneration from shoot apices induced callus

All of the 8 callus lines induced from shoot apices were

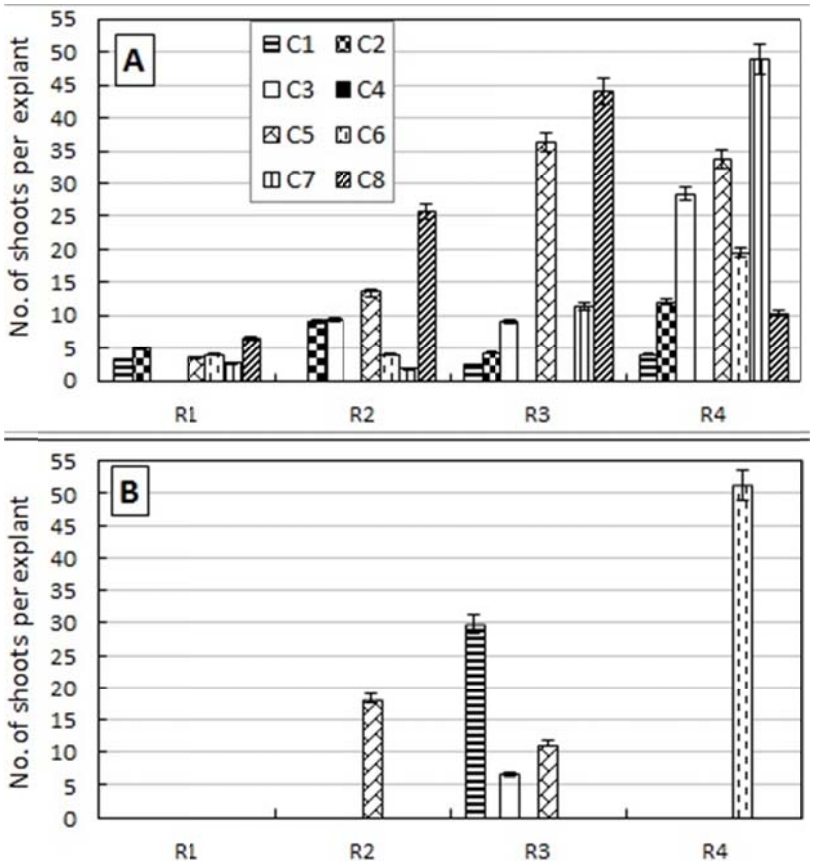

Figure 4. Adventitious shoot regeneration of eight callus lines induced from root apices of Balady cultivar (A) and VFG 180 (3 -1) (B). Regeneration media were supplemented with various PGRs: R1 (1.0 mg l^{-1} BA + 0.5 mg l^{-1}NAA); R2 (2.0 mg l^{-1}BA + 0.5 mg l^{-1}NAA); R3 (2.0 mg l^{-1}BA + 1.0 mg l^{-1}NAA); R4 (10.0 mg l^{-1}Kin + 2.0 mg l^{-1} IAA).

also transferred into four regeneration media rendering a group of 32 treatments for each cultivar as well as the wild type. The eight callus lines for both Sids 40 cultivar and the wild type failed to regenerate shoots. The cultivar VFG 180 (3 -1) showed very low regeneration efficiency. Only 1 treatment (C5 callus line cultured into R1 medium) produced 40.3 shoots per explant (data not shown). The Balady cultivar showed the highest regeneration efficiency among other cultivars. Thirty one out of 32 treatments regenerated shoots and the highest shoot number per explant (39) was obtained from C5 callus line cultured into R4 medium (Figure 5). Although shoot apices induced callus had higher regeneration efficiency than root apices induced callus but the number of regenerated shoots per explant was lower than that obtained from root apices induced callus. Six treatments for shoot apices induced callus lines produced more than 20 shoots per explant compared to one treatment for root apices induced callus lines (Figures 4A and 5).

In vitro cultures of garlic are greatly influenced by the genotype (Barandiaran et al., 1999a). It has been reported that using root tips as explant greatly increases the regeneration potential that can be achieved over other explant types such as shoot tips (Nagakubo et al., 1993; Mohamed-Yassen et al., 1994). Root tip explants are commonly used for the development of garlic regeneration system (Haque et al., 1997; Barandiaran et al., 1999b; Robledo-Paz et al., 2000; Keller and Senula, 2013).

Different genotypes of garlic also showed variations in time required for plant regeneration. It could be achieved within 4 and 9 months by Barandiaran et al. (1999a) and Myers and Simon (1998), respectively. In the present study, plant regeneration was obtained after 8 weeks in Balady cultivar and 12 months for Sids 40 and VFG 180 (3 - 1) cultivars while no regeneration could be obtained from the wild type.

In vitro rooting and acclimatization

The regenerated garlic shoots formed a well-developed root system within seven to eight days upon their culture on MS medium without PGRs (Figure 3C and D). Each

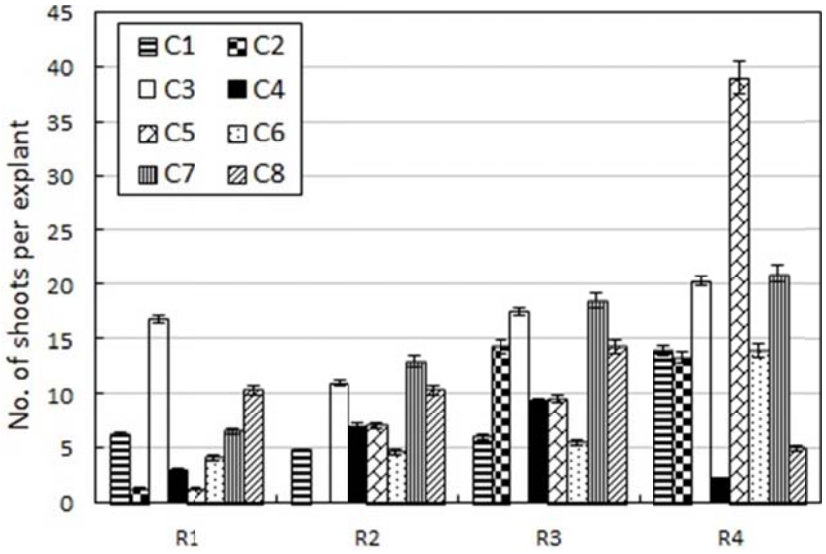

Figure 5. Adventitious shoot regeneration of eight callus lines induced from shoot apices of Balady cultivar and cultured into four regeneration media supplemented with various PGRs: R1 (1.0 mg l^{-1} BA + 0.5 mg l^{-1}NAA); R2 (2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA); R3 (2.0 mg l^{-1} BA + 1.0 mg l^{-1}NAA); R4 (10.0 mg l^{-1} Kin + 2.0 mg l^{-1} IAA).

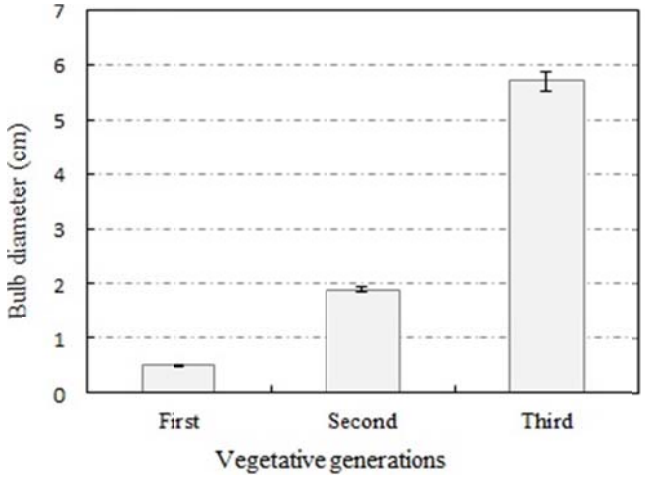

Figure 6. Development of bulb diameter during three successive vegetative generations of micropropagated garlic Balady cultivar.

plantlet had 2 - 5 roots with 2 - 3 cm in length. Previous reports indicated that *in vitro* rooting of garlic is easily achieved on MS medium without PGRs (Metwally and Zanata, 1996; Metwally et al., 2012; Keller and Senula, 2013). However, Tapia (1987) reported that garlic roots formed well on MS medium supplemented with Kin and IAA. Exposure of garlic plantlets to 50 µ mol m^{-2} s^{-1} PPF for two weeks permitted its acclimatization with 100% survival rate. It is well known that increasing light intensity reduces leaf length, length and width of cell and stomata index while increases leaf thickness (Rahim and Fordham, 1991). It has been reported that bulbs transferred to the soil sprouted in the first 3 months of culture with a 60% survival rate (Barandiaran et al., 1999a). At the end of the acclimatization stage, each plantlet gave a small and a non-divided bulb ranging from 0.2 - 1.2 cm in diameter (Figure 3E and F). The obtained plantlets differed in the shoot size; green color darkness, number of leaves and skin color of bulblet (white, light purple and purple). Such observations were also reported by Metwally and Zanata (1996) and Metwally et al. (2012). The size of the bulblets formed *in vitro* determines the quality of the plants developed in the field and the period of time required for commercial-sized cloves. In the present study, *in vitro* bulblets (0.5 cm in diameter) reached 1.9 and 5.7 cm in diameter in the second and the third vegetative generations, respectively (Figure 6). Thus, 3 years were required to obtain the commercial-sized cloves in Balady cultivar.

The present study investigates *in vitro* regeneration of three garlic cultivars that is Balady, Sids 40 and VFG 180 (3 - 1) as well as a garlic wild type through adventitious shoot organogenesis. Shoot apices showed higher frequency of callus induction than root apices. Balady cultivar showed the highest frequency of callus induction while Sids 40 showed the lowest values. There were large variations in the regeneration efficiency among the eight callus clones as well as different garlic cultivars. plant regeneration was obtained after eight weeks in Balady cultivar and 12 months for Sids 40 and VFG 180 (3 - 1) cultivars while no regeneration could be obtained from the wild type. The Balady cultivar showed the highest regeneration efficiency with 39 shoots per explant.

Garlic plantlets of Balady cultivar derived through tissue culture required three vegetative generations to produce bulbs of commercial size.

Conflict of Interests

The authors declare no conflict of interests.

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