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Somatic Embryogenesis and Direct Shoot Bud Formation from *In Vitro* Root Segments of Garlic (*Allium sativum* L.)

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Abstract

In vitro techniques are unconventionally used for garlic improvement. In garlic tissue culture, numerous roots are produced *in vitro*. Segments (1 cm) of those *in vitro* roots were cultivated on MS medium with 5.0 μM 2, 4-D for callus initiation followed by transfer to 0.5 μM 2, 4-D for somatic embryogenesis. Root segments had 51.67% callus and somatic embryo formation. Plantlet regeneration was obtained from 90% calli after being transferred to MS medium supplemented with 5.0 μM kinetin. Within 6 months, 54.8 plants per root segment and 42.2 plants per root tip explant were found. MS media supplemented with 1.0 or 5.0 μM NAA in combination with 10.0 or 50.0 μM BA were used for direct shoot bud induction from root segments. The highest percentage (23.33 %) of direct shoot bud regeneration was found in 5.0 μM NAA with 50.0 μM BA within 2 months. The number of shoots per explant was varied from 1-81 with the highest average of 15.75 shoots per explant. The shoots produced bulblets upon transfer to a medium containing higher amount of sucrose (12%). The largest bulblets weighed 356.57 g in the medium with 5.0 μM NAA and 50.0 μM BA. In both pathways, regeneration occurred from only the apical (distal) end of the root segments. The protocols are promising for continuous regeneration of propagules and genetic transformation study for improvement of garlic.

Introduction

Cultivated garlic (*Allium sativum* L.) is sexually sterile and propagated exclusively by vegetative cloves. It is cultivated throughout the world as a spice crop having many health benefits including its antibiotic, antitumor, cholesterol-lowering, and antithrombic effects on animal cells (Fujiwara and Natata 1967). Sexual sterility has given a very low

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rate of field multiplication and imposed a barrier on the genetic improvement of this important crop. Therefore, the improvement of this species is limited to clonal selection. Tissue culture and genetic transformation techniques are efficient tools for the breeding of many crops and could be especially applicable in the future improvement of the " genetically closed" garlic plant. However, these tools need appropriate explants and an efficient protocol for regeneration from those explants. *In vitro* regeneration systems described in garlic include direct and indirect organogenesis and somatic embryogenesis using a wide variety of explants like young leaves (Nagasawa and Finer 1988), shoot tips (Kehr and Schaeffer 1976, Fišerová et al. 2016, Ayed et al. 2018, Karn et al. 2022), basal plates (Koch et al. 1995) and flower organs (Suh and Park 1993). Research conducted by our group has first shown that root tips are the most advantageous explant to be used in the regeneration system (Haque et al. 1997). Subsequently, several reports confirmed the efficient use of root tips in garlic propagation (Haque et al. 1998b, Myers and Simon 1998, Haque et al. 1999, Barandiaran et al. 1999, Robledo-Paz et al. 2000). Using root tips of garlic cv. White Roppen was reported on regeneration of plantlets by somatic embryogenesis (Haque et al. 1998a) and organogenesis from root tip callus (Haque et al. 2000). However, root segments were recalcitrant.

In our previous studies, we observed that numerous roots are developed in the culture compared to the limited number of roots per sprouted clove; although this number varied with the cultivar character (Haque et al. 2000). The roots developed from sprouted cloves were without any branches while those from *in vitro* culture were with numerous branches. The branching nature of the *in vitro* roots gave us an idea that it might be possible to get morphogenesis from the segment of these *in vitro* roots although we have previously failed to get any regeneration from the roots developed from sprouted cloves. These *in vitro* roots that had branches could be used as explants if their totipotency is exploited by the application of proper growth regulators in the tissue culture medium. In the present investigation, we have established regeneration protocols using *in vitro* root segments via somatic embryogenesis and direct shoot bud formation.

Materials and Methods

Bulbs of a Japanese garlic cv. White Roppen were used in this study. Cloves were separated and the outer scales were removed. The cloves were surface sterilized with 70% ethanol for 30 sec and 0.1% sodium hypochlorite for 20 minutes followed by washing at least three times with sterile distilled water. The cloves were then sprouted in a sterilized semisolid medium composed of distilled water containing agar (0.8%) (Fig. 1A).

The root tips were cultured on MS medium supplemented with 1 µM NAA and 10 µM BA following the standard protocol of Haque et al. (1997). After regeneration and cultivation for two months on the same medium, lots of roots were developed in the culture vessels (Fig. 1B). Root tips and segments (1.0 cm) were cut off from *in vitro*

regenerated plants with a scalpel under sterile conditions. Explants were cultivated in Petri dishes (10 cm diameter) containing 25 ml of medium. MS basal medium was used with supplementation of 3% sucrose and solidified with 0.8% agar. For callus initiation, the medium was further supplemented with 5.0 and 0.5 µM 2, 4-D. Calli developed on root segments cultured only with 5.0 µM 2, 4-D. For somatic embryo formation, the calli were transferred to MS medium supplemented with 0.5 µM 2, 4-D, and the regeneration of plantlets from somatic embryos was accomplished on medium supplemented with 5.0 µM kinetin (Haque et al. 1998a). For direct regeneration from root segments, the explants were cultured on the medium supplemented with a combination of NAA (1.0 and 5.0 µM) and BA (10.0 and 50.0 µM). For rooting of shoots, the shoots were transferred to a growth regulator-free MS medium. For *in vitro* bulblets formation, the shoots were transferred to a growth regulator-free MS medium fortified with 12% sucrose (Haque et al. 1998b).

All the media were supplemented with 3% sucrose and 0.8% Bacto agar and adjusted to pH 5.8 before autoclaving. The media were refreshed every month. Cultures were incubated in a growth chamber at 25 ± 2°C under constant cool-white, fluorescent light. Data were collected on the basis of percentage of callus, plant regeneration, and number of shoots or plants per explant. The experimental design was completely randomized, with three replications per treatment. One replication consisted of twenty explants each. The data obtained were compared by DMRT.

Results and Discussion

The present study was designed to regenerate plants from *in vitro* root segments and compare the protocol with those we previously developed from root meristems of aseptically sprouted garlic plantlets. Callus formation was found with both root meristem and root segments from *in vitro* roots. However, the rate of callus formation was comparatively lower. Callus formation frequency was significantly different between root tip and root segment explants. Root tips had a significantly higher percentage (94.33%) of callus formation than that from root segments (51.67%) (Table 1). Callus initiation started within two weeks of culture and callus of nodular size was found in two months of culture on the medium containing 5.0 µM 2, 4-D (Fig. 1C, D). At this stage, the calli were compact in nature. No calli developed in root segments cultured on 0.5 µM 2, 4-D while callus formation was suppressed in root tips cultured on a medium containing 0.5 µM 2, 4-D. Callus initiation was confined to only one end of the root segments. Calli were induced on the cut surface of the apical end of the *in vitro* root segments. The proximal part of the root segment did not show any callusing (Fig 1C, D). After 2 months on the initiation medium with a high 2, 4-D level, the explants with embryogenic calli were transferred to a medium supplemented with a lower content of (0.5 µM) 2, 4-D for somatic embryo formation. With the transfer of the calli from 5.0 µM 2, 4-D to 0.5 µM 2, 4-D, the calli gradually turned friable in nature with a greenish-white

colour. Numerous somatic embryos were developed on the surface of the calli within another 2 months (Fig. 1E). Callus formation followed by either shoot bud formation or somatic embryogenesis from root tips of garlic was achieved at high frequency in our previous studies (Haque et al. 1998a, 2000). Direct organogenesis from root tips was also found (Haque et al. 1997). In all these cases, root tips were excised from sprouted cloves. The somatic embryos resembled with those we reported earlier (Haque et al. 1998a). The reason for callusing on one end of the root segment is obscure but might be related to the auxin gradient of the root. The failure of the root segment to induce callus at a lower concentration of 2, 4-D might be due to its inappropriate concentration while the same in high concentration of root tips to develop a usable callus might be due to the supra-optimal level for the root tips. A similar result was found in our previous study with root tips (Haque et al. 1998a).

Table 1. Callus initiation, somatic embryo formation and plantlet regeneration from *in vitro* root segments of garlic.

Explant type	% of callus formation	% of regeneration	Number of plant/explants
Root segments	51.67b	91.33b	54.8a
Root tips	94.33a	95.62a	42.2b

Figures followed by different letters differ significantly by DMRT at 5% level.

The somatic embryos were transferred to a medium supplemented with 5.0 µM kinetin for somatic embryo germination and plantlet formation. After about one month of transfer, the somatic embryos started to germinate. Plantlet regeneration was found from all the calli and there was a significant difference between calli from two explants in their potential for regeneration and a higher rate of regeneration was accounted for root tip explant (95.62%) compared to 91.33% in root segments. The germination was characterized by gradual greening of the mature somatic embryos (Fig. 1F) and different stages of germination were observed in each mass of callus (Fig. 1 G, H). The germinating somatic embryos could be easily detached from each other and also from the mother calli. They were characterized by a bipolar structure with distinct shoot and root poles (Fig. 1I). The germinating plants were transferred to a growth regulator-free medium for proper development of the plants and it was observed that the profuse regeneration of plants covered the entire surface of the conical flask (Fig. 1J). The root segment explants had a higher number of plants (54.8) regenerated per explant than the other explants had 42.2 plants regeneration per explant (Table 1) as recorded after two months of transfer of the somatic embryos to the medium. The regeneration process took about 6 months which was shorter than our previous report (Haque et al. 1998a). This difference might be due to the type of explants, their physiological condition, and the use of two different 2, 4-D levels for callus initiation and somatic embryo formation.

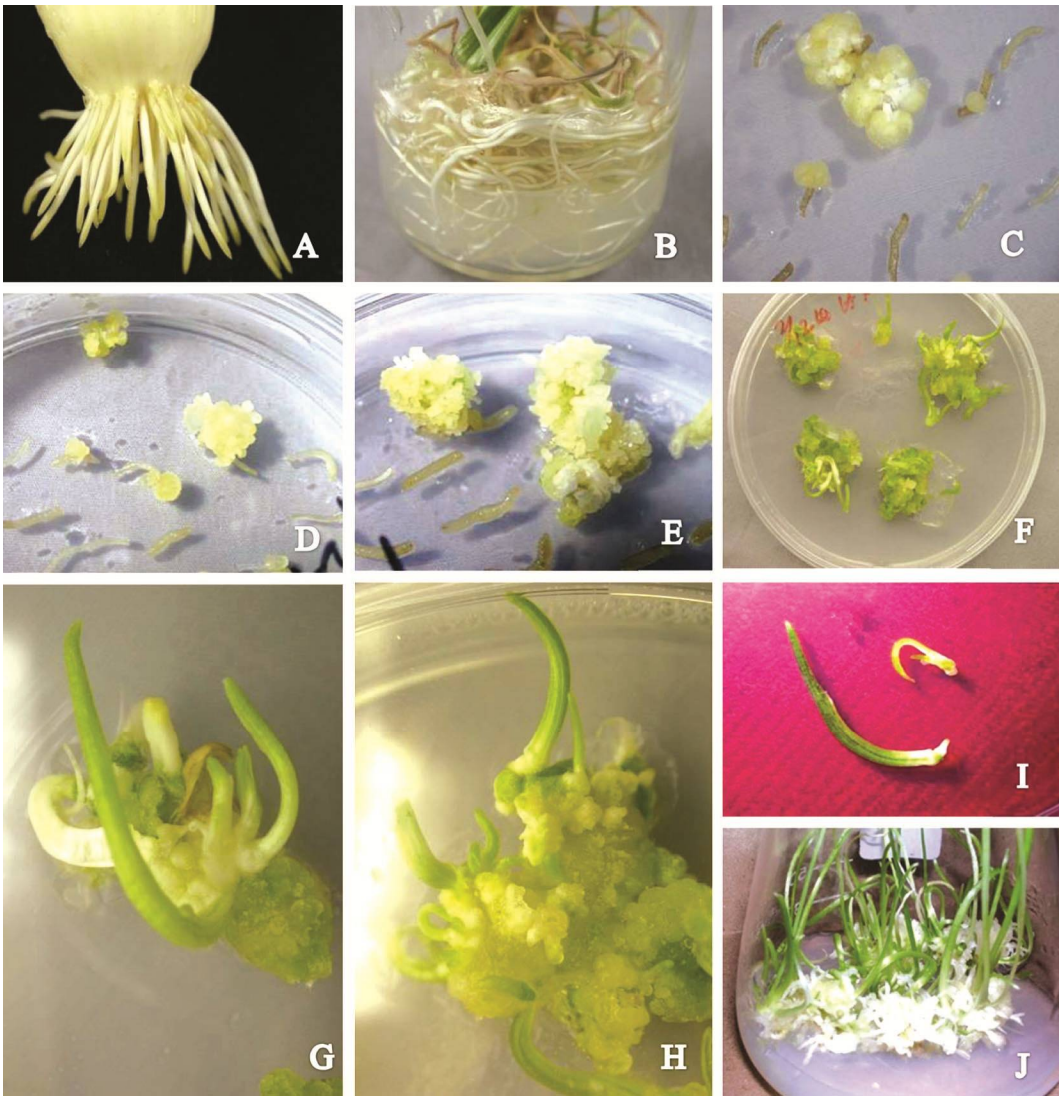

Fig. 1 (A-J). Callus induction, somatic embryo development and plantlet formation from *in vitro* root segments of garlic. Sprouted clove (A) and *in vitro* roots (B). Nodular callus formed at one end of the cultured explants (C-D). Somatic embryos formed after two months of culture (F). The embryos germinated (G-H), could be isolated from the calli and seen to have bipolar structure (I). The germinated somatic embryos were transferred to hormone free medium for further development of shoot and root (J).

Root segments were cultured on MS medium supplemented with 1.0 or 5.0 μM NAA and 10.0 or 50.0 μM BA for the induction of direct shoot buds from root segments. All the media supplements showed direct shoot bud initiation, although the frequency was relatively lower. The frequency of regeneration and the number of shoots per explant varied among the treatments. The Highest percentage (23.33%) of direct shoot bud

regeneration was found in a medium supplemented with 5.0 μM NAA and 50.0 μM BA (Table 2). There was no callus phage in the regeneration process (Fig. 2A-C). Like somatic embryogenesis, the regeneration was confined to one end of the explants only. The number of shoots per explant varied greatly and ranged from 2.33 shoots/explants in 1.0 μM NAA and 10.0 μM BA to a maximum 15.75 shoots/explants in 5.0 μM NAA and 50.0 μM BA (Table 2). Sometimes there was a single shoot (Fig. 2A), sometimes a few shoots (Fig. 2B) while in others a clump of many shoots was observed (Fig. 1C) with occasional development of roots. Rooted plants were transferred to plastic pots containing soil (Fig. 2D). The number of shoots regenerated per root segment varied from only one to as

Table 2. Effects of NAA and BA on direct shoot regeneration from *in vitro* root segments of garlic.

Concentration (μM)		% of regeneration	Number of plants/ explants	Average bulblet wt. (mg)
NAA	BA			
1.0	10.0	15.00b	2.33c	132.12a
1.0	50.0	17.50b	2.89c	101.00bc
5.0	10.0	21.67a	7.88b	356.57a
5.0	50.0	23.33a	15.75a	88.13c

Figures followed by different letters differ significantly by DMRT at 5% level.

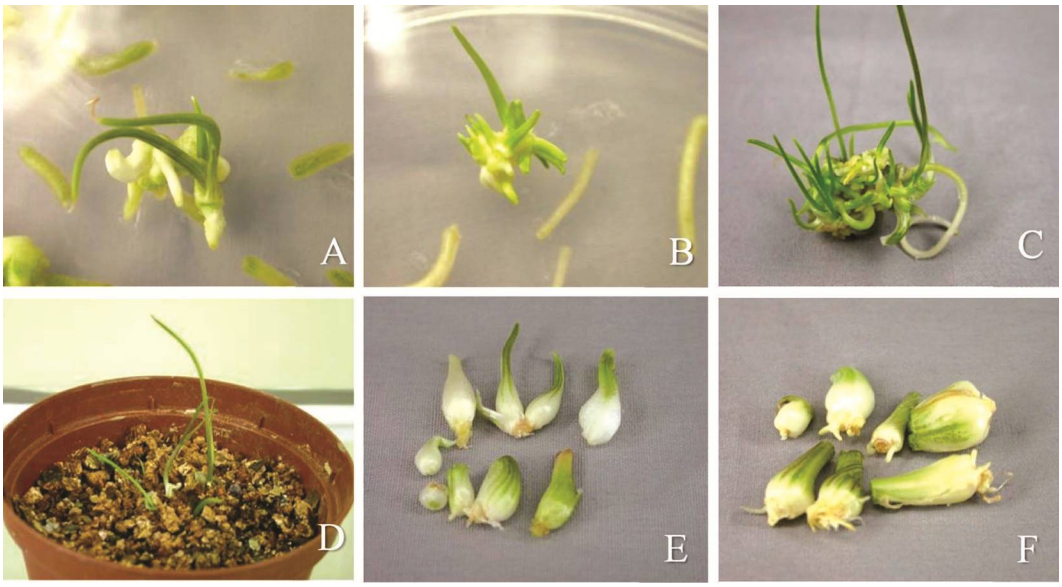

Fig. 2 (A-F). Direct shoot regeneration and bulblet formation from *in vitro* root segments of garlic. Each root segment explant regenerated a single (A) or few (B) or many (C) shoots. Rooted plantlets were transferred to plastic pots (D). The regenerated shoots produced bulblets (E, F).

many as 81. Shoots were transferred to medium without growth regulators but with higher (12%) sucrose where they proliferated and formed roots and bulblets. The individual bullet weight varied considerably. The larger bulblets were found in 5.0 μM NAA and 10.0 μM BA (Fig. 1F) while the smaller ones were from in 5.0 μM NAA and 50.0 μM BA (Fig. 1E). Direct shoot regeneration took only 2 months compared to 6 months in somatic embryogenesis which had higher percentage of regeneration (Table 1). The features of direct regeneration were the absence of a callus phase and shorter duration. These features are important for the rapid propagation of elite variety without changing the genetic fidelity (Haque et al. 2000). The production of vegetative propagules is desirable because these could be stored and planted at any time. They also avoid an acclimatization phase which is a difficult and essential process in micropropagation. Despite the relatively lower regeneration frequency, this process can become a preferable pathway as there is lots of root in the *in vitro* culture and many root segment explants can be possible from a single root. Moreover, the use of roots can give a cyclic process of harvesting the bulblets and recycling the root for the next cycle. A similar process has been developed by our group in a previous study using root tips as an explant. The present one is advantageous because of the much higher number of *in vitro* roots and the higher number of root segment explants per root (Haque et al. 1998b).

In both pathways, callus formation and direct regeneration occurred from only the apical (distal) end of the root segments. Despite the lower frequency of regeneration, the results are significant as lots of roots are regenerated from a single explant *in vitro* and several root segment explants (compared to a single root tip explant) can be excised from one root (Haque et al. 1998b, Myers and Simon 1998). The shoot regeneration system from *in vitro* root segments is promising for a year-round continuous regeneration of shoots and bulblets and use in genetic transformation studies.

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