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Shoot Regeneration and Bulblet Formation from Shoot and Root Meristem of Garlic Cv Bangladesh Local

[1]Muhammad Shahidul Haque, Tomikichi Wada and Kazumi Hattori
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
[1]Department of Crop Botany, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

Abstract: Tissue culture is considered as an alternative tool for garlic breeding as traditional breeding is not possible due to its sterility. A study was carried out to develop a protocol of regeneration in Bangladesh local garlic. Shoot and root meristems of garlic cv. Bangladesh local were cultured on Murashige and Skoog (MS) medium containing various growth regulator combinations. Regeneration from shoot meristem was found with all medium composition but highest (95.55%) in growth regulator-free medium. Shoots were transferred onto media containing BA or high level of sucrose. Forty per cent (40.00%) of the root meristem regenerated shoots on MS medium supplemented with 1 μM NAA and 10 μM BA. The shoots were multiplied on MS medium containing 0.5 μM BA. Bulblets were obtained from the regenerated shoots of garlic in all the media. It is the first to report regeneration from any Bangladeshi garlic.

Key words: *Allium sativum* L., bulblets, meristem culture, regeneration, root tip culture

Introduction

Garlic (*Allium sativum* L.) is an important and widely cultivated crop used for food and medicinal purposes. It represents a significant crop in many regions of Bangladesh. It is cultivated in 13,000 ha of land mostly after the receding of floodwater from the field. In most of the years, planting is delayed due to the prolonged flooding. Delayed planting greatly reduces yield due to the high temperature shortly after the planting and during the vegetative growth. Among the Asian countries, Bangladesh has the lowest garlic yield (Haque *et al*., 1997a) and the yield is gradually decreasing probably due to viral infection (Rahim *et al*., 1993). For the vegetative propagation, garlic is infected by a number of viruses that caused 70% yield reduction in Japan (Nagakubo *et al*., 1993). To improve the yield of garlic in Bangladesh, there is a practical need of some new cultivars suitable for relatively high temperature and either resistant to or free from viruses. Due to the difficulties of inducing flowering in this species, breeding programs have been limited to clonal selection and production of virus-free stocks via meristem culture (Barandiaran *et al*., 1998). Shoot tip culture is widely used as a mean of producing virus-free garlic. However, there is no report of regeneration of garlic plants *in vitro* in Bangladesh.

In fact, research with garlic is very limited and until now, there is no recommended variety of garlic in Bangladesh. However, production of garlic clones of high quality and productivity is urgently needed. Production of virus-free clones through meristem tip culture and virus resistant garlic by genetic engineering; induction of somaclonal variation through callus culture and production of new clones through somatic hybridization are the possible steps that can be considered for the varietal improvement of garlic in Bangladesh. Production of virus-free clones through meristem tip culture has been reported elsewhere in the world, however, production of virus resistant garlic by genetic engineering is yet to be possible. For the production somatic hybrid, established regeneration protocol is needed for both the donor and recipient cultivars. A good regeneration protocol is a prerequisite for genetic engineering also. Protocol of regeneration has been reported in most of the countries except for Bangladesh, so far the authors know. Therefore, the present study was under taken to establish a protocol of regeneration in garlic cv Bangladesh local using shoot and root meristem as explants.

Materials and Methods

The study was carried out at the Laboratory of Plant Genetics and Breeding, Graduate School of Bioagricultural Sciences, Nagoya University, Japan during 1997-98. Garlic (cv. Bangladesh Local) bulbs, stored at 4°C were separated into single cloves. The outer, dry, papery bulb scales of the cloves were removed. Healthy cloves were surface-sterilized by placing them in 70% ethanol for 30 sec and 2.0% sodium hypochlorite solution, containing two drops of Tween 20 per 100 ml, for 20 min with frequent agitation. Then the cloves were washed three times for at least 2 min in sterile distilled water. Shoot meristems measuring about 0.5 mm were excised from the cloves aseptically inside a flow cabinet, under a microscope with

the help of sterile surgical blades. The explant consisted of the shoot meristem and one or two leaf primordia.

For root tip culture, the cloves, after surface-sterilization were cultured on water-agar (0.7% agar in distilled water) medium. Within 10-15 days, the cloves sprouted and developed into plantlets with more than 20 roots and two to three leaves on the water-agar medium. Root tips measuring 2-3 mm were excised from these plantlets with the help of sterile knife inside clean bench (Haque *et al.*, 1997b).

Shoot regeneration: The shoot tip explants were cultured on five different media compositions:

1. MS growth regulator-free medium
2. MS plus 1 μM BA
3. MS plus 1 μM NAA and 1 μM BA
4. MS plus 1 μM NAA and 10 μM BA
5. MS plus 5 μM NAA and 10 μM BA

All are based on MS basal medium (Murashige and Skoog, 1962).The root tip explants were cultured on MS medium supplemented with 1 μM NAA and 10 μM BA, the best growth regulator combination for root tip culture of garlic cv. White roppen (Haque *et al.*, 1997b).

Development of the shoots and bulblet formation: Shoots developed on growth regulator-free medium were transferred to a medium that was supplemented with 0.5 μM BA and 3% sucrose for further development of the shoots. Shoots developed on media containing growth regulator(s) were transferred to growth regulator-free media containing 3 or 12% sucrose. The root tip derived shoots were transferred to growth regulator free medium containing 12% sucrose (Haque, 1996).

Media preparation and culture condition: The medium pH was adjusted to 5.8. The media were solidified by the addition of 0.8% agar. They were then autoclaved at 121°C for 20 min. Five explants were cultured in each bottle that contained 40 ml of the medium. The culture bottles were closed with polypropylene lids and additionally sealed with parafilm (Haque *et al.*, 1997b). All cultures were incubated in a growth cabinet maintained at 28°C constant temperature and illuminated with continuous light (15 μmol m^{-2} s^{-1}) provided with cool white fluorescent tubes. Each treatment was replicated thrice. Statistical analysis was done by the determination of variance analysis and Duncan's multiple range test (DMRT).

Results

Shoot regeneration: Shoot buds started to develop on the growth regulator-free medium within two weeks of culture. Shoot development was delayed on the other media that contained either BA or BA and NAA. The shoot buds on growth regulator-free medium developed into shoots approximately 2 cm long within 3 weeks. Regeneration occurred on all media tested. The effects of growth regulators were monitored by scoring the number of regenerated shoots and observing the morphological differences among the regenerants on media containing the growth regulators. The percentage of shoot regeneration varied from 2.11 to 95.55% (Table 1). No particular growth regulator supplement was observed to be distinctly better than the growth regulator-free medium. The highest regeneration was found on hormone-free medium (95.55%). Addition of 1 μM BA lowered the shoot regeneration to only 58.33%. Inclusion of both NAA and BA, at a concentration of 1 μM each, increased the percentage considerably. Increasing the BA concentration further gave a frequency comparable to the growth regulator-free medium. The lowest regeneration was found with the medium containing 5 μM NAA and 10 μM BA (Table 1). The supplements of BA or BA and NAA suppressed the regeneration of shoot buds in a dose-dependent manner. The inhibitory effect of NAA was more pronounced than that of BA, and an addition of

Table 1: Frequency of shoot regeneration from shoot meristem of garlic cv. Bangladesh local as influenced by growth regulators

Media composition	Percentage of explant regenerating shoot
Growth regulator-free MS	95.55±3.85a
MS + 1 μM BA	58.33±8.33c
MS + 1 μM NAA and 1 μM BA	80.55±4.81b
MS + 1 μM NAA and 10 μM BA	87.22±4.20ab
MS + 5 μM NAA and 10 μM BA	2.11±3.66d

Mean values (± standard deviation) followed by different letters are significantly different at $P< 0.05$.

Table 2: Shoot organogenesis from root tip explant of garlic cv. Bangladesh local*

Regenerative characters	Frequency
% Explant with shoot	40.0
%Explant with Callus	33.0
Number of shoot/explant	4.0
% Explant with bulblet	60.0

*Root tips were cultured on MS medium containing 1 μM NAA and 10 μM BA.

Table 3: Bulblet formation from shoot and root tips of garlic cv. Bangladesh local on different media

Medium used	Bulblet		
	weight (g)	diameter (cm)	length(cm)
Shoot tips			
Hormone-free MS[a]	0.27a	0.66	1.17a
MS+12% sucrose[a]	0.30a	0.63	1.09ab
MS+0.5 μM BA[b]	0.13bc	0.55	0.81bc
Root tips			
MS+12% sucrose[a]	0.17b	0.50	0.62c

[a] Shoot regeneration occurred on MS medium containing 1 μM NAA and 10 μM BA [b] Shoot regeneration occurred on hormone-free MS medium. Mean values followed by different letters are significantly different at 5% level by DMRT.

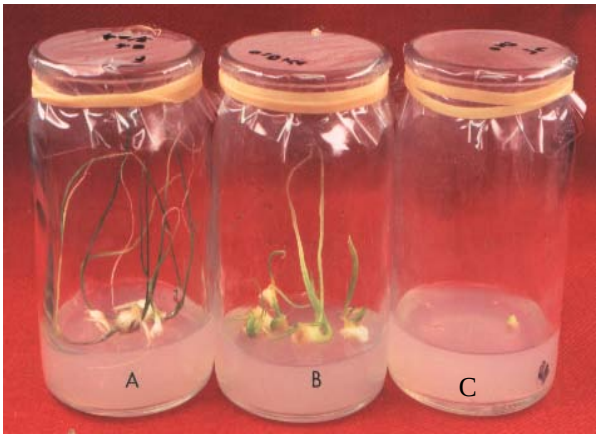

Fig. 1: Effect of growth regulators on shoot regeneration from shoot meristem of garlic cv. Bangladesh local. (A) Shoot regeneration on growth regulator-free medium. (B) Shoot regeneration on medium containing 1 μM NAA and 10 μM BA. © Shoot meristems on medium containing 5 μM NAA and 10 μM BA either failed to grow or formed small callus

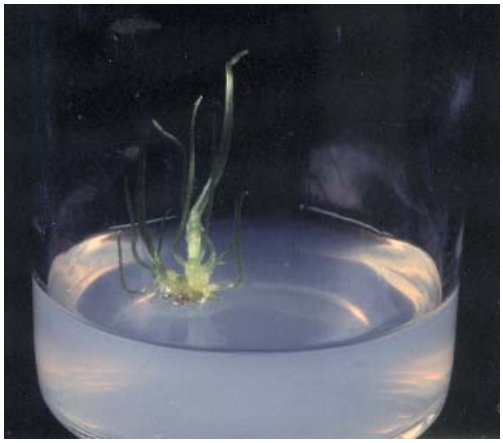

Fig. 2: Shoot regeneration from root tip explant. Root tips were cultured on MS medium containing 1 μM NAA and 10 μM BA.

5 μM NAA nearly blocked bud growth and differentiation. However, a balance between BA and NAA gave better performance and a higher BA:NAA ratio was suitable combination, which was still inferior to the growth regulator-free medium in term of their ability to induce the shoot buds.

As for the morphological changes, shoots regenerated on growth regulator-free medium were weak (Fig. 1A), pale and long, whereas those on media containing BA or BA and NAA were thick, deep green and shorter (Fig. 1B). On the growth regulator-free medium, there was only one shoot developed from one shoot meristem. Media containing growth regulators had more than one shoots per explant. The medium containing 5 μM NAA and 10 μM BA had rare shoot differentiation (Fig. 1C). In stead the explants on this medium had callus formation to a small extent. However, no shoot regeneration occurred from the calli. Shoots on growth regulator-free medium had swelled at the base indicating bulblet formation. Shoots on other media did not have any swelling. There was no root development on any of the media.

Shoot regeneration was achieved directly from the root tips cultured on MS medium supplemented with NAA and BA (Fig. 2). The percentage of shoot regenerating root tip explants was only 40.00% (Table 2). This percentage is much lower than that of shoot meristem. However, there were more than 20 root tip explants compared to only one shoot tip explant per clove. There was a considerable percentage (33.00%) of callus formation from root tip explants that did not have direct shoot formation. However, shoot formation from these calli was not possible. The root tips had many shoots per explant (Table 2, Fig. 2). Regeneration from root tip occurred within one month of culture on the medium. Like regeneration from shoot meristem, regeneration from root tips did not involve callus formation.

Shoot development and bulblet formation: The regenerated shoots were transferred to a proliferation and bulblet formation medium. Shoots developed on growth regulator-free medium were transferred to a medium that was supplemented with 0.5 μM BA and 3% sucrose for further development. On the contrary, shoots developed on media containing growth regulator(s) were transferred to growth regulator-free media containing 3 or 12% sucrose. The shoots were growing well on all media tested. Shoots developed on growth regulator-free medium were elongating quickly on the medium containing 0.5 μM BA. The shoots developed on media containing BA or NAA and BA were also developing well on growth regulator free medium containing 3% sucrose. Shoot development was restricted on the medium containing 12% sucrose. Shoots were shorter and thicker. Shoots from growth regulator-free medium developed fewer leaves.

After about 3 weeks of culture on these media, there was bulb-like swelling at the bases of the shoots. Shoots that were taken from growth regulator-free medium and cultured on medium containing 0.5 μM BA were forming bulblets earlier. It was evident that there was bulblet formation in all media. However, bulblet size varied on different media. The variation was the most pronounced in the bulblet fresh weight. A medium containing 12%

sucrose had higher fresh weight of the bulblets (0.30 g) while medium containing 0.5 μM BA had the lowest bulblet weight (0.13 g) (Table 3). Diameter of bulblets produced on different media did not differ significantly. However, the hormone-free medium supplemented with 3% sucrose had slight superiority over the other two media. The hormone-free medium supplemented with 3% sucrose had significantly higher bulb length (Table 3). The shoots regenerated from root tips were transferred to MS medium containing 12% sucrose. There was bulblet formation from 60.00% explants (Table 2). The root tip derived bulblets were comparatively smaller than those derived from shoot tips.

Discussion

This result indicates that a high frequency of shoot regeneration can be obtained in shoot meristem culture of garlic cv. Bangladesh local. There was a strong influence of the growth regulators on the percentage of shoot formation (Table 1). All the growth regulator combinations used were inferior to the growth regulator-free medium. The medium without any growth regulator had the highest shoot regeneration frequency of 95.55% (Table 1). There was a gradual decrease in the regeneration frequency with increasing growth regulator concentration. Regeneration was almost suppressed by the addition of 5 μM NAA and 10 μM BA to the medium. Ayabe and Sumi (1998) also reported similar dose-dependent suppression of bud formation from stem-disc of garlic due to the additions of NAA and BA. However, higher growth regulator concentrations were suitable for shoot regeneration from other explants. There was 40% shoot regeneration from the root tips. In spite of the lower rate of regeneration from root tip compared to shoot tip, it is very significant because a single clove gives more than 20 root tip explants in this cultivar and we found more than 40 root tips in another cultivar (Haque *et al.,* 1998).

Shoot bud differentiation was direct and without the formation of any callus in both the explant types. A large callus formation can induce somaclonal variation in the regenerants from garlic tissue culture (Maggioni *et al*, 1989; Novak, 1980). Thus, a method of regeneration involving no callus formation phase is preferred for the production of clones of true-to-type (Haque *et al*., 1997b; Haque *et al*., 1999).

Table 3 shows that 12% sucrose is best for bulblet weight only and hormone free MS medium is best for bulblet diameter and length. However, the medium containing 3% sucrose also had bulblet formation comparable to the 12% sucrose. In a previous study with root tip of 'White roppen', bulblet formation was rare on the medium containing 3% sucrose and best on 12% sucrose (Haque *et al*., 1998). This differential response of shoot tip of cv. Bangladesh local might be due to the cultivar and explant type.

Number of bulblets produced per explant was almost similar on all media tested. There was an apparent effect of the initiation media on the number of shoots and bulblets produced per shoot meristem. Shoots that were taken from growth regulator-free medium and subsequently cultured on medium containing 0.5 μM BA formed only one bulblet per explant. While shoots that were taken from BA and NAA supplemented media and subsequently cultured on growth regulator-free media containing 3 or 12% sucrose formed more than one bulblet per shoot tip.

In this study, all the shoots had bulblet formation. *In vitro* bulblets are preferred to plantlets that need acclimatization step before their transfer to field. Bulblets are easy to handle, store, transport and maintain. They are easy to transfer to the field and 80 to 100% sprouting of the bulblets was possible.

Almost all commercially cultivated garlic plants have been reported to be infected with a complex of viruses (Walkey, 1990; Sako *et al*., 1991; Van Dijk and Sutarya, 1992; Sumi *et al*., 1993; Barg *et al*., 1994; Tsuneyoshi and Sumi, 1996). Shoot tip culture alone or in combination with thermo-therapy has been used globally for eliminating viruses from infected plantlets and for producing virus-free seedlings (Ayabe and Sumi, 1998). Unfortunately no step has been taken for the detection and elimination of viruses in Bangladesh. Garlic yield is gradually decreasing due probably to the infection by viruses like other countries (Rahim *et al.,* 1993). This is the first report of regeneration from Bangladeshi garlic and the present study can be considered as an important step toward the elimination of viruses and production of virus-free garlic in Bangladesh. This report of bulblet formation from shoot meristem can be used as a protocol of producing virus-free garlic in Bangladesh and regeneration from root tips has the potential for the multiplication of the virus-free clones. Virus detection in the mother plant and the regenerated plantlets remains for future study.

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