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Detection of viruses of Bangladeshi and Japanese garlic and their elimination through root meristem culture

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Abstract

A number of viruses cause considerable yield loss and quality deterioration in garlic. Root meristems of virus infected plants are known to be free from detectable viruses. This potentiality could be exploited to obtain virus free clones at a high frequency by culturing excised root meristems *in vitro*. We have developed efficient methods of direct and somatic embryo derived shoot regeneration from root meristems of garlic. The objectives of this work were to detect viruses infecting Bangladeshi and Japanese garlic clones and find an easy and efficient method of eliminating the viruses for the improvement of both yield and quality of garlic. At first, we confirmed the presence of detectable viruses in three Bangladeshi and one Japanese clones. The clones were infected with four different types of viruses: Garlic viruses (GarVs), Onion yellow dwarf virus (OYDV), Leek yellow stripe virus (LYSV), and Garlic common latent virus (GCLV). To eliminate those viruses, as per our previous method, root meristems were cultured on MS medium supplemented with 1.0 µM NAA and 10.0 µM BA. Shoot primordia developed from the cultured explants within 1 month. The regenerated individual shoot buds (2-5 mm) were separated from the mother explants and transferred to growth regulators free medium. RT-PCR confirmed that the viruses present in the mother garlic plants were absent in the shoots found after two-step culture. The regenerated shoots were rooted on growth regulator free medium and transferred to pots. Results indicated that the plants remained free from LYSV. Virus elimination through root meristem culture emerged as an efficient novel technique for the eradication of multiple viruses as confirmed by RT-PCR in this study. This technique has the potential for the production and supply of virus free propagules (plants/bulblets) for the yield and quality improvement of garlic.

Key words: Root meristem, RT-PCR, two-step culture, virus detection, virus elimination

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Introduction

Garlic (*Allium sativum* L.) is one of the most important spicy vegetable crop as well as a medicinal herb with numerous health benefits like antibiotic, antitumor, cholesterol lowering, and antithrombic effects on animal cell (Fujiwara *et al*., 1967). Almost all garlic clones cultivated throughout the world are infected with a variety of viruses that cause significant reduction in yield and quality (Fujiwan, 1989; Walkey, 1990; Lot *et al*., 1998). The viruses infecting garlic include leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV), shallot (garlic) latent virus (SLV, GLV), and garlic common latent virus (GCLV) (Walkey, 1990; Sako *et al*., 1991; van Dijk, 1991, 1993a, b; Conci *et al*., 1992; van Dijk *et al*., 1992; Barg *et al*., 1994;

Tsuneyoshi *et al*., 1996). In spite of extensive studies on detection and elimination of viruses in the other countries of the world, there have been limited efforts in Bangladesh.

Given that there are no available antivirus remedies in garlic; meristem-tip culture in many cases aided by thermotherapy is considered to be the main and well established way of producing virus-free plants (Walkey, 1990). In spite of the possibility of re-infection, garlic shoot meristem culture has long been practiced for virus elimination (Bhojwani, 1980; Verbeek *et al*., 1995) and will continue, since source of virus resistance among garlic and its wild relative is yet to be found to develop virus resistant garlic using biotechnological approaches. However, the production and propagation rate of virus-free plantlets by shoot meristem culture is very low (Ayabe *et al*., 2001). Moreover, excision of meristems containing only two primordial leaves under microscope is a laborious, expensive, inefficient and time-consuming procedure. Therefore, development of an alternative easy method would provide sustainability in and improve the efficiency of virus elimination.

A zone of variable length near the shoot or root tip has long been reported to be free of virus or contains very little virus (Mori *et al*., 1982). Root tips of virus infected plants were also reported to be free from detectable virus (Appiano *et al*., 1983) and the first 400 µm of the root tip, which included the root cap and the meristem, were virus free. This potentiality could be exploited to obtain virus free clones at a high frequency by culturing excised root meristems *in vitro*.

We have developed a method of direct shoot regeneration from root tips in garlic (Haque *et al*., 1997) which is more practical and efficient than any other previous methods (Havrenek *et al*., 1973; Kehr *et al*., 1976; Abo El-Nil, 1977; Nagakubo *et al*., 1993). Histological study revealed that the regeneration occurred from the root meristems and it was interesting to observe that the regenerating buds had no vascular connection with the root explants which could block the transmission of virus from the infected mature tissues of the explants to the regenerating buds (Haque *et al.*, 1999). With this background, we attempted to produce virus free plants by root meristem culture. The objectives of this study were to detect viruses in Bangladeshi and Japanese garlic using RT-PCR, produce virus free garlic plants by root meristem culture.

Materials and Methods

***Plant material*:** Garlic (*Allium sativum* L.) bulbs of a Japanese typical cultivar, white roppen were collected from a seed company. Bulbs of three Bangladeshi garlic clones (G1, G3 and G14) were carried to Nagoya University, Japan. The cloves were separated, outer dry scale leaves removed and surface sterilized first with 70% ethanol for 30 sec and then with 0.1% sodium hypochlorite for 20 min followed by three times washing with sterile distilled water. The cloves were then cultivated on 0.7% agar for sprouting and plantlet formation (Figure 1a). Leaf samples were collected from these *in vitro* grown plantlets. Root tips were excised from theses plantlets after 10 to 15 days. Axenic root-tips were obtained from *in vitro* garlic shoots cultured following the protocol of Haque *et al*. (1998).

***Root tip culture*:** Root-tips (2-3 mm long) including the meristems were cut off from the plantlets obtained after culture of the sterilized cloves on agar and also from the micropropagated plantlets with a scalpel under sterile conditions. Explants were cultivated in petri dishes containing 25 ml of MS (Murashige and Skoog, 1962) medium supplemented with 1 µM NAA and 10 µM BA (Haque *et al*., 1997). All media were fortified with 3% sucrose and solidified with 0.8% agar (BA 30) and adjusted to pH 5.8 prior to autoclaving. After 35 to 40 days when the shoot became ca. 5 mm, they were cut off from the mother explants and transferred to growth regulator free MS medium for their further development and rooting. The media were neither changed nor refreshed during the culture period. Cultures were incubated in a growth chamber at 28 ± 2^{O}C under a constant light condition.

***Molecular method of virus detection*:** RT-PCR was used to detect garlic viruses according to the published protocol (Tsuneyoshi *et al*., 1996). In the RT-PCR analyses, reverse transcription (RT) was performed using a first-strand cDNA Synthesis Kit (Pharmacia) with approximately 1-2 µl of the RT mix was added to 100 µg of a polymerase reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 2.5 U Taq polymerase (GIbco BRL), and 100 ng each of the upstream and downstream primers. Thirty reaction cycles were used, with periods of 30 s for annealing at 50^{O}C, 45 s for synthesis at 72^{O}C and 45 s for melting at 94^{O}C. Following PCR, 10 µl portions of the reaction mixtures were analyzed on 1.5% agarose gels. The primers and the sequences used for detecting the respective viruses are presented in Table 1. The primer sequences for allin lyase, used as the positive control of the RT-PCR experiment, are 5-TCTGGTAGTCGATTTGGGTCG-3′ and 5-GCCGTAGCATTAGGATGTATG-3′.

***Plantlet establishment*:** Rooted shoots from virus free culture were washed thoroughly to remove media. Then they were planted in small plastic pots containing vermiculite and kept in an incubation room at 23 ± 2^{O}C under a constant light condition for acclimatization.

Results and Discussion

Four primers were selected for garlic virus detection (Table 1). Plants grown from garlic cloves *in vitro* were tested for the presence of leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV), garlic common latent virus (GCLV) and garlic viruses (GarVs), all of which have been reported to be present in garlic elsewhere in the world. Randomly selected plants from each clone were tested for four virus infection. RT-PCR result indicated that all three garlic clones of Bangladesh were infected by viruses. A total of four viruses belonging to three distinct genera were identified from infected garlic plants based on partial cDNA cloning and sequencing of their genomes (Figure 1). Onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV) were detected in all three clones of garlic (G1, G3 and G14). The garlic viruses were detected from only one (G1) clone, while garlic common latent virus (GCLV) was detected from clone G3 and clone G14. Allinase and actin were used as a positive control and they were found to be present in all the three clones. RT-PCR analysis of the samples indicated that no amplified DNA fragment was detected in the clone G1 for GCLV and in clones G3 and G14 for GarVs (Figure 1). Each of the three clones showed to bear at least three viruses. Figure 1 indicates also that there was strong allinase activity in the garlic clones, although the intensity of banding varied among the clones. Clone G14 had the most prominent band, while the clone G3 had the least prominent banding. The presence of allinase proves that the samples were garlic. We detected four different types of viruses in Bangladeshi garlic clones.

The yield and quality of garlic are gradually decreasing in Bangladesh. The impact of viruses on garlic production may be significant here, but little is known about the occurrence or identity of specific viruses. Farmers keep garlic bulbs to use as seed bulb in the following growing season. The cloves act as a carrier of viruses. There are limited reports of the development of symptoms indicative of viral infection, primarily chlorotic spots and yellow stripes on leaves. In spite of extensive studies in Italy (Bellardi *et al*., 1995), France (Messiaen, 1994), Japan (Nagakubo *et al*., 1994), Java (Sutarya, 1994), Australia (Sward *et al*., 1994) and some other Asian countries (Barg *et al*., 1994), no study has been conducted leading to detection and identification of garlic viruses in Bangladesh. The detection of viruses in this study will provide important information and work as a guide line for the future researches aiming at garlic improvement.

***Shoot regeneration from root meristem*:** The apical 2 mm of the garlic root tips that contained the meristems were excised from aseptically sprouted garlic cloves (Figure 2A). The explants were cultured on MS medium supplemented with 1.0 µM NAA and 10.0 µM BA for shoot initiation (Haque *et al*., 1997). Shoot regeneration occurred from garlic root meristems

within two weeks (Figure 2C). Root tip explants derived from *in vitro* plantlets (Figure 2B) were also excised and cultured similarly to assess their regeneration capacity and suitability of virus elimination. Shoot bud regeneration occurred from *in vitro* root meristems (Figure 2D) also but took a slightly longer time than that from root meristems of sprouted cloves. It is evident from the result that sprouted clove had relatively higher percentage of shoot regeneration (80%) comparative to the *in vitro* root meristems that had 75% regeneration of shoots (Table 2). However, *in vitro* root meristems were found to be superior to the root meristems of sprouted cloves in number of shoots per explants and the rooting ability of the regenerated shoots. *In vitro* root meristems regenerated 13.8 shoots/explant while root meristems from sprouted cloves had 12.6 shoots/explant. Ninety per cent of the regenerated shoots from *in vitro* root meristems rooted on growth regulator free medium compared to 85% rooting of shoots from root meristems of sprouted cloves (Table 2). Shoot initiation was evident directly from the meristematic region of the root, and neither shoots nor callus developed from the cut surface or non-meristematic part of the explant. There was no intervening callus phase in the regeneration process as was also confirmed by histological study previously (Haque *et al*., 1999). It is worth mentioning that the number of shoots produced per explant was appreciable. The regenerated shoots were rooted on growth regulator free MS medium where the shoots proliferated and produced numerous roots.

Table 1. Primer sequences for RT-PCR detection of garlic viruses

Target viruses	Primer sequence	Corresponding regions of the primer	Amplified size
GarVs	N-RT1: 5′-CCTGCTAAGCTATATGCTGA-3′ N-RT2: 5′-GTAAGTTTAGCGATATCAAC-3′	3′-terminal ORF 3′-non coding	182-185bp
OYDV	ON-RT1: 5′-GAAGCGCACATGCAAATGAAG-3′ ON-RT2: 5′-CGCCACAACTAGTGGTACAC-3′	CP gene 3′-non coding	290bp
LYSV	P-RT3: 5′-AAGATCAACACTTGGTTTG-3′ P-RT4: 5′-GGTCTCAATCCTAGCTAGTC-3′	3′-non coding 3′-non coding	191bp
GLV/SLV	GS-RT1: 5′-TATGCTCGAGCTCGTAGAGC-3′ GS-RT2: 5′-GGGTTTCACATTGTTACACC-3′	3′-terminal ORF 3′-terminal ORF	170bp

Table 2. Shoot regeneration and multiplication efficiency of garlic rot meristem

Source of root	% Regeneration	No. of shoot/explant	Rooting (%)
Sprouted clove	80	12.6	85
In vitro roots	75	13.8	90

***Virus detection*:** In our study, we used RT-PCR, which has routinely been used for detection of viruses in many plants including garlic, to detect viruses from the regenerated shoots from garlic root meristem explants. The regenerated shoots as well as the mother plantlets showed the presence of viruses (Table 3 and Figure 1). The mother plant showed the presence of three (OYDV, LYSV and GCLV); while the regenerated shoots indicated the presence of LYSV. OYDV and GCLV were eliminated by this process of one step culture. Since, two viruses were eliminated by the one step culture was possible as indicated by RT-PCR detection method, success of total elimination is expected through additional measures. We followed a two-step method of culture where the tiny regenerated shoot buds (<5 mm in size) were separated out by cutting with the help of a knife and transferred to another media. They grew well in growth regulator free

MS medium (Figure 2E & F) and formed well developed roots. The shoots developed roots within 3 weeks and formed rooted plantlets (Figure 2G & H).

Table 3. Summary of RT-PCR detection of viruses from mother plants and regenerated shoots of garlic from root meristem

	Samples						
Viruses	G1	G3	G14	WR	CR	CRII	IRII
GarVs	+	-	-	-	-	-	-
OYDV	+	+	+	+	-	-	-
LYSV	+	+	+	+	+	-	-
GCLV	-	+	+	+	-	-	-
Allinase	+	+	+	+	+	+	+
Actin	+	+	+	+	+	+	+

+: Positive; -: Negative; GarVs: Garlic Viruses; OYDV: Onion yellow dwarf virus; LYSV: Leek yellow stripe virus; GCLV: Garlic common latent virus; G1, G3 and G14 are three Bangladeshi clones; WR: White roppen, a typical Japanese garlic; CR: Regenerants from clove derived roots; CRII: Regeneratnts from clove derived roots and separated at the early stage; IRII: Regenerants from *in vitro* roots and separated at the early stage.

The process completed within 2 month period and it was possible to obtain up 12 plantlets per root tip explant and over 300 plants per garlic clove. These shoots after two-step culture were examined using RT-PCR for the total elimination of four viruses. It was interesting to note that the test was negative for all the four viruses indicating that the shoots were free from all viruses tested. The root tip culture followed by separation of the regenerated tiny shoots (<5 mm) from the mother explants (the two step process) was confirmed as an efficient method of virus elimination using root meristem (Haque *et al*., 2007). This result proves that root tip culture can be an efficient technique for the eradication of multiple viruses from garlic.

As we reported earlier, shoot buds regenerated from root meristems and had no vascular connection with

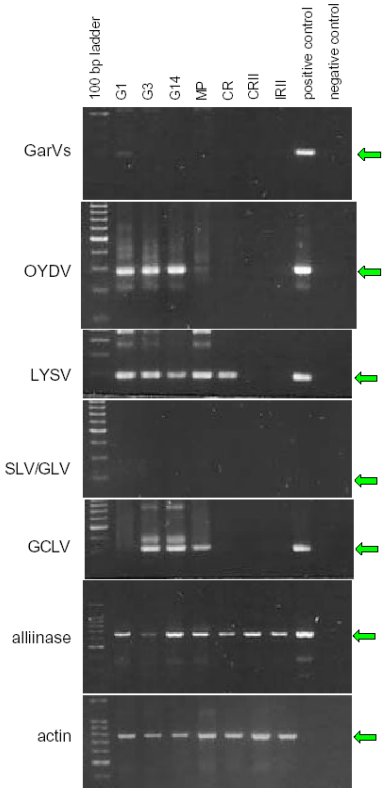

Figure 1. RT-PCR amplification of viral cDNA. Arrows indicated precise amplicons. Alliinase and actin were used as internal control. GarVs: Garlic Viruses; OYDV: Onion yellow dwarf virus; LYSV: Leek yellow stripe virus; GCLV: Garlic common latent virus. G1, G3 and G14: three Bangladeshi clones; CR: Regenerants from clove derived roots; CRII: Regeneratnts from clove derived roots and separated at the early stage; IRII: Regenerants from *in vitro* roots and separated at the early stage. cDNA clone of SLV/GLV was not available.

the mother explant (Haque *et al.*, 1999). We hypothesized that the shoots might be free from viruses. To test the hypothesis, first we examined the regenerated shoots that had not been separated from the mother explants at the early stage of development. The mother plants from which the shoots were regenerated were also tested in the same process to find out whether the mother plant was infected by virus at all, and if infected, what were the viruses. We performed

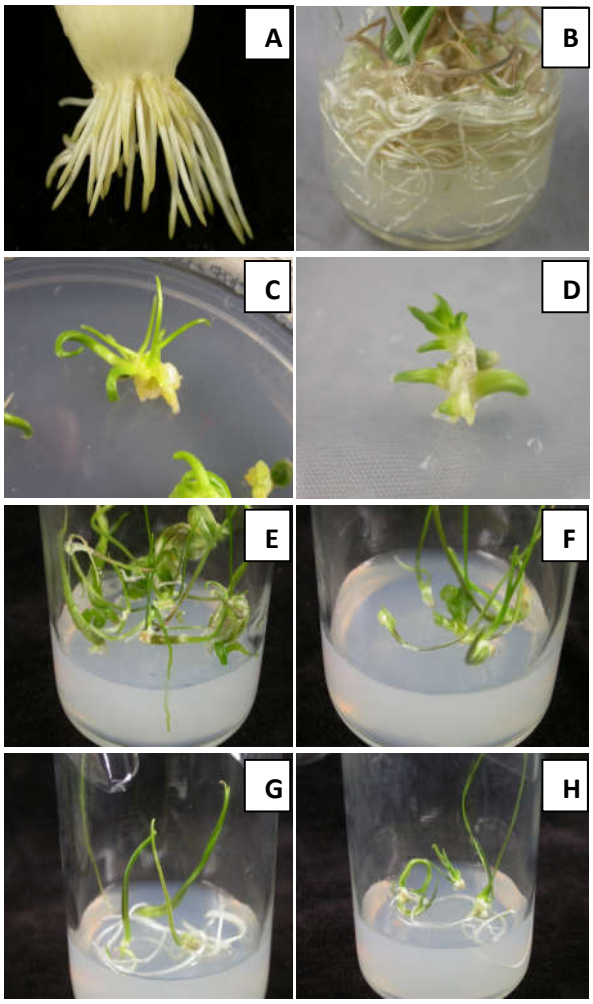

Figure 2. Regeneration from garlic root meristem and production of virus free plantlets. A) A sprouted clove with numerous roots, B) *In vitro* roots, C) Regenerated shoot buds from root meristem of a sprouted clove, D) Regenerated shoot buds from *in vitro* root meristem, E) Shoots buds from root meristem of a sprouted clove transferred to growth regulator free medium, F) Shoots buds from *in vitro* root meristem transferred to growth regulator free medium, G) Rooted shoots from root meristem of a sprouted clove transferred to growth regulator free medium, H) Rooted shoots from *in vitro* roots transferred to growth regulator free medium.

RT-PCR to test the presence or absence of four frequently infecting garlic viruses in the mother plants and to know whether the viruses were completely or partially eliminated by root meristem culture. The study confirmed that the mother plants were infected with OYDV, LYSV, and GLV while GarVs were not found in the sample. It was evident that the regenerated shoots were partially free from the viruses fro which the tests were performed. It means that OYDV and GLV were eliminated but LYSV could not be. Hence, our hypothesis was partially true and needs some additional precautionary measures. It proved once again that the root meristems were free from viruses (Appiano *et al*., 1983). The presence of LYSV in the regenerated shoot indicates that the regenerated shoots might be free from all viruses but infected later on during the shoot development.

The regenerated shoots were rooted on growth regulator free medium. They were hardened and transferred to pots containing vermiculite. They were taken proper care to grow until maturity. All the samples taken from IRII remained free from LYSV. This finding indicated that the virus free plantlets obtained from garlic root meristem culture by a two-step process remained virus free until maturity. This also indicates the applicability of this process for the rapid and cyclic production (Figure 3) of virus free propagules.

Conclusion

Like shoot meristems, root meristems are also known to be free from viruses but virus elimination and production of virus free plants through root meristem culture has not been possible due to the recalcitrant nature of root meristem to *in vitro* regeneration. In this study, we developed an efficient novel technique for the eradication of multiple viruses and confirmed it by RT-PCR. It is simple and easier than the previous methods of virus elimination through shoot meristem culture coupled with thermotherapy. This technique has the potential for the year round production and supply of virus free propagules (plants/bulblets) of garlic for the improvement of its yield, quality and rate of multiplication. This information would be useful for virus eradication in other plants in future.

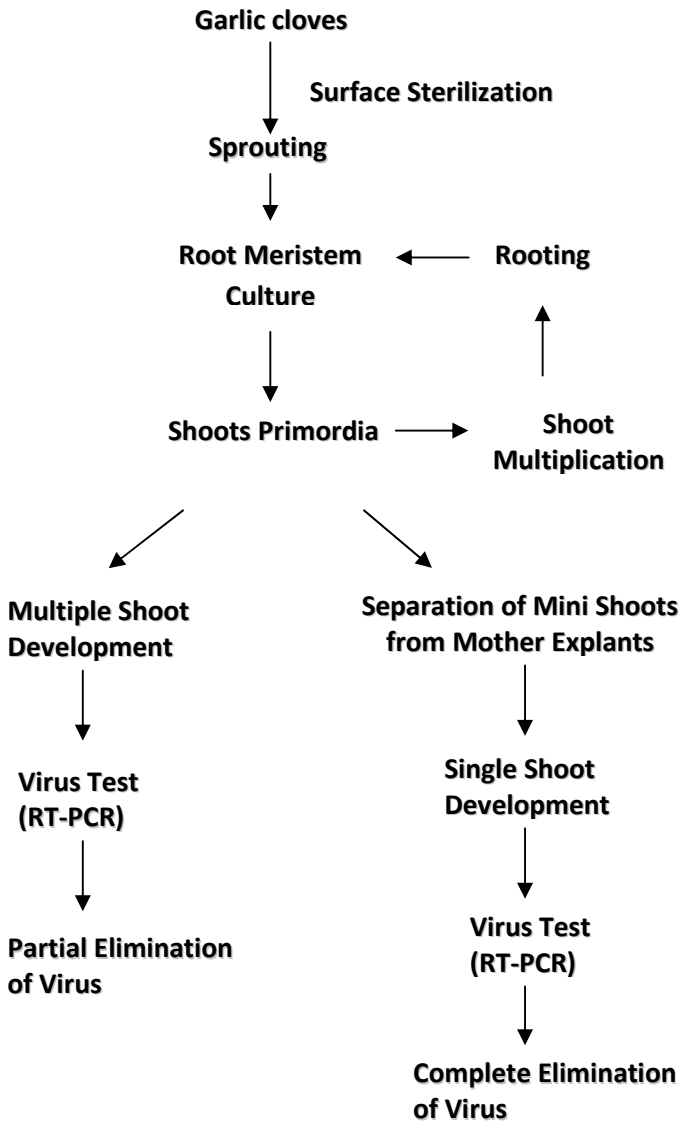

Figure 3. Diagram showing the elimination of different viruses of garlic by root meristem culture

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