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A novel and efficient tissue culture method – "stem-disc dome culture" – for producing virus-free garlic (*Allium sativum* L.)

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Abstract Using our previously reported stem-disc culture method as a basis, we have developed an efficient tissue culture method, "stem-disc dome (SD-Dome) culture", to eliminate viruses from infected garlic plants. Fifteen to 25 dome-shaped structures that formed on stem-disc explants from a single garlic clove were excised and maintained on phytohormone-free Linsmaier and Skoog medium, as in the usual shoot-tip culture. These excised dome-shaped structures grew independently into more than 5-cm-long shoots, rooted after 8 weeks, and were successfully transplanted to soil. The resulting plants showed no viral symptoms on their leaves, even when cloves of garlic plants with severe mosaic and yellow streak symptoms were used for the cultures. Examinations of virus infection in three generations of the progenies using the direct tissue blotting immunoassay and reverse transcription polymerase chain reaction showed that viruses were excluded during culture. Microscopic observations of transverse sections of tissues in different developmental stages during stem-disc culture – dome-shaped structures, shoot buds, and shoots – suggested that the elimination of viruses may be correlated with the stage of development and organization of vascular-bundle structures.

Keywords *Allium sativum* · Garlic · Stem disc · Tissue culture · Virus-free

Introduction

Garlic (*Allium sativum* L.), an economically important vegetable with wide culinary and medicinal applications, is cultivated throughout the world. Traditionally it has been cultivated vegetatively because of sexual sterility and, as a result, viral infection constitutes a very serious problem. Almost all commercial garlic plants have been shown to be infected with a complex of viruses that 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 and Sutarya 1992; Barg et al. 1994; Tsuneyoshi and Sumi 1996). Recently, Sumi et al. (1993, 1999) identified novel rod-shaped, garlic viruses (GarVs) A–D, which are classified in the newly ratified genus, *Allexivirus*.

Tissue culture provides a useful technique for eliminating viruses from infected plantlets and for producing virus-free garlic seedlings. Although shoot-tip culture has been used for this purpose (Bhojwani 1980; Walkey et al. 1987), the propagation rate of virus-free plantlets is very low. Moreover, it is a laborious, time-consuming procedure. While various tissue culture techniques have been reported to improve the efficiency of propagation (Havránek and Novák 1973; Kehr and Schaeffer 1976; Abo El-Nil 1977; Nagakubo et al. 1993), all have drawbacks as practical methods: the need for long-term cultivation, relatively low propagation rates, and the need for skilled techniques.

We recently established a novel, practical tissue culture method for garlic in which the stem disc is used as an explant (Ayabe and Sumi 1998). During the early stage of "stem-disc culture", multiple, dome-shaped structures appear on the surface of the stem-disc explant. Histological observations show that both the internal cell organization and formation process of these structures are similar to those in the shoot-tip of the garlic clove. These findings suggest that individual dome-shaped structures have properties equivalent to those of shoot tips, including the property of being virus-free.

Here we report that the dome-shaped structure of garlic can replace the shoot tip as the tissue-cultured explant used to produce virus-free seed plants. We speculate that

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the stem-disc culture method will prove to be a useful experimental tool for studying the protection mechanism of the shoot tip that functions against virus invasion.

Materials and methods

Plant material

The two garlic (*Allium sativum* L.) cultivars, Fukuchi-howaito and Hokkaido-zairai, grown at our experimental farm in Hokkaido, Japan were used. We had earlier checked for virus infection by the reverse transcription polymerase chain reaction (RT-PCR), so that only virus-infected garlic individuals were used in this experiment.

Preparation and culture of the dome-shaped structure

Stem-disc culture was carried out as reported by Ayabe and Sumi (1998), and the dome-shaped structures that appeared on the surfaces of stem-disc explants were excised. The separated dome-shaped structures were re-cultured on solid LS medium (Linsmaier and Skoog l965) under the same conditions and grew into shoots with roots. These shoots were transferred to pots filled with soil and cultivated in a screen house.

Detection of garlic viruses

The direct tissue blotting immunoassay (DTBIA) and RT-PCR (Tsuneyoshi and Sumi 1996) were used to detect garlic viruses. In the RT-PCR analyses, reverse transcription (RT) was performed using a First-strand cDNA Synthesis Kit (Pharmacia) with approximately 1–2 µg of garlic total RNA in a reaction volume of 15 µl. For PCR, 1 µl of the RT mix was added to 100 µl of a polymerase reaction mixture containing 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* 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°C, 45 s for synthesis at 72°C, and 45 s for melting at 94°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 as follows: N-RT1/N-RT2 (GarVs) (5′-CCTGCTAAGCTATATGCTGA-3′ and 5′-GTAAGTTTAGCGATATCAAC-3′); P-RT3/P-RT4 (LYSV) (5′-AAGAGTCAACACTTGGTTTG-3′ and 5′-GGTCTCAATCCTAGCTAGTC-3′); ON-RT1/ON-RT2 (OYDV) (5′-GAAGCGCACATGCAAATGAAG-3′ and 5′-CGCCACAACTAGTGGTACAC-3′); GS-RT1/GS-RT2 (GLV/SLV) (5′-TATGCTCGAGCTCGTAGAGC-3′ and 5′-GGGTTTCACATTGTTACACC-3′). The primer sequences for alliin lyase, used as the positive control of the RT PCR experiment, are 5′-TCTGGTAGTCGATTTGGGTGGGCG-3′ and 5′-GCCGTAGCATTAGGATGTATG-3′.

Results and discussion

Development of plantlets from the dome-shaped structures

In a previous report on the novel stem-disc tissue culture method (Ayabe and Sumi l998), we showed that (1) within 5 days of culture, 15–25 dome-shaped structures appeared on the surface of each stem-disc explant prepared from a single clove (Fig. 1A) and (2) both the external appearance and internal cell organization of these structures were similar to those of the shoot tip (Fig. 1B, C). These findings suggested that these dome-shaped structures could be used in place of shoot tips in tissue culture to produce large numbers of virus-free garlic plants.

To test this hypothesis, we first determined whether the dome-shaped structures could be cultured when separated from the stem-disc explant. Dome-shaped structures that had been excised (Fig. 2A) and placed on LS media without phytohormones grew vigorously and developed into in vitro shoots approximately 1 cm long after 2 weeks (Fig. 2B), just as observed in the previously reported stem disc culture. This was evidence that an isolated dome-shaped structure can be used as an explant for in vitro culture. We have tentatively named this in vitro culture method “stem-disc dome (SD-Dome) culture”.

In addition, on the same medium the shoots produced roots (Fig. 2C), and these rooted shoots were subsequently transferred successfully to pots filled with soil (Fig. 2D).

Disappearance of virus symptoms

Leaves of garlic plants infected with viruses develop yellowish mosaic and/or streak symptoms, whereas leaves

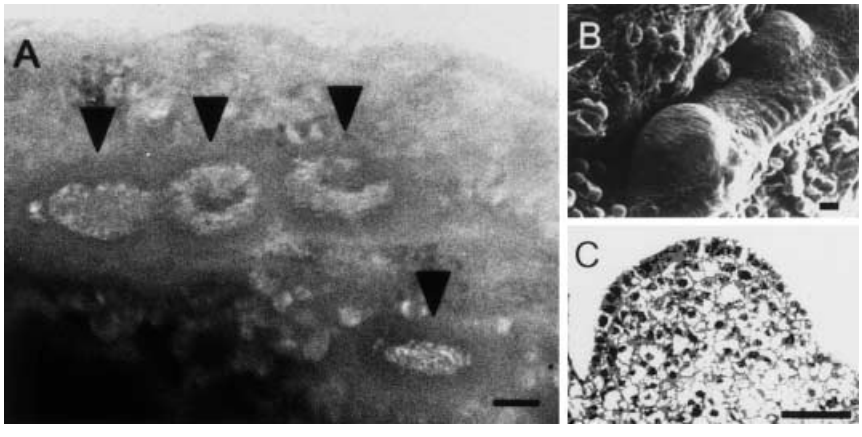

Fig. 1A–C Microscopic observations of dome-shaped structures that developed on a garlic stem disc after 5 days of culture. **A** Dome-shaped structures as seen under a stereoscopic microscope (*arrowheads*). **B** Scanning electronic microscopy observation of dome-shaped structures on a stem-disc explant. **C** Histological observation of a dome-shaped structure. *Bars*: 0.1 mm

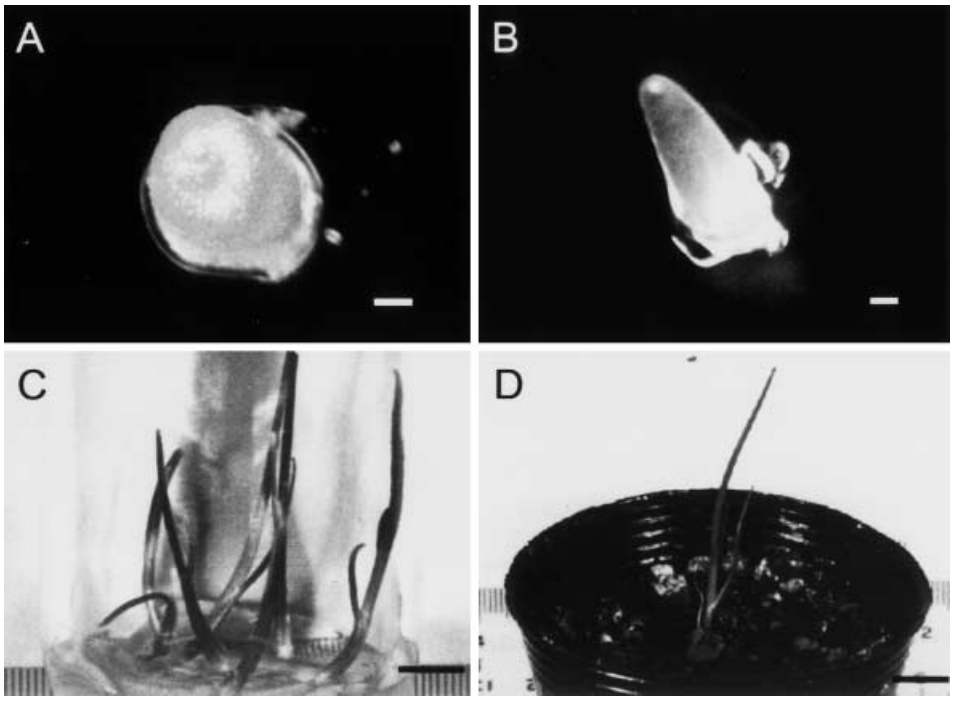

Fig. 2A–D Growth of an isolated dome-shaped structure into a garlic plantlet. **A** The structure just after excision from a stem-disc explant. **B** A shoot developed from an isolated dome-shaped structure after 2 weeks of culture. **C** Rooted garlic plantlets after 8 weeks of culture. **D** Growing garlic plantlet after transfer to a pot. *Bars*: 0.1 mm (**A, B**) or 1.0 cm (**C, D**)

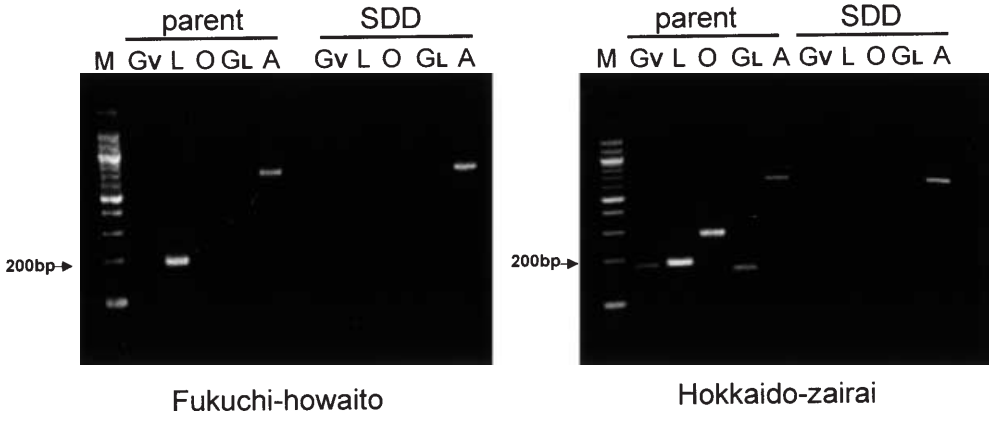

Fig. 3 Elimination of viruses through SD-Dome culture of garlic plants infected with a complex of viruses. The RT-PCR was done on the parent garlic (*parent*) and plants obtained by SD-Dome culture (*SDD*) using primers specific to each of the four garlic viruses, GarVs, LYSV, OYDV, and GLV. The amplified DNA fragments were analyzed on a 1.5% agarose gel. *Gv, L, O, GL,* and *A above* the lanes indicate the respective sets of primers for GarVs, LYSV, OYDV, GLV, and the alliin lyase cDNA sequence. An RT-PCR using the primer set for alliin lyase was the positive control. *Lane M* 100-bp ladder size marker

of healthy plants are of a deep-green color and do not show these symptoms. Healthy plants can therefore be easily distinguished from infected ones by their appearance.

We compared the appearances of developing leaves from garlic plants produced by SD-Dome culture with those from parent plants infected with LYSV. Leaves of the parent garlic plant clearly showed yellowish mosaic and streak symptoms, whereas those of garlic plants differentiated from the separated dome-shaped structures showed no symptoms of infection and were of a deep-green color, indicative that the viruses were completely or partially eliminated from the garlic plants during in vitro SD-Dome culture.

Detection of viruses by DTBIA and RT-PCR

To determine whether the viruses were completely or partially eliminated from the affected parent garlic plants through in vitro SD-Dome culture, we conducted RT-PCR and DTBIA on differentiated garlic plants from the separated dome-shaped structures. Three garlic individuals infected with LYSV, LYSV, OYDV, and a complex of viruses that included LYSV, OYDV, GLV, and GarVs, were the starting materials for SD-Dome culture (Fig. 3, Table 1). The first two plants were of Japanese cv. Fukuchi-howaito and the third plant was of cv. Hokkaido-zairai. As the control experiment, we made the usual

Table 1 Detection of garlic-infecting viruses in the plants produced by the stem-disc culture (*SD*) or stem-disc dome culture (*SD-Dome*) methods

		Detection of PCR product:			
		GarVs	LYSV	OYDN	GLV
Fukuchi-howaito	Parent[a]	–	+	–	–
	SD	–	+	–	–
	SD-Dome	–	–	–	–
Fukuchi-howaito	Parent	–	+	+	–
	SD	–	+	+	–
	SD-Dome	–	–	–	–
Hokkaido-zairai	Parent	+	+	+	+
	SD	+	+	+	+
	SD-Dome	–	–	–	–

[a] *Parent* Plant used for stem-disc culture

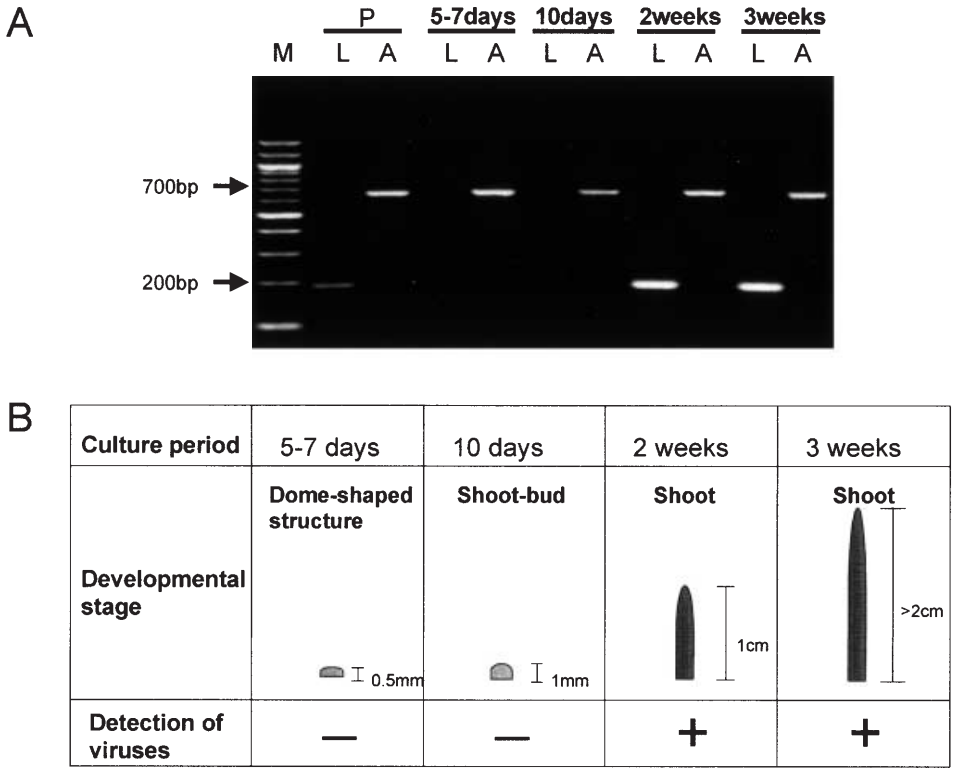

Fig. 4A, B Effect of the developmental stage of the dome-shaped structure on virus elimination. **A** Amplification of DNA fragments by RT-PCR using the primers specific to LYSV. Dome-shaped structures derived from the Japanese garlic, cv. Fukuchi-howaito, plant infected with LYSV were excised at different developmental stages: the dome-shaped structure (5–7 days of culture), shoot-bud (10 days of culture), or shoot (2–3 weeks of culture). PCR analyses were conducted on growing shoots approximately 5 cm in length. *P* The parent garlic plant. *Lanes*: *L* and *A* The respective specific primer sets for LYSV and alliin lyase cDNA, *M* 100-bp ladder size marker. **B** Timing of dome-shaped structure excision from the stem-disc explant for elimination of viruses by the SD-Dome culture method

stem-disc culture with cloves of the garlic individuals used in the SD-Dome culture and conducted RT-PCR and DTBIA on the plants obtained. No amplified DNA fragment was detected in the RT-PCR analysis carried out on garlic plants that had differentiated from the separated dome-shaped structures, whereas there were specific PCR products for the parent plants (Fig. 3). The DTBIA analyses gave results consistent with those of RT-PCR detection (data not shown). In contrast, garlic plants produced by the usual stem-disc culture method were infected with the same viruses as their parents. The results of these experiments are shown in Table 1. We continued viral surveillance through three generations of progenies obtained by SD-Dome culture and detected no evidence of virus infection. These findings provide evidence that the viruses were completely eliminated by the use of SD-Dome culture. Moreover, the dome-shaped structures remained free of viral infection, with separation from the stem-disc explants being crucial for virus elimination.

Developmental stages of the dome-shaped structures and virus invasion

To determine the appropriate time for separating the dome-shaped structures from the stem discs, we excised them at different developmental stages: at formation of the dome-shaped structure and at the shoot-bud and shoot stages (see Fig. 4). All grew into shoots approximately 5 cm in length. In this experiment, cv. Fukuchi-howaito infected with LYSV was used.

In RT-PCR analyses of garlic samples grown from immature shoots separated 2 or 3 weeks after the onset of stem-disc culture, the specific DNA fragment was amplified, thereby providing evidence of infection by

LYSV (Fig. 4A). In contrast, no fragment was amplified in the analyses of shoots derived from the dome-shaped structures or shoot buds excised on days 7 to 10 of culture (Fig. 4A). These findings show that the dome-shaped structures, like the shoot tip, were free from viruses, whereas viruses had invaded the tissues when the dome-shaped structures were developing into shoots.

Viruses are reported to spread throughout the plant body by cell-to-cell mechanisms and long-distance movement (Maule 1991; Leisner and Howell 1993; Lucas and Gilbertson 1994). In cell-to-cell movement, viruses move into surrounding cells through the plasmodesmata. Vascular-bundle systems function in long-distance movement. We therefore investigated the internal configurations of the dome-shaped structures, shoot bud, and shoot by microscopic observation of transverse sections stained with an aceto-orcein solution. Each dome-shaped structure and shoot bud consisted of small cells, with no organized internal structures present (data not shown). In contrast, large cells were present in the internal portion of a section prepared from a shoot after 2 weeks of culture. In addition, some deeply stained cell clusters were seen in the section prepared from the shoot (data not shown). The clusters might be procambia, which differentiate into vascular bundle systems. These findings imply that neither the plasmodesmata nor vascular bundle systems are fully differentiated in the early developmental stage of the dome-shaped structures and that this is the reason why viruses from neighboring infected cells can not invade them. Once the connecting systems are formed, however, virus invasion immediately appears to be initiated. The process by which viruses are completely eliminated during dome-shaped structure formation, however, is not yet clear.

In conclusion, we have developed a novel, practical culture method for producing virus-free garlic – SD-Dome culture – which has a much higher efficiency than the usual shoot-tip culture. In this method, 15–25 virus-free plants can be obtained consistently from a single clove in the same period as used in shoot-tip culture. Furthermore, SD-Dome culture provides a useful experimental tool for studying the mechanism by which the shoot tip is protected against virus invasion when used in combination with such molecular biology and immunochemical techniques as in situ hybridization, PCR and immuno-staining.

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