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Establishment of a novel tissue culture method, stem-disc culture, and its practical application to micropropagation of garlic (*Allium sativum* L.)

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Abstract A restricted part of the undeveloped stem of the garlic clove, called the "stem disc", which is just under the basement of the immature foliage leaves, proved to be a very potent explant for the micropropagation of garlic. Twenty to thirty tissue-cultured shoots consistently were differentiated from a single clove during 1 month of culture on phytohormone-free Linsmaier and Skoog medium. In addition, more than 90% of the shoots formed bulblets in vitro during an additional 1 month of culture. Pretreatment of the garlic bulbs at 4 °C for approximately 8 weeks before preparing the stem discs enhanced both shoot development and bulblet formation. This novel method for culturing garlic was designated the stem-disc culture method. Shoot development in this type of in vitro culture apparently is divided into four stages: expansion of tissue zones surrounded by the basal parts of the immature foliage leaves, formation of dome-shaped structures, bud differentiation directly from each dome, and development into shoots and bulblets. The dome-shaped structures appeared within 5 days of the onset of culture and had developed independently into shoots approximately 1 cm high 3 weeks later. Histological observations showed that both the internal cell organization and formation process of the dome-shaped structures were similar to those in the meristem. In addition, events leading to the formation of these dome-shaped structures appeared to be initiated by vigorous cell division in the epidermis of concentric tissue zones surrounded by the basements of immature foliage leaves. The results of several field trials showed that the stem-disc culture method is useful for the production of garlic seed plants, including virus-free plantlets. Furthermore, it is a novel field cultivation system for garlic in that the seedlings produced by in vitro-induced bulblets are used as seed instead of the usual cloves.

Key words *Allium sativum* · Garlic · Micropropagation · Stem-disc culture · Tissue culture

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Introduction

Garlic (*Allium sativum* L.) is an important plant widely used for both culinary and medicinal purposes because of its ability to improve the taste of food and its biological activities that include antibiotic, antitumor, cholesterol-lowering, and antithrombic effects on animal cells (Fujiwara and Natata 1967). Garlic traditionally has been cultivated vegetatively because of its sexual sterility; consequently, viral diseases are a very serious problem. Almost all commercial garlic plants have been shown to be infected with a complex of viruses such as leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV), shallot latent virus (SLV), and garlic common latent virus (GCLV) (Walkey 1990; Sako et al. 1991; Van Dijk 1991; Conci et al. 1992; Van Dijk 1993a, b, Van Dijk and Sutarya 1992; Barg et al. 1994; Tsuneyoshi and Sumi 1996), as well as by unclassified novel rod-shaped viruses, called garlic viruses (GarVs) A–D, that we have identified (Sumi et al. 1993).

Tissue culture is 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 and it is a laborious, time-consuming process. 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), but all have inherent defects as practical methods – the need for long-term cultivation, relatively low propagation rates, and the necessity of mastering skillful techniques.

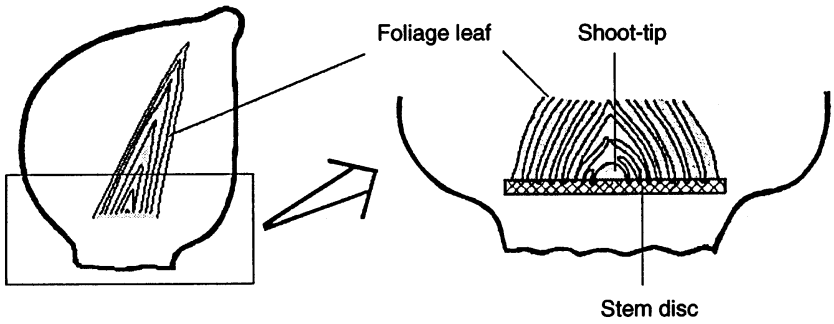


Fig. 1 Schematic depiction of garlic tissue designated the "stem disc". This is a restricted tissue (*box*) just under the basements of the immature foliage leaves approximately 1 mm thick

Xeu et al. (1991a, b) examined the effects of garlic explants cultured in vitro on embryogenic callus formation and reported that the basal part of the clove, with or without the shoot apex, is much more potent than the storage leaf. This finding agrees with the report of Nagasawa and Finer (1988) that the basal section of in vitro garlic shoots produced by shoot-tip culture induced morphogenic callus formation. In a preliminary experiment, we observed in vitro shoots developing directly from the basal part of the foliage leaf. Taken together, these findings suggest that the basal part of the garlic clove is advantageous explant material for the micropropagation of garlic. We subsequently established a novel tissue culture method for garlic that uses the stem disc as an explant. We describe here that method and our histological observations of the shoot formation process.

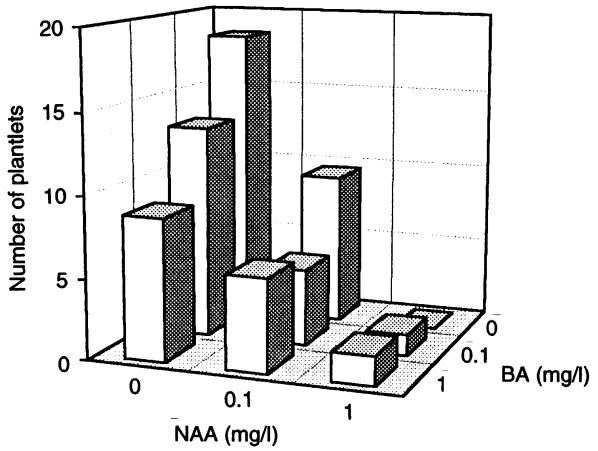


Fig. 2 Effect of phytohormone on development of in vitro shoots from a stem disc. The numbers of shoots differentiated from each stem disc explant were scored after 2 weeks of culture on solid LS media containing NAA and BA in various combinations

Materials and methods

Plant material

Garlic (*Allium sativum* L. cv 'Fukuchi-howaito') cultivated at our experimental farm in Hokkaido, Japan was used in all the experiments.

Preparation and culture of the stem disc

Garlic cloves cut into small cubes that contained the basal part of the stem were sterilized for 5 min in 70% ethanol. After removing the residual storage and foliage leaves, we excised a portion of the base approximately 1 mm thick, the stem disc (see Fig. 1). Each disc was cut into four pieces which were placed on solid Linsmaier-Skoog (LS) medium (Linsmaier and Skoog 1965) in petri dishes. The dishes were then incubated at 25 °C under a 16-h photoperiod with fluorescent illumination at 3000 lux.

Observation of the dome-shaped structure

We observed the process of shoot development both under a stereoscopic microscope and with a scanning electron microscope (SEM). For the SEM observations, stem-disc samples, taken just after preparation and cultured for 3, 4, or 5 days on LS medium, were fixed in a mixture of 5% glutaraldehyde and 4% formaldehyde. The fixed samples were dehydrated in a graded ethanol series. The critical point drying was done in liquid carbon dioxide. The dried samples were mounted on stubs, sputter-coated with gold, then observed with a Hitachi S-510 scanning electron microscope at the accelerating voltage of 15 kV.

Histological observations of the dome-shaped structure

Stem discs were fixed and dehydrated as for the SEM observations, after which they were embedded in paraffin then cut into 5-μm sections in an ultramicrotome with a diamond knife (LKB Ultratome III). The sections were double-stained with hematoxylin and eosin. We observed these samples under a stereoscopic microscope.

Results and discussion

Shoot development from stem-disc explant

Pieces of the stem disc were cultured on phytohormone-free solid LS medium or the same medium supplemented with 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 0.1 mg/l 6-benzylaminopurine (BA). After 2 weeks, multiple shoot buds had formed on each explant. These buds developed into shoots approximately 1 cm high after an additional 2 weeks of culture. These shoots appeared to differentiate directly from the explant surface (data not shown).

Next, we investigated the effects of phytohormones on the differentiation of in vitro shoots. Stem-disc explants from each of five cloves were cultured on solid LS medium containing various combinations of NAA and BA at con-

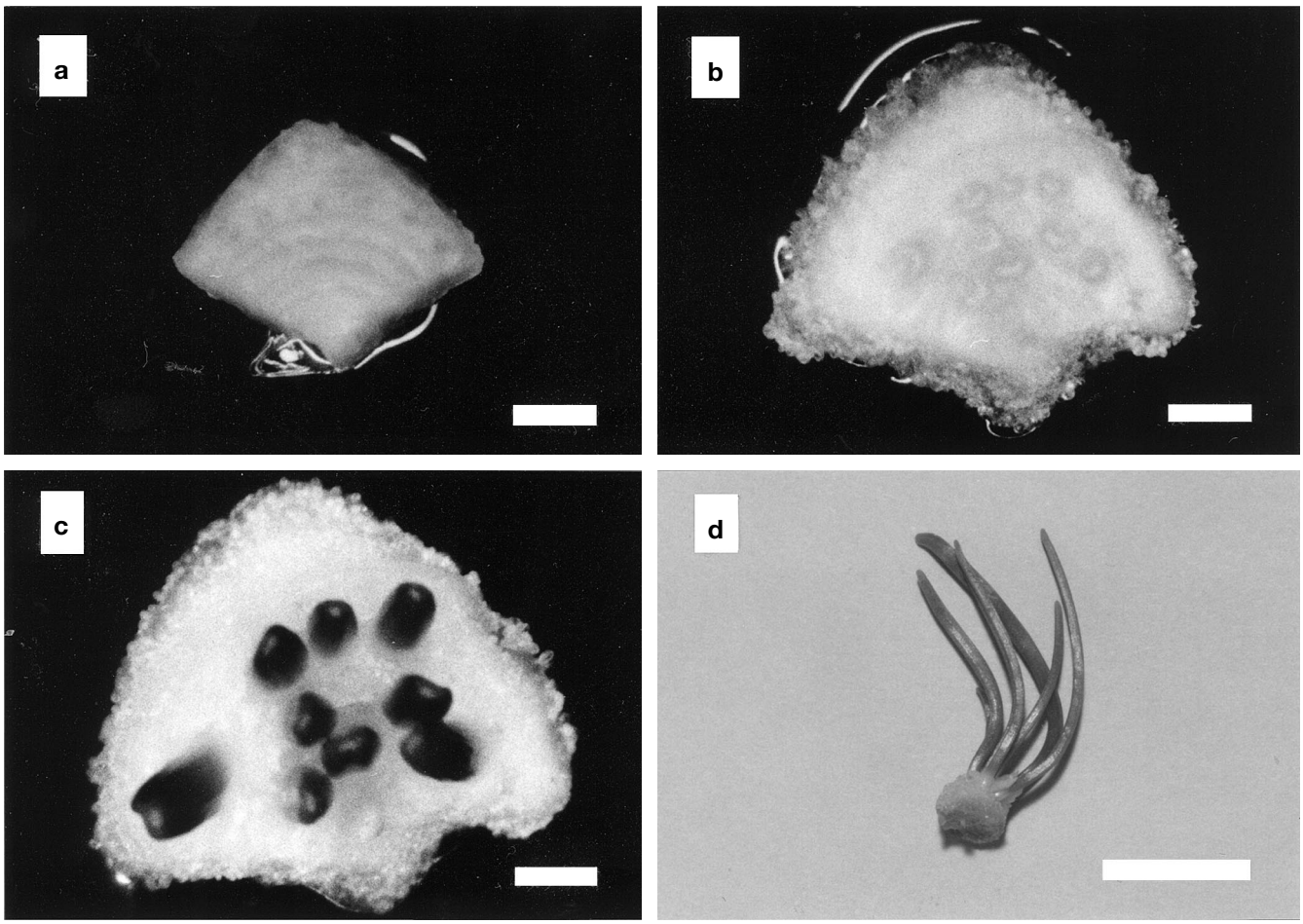


Fig. 3a–d Progressive development of in vitro shoots from a stem-disc explant. Morphological changes in one piece of a stem-disc explant cut into four pieces were observed by microscopy: **a** 1 day after culture, **b** dome-shaped structures after 1 week of culture, **c** green buds after 2 weeks of culture, **d** in vitro shoots after 3 weeks of culture. *Bar* (**a**–**c**): 1 mm; *bar* (**d**): 1 cm

centrations of 0, 0.1, and 1.0 mg/l. The phytohormone effects were monitored by scoring the number of differentiating shoots and observing the morphological changes that occurred in the explants during in vitro culture. Shoot scores 2 weeks after culture in the various combinations and concentrations of phytohormone are shown in Fig. 2. No one particular phytohormone supplement was observed to be distinctly better than the others under the test conditions, and more than 15 shoots, on average, differentiated from a single stem disc. Additions of NAA or BA suppressed bud formation in a dose-dependent manner. The inhibitory effect of NAA was much greater than that of BA, and an addition of 1 mg/l of NAA blocked bud differentiation almost completely. As for the morphological changes, an NAA supplement tended to promote callus formation, whereas BA elongated the residual basal parts of the foliage leaves on the stem-disc explant.

We call this novel tissue culture method for garlic "stem-disc culture". The use of the stem disc as an explant is unique and in vitro shoots differentiate with much higher efficiency and in a shorter culture period than that previously reported in other methods (Havránek and Novák 1973; Kehr and Schaeffer 1976; Abo El-Nil 1977; Nagakubo et al. 1993).

Observation of the process of shoot development from stem discs

We investigated the developmental process of in vitro shoots from stem-disc explants using microscopy. Figure 3 shows the progressive development of in vitro shoots from the stem disc. Multiple, dome-shaped structures first appeared on the surface of the stem-disc explant 1 week after culture initiation (Fig. 3b). These structures appeared concentrically on the explant, and callus was present exclusively in the regions between the developing zones of the dome structures. The structures developed rapidly and produced green buds after approximately 2 weeks of culture (Fig. 3c). The buds grew vigorously and developed into in vitro shoots approximately 1 cm high after approximately 3 weeks (Fig. 3d).

We also made SEM observations of stem discs sampled serially 0–5 days after culture to determine the morphological events that occur during the early formative stages

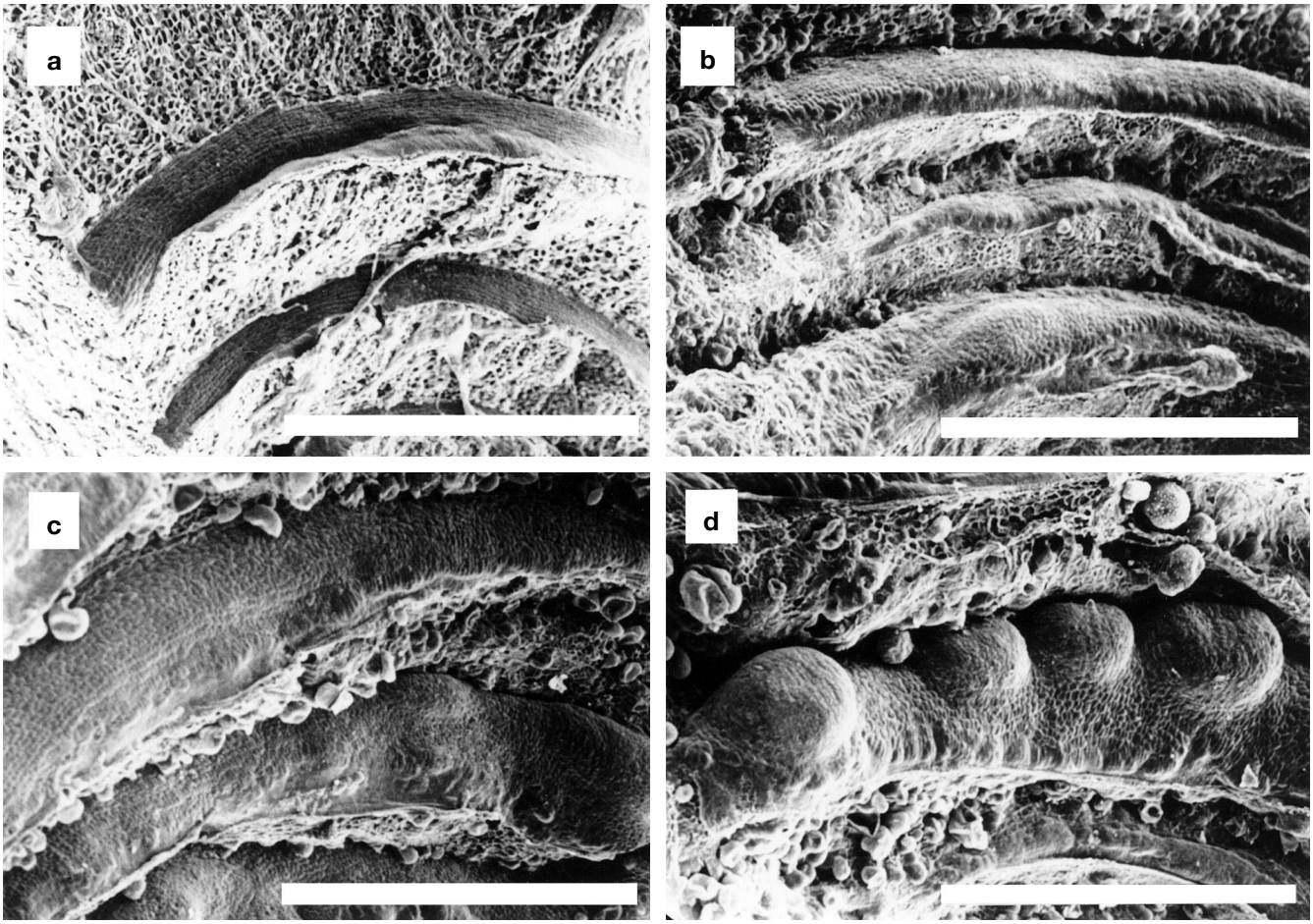


Fig. 4a–d SEM observations of the early formative stages of the dome-shaped structures on a stem-disc explant. **a** The stem-disc explant just after preparation, **b** the explant after 3 days of culture, **c** the explant after 4 days of culture, **d** the explant after 5 days of culture. *Bars:* 1 mm

of the dome-shaped structures. These changes are shown in Fig. 4. Concentric grooves of high density with smooth surfaces (Fig. 4a) are present among reticula with rough, rugged surfaces which correspond to the residual basements of the foliage leaves (see Fig. 1). Three to four days after culture, these zones were slightly enlarged, and some parts appeared protuberant (Fig. 4b, c). On day 5, the protuberances developed into dome-shaped structures approximately 0.5 mm in diameter (Fig. 4d). SEM showed that development of the in vitro shoots was restricted to regions surrounded by the basements of foliage leaves and that the morphological changes apparently occurred within the first 3 days of culture. Moreover, shoot formation events seemed to occur through differentiation rather than dedifferentiation.

Histological observations of the dome-shaped structures

To characterize the dome-shaped structures formed during stem-disc culture, we conducted histological observations of these structures at different developmental stages. Figure 5a shows a section from a stem disc just after preparation. A single distinctive cell layer composed of relatively small cells that are intensively stained with hematoxylin and eosin is present in the epidermal tissue between the basements of the foliage leaves (arrowhead). These small cells vigorously divided, multiple layers composed of intensively stained small cells appearing on day 3 of culture (Fig. 5b). As a result, the epidermal tissue bulges slightly. On day 5, a dome-shaped structure which is similar histologically to the shoot tip is present (Fig. 5c). These findings show that the dome-shaped structures apparently develop from the epidermal single-cell layer, which consists of small cells with a high potential for cell division, between the basements of the foliage leaves.

These observations and findings support our conclusion that the in vitro shoots differentiated directly from the stem-disc explants and were not produced through dedifferentiation. In addition, both the internal cell organization and formation process of the dome-shaped structures were similar to those of the meristem.

Effects of low-temperature pretreatment on stem-disc culture

It has been reported that exposure of in vitro shoots to a low temperature (5°C) for more than 4 months promoted

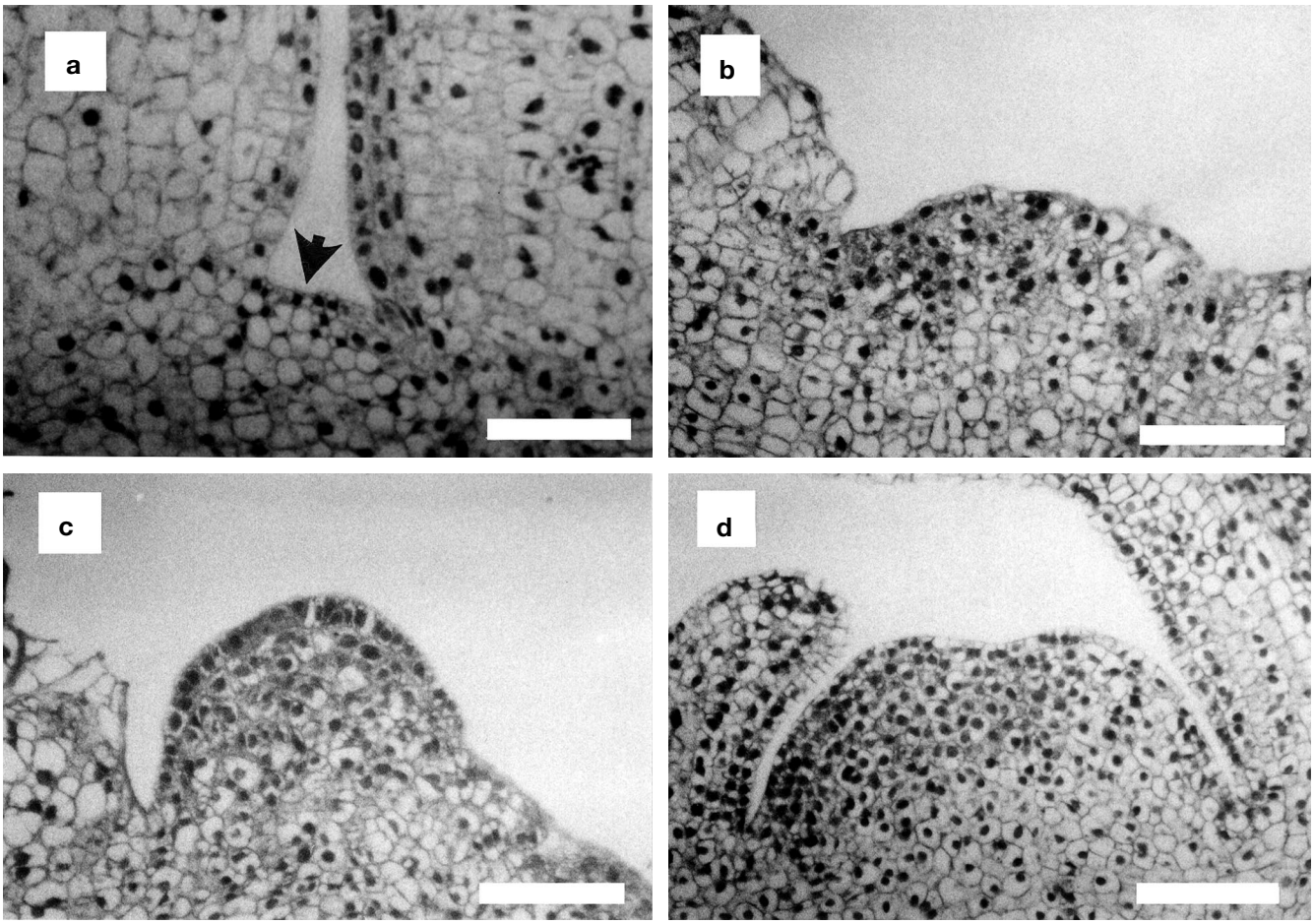


Fig. 5a–d Histological observations of the dome-shaped structures during formation. Stem-disc explants were sampled at various times during culture, and thin sections were prepared from these samples to observe histological changes. **a** Thin section from a stem disc before culture (arrowhead, see text), **b** the same section after 3 days of culture, **c** the same section after 5 days of culture, **d** the garlic shoot apex. *Bars:* 0.1 mm

garlic bulblet formation in in vitro culture (Nagakubo et al. 1993; 1997; Takagi 1990). We also found that pretreatment of garlic bulbs at 4 °C promoted bulbing in in vitro shoots produced by meristem culture. Based on these findings, we examined the effect of low-temperature pretreatment of garlic bulbs on the formation of in vitro bulblets during stem-disc culture.

Garlic bulbs were stored at either 4 °C or room temperature for 2–12 weeks before preparing the stem-disc explants. For the statistical evaluation, 25–50 cloves were examined in the respective storage conditions. We scored the total numbers of in vitro shoots differentiated from each clove after 3 weeks of culture and in vitro bulblets formed on the shoots after 8 weeks of culture. The numbers of in vitro shoots as well as in vitro bulblets were markedly increased by pretreatment at 4 °C (Fig. 6). The gradual increase in shoot number paralleled the duration of the low-temperature pretreatment, with an average of 25 shoots (SD

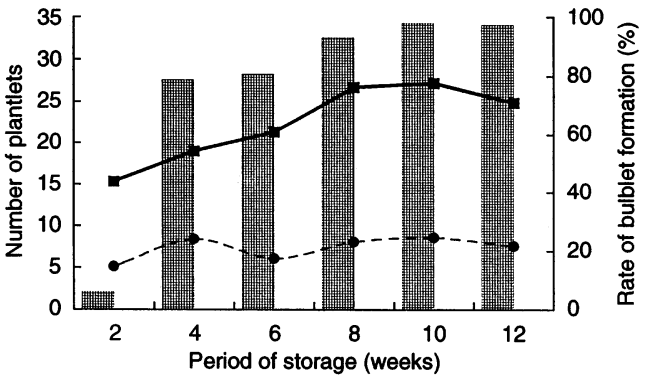


Fig. 6 Effects of low-temperature pretreatment of the differentiation of in vitro shoots and formation of in vitro bulblets. Stem-disc explants were prepared from garlic bulbs stored at either 4 °C or room temperature for 2–12 weeks, then cultured on phytohormone-free LS media. The total number of in vitro shoots was scored after 3 weeks of culture and in vitro bulblets after 8 weeks. *Solid line* stored at 4 °C, *dotted line* stored at room temperature, *haschured bar* rate of in vitro bulblet formation (average %) in explants stored at 4 °C. Explants stored at room temperature did not form any in vitro bulblets. The rate of bulblet formation was calculated by dividing the number of shoots with bulblets by the total number of in vitro shoots

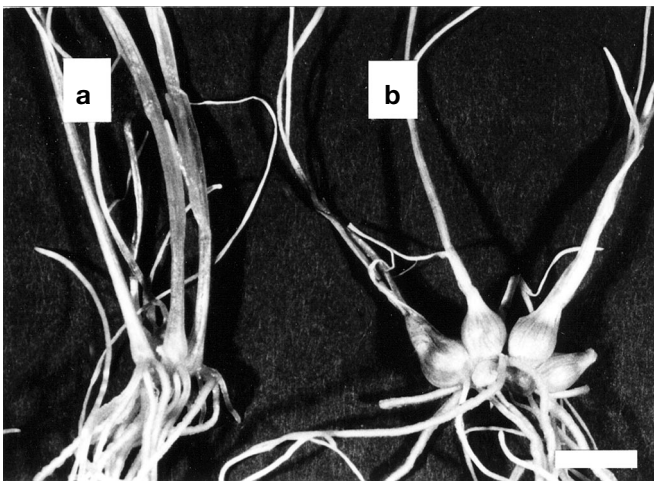


Fig. 7 a, b Representative in vitro shoots produced from stem discs cultured for 2 months. **a** In vitro shoots from explants stored at room temperature, **b** in vitro shoots from explants stored at 4 °C. *Bar:* 1 cm

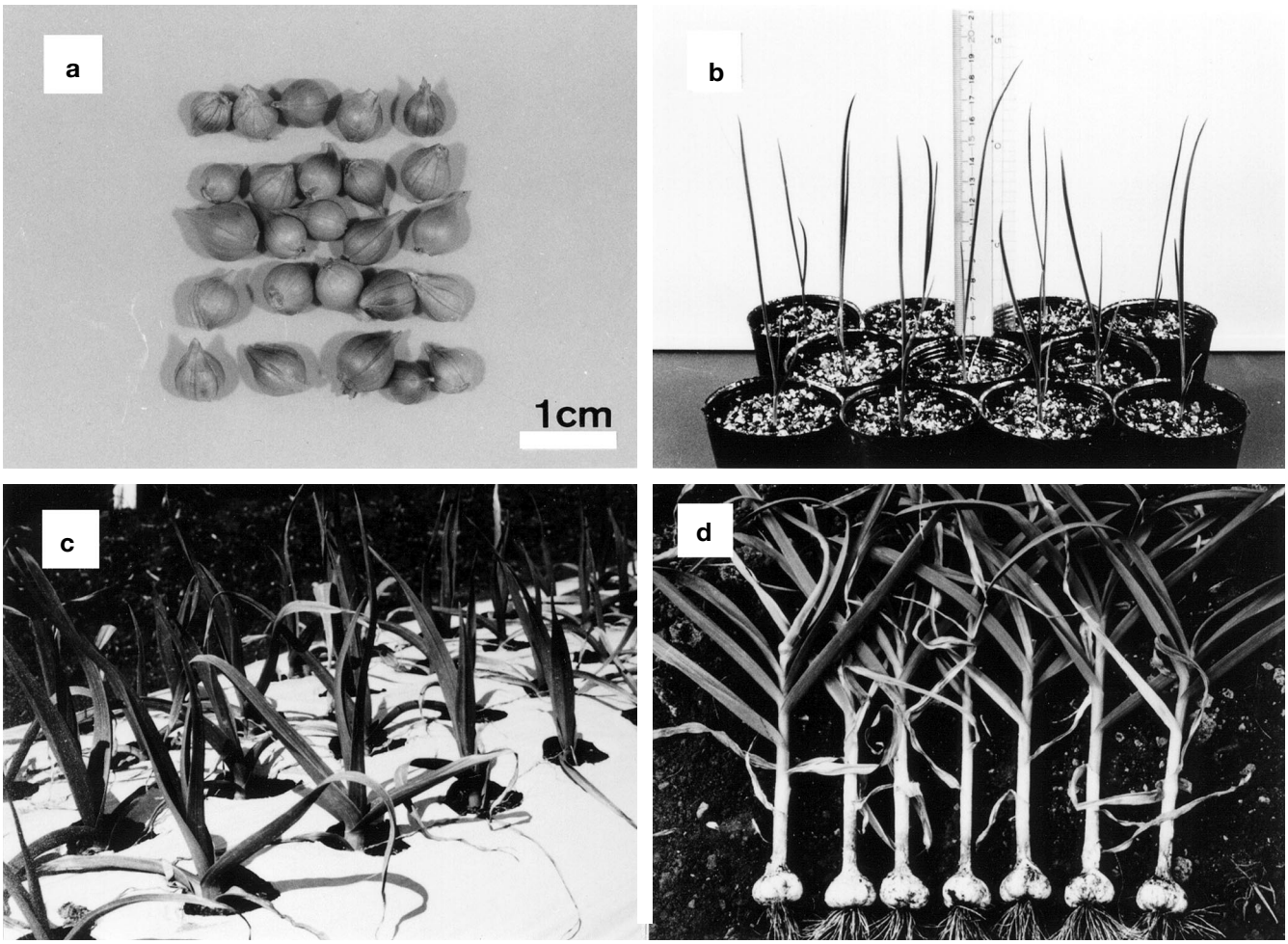
Fig. 8 a–d Field cultivation of garlic shoots germinated from in vitro bulblets. **a** In vitro bulblets obtained from a single clove, **b** garlic shoots germinated from in vitro bulblets, **c** garlic plants growing in the field, **d** harvested garlic bulbs

ranges ±3.0 to ±5.1) differentiating from a single garlic clove stored for more than 8 weeks at 4 °C. The formation of in vitro bulblets was also enhanced, with more than 95% of the shoots forming bulblets after low-temperature pretreatment for more than 8 weeks. In contrast, garlic stored at room temperature showed no enhancement of differentiation of in vitro shoots. Furthermore, the shoots never formed in vitro bulblets (Fig. 7).

The stem-disc culture method combined with the pretreatment of garlic at 4 °C for approximately 8 weeks consistently produced more than 100 in vitro bulblets from each bulb, this in comparison to the bulb of the Japanese garlic cultivar 'Fukuchi-howaito' which ordinarily has only 5–6 cloves.

Field cultivation of in vitro bulblets

To evaluate the biological activities of the in vitro bulblets and their possible use as seeds in garlic production, we germinated then cultivated them in the field (Fig. 8). Sprouting shoots planted in late August to early September produced garlic bulbs, late the next June, of comparable size and weight to those obtained by the usual clove cultivation method. There were no apparent abnormalities in shoot



growth or in the resulting bulb morphology throughout the cultivation period. It should be noted that storage of the in vitro bulblets for up to 6 months did not affect their germination activities (data not shown). These findings indicate that the stem-disc culture method is of practical use for the micropropagation of garlic plants, in particular as virus-free seed plants produced by shoot-tip culture. Furthermore, this culture method has produced a novel, epoch-making garlic cultivation system, in which seedlings instead of the usual cloves are used for propagation. Because seedlings are much more easily planted by machinery than cloves, this culture system is efficacious for practical garlic cultivation, in particular large-scale cultivation.

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