Development of a New Process for Mass-Production of Virus-Free Seed Bulbs of Garlic(*Allium sativum* L.) Through Plant Tissue Culture Technique

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I. Introduction

A garlic (*Allium sativum* L.) is an important and useful vegetable such that it is not only most used for spices but also exploited for health foods and pharmaceuticals.

The garlic is a kind of vegetable propagated vegetatively through cloves. Therefore, development of new varieties of garlics is possible only through selection breeding because cross breeding is impossible. Like other plants propagated vegetatively, garlics suffer from viral diseases seriously. Usually garlics are readily infected by viruses known as garlic mosaic virus(GMV) and garlic latent virus(GLV), which cause a decrease in the yield and a degradation in the quality. Agricultural chemicals that have been developed thus far cannot treat such viral diseases effectively. Thus, in order to solve the viral problems, there have been made many attempts through improvement of quality in the seed bulbs. Until the present, a pathogen-free seed garlic is known as a best means for overcoming the problems attributable to the viral diseases. There were several reports that infection by garlic viruses caused a 40-50 % yield reduction in Korea (Table 2).

Although use of virus-free seed garlics is an effective means for reducing the damages attributable to the viral diseases, farmers cannot readily utilize them because there has not been developed an effective method for the mass-production of a virus-free seed garlics economically. The production of virus-free seed bulbs is possible only through plant tissue culture technique. Several processes for the production of virus-free seed garlic through plant tissue culture have been developed so far, and they can be classified into four types as follows; first, an apical meristem is separated from a clove of garlic and cultured on a suitable medium to derive one to several shoots therefrom, as described in Rumanian Patent No. 96914, yielded on May 30, 1989, (hereinafter referred to as "apical meristem culture process"); second, a callus is derived from a separated apical meristem and multiplicated, and differentiated into garlic shoots, as described in Japanese Pat. Appln. No. Heisei 1-300771 applied

by Aginomoto K. K., on Nov. 21 1989 (hereinafter referred to as "callus culture process"); third, a process, reported by Sumitomo Chemical Co., Japan (Plant Cell Tiss. Org. Cult. 32:175-183, 1993), which is similar to the apical meristem culture process but further comprises multiplicating shoots derived from an apical meristem through subcultures (hereinafter referred to as "sumitomo process"); fourth, a process described in Japanese Pat. Appln. No. Heisei 1-19850 applied on Jan. 31, 1989 by Shin Nippon Seitetsu K. K., in which an undifferentiated shoot mass, called shoot primordia, is derived from an apical meristem of a garlic clove and multiplicated, and developed to shoots (hereinafter referred to as "shoot primordium process"). In order to better understand the characteristics of methods, a description of the above-mentioned processes will be given in connection with some drawings (Fig. 3).

II. Characteristics of conventional processes for the production of virus-free garlic

There is a flow chart illustrating the apical meristem culture process in Fig. 3-1 As shown in this figure, shoots are derived from the apical meristem of clove, and then microbulbs were formed from them.

This process is disadvantageous in several aspects. First, because the apical meristems of clove, having a size of about 0.3 mm, are continually separated under an microscope in order to get a large number of shoots, the workability is very slow. Second, viability of the separated shoot meristems is very low at early stage. Another significant disadvantage of the apical meristem culture process is that explants of garlic cloves are necessary as many as the microbulbs to be produced. Furthermore, it is virtually impossible to obtain genetically homogeneous microbulbs because they are originated from varied explants

Referring to Fig. 3-2, it illustrates the callus culture process, which marks a substantial improvement, compared with the shoot meristem culture process. As shown in the figure, it consists mainly of deriving a callus from an apical meristem of a garlic clove, multiplicating the callus through repetitive subcultures, regenerating shoots from callus and forming the shoots into microbulbs. Likewise, this process has also several disadvantages. It is poor in production efficiency because of limitation of the number of the subculturing. That is, it is necessary for the subculture of the callus to be carried out many times in order to secure enough amount of callus, but the number of subcultures are limited to about 8 times in this process because shoot regeneration rate decreases very sharply after subcultures continuous more than 8 times. Another significant disadvantage is that the number of the regenerated shoots is difficult to predetermine owing to fluctuation in the regeneration rate and a large portion of the regenerated shoots are likely to be

vitrified, resulting in difficulty in controlling the number of the shoots. Also the multiplication rate of callus is low (4 folds/month). Four steps are needed from separation of apical meristem to formation of microbulbs, and a long time, about 8 months, is taken for this. Besides, the callus culture process has somewhat difficulty in obtaining genetically homogeneous shoots. Only limited number of virus-free microbulbs of garlic can be produced by both of the two processes, shoot meristem culture and callus culture process. Because a large amount of the virus-free microbulbs can not be produced through these processes, they should be propagated in open fields for the purpose of mass-propagation of the garlics. However, the multiplication rate of garlic in open field is low (5-6 folds/year), and also bulbs are gradually reinfected by virus through insect vectors whenever they are cultivated in open field.

Sumitomo process are showed in Fig. 3-3, which is recognized as better one than the above-mentioned processes. As shown in the figure, sumitomo process comprises deriving shoots from a shoot meristem of a garlic clove, propagating the shoots through repetition of subcultures, and finally forming microbulbs from the proliferated shoots. Even if sumitomo process might be improved process, it also has several limitations. The multiplication rate of shoot is also low (about 2.5 folds/month), and it takes long time for low temperature treatment necessary for enhancing garlic microbulb formation from shoots (about 6 months). Because it is difficult to obtain several millions of microbulbs from a single shoot meristem, a lots of explants are needed for it. Thus varied explants make it virtually impossible to obtain genetically homogeneous shoots. It seems likely that sumitomo process is not so economically proper for mass-production of several hundred million seed garlics.

The shoot primordium process is showed in Fig. 3-4. It comprises deriving shoot primordia from the apical meristems of garlic cloves, proliferating the shoot primordia, and forming garlic microbulbs. A liquid culture with a special apparatus is necessary at the stage of the induction and proliferation of shoot primordia. Owing to limitation on the liquid culture facility such as rotary shaker, it is virtually difficult to mass-produce the shoot primordia. In addition, the prolificacy of shoot primordia is low (2-5 folds/month). A low-temperature treatment is necessary for forming the microbulbs. Another disadvantage of the shoot primordium process is that the time from the initial culture to the formation of microbulb is long (12 months). Further, it is difficult to obtain genetically homogeneous shoots because they are originated from diverse explants.

Above-mentioned conventional processes are suitable for small scale production of virus-free microbulbs of garlic and not proper processes for the mass-production of several hundred million virus-free and genetically homogeneous microbulbs. In other words, the conventional processes are so high in production cost that they can not be applied for commercial production of virus-free microbulbs in Korea.

Ⅲ. Characteristics of a new processes(multishoots process) for the production of virus-free garlic

A new process, called multishoots process developed recently in Tong Yang Moolsan Co. can provide solutions to overcome the problems of the conventional processes. Accordingly, it is a principal object of the new process to provide a novel method for mass-producing virus-free microbulbs of garlic through plant tissue culture technique. In order to achieve this objective, the production yield is much improved, and the process steps are reduced as shown in Fig. 3-5.

This new process consists of 4 steps; deriving callus from the apical meristems of garlic cloves and propagating the callus; regenerating the shoot from callus; multiplicating multishoots through repetitive subcultures; and forming virus-free microbulbs from the multishoots. Once multishoots are induced, they can be multiplicated continuousely for several years through subcultures, and more than several hundred millions of shoots can be multiplicated from a clump of multishoots. That means the first two steps, callus induction and propagation, and shoot regeneration, do not need to be repeated again any more. The comparison of main characteristics between the new process and the conventional processes are summarized in Table 1.

The multishoots process has several advantages over the conventional processes; more than 300 shoots can be multiplicated in a small vessel (Fig. 2); multiplication rate of the multishoots is more than 10-fold per month; it takes less than 3 months from first step to the last because steps of culture are reduced to two; the multishoots can be multiplicated for more than several years without any variations during subcultures (subcultured for 3.5 years already); finally, a large amount of bulbs which are exactly the same as mother plant genetically can be produced because more than 10 billion shoots can be proliferated from a single shoot in a year. So, a unit cost of microbulbs is able to decrease to 1/5 - 1/10 compared with those of the conventional processes owing to the above-mentioned advantages.

Ⅳ. Brief description of method of the multishoots process

A variety of Korean garlics are planted in soil which is contained in a growth chamber with a temperature of 24-26℃ and cultivated therein. At the time that the garlics grow vigorously, they are cultured at about 30-31 ℃ for 7 to 10 days and then, at about 36-37 ℃ for 10 to 14 days, so as to deactivate the viruses present in the plant. After heat treatment, the apical meristem-containing parts are cut under a microscope to separate apical meristems (about 0.3 mm). In order to derive callus from them, these apical meristems are cultured on Murashige and Skoog (MS) solid

medium containing 0.1 to 0.2 ppm (mg/L) of 2,4-D (2,4-dichlorophenoxyacetic acid). A medium for shoot regeneration from callus (hereinafter referred to as "shoot regeneration medium") consists of MS basal medium containing 2-3 % sucrose, 0.3-3.0 mg/L benzylaminopurine(BA) and 0.7 % agar.

The regenerated shoots are transferred to the multiplication medium and cultured. After they are cultured for a month, multishoots are multiplicated into about 100 shoots per a clump, and 4 clumps of multishoots are placed in a standard petridish with a size of 10 x 1.5 cm. Based on the fact that subcultures can be continued more than 3.5 years without any degeneration or vitrification of the shoots, it is apparent that the multishoots can be subcultured semi-permanently.

One fifteenth of the multishoots propagated on the medium is transferred to another fresh shoot multipliaction medium and subcultured therein, with the aim of reproducing shoots. The rest, that is, fourteen fifteenths of the multishoots are transferred into a medium for forming microbulbs of garlic. The rate of bulb formation increase after being stored at 4 ℃ for 2 months.

In order to produce minibulbs in open field from the multishoots, a large clump of the propagated multishoots, consisting of 100 shoots, is divided into small clump, each consisting of about 5 shoots, which are planted in open field in fall. Since the shoots propagated in vitro are feeble, they are planted earlier than general garlic sowing period in order to secure enough time to develop the root system.

Extract of crushed calli, multishoots, and microbulbs are used for analysis of virus. ELISA analysis is applied to examine whether viruses are present in the samples or not. These analyses show that the microbulbs including callus, multishoots, and microbulbs produced through the multishoots process have little or no virus.

V. References

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Table 1. Characteristics of multishoots process and conventional processes

Process	Genetic Homogeneity	Proliferation Intermediate	Multiplication Rate (folds/mo)	Limitation of Subcultures	Number of Steps
Apical Culture	heterogeneity	none	-	-	3-4
Callus Culture	homogeneity possible	callus	4-5	limited (~8 times)	3-4
Sumitomo	heterogeneity	shoot	2-5	limited (~4 times)	3-4
Shoot Primodium	homogeneity possible	shoot primodium	2-5	-	3-4
Multishoots	homogeneity	multishoots	10-15	semi-permanent	2

Table 2. Comparison of yield between conventionally propagated cloves and *in vitro* cultured cloves in garlic 'Danyang'

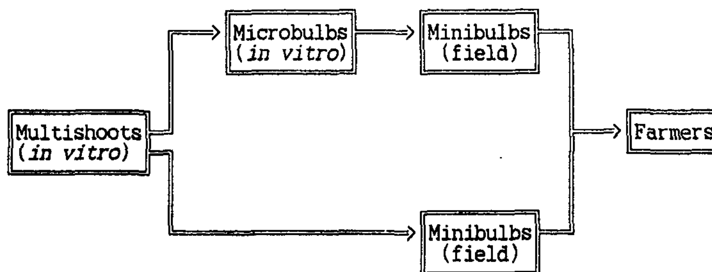
Cultured Garlic Source			Bulb					Yield (kg/10a)	Yield Increase (%)
			Height (cm)	Diameter (cm)	Weight (g)	No.of Cloves	Wt/Clv (g)		
Conventionally Propagated Cloves			3.5ca	4.1c	22.7e	6.3e	3.6	707e	100
Tissue Cultured Cloves	Screening House		4.1a	5.2a	39.4a	8.6a	4.6	1,102a	156
	Open Field *	1st year	3.9b	5.1a	32.7b	8.0b	4.1	1,036b	147
		2nd year	3.9b	4.8b	30.6c	7.6c	4.0	969c	137
		3rd year	3.8b	4.7b	27.3d	7.3d	3.7	895d	127

Means within columns seperated by DMRT at p=0.05.
*Garlic obtained from apical meristem culture and grown in insect-proof house for 3 years.

Table 3. Agricultural Industry of Garlic in Korea

Year	Acreage (ha)	Productivity (kg/10a)	Production (M/T)	Consumption (kg/prs/yr)
1970	15,351	508	78,009	1.5
1980	37,000	684	253,000	3.9
1985	39,015	657	256,201	3.8
1990	43,643	955	416,774	5.7
1991	49,160	977	480,513	7.2
1992	43,494	1,068	464,649	6.9

Fig. 1. Production system of minibulbs(2-3g/clove)



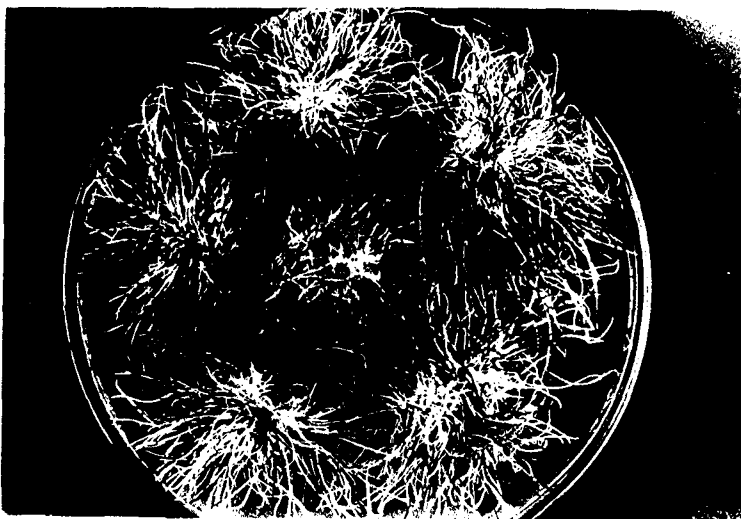


Fig. 2. Multishoots growing in a petridish

Fig. 3. A flow chart of processes

Fig. 3-1. Apical meristem culture process

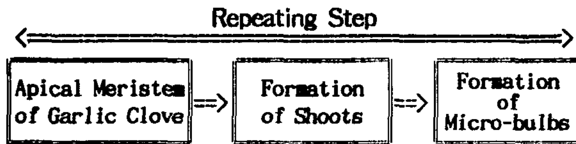


Fig. 3-2. Callus culture process

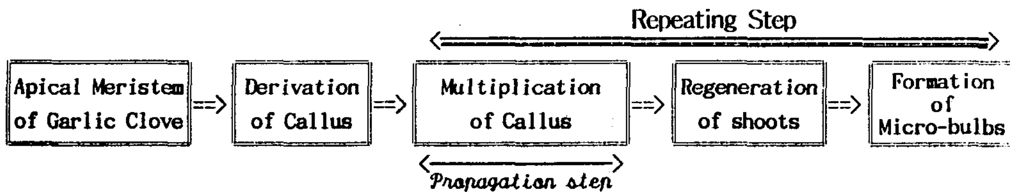


Fig. 3-3. Sumitomo process (Aginomoto K.K.)

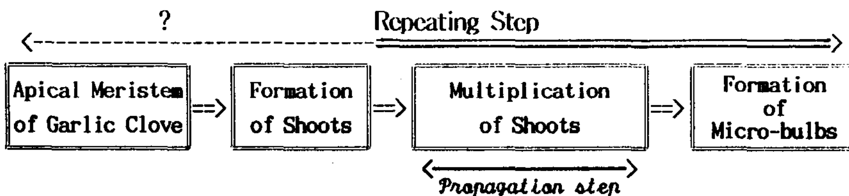


Fig. 3-4: Shoot primodium process (Shin Nippon Seitetsu K.K.)

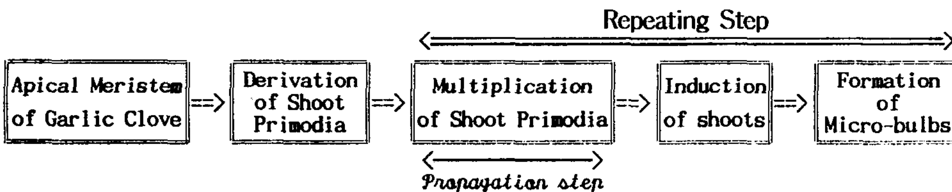


Fig. 3-5. Multishoots process (Tong Yang Moolsan Co.)