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Systematic Propagation of High Quality Garlic (*Allium sativum* L.) Through Shoot Apical Meristem Culture
I. Organogenesis from in Vitro Cultured Shoot-tips

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생장점배양에 의한 우량마늘 체계적 증식
I.생장점배양으로부터 기관형성

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Since garlics (*Allium sativum* L.) are propagated through cloves, infection by virus or other pathogens may become severe problem if not using high quality seed bulbs every year, resulting in the reduction of yield and bulb quality. In order to solve this problem, the establishment of virus-free bulb production and its supply system have been required because no chemicals were found to eliminate viruses from seed bulbs. This experiment was conducted to develop an effective production technique of high quality seed bulbs using shoot-tip culture. Over 90% of shoot-tips explanted on January 4, 1990 were survived at constant temperature of either 20, 24, or 28° C, wheres 88% at alternate temperature (28/20° C). The growth of shoot and root was most vigorous at constant 24° C, and least at alternate temperature (28/20° C) condition. When shoot-tips were explanted June 21 to August 1, 1991, survival and growth of shoot-tips was most vigorous on MS medium supplymented with 0.1 mg/L NAA and 2 mg/L kinetin and least 1 mg/L GA_3. The shoot-tips taken from the seed bulbs stored at 4° C for 15 to 60 days were placed on MS medium, shoot growth and in vitro bulblet formation increased slightly as affected by the increase of cold treatment period at 4° C.

Key words: cold treatment, cultural temperature, growth regulators, shoot-tips

Garlic, one of the most important economical crops in Korea, has been produced on a large scale for the last decades that the cultivated area increased to 43,494 ha and the total yield reached 464,649 M/T in 1992 (Ministry of Agriculture, Forestry and Fisheries, Rep Korea, 1992).

Common garlic, a diploid plant, has sterile flowers and can be propagated only vegetatively by means of cloves and bulbils. Development of sexual cells in garlic flowers stops at early stages of both female (Weber, 1929) and male gametophytes (Weber, 1929: Koul and Gohil, 1970). Tapetal layer persists and becomes hypertrophic in sterile anthers (Novak, 1972), and only uninuclear pollens are produced in them (Koul and Gohil, 1970). Accordingly, its propagation relies on the slow natural multiplication: only 6 to 9 daughter cloves are produced by a mother clove in a year. Most of the crops such as strawberry, potato, carnation and gerbera etc. propagated with vegetative organs for several years are systemically infected with virus or pathogens, and it must be essential to replace the mother plants every year (Boxus, 1976). Pathogen attack does not always lead to death of the plant. Many viruses may not even show visible symptoms, however, the presence of viruses in plants can reduce the vigour, quality and yield of crops (Wang and Hu, 1980).

It is revealed that garlics observed at 50 sites on 20

locations in Korea were infected with virus (Chung and Chang, 1979). In this viewpoint, it is neccessary to produce virus-free plants for the increase of bulb quality and yield. Propagators and farmers require the development of rapid vegetative propagation system for virus-free garlic, but unfortunately the attempts to obtain virus-free garlics for commercial use have not been successed in Korea. The virus-free plants can be distributed to farmers by supply organizations, for example, the Rural Development Administration or seed companies.

Virus-free garlic could be obtaind by shoot-tip and callus culture. In shoot-tip culture, untill now, one shoot-tip of garlic usually produces only 1 to 3 shoots (Lee et al., 1988). The shoot-tip culture, however, is considerd to be more suitable than callus culture for propagation of virus-free plants with normal karyotype (Novak, 1974: 1978: 1980).

This experiment was carried out to investigate the factors affecting organogenesis from in vitro cultured shoot-tips in order to describes the efficient and systematic propagation of high qulity garlic through shoot apical meristem culture.

MATERIALS AND METHODS

The shoot-tips of a local cultivar Seosan garlic (*Allium sativum* L.) cloves of 4 to 5 g in weight were excised. The protecting leaves were removed and the cloves were surface-sterilized by immersion for 10 s in 98% ethanol followed by a 15 mim immersion in diluted solution of sodium hypochlorite (active chlorine 0.7%) and washed 3 times with sterile distilled water. The shoot-tips (0.3-0.5 mm in diameter) including one leaf primordium were excised under stereomicroscope and placed onto medium. For shoot-tip culture of clove, MS (Murashige and Skoog, 1962) medium supplemented with 0.1 mg/L naphthaleneacetic acid (NAA) and 2 mg/L kinetin was used. The media were autoclaved at 121°C for 15 min. The pH of the medium was adjusted to 5.8 with 1 N NaOH before autoclaving. And they were cultured under the light conditions of 2,500 Lux and 16 h photoperiod.

Effect of Temperature

The following ranges of temperature were given: (a) alternate temperature (28/20°C), (b) 20 ± 0.5°C, (c) 24 ± 0.5°C, and (d) 28 ± 0.5°C. Bulbs were harvested on June 1989, stored at room temperature and shoot-tips were excised on January 4, 1990. Forty explants were cultured per treatment and survival rate, number of shoots, leaves and roots, and shoot height were measured after 8 weeks in culture.

Explanting Dates and Growth Regulators

The shoot-tips were excised from June 21 to August 1 at 10 day intervals, 1991 and placed onto MS medium supplemented with 0, 0.1 mg/L NAA and 2 mg/L kinetin, and 1 mg/L GA_3. Survival, organogenesis of shoot-tips were observed on 115 days after culture. Cultural temperature was 24 ± 3°C.

Cold Treatment to Seed Bulbs and Explanting Date

In cold treatment of seed bulb the starting date of the treatment and the tratment period is one of the most important factors in this experiment. Treatment were classified into two groups depending on the starting date of the cold treatment. In one group (5 treatments) cold treatment has been started at the same time, on July 10, 1990 and each sample has been stored in 4°C for 0, 15, 30, 45 or 60 days respectively. In the other group (5 treatment) cold treatemt has been started at five different dates and finished at the same time, on September 10. This also resulted the five different storage periods of 0, 15, 30, 45 or 60 days as in the first group. After the cold treatment, shoot-tips were excised from each sample placed onto the medium. Figure 1 shows

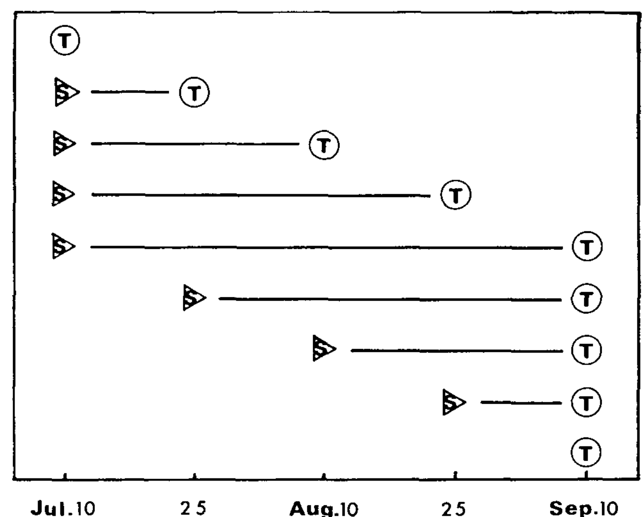

Figure 1. Diagram showing the periods of cold treatment of seed bulbs. S:starting dates of cold treatments: T:explanting dates of shoot-tip.

the schematic diagram of the details of startiing date and explanting date of each sample. Organogenesis of shoot-tips were observed on December 21, 1990. The plantlets obtained were subcultured on MS medium containing 8% sucrose and supplemented with 0.1 mg/L NAA at 4°C under darkness for 40 days from Janury 18 and again recultured at 24 ± 3°C under 16 h daylength for 45 days. The formation ratio of in vitro bulblet and weight of in vitro bulblet were measured.

RESULTS

Effect of Temperature

Over 90% of explants were survived at constant temperature of either 20, 24, or 28°C, whereas 88% at alternate temperature (28/20°C) (Table 1). However, the differences in number of shoots per survived explant among cultural temperature were not significant. The growth of shoot and root was most vigorous at constant 24°C, and least at alternate temperature (28/20°C) condition. The leaf formation was better at 24°C than at the other temperature treatment. Constant temperature at 24°C was seemingly effective for the growth and development of shoot-tips.

Table 1. Effects of culture temperature on organogenesis in shoot-tip culture of garlic after 8 weeks in culture.

Temp. (°C)	Survival (%)	No. shoots/surv. explant	Shoot hight (cm)	No. leaves	No. roots
28/20	88A[a]	1.6A	2.9B	2.7A	0.1A
20	93A	1.6A	6.7A	2.7A	0.4A
24	95A	1.5A	7.3A	3.3A	0.5A
28	90A	1.5A	7.0A	3.0A	0.5A

[a]Means within columns followed by the same letter are not singificantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Effect of Explanting Dates of Shoot-tips and Growth Requlators

As shown in table 2, 47% to 60% of shoot-tips placed onto medium supplmented with 0.1 mg/L NAA and 2 mg/L kinetin on June 21 to August 1 were survived, 25% to 53% onto MS basal medium, whereas 7% to 47% onto medium supplemented with 1 mg/L GA_3. The shoot-tips cultured on August 1 produced 1.6 shoots by cultureing for 115 days in medium containing 0.1 mg/L NAA and 2 mg/L kinetin, but the shoot formation was little difference between explanting dates and growth regulators added to medium. The produced shoots onto medium containing 0.1 mg/L NAA and 2 mg/L kinetin were the highest in their growth increasement and the growth of shoot was increased as delaying the explanting dates regardless growth regulators. The formation of root was vigorous onto medium containing with or without 0.1 mg/L NAA and 2 mg/L kinetin, and the least 1 mg/L GA_3.

Table 2. Survival, formation and growth of shoot by explanting date of shoot-tip and growth requlators.[a]

Explanting date	Growth requlators (mg/L)	Survival (%)	No. shoots	Shoot height (cm)	No. roots	Root length (cm)
June 21	0	25	1.1	3.9	1.3	2.1
	K 2+N 0.1[b]	47	1.3	5.1	1.2	1.9
	GA_3 1	7	1.0	0.3	0	-
July 1	0	47	1.2	2.8	0.2	4.6
	K 2+N 0.1	50	1.4	4.2	1.5	1.2
	GA_3 1	20	1.0	3.8	0.3	0.5
July 11	0	53	1.3	6.7	1.1	2.8
	K 2+N 0.1	60	1.3	7.0	2.1	2.5
	GA_3 1	23	1.0	3.6	0.6	1.6
July 21	0	50	1.0	8.6	2.3	2.4
	K 2+N 0.1	57	1.1	8.3	1.0	2.4
	GA_3 1	30	1.0	4.5	0.7	1.2
Aug. 1	0	33	1.0	9.0	2.0	2.1
	K 2+N 0.1	53	1.6	13.0	2.6	2.5
	GA_3 1	47	1.4	9.5	2.1	2.1

[a]Datas were collected on 115 days after explanting of shoot-tip.
[b]Kinetin 2 mg/L + NAA 0.1 mg/L.

Effects of Cold Treatment to Seed Bulb and Explanting Dates

The shoot-tips taken from the bulbs which had been stored at 4°C for 0, 15, 30, 45 or 60 days were cultured from July 10 and shoot formation and growth were compared. The number of shoot formed from shoot-tip without cold treatment was about 1.5 shoots per explant cultured on July 10, August 16 and 28, and September 10, whereas those cold treatment about 1.6 shoots (Figure 2A). And it was 1.7 shoots that were formed from the shoot-tips of seed bulbs which had been stored at 4°C for 0, 15, 30, 45, 60 days till September 10 (Figure 3A).

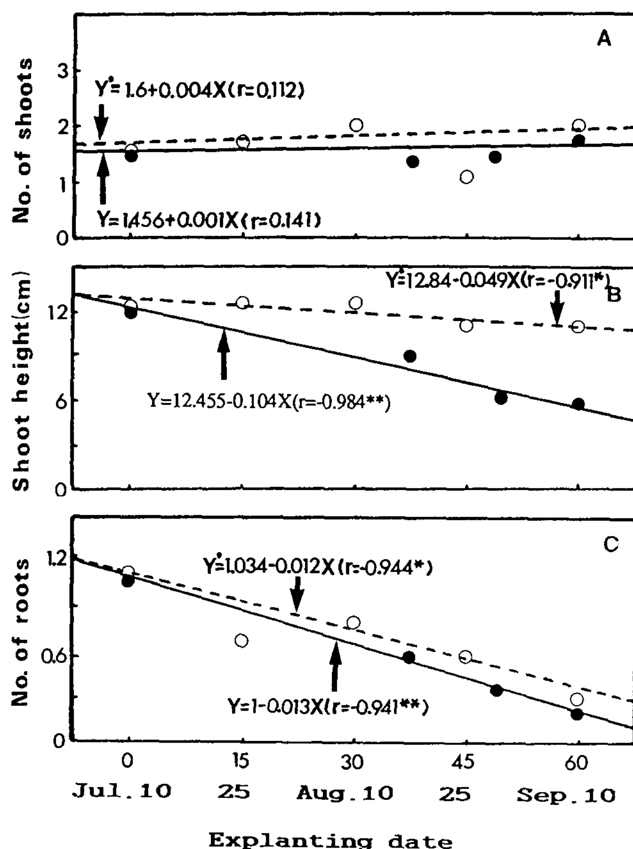

Figure 2. Relationship between number of shoots (A), shoot height (B), number of roots (C), and explanting date of shoot-tip. Seed bulbs were treated with chilling (Y') and without chilling (Y). X axis value indicates 0 as explanting date of shoot-tip on July 10.

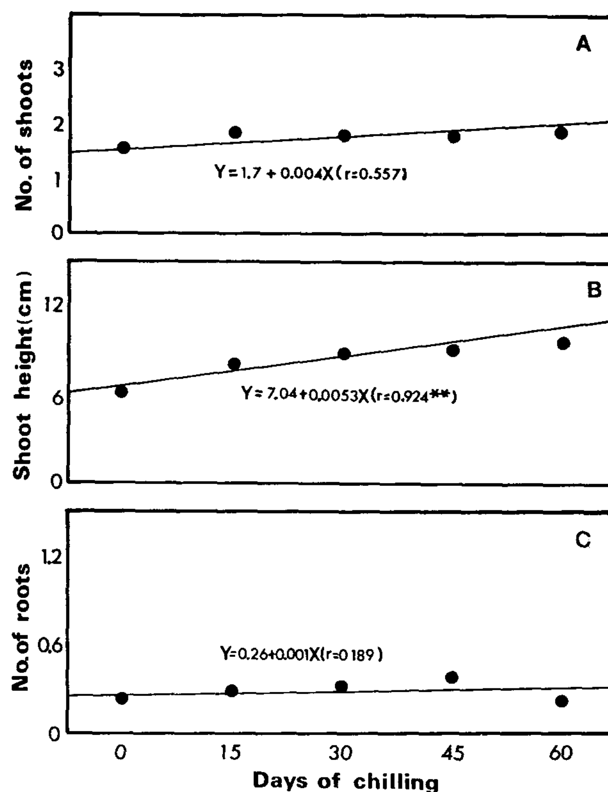

Figure 3. Relationship between number of shoots (A), shoot height (B), number of roots (C), and periods of cold treatment. Shoot-tips were explanted on September 10, 1990.

The height of shoot derived from the explants without cold treatment was decreased as delaying the explanting date, but those cold treatment was no difference between explanting date (Figure 2B). However, when the shoot-tips taken from the bulbs stored at 4°C for 0, 15, 30, 45 or 60 days were cultured on September 10, shoot height increased slightly along with the increase of cold treatment period at 4°C (Figure 3B).

The number of roots from shoot was highest in the explant explanted on July 10, middle on August 25, and lowest on September 10, and root number of shoot without cold treatment was less than those of shoot experienced cold treatment (Figure 2C). When shoot-tips were explanted on September 10, the differences in number of roots among cold treatment were not significant (Figure 3C).

The plantlets obtained from above experiments were subcultured on MS medium for in vitro bulblet formation. As shown in table 3, the effects of cold treatment of seed bulb on in vitro formation of bulblet was somewhat different

Table 3. Effects of cold treatment of seed bulb and explanting date of shoot-tip on the in vitro bulblet formation.[a]

Chilling (days)	Explanting date	Bulbing (%)	No. bulblets/explant	Bulb weight(mg)[b]
0	Jul. 10	18(0)[c]	0	-
0	Aug. 18	36(9)	0.1	110
0	Aug. 28	21(0)	0	-
0	Sep. 10	37(17)	0.2	105
15	Jul. 25	37(0)	0	-
15	Sep. 10	100(58)	0.5	222
30	Aug. 10	75(31)	0.3	162
30	Sep. 10	100(62)	0.6	230
45	Aug. 25	94(56)	0.9	150
45	Sep. 10	100(67)	0.7	200
60	Sep. 10	100(85)	1.2	243

[a]The plantlets obtained though shoot-tip culture were subcultured on MS medium containing 8% sucrose and supplemented with 0.1 mg/L NAA.

[b]Bulblet weight presented only normal bulblet.

[c]Number in parenthesis means the percentage of normal bulblet having senescenced leaves.

from that of explanting date of shoot-tip. The formation of in vitro bulblet induced from plantlets and normal bulblets were proportionally increased with the increment of the cold treatment period or in the case of plantlet explanted lately.

Weight of bulblets were also increased with the increment of cold treatment to seed bulb. The maximum formation, number and weight of bulblet were obtained at cold treatment of seed bulb for 60 days when shoot-tip were explanted on September 10.

DISCUSSION

In virus infected plants, the apical meristem are generally free or carry a very low concentration of the viruses (Quak, 1977: Wang and Hu, 1980). Morel and Martin (1952) developed the technique of shoot-tip culture for in vivo virus eradication. Since then, the advances in shoot-tip culture have been regarded as the most efficient technique in obtaining completely virus-free plants (Boxus et al., 1977), and this has been succeessfully applied to a wide range of plants. Although it is mainly used for virus elimination, shoot-tip culture has also enabled plants to be freed from other pathogens including viroids, mycoplasmas, bacteria and fungi. Factors such as culture conditiion, explant size, physiological stage of the explants and culture time which affect plant regeneration from small excised shoot-tips (0.1 to 1 mm), will considerably affect virus eradication by this method.

The cultures are generally conducted under standard culture-room temperatures of 25 ± 2°C. The temperature of 24°C is also effective in meristem culture of lily (Kawarabayashi and Asahira, 1988) and Seabrook and Cumming (1982) tested a range of temperature requiring for the shoot and bulbil formation in leaf base-tissue cultures of *Narcissus* and found that more shoots were formed at a constant temperature of 25°C. Information is limited about the effect of temperature on the organ formation of plants from excised shoot-tip of garlic. In this shoot-tip culture of garlic, temperature for the incubation of culture affected the development of the meristem tissue. Under the temperature range of 20 to 28°C, the optimal condition was 24°C for improving leaf formation and shoot growth but alternative temperature of 28/20°C was not effective because small tissue of shoot-tip seemed to accept the stress by alternate temperature.

One of the most important factors affecting and controlling shoot regeneration is the development stage of a donor plant at the time of the removal of explant (Novak et al., 1986). Shoot-tips should be taken from actively growing buds. Garlic bulbs enter dormant stage from the late in April, reaches into deep dormancy in the middle in June and stays dormant for a long time to planting date (Moon et al., 1983). It seems that shoot-tip culture of garlic during deep dormancy is difficult (Moriaki et al., 1989). The dormancy is broken by cold treatment or high temperature treatment to seed bulbs (Hwang, 1988). Boxus and Quoirin (1974) have shown that in *Parious pranus* species the stem must be maintained at 4°C for 6 months before excising the shoot-tips. In *Narcissus* shoot-tips taken from bulbs after cold treatment at 11°C for 6 to 8 weeks grow faster and survive better in vitro than those from untreated bulbs (Seabrook et al., 1976). For tulip and Iris, a pretreatment of bulb at 30°C for 4 to 8 weeks germinate more rapidly and sychronizes than bulb without such a treament. Explants from actively growing shoots at the beginning of the growing season generally give better results on shoot differentiation and growth, and bulb formation (Seabrook et al., 1976: Alderson et al., 1983). For seasonal influence on shoot-tip culture processes, Stone (1963) observed better survival of carnation meristem in early spring and early autumn than in either winter or summer. With bulbs and corms, the best results may be expected when they are dissected at the end of their dormancy period. In garlic, the period of cold treatment on seed bulb and the excision time of shoot-tip were important factors. The shoot formation, callus proliferation and shoot formation from proliferated calli are more effective in cold treatment at 4°C for 2 months than room temperature treatment to seed bulb in case of shoot-tip culture at a short period of autumn (Lee et al., 1988: Min et al., 1991). And, in deep dormancy in July to August, shoot formation and its growth, and in vitro bulblet formation were stimulated by cold treatment at 4°C for 15 to 60 days (Figures 2, 3 and Table 4). It is similar to the results of Moriaki et al. (1989).

It seems that the dormancy of garlic could be overcome by growth regulator application. The shoot-tip culture of the dormant garlic were successful only 25% to 53% without any supplement of plant growth regulators. On the other hand, 47% to 60% of shoot-tips placed onto medium supplemented with 0.1 mg/L NAA and 2 mg/L kinetin were survived. But GA_3 application tends to decrease the survival and shoot formation. A small amount of GA_3, is often added to media for shoot-tip cultures. It may not always be beneficial or absoutely necessary (George and Sherrington, 1984).

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적 요

본 연구은 마늘 무병우량종구의 효율적인 생산, 증식 및 보급체계를 확립하기 위한 기초 자료를 얻기 위하여 한지형 瑞山種 마늘의 생장점 배양시 기관분화에 미치는 배양온도, 배양시기, 생장조절물질 및 저온처리 영향을 검토하기 위하여 실시하였다. 동절기의 생장점 배양은 28/20°C의 변온 조건에서 배양하는 것보다 24°C 항온조건에서 배양하는 것이 생존율 및 shoot 형성과 생장이 양호하였다. 마늘인편 휴면기인 7, 8월에는 kinetin 2 mg/L와 NAA 0.1 mg/L을 첨가한 배지에서 생존율 및 shoot의 생장이 양호한 반면에, GA_3 1 mg/L 첨가한 배지는 억제되었다. 또한 휴면기에는 생장점 배양 전 종구를 15-60일간 4°C에서 저온처리 함으로서 shoot 생장 및 발근을 촉진시켰으며, 또한 구형성 배지에 계대배양시 저온처리기간이 증가할수록 기내인경구 형성 및 비대를 촉진하였다.

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