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Multiple Shoots Regeneration and *In vitro* Bulblet Formation from Garlic Callus

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Abstract

The leaf segments of garlic (*Allium sativum* L.) were cultured *in vitro* and determined optimal concentration of plant growth regulators and sugars for callus induction, multiple shoots regeneration and *in vitro* bulblet formation. Highest yield of callus was observed in the leaf segment culture on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 2,4-D, 30 g/L sucrose and 8 g/L agar. Regeneration rate of multiple shoots from callus was high in the MS medium supplemented with kinetin 3.0 + NAA 3.0 mg/L or BA 1.0 + NAA 3.0 mg/L, containing 30 g/L sucrose. High rate of bulblet formation was observed as the concentration of jasmonic acid increased from 0.5 to 2.0 mg/L in medium, whereas addition of gibberellic acid significantly suppressed bulblet formation. The rate of *in vitro* bulbing was as high as 96% in MS medium supplemented with 2.0 mg/L jasmonic acid and 120 g/L sucrose after two month culture at 25 ± 1°C under 16 hours day light.

Key words: Callus induction, garlic (*Allium sativum* L.), jasmonic acid, micropropagation, plant growth regulators

Introduction

The propagation of garlic through *in vitro* technique has been studied by several researchers (Abo 1977; Bhojwani 1980; Nagakubo et al. 1993; Barandiaran et al. 1999). Although various explants such as shoot tip (Abo 1977; Nagasawa and Finer 1988; Novak 1981), stem disc (Koch et al. 1995; Ayabe and Sumi 1998) and roots (Mayer and Simon 1998; Ali and Metwally 1992; Shuto et al. 1993) were successfully used to induce callus and subsequent shoot regeneration, the propagation rate of shoot from callus is still low. Recently, the improvement of shoot regeneration by adding various combinations of plant growth regulators to media have been reported (Koch et al. 1995; Barandiaran et al. 1999), and encouraging results are shown to be helpful in obtaining a large quantity of regenerated shoots for commercial use. However, a systematic experiment on the effects of combinations of plant growth regulators on the shoot proliferation are not still enough, which may overlook the potential combinations of certain plant growth regulators that are more suitable for shoot multiplication.

In vitro bulblet formation of garlic shoots are largely dependent on growth regulators and sucrose in media (Abo 1977; Matsubara and Chen 1989; Nagakubo et al. 1993), as well as other conditions such as cultivar, photoperiod, temperature etc. BA and jasmonic acid are reported more effective factors promoting *in vitro* bulbing of garlic shoot, despite a stimulatory role of the higher concentration of sucrose *in vitro* bulblet formation (Nagakubo et al. 1993). Gibberellic acid is a known growth regulator which promotes elongative growth of a plant organ, therefore it might suppress bulbing of garlic thereby altering the balance of growth substances needed for bulbing in shoot.

This study was conducted to understand the possible role of plant growth regulators and sugars in the multiple shoot regeneration and *in vitro* bulblet formation using garlic callus, and to obtain basic information on the practical application of *in vitro* bulblet as seed bulb of the garlic.

Materials and Methods

Plant materials

A Korean local garlic (*Allium sativum* L. cv Euisung), a late maturing variety, commonly cultivated in the central regions of

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Korea, was used in the experiment. Garlic cloves were surface sterilized in 1.0% sodium hypochlorite solution for 15 min after removing the outer protective leaf sheaths, and then rinsed three times with sterilized water. The cloves were sprouted aseptically in the 25±1°C growth chamber. The leaves emerged out from stored shoot were isolated and cultured.

Callus induction and proliferation

To determine the optimal concentration of plant growth regulator for callus induction from garlic leaf explant, six leaf segments (5×5 mm) with ten replications were placed in 9 cm plastic petridishes containing 25 mL of MS (Murashige and Skoog 1962) media supplemented with various combinations of plant growth regulators, such as kinetin, BA (6-benzyladenine), 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA (1-napthaleneacetic acid), 30 g/L sucrose, 8 g/L agar at pH 5.8. Cultures were incubated at 25±1°C in darkness for 8 weeks, and measured fresh weight of callus derived from the leaf segment. Callus was subcultured to fresh medium for another 4 weeks before being transferred to shoot regeneration medium.

Regeneration of multiple shoots

After 4 weeks incubation, callus (ca. 50 mg) was transferred to the regeneration medium, consisting of MS basal medium with various combinations of cytokinins and auxins. Cultures were incubated at 25±1°C, under cool white fluorescent lights with a 12-hour photoperiod. At least 15 replicate petridishes, each with six calli were used for each treatment of regeneration media. To determine the effect of sugars, such as sucrose and glucose, callus was treated with various concentrations of sugars (10 to 90 g/L). After 8 weeks culture, regeneration rates were measured by counting the number of shoots per petridish, and shoots were used to evaluate the effect of plant growth regulators on *in vitro* bulblet formation.

In vitro bulblet formation

The clusters of multiple shoots, proliferated in MS medium with kinetin 3.0+BA 3.0 mg/L, were divided into single shoots, and transferred to media containing various concentrations of jasmonic acid, gibberellic acid and/or sucrose for *in vitro* bulblet formation. Cultures were incubated at 25±1°C under fluorescent lights with a 16-hour photoperiod. At least 20 replicate petridishes, each with 4 shoots were tested for each treatment of the bulbing media. After 2 months culture, the percentage of shoots forming bulblets and their fresh weight were recorded.

Results and Discussion

Callus induction

Based on the fresh weight of callus derived from garlic leaf segment, 2,4-D was more effective than NAA regardless of concentrations and combinations of growth regulators tested (Table 1). Plant growth regulator initiating friable callus on garlic leaf segment was 1.0 mg/L 2,4-D in MS medium supplemented with 30 g/L sucrose and 8.0 g/L agar. Callus induction rates in the media containing 2,4-D less than 1.0 mg/L, such as 0.5 mg/L or less, were negligible (data not shown). Addition of cytokinins (kinetin and BA) to 1.0 mg/L 2,4-D treated media significantly inhibited the induction of callus on garlic leaf segment (Table 1). Mayer and Simon (1998) suggested that 2,4-D is effective for callus initiation from garlic leaf segment.

Shoot regeneration

Shoot regeneration rates were compared in different combinations of cytokinins (BA or kinetin) and auxins (IAA or NAA). Multiple shoot regeneration was better in NAA treatment than that of IAA, irrespective of cytokinin types combined. It was well consistent with the results of Ali and Metwally (1992) and Barandiaran et al. (1999).

The different effects of combinations of growth regulators on shoot regeneration were observed. Media supplemented with kinetin and NAA showed better multiple shoot regeneration rate than that of BA and NAA. High concentration of kinetin+NAA (3.0+3.0 mg/L) showed the best shoot regeneration rate among all the treatments (Table 2, 3 and Figure 3).

The treatment of sugars, sucrose or glucose, higher than 30 g/L markedly inhibited multiple shoot regeneration from garlic callus. Sucrose was more effective than glucose for shoot

Table 1. Effect of plant growth regulators (2,4-D, NAA, kinetin and BA) on fresh callus weight (mg) derived from leaf segment of garlic (*A. sativum* L.)[a]

Treatments (mg/L)		2,4-D		NAA	
		1.0	2.0	1.0	2.0
	0	789.1	359.1	31.1	93.1
Kinetin	0.1	685.6	297.3	37.2	117.2
	1.0	490.3	363.8	35.2	74.6
BA	0.1	652.4	382.1	42.9	60.3
	1.0	470.5	371.8	124.2	47.1
LSD (0.05)		114.4	87.2	32.4	44.5

[a]Total fresh weight (mg) of callus initiated from six leaf segments in one petridish, determined at 8 weeks after inoculation

regeneration in all concentrations, and highest regeneration rate was observed in 30 g/L sucrose medium (Figure 1).

Barandiaran et al. (1999) observed the possible detrimental effect of 2,4-D on callus induction and regeneration. However, such an adverse effect of 2,4-D on callus formation was not found when used in a concentration of 1.0 mg/L. In this study, 1.0 mg/L 2,4-D showed the best effect on callus production and proliferation. Even though the callus was multiplied in the same medium and growth regulator, callus quality was varied, and the quality of callus, such as callus color, compactness, friability etc., was closely related to the potential of shoot regeneration. In this regard, the continuous selection of high quality callus during the subculture was also found to be important for efficient shoot regeneration from the callus.

In vitro bulblet formation

In vitro bulblet formation of garlic shoot was investigated. After 2 months culture under 16 hours light at 25±1°C, bulblets of 20-193 mg were formed on the basis of shoot. The frequency of bulblet formation was closely associated with the concentration of jasmonic acid in media. The highest percentage of bulblet formation was found in the 2.0 mg/L jasmonic acid media (Table 4, and Figure 3). Bulblet formation also occurred in jasmonic acid free media. This indicated that garlic bulblet formation *in vitro* was not only induced by plant growth regulator, but also largely dependent on the size and quality of regenerated shoots. Nagakubo et al. (1993) also observed the *in vitro* bulblet formation on hormone free media. The frequency of bulblet formation was significantly suppressed by addition of gibberellic acid to jasmonic acid containing media. Single treatment of 2.0 mg/L jasmonic acid formed bulblets at a rate of 77.6%, whereas jasmonic acid 2.0+gibberellic acid 1.0 mg/L or jasmonic acid 2.0+gibberellic acid 3.0 mg/L treatments showed frequency of 55.6 or 32.0% bulblet formation, respectively. This result suggests that gibberellic acid suppressed *in vitro* bulblet formation in the media containing jasmonic acid. However, fresh weight of a bulblet was markedly increased by addition of 1.0 mg/L gibberellic acid to jasmonic acid treated media, and 3.0 mg/L gibberellic acid to 1.0, 1.5 and 2.0 mg/L jasmonic acid media. This suggests that the growth of *in vitro* bulblet was promoted by the action of exogeneous gibberellic acid in the medium. Even 2.0 mg/L jasmonic acid treatment showed the highest rate of *in vitro*

Table 2. Effects of plant growth regulators (BA+IAA or NAA) on multiple shoot regeneration from the garlic callus[a].

BA (mg/L)	IAA (mg/L)				NAA (mg/L)		
	0	0.1	1.0	3.0	0.1	1.0	3.0
0	0.0	0.2	0.0	0.0	0.0	2.2	2.4
0.1	0.0	0.0	0.0	0.0	0.0	11.5	19.8
1.0	0.0	5.4	5.4	0.0	9.8	31.7	63.5
3.0	25.6	11.0	3.0	6.0	4.6	11.0	46.4

[a]Total number of shoots regenerated from six calli in one petridish, determined at eight weeks after treatment.

Table 3. Effects of plant growth regulators (kinetin+IAA or NAA) on multiple shoot regeneration from the garlic callus[a].

Kinetin (mg/L)	IAA (mg/L)				NAA (mg/L)		
	0	0.1	1.0	3.0	0.1	1.0	3.0
0	0.0	0.2	0.0	0.0	0.0	2.2	2.4
0.1	0.0	0.8	0.0	0.8	9.3	7.4	39.8
1.0	2.2	0.3	0.0	0.0	9.4	42.2	34.0
3.0	2.8	0.0	0.5	0.0	7.3	39.2	152.8

[a]Total number of shoots regenerated from six calli in one petridish, determined at eight weeks after treatment.

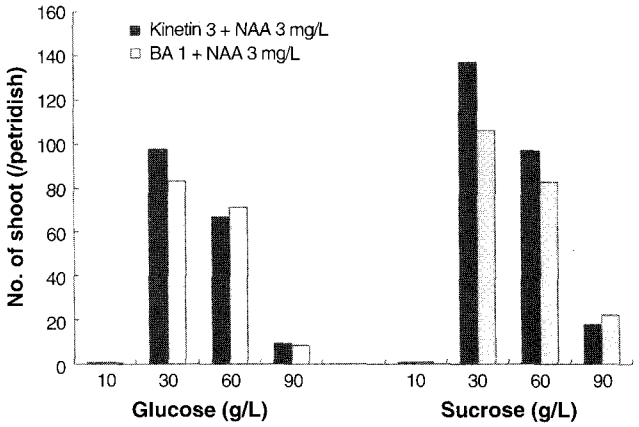

Figure 1. Effects of various concentrations of glucose and sucrose on the regeneration of multiple shoots from garlic callus. Six calli (50 mg) were placed on 9 cm petridish containing 25 mL medium with kinetin 3.0+NAA 3.0 mg/L or BA 1.0+NAA 3.0 mg/L. Data were collected at eight weeks after inoculation.

Table 4. Effect of jasmonic acid (JA) with gibberellic acid (GA) on *in vitro* bulblet formation in shoots regenerated from callus[a].

Treatment (mg/L)	Percentage of bulbing			Bulblet fresh wt (mg/bulb)		
	GA 0.0	GA 1.0	GA 3.0	GA 0.0	GA 1.0	GA 3.0
JA 0.0	8.3	6.8	2.0	73	114	42
0.5	36.8	18.7	13.0	111	144	73
1.0	40.2	30.4	15.0	102	154	175
1.5	50.0	37.0	28.0	104	193	187
2.0	77.6	55.6	32.0	63	128	145
3.0	64.7	53.0	34.9	82	88	52
4.0	34.7	47.8	39.0	22	32	57

[a]Number of *in vitro* bulblets and fresh weigh were determined at two months after treatment.

bulblet formation, fresh weight (63 mg) of a bulblet was low as compared with 0.5, 1.0 or 1.5 mg/L jasmonic acid treatment showing 111, 102 and 104 mg, respectively. The size of *in vitro* bulblet is one of the most important factors for practical application of the bulblet as seed bulb of the garlic. Further studies are needed regarding on the adaptable size of *in vitro* bulblet suitable for seed bulb as well as on the various conditions for high rate of *in vitro* bulbing with resonable size. The effects of sucrose concentrations in the media with or without jasmonic acid on *in vitro* bulbing were also investigated. Effects of sucrose concentrations on *in vitro* bulbing were not significant in the media without jasmonic acid because all concentrations of sucrose showed less bulblet formation rate than 25% (Figure 2). However, addition of sucrose to the media containing 2.0 mg/L jasmonic acid markedly increased the rate of bulbing up to 96 % in 120 g/L sucrose + jasmonic acid 2.0 mg/L medium.

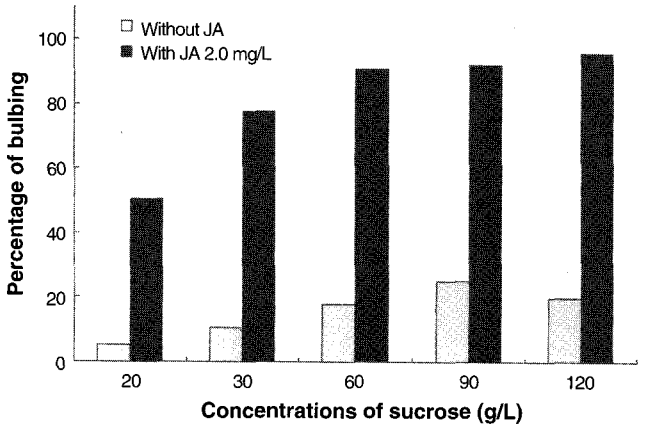

Figure 2. Effects of various concentrations of sucrose with or without jasmonic acid (JA) on *in vitro* bulblet formation from garlic shoot. Four shoots with 20 replications were inoculated on 9 cm petridish containing 25 mL medium. Data were collected at 2 months after incubation under 16 h photoperiod at 25±1°C.

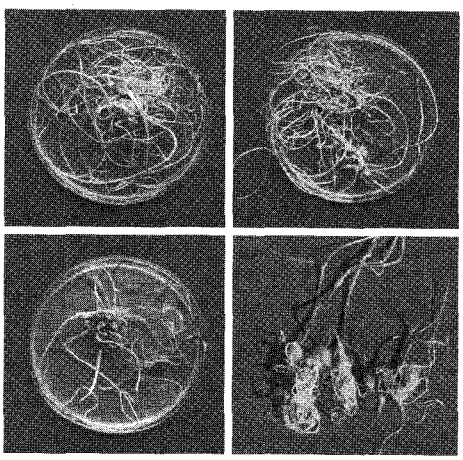

Figure 3. Multiple shoot regeneration from garlic callus on kinetin 3.0 + NAA 3.0 mg/L (upper left) and BA 1.0 + NAA 3.0 mg/L (upper right) media, *in vitro* bulblet formation (lower left) on jasmonic acid treated medium, and bulblets harvested from petridishes (lower right).

In vitro bulbing for a late maturing cultivar required more than 4 months of the low temperature treatment at 5°C (Nagakubo et al. 1993). In this study using cultivar 'Euisung', a late maturing cultivar, *in vitro* bulblets were effectively formed in jasmonic acid treated media without a low temperature pretreatment of shoot, and the bulblet also even formed in the medium without jasmonic acid (Table 4).

An efficient micropropagation method which was optimized using a late maturing cultivar, 'Euisung' was developed through this study. This method would be able to apply to other garlic cultivars with slight modification in the processes of multiple shoot regeneration or *in vitro* bulblet formation. However, a high rate of vitrification in multiple shoots by continuous subculture seriously inhibited bulblet formation (data not shown). A further study is needed to increase the efficacy of *in vitro* proliferation of garlic, and to identify factors that induce or inhibit invitro bulblet formation. For the purpose of practical application of *in vitro* bulblets as seed bulb of the garlic, several problem, such as the habituation of bulblets for field condition, control of dormancy and sprouting, and proper storage of bulblets, are to be solved.

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