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Systematic Propagation of High Quality Garlic (*Allium sativum* L.) Through Shoot Apical Meristem Culture
II. Effects of Sucrose Concentration and Nitrogen Source on In Vitro Formation of Bulblets

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생장점배양에 의한 우량마늘 체계적 증식
II. 기내 인경 비대에 미치는 질소 및 Sucrose의 영향

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The effects of sucrose concentration and nitrogen source on shoot growth and in vitro formation of garlic (*Allium sativum* L. cv Seosan) bulblet were investigated in order to systematize propagation of high quality garlic through a shoot apical meristem culture. Shoot differentiation was not affected by sucrose concentration and nitrogen source, but plantlets which contain medium of NH_4 − N or NH_4 + NO_3 were vigorous and healthy in appearance. Shoot growth was vigorous in changeing of nitrogen source. The best quality of in vitro bulblets was obtained in culture on the medium containing 8% sucrose and NH_4 − N, and the formation of bulblet was more effective when plantlets were subjected to cold treatment before use. NH_4 − N was a major factor for shoot growth and bulblet development, but NO_3 − N was not and suppressed K^+ absorption. The level of ethylene production was not affected by different nitrogen sources, however this production was enhanced in medium containing a higher concentration of sucrose.

Key words: cold treatment, ethylene, inorganic compounds

Virus-free garlic could obtained by meristem-tip or callus cultures up to now. However, one meristem-tip of garlic usually produces only 1 to 3 shoots (Lee and Lee, 1994) and the transplantation of induced plantlets into soil requires a careful and step-wise procedure and this process is limited to a season of spring or autumn. Plants forming rest organs should be allowed to produce them in tissue culture. As a result, bulblets, cormlets, tubers and rhizomes are easy to handle for transplantation or transportation than the above delicate plants. Bulblets or cormlets are regarded as the final product of propagation schemes, and this bulbing process is generally thought to coincide with a gradual deeping of the dormant state. Various factors have been reported to stimulate bulbing of shoots, generally at the expense of further leaflet formation. These factors include higher culture temperature of 15 to 30°C (Nishiuch, 1980: Stimart and Ascher, 1978), such as a chilling (5°C) for 2 to 10 weeks (Nishiuchi, 1980), darkness (Chung et al., 1983: Leshem et al., 1982: Steimitz and Yahel, 1982: Stimart and Ascher, 1978: Ziv, 1979), high sucrose levels (Nishiuch, 1980: Takayama and Misawa, 1980: Ziv, 1979), relatively low pH about 5.0 (Chung et al., 1983: Nishiuchi, 1980), the presence

of a low concentration of auxin (Nishiuchi, 1980: Stimart and Ascher, 1978), and ABA, CCC or cumaric acid in combination with a cold treatment (Han, 1987). On the contrary, light, cytokinins, low sucrose, and low temperature treatments are regarded to promote shoot or leaflet growth or to inhibit or retard the onset of bulbing (Nishiuchi, 1980: Stimart and Acher, 1978, 1981a, 1981b: Takayama and Misawa, 1980: Ziv, 1979).

In garlic, the nutritional requirements on the regulation of bulblet growth and development has not been studied. This experiment was carried out to investigate the effects of sucrose concentration and nitrogen source on in vitro formation of garlic bulblet.

MATERIALS AND METHODS

Shoot-tips including one leaf primordium were cultured on MS medium supplemented with 0.1 mg/L NAA and 2 mg/L kinetin. For rooting, the induced shoot were subcultured on MS medium supplemented with 0.1 mg/L NAA for 55 days. For the stimulation of in virto bulblet formation, the rooted plantlets were placed at 4°C under darkness for 35 days. After cold treatment, plantlets which are uniform, in height and growth, were selected and were subcultured to investigate the responses of sucrose concentration and nitogen source during the plantlet growth and bulblet formation in vitro culture. Two levels of sucrose, 30 and 80 g/L, were examined with the three treatments of nitrogen sources which consiste of: 1) NO_3-N, 2) NH_4-N, 3) NH_4 + NO_3. The media were solidified with 8 g/L Bacto agar (Difco). Plantlet growth and bulblet formation were observed after 35 days culture.

Likewise plantlets without cold treatment were subcultured by a filter paper bridge culture on liquid media consisted of sucrose 30 g/L and 80 g/L, and nitrogen treatment 1, 2 or 3 for 45 days and then transferred to the medium consisted of : 1) NO_3-N, 2) NH_4-N, 3) NH_4 + NO_3, 4) NO_3-N followed by NH_4-N (NO_3-N to NH_4-N), 5) NH_4-N followed by NO_3-N (NH_4-N to NO_3-N), 6) NH_4-N followed by NH_4 + NO_3 (NH_4-N to NH_4 + NO_3), or 7) NH_4 + NO_3 followed by NH_4-N (NH_4 + NO_3 to NH_4-N). Those were cultured for 45 days. The media contained 20 mM total nitrogen compounds (Table 1) and the pH of the media were adjusted to 5.8 with 1 N NaOH or 1 N HCl. The cultures were incubated at 24 ± 3°C.

The residual media after culture were analysed for NH_4-N, NO_3-N, $H_2PO_4^-$, K^+, Ca^{+2}, Mg^{+2}, Cl^-, and SO_4^{-2}. NH_4-N and NO_3-N were analysed by steam distillation method added with Mgo-Devart's alloy (Keeney and Nelson, 1982) and Ammonium metavanadate coloric method was applied for phosphorus analysis (Olsen and Sommers, 1982). In addition, K^+, Ca^{+2}, and Mg^{+2} were measured by Perkim Elmer 2380 automatic absorption spectrophotometer, Cl^- by Mohr method and SO_4^{-2} by Turbidity method (Japan Soc. Analytical Chemistry, 1981). During the culture in nitrogen treatments 1, 2, 3, 4, 5, 6, or 7 for 63 days, the amounts of ethylene were measured at one week interval.

Table 1. Composition of nitrogen treatment solutions used to grow garlic plantlets in mediaa.

Salts	Nitrogen treatments (mM)		
	NO_3-N	NH_4-N	NH_4 + NO_3
NH_4HCO_3	-	20.0	-
KNO_3	10	-	-
$Ca(NO_3)_2$ $4H_2O$	5.0	-	-
$CaCl_2$ $2H_2O$	-	5.0	5.0
KCl	-	10.0	10.0
NH_4NO_3	-	-	10.0
KH_2PO_4	2.5	2.5	2.5
$MgSO_4$ $7H_2O$	5.0	5.0	5.0

[a]Micronutrients were supplied according to Murashige and Skoog (1962).

RESULTS

The number of shoot on plantlet with cold treatment was not different among various sucrose concentration and nitrogen source. Explants cultured in medium containing NH_4 + NO_3 with 3% sucrose developed 1.0 shoot, but the number of differentiated shoot was 1.4 or 1.7, respectively in either NO_3-N or NH_4-N alone as nitrogen source after 35 days of culture. In contrast to that in medium containing either NO_3-N or NH_4 + NO_3 with 8% sucrose showed 1.4 and 1.7 shoots, but it was 1.0 in NH_4-N with the same sucrose concentration(Table 2). In medium containing NH_4-N with 3% or 8% sucrose, shoot growth was restricted and shoot apex blighted. The height of shoot was greatest in NH_4 + NO_3 regardless of sucrose concentration, intermediate in NO_3-N, and lowest in NH_4-N, but shoots appeared less

Table 2. Effects of sucrose concentration and nitrogen source on plantlet[a] growth and bulbing on solid media.

Sucrose (%)	Nitrogen source	No. shoots	Shoot height (cm)	Bulbing[b] (%)
3	NO_3-N	1.4	11.9	67.0
	NH_4-N	1.7	8.3	73.9
	NH_4 + NO_3	1.0	14.7	90.0
8	NO_3-N	1.4	12.6	79.6
	NH_4-N	1.0	6.9	100.0
	NH_4 + NO_3	1.7	15.4	91.0
			F value	
	Sucrose	NS	NS	4.63*
	Nitrogen	NS	4.98**	NS
	Sucrose × Nitrogen	NS	NS	NS

[a]Plantlets were treated at 4°C for 35 days before culture.

[b]Bulbing presented bulblet with bulb index more than 2.

NS, *, ** Nonsignificant and significant at P=0.05 or P=0.01, respectively.

Table 3. Effects of sucrose concentration and nitrogen source on plantlet[a] growth and bulbing in liquid media.

Sucrose (%)	Nitrogen source	No. shoots	Shoot height(cm)	Bulbing[b] (%)
3	NO_3-N	1.5	12.8	18.8
	NH_4-N	1.6	17.4	11.9
	NH_4 + NO_3	1.5	17.3	8.4
8	NO_3-N	1.5	16.3	24.5
	NH_4-N	1.8	12.0	37.8
	NH_4 + NO_3	1.6	13.9	20.0
			F value	
	Sucrose	NS	NS	14.33**
	Nitrogen	NS	NS	NS
	Sucrose × Nitrogen	NS	4.56**	NS

[a]Plantlets were not subjected to cold treatment.

[b]Bulbing presented bulblet with bulb index more than 2.

NS, *, ** Nonsignificant and significant at P=0.05 or P=0.01, respectively.

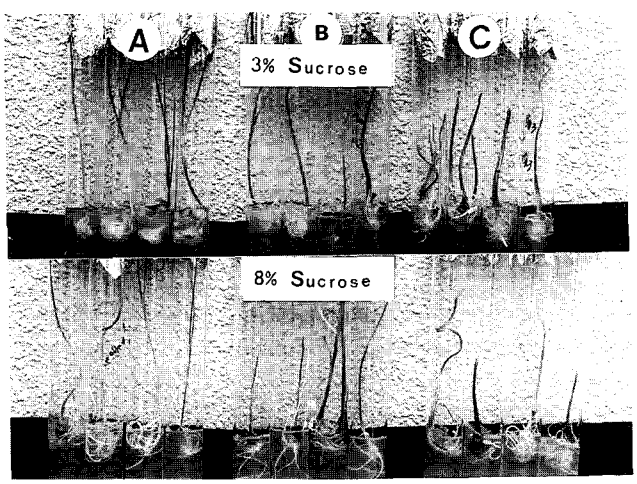

Figure 1. Growth of plantlet and bulblet on MS media with 3% and 8% sucrose, and various nitrogen sources. Plantlets were treated at 4°C for 35 days before culture. A: NO_3-N; B: NH_4 + NO_3; C:NH_4-N.

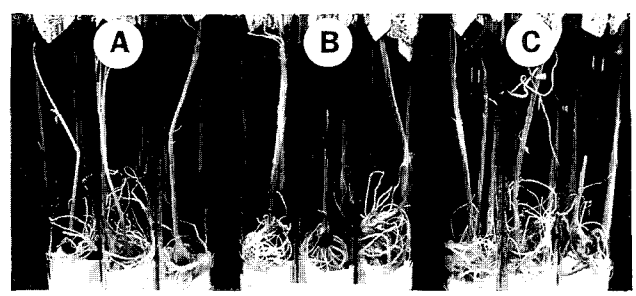

Figure 2. Growth of plantlet and in vitro bulblet on various nitrogen sources in MS liquid media contaning 8% sucrose. A: NH_4-N to NH_4-N; B: NH_4-N to NH_4 + NO_3; C: NH_4-N to NO_3-N.

vigorous in NO_3-N. Numerous bulblets were formed in medium containing NH_4-N with 8% sucrose, at a frequency of 91% in NH_4 + NO_3 with 8% sucrose and 90% in NH_4 + NO_3 with 3% sucrose (Figure 1 and Table 2). The reason of good bulblet formation regardless of sucrose concentration and nitrogen source seemed owing to cold treatment of explant before culture.

To investigate the growth of plantlet and the formation of bulblet, plantlets without cold treatment before culture were cultured on the media with two levels of sucrose and various nitrogen sources. There was no difference in shoot differentiation among various sucrose concentrations and nitrogen sources (Table 3). The growth of shoot was inhibited in NH_4-N with 8% sucrose and it reached 16.3 cm in NO_3-N. The frequency of bulblet formation on shoot was 11.9% or 8.4%, respectively in NH_4-N or NH_4 + NO_3 with 3% sucrose. Although shoot growth was restricted in NH_4-N with 8% sucrose, bulbing frequency was as high as 37.8%.

Table 4. Effects of sucrose concentration and change in nitrogen source on plantlet growth and bulbing in liquid media in second subculture.

Sucrose (%)	Changes in nitrogen sources	No. shoots	Shoot height (cm)	Bulbing[a] (%)	Bulb weight (mg)
3	NO_3-N -> NO_3-N	1.2	14.2	0	-
	NO_3-N -> NH_4-N	1.5	22.3	0	-
	NH_4-N -> NO_3-N	1.9	29.9	8.3	190
	NH_4-N -> NH_4-N	1.2	17.5	0	-
	NH_4-N -> NH_4 + NO_3	1.4	22.6	0	-
	NH_4 + NO_3 -> NH_4 + NO_3	1.5	26.5	0	-
	NH_4 + NO3 -> NH_4-N	1.9	26.4	0	-
8	NO_3-N -> NO_3-N	1.3	17.3	0	-
	NO_3-N -> NH_4-N	2.2	21.1	45.8	260
	NH_4-N -> NO_3-N	1.7	20.8	22.0	550
	NH_4-N -> NH_4-N	2.5	20.6	62.5	238
	NH_4-N -> NH_4 + NO_3	1.8	28.6	8.3	400
	NH_4 + NO_3 -> NH_4 + NO_3	1.8	19.7	54.2	233
	NH_4 + NO_3 -> NH_4-N	1.3	22.5	87.5	216
			F value		
Sucrose		NS	NS	61.74**	-
Nitrogen		NS	2.93*	5.61**	-
Sucrose × Nitrogen		NS	NS	6.24**	-

[a]Bulbing presented normal bulblet with bulb index more than 4.

NS, *, **Nonsignificant and significant at P=0.05 or P=0.01, respectively.

Table 5. Analaysis of macronutrients in residual media after culture.

Sucrose(%)	Nitrogen source	$NA4^+$	$NO3^-$	Total N	CA^{++}	Mg^{++}	K^+	$H2PO4^-$	$SO4^{--}$	Cl^{--}	TIC[a]	pH
						Original media						
3 or 8	NO_3^-	0	20	20	5.0	5.0	12.5	2.5	5.1	0.2	50.3	5.8
	NH_4^+	20	0	20	5.0	5.0	12.5	2.5	5.1	20.0	70.1	5.8
	$NH_4^{++}NO_3^-$	10	10	20	5.0	5.0	12.5	2.5	5.1	20.0	70.1	5.8
						45 days after 1st subculture						
3	NO_3^-	0.5	18.6	19.1	2.2	2.2	12.1	0.8	2.1	1.7	40.2	5.6
	NH_4^+	10.4	0.8	11.2	2.4	1.5	1.0	0.6	1.5	14.3	32.3	4.4
	$NH_4^{++}NO_3^-$	10.0	11.0	21.0	2.5	1.7	1.1	0.4	1.7	8.6	37.1	4.4
8	NO_3^-	0.4	18.0	18.4	2.2	2.0	13.3	0.9	2.2	0.8	39.8	5.0
	NH_4^+	12.4	1.0	13.4	2.8	1.8	1.3	0.8	2.1	17.1	39.3	4.4
	$NH_4^{++}NO_3^-$	11.0	13.0	24.0	2.8	2.1	2.1	0.9	2.3	7.4	41.6	4.4

[a]Total ionic concentration (mM).

In media containing 3% sucrose, the number of shoot was enhanced by transfer to medium containing different nitrogen source (Table 4). However, there were no remarkable differences in number of shoots among different sucrose concentration and the nitrogen source. Shoot growth was retarded in continous NO_3-N fed medium with 3% and 8% sucrose. The heights of shoots in continuous NO_3-N fed medium with 3% sucrose or in that with 8% sucrose were

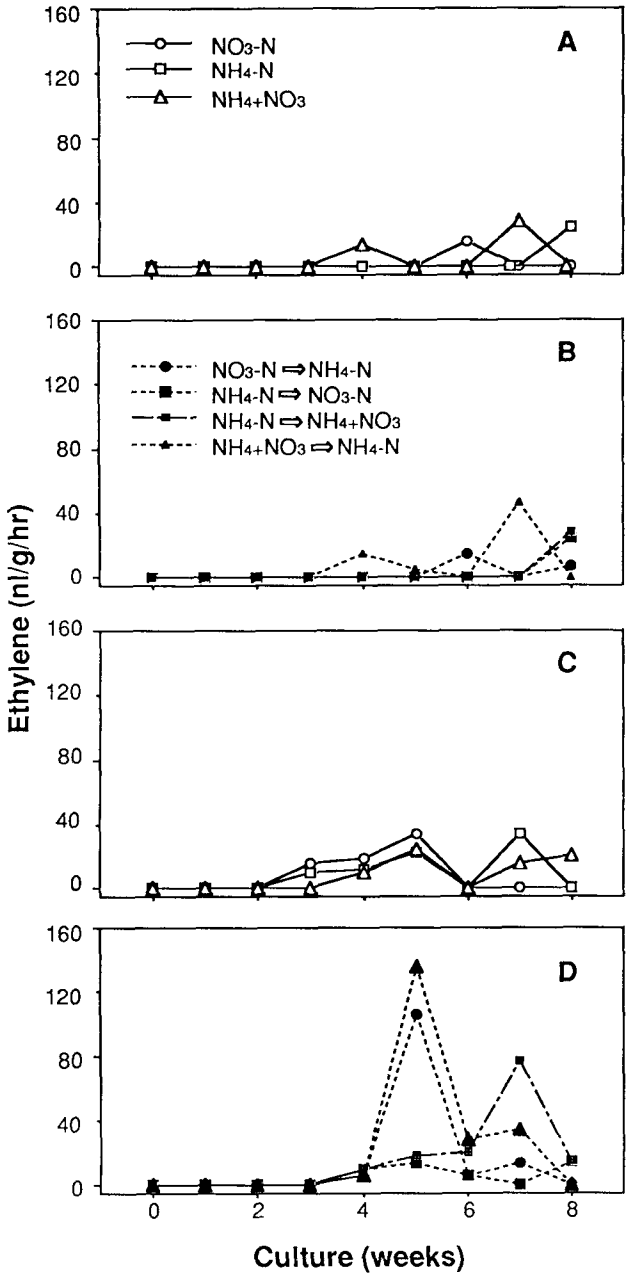

Figure 3. Production of ethylene by 3% (A, B) or 8% (C, D) sucrose and change in nitrogn source in garic plantlet culture.

14.2 or 17.3 cm, respectively. But those were vigorous in NH_4-N to NO_3-N with 3% sucrose and in NH_4-N to $NH_4 + NO_3$ with 8% sucrose. Rooting was inhibited more in liquid media when compared with on solid medium in all treatments (Figures 1 and 2). Bulb formation was not observed at 3% sucrose regardless of nitrogen source, but its frequencies in continous NH_4-N fed medium with 8% sucrose, $NH_4 + NO_3$ and $NH_4 + NO_3$ to NH_4-N were 62.5%, 54.2% and 87.5%, respectively.

The residual medium after first and second subculture was anaiyzed for NO_3-N, NH_4-N, Ca^{+2}, Mg^{+2}, K^+, $H_2PO_4^-$, SO_4^{-2}, and Cl^-. The nitrogen in NO_3-N fed medium containing 3% sucrose in first subculture was not absorbed to plant, but the NH_4-N was absorbed about half amount of original level, and the $NH_4 + NO_3$ was not absorbed (Table 5). And also, in medium containing 8% sucrose, the absorption according to nitrogen source was simlar to that of 3% sucrose. In the NO_3-N or $NH_4 + NO_3$ fed medium, remained nitrogen was 18.0 mM NO_3-N or 11 mM NH_4-N + 13 mM NO_3-N in medium but in NH_4-N fed medium was 12.4 mM NH_4-N in residual medium. The amount of Ca^{+2} in residual medium was lower than that of original medium, 5.0 mM Ca^{+2}. The remained Mg^{+2} was a little more in NO_3-N fed treatment than in NH_4-N, and $NH_4 + NO_3$. K^+ concentration, however, remained largely unchanged in NO_3-N with 3% and 8% sucrose and it was absorbed much in NH_4-N and $NH_4 + NO_3$. The $H_2PO_4^-$ levels were higher in NO_3-N than in NH_4-N and $NH_4 + NO_3$ in residual medium. Cl^- was absorbed more in $NH_4 + NO_3$ treatment than in NH_4-N. The overall results suggest that the absorption of inorganic compounds were higher in the media with NH_4-N and $NH_4 + NO_3$ than in NO_3-N in two levels of 3% and 8% sucrose. When the nitrogen source was changed in second subculture, the lots of NO_3-N remained in residual medium but NH_4-N feeding treatment did a little. However, the nitrogen was absorbed in $NH_4 + NO_3$ medium unlike first subculture. That reason may be owing to the stage of plantlet development. The amounts of Ca^{+2}, Mg^{+2}, K^+, $H_2PO_4^-$, SO_4^{-2}, and Cl^- remained in medium similarly to first subculture (data not shown).

When plantlets of garlic were subcultured on MS medium with 3% sucrose and various nitrogen sources, ethylene was not produced till 3 weeks after culture, but was increased a little from 4 to 6 weeks (Figure 3). The levels of ethylene were 46.4 and 28.2 nl/g/hr in $NH_4 + NO_3$ to NH_4-N and in $NH_4 + NO_3$ after 7 weeks of culture, respectively. In changing nitrogen source with 8% sucrose, ethylene was not produced till 2 weeks after culture and it was increased continousely as 34.1 nl/g/hr in NO_3-N, 105 nl/g/hr in NO_3-N to NH_4-N and 135.4 nl/g/hr in NH_4-N to NO_3-N and that was decreased by 8 weeks. The ethylene was produced more in medium with 8% sucrose than in 3% sucrose.

DISCUSSION

Shoot differentiation was not affected by sucrose concentration and nitrogen source (Tables 2 and 3). But plantlets cultured in medium containing NO_3-N were small and less vigorous (Figure 1) becouse of suppression of NO_3-N and K^+ absorption (Table 5). In contrast, plantlets cultured on medium containing NH_4 + NO_3 or NH_4-N with 3% sucrose were more vigorous and healthy in appearance. Thus, some NH_4-N was beneficial for garlic growth.

The last step is an acclimation in the propagation of plants through plant tissue culture. In culture conditions of Korea, the acclimatization of garlic must be done by February to cultivate in field. For that reason it seems to be efficient to plant at a proper time after bulblet formation in vitro. And its formation is influenced by the environmental factors such as light intensity, photoperiod, temperature, phytohormone, and nutrition. Bulblets were formed when plantlets were subjected to cold treatment regardless of sucrose concentration and nitrogen source in culture medium (Table 2). The best quality of bulbs was obtained on the media containing 8% sucrose with NH_4-N or NH_4 + NO_3. However, when plantlet cultured without cold treatment, in vitro bulblets were formed only 20% to 40% (Table 3) and bulblets produced only on medium containing 8% sucrose (Table 4). Consequently, cold treatment is thought to be essential for bulb formation. In tulip, cold treatment (4°C) enhances bulb development, and this may have been due to an increase in the sucrose level in shoots before transfer for final incubation at 20°C or 25°C, or to an increase in endogenous gibberellin following the cold treatment. Alpi and De Hertogh (1972) found that low temperature increased the level of gibberellin in tulip flower stalks. Also gibberellin-like compounds were accumulated when tulip bulbs received a cold treatment (Aung and De Hertogh, 1968). Rice et al.(1983) observed on enhanced bulb production by soaking adventitious shoots in GA_3 solutions. Increasing the sucrose concentration in the medium and low-temperature treatment enhanced bulb development of tulip, which is in agreement with the results of Nishiuchi (1980) and Rice et al. (1983). The formation frequency and weight of bulblets were more effective when the seed bulb and plantlets were treated with chilling (4°C) for 60 days (Min et al., 1992). Similar results were obtained in this study.

High sucrose concentration stimulates shoots to form bulbs in bulbing of shoots (Tabe and Alderson, 1990). The plantlet directly uptakes the nutrient from medium and store in the basal part. And plantlets may be stimulated for the bulb development because of the higher osmotic potential achieved in the medium by high sucrose level, the unsuitable environmental condition and the induction of dormancy. Sucrose may be a carbon soruce and have an osmoregulatory function. This suggestion was confirmed by Rice (1984) who reported that in tulip, the increment of sucrose in the medium from 3% to 6% enhanced the number of bulbing shoot, whereas a greater concentration inhibited.

Bulblet formation was not affected by nitrogen source but bulblet was developed on the medium containing 8% sucrose (Tables 2 and 3). And some NH_4-N was beneficial for bulb development on the medium containing 8% sucrose, but NO3-N did not improved them and suppressed K^+ absorption. In shoot growth similarly, NH_4-N would seem to be more favorable nitrogen source unlike NO_3-N, because it does not need to be reduced before it was assimilated. The NH_4-N were absorved more than NO_3-N because in vitro plantlets were cultured in liquid media under low intensity of light. Thus, plants assimilated NO_3-N under field condition but did NH_4-N under shadding condition and low intensity of light (Barker and Mills, 1980). NH_4-N uptake of plant cultured in field is dependent on carbohydrate reservoir (Haynes and Goh, 1978: Barker and Mills, 1980). When NO_3-N uptake exceeds assimmilation, it can be stored in the plant. Mature plants with adequate carbohydrate reservoir may be more tolerant of NH_4-N than young plants with low carbohydrate levels. Thus, NH_4-N should be available to garlic for proper growth and development.

Ethylene is involved in growth, development and senescence of tissue culture in vitro (George and Sherrington, 1984). Its production was not difference among the nitrogen sources and it was increased in medium containing 8% sucrose than 3% (Figure 3). In this study, the objective was to investigate the factors of bulb formation during in vitro culture. But there was no correlation between the production of ethylene and the growth of shoot and bulblet. However, additional works will be required to gain a better understanding of physiological state of plant and ratio of carbon to nitrogen in medium, and to study on growth regulators affected on bulb formation and dormancy.

적 요

마늘 생장점 배양을 통한 효율적인 우량종구 증식방법을 확립하기 위하여 기내배양시 유식물체 분화 및 기내인경 비대에 미치는 영향을 조사하였다. 유식물체 분화는 sucrose 및 질소 공급형태에 따라서 차이가 없었으나, 유식물체의

생장은 NO_3-N 공급시 저조하고, NH_4-N 및 NH_4 + NO_3 공급형태에서 양호하였으며 특히 2차 계대배양시 질소형태를 바꾸워 공급할 경우 더욱 촉진되었다. 기내인경 비대는 NH_4-N를 공급하였을 때와 고농도의 sucrose을 첨가하였을 때 촉진되었으며, 특히 유식물체에 저온처리를 하면 그 효과가 더 상승되었다. 기내배양에서 마늘의 생장에 이용되는 질소형태는 NH_4-N 이며, NO_3-N을 공급하면 흡수가 거의 되지 않고, K^+ 흡수도 저조하였다. Ethylene의 발생은 질소 공급 형태와 관계없이 8% sucrose 고농도에서 더 많이 발생되었다.

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