J. Plant Biotechnology(2001) *Vol. 3(3). pp. 141~144*

Journal of Plant Biotechnology

Factors Affecting Efficiency of Shoot Induction in *Citrus junos* Sieb.

Mi-Hye Kim[1], Hyoshin Lee[1], Min-Sup Chung[2], Jinki Jo[1*]

[1]Department of Animal Science and Biotechnology, Kyungpook National University, Daegu, 702-701, Korea; [2]Department of Agricultural Biology, Kyungpook National University, Daegu, 702-701, Korea.

Key words: *Citrus*, nodal stem, shoot induction, yooza

Abstract

To enhance the shoot induction efficiency from nodal stem of yooza, the culture conditions such as basal medium, carbohydrate source, solidifying agent and the optimum concentration of plant growth regulators for shoot induction were investigated. The nodal explants were cultured better on MS medium than on MT, SH, B5 or W media in considering of shoot induction rate and mean shoot length. Solidifying agent in medium was better with 0.8% agar than with 0.3% agar, 1.2% agarose or 0.2% gelrite. Carbohydrate source in shoot induction medium was efficient with 30 g/L sucrose. The optimum concentrations of plant growth regulators were determined that 0.1 mg/L NAA as auxin was effective on the shoot induction, and 1.0 mg/L BAP as cytokinin induced mutiple shoots efficiently. Shoot induction was the most effective on MS medium supplemented with 4 mg/L GA_3 in yooza.

Introduction

There are over 12 local species of *Citrus* being grown in the southern coastal areas and Jeju island in Korea for centuries. On Jeju island, *Citrus* has been a major crop for the horticultural industry since the 1960's (Lee and Han, 1991). Among them, yooza (*Citrus junos* Sieb. *et* Tanaka) is commercially cultured for its processing quality and culinary purposes. Yooza tea is very popular in Korea because of its aromatic flavor and fresh taste. Recently, yooza plantations have increased for their economical value, although some problems exist in the yooza culture such as large plant body, long juvenility and strong thorns. Thorns which hurt their fruits when blown by wind or storms are financial problem to farmers and give difficulty in tree management.

In vitro propagation has, therefore, been a great potential factor in avoiding problems related with the field culture for such species (Hidaka and Omura, 1989). In *Citrus*, entire plant regeneration has been achieved from embryogenic callus (Hidaka and Kajiura, 1988; Song et al., 1990; Vardi and Spiegel·Roy, 1982). However, there have been limited studies of shoot induction from stem segments in *Citrus*. The shoot tip culture of *Citrus* has only been initiated from explants of shoots trimmed to a length of more than 5 mm of young seedlings (Kitto and Young, 1981; Starrantio and Caruso, 1987). But it was difficult to recover plantlets from the shoot tips with a few leaf primordia in mature trees (Murashige et al., 1972). In this paper, we have described to determine the conditions of culture media and the effects of plant growth regulators on shoot induction by nodal culture.

Materials and Methods

Experimental materials and culture conditions

The seeds of yooza (*Citrus junos* Sieb. *et* Tanaka cv. Namhae No.1) were kindly distributed from the Namhae Horticulture Research Institute. Yooza plants were cultivated in a greenhouse under 16h-light/8h-dark (27℃/18℃) photoperiod with a light intensity of 350 $\mu molm^{-2}s^{-1}$ provided by white fluorescent lamps. Stem segments of 10 to 15 cm in length were removed from the two-year-old

*Corresponding author, E-mail; jkjo@knu.ac.kr
Received Jul. 6, 2001; accepted Aug. 10, 2001.

plants, and leaves and thorns were stripped off from them. Stem explants of 1 to 1.5 cm long were split longitudinally containing one axillary bud. The stem explants were surface sterilized with 70% ethanol for 1 to 2 sec and with 1.0% (v/v) sodium hypochlorite solution containing 0.01% (v/v) Tween-20 for 15 min, and then rinsed 4 times with sterile deionized water. At least 20 stem explants were placed with the longitudinal cut surface contacting with the medium and incubated under 16h-light/8h-dark photoperiod at 25±2℃.

The five different basal media were compared among MS (Murashige and Skoog, 1962), MT (Murashige and Tucker, 1969), SH (Schenk and Hildebrandts, 1972), B5 (Gamborg et al., 1968) and W (White, 1963). Every medium was supplemented with 0.1 mg/L NAA and 1.0 mg/L BAP as plant growth regulators. Various saccharides were supplemented into MS basal medium containing 0.1 mg/L NAA, 1.0 mg/L BAP and 10 mg/L GA_3 to investigate the shoot induction rate. Monosaccharides such as galactose, glucose, fructose, mannitol and sorbitol were filter-sterilized and added to the medium. Sucrose, lactose, cellobiose and maltose were sterilized by autoclaving for 15 min at 121℃.

Effects of plant growth regulators on shoot induction

To determine the optimum concentration of auxin for shoot induction, 2,4-D, NAA, IAA or IBA was added to MS medium at 0.05 to 2.0 mg/L level. Cytokinins such as BAP, 2*i*P and kinetin were investigated at the range of 0.1 to 20.0 mg/L. Shoot induction was tested on the various concentration with 2.0 to 20.0 mg/L GA_3. All the plant growth regulators were filter-sterilized (Millpore Millex-HA 0.45 μm) and were added while the medium was still warm (approximately 45 to 50℃). The culture were incubated in a growth chamber at 25±2℃ under 16h-light/8h-dark photoperiod.

Results and Discussion

Establishment of shoot induction medium

Nodal stem explants were cultured on the five different media (MS, MT, SH, B5 and W) to evaluate the effect on shoot induction in yooza (Table 1). MT medium was more efficient than MS or SH medium for shoot induction and adventitious shoot development. However, nodal explants produced much more shoots in MS medium than in MT or SH medium. Both B5 and W media had not only low efficiency for shoot induction but also produced brown-color shoots, and they were finally etiolated within 2 subculture cycles. Based upon the results, MS was the most efficient culture medium for shoot induction from nodal explants in yooza.

Carbohydrates are usually supplied as carbon source in tissue or cell culture and the most commonly used one is sucrose (Strickland et al., 1987). We compared the several monosaccharides and disaccharides for the efficiency of shoot induction in yooza (Table 2). When we examined them as groups, disaccharides had better effect than monosaccharides in shoot induction though variation of the values among disaccarides was large. Within disaccharides, sucrose was the most effective carbon source for shoot induction as we expected.

Effect of plant growth regulators on shoot induction from nodal stem segments

Nodal stem segments containing one axillary bud were cultured in MS medium containing different concentrations of auxins and cytokinins (Durán-Vila et al., 1989). Four auxins (2,4-D, NAA, IAA and IBA) were investigated for the effect on shoot induction. Among them, NAA was

Table 1. Effect of five different media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA on the shoot induction rate from nodal stem segments in yooza.

Medium	Week of culture		
	1	3	5
MS	±[a]	+	++
MT	+	+	+++
SH	±	±	±
B5	±	±	±
W	±	±	±

[a]Shoot induction rate: ± (0-39%); rate, + (40-59%); moderate, ++ (60-79%); good, +++(80-100%); excellent.

Table 2. Effect of saccharides on shoot induction of yooza[a].

Monosaccharids (0.1 M)	Shoot induction[b]	Disaccharids (0.1 M)	Shoot induction[b]
Galactose	-	Sucrose	++
Glucose	±	Lactose	±
Fructose	±	Cellobiose	+
Mannitol	-	Maltose	+
Sorbitol	-	None	-

[a]Explants were cultured on MS medium supplemented with 10 mg/L GA_3, 1.0 mg/L BAP and 0.1 mg/L NAA for 1 month.
[b]Visual estimation: - (none), ± (poor), + (good), ++ (excellent).

much more effective than IAA, IBA or 2,4-D (data not shown). When investigated for more detailed concentrations of NAA, 0.1 mg/L of it was the optimum concentration (Figure 1).

Cytokinin types had more profound effect on the shoot induction. When we compared cytokinins, BAP was more effective than 2*i*P or kinetin for shoot multiplication in yooza (Table 3). It is possible that cytokinins are readily broken down due to the cytokinin oxidase operating in *Citrus* (Norton and Norton, 1985). A number of shoots were developed from the buds on MS medium in the concentration of 1.0 mg/L of BAP. Higher concentrations of BAP, on the contrary, resulted in inhibition of shoot induction (Figure 2). At 1.0 mg/L BAP, 4 to 5 multiple shoots were produced from a single bud in which a main shoot grew very fast and it reached a size of 2 cm within 4 weeks. The rest of the soots grew slowly or did not reach more than a few millimeters. These small shoots grew only when the main shoot was removed from the original explant. Similar results that BAP among cytokinins (BAP, 2*i*P and kinetin) was the most effective stimulator for multi-shoot production from shoot-tip culture were reported in *Satsuma mandarin* and tangor species (Durán-Vila et al., 1989; Omura and Hidaka, 1992). The development of a single shoot reaching a large size may be related to the phenomenon of apical dominance described by Grimblat (Kitto and Young, 1981), who explained that single buds on a given explant developed into shoots faster but inhibited the remaining buds.

Figure 2 indicates that most of the shoots are produced more vigorously at the treatment of 0.5 or 1.0 mg/L BAP than at control or at more than 5.0 mg/L. However, no significant difference in shoot induction is observed between 0.5 and 1.0 mg/L BAP. The optimum concentration of BAP obtained in this study, 0.5 or 1.0 mg/L for shoot induction was significantly lower compared with 10 mg/L BAP in *Carrizo citrange* (Kitto and Young, 1981). This difference of optimum concentrations of BAP might be explained by species dependence.

Among the known plant growth regulators, cytokinin and gibberellin are known to be the key substances for the growth of yooza shoot-tips and buds in *in vitro* culture (Kitto and Young, 1981; Mori et al., 1987). GA_3 has been shown to play an essential role in the regulation of internode elongation (Katsumi and Kazama, 1978). So, it was tested for nodal stem segments in which they were cultured on the medium containing 2 to 20 mg/L GA_3 (Table 4). GA_3 stimulated the initial growth of the shoot-tips, and it was more effective at 10 mg/L than at 2 to 8 mg/L GA_3. However, GA_3 at high concentrations, such as 10 and 20 mg/L induced abnormally twisted growth accompanied

Table 3. Effect of cytokinins on shoot induction rate from explants[a] on MS medium.

Cytokinins	Concentration (mg/L)			
	0.1	1.0	10.0	20.0
BAP	+[b]	±	±	±
2*i*P	+	+	±	+
Kinetin	+	±	-	±

[a]Explants were cultured on MS medium for 1 month.
[b]Shoot induction rate : ± (0-39%); rare, + (40-59%); moderate, ++ (60-79%); good, +++ (80-100%); excellent.

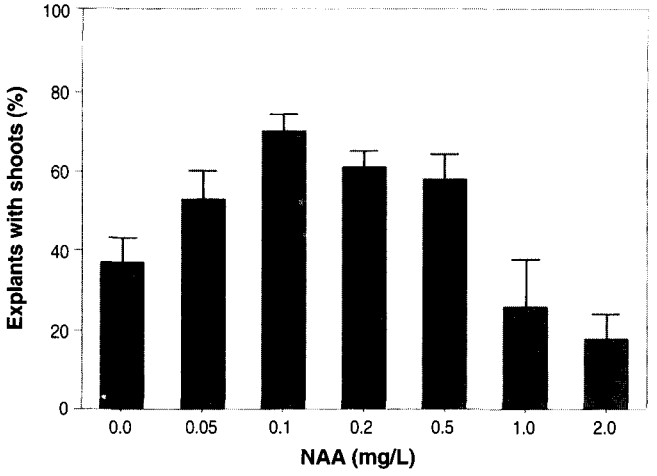

Figure 1. Effect of the different concentrations of NAA on shoot induction rate from explants on MS medium. Vertical bars indicate standard deviations.

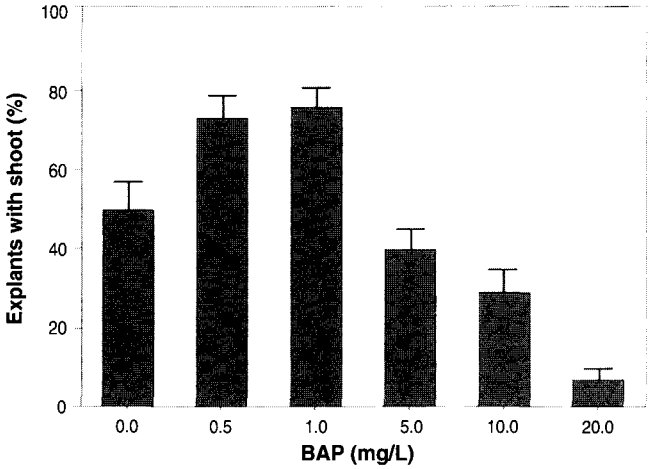

Figure 2. Effect of the different concentrations of BAP on shoot induction rate from explants on MS medium. Vertical bars indicate standard deviations.

Table 4. Effect of the different concentrations of GA_3[a] on shoot regeration from explants on MS medium.

GA_3 Conc. (mg/L)	Survival rate (%)	Shoot length (mm)	No. of shoots[b]	Multishooting rate
0	80	5	3	+[c]
2	92	10	6	++
4	100	10	8	+++
8	100	15	7	+++
10	80	15	4	++
20	64	10	2	+

[a]Explants were cultured on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA.
[b]The number of shoots per explant was calculated dividing the total number of shoots by the number of explants with shoots.
[c]Multishooting rate: No. of multishoots/explant ; + (1-3), ++ (4-6), +++ (7-9)

by some etiolation. Based upon these results, 4 mg/L GA_3 was added to the subculture medium and 10 mg/L GA_3 was applied only at the initial shoot induction stage. Omura and Hidaka (1992) also reported a similar result in *Citrus* where 10 mg/L GA_3 in MS medium was effective for the initiation of multiple shoots from nodal stem segments.

Acknowledgements

This work was supported by a grant from Korea Research Foundation (KRF2000-005-G00003).

References

Durán-Vila N, Ortega V, Navarro L (1989) Morphogenesis and tissue culture of three *Citrus* species. Plant Cell Tiss Org Cult 16: 123-133.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp Cell Res 50: 151-159.

Hidaka T, Kajiura I (1988) Plantlet differentiation from callus protoplasts induced from *Citrus* embryo. Scientia Horticulturae 34: 85-92.

Hidaka T, Omura M (1989) Control of embryogenesis in *Citrus* cell culture: Regeneration from protoplasts and attempts to callus bank. Bull Fruit Tree Res Stn B 16: 1-17.

Katsumi M, Kazama H (1978) Gibberellin control of cell elongation in cucumber hypocotyl sections. Bot Mag Tokyo Special Issue 1: 141-158.

Kitto SI, Young MJ (1981) *In vitro* propagation of *Carrizo citrange*. Hortscience 16: 305-306.

Lee H, Han K (1991) *In vitro* germination of *Citrus* spp. and culture of *Citrus benikoji* 'Kamja' for *Citrus* breeding. J Nat Sci Inst, Seoul Woman's Univ 2: 109-115.

Mori S, Hosoi T, Ooishi A (1987) Investigation of bud culture in *Citrus unshiu* Mark. Bull. Tokushima Hort Exp Stn 15: 7-17.

Murashige T, Tucker DPH (1969) Growth factor requirements of *Citrus* tissue culture. Proc. 1st Intern. *Citrus* Symp 3: 1155-1161.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 5: 473-479.

Murashige T, Bitters WP, Rangan EM, Nauer EM, Roistacher CN, Holliday PB (1972) A technique of shoot apex grafting and its utilization towards recovering virus-free *Citrus* clones. Hortscience 7: 118-119.

Norton ME, Norton CR (1985) *In vitro* propagation of Ericaceae: a comparison of the activity of cytokinins N^6-benzyl adenine and N^6-isogentenyl adenine in shoot proliferation. Scienta Horticulturae 27: 335-340.

Omura M, Hidaka T (1992) Shoot tip culture of *Citrus*. I. Culture condition. Bull Fruit Tree Res Stn 22: 23-36.

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous plant cell culture. Can J Bot 50: 199-204.

Song WS, Kim CS, Eun JS, Park EH, Yoo SO (1990) Studies on the somatic embryogenesis in yooza (*Citrus junos* Sieb. *et* Tanaka). I. Somatic embryogenesis from ovule culture. Kor J Plant Tiss Cult 17: 255-265.

Starrantio A, Caruso A (1987) Experience on the *'in vitro'* propagation of some *Citrus* rootstock. Acta Horticulturae 212: 471-478.

Strickland SG, Nichol JW, McCall CM, Stuart DA (1987) Effect of carbohydrate sucrose of alfalfa somatic embryogenesis. Plant Science 48: 113-121.

Vardi A, Spiegel, Roy P (1982) Plant regeneration from *Citrus* protoplasts: Variability in methodological requirements among cultivars and species. Theor Appl Genet 62: 171-176.

White PR (1963) The Cultivation of Animal and Plant Cells. pp. 199-203. Romald Press, New York.