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Effect of Cytokinins on Nodal Cultures of *Citrus* Species

Kyung chul HAN* and Youn Hwa HAN[1]

Dept. of Applied Science, Cheongju Nat'l Univ. of Education, 135 Sukog-dong, Cheongju, Choongbuk 360-150; and [1]Dept. of Horticulture, Cheju Technical Junior College, Cheju, 690-751. *Corresponding author.

감귤류의 마디배양에서 싸이토키닌류가 기내 대량증식에 미치는 영향

한경철* · 한윤화[1]

청주교육대학교 실과 교육과, [1]제주실업전문대학 원예학과

This study was conducted to determine the effects of 3 cytokinins (BA, 2iP, and kinetin) and their concentrations (0, 0.1, 0.5, 1.0, and 2.0 mg/L) on multiple shoot production of *Citrus* spp. 'Sambokam' and 'Byungkyool' by nodal culture. Nodal explants were obtained from in vitro germinated seedlings of both cultivars. 'Sambokam' produced more multiple shoots than did 'Byungkyool' by nodal culture. Among the 3 cytokinins tested in this study, BA supplemented in semi-solid MS basal medium was the most effective stimulator for multiple shoot production, and an optimal concentration was determined to be 1.0 mg/L. Shoot elongation and root formation were inhibited by increasing cytokinin concentration, regardless of cytokinin types. BA at 1.0 mg/L produced the most multiple shoots and the highest number of leaves in 'Sambokam', whereas any cytokinin and concentration studied in this experiment did not affect any scored variables such as shoot and leaf numbers, etc. in 'Byungkyool'.

Key words: growth regulator, shoot multiplication, tissue culture

Citrus has been a major crop for the horticultural industry in Cheju-do, Korea since 1960's (Lee and Han, 1992). The total growing area of citrus species was over 17,000 ha in Cheju-do in 1987. The growing area has continued to increase slightly afterwards. However, it might be expected that citrus industry in Cheju-do should be severely damaged by the recent solution of the Uruguay Round.

Citrus propagation has normally been done by grafting scions onto known rootstocks (*Poncirus trifoliata*) since no other method was developed. In vitro methods might be possibly applied to the propagation of citrus rootstocks. In vitro culture has been mainly developed on Murashige and Skoog medium (1962) or Murashige and Tucker medium (1969) which was devised specially for citrus. According to Kitto and Young (1981), in vitro propagation of citrus from non-nucellar vegetative tissue could be more beneficial than the conventional grafting method. Besides shoot-tip culture, single nodal culture is another possible in vitro technique that can be used for plant propagation from axillary buds.

Few researches have been done in Korea by using in vitro techniques for citrus micropropagation. Thus, an attempt was made to determine the effects of 3 cytokinins and their concentrations on micropropagation of citrus by nodal culture in this study.

MATERIALS AND METHOD

The seeds of two cultivars of *Citrus* species (*Citrus* spp. 'Sambokam' and 'Byungkyool') were obtained from the Cheju *Citrus* Research Institute. The seeds were put in a 300 ml flask containing 2% sodium hypochlorite (act. Cl. 12%)

and swirled continuously for 15 minutes, after which all steps were performed aseptically in a laminar-flow hood. After four rinses in sterile water, the prepared seeds were plated onto 30 ml of semi-solid MS basal medium in 100 ml flasks for in vitro germination. The original growth regulators, kinetin and IAA, on MS medium were deleted, and no other plant growth regulators were added into the cultural medium. The pH of the medium was adjusted to 5.8 ± 0.05 before adding 7.5 g/L Junsei agar. The medium was autoclaved 15 minutes at 1.1 kg Cm^{-2} and 121°C.

For micropropagation, nodes of two Citrus spp. 'Sambokam' and 'Byungkyoo' grown in vitro were used as explants. Each node was cut to a length of 5-7 mm, and three explants were plated onto 30 ml of semi-solid MS medium in which three different cytokinins with various concentrations were supplemented in 100 ml flasks.

Three cytokinins, BA, kinetin, and 2iP, were determined to be singly added to the MS basal medium since Lee and Han (1992) reported that 3 auxins, NAA, IBA, and 2,4-D, were not effective for micropropagation of *Citrus* sp. 'Kamja'. Five concentrations of each cytokinin, 0, 0.1, 0.5, 1.0, and 2.0 mg/L, were used in this study. The medium preparation was same as early mentioned above for the MS basal medium. The culture were incubated in a culture growing room at 23 ± 3 °C under approximately 2,000 lux with cool white fluorescent lamps.

Data on shoot, leaf, and root numbers, and mean shoot length were taken after 10 weeks in culture. The experiment was designed as a Complete Random Design (CRD) with 30 treatments (2 cultivars × 3 cytokinins × 5 concentrations) and five replications per treatment. Data were statistically analyzed by using the Genereal Linear Models Procedure of the Statistical Analysis System (SAS Institute, Inc, 1987) at the end of experiment. Least significant difference (LSD) and standard deviation (SD) were applied to interpret the data.

RESULTS AND DISCUSSION

Mean shoot and leaf numbers and shoot length per explant were significantly different between the 2 citrus cultivars tested in this study. 'Sambokam' produced more shoots (4.3) per explant in average than did 'Byungkyool' (2.7). More leaves were also formed from 'Sambokam' than from 'Byungkyool', and shoots were longer in 'Sambokam' than in 'Byungkyool' (Table 1). Based upon the results from this study, shoot multiplication of *Citrus* species seemed to be cultivar-dependent, as reported in other plant species (Stapfer, et al., 1985: Fordham and Stimart, 1982: Norton and Boe, 1982).

Table 1. Effect of *Citrus* cultivars on shoot, leaf, and root numbers, and mean shoot length in nodal culture after 10 weeks in culture.

Cultivar	Shoot number	Leaf number	Root number	Mean shoot length(cm)
'Sambokam'	4.3	19.0	0.4	0.6
'Byungkyool'	2.7	12.0	0.3	0.4
LSD, 0.05	0.7	3.6	0.2	0.1

Table 2. Effect of cytokinin type on shoot, leaf, and root numbers, and mean shoot length of *Citrus* species in nodal culture after 10 weeks in culture.

Cytokinins	Shoot number	Leaf number	Root number	Mean shoot length(cm)
BA	4.3	18.4	0.3	0.5
2iP	3.1	13.1	0.3	0.5
Kinetin	3.0	15.0	0.4	0.5
LSD, 0.05	0.8	4.3	0.2	0.1

Cytokinin types also affected shoot and leaf formation significantly (Table 2). Compared to other cytokinins tested, BA produced most shoots per explant. However, root formation and shoot elongation were not statistically significant. Kitto and Young (1981) reported that they obtained appreciable shoot multiplication from the shoot-tip culture of Carrizo citrange on a medium consisting of Knop's macro-elements, MS micro-elements, increased vitamins and adenine sulfate plus 10 mg/L of BA. Similar results that BA among cytokinins (BA, kinetin, and 2iP) was the most effective stimulator for multiple-shoot production from shoot-tip culture were reported in *Veronica* species (Stapfer, et al., 1985), Rosacea (Norton and Boe, 1982), and *Rhododendron* (Fordham and Stimart, 1982). However, Lee and Han (1991) reported that cytokinins, BA and kinetin, did not stimulate

multiple shoot formation from shoot-tip, root-tip, and nodal cultures of *Citrus* sp. 'Kamja'. Rather, an auxin, IBA, was found most effective for shoot multiplication from both shoot-tip and nodal explants.

Different cytokinin concentrations significantly affected shoot, leaf, and root formation, and shoot elongation (Table 3). More shoots were produced at 0.1-1.0 mg/L compared with the control and 2.0 mg/L. However, no significant difference in shoot formation was observed between 0.1 and 1.0 mg/L. The optimal concentration of cytokinin obtained in this study which was 0.1-1.0 mg/L for shoot multiplication was significantly lower compared with 10 mg/L BA in Carrizo citrange (Kitto and Young, 1981) and 10-12 mg/L 2iP in blueherries (George and sherrington, 1984). Leaf number was least at the highest concentration (2 mg/L), but was not significantly different from the other cytokinin concentrations tested. Root formation was strongly inhibited by addition of cytokinins, and root number decreased significantly as cytokinin concentration increased. Shoot elongation, on the other hand, was found to be inversely proportional to cytokinin concentration in this study.

Highly significant interaction between cytokinin type and cultivar was observed on shoot, leaf, and root numbers, and shoot elongation (Table 4). Among the three cytokinins tested, BA was most effective for shoot and root formation in 'Sambokam', whereas any scored variable was not significantly affected by cytokinins in 'Byunkyool'. Similar results were reported that BA was the most effective cytokinin from shoot-tip or nodal cultures in most fruit crops (George and Sherrington, 1984) and from nodal culture in pecan (Wood, 1982), whereas 2iP was most effective in blueberries (George and Sherrington, 1984).

Shoot and leaf formations were significantly affected by an interaction between cytokinin type and its concentration (Table 5). BA at 1.0 mg/L produced most shoots and leaves compared with any other treatment. Both shoot and leaf numbers continued to increase as BA concentration increased up to 1.0 mg/L and then rapidly decreased at 2.0 mg/L. This optimal concentration of BA was much lower than that reported in Carrizo citrange (Kitto and Young, 1981). This different requirement of cytokinin concentration might be explained by genotype-dependence.

Fig. 1 showed a significant interaction effect between cultivar and cytokinin concentration on multiple shoot production. Regardless of cytokinin concentration, 'Sambokam' generally produced more multiple shoots than did 'Byungkyool'. Most shoots were produced in 'Sambokam' at 1.0 mg/L among all tested concentrations of three cytokinins combined, whereas shoot multiplication was not affected by an increase in cytokinin concentration in 'Byungkyool'.

Table 3. Effect of cytokinin concentrations on shoot, leaf, and root numbers, and mean shoot length of *Citrus* species in nodal culture after 10 weeks in culture.

Conc.(mg/L)	Shoot number	Leaf number	Root number	Mean shoot length(cm)
0	2.9	15.9	1.5	0.7
0.1	3.8	18.8	0.1	0.6
0.5	3.9	16.2	0.1	0.4
1.0	4.2	17.8	0	0.4
2.0	2.6	8.8	0	0.3
LSD, 0.05	1.0	5.6	0.3	0.1

Table 4. An interaction effect between cultivar and cytokinin type on shoot, leaf, and root numbers, and mean shoot length of *Citrus* species in nodal culture after 10 weeks in culture.

Cytokinin	'Sambokam'				'Byungkyool'			
	Shoot number	Leaf number	Root number	Mean shoot length(cm)	Shoot number	Leaf number	Root number	Mean shoot length(cm)
BA	5.8 ± 3.6[a]	25.4 ± 15.1	0.5 ± 1.0	0.5 ± 0.3	2.9 ± 2.4	11.4 ± 8.6	0.1 ± 0.2	0.4 ± 0.3
2iP	3.7 ± 1.5	14.9 ± 8.5	0.4 ± 0.8	0.6 ± 0.3	2.5 ± 1.6	11.3 ± 8.9	0.3 ± 0.7	0.4 ± 0.3
Kinetin	3.3 ± 1.5	16.7 ± 12.9	0.4 ± 0.8	0.6 ± 0.3	2.7 ± 1.9	13.2 ± 10.4	0.5 ± 0.9	0.4 ± 0.3

[a]Standard deviation.

Table 5. An interaction effect between cytokinin type and concentration on shoot and leaf numbers of Citrus species on nodal culture after 10 weeks in culture.

	BA		2iP		Kinetin	
Conc (mg/L)	Shoot number	Leaf number	Shoot number	Leaf number	Shoot number	Leaf number
0	2.3 ± 1.7[a]	12.1 ± 9.7	3.3 ± 1.6	17.5 ± 9.3	3.3 ± 1.6	17.8 ± 9.3
0.1	3.4 ± 2.5	17.3 ± 14.0	3.9 ± 1.2	15.8 ± 10.3	4.0 ± 1.2	23.3 ± 14.5
0.5	6.0 ± 3.3	24.1 ± 15.5	0.3 ± 1.2	12.1 ± 5.4	2.8 ± 2.3	12.4 ± 13.0
1.0	6.9 ± 3.9	28.0 ± 14.6	2.9 ± 2.4	11.9 ± 10.6	2.8 ± 1.8	13.5 ± 10.3
2.0	3.1 ± 3.0	10.6 ± 9.8	2.5 ± 1.6	8.0 ± 5.7	2.3 ± 1.4	7.8 ± 6.0

[a]Standard deviation.

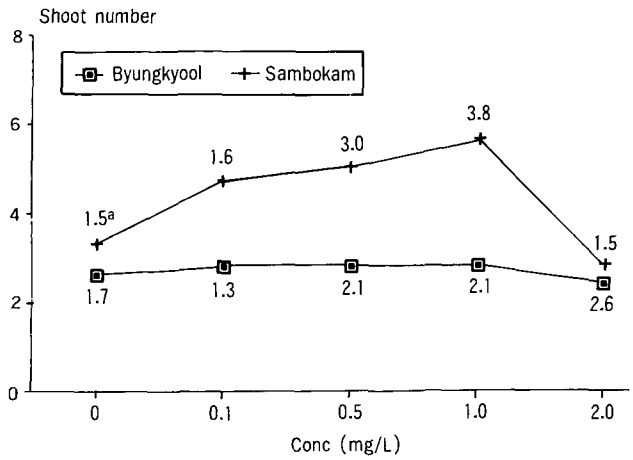

Figure 1. An interaction effect between cytokinin concentration and cultivar on shoot formation of *citrus* species by nodal culture.
[a]Standard deviation.

Different influences of cytokinin concentrations on cultivars might be possibly explained by different effectiveness or uptake efficiencies of the same concentration in given species.

Among the three cytokinins, both shoot and leaf numbers were significantly increased by BA in 'Sambokam', whereas root number and mean shoot length were not significantly affected by cytokinin types. Most shoots (5.7/explant) and leaves (25.4/explant) were formed by BA rather than 2iP and kinetin (Table 6). Apparently, BA was more effective than 2iP or kinetin for shoot multiplication in 'Sambokam'. Perhaps uptakes of 2iP and kinetin are less efficient than BA. Another possibility is that cytokinins are preferentially broken down, depending on the cytokinin oxidase operating in a given species or family (Norton and Norton, 1985).

Different cytokinin concentrations significantly affected all variables scored in this study such as shoot, leaf, and root numbers, and mean shoot length of 'Sambokam' (Table 7). Cytokinin concentration at 1.0 mg/L produced most shoots, whereas the highest leaf number was obtained at a

Table 6. Effect of cytokinin type on shoot, leaf, and root numbers, and mean shoot length of *Citrus* sp. 'Sambokam' in nodal culture after 10 weeks in culture.

Cytokinin	Shoot number	Leaf number	Root number	Mean shoot length(cm)
BA	5.8	25.4	0.5	0.5
2iP	3.7	14.9	0.4	0.6
Kinetin	3.3	16.7	0.4	0.5
LSD, 0.05	1.1	6.7	0.4	0.2

Table 7. Effect of cytokinin concentration on shoot, leaf, and root numbers, and mean shoot length of *Citrus* sp. 'Sambokam' in nodal culture after 10 weeks in culture.

Conc(mg/L)	Shoot number	Leaf number	Root number	Mean shoot length(cm)
0	3.3	17.8	1.8	0.7
0.1	4.7	25.3	0.3	0.8
0.5	5.0	20.3	0	0.6
1.0	5.6	21.4	0	0.4
2.0	2.8	10.1	0	0.3
LSD, 0.05	1.4	8.6	0.5	0.2

Table 8. An interaction effect between cytokinin type and concentraction on shoot and leaf numbers of Citrus sp. 'Sambokam' in nodal culture after 10 weeks in culture.

Conc (mg/L)	BA		2iP		Kinetin	
	Shoot number	Leaf number	Shoot number	Leaf number	Shoot number	Leaf number
0	3.3 ± 1.7[a]	17.8 ± 10.1	3.3 ± 1.7	17.8 ± 10.1	3.3 ± 1.7	17.8 ± 10.1
0.1	5.0 ± 2.2	26.5 ± 12.9	4.3 ± 1.7	17.3 ± 12.5	4.8 ± 1.0	32.0 ± 15.3
0.5	8.8 ± 1.0	35.3 ± 12.0	3.3 ± 1.3	12.8 ± 5.6	3.0 ± 1.6	13.0 ± 14.5
1.0	10.0 ± 2.9	39.3 ± 9.5	3.5 ± 0.6	10.8 ± 5.1	3.0 ± 1.2	11.5 ± 6.1
2.0	3.1 ± 3.0	10.6 ± 9.8	2.5 ± 1.6	8.0 ± 5.7	2.3 ± 1.4	7.8 ± 6.0

[a]Standard deviation.

concentration of 2.0 mg/L. However, root number and mean shoot length decreased as the concentration increased. Several results that root formation and shoot elongation were inhibited by increased concentrations of cytokinins were reported in citrus species (George and Sherrington, 1984: Chun, 1984: Kitto and Young, 1981).

Based upon an interaction effect between cytokinin type and its concentration on shoot and leaf formation in 'Sambokam', BA at 1.0 mg/L was most effective for both shoot and leaf numbers, and produced most shoots (10/explant) and leaves (39.3/explant), compared with any other treatment (Table 8). Kinetin and 2iP at any concentration were much inferior to BA at 1.0 mg/L for both shoot and leaf numbers. Based upon the results from this study, BA was most effective stimulator for shoot multiplication in 'Sambokam' as reported in other plant species (Stapfer, et al., 1985: Norton and Boe, 1982: Fordham and Stimart, 1982). An optimal BA concentration was considered to be 1.0 mg/L for multiple shoot production.

All shoots longer than 0.5 cm in length were transferred to MS basal medium containing 1.0 mg/L NAA, and were successfully rooted after 5 weeks in culture, regardless of cultivars tested. This result was very similar to the reports that the medium containing 1.0 mg/L NAA resulted in good root formation from *Citrus* shoot-tip cultures (Chun, 1984: Kitto and Young, 1981). Barlass and Skene (1983) also reported a similar result that average 5 roots per explant were successfully obtained from the MS medium supplemented with 5.0 mg/L NAA in *Citrus* species and hybrids. However, Lee and Han (1991) reported that IBA was more effective rather than NAA for root formation from nodal culture of *Citrus benikoji*, and most roots were formed on the MS medium supplemented with 4.0 mg/L IBA. Lits and Conover (1978) also reported that root formation was best initiated on the medium containing IBA by shoot-tip culture of *Carica* species.

Multiple shoot production was successfully achieved from nodal explants obtained from in vitro germinated seedlings of *Citrus* species in semi-solid MS basal medium supplemented with BA at 0.1-1.0 mg/L. Even if this modified MS medium may be useful for shoot multiplication of 'Sambokam', further modifications may be necessary for 'Byungkyool' and other *Citrus* species in the future.

적 요

본 연구는 감귤 품종인 '삼보감'과 '병귤'에서 3종류의 싸이토키닌(BA, 2iP, kinetin)과 그들의 농도(0, 0.1, 0.5, 1.0, 2.0 mg/L)가 마디배양에 의한 대량 증식에 미치는 영향을 구명하고자 수행되었다. 마디의 절편체는 기내 발아된 두 품종의 유묘에서 얻어졌다. 두 품종을 비교하면 신초의 수는 '병귤'에서보다 '삼보감'에서 더 많이 형성되었다. 본 실험에서 사용된 3종류의 싸이토키닌중에 고체 MS 기본배지에 첨가된 BA가 신초의 대량 생산에 가장 효과적인 촉진제였으며 그 적정농도는 1.0 mg/L인 것으로 나타났다. 줄기의 신장 뿌리의 형성은 싸이토키닌의 종류에 관계없이 싸이토키닌의 농도가 증가함에 따라 억제되었다. '삼보감'에서는 BA 1.0 mg/L에서 가장 많은 신초 및 엽수를 형성한 반면 '병귤'에서는 어떠한 싸이토키닌의 종류나 농도도 본 실험에서 신초 및 뿌리 등의 형성에 커다란 영향이 없었다.

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