Effect of CCC and BA on the Formation of Potato Tuber *in vitro**

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Abstract : Using the micropropagated potato shoots of 'Benimaru', 'May Queen', and 'Danshakuimo', originating from a meristem culture, the effects of 2-chloroethyltrimethylammonium chloride (CCC) and benzyladenine (BA) on the formation of microtuber were investigated. The number and fresh weight of microtubers after 28 days of culturing were largest in the Murashige and Skoog (MS) solid medium supplemented with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹) under dark conditions. This indicates that the tuberization medium proposed by the International Potato Center (CIP) is available for the formation of microtuber of the three cultivars described above. It was also found that CCC was effective for increasing the number of microtubers at the early stage of culturing, and that the fresh weight of microtuber of 'May Queen' was relatively small as compared to those of the other two cultivars. Scanning electron microscopy (SEM) revealed an external morphology at the stage of transforming into tuber from axillary bud after two days of culturing on a medium supplemented with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹) in the dark at 20°C.

Key words : BA, CCC, Microtuber, Potato, Tissue culture.

培養容器内でのジャガイモの塊茎形成に及ぼすCCCとBAの影響：山本友英・中田和男**（南九州大学園芸学部，** 東京農業大学総合研究所）

要 旨：「紅丸」，「メークイン」，および「男爵薯」の生長点培養由来の増殖シュートを用い，マイクロチューバー形成に及ぼすMS固型培地中のCCCとBAの影響を調べた．28日間の培養で形成されたマイクロチューバーの数と生体重は，いずれも暗黒条件下で，CCC (500 mg L⁻¹) 及びBA (5 mg L⁻¹) を含む培地の場合にもっとも高い値であった．この結果は，マイクロチューバーの生産のためにInternational Potato Center (CIP) が提唱した塊茎形成培地が日本のこれら三品種にも適用できることを示している．この場合，CCCは特に培養初期におけるマイクロチューバー形成に効果的であり，また，「メークイン」のマイクロチューバーの生体重は，他の二品種のものに比べて低い値であることが認められた．20°Cの暗黒条件でCCC (500 mg L⁻¹) とBA (5 mg L⁻¹) を含む培地で2日間培養したシュートの腋芽基部が膨らみはじめ，塊茎に転化する様相がSEMによって観察された．

キーワード：CCC，ジャガイモ，組織培養，BA，マイクロチューバー．

There have been reports addressing induction of tubers in the shoots of *in vitro* potato plants since Baker's demonstration²⁾. In the early 1980's, Wang and Hu^{16,17)} showed the procedures for mass production of *in vitro* tuber and its application to potato production. The *in vitro* tuber, because its size is usually in the range of 7~10 mm in diameter, acquired an alias, that of microtuber. The tuber is believed to be convenient for storage, long distance transport and circulation of elite clones, such as virus-free potato.

Although the optimum conditions for formation of microtuber vary among different reported studies^{1,17)}, it has been widely accepted that the formation of microtuber is promoted by cytokinins in various mediums, e.

g. zeatin^{8,10)}, kinetin^{11,13,14)} and benzyladenine (BA)^{3,4,7,15)}. In addition, some growth retardants, 2-chloroethyltrimethylammonium (CCC)^{3,7,15)} and coumarine¹⁷⁾ are reported to be effective for the formation of microtuber. On the other hand, Leclerc et al.⁹⁾ reported that CCC, BA and coumarine failed to trigger any increase in fresh weight or the number of microtubers per shoot. Garner et al.⁶⁾ demonstrated a reliable method of microtuber production on a medium free of any growth-regulating agent. As for the effect of abscisic acid (ABA) on tuberization *in vitro*, some contradictory results have been reported, i.e. promotive effect¹⁷⁾ and ineffective role^{7,8)}. The International Potato Center proposed a method for obtaining microtubers using the tuberization medium supplemented with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹)¹⁵⁾, and the

* A part of this study was presented at the 185th meeting of the Crop Science Society of Japan in 1988.

production of microtuber in over 50 different genotypes was tested by the method¹⁵⁾. However, there has been no report on the availability of the method for cultivars used in Japan.

In this study, using three cultivars popularized in Japan, we report on the effect of CCC and BA on an increase in the number and fresh weight of microtuber formed in a solid medium, and an observation of external morphology at the initial stage of formation by scanning electron microscope (SEM).

Materials and Methods

Three cultivars of potato (*Solanum tuberosum* L.), 'Benimaru', 'May Queen' and 'Danshaku-imo' grown in the field of Minami-Kyushu University were used. Potato tubers were obtained from plants cultivated in the field. The culture system for obtaining microtubers was composed of the following four steps, meristem culture, nodal segment culture, shoot propagation culture and tuberization culture. At first, shoot apices (about 4 mm) were excised from small plants (4~5 cm in height) which had been sprouted from seed potatoes. They were surface sterilized with 0.1% benzalkonium chloride for 10 min., and with 70% ethanol for 20 sec., then with 1% sodium hypochlorite solution for 15 min. After being rinsed with sterilized water three times, apical meristems were excised from the shoot apices under a stereomicroscope, and placed onto the Murashige & Skoog (MS) medium supplemented with 2 mg L⁻¹ kinetin and 0.02 mg L⁻¹ naphthaleneacetic acid (NAA). All media were adjusted to pH 5.75 before autoclaving, and solidified with 0.2% Gelrite. Sucrose contents were 3% in the media, except for the tuberization medium which contained 8% sucrose. After two months, the plantlets emerged from the meristem reached a height of about 7 cm. Nodal cuttings were removed from the plantlets and placed onto the MS medium supplemented with 0.01 mg L⁻¹ NAA and 0.25 mg L⁻¹ gibberellic acid (GA₃) to allow shoot development. The axillary bud rapidly grew out and in 15 days developed into a plantlet (about 5 cm in height). Then, the roots and apices were carefully removed and the shoots with 3~4 nodes were horizontally placed on the MS medium without hormones. When the new shoots induced from each node attained 4~5 cm in height, these

were decapitated and placed horizontally on the tuberization medium. The combinations of CCC (0, 200 and 500 mg L⁻¹) and BA (0 and 5 mg L⁻¹) were used for the tuberization culture medium. The combinations of hormones in each step of the culture described above were the same as those proposed by The International Potato Center¹⁵⁾. The conditions of incubation for tuberization were total darkness at 20° and 25°C, or over a 10 h photoperiod provided by fluorescent tubes at 20°C and 25°C.

For scanning electron microscopy (SEM), the shoot segments with microtuber were fixed in 2% glutaraldehyde for 2 h and post-fixed in osmium tetroxid for 3 h. After ethanol dehydration, the samples were critical-point dried and coated with gold. Observations were made with a JEM scanning electron microscope at 50KV.

After 28 days of tuberization culture, the microtubers were harvested and washed carefully with water. The fresh weight of tubers decreased with time because of the evaporation of water from the surface of tubers, but began to decline more slowly after 24 h. Therefore, the fresh weight was scored after 24 h of preservation in Petri dishes at room temperature. The tubers smaller than about 2 mm in diameter were discarded from the measurement of fresh weight. Prior to the transference of the microtubers to a vermiculite medium in pots, the tubers were soaked in a 10 ppm solution of GA₃ for several hours in order to break endogenous rest. When the plants reached approximately 5 cm in height, they were transplanted into the small pots (12 cm in diameter) filled with a sterilized soil and allowed to grow in a greenhouse.

Results and Discussion

Fig. 1 shows the effects of CCC (500 mg L⁻¹) on the number of microtubers formed on the shoots of 'Benimaru' in condition of total darkness at 20°C or 25°C, and 10 h photoperiod at 20°C or 25°C. In the total darkness, the formation of small microtubers was observed two days after the beginning of culture on the medium with CCC and BA. The number increased rapidly, attaining an almost constant level after 12 days of culture. The increase in the number of microtubers on the

medium was more rapid than that on the medium with BA alone. This indicates that CCC accelerated tuberization at the early stage of culture.

The number and fresh weight of microtubers of 'Benimaru' after 28 days in culture are shown in Table 1. The maximum number of microtubers was obtained in the medium with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹) at 20°C in total darkness conditions. The temperature of 20°C was more effective in increasing the mean value of fresh weight of microtubers than that of 25°C except on the medium with CCC and BA in the dark. The temperature effect on the mean value of fresh weight was more remarkable in the 10 h photoperiod than in total darkness. Wang and Hu¹⁶⁾ and Nowak and Colborne¹²⁾ reported that the optimum temperature for tuberiza-

tion *in vitro* was 20°C. Tovar et al.¹⁵⁾ proposed the darkness conditions at 22°C with CCC and BA for tuberization *in vitro*. The effects of temperature on tuberization *in vitro* shown in Fig. 1 and Table 1 seem to be in accordance with the results of these reports^{12,15,16)}.

Further experiments were carried out on the effect of CCC and BA on the formation of microtubers in total darkness at 20°C, using the three cultivars 'Benimaru', 'May Queen' and 'Danshakuimo'. Table 2 shows as a common tendency of the three cultivars the medium supplemented with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹) gave the highest values in the number and fresh weight of microtubers produced per 10 shoots. In this case, the mean of the fresh weight of microtuber of 'May Queen' was far lower than those of the other two cultivars. This is consid-

Fig. 1. Effect of CCC on the formation of *in vitro* tuber.

●—●: CCC (500 mg L⁻¹) and BA (5 mg L⁻¹), ○—○: BA (5 mg L⁻¹)
Cultivar: Benimaru.

Table 1. Effect of CCC, BA and light-dark conditions on the number and fresh weight of microtubers.

	(mg L ⁻¹)		Temperature	Number of tubers per 10 shoots	Fresh weight (mg)	
	CCC	BA			Means	mg/10 shoots
Dark	0	5	20°C	7.0	72	504
	0	5	25°C	11.3	37	418
	500	5	20°C	16.2	133	2155
	500	5	25°C	15.2	171	2599
10h Light	0	5	20°C	8.5	109	927
	0	5	25°C	4.0	47	188
	500	5	20°C	10.0	208	2080
	500	5	25°C	5.5	74	407

Cultivar used is Benimaru. Each value was scored after 28 days in tuberization culture. 18 shoots were used in each treatment.

ered to be a varietal difference. In all three cultivars, the means of fresh weight of microtuber after 28 days in culture were higher in the medium with BA alone than that with CCC alone. This suggests that BA also contributed an increase in fresh weight of microtuber. As shown in the Table 2, the number of microtuber per 10 shoots was far greater in the medium with CCC alone than that with BA alone after 12 days in culture. After 28 days in culture, however, little difference in such effect was found between CCC and BA. The facts and results shown in Fig. 1 suggest that CCC (500 mg L⁻¹) markedly promoted tuber initiation at the early stage of tuberization culture. Therefore, we tried to elucidate the role of CCC for the initiation of sink from the change of the external morphology of axillary bud.

The SEM micrograph (Fig. 2) revealed a small organized structure on the shoots of 'Benimaru' after two days in culture on the medium supplemented with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹) in the dark at 20°C. As can be seen in the Fig., this small organ consisted of leaves in the distal part and a swelled basal part. Some stomata were observed on the organ as a whole. This suggests that the swelling of the basal part is the first

sign of tuberization, and that such a rapid change of morphology is induced by CCC which prevent biosynthesis of gibberellic acid. This enlargement of microtuber proceeded through the swelling of the basal part with degeneration of leaves. Fig. 3 shows an example of microtuber formed for 6 days in the same conditions as Fig. 2. The remains of buds degenerated could be clearly observed in Fig. 3.

Leclerc et al.⁹⁾ reported that CCC (500 mg L⁻¹), BA (5 mg L⁻¹) and coumarine (50 mg L⁻¹) failed to trigger any increase in fresh weight or number of microtubers per shoot. Garner et al.⁶⁾ demonstrated a reliable microtuber production method on a medium free of any growth regulating agent. The discrepancy between their results and ours can be considered to be due to the difference of culture conditions, type of explant, and genotype. Leclerc et al.⁹⁾, using layered shoots, produced microtuber with a liquid medium for tuberization at 15°C of 8 h photoperiod. Garner et al.⁶⁾ used nodal explants obtained from potato cultivars widely grown in Britain. We may ascribe the main reason for the discrepancy among the results regarding the role of CCC and BA to the endogenous hormonal content inherent to the potato

Table 2. Effect of CCC and BA on the number and fresh weight of microtubers.

Cultivars	(mg L ⁻¹)		Number of tubers/10 shoots		Fresh weight (28 days) *	
	CCC	BA	12	28 (days) *	Means (mg)	mg/10 shoots
Benimaru	0	0	0	5.7	7	40
	0	5	4.5	7.0	72	504
	200	5	4.1	11.7	83	971
	500	0	8.9	11.0	58	638
	500	5	10.1	16.2	133	2155
May Queen	0	0	2.0	4.7	29	136
	0	5	4.0	11.3	93	1051
	200	5	2.7	14.0	75	1050
	500	0	10.0	10.7	61	653
	500	5	12.3	16.7	78	1303
Danshakuimo	0	0	1.3	3.3	49	162
	0	5	6.7	11.6	79	916
	200	5	6.7	14.0	78	1092
	500	0	10.0	11.3	62	700
	500	5	11.7	14.7	106	1558

* Days in tuberization culture. Cultures were kept in the total dark at 20°C. 18 shoots were used in each treatment.

Fig. 2. SEM of *in vitro* tuber after 2 days in culture on tuberization medium.
Cultivar ; Benimaru.
(Bar=1 mm).

Fig. 3. Sessile tuber formed on the shoot.
The picture was taken after 6 days in culture.
Cultiver : Benimaru.
(Bar=1 mm).

genotype.

The means of fresh weight of microtubers shown in Table 2 were comparable to the range of means reported by Estrada et al.⁵⁾, who used the tuberization medium with CCC (500 mg L⁻¹) and BA (5 mg L⁻¹). The plants regenerated through sprouting of microtubers grew normally in greenhouse, and a considerable yield of potato tuber was obtained.

In conclusion, it became clear that the method for formation of microtuber proposed by the International Potato Center (CIP)¹⁵⁾ is available to the three cultivars, 'Benimaru', 'May Queen' and 'Danshakuimo', and that the fresh weight of microtuber of 'May Queen' was relatively small compared to those of the other two cultivars.

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