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# STUDIES ON THE BREEDING OF ALL-MALE CULTIVAR IN ASPARAGUS

## III. Response to the *in vitro* culture in a supermale plant 'MM-1'

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### Introduction

As reported in the previous papers<sup>6,8)</sup>, six male plants bearing some berries with germinative seeds were found in cv. Zuiyoh asparagus fields at Date City in Hokkaido. From plants developed from the seeds, twelve supermale plants, which have the genotype (MM) including dominant genes alone in sex determination, were obtained by crossing test.

Subsequently, their growth habit, yielding ability, characters of spears and seed productivity were observed, and a supermale plant which has superior characteristics was selected as a pollinizer to breed an all-male cultivar. This selected plant was named "MM-1" in 1976. In this paper, the response of 'MM-1' in the *in vitro* culture was observed to obtain basic knowledge to establish a vegetative propagation method as a routine method of multiplying 'MM-1'.

### Materials and Methods

#### Experiment I. Callus and organ formation in the *in vitro* culture of apices or shoot segments of stock shoots

The small predeveloping lateral shoots in a head of young spears of supermale plant 'MM-1' were aseptically cultured *in vitro* to produce stock shoots. In this culture, a medium containing MURASHIGE and SKOOG's (MS) organic and inorganic substances<sup>9)</sup>, 20 g/l sucrose,  $10^{-7}$  M of N<sup>6</sup>-benzyladenine (BA),  $10^{-6}$  M of NAA and 7 g/l agar were used. The pH of the medium was adjusted to 5.5 with NaOH or HCl before autoclaving.

After three months of culture, the apices (0.5 mm in thickness) and 7 mm-length stem segments with a node were excised from the stock shoots

and were planted onto solid medium in which the constituents were similar to the medium mentioned above except that it contained  $10^{-7}$  and  $10^{-6}$  M of IBA or NAA separately or in combination with  $10^{-7}$  M of BA. In this case, four pieces of the apices or stem segments were planted onto the medium (poured by 25 ml per 100-ml Erlenmeyer flask) and ten flasks were used in one treatment.

The cultures were incubated under temperature conditions ( $25^{\circ}\text{C}$ ) and light conditions (4,000 lx, 16-hour illumination per day with a 40-watt daylight fluorescent lamp).

#### **Experiment II. Proliferation and organ formation in culturing the calluses initiated from the stem segments of stock shoots**

In the culture of stem segments of stock shoots, a line of callus which has an ability of organ redifferentiation was obtained. This line was proliferated on the MS medium containing  $10^{-7}$  M of BA and  $10^{-6}$  M of NAA and was used in this experiment. Namely, the calluses which grew to Azuki bean size to soybean size were cut to rice grain size, and three of those were planted onto the medium in 100-ml Erlenmeyer flasks.

The concentration of growth regulators (BA and IBA) and organic and inorganic substances in MS medium in each batch were shown in Table 1. In batches of A and B, the calluses were cultured on solid medium containing growth regulators, although in the other four batches (C-F) calluses were precultured for seven days in liquid medium containing growth regulators, and then they were transferred onto solid medium without growth regulators.

The cultures were incubated under conditions of  $25^{\circ}\text{C}$  and 1,000 lx (16 hr. per day).

For observation of chromosomes, root tips of the regenerated plants were kept in the distilled water at  $0^{\circ}\text{C}$  for 24 hours and fixed in Carnoy's solution for 24 hours. They were stained with acetocarmin and observed by the squash method.

### **Results and Discussion**

#### **Experiment I. Callus and organ formation in the *in vitro* culture of apices or shoot segments of stock shoots**

In the culture of apices, shoot development was observed within two weeks after the beginning of the culture, and almost all of apices developed shoots without exception. Root formation on the basal portion of the apex began to be observed after a month of incubation.

The percentage of apices forming roots is shown in Fig. 1. Namely, it was the highest (60%) on the medium containing both  $10^{-7}$  M of BA and  $10^{-6}$  M of IBA, and on the medium containing  $10^{-6}$  M of NAA alone, followed by that on the three kinds of media containing  $10^{-7}$  M or  $10^{-6}$  M of IBA alone, or  $10^{-7}$  M of NAA alone.

The percentage of apices forming white roots was highest (40%) on the medium containing both  $10^{-7}$  M of BA and  $10^{-6}$  M of IBA, and followed by that on the two media containing  $10^{-7}$  M of IBA alone or  $10^{-6}$  M of IBA alone.

Some of the apices which developed both shoots and roots (the majority were white roots) grew into whole plantlets. However, it was not so easy to obtain whole plantlets from apices, because it is difficult to form a rhizome.

In the culture of stem segments, lateral shoots began to develop from the node within a week. Root formation was observed on part of the node of stem segments in four or five weeks of incubation, but the percentage of the segments forming roots were lower than that in apex culture as shown in Fig. 2. Namely, the highest was only 17.5% at  $10^{-7}$  M of NAA. In this case, stem segments which developed both shoots and white roots grew into complete plantlets and were transplanted in a pot successfully, because rhizomes were formed on the basal portion between shoots and roots and the vascular-bundles of shoots and roots were connected smoothly.

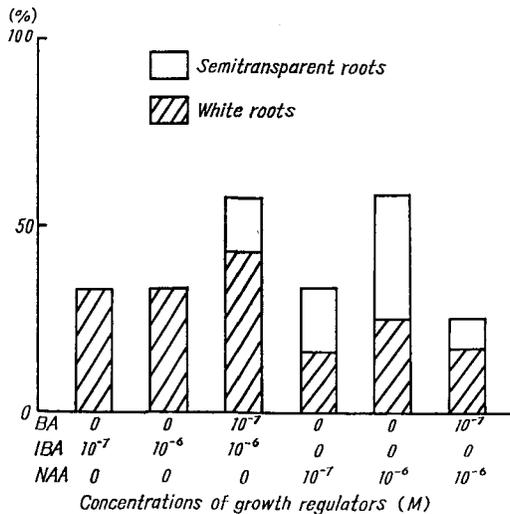


Fig. 1. Percentage of apices forming roots in culturing apices excised from the stock shoots of 'MM-1' after 12 weeks of culture.

In this experiment, callus formation was observed in all batches in the culture of both apices and stem segments, and its percentage was relatively high in apex culture (Fig. 3) and low in stem segment culture (Fig. 4).

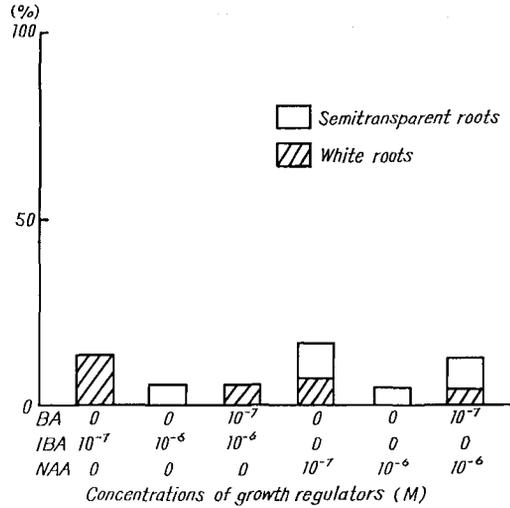


Fig. 2. Percentage of stem segments forming roots in culturing stem segments with a node excised from the stock shoots of 'MM-1' after 12 weeks of culture.

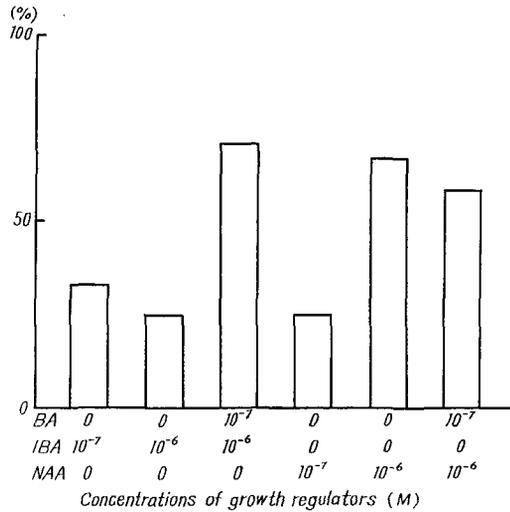


Fig. 3. Percentage of apices forming callus in culturing apices excised from the stock shoots of 'MM-1' after 12 weeks of culture.

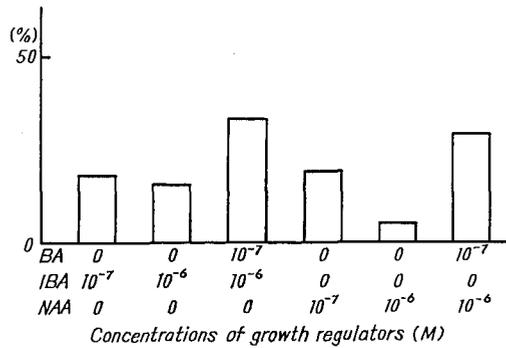


Fig. 4. Percentage of stem segments forming callus in culturing stem segments with a node excised from the stock shoots of 'MM-1' after 12 weeks of culture.

TABLE 1. Effect of growth regulators and transplanting on organ formation in culturing calluses formed from stem segments

Mark of batches	Conc. of growth regulators				Conc. of MS medium	Degree of callus growth*			
	Liquid medium		Solid medium			—	+	#	##**
	BA (M)	IBA (M)	BA (M)	IBA (M)					
A	—	—	0	$10^{-6}$	1	19.6	34.8	27.6	18.0
B	—	—	$10^{-7}$	$10^{-6}$	1	13.3	20.0	41.7	25.0
C	0	$10^{-5}$	0	0	1	29.2	41.6	29.2	0
D	0	$10^{-5}$	0	0	1/2	39.3	51.7	9.0	0
E	$10^{-7}$	$10^{-6}$	0	0	1	40.0	40.0	15.0	5.0
F	$10^{-7}$	$10^{-6}$	0	0	1/2	72.9	25.0	2.1	0

Mark of batches	Organ formation				Number of calluses measured
	Shoot+root (%)	Shoot alone (%)	Root alone (%)	Callus regenerated plantlet (%)	
A	20.5	8.9	10.7	8.0	112
B	60.0	30.0	8.3	23.3	60
C	16.6	54.2	4.2	8.3	24
D	26.8	7.1	10.7	12.5	56
E	5.0	25.0	10.0	0	20
F	4.2	14.6	8.3	2.1	48

\* Figures represent percentage of the calluses showing the growth degree in the batch.

\*\* Symbol indicates the degree of callus growth

— no callus + a trace # good ## excellent

The chromosome numbers observed were all diploid ( $2n=20$ ) in the plants regenerated from apices and stem segments with a node.

**Experiment II. Proliferation and organ formation in culturing the calluses initiated from the stem segments of stock shoots**

As shown in Table 1, callus growth and organ formation were generally good in the batch of A and B in which calluses were cultured continuously on solid medium containing growth regulators from the beginning of the culture. The percentage of the callus regenerated plantlets was the highest (23.3%) in the batch of B ( $10^{-7}$  M of BA,  $10^{-6}$  M of IBA). In this batch, the percentage of calluses forming both shoots and roots was highest (60%) likewise. On the whole, it is expected that about 60% or more of calluses regenerate to intact plantlets after several weeks of culture.

In the batch of A, in which no BA and  $10^{-6}$  M of IBA was added, 20% of calluses cultured formed both shoots and roots.

When calluses were precultured in the liquid medium containing growth regulators for seven days and then transferred onto solid medium containing no growth regulator, the percentage of calluses forming both shoots and roots was highest (26.8%) in the batch of D (no BA and  $10^{-5}$  M of IBA in 1/2 MS medium) and second highest (16.6%) in the batch of C (no BA and  $10^{-5}$  M of IBA).

In the calluses which developed both shoots and roots, rhizomes was not formed smoothly. However, about fifty plantlets were obtained in this

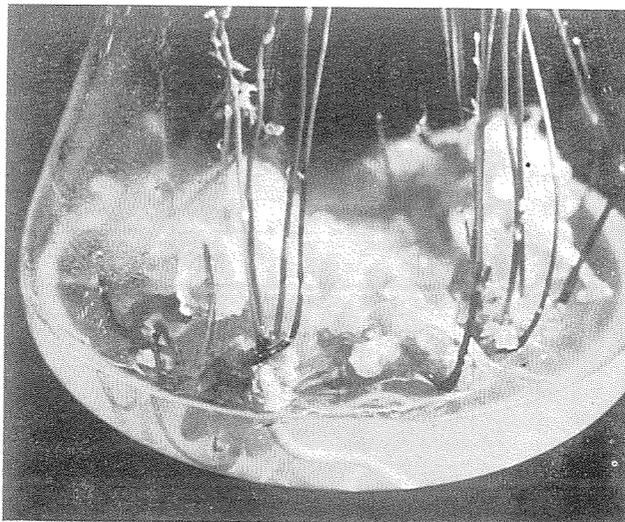


Fig. 5. Shoots and roots formed from the callus in Experiment 2.

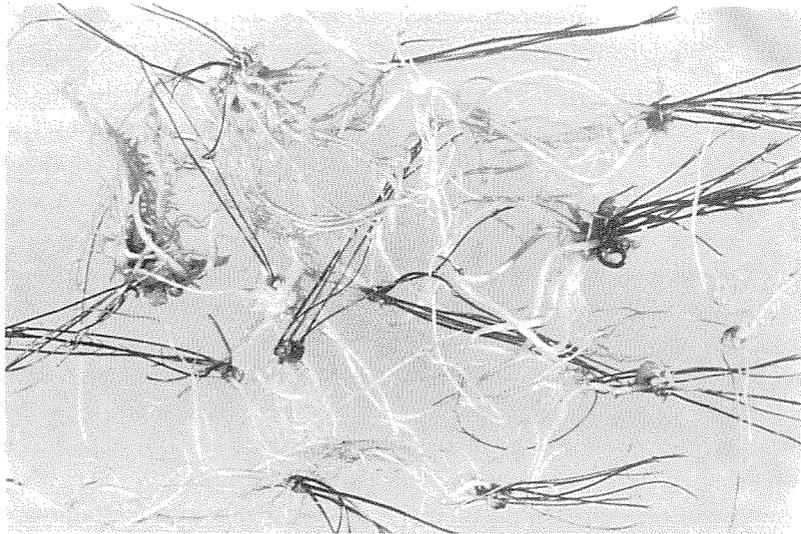


Fig. 6. Transplantable plantlets of 'MM-1' developed from the calluses.

experiment, and the chromosome numbers were observed on these plantlets (Fig. 5, 6, 7). In this observation, six tetraploids ( $4n=40$ ) and one aneuploid ( $2n=26$ ) were observed, and forty three other plants were diploid ( $2n=20$ ).

In the previous papers describing the experiments using 'Mary Washington 500', we reported that asparagus plantlets were obtained through tissue culture of an apex or a stem segment excised from the stock shoots<sup>5,9</sup>. Namely, in apex culture, good shoot formation was recognized at  $5 \times 10^{-7} - 10^{-5}$  M of IBA or  $5 \times 10^{-7} - 10^{-5}$  M of NAA, and a high percentage of root development was recognized at  $10^{-6} - 10^{-5}$  M of IBA or  $5 \times 10^{-7} - 10^{-5}$  M of NAA. The percentage of root



Fig. 7. Cloned plants propagated from the supermale plant 'MM-1' through tissue culture.

development in the stem segment culture was still lower than that in apex culture. These results are almost similar to that in this report and other papers<sup>1,10,11</sup>.

In culturing the stem segments with a node, studies on transferring should be carried out because the effectiveness of transferring on root formation was clearly recognized in the previous experiments<sup>7</sup>.

From the results mentioned above, it seems that the response of a 'MM-1' in the *in vitro* culture are similar to that of 'Mary Washington 500'. To establish an useful method for mass propagation of 'MM-1', further studies are required in connection with the formation of rhizome.

### Summary

The response of the supermale plant 'MM-1' in the *in vitro* culture was observed to obtain basic knowledge for establishing a vegetative propagation method of the line.

The media contained MS medium, 20 g/l sucrose, growth regulators (BA, IBA, NAA), and were solidified with 7 g/l agar after pH adjustment to 5.5. The growth regulators used were added separately or in combination according to the objectives of the experiments. The cultures were incubated under constant temperature conditions (25°C) and light conditions (4,000 or 1,000 lx, 16 hour illumination per day). The results obtained are summarized as follows :

1. In culturing the apex excised from stock shoot, the highest percentage of apices forming both shoots and white roots was 40% on the medium containing both  $10^{-7}$  M of BA and  $10^{-6}$  M of IBA, and  $10^{-6}$  M of NAA alone. Some of these apices grew into whole plantlets, however it was not easy to transplant these plantlets into a pot, because it was difficult to form a rhizome.

2. In culturing stem segments with a node, the percentage of segments developing shoots and roots was lower than that in apex culture. However, in this case, stem segments which developed both shoots and white roots grew into whole plantlets and were transplanted into a pot successfully, because rhizomes were formed on the basal portion between shoots and roots and the vascular bundles of shoots and roots were connected readily.

3. A line of callus which has an ability of organ redifferentiation was obtained in the culture of stem segments of stock shoots. Callus growth and organ formation was generally good, and fifty whole plantlets were obtained in the culturing of this line. However, six tetraploids ( $4n=40$ ) and an aneuploid ( $2n=26$ ) were observed in these plantlets.

4. From these results, it appears that the tissue culture response of 'MM-1' are similar to that of 'Mary Washington 500'. To establish an useful method for mass propagation of 'MM-1', further studies are required in connection with the formation of a rhizome.

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