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STUDIES ON THE ANTHER CULTURE OF HORTICULTURAL CROPS

VII: Investigation of optimal conditions for obtaining pollen-originating callus and its characteristics in *Asparagus officinalis* L.

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Introduction

In the anther culture of some higher plant species such as genus *Datura* and *Nicotiana*, it is well known that embryoids develop directly from pollen grains, and grow into intact plants. However, in most species including asparagus, the process mentioned above has not been established to the present, and following pathways alone is known in relation to obtaining pollen-originating plants through anther culture. Namely, calluses are induced from pollen grains, and shoots and roots redifferentiate from the calluses, and then intact plants regenerate. In the latter case, one of the most important problems is to induce haploid calluses originating from pollens. In the anther culture of asparagus, various types of calluses induced from both pollens and an anther wall proliferate to form callus clumps, and it is very difficult to determine the origin of the calluses by appearance alone.

In this experiment, the authors attempted to clarify the origin of the calluses by determining the haploidy callus cells and observing linkage among pollens, an anther wall and callus cells through histological observation, in addition, we attempted to identify the characteristics of the calluses and clarify the conditions suitable for induction and proliferation of them.

Materials and Methods

The anthers cultured in this experiment were derived from flowers of an male intact plant of cv. 'Mary Washington 500' and cv. 'Zuiyo'. These

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anthers were at the developmental stage with uninuclear to tetrad-pollens. The media used contained MURASHIGE and SKOOG's inorganic and organic substances, 2% glucose, 0.1 and 1.0 mg/l of both NAA and BA, and 0.7% agar. pH of the above were adjusted to 5.5 before autoclaving.

The cultures were incubated under the light conditions (in a 16-hr day length at 400-500 lx with a fluorescent lamp) at 27-28°C.

Origin of callus cells formed from anther was identified by determination of the chromosome number and histological observation on the linkages of various parts such as pollens, an anther wall and calluses of the cultures.

The samples for histological observation were fixed in FAA solution (Formalin : 5, Acetic acid : 5, 70% ethyl alcohol : 90) for 48 hrs, dehydrated by ethyl alcohol series, penetrated by xylol or telpineol and embedded in paraffin. Those for light microscopy were sectioned at 10 μ m and stained with Mayer's acid haemalaun.

Calluses used for observation of chromosomes were kept in the distilled water at 0°C for 24 hours and fixed in Carnoy's solution for 24 hours. They were stained with Feulgen's reaction and observed by the squash method.

Results

Callus formation from anthers was observed in cv. 'Mary Washington 500' and cv. 'Zuiyo', but the percentages of anthers forming callus and amount of callus growth were different according to the concentrations of growth regulators (Table 1.)

TABLE 1. Effect of concentrations of BA and NAA on callus formation from anthers

Cultivers	Growth regulators		Culture conditions		Number of anthers with callus	% of anthers with callus (%)	Degree of callus growth
	BA (mg/l)	NAA (mg/l)	Temperature (°C)	light (lx)	Number of anthers in culture		
MW 500	0.1	0.1	27-28	400-500	4/27	14.8	+
MW 500	1.0	1.0	27-28	400-500	75/110	68.2	##
Zuiyo	0.1	0.1	27-28	400-500	21/84	25.0	+
Zuiyo	1.0	1.0	27-28	400-500	137/157	87.3	##

In both the cultivars, anthers cultured on the medium containing 0.1 mg/l BA and 0.1 mg/l NAA, turned brown after 7 to 10 days of incubation and some of them formed calluses after 4-6 weeks of incubation (Fig. 1). The inside of these anthers was filled with callus cells proliferating produced

by dedifferentiation of pollens, and some of the calluses emerged toward the outside of the anthers (Fig. 2, 3 and 4). These callus cells were so enlarged and vacuolated that chromosome numbers could not be determined by the squash method. For this reason, some of these calluses were transferred onto the callus-proliferating medium containing 1.0 mg/l BA and 1.0 mg/l NAA. After 7 to 10 days incubation, haploid cells with 10 chromosomes were observed in some of the calluses, while after 20 or more days of incubation, no haploidy callus cell was observed. Especially in the later stage, some polyploid and aneuploid cells were observed beside diploid (Fig. 5, 6, 7).

In the medium containing 0.1 mg/l of BA and NAA, the growth of callus was slow and the size of callus reached rice grain to Azuki bean size after two-month incubation.

Color of these calluses was light yellow or light green and the characteristics of the calluses emerging from inside of the brown anther was soft and friable.

In the medium containing 1.0 mg/l of BA and NAA, calluses were formed from enlarged and yellowish green anthers after 10 to 14 days of incubation. In this case, the anther wall was covered with calluses within two or three weeks after callus induction, because callus growth was very rapid, and no haploid cell was observed in these calluses. Namely, in the early stage of incubation, many diploid and some tetraploid cells were observed, whereas many kinds of aneuploid cells were mixed in the later stage of incubation (Fig. 6). These calluses reached the size of soybean after two-month incubation. Color of these calluses was light yellow or green, and sometimes, white calluses were observed. The characteristic of calluses growing in medium containing 1.0 mg/l of BA and NAA was hard and compact. However, in the medium mentioned above, the calluses formed from brown anthers after 4 to 8 weeks of incubation were shown to be pollen-originating calluses.

Discussion

Concerning the induction of pollen-originating callus in anther culture of asparagus, PELLETIER *et al*⁴⁾ and DORE¹⁾ reported that a pollen-originating callus could be obtained on the modified MS medium to which BA, NAA and 2, 4-D were added in combination over a range of 0.2-1.0, 0.02-1.0 and 0.2-1.0 mg/l respectively. However, they did not make detailed observations on the cultured anthers with calluses, so it is not clear whether somatic cell-originating calluses could be formed or not in these medium.

In this paper, the authors confirmed that the growth and origin of calluses were different according to the concentrations of growth regulators.

Namely, in the medium containing a relatively high concentration (1.0 mg/l) of BA and NAA, calluses were formed from the anther in relatively early stage (after 10 to 14 days of incubation) and grew very rapidly. But, in this case, they were not induced from a pollen except for the calluses formed from brown anthers after 4 to 8 weeks of incubation. On the other hand, in the medium containing relatively low concentrations (0.1 mg/l) of BA and NAA, the percentage of callus formation was very low and callus growth was very slow. However, in this case, there is a high possibility of obtaining pollen-originating calluses. These differences were proved by chromosome number and cytohistological observation. Namely, under the former conditions (high concentration of BA and NAA), diploid, tetraploid and aneuploid cells were observed, but no haploid cells was observed. On the contrary, the callus obtained under the later conditions (low concentration of BA and NAA) included haploid cells in the early stage of callus formation and the interior of anthers was filled with calluses which dedifferentiated from the pollen. But, no haploid cell was observed in the later stage in this case, likewise. Thus, it is imperative in confirming pollen-originating calluses that the observation on the chromosome number of callus cells should be done in the early stage of callus formation.

As reported in the previous paper, when the concentrations of BA and NAA were lower than 0.1 mg/l, the percentage of anthers with calluses gradually decreased with a decrease of these concentrations. Also the concentrations of BA and NAA higher than 1.0 mg/l is not desirable because of high frequency of induction of the somatic cell-originating calluses.⁹⁾

The results of our experiments on the effect of NAA or 2, 4-D with BA on callus formation from anther showed that 2, 4-D was less effective than NAA in callus formation.²⁾ The similarity of the characteristics of the calluses formed on both medium with 2, 4-D and NAA would indicate that those calluses originated from the same type of tissue.

From the results of the present experiment and previous experiments mentioned above, it became obvious that the medium containing relatively low concentrations of BA and NAA and the conditions of culturing with relatively high temperature (27-28°C) would be suitable for inducing pollen-originating callus. However, all the calluses formed under the culture condition mentioned above are not pollen-originating calluses.

Hereafter, for the establishment of the method for obtaining pollen-originating calluses, a more detailed observation on the inside of the cultured anthers as well as the confirmation of the chromosome number of callus cells would be required to clarify the difference between the two types of

TABLE 2. The characteristics of pollen-originating or somatic cell-originating callus

	Pollen-originating callus	Somatic cell-originating callus
Time of callus formation	after 4-6 weeks of incubation	after 10-14 days of incubation
Color and size of the anthers from which calluses were formed	turned brown and their size was the same as the initial anthers before using for incubation	yellowish green or light green and enlarged
Growth of callus	slow	rapid
Color of callus	light yellow-dark yellow in dark condition and light yellow-light green under light conditions many calluses were light yellow	light yellow-dark yellow in dark condition and light yellow-green in light conditions sometimes white calluses were formed
Characteristics of callus	soft and friable	hard and compact

path ways for obtaining a pollen-originating callus or a somatic cell-originating callus.

In conclusion, the characteristics of the pollen-originating or somatic cell-originating callus are summarized in Table 2.

Summary

For the purpose of obtaining pollen-originating calluses from the anther of *Asparagus officinalis* L., experiments on the concentrations of BA and NAA was carried out using 'Mary Washington 500' and 'Zuiyo'. Also, the cytohistological observation on cultured anthers and calluses were attempted to investigate the characteristics of pollen-originating calluses. The results obtained are summarized as follows :

1. Haploid callus cell with 10 chromosome were observed in some of callus clumps formed on the medium containing relatively low concentrations of BA and NAA (0.1 mg/l) restrictedly in the early stage of incubation, while no haploid cell was observed in later stage of incubation.

The cytohistological observations on these anthers showed that the interior of those anthers was filled with callus cells which dedifferentiated from pollens.

2. In the medium containing relatively high concentrations of BA and NAA (1.0 mg/l), calluses were formed from anthers after 10 to 14 days of incubation and the anther wall was covered with calluses within 2 or 3 weeks after callus formation. In these calluses, no haploid cell was ob-

served, while diploid, tetraploid and aneuploid cells were observed. However, in the case of these mediums mentioned above, the calluses formed from a brown anther after 4 to 8 weeks of incubation were clarified to be pollen-originating calluses.

3. From the results of this experiment, it was concluded that pollen-originating calluses could be obtained from anthers cultured on the medium containing relatively low concentrations of 0.1 mg/l of both BA and NAA at 27–28°C.

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Explanation of Plate

- Fig. 1. Callus formed from anthers cultured on MS medium with 0.1 mg/l of BA and NAA. cv. 'Mary Washington 500'.
- Fig. 2. Dedifferentiation of pollens in anther locus.
- Fig. 3. Early stage of callus formation from pollens in the anther locus.
- Fig. 4. Longitudinal section of cultured anthers with the callus showed in Fig. 1. Interior of anther locus was filled with callus cells, and some of the callus cells were growing toward the outside of anther locus.
- Fig. 5. Haploid cells observed in the callus cells.
- Figs. 6, 7. Aneuploid cells observed in the callus cells.

