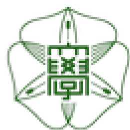
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STUDIES ON THE ANTHER CULTURE OF HORTICULTURAL CROPS

IV. Effect of growth regulators on organ formation from anther-derived callus of *Asparagus officinalis* L.

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Introduction

Generally, two pathways have been clarified in relation to obtaining pollen-origined plants through anther culture. In one pathway, embryoids develop directly from pollen grains, and grow into intact plants. In the other pathway, callus is induced from pollen grains, and shoots and roots differentiate from the callus, and then intact plant regenerate.

The latter pathway alone is known in anther culture of asparagus.

In our previous papers[4,5], we described the optimum conditions for the callus induction from anthers of several cultivars of asparagus and the growth of the calluses.

In the present experiment, effects of growth regulators such as auxin, cytokinin and gibberellin on organ formation from anther-derived callus were investigated.

Materials and Methods

Callus used in this experiment was prepared by the following method.

The anthers at uninuclear stage of pollen development of cv. Mary washington 500 (Experiment I) and Grüne Krone (Experiment II, III) were placed on Murashige and Skoog's medium (M. S. medium) solidified with 0.7% agar to which 1.0 mg/l BA, 1.0 mg/l NAA and 20 g/l sucrose were added. The cultures were incubated under the conditions of 25～27°C and 4000 lx (16 hr per day).

When callus formed from anthers grew into the size of Azuki bean

to soybean, each callus was cut into the size of a rice grain. Three of those were transferred onto the medium (poured by 25 ml per 100 ml Erlenmeyer flask) for organ formation and 10 flasks were used in one treatment.

The three experiments mentioned below were carried out using the solid medium with M. S medium, 0.7% agar, 2% sucrose (Experiment I) or 2% glucose (Experiment II, III) and various growth regulators for organ formation. The pH of the medium was adjusted to 5.5. The cultures were incubated under the condition of 4000 lx (16 hr per day) and 25~27°C.

Experiment I: Effect of BA and NAA on organ formation from anther-derived callus.

Nine kinds of media were prepared by addition of 0.5, 1.0 and 5.0 mg/l BA combined with 0.1, 0.5 and 1.0 mg/l NAA respectively. Rice grain-sized calluses were placed onto the medium described above for testing effects of BA and NAA on organ formation, especially on shoot formation.

Experiment II: Effect of BA and IBA on organ formation from anther-derived callus.

Nine kinds of media were prepared by the addition of 0, 0.1 and 0.5 mg/l BA combined with 0, 0.1 and 0.5 mg/l IBA respectively. Rice grain-sized calluses were placed onto the medium described above for testing the effect of BA and IBA on organ formation, especially on root formation.

Experiment III: Effect of BA, NAA and gibberellin (GA) on organ formation from anther-derived callus.

Twelve kinds of media were prepared by the addition of BA 0+NAA 0 mg/l, BA 0.2+NAA 0.1 mg/l and BA 1.0+NAA 0.5 mg/l combined with 0, 0.01, 0.1 and 1.0 mg/l GA, respectively for especially examining the effect of gibberellin on growth of callus pieces divided into the size of rice grain and organ formation.

For histological observation, calluses and organs were embedded in paraffin, sectioned at 10 μ and stained with Mayer's acid hæmalaun.

Callus growth was indexed as follows; Index of callus growth: 0: no callus growth, 0.5: a trace growth, 1.0: as large as a rapeseed, 1.5: as large as a rice grain, 2.0: as large as an Azuki bean, 3.0: as large as a soybean.

Index of more than 4 was based on the size of a soybean.

Example: Index 4 is twice as large as a soybean.
Index 5 is three times as large as a soybean.

Results

The results of these experiments are as follows;

Experiment I: Effect of BA and NAA on organ formation from anther-derived callus.

Cells in outer layers of the callus were enriched in cytoplasm and had a high potential of cell division. The cells in the inner part of the callus were larger than those in the outer layers and had a large vacuole.

Callus growth after transferring onto the medium for organ formation is shown in Fig. 1. The growth of the calluses was good on all media used in this experiment. Callus growth was especially enhanced on the medium containing 0.5 to 1.0 mg/l BA combined with 0.5 to 1.0 mg/l NAA respectively. However, a long term of incubation made the calluses brown and dead. This phenomenon was more marked at higher concentrations of NAA.

Shoot formation began to be observed in about a month of incubation.

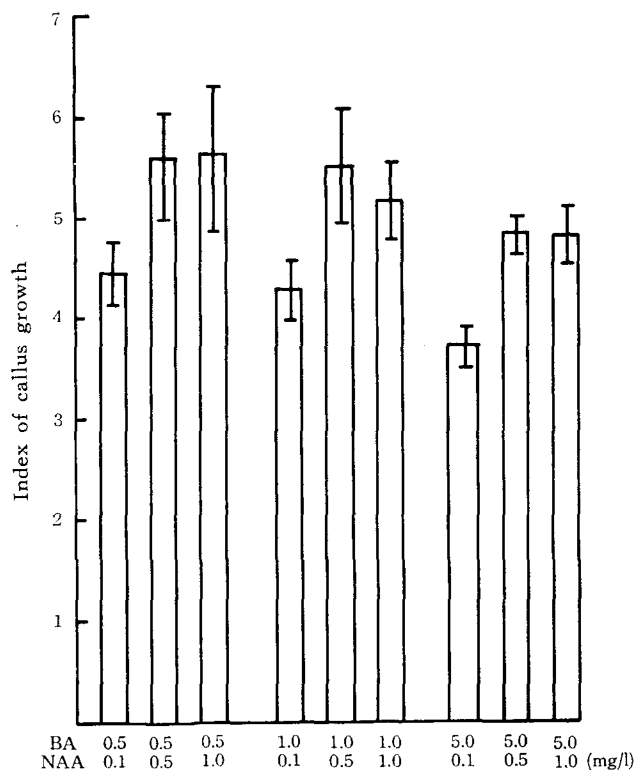

Fig. 1. Effect of BA and NAA on callus growth. (incubated 28 weeks at 25-27°C).

I: Bar indicates confidence limits at 95%.

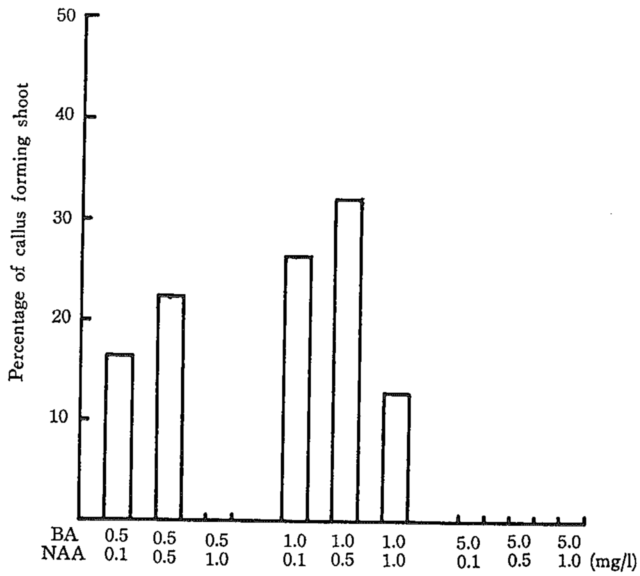

Fig. 2. Effect of BA and NAA on shoot formation from callus. (incubated 28 weeks at 25–27°C).

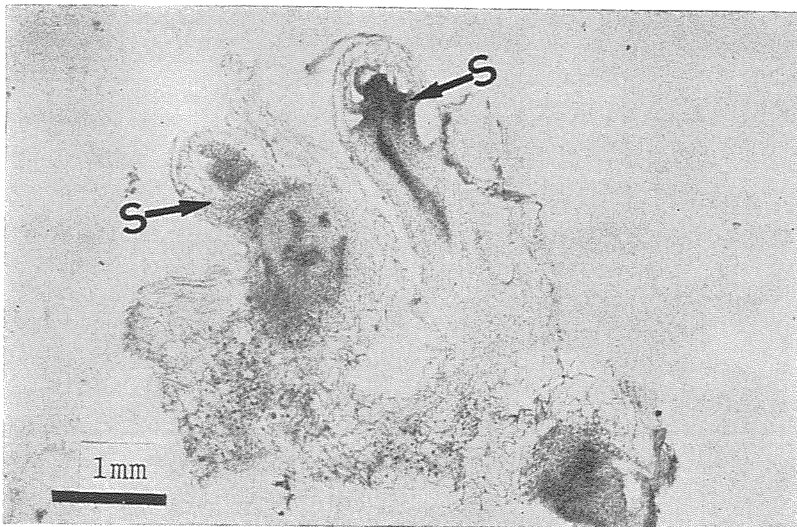

Fig. 3. Shoot differentiation from anther-derived callus of asparagus. S: Shoot differentiating from callus.

Most of the calluses which formed shoots grew into the size of an Azuki bean to a soybean.

Percentage of the callus forming shoots is shown in Fig. 2. High percentage of the callus forming shoots was obtained on the medium containing 0.1 to 0.5 mg/l NAA combined with 0.5 to 1.0 mg/l BA respectively and, shoot formation was good in a high ratio of BA relative to NAA.

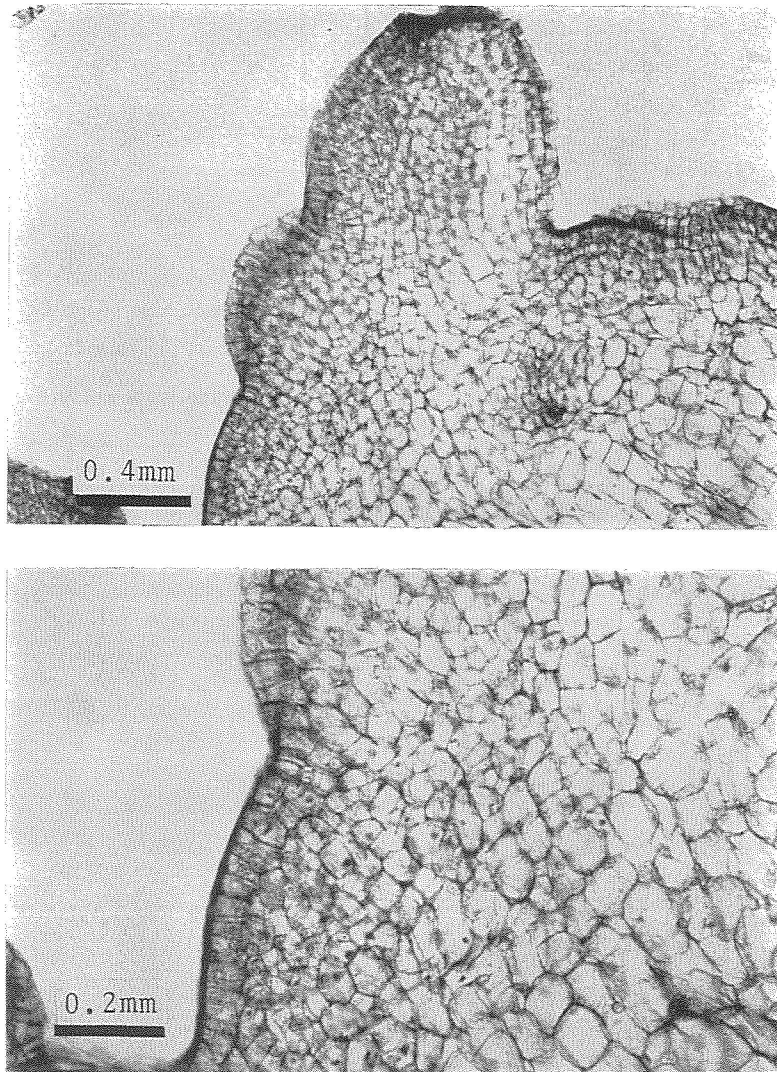

Fig. 4. Differentiation of shoot primordia from anther-derived callus. Longitudinal section of callus with shoot primordia.

The highest percentage of the callus forming shoots (32%) was obtained on the medium containing 1.0 mg/l BA and 0.5 mg/l NAA.

Fig. 3 and Fig. 4 represent the histological observation of the callus cultured on the medium for organ formation, and shoot primordia and shoots obtained on the same medium.

Shoot formation was observed in the outer layers of several compact callus with high potential of cell division when the transferred callus grew into a proper size of callus (as large as a soybean). No root formation was observed in this experiment, because concentrations of BA and NAA were too high for root formation.

Experiment II: Effect of BA and IBA on organ formation from anther-derived callus.

The growth of transferred callus is shown in Fig. 5. The growth of callus was observed in all media used in this experiment, and especially enhanced in the medium containing 0.1 and 0.5 mg/l IBA with 0.5 mg/l BA,

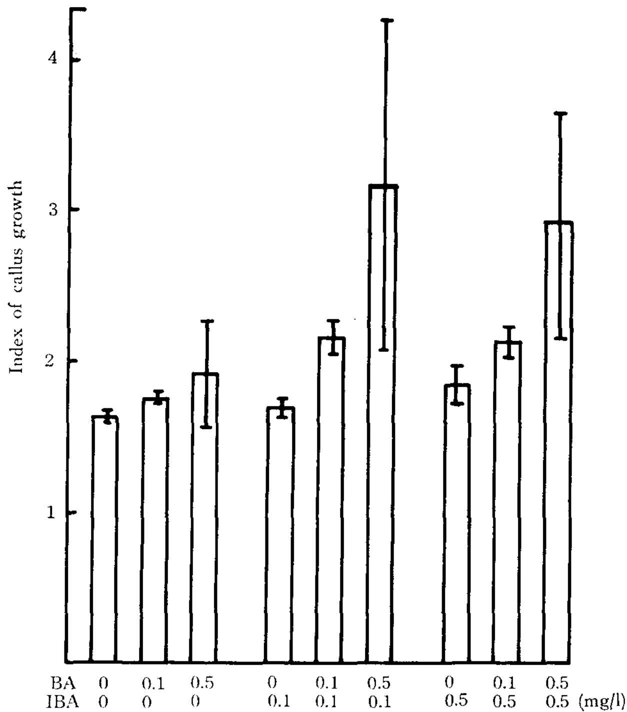

Fig. 5. Effect of BA and IBA on callus growth on the organ-forming medium. (incubated 14 weeks at 25–27°C).

I: Bar indicates confidence limits at 95%.

however, the growth of callus was inferior to that of experiment I in which NAA was substituted for IBA. From the result, it was considered that IBA was inferior to NAA in callus growth. The growth of callus on each medium containing no BA, or no IBA was little and stopped within two or three weeks after transferring.

Root formation from transferred callus was observed within a month after transferring. The percentage of callus forming roots was the highest with the medium containing 0.1 mg/l IBA, and followed by that on the medium containing 0.1 mg/l BA and 0.5 mg/l IBA (Fig. 6). It appeared that

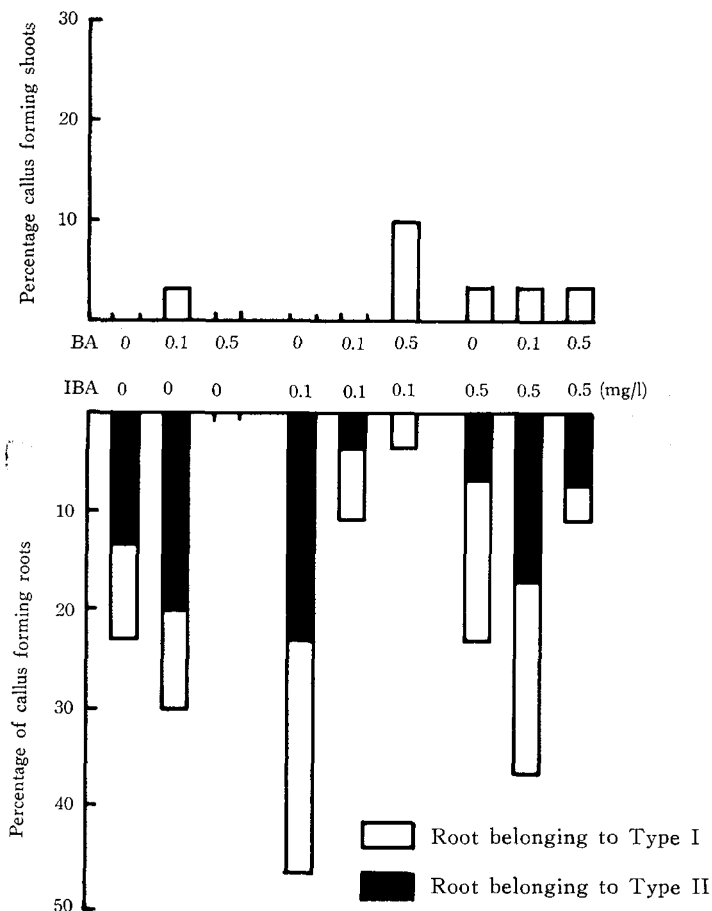

Fig. 6. Effect of BA and IBA on shoot and root formation from callus. (incubated 14 weeks at 25-27°C).
Vascular system of root in Type I is undeveloped. Root in Type II is normal.

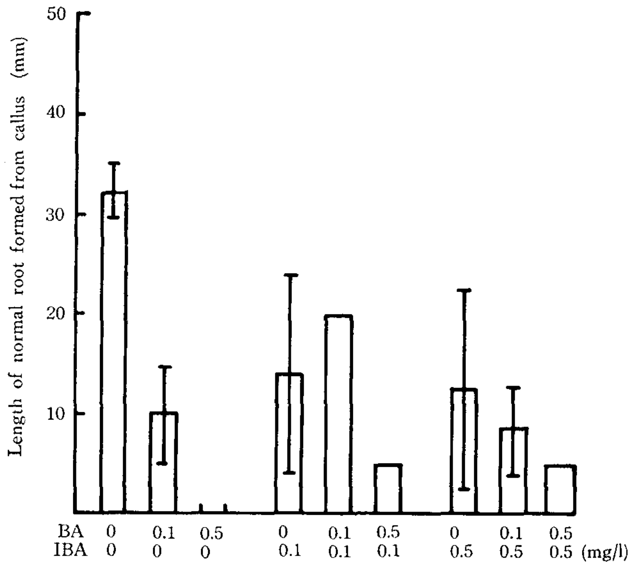

Fig. 7. Effect of BA and IBA on the elongation of normal root formed from callus. (incubated 14 weeks at 25–27°C).
I: Bar indicates confidence limits at 95%.

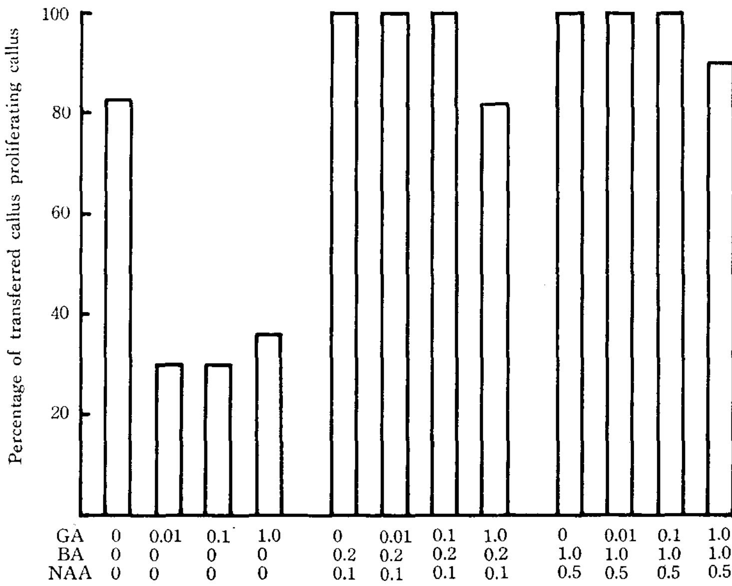

Fig. 8. Effect of GA, BA and NAAon proliferation of transferred callus. (incubated 14 weeks at 25–27°C).

in relation to growth of callus root formation was observed in the callus of which growth was poor. In addition, calluses with roots hardly grew after forming roots.

Root elongation was good on the medium without BA and IBA. It was assumed that root elongation was inhibited by addition of BA and IBA (Fig. 7).

Percentage of callus forming shoots was low in this experiment. Namely, the highest percentage of callus forming shoots was 10% at 0.5 mg/l BA and 0.1 mg/l IBA (Fig. 6). Calluses with shoots grew rapidly, of which growth index was more than 3.0 (the same size as soybean). On the other hand, the growth of callus forming no shoots was less than that of callus forming shoots. Even on the same medium, the character of calluses forming no shoots was different from that of calluses forming shoots. Especially

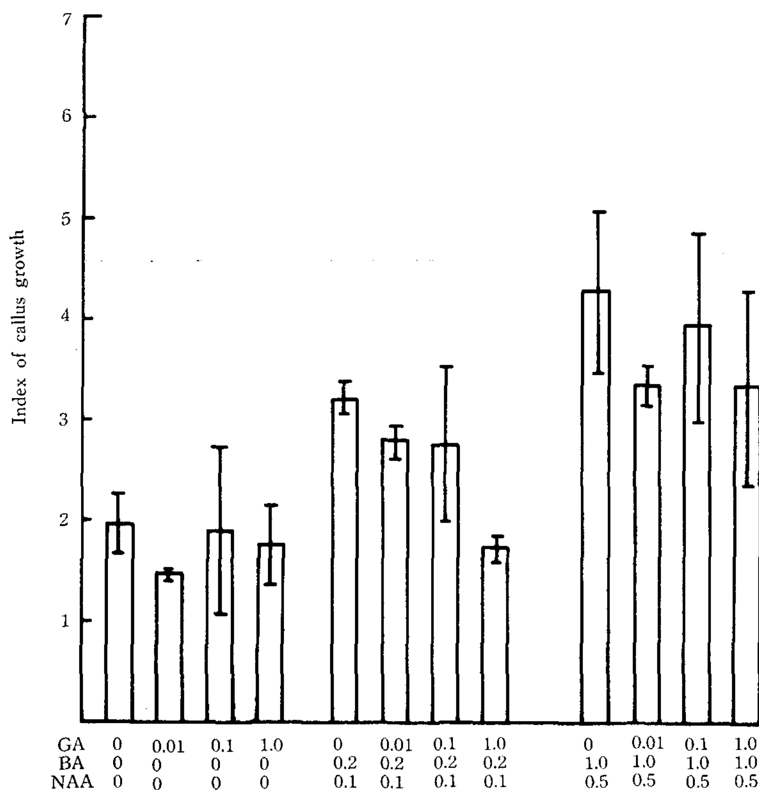

Fig. 9. Effect of GA, BA and NAA on callus growth. (incubated 14 weeks at 25–27°C).

I: Bar indicates confidence limits at 95%.

relating to calluses grown on the medium containing 0.1 mg/l BA, or 0.5 mg/l IBA, most calluses hardly grew except for the calluses forming shoots.

Experiment III: Effect of BA, NAA and gibberellin on organ formation from anther-derived callus.

Percentage of callus regrowing after transferring is shown in Fig. 8. It was observed that more than 80% of the transferred calluses regrew on the medium without BA and NAA, and that addition of GA_3 to the same medium considerably inhibited callus proliferation. Inhibition of callus proliferation by addition of GA_3 was also observed a little on the medium containing 1.0 mg/l GA_3 combined with BA and NAA. GA_3 also had a tendency to inhibit the growth of callus, and its tendency was especially marked in the case of addition of 1.0 mg/l GA_3 (Fig. 9). Most calluses grown on the medium containing 0.1 mg/l or 1.0 mg/l GA_3 were lacking in chlorophyll, slightly dried and turned brown.

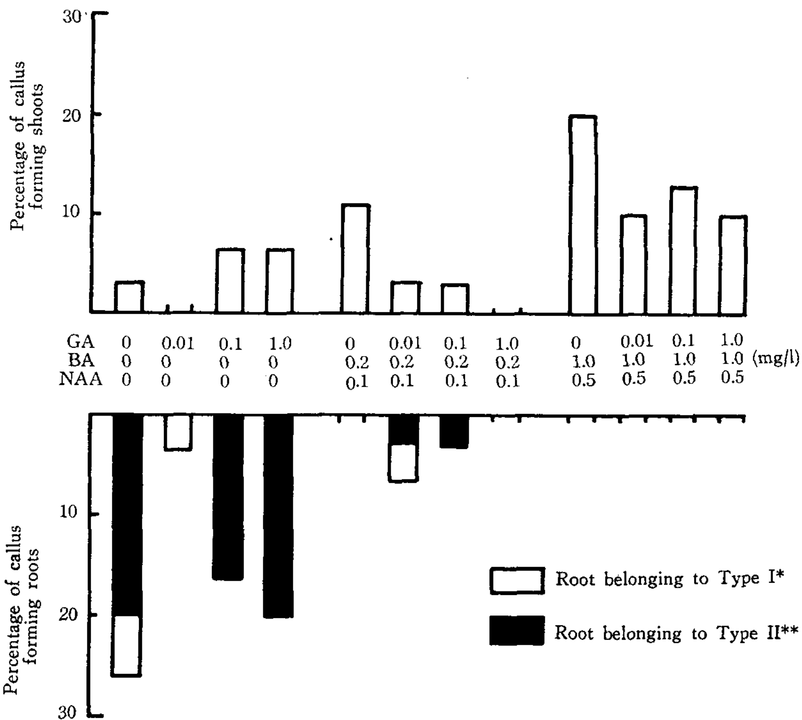

Fig. 10. Effect of GA, BA and NAA on shoot formation from transferred callus. (incubated 14 weeks at 25–27°C).

* Roots belonging to Type I do not function as normal roots.

** Roots belonging to Type II are normal white roots.

Percentages of callus forming roots and callus forming shoots are shown in Fig. 10. Percentage of callus forming roots was the highest on the medium without growth regulators. The elongation of roots was good in the same medium, too. Percentage of callus forming roots was about 20% on the medium containing 0.1 mg/l or 1.0 mg/l GA_3 alone, but the roots were very slender and short.

From this result, it was considered that GA_3 inhibited the formation and elongation of roots. Shoot formation was most enhanced on the medium containing 1.0 mg/l BA and 0.5 mg/l NAA, but was inhibited by addition of GA_3 to the same medium. Inhibition of shoot formation by addition of GA_3 was also observed on the medium containing 0.2 mg/l BA and 0.1 mg/l NAA with GA_3.

Discussion

In general, for the purpose of differentiation of organs such as shoot and root, and the regeneration of plantlet, callus induced from anther is usually transferred onto the medium different from that for the induction and proliferation of callus in relation to the kind or concentration of growth regulators[2,6,7,8,10]. In anther culture of asparagus too, the above method was used for organ formation.

In anther culture of asparagus, DORÉ suggested that callus formed from anthers should be transferred to the medium with the concentrations of growth regulators (auxin and cytokinin) lower than those used for callus formation[1]. PELLETIÈR *et al.* also reported that it would be possible to form shoots from anther-derived calluses by transferring their calluses to the same medium as that used for callus formation except that auxin was lacking[9].

However, our experimental results showed that it was not necessarily suitable for shoot formation that calluses were transferred onto the medium in which cytokinin and auxin were decreased or auxin was excluded as compared with that used for callus formation. Since the highest percentage of callus forming shoots was observed on the medium containing 0.5 mg/l NAA with 1.0 mg/l BA, it was considered that the medium containing cytokinin and auxin was better than that containing cytokinin only in shoot formation. From our experimental results, it was found that the optimum range of concentrations of BA and NAA for shoot formation from anther-derived callus of asparagus was rather wider than that DORÉ and PELLETIÉR *et al.* used.

In experiment II, in which IBA was substituted for NAA, IBA was

inferior to NAA in callus growth and shoot formation, but was good in root formation. The highest percentage of callus forming roots was observed on the medium containing 0.1 mg/l IBA, and 0.5 mg/l IBA with 0.1 mg/l BA followed. Concerning root formation from callus, HARADA *et al.* reported that root formation from callus formed from hypocotyl of asparagus seedling was frequently observed on the medium containing 0.1 to 1.0 mg/l NAA with 0.01 mg/l BA[3]. Results like these are also obtained in other plants, and it is generally known that root formation occurs at a high ratio of auxin relative to cytokinin, while shoot formation occurs at a high ratio of cytokinin relative to auxin.

It was considered that optimum concentrations of BA and NAA for root formation was lower than that for shoot formation. The ratio of auxin to cytokinin for root formation appered to be the very opposite of that for shoot formation. Root elongation seemed to be inhibited by addition of auxin and cytokinin, and it seemed that cytokinin had a tendency to thicken roots.

The effect of gibberellin was also observed as well as that of cytokinin and auxin on callus growth and shoot formation. GA_3 showed a tendency to inhibit callus growth and shoot formation. Callus grown on the medium containing 1.0 mg/l GA_3 was deficient in water, gradually lacking in chlorophyll and had a tendency to turn brown. These calluses were rather different from those grown on the medium without GA_3, which were sodden and turned brown after a long term of growth.

THORPE *et al.*[11] reported that inhibition of shoot formation by gibberellin was caused by disturbance of starch accumulation that seemed to be essential for shoot formation. In our experiment, starch accumulation was partially observed in the callus grown on the medium for shoot formation, but a clear relationship between shoot formation and starch accumulation could not be detected. Further reserch is needed to clarify the relationship between them.

Morphological observation on the shoot and root formation from callus showed the following results.

(1) Shoot formation was hardly observed before a rice grain-sized callus divided for transferring grew into an Azuki bean to a soybean-sized callus on the medium for shoot formation. Shoot formation was observed in the outer layers of high cell dividing potential and compact callus.

(2) Root formation was observed in calluses that hardly grew as well as those that grew actively after transferring to the medium for organ formation. Root formed from callus that hardly grew continued to elongate

even after being isolated from callus.

In anther culture of asparagus, it was difficult to form both shoot and root on a callus and regenerate an intact plantlet. Hereafter, for the purpose of regenerating plantlets from anther-derived callus, shoot formation from callus is needed to be attempted first, and then culture of shoot tips or stem section with a node is needed to be attempted.

Summary

Effect of growth regulators (cytokinin, auxin, gibberellin) on organ formation from anther-derived callus were investigated.

The anther-derived calluses, which were the same size as a soybean were divided into the size of a rice grain before transferring to the medium for organ formation.

The following experiments were carried out and the results obtained are summarized as follows:

1) Effect of BA and NAA on shoot formation.

Shoot formation was good on the medium containing 0.5 and 1.0 mg/l BA combined with 0.1 and 0.5 mg/l NAA respectively. The highest percentage of callus forming shoots was observed on the medium containing 1.0 mg/l BA and 0.5 mg/l NAA.

2) Effect of BA and IBA on organ formation.

Callus growth was good on the medium containing 0.5 mg/l BA and 0.1 to 0.5 mg/l IBA. Callus growth was better with NAA than IBA. The highest percentage of callus forming shoots was obtained on the medium containing 0.5 mg/l with 0.1 mg/l IBA and that of callus forming roots was obtained on the medium containing 0.1 mg/l IBA alone. Root elongation was inhibited by addition of BA and IBA.

3) Effect of BA, NAA and gibberellin on organ formation.

Gibberellin inhibited proliferation of callus transferred to the medium for organ formation.

Addition of gibberellin to the medium containing 0.2 mg/l BA and 0.1 mg/l NAA, or 1.0 mg/l BA and 0.5 mg/l NAA, which were favorable for shoot formation, inhibited shoot formation from callus.

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