STUDIES ON THE MORPHOGENESIS OF ASPARAGUS

V. Organ formation in the *in vitro* culture of segments with a node excised from the shoots of seedlings

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Introduction

Seed propagation has been carried out in usual commercial cultivation of asparagus, whereas the plants obtained are individually different in the characteristic such as productivity. An efficient asexual reproduction method is necessary to obtain numerous plants with the same excellent characteristics. In recent years, to establish a reliable asexual propagation method, studies on tissue culture of asparagus have been carried out using various kinds of tissues, artificial culture medium and other culture conditions[1,2,4,7,9,10].

Generally in a tissue culture, the formation of callus and organs such as shoots and roots is related closely to the developmental stage of the tissue cultured or the age of plants concerned.

In the present experiment, the segment with a node derived from a shoot of an asparagus seedling were aseptically cultured to clarify the conditions suitable for organ and callus formation, and to obtain some fundamental knowledges in relation to the vegetative propagation and the morphogenesis in asparagus.

Materials and Methods

Plant materials. Seeds of asparagus (*Asparagus officinalis* L. cv. 'Mary Washington 500') were immersed in 70% ethanol momentarily, and twice in sodium hypochloride solution (available chlorine 1%, a few drops of Tween 20 were added) for 30 min., and were washed thoroughly with sterile distilled water. Then, the seeds were aseptically incubated at 25°C in the dark using solid agar medium containing 20 g/l sucrose and 6 g/l agar. After

2 weeks of incubation, the first shoot without a chloroplast and a cladophyll elongated, and the 1 cm-long shoot segments, which had a node in the center, were excised from the portion of the first node of the first shoot 5 cm in length and 1 mm in diameter.

Culture media and culture methods. Culture media were prepared by additions of 0, 0.1, 1 and 10 mg/l N^6-benzyladenine (BA) combined with 0, 0.001, 0.01, 0.1, 1 and 10 mg/l α-naphtaleneacetic acid (NAA) to MURASHIGE and SKOOG's medium. To these media, 20 g/l sucrose and 7 g/l agar were added. The initial pH was adjusted to 5.5 with HCl or NaOH. After heating to melt the agar, 25 ml of medium was pipeted into 100-ml Erlenmeyer flasks, and each flask was sealed with alminium foil. Then, all the media were sterilized by autoclaving at 120°C for 15 minutes. Three of segments mentioned above were planted horizontally on the medium in each flask and each of 24 treatments depending on the differences in concentrations of BA and NAA was consisted of 20 flasks. A harf of the flasks with each treatments were maintained at 25°C under darkness and the others at the same temparature were under light (16-hr light period under artificial illumination with white fluorescent light, 4,000 lx).

Scoring of the results. The average number of shoots in a treatment was determined by the average value of number of all shoots in the treatment, and the average length of shoots in a treatment was shown by the average value of the length of all shoots belonging to the treatment. The thickness of a shoot and the size of a callus clump were represented by indices. Also, percentage of callus-inducing segments was calculated from the ratio of number of those to that of all segments observed. The percentage of root-inducing callus clumps was calculated from the ratio of those against all callus clumps observed.

Results

Formation of shoots. Shoots began to elongate directly from the node of the shoot segments after two to three days of culture and those elongating most rapidly became approximately 3 cm long after 5 days both under darkness and light. The shoots developing under light became green and, after a certain period of culture, had cladophylls on the nodes, while those under darkness remained white and had no cladophyll. Redifferentiation of shoots from callus, which had been observed in the culture of segments excised from an internode of shoots of the seedlings as in the previous reports,[6] were not observed in the present experiment.

Fig. 1 shows percentage of segments with elongating shoots after 11

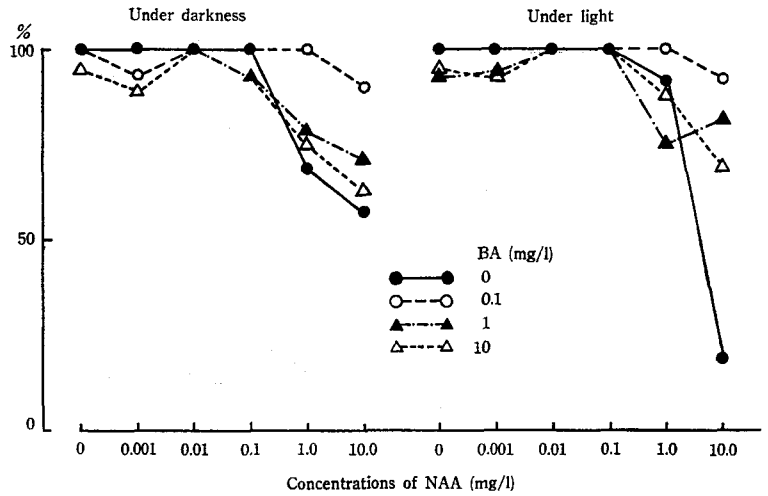

Fig. 1. Percentage of callus-forming segments after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings.

weeks of culture. It was 85 to 100% in both the absence and the presence (below 0.1 mg/l) of NAA regardless of BA, but became lower with the increasing of concentrations of NAA ranging from 1.0 to 10 mg/l. It was noteworthy that shoots had elongated in the absence of both NAA and BA, and the percentage was 100%. With exception of the case where it was lower in the cultures under light than in the dark with no BA and 10 mg/l of NAA added to the culture media, the same tendency was recognized in cultures both under light and darkness.

Fig. 2 shows the number of shoots after 11 weeks of culture. Generally, the number of the shoots were much larger under light than in the dark. In culture under light, it was 1 to 6 and varied with concentrations of NAA and BA. Namely, it was the largest at 0.1 mg/l of BA, and was the smallest at 10 mg/l of BA regardless of the presence or absence of NAA. In both the absence and the presence of BA, the number of shoots was the smallest at 10 mg/l of NAA. When NAA alone was added, it was the largest at 0.1 mg/l of NAA. The largest number of shoots in a segment approximately six, was seen at 0.1 mg/l of both NAA and BA in the culture under light. On the other hand, under darkness, 1 to 3 shoots elongated in each treatment, and only a small difference in number of the shoots was observed depending upon the presence or absence of NAA and BA.

Length of the shoots after 2 weeks of culture is shown in Fig. 3. It

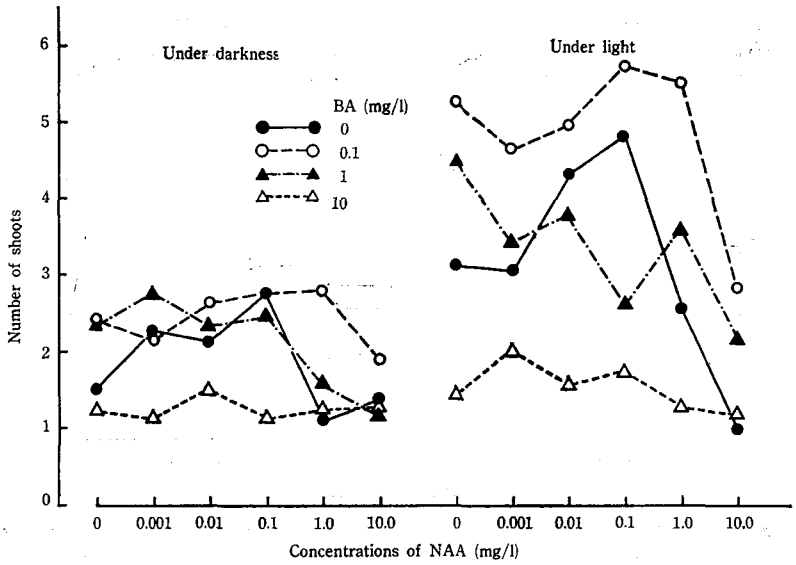

Fig. 2. Number of shoots elongating on the node of segments after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings.

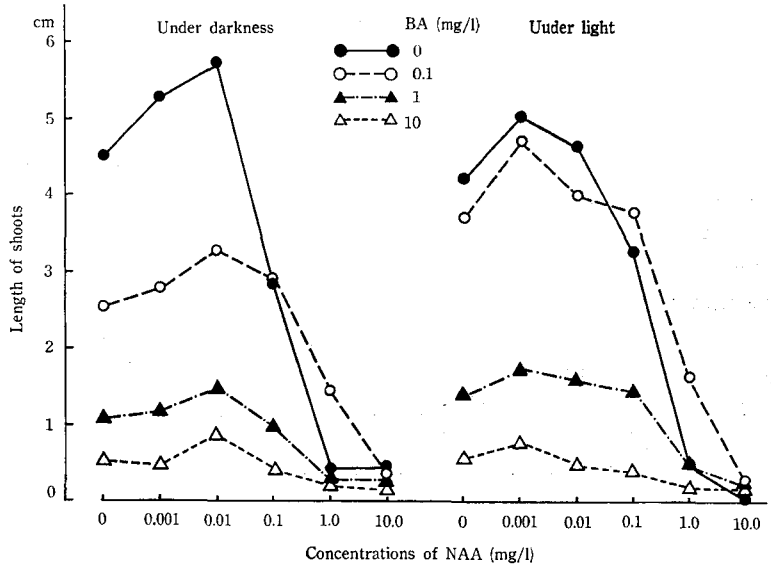

Fig. 3. Length of shoots after 2 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings.

was larger at 0.001 mg/l of NAA under light and at 0.01 mg/l of NAA in the dark in both the absence and the presence of BA. Also, it was the smallest at high concentrations of NAA without BA both under light and in the dark. When none or low concentrations of NAA were added, a considerable difference was recognized between in the absence and the presence of BA. For instance, the shoots were the longest in the absence of BA, and when BA was added, they gradually became shorter with the increasing of concentrations of BA.

The thickness of the shoots is shown in Fig. 4. When no BA was added to media, it was as large as that of normal regardless of the presence and the absence of NAA both under light and in the dark. When BA was added, however, the shoots became thicker both under light and in the dark. And, it was much thicker at high concentrations of BA. Also, additions of both NAA and BA had made shoots thicker than those in the presence of BA alone, but, such a tendency was not seen at high concentrations of BA. In addition, when BA was added at a low concentration, shoots became thicker with the increasing of NAA concentrations. When BA was added at high concentrations, however, the thickness of shoots was small at high concentrations of NAA.

Formation of roots. Two types of roots were observed: one was a long, branched and opaque root akin to a storage root, which differentiate directly

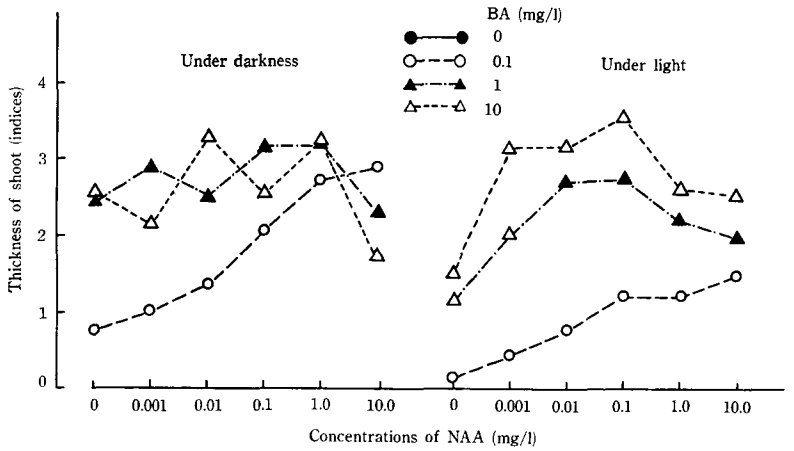

Fig. 4. The thickness of shoot after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings. Indices; 0: approximately 1 mm in diameter as thick as a normal shoot of the seedling, 1: 1.5 folds of normal thickness, 2: 2 folds, 3: 2.5 folds, 4: 3 folds.

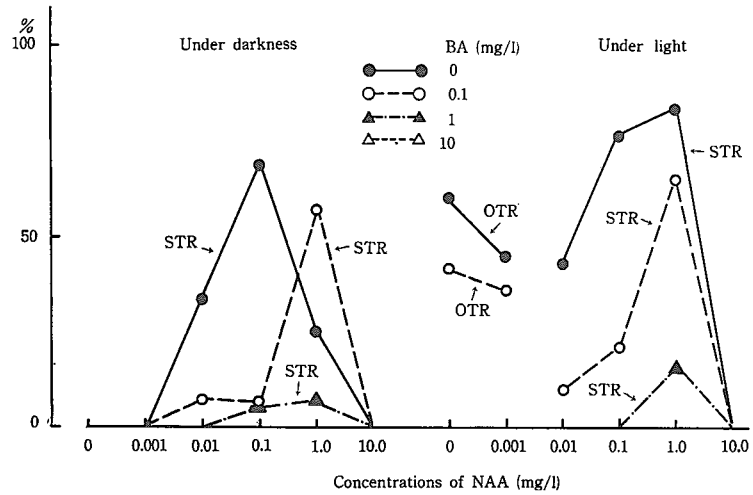

Fig. 5. Percentage of root-forming segments after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings. OTR: opaque-type root, STR: semi-transparant-type root.

from a node of the cultured segments, and the other was a semitransparent and non-branched root which redifferentiated from a callus. The so-called opaque-type roots were formed only in the culture under light. However, the semitransparent-type roots were redifferentiated both under light and in the dark. The percentage of root-forming segments is shown in Fig. 5.

Regarding the opaque-type roots, it was formed only in the absence of growth regulators or in the presence of low concentrations of both NAA and BA, namely at 0.001 mg/l of NAA alone and at 0.1 mg/l of BA alone (Fig. 6), but was not observed at over 0.01 mg/l of NAA or at over 1.0 mg/l of BA. It was noteworthy that the opaque-type roots were observed even in the absence of both NAA and BA.

On the contrary, in the case of the semitransparent-type roots, two peaks of

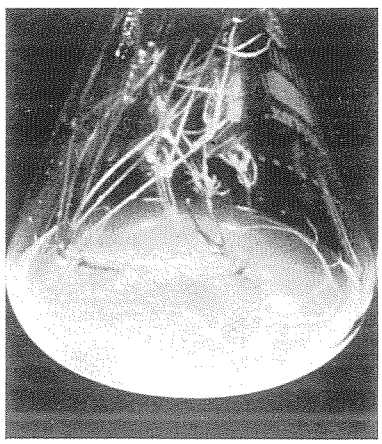

Fig. 6. The shoots and roots formed on a node of shoot segments of asparagus seedlings in the media without both NAA and BA under light. The shoot is as thick as those of a seedling, and the roots is long, branched and opaque.

percentage of root-forming segments were recognized in the dark : one was at 1 mg/l of NAA in the presence of BA, and the other was at 0.1 mg/l of NAA alone. On the other hand, under light, one peak of the percentage appeared at 1.0 mg/l of NAA in the absence or presence of BA. The percentage was the highest of 83% at 1.0 mg/l of NAA alone, and was relatively higher in the presence of NAA alone. The roots could not been observed at 10.0 mg/l of NAA combined with or without BA, and at 10.0 mg/l of BA combined with or without NAA. This tendency was recognized both under light and in the dark.

Formation of callus. In the media containing 0.1 to 10.0 mg/l of BA and 0.1 to 1.0 mg/l of NAA, callus formation was induced after 1 to 2 weeks of culture.

The percentage of callus-forming segments is shown in Fig. 7. Regarding the cultures under darkness, it was 90 to 100% at 0.1 to 10.0 mg/l of NAA in the absence or presence of BA. When none or low concentrations of NAA were added, callus formation varied with concentrations of BA. Even in the absence of NAA, the calluses were formed in the medium containing BA.

For example, the percentage of callus-forming segments was over 90% at 1.0 mg/l of BA, when no NAA was added. Of course, it was not observed on the media without both NAA and BA. Generally speaking, the callus formation was slightly lower in the cultures under light than in those

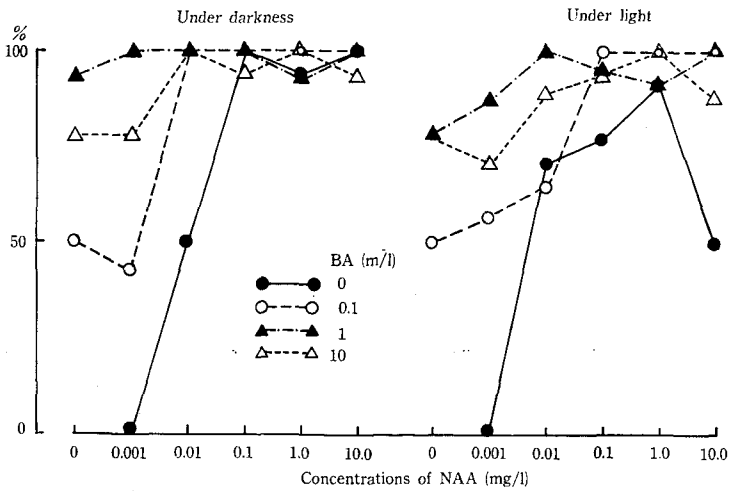

Fig. 7. Percentage of callus-forming segments after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings.

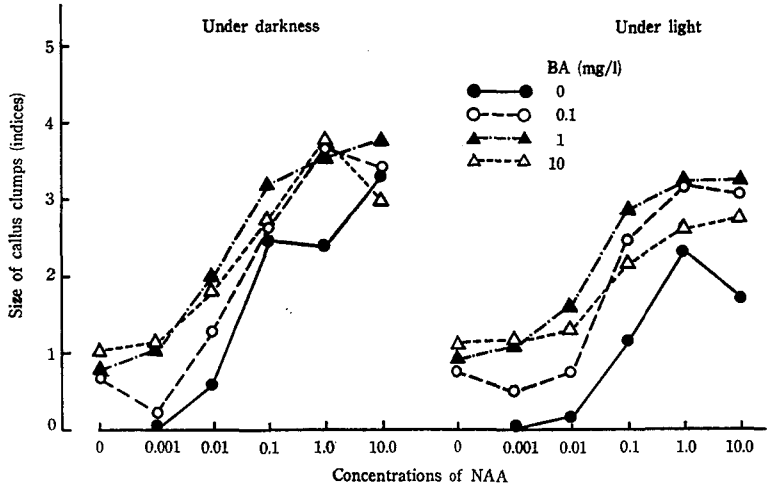

Fig. 8. Growth of callus after 11 weeks of culture in the *in vitro* culture of shoot segments with a node excised from asparagus seedlings. Indices; 1: a size as large as a sphere with a diameter 3 mm long, 2: 5 mm, 3: 7 mm, 4: 9 mm, respectively.

under darkness.

Callus growth was illustrated in Fig. 8. After 11 weeks of culture, the size of the callus clumps was large at high concentrations of NAA in the presence of BA. Also, it became considerably large in the presence of NAA alone, but was small in the presence of BA alone. Green or nongreen calluses were induced under light, while nongreen callus alone was formed under darkness.

Regeneration of plantlets. The cultures with the shoots and the opaquetype roots such as seen in storage roots grew into intact mature plants which could be transplanted to a medium in a pot. It was recognized that a crown was formed at the node to produce shoots and roots.

Discussion

As described in the introduction, the authors aseptically cultured segments with a node excised from the first shoot of asparagus seedlings to clarify the conditions of organ formation in the asparagus plant.

In the present experiment, even in the case of adding no growth regulators, shoots emerged from the node and the percentage of segments with the shoots was very high (100%). The addition of growth regulators such as NAA or BA showed no stimulatory effect in the increasing of the incidence

of shoot initiation, while it showed an inhibitory effect at high concentrations. In addition, the inhibitory effect of high concentrations of NAA decreased with the addition of BA.

The number of shoots elongating from the node of the segments generally was larger and the responses of the shoots to growth regulators, especially to BA, were more sensitive under light than in the dark. In some experiments,[7,9,10] as well as in this experiments, the shoot initiation was better under the addition of none or comparatively low concentrations of auxins and cytokinins and the irradiation of light, slightly varying according to the kinds of the tissues cultured.

On the other hand, regarding shoot elongation, NAA added separately at a limited low concentration enhanced it somewhat, but the separately added BA and both the growth regulators in combination have shown a inhibitory effect rather than a stimulatory effect.

Thickening of a shoot was influenced mainly by BA rather than NAA. The authors have recognized that the phenomenon was given by an addition of high concentrations of cytokinins in the *in vitro* culture of various tissues excised from the asparagus plant.[5,6] From the results of the present experiment, it is assumed that a correlation exists between the elongating and thickening of the shoots.

On the root formation, the following facts were recognized, i. e., the differentiation of so-called opaque-type roots appeared in a higher frequency without growth regulators especially under light, and decreased with an addition of NAA or BA to disappear with an increase of NAA and BA concentrations.

Related to semitransparent roots redifferentiated from callus, the following was recognized and consideration were made ; As we reported in the previous experiment[6] using internode segments of shoots of a seedling, separately added NAA showed a peak at 0.1 mg/l both under darkness and light in the curves of percentage of root-initiating segments, but NAA combined with 0.1 mg/l of BA gave a peak at 1.0 mg/l. In other words, the peak shifted toward 10 fold higher concentrations of NAA.

On the other hand, culturing segments as in this experiment, the tendency described above appeared only in the culture in darkness. Especially under light, however, the separately added NAA gives the highest percentage and shows a peak at 1.0 mg/l. The fact such as seen in this experiment is considerably different from that in the culturing of the segments from internode of seedlings. KODA and OKAZAWA[3] recognized through shoot tip culture that cytokinins are produced in a shoot tip of asparagus seedlings

and move basipetally. It might be that certain metabolic products were related to the fact mentioned above, because the cultures in this experiment have the shoots which have elongated prior to root redifferentiation.

In the *in vitro* culture of the various tissues of asparagus, which were a segment excised from the shoots of seedlings and a small pith tissue from the spears of mature plants, callus could be induced and grew to a certain extent in a medium containing BA alone, of course a callus could be sufficiently formed with NAA alone and with optimal concentrations of NAA and BA in combination. This suggests that BA plays an important role and a kind of auxin may be produced endogenously.

It is interesting to observe the organ formation in this experiment from a viewpoint of organization or morphogenesis. An asparagus plant is perennial, and usually grows to become an adult plant after 3 years, growing through juvenile stages and continuing to live for several decades through shooting and rooting from a rhizome. When an intact seedling growing in a field exsists, several cladophylls are usually formed on the node of the first shoot. However, in the present experiment, shoots, which correspond to the lateral shoot in an intact plant, develops and then a crown is formed on the same node.

YANG and CLORE[7,9,10] reported that segments with a node, which were derived from the shoots of the stock plants obtained through tissue culture, formed shoots and roots with culturing in an artificial medium containing NAA and kinetin. Also, it was described by YANG and CLORE[8] that nodes, which had a lateral bud in the basal portion of shoots (0.9 to 1.3 m in height and 1.5 to 2.2 mm in diameter) of an asparagus plant growing in pots, were treated with IAA or kintein in lanolin paste, and a lateral shoot and an aerial crown were formed on the node. These facts point to a ramification type and the organization of the asparagus plant which is of considerable interest.

Summary

Segments (1 cm in length) with a node derived from the first shoot of asparagus seedlings were aseptically cultured to clarify the conditions suitable for organ and callus formation, and to obtain some fundamental knowledge in relation to the vegetative propagation and morphogenesis of the asparagus plant.

The culture medium contained MURASHIGE and SKOOG's inorganic and organic substances, 2% sucrose, growth regulators and 0.7% agar. The concentrations of growth regulators were 0, 0.001, 0.01, 0.1, 1.0 and 10.0

mg/l of NAA and 0, 0.01, 0.1, 1.0 and 10.0 mg/l of BA, both of which were added separately or in combination. The culture conditions were defined as follows, i.e., pH of the media were adjusted to 5.5, and the cultures were maintained at 25°C in darkness and under light (16-hour-a day illumination of 4,000 lx with a white fluorescent lamp). The results obtained are summarized as follows:

Shoots began to elongate directly from the node of the shoot segments. The percentage of segments with elongating shoots was 90 to 100% both in the absence or presence (below 0.1 mg/l) of NAA, regardless of BA, under light as well as in darkness. The number of the shoots was the largest (six) at 0.1 mg/l of both NAA and BA under light, and it was much larger under light than in darkness. The length of shoots was larger at 0.001 mg/l of NAA under light and at 0.01 mg/l of NAA in darkness in both the absence and presence of BA. The shoots were thicker both under light and in darkness with an addition of BA. The fact is characteristic in the *in vitro* culture of some tissues of an asparagus plant.

Two type of roots, which are a long, branched and opaque root differentiating directly from the node and an semitransparent and non-branched roots redifferentiating from a callus, were formed, i.e., the former were observed only in the absence of growth regulators or in the presence of low concentrations of both NAA and BA only under light, and the latter in the presence of 0.01 to 1.0 mg/l NAA and 0 to 1.0 mg/l BA both under light and in darkness.

The percentage of callus-forming segments was 90 to 100% at 0.1 to 10 mg/l of NAA in the presence of BA. Callus formation was not observed on the media without growth regulators, but was recognized in the medium containing BA alone and devoid of NAA.

The opaque-type roots such as storage roots were considered to have a normal function akin to that of a seedling, and the cultures both with the shoots and the opaque-type roots could grow to become an intact mature plant.

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