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STUDIES ON THE MORPHOGENESIS OF ASPARAGUS

X. Influence of light and growth regulators on callus and organ formation in the *in vitro* culture of segments from seedling shoots

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Introduction

Various kinds of *in vitro* cultures have been carried out to clarify the mechanism of morphogenesis in asparagus and to obtain a fundamental knowledge for establishing a vegetative propagation method.^{1-3,6,7,9-14} As reported in the previous paper,⁴ the initiation and growth of callus and the formation of organs were observed in culturing segments which had a node and were excised from the first shoot of asparagus seedlings (*Asparagus officinalis*, L. cv. Mary Washington 500). In this case, the segments of 1-cm length and with a node formed shoots and roots, and initiated callus with vigorous growth on a cut surface. In contrast, the 1-cm length segments, which had no node, first formed callus on the cut surface, and then differentiated shoots and roots. In these cases, light conditions play certain important roles in callus and organ formation as described before.⁴

On the other hand, especially in the *in vitro* culture of tissues derived from seedlings, it is speculated that light conditions during seedling development provide a certain effect on the callus and organ formation. In this report, a few results obtained will be described with regard to these points.

Materials and Methods

Asparagus seeds (*Asparagus officinalis* L. cv. Mary Washington 500) were germinated on the medium solidified with agar under the aseptic conditions and light conditions mentioned below.

Surface sterilization of the seeds are made with sodium hypochloride solution (active chlorine 1%, with a few drops of Tween 20). When seedlings

attained 5 cm in height, two different segments — one with a node in middle portion, the other without it — were excised from the first shoot and cultured *in vitro*. These segments were horizontally planted with 3 pieces on the medium 25 ml in volume in a 100-ml Erlenmeyer flask, and used with 30 pieces to a batch.

The media used uniformly contained MURASHIGE and SKOOG's prescription, 20 g/l of sucrose, 0.7 g/l of agar, and the media with growth regulators contained both 1.0 mg/l of NAA and 0.1 mg/l of BA. The pH of media was adjusted to 5.5 before autoclaving. The cultures were maintained at 25°C.

The light conditions were adjusted to 3,000 or 6,000 lx amid germination and to 3,000 lx in the *in vitro* culture, respectively at flask level, and the length of the illumination was 16 hours per day — darkness conditions were also prepared.

The seedlings developed under darkness condition had a white shoot without cladophyll, while those under 3,000 lx had a slightly green-colored shoot without cladophyll. In contrast, those under 6,000 lx had a deep green shoot with cladophylls elongated to certain extent. The size of callus was indexed as follows: Index '1' stands for the the volume of sphere 2 mm in diameter, Index '2' for that 4 mm in diameter, and '3' for 6 mm, '4' for 8 mm, '5' for 10 mm, respectively.

Results

Callus initiation began to be observed after 7 to 10 days of culture only in the cultures on the media with the growth regulators (1.0 mg/l of NAA and 0.1 mg/l of BA). The percentage of the segments forming callus after 16 weeks of culture is shown in Fig. 1. In culturing the segments with a node, percentage of the segments forming callus were 95 to 100%, and no difference was recognized among batches, regardless of light condition. In contrast, in the segments without a node, they were 90 to 100% in the segments excised from the seedlings developed under light, while were 75 to 80% in the segments from the seedlings under darkness. In general, the callus initiation especially depended on the addition of the growth regulators, and seemed to be independent on light condition. No difference was recognized in the percentage of segments with a callus according to light conditions of 3,000 lx or darkness during seedling development.

Callus growth after 16 weeks of culture is shown in Fig. 2. In the case of the segments with a node, the size of the calli was approximately Index 3 in the segments from the seedling under light, while they were

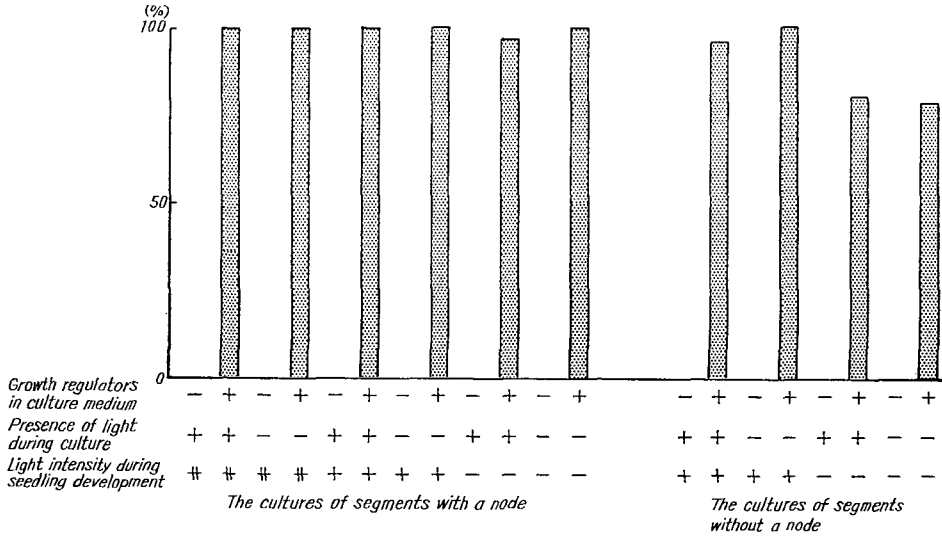


Fig. 1. Percentage of the explants initiating callus in the *in vitro* culture of seedling shoot segments of asparagus related to both the light conditions during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are represented as follows: - stands for darkness, + for 3,000 lx and # for 6,000 lx.

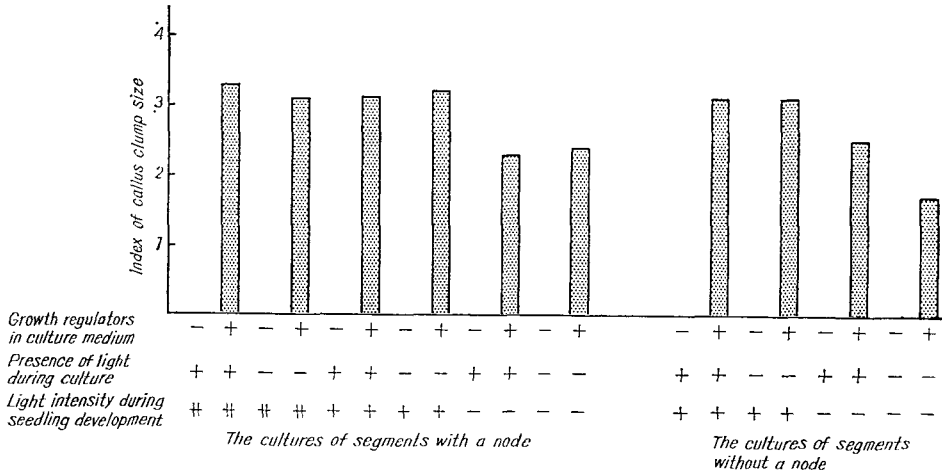


Fig. 2. Callus growth in the *in vitro* culture of seedling shoot segments of asparagus related to both the light conditions during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are also represented as follows: - stands for darkness, + for 3,000 lx and # for 6,000 lx. Index 1 stands for the volume of a sphere 2 mm in diameter, Index 2 for that 4 mm in diameter, and 3 for 6 mm, 4 for 8 mm, 5 for 10 mm, respectively.

approximately 2.5 of the index in the segments from the seedling under darkness. Likewise, in the case of the segments without a node, the sizes of the calli were about Index 3 in the segments from the seedlings under light, and approximately within a range of 1.5 to 2.5 of the Index under darkness. In other words, a similar tendency concerning callus growth was recognized in the cultures of both 2 different segments produced under different conditions. Related to the influence of light conditions (3,000 lx of illumination or darkness) in the *in vitro* culture, no difference was recognized in the segments from the seedlings under light, while a slight difference was observed in the segments from the seedlings under darkness.

Concerning shoot formation, two types of it were observed: one was the direct emergence from the node, the other was the redifferentiation from the callus. The shoots in the former case could be considered to correspond to a lateral branch in morphogenesis of an intact plant of asparagus. Shoots emerging on the node — a branch in other words — began to elongate after 7 days of culture, while shoots redifferentiated appeared after 8 weeks of culture. The shoots emerging directly on the node elongated to become

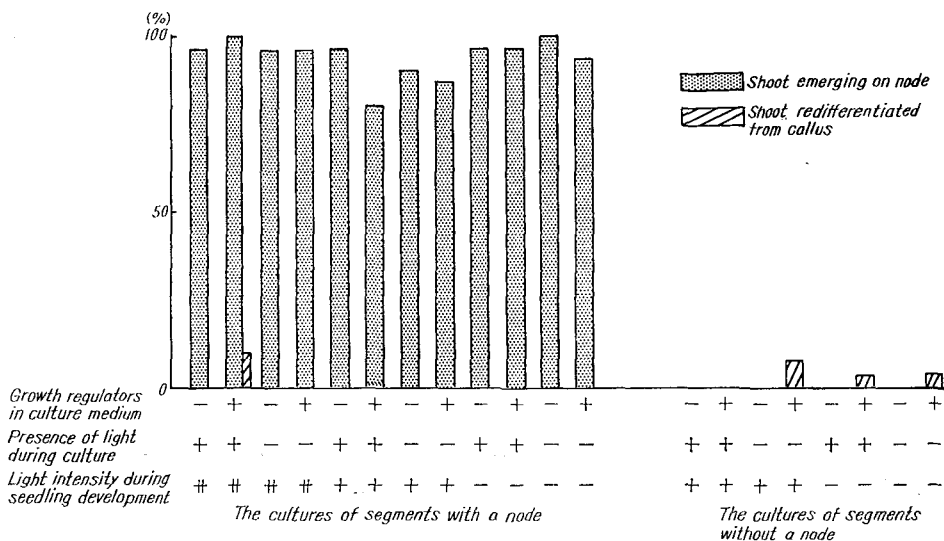


Fig. 3. Percentage of the explants forming shoots in the *in vitro* culture of seedling shoot segments of asparagus related to both the light conditions during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are represented as follows: - stands for darkness, + for 3,000 lx and # for 6,000 lx.

over 10 cm in length, while those redifferentiating from the calli also reached 7 cm, respectively in maximum.

The percentage of segments forming shoots after 16 weeks of culture is shown in Fig. 3. The shoots emerging on the node were observed only in the segments with a node, and the percentages of those segments were above 80%, regardless of both the light conditions during seedling development and *in vitro* culture and the presence or absence of growth regulators in the media used.

With regard to the redifferentiated shoots, in the case of the segments with a node, they were observed only in the segments with the conditions of 6,000 lx- illumination throughout seedling development, 3,000 lx-illumination throughout culturing and the growth regulator addition to culture media, whereas the percentage of the segments were fairly low (approximately 10%).

On the other hand, in the segments without a node, the redifferentiated shoots were obtained only with the growth regulator-containing media, regardless of light or darkness conditions in culturing, and the percentages were low (below 10%) as well as in the case of the segments with a node.

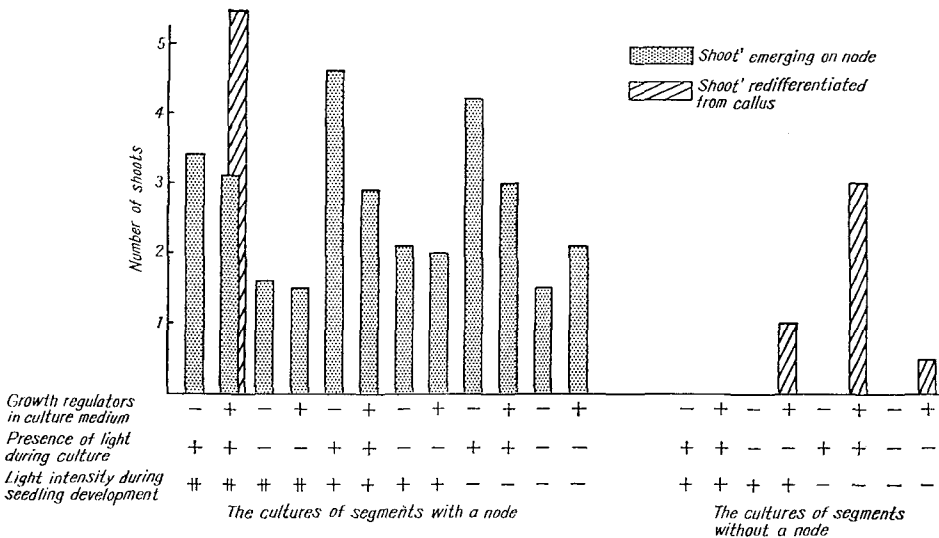


Fig. 4. Number of shoots emerging in the *in vitro* culture of seedling shoot segments of asparagus related to both the light condition during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are also represented as follows: - stands for darkness, + for 3,000 lx and # for 6,000 lx.

The number of the shoots directly emerging on the node after 16 weeks of culture is shown in Fig. 4. First, concerning the shoots emerging from the node in culturing the segments with a node, the number of the shoots were larger in the segments cultured under light than in those under darkness in both the media with or without growth regulators. No clear tendency was recognized in connection with the presence or absence of light during seedling development. On the effect of growth regulators, the absence of them was appropriate to the increasing of the number, with a few exceptions. On the other hand, in the case of the redifferentiated shoots, the numbers were large in the cultures under illumination.

Related to root formation, two types of it were observed: one was a storage root-like white root emerging on the node, and the other was a semitransparent root redifferentiated from the callus. The storage root-like white roots, also in this experiment, emerged in the segments with a node only in the batch under the conditions of no growth regulator, 3,000 lx throughout culture period and 6,000 lx during seedling development (about 10%). This result confirmed the fact that in the experiment reported

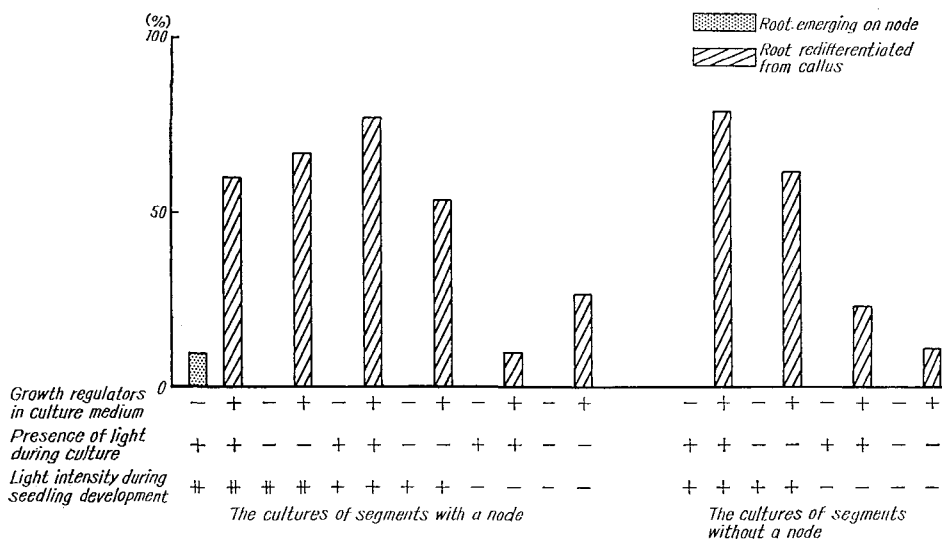


Fig. 5. Percentage of the explants forming roots in the *in vitro* culture of seedling shoot segments of asparagus related to the both light conditions during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are also represented as follows: - stands for darkness, + for 3,000 lx, + for 6,000 lx.

previously,⁴ a storage root-like white root emerged only on the node of nodal segments cultured under no growth regulator and the illumination. Redifferentiated roots were obtained in the cultures of both the nodal segments and the internodal segments. As matter of course, they were obtained on the medium, in which growth regulators were contained and callus were formed. The roots emerging directly on the node elongated to over 3 cm, and also those redifferentiating from callus reached 5 to 12 mm.

The percentage of rooting explants after 16 weeks of culture was shown in Fig. 5. Percentage of cultures with redifferentiated roots was comparatively high in the batch in which seedlings were prepared under light, and was low in those with seedlings under darkness. Referring to the light condition during the seedling development, the percentages were higher (above 50%) in the batches under illumination than in those under darkness in the case of the internodal segments, while they showed no clear tendency in the case of nodal segments.

On the whole, the highest percentage of the cultures redifferentiating roots was obtained under optimal light intensity — 3,000 lx in this experiment — during both the seedling development and the *in vitro* culture.

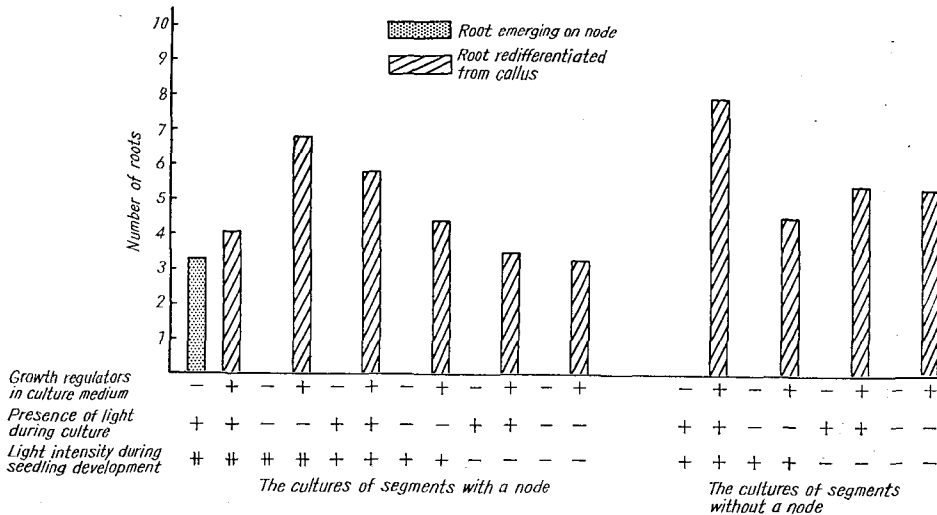


Fig. 6. Number of roots formed in the *in vitro* culture of seedling shoot segments of asparagus related to both the light condition during seedling development and culture period and to the growth regulators in culture medium. Presence of growth regulators is represented as follows: symbol + stands for the addition of both 1 mg/l of NAA and 0.1 mg/l of BA; - for no growth regulator. Light conditions are represented as follows: - stands for darkness, + for 3,000 lx and # for 6,000 lx.

Fig. 6 shows the number of roots after 16 weeks of culture. The storage root-like white roots were over 3 in number in the nodal segments in the batch under the conditions of no growth regulator. Associated with the redifferentiated roots, the numbers were 3 to 8 in both nodal and internodal segments. In the nodal segments, the number of those roots were largest under 6,000 lx-illumination during seedling development, while in the internodal segments the numbers were largest under 3,000 lx in both the seedling development and the *in vitro* culture. However, no clear-cut tendency was shown in connection with the presence or intensity of light.

Discussion

Some papers have been published associated with light requirement in the *in vitro* culture of certain kinds of asparagus tissues, *e. g.*, apices of adult plant spears, small undeveloped lateral shoots, segments from stock plant shoots cultured *in vitro*. HASEGAWA *et al.*⁵⁾ described several facts in relation to light requirement in the *in vitro* culture of asparagus shoot apex: the optimum light intensity for a shoot as well as a root initiation was approx. 1,000 lx; root initiation was equally satisfactory under light intensities ranging from 0 to 3,000 lx, and 10,000 lx of light intensity was excessive for the initiation of both shoots and roots. Also, 1,000 to 2,000 lx of light intensities were used in other reports,^{2,3,8,15,16)} which described the *in vitro* culture of various segments such as shoot apices and a shoot segments from stock plants cultured *in vitro*.

As reported previously,⁴⁾ the authors have carried out *in vitro* culture of segments excised from the shoots of asparagus seedlings for the purpose of applying the results obtained from it to the clarification of shooting and rooting and to the regeneration of plantlets. In contrast to the experimental design described in HASEGAWA's report⁵⁾, in the present experiment, the *in vitro* culture was made under 0 or 3,000 lx of light intensities and the seedlings were developed under 0, 3,000 and 6,000 lx of light. This trial is based on the followings.

The authors paid attention particularly to light conditions during seedling development, regarding how the light intensities influence cladophylls emerging in seedlings and what effect the cladophylls emerging on the node had on the organ formation of the cultured segments.

Generally in asparagus plants growing in a field, they develop into an adult plant approximately 4 years after planting, because of its perenniality. In the course of growth of a seedling in the field, the first shoot develops into 15 to 20 cm length, and bears cladophylls on nodes, because of sufficient

presence of light. On the other hand, under artificial preparation of sufficiently high light intensity in an experiment, the first shoot can grow into a length similar to that in the field and can develop the cladophylls in certain number and length. However, in the dark, the first shoot elongates far more longer and appears as white, and has no cladophyll developing, in contrast to those with high light intensity. While, under low light intensity, the first shoots are more longer than those under high light intensity, and shorter than those in the dark, and have a slight green color and bear cladophylls shorter than that under high light intensity. SATO *et al.*⁹ described that in the *in vitro* culture of apices excised from lateral branches of an adult plant of asparagus, the developed shoots of the cultures had no cladophyll under 500 and 1,000 lx at flask level, but had cladophylls of a short length under 5,000 lx at flask level. Also, in the *in vitro* culture in this experiment, both the shoots emerging directly from a nodal portion of the shoot segments and the shoot redifferentiating from calli developed no cladophyll. On the other hand, speaking morphologically, it is considered on the basis of the author's observation that an alternativity may exist between the emerging of cladophylls or lateral shoots and the differentiation of shoots and roots.

As described in the previous paper⁹, in which segments with a node were cultured, shoots — corresponding to a lateral shoot in organization — emerged from the nodal portion of the segments, and white roots akin to a storage root were formed directly from the node under the conditions of light presence and no growth regulator. Also, in this study, such phenomena were observed in connection with the rooting directly from the node, namely it appeared only in the presence of light, and was not induced in the absence of light. This may offer a key to clarify the mechanism of rooting directly from the node. In addition, a root redifferentiated from callus was obtained both in the dark and under light similarly in the culturing of the segments with and without a node. From this result, it seemed that the presence or absence of light give no definite effect on the root redifferentiation from callus.

This experiment, in other words, intends to examine the influence of culture conditions on shooting and rooting on the basis of a juvenility existing in the seedlings. The juvenility is considered to be of fair importance for the formation of a shoot and a root in various situations, *i.e.* the culture of lateral shoot segments from adult plant spears and the culture of redifferentiated shoot segments from stock plants cultured. It is desirable that further more investigations are carried out in connection with shooting and rooting, using various materials from an asparagus plant and based on a wider viewpoint.

Summary

Shoot segments excised from seedlings of asparagus (*Asparagus officinalis* L. cv. Mary Washington 500) were aseptically cultured on the medium containing MURASHIGE and SKOOG's inorganic and organic substances, 2% sucrose, none or 2 kinds of growth regulators (1.0 mg/l of NAA and 0.1 mg/l of BA) and 0.7 g/l of agar. The cultures were maintained at 25°C under 16 hour-day length illuminating conditions (0, 3,000 and 6,000 lx during seedling development, and 0 and 3,000 lx during *in vitro* culture.

1. Callus formation was observed only in the cultures on the media with the growth regulators. In culturing segments with a node, the ratio of the segments forming callus was 95 to 100%, and also in segments without a node was 90 to 100%, whereas it was 75 to 80% in the segments from the seedling developed under darkness. Considerably vigorous callus proliferation was observed in all batches with the culture conditions prepared in connection with light and growth regulators.

2. Two types of shoot formation were recognized: one was the direct emergence from the node and the other was the redifferentiation from the callus. The percentage of cultures with a node-emerging shoot was above 80% regardless of both the light conditions during seedling development and *in vitro* culture and the use of growth regulators. Redifferentiated shoots emerged as follows: in nodal segments, they were observed only under conditions of 6,000 lx illumination throughout seedling development, 3,000 lx illumination throughout culturing and growth regulator addition to media (approximately 10% at segment level); in internodal segments, they appeared in growth regulator-containing media regardless of light or darkness condition in the culturing,

3. Two types of root formation were observed: one was a storage root-like white root emerging from the node; the other was a semitransparent root redifferentiated from the callus. The storage root-like white roots emerged in the nodal segments only in the batch under the conditions of no growth regulator, 3,000 lx during culturing and 6,000 lx during seedling development (about 10%). Redifferentiated roots was obtained in higher frequency in the batch under illumination during seedling development (above 50%).

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