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STUDIES ON THE ANTHER CULTURE OF HORTICULTURAL CROPS

IV: Regeneration of plantlets from shoots obtained through the anther culture of *Asparagus officinalis* L.

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Introduction

As described in previous papers, the obtaining of plantlets through anther culture of asparagus requires the following three pathways.

- (1) Callus formation from anthers.
- (2) Shoot formation from this callus.
- (3) Regeneration of plantlets from these shoots through the culture of shoot tips or stem segments.

In previous papers, pathways (1) and (2) have been reported.^{8,7,9,9,10}

In the present paper, effects of growth regulators and light intensity on the regeneration of plantlets from shoots obtained through anther culture were investigated.

Materials and Methods

Anthers of Mary Washington 500 at uninuclear stage of pollen were cultured on Murashige and Skoog's medium containing 1.0 mg/l NAA with 1.0 mg/l BA and 20 g/l sucrose to produce calluses. Calluses obtained were divided into the size of a rice grain and transferred onto M. S medium containing 0.5 mg/l NAA with 1.0 mg/l BA for shoot formation.

Shoots obtained by the above procedure were used for regeneration of plantlets through cultures of shoot tips and stem segments with a node.

The following three experiments were carried out to investigate optimal conditions for regenerating plantlets from shoots.

Experiment I: Effects of BA, IAA and NAA on regeneration of plantlets from stem segments with a node.

In this experiment, twelve types of media were prepared by additions of 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 mg/l IAA or NAA combined with 0 and 0.01 mg/l BA. Each of the media contained 20 g/l sucrose, 7 g/l agar, and was adjusted to pH 5.5. Stem segments 0.5 to 1.0 cm long with a node were placed horizontally in 100 ml Erlenmyer flasks containing 25 ml medium. The cultures were incubated under 16 hr illumination (4000 lux) a day.

Experiment II: Effects of BA and NAA on regeneration of plantlets from shoot tips.

Nine types of media were prepared by additions of 0.01, 0.1 and 1.0 mg/l NAA combined with 0, 0.01 and 0.1 mg/l BA. Each of the media contained 20 g/l sucrose and 7 g/l agar, and was adjusted to pH 5.5. Shoot tips 0.5 to 1.0 cm long were placed upright in 25 ml medium. The culture condition was the same as that of experiment I.

Experiment III: Effect of light intensity on regeneration of plantlets from stem segments with a node.

Four different light intensities, 10,000, 4,000, 1,600 and 480 lux, were tested for their effects on regeneration of plantlets from stem segments. The media used in this experiment contained 0.1 mg/l NAA and 20 g/l sucrose and 7 g/l agar, and were adjusted to pH 5.5. The length of stem segments and the cultural conditions were the same as in experiment I, except for light conditions.

Results

Experiment I: Effects of BA, IAA and NAA on regeneration of plantlets from stem segments with a node.

(1) *Effects of BA and IAA (Table 1)*

Callus formation of stem segments was mainly observed on the cut surfaces, and the growth of callus was stimulated a little by the addition of BA at 0.01 mg/l with IAA. Shoot development from nodes of stem segment was observed on all media, and the percentages of stem segments developing shoots ranged from 17% to 63%, owing to concentrations of BA and IAA. A relationship between the percentage of stem segments developing shoots and BA or IAA was not observed in this experiment. Shoot development was observed only on the node of stem segments.

Differentiation of normal white roots (Type II) was observed on the

TABLE 1. Effects of BA and IAA on regeneration of plantlets from stem segments with a node

BA (mg/l)	IAA (mg/l)	Stem segments inducing callus	Degree of callus growth	Stem segments producing shoots	Stem segments producing roots		Stem segments regenerating plantlets
					Type I	Type II	
0	0.01	20% (6/30)	+	43% (13/30)	3.3% (1/30)	0%	0%
	0.05	33 (10/30)	+	43 (13/30)	3.3 (1/30)	6.7 (2/30)	6.7 (2/30)
	0.1	3.3 (1/30)	+	47 (14/30)	0	0	0
	0.5	0	-	17 (5/30)	0	0	0
	1.0	3.3 (1/30)	+	33 (10/30)	0	0	0
	5.0	0	-	23 (7/30)	10 (3/30)	0	0
0.01	0.01	3.3 (1/30)	+	20 (6/30)	0	0	0
	0.05	57 (17/30)	+	40 (12/30)	3.3 (1/30)	3.3 (1/30)	3.3 (1/30)
	0.1	47 (14/30)	+	63 (19/30)	0	0	0
	0.5	80 (24/30)	+	47 (14/30)	3.3 (1/30)	6.7 (2/30)	6.7 (2/30)
	1.0	77 (23/30)	+	37 (11/30)	3.3 (1/30)	0	0
	5.0	23 (7/30)	+	47 (14/30)	6.6 (2/30)	0	0

Sign means the degree of callus growth

- no callus

+ a trace

† good

‡ excellent

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

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

medium containing 0.05 mg/l IAA alone, 0.01 mg/l BA+0.05 mg/l IAA, and 0.01 mg/l BA+0.5 mg/l IAA. The percentages of stem segments with a node differentiating roots on the above media were 6.6%, 3.3% and 6.6% respectively, and these stem segments developed complete plantlets. Semi-transparent roots, which did not function as normal roots, differentiated from calluses which were formed on the cut surface of stem segments.

(2) *Effects of BA and NAA* (Table 2)

Callus formation was mainly observed on the cut surface of stem segments, and partially on the epidermis of stem segments which thickened. Callus growth was enhanced on the medium containing 0.1 mg/l or more NAA.

Shoot development was enhanced on the medium containing 0.01 and 0.1 mg/l NAA, and was inhibited on the medium containing 0.5 mg/l or more NAA. Shoots developed from each node of stem segments.

TABLE 2. Effects of BA and NAA on regeneration of plantlets from stem segments with a node

BA (mg/l)	NAA (mg/l)	Stem segments inducing callus	Degree of callus growth	Stem segments producing shoots	Stem segments producing roots		Stem segments regenerating plants
					Type I	Type II	
0	0.01	27% (8/30)	+	43% (13/30)	3.3% (1/30)	3.3% (1/30)	3.3% (1/30)
	0.05	57 (17/30)	+	53 (16/30)	23 (7/30)	10 (3/30)	10 (3/30)
	0.1	47 (14/30)	##	33 (10/30)	47 (14/30)	10 (3/30)	10 (3/30)
	0.5	50 (15/30)	##	13 (4/30)	13 (4/30)	3.3 (1/30)	0
	1.0	70 (21/30)	##	17 (5/30)	50 (15/30)	0	0
	5.0	20 (6/30)	##	0	0	0	0
0.01	0.01	67 (20/30)	+	73 (22/30)	3.3 (1/30)	0	0
	0.05	73 (22/30)	+	57 (17/30)	33 (10/30)	10 (3/30)	6.7 (2/30)
	0.1	40 (12/30)	##	47 (14/30)	70 (21/30)	3.3 (1/30)	0
	0.5	67 (20/30)	##	33 (10/30)	67 (20/30)	0	0
	1.0	83 (25/30)	##	30 (9/30)	93 (28/30)	0	0
	5.0	27 (8/30)	##	0	0	0	0

Differentiation of normal roots was observed on the medium containing 0.01 to 0.5 mg/l NAA alone, and 0.05 to 0.1 mg/l NAA with 0.01 mg/l BA. Addition of BA, however, was not effective in root formation. Most semi-transparent roots (Type I) which did not function as normal roots differentiated from callus.

The results obtained from (1) and (2) show that regeneration of plantlets from stem segments with a node was effective on the medium containing 0.05 to 0.1 mg/l NAA or IAA. NAA was superior to IAA in normal root formation and regeneration of plantlets. BA stimulated callus formation from stem segments and inhibited regeneration of plantlets.

Experiment II: Effects of BA and NAA on regeneration of plantlets from shoot tips.

Callus formation, root formation and regeneration of plantlets from shoot tips are shown in Table 3. Callus formation was observed on all media except for the medium containing 0.01 mg/l NAA alone, and 0.01 mg/l NAA+0.01 mg/l BA. Callus growth was enhanced on the medium containing 0.1 mg/l NAA.

Development of shoot tips was observed in all treatments. BA at 0.1 mg/l, however, thickened and deformed shoots obtained from shoot tip cultures.

TABLE 3. Effects of BA and NAA on regeneration of plantlets from shoot tips

BA (mg/l)	NAA (mg/l)	Shoot tips inducing callus	Degree of callus growth	Shoot tips producing shoots	Shoot tips producing roots		Shoot tips regenerating plantlets		
					Type I	Type II			
0	0.01	0%(0/30)	—	93%(28/30)	13%(4/30)	3.3%(1/30)	3.3%(1/30)		
	0.1	50 (15/30)	+	77 (23/30)	20 (6/30)	13 (4/30)	13 (4/30)		
	1.0	47 (14/30)	##	40 (12/30)	37 (11/30)	0 (0/30)	0 (0/30)		
0.01	0.01	0 (0/30)	—	87 (26/30)	13 (4/30)	0 (0/30)	0 (0/30)		
	0.1	63 (19/30)	+	90 (27/30)	60 (18/30)	6.7 (2/30)	6.7 (2/30)		
	1.0	43 (13/30)	##	57 (17/30)	43 (13/30)	0 (0/30)	0 (0/30)		
0.1	0.01	30 (9/30)	+	97 (29/30)	0 (0/30)	0 (0/30)	0 (0/30)		
	0.1	97 (29/30)	+	97 (29/30)	10 (3/30)	6.7 (2/30)	6.7 (2/30)*		
	1.0	97 (29/30)	##	100 (30/30)	47 (14/30)	17 (5/30)	17 (5/30)*		

* Shoots of regenerated plantlets were thick and not normal compared with those of plantlet from seed.

The formation of normal white roots (Type II) was observed on the medium containing 0.01 to 0.1 mg/l NAA alone, 0.01 mg/l BA+0.1 mg/l NAA and 0.1 mg/l BA+0.1 to 1.0 mg/l NAA. Semi-transparent roots (Type I) without vascular system were mostly formed from callus derived from shoot tips and did not function as normal roots. Most semi-transparent roots were observed on the medium on which callus grew rapidly. From the above results, it was considered that regeneration of plantlets from shoot tips was successfully obtained on the medium containing 0.1 mg/l NAA alone on which shoot development and root formation were stimulated.

Experiment III: Effect of light intensity on regeneration of plantlets from stem segments with a node.

As shown in Table 4, among 4 different light intensities tested at 10,000, 4,000, 1,600 and 480 lux, shoot development was enhanced under light intensities at 4,000 and 1,600 lux. Light intensity of 10,000 lux inhibited shoot development, because cladophyllus developed instead of shoots at the nodes of stem segments, and chlorosis and antocyanin formation was observed in the shoots. Light intensity of 480 lux was more effective in shoot development than 10,000 lux light intensity, but shoots developing under 480 lux were poor in chlorophyll and elongated uselessly.

The optimum intensities for root formation from nodes of stem seg-

TABLE 4. Effect of light intensity on regeneration of plantlets from stem segments with a node

Light intensity (lx)	Stem segments inducing callus	Degree of callus growth	Stem segments producing shoots	Degree of shoot elongation	Stem segments producing roots		Stem segments regenerating plantlets
					Type I	Type II	
10,000	26%(7/27)	+	74%(20/27)	+	22%(6/27)	3.7%(1/27)	3.7%(1/27)
4,000	57 (17/30)	+	53 (16/30)	≠	23 (7/30)	10 (3/30)	10 (3/30)
1,600	66 (18/27)	≠	96 (26/27)	≠	22 (6/27)	26 (7/27)	26 (7/27)
480	62 (18/29)	≠	83 (24/29)	≠	27.5(8/29)	0	0

ments were 1,600 and 4,000 lux, and regeneration of plantlets was obtained in these treatments. No root formation occurred at 480 lux, and only one root formed at 10,000 lux.

Discussion

Regeneration of plantlets through shoot tip cultures has been attempted to obtain virus free plants or vegetative mass production in many species.

In asparagus, since LOO^{10,11} successfully grew excised shoot tips of seedling unlimitedly through successive transfers, MURASIGE *et al.*,^{2,3} GORTER,⁴ YANG *et al.*¹⁵ and ANDREASSON *et al.*¹⁶ have attempted shoot tip culture of asparagus for vegetative mass production, and YANG *et al.*¹⁷ have attempted it to obtain virus free plants. For obtaining virus free plants, 0.1 to 0.3 mm long shoot tips are usually cultured as sources of explants, whereas for vegetative mass production 5 to 10 mm long shoot tips are often used.

We also tested the effects of cytokinin (BA) and auxins (NAA, IAA) on regeneration of plantlets from 5 to 10 mm long shoot tips. NAA effectively stimulated root formation at concentrations ranging from 0.01 to 0.1 mg/l. On the other hand, BA was not effective for root formation, but stimulated shoot development effectively at less than 0.1 mg/l.

Since concentrations of 0.1 mg/l or more BA thickened shoots, plantlets regenerated on the medium containing 0.1 mg/l or more had a tendency to become abnormal. So it was difficult to transplant these plantlets to the soil.

Regarding length of shoot tips used as sources of explants for regeneration of plantlets, GORTER⁴ cultured shoot tips ranging from 5 to 34 mm in length which were isolated from lateral green shoots or etiolated lateral shoots. She reported in her paper that root formation was better in short shoot tips than long shoot tips in both green and etiolated lateral shoots, and also the optimum concentration of NAA and IBA for root formation

from shoot tips of seedlings or green lateral shoots was 0.1 to 2 mg/l and 1 to 5 mg/l respectively. She also reported that root formation from shoot tips of etiolated lateral shoots was best at 5 mg/l NAA.

On the other hand, MURASHIGE *et al.*^{5,12,13} reported that the cultures of 0.15 mm long shoot tips successfully regenerated plantlets on the medium containing 0.1 mg/l kinetin and 0.3 mg/l NAA.

In our experiment on the culture of shoot tips from shoots obtained through anther culture of asparagus, root formation from shoot tips was enhanced on the medium containing 0.01 to 1.0 mg/l NAA combined with 0 to 0.01 mg/l BA.

From the above results, it seems likely that optimum concentration of cytokinin and auxin for root formation from shoot tips depends on the length of shoot tips.

Gorter reported that the optimum concentration for root formation from shoot tips was different from that for root growth, and auxin was not essential to root growth. Concerning the growth of roots formed from callus, the growth of roots was found to be enhanced on the medium without growth regulators. However, for root formation from shoot tips, it seemed important that they were transferred onto the medium without auxin or with reduced auxin soon after root primordia emerged from the base of the shoot tips.

Besides culture of shoot tips, culture of stem segments with a node also can be used for regeneration of plantlets.

This procedure enables us to get a number of stem segments under suitable conditions, and a number plantlets might be regenerated on the medium suitable for root formation.

However, the culture of stem segments with a node is not superior to that of shoot tips in regeneration of plantlets. At present, the percentage of stem segments regenerating plantlets is less than that of shoot tips regenerating plantlets. Concerning the effect of light intensity on regeneration of plantlets from shoot tip culture, YANG *et al.*¹⁰ obtained good results at 1,600 lux, and MURASHIGE *et al.*⁹ obtained good results at 1,000 lux. We also obtained good result with light intensity of 1,600 lux. Concerning the effects of temperature, sugars, pH, and so on, further reserch would be needed to obtain higher percentages of regeneration of plantlets.

In regeneration of plantlets from shoot tips or stem segments with a node, the nutrient states of shoots as sources of explants would be considered to affect it, therefore uniform and healthy shoots would be needed to be prepared for regeneration of plantlets.

Summary

Effects of growth regulators (cytokinin and auxin) and light intensity on the regeneration of plantlets from shoots obtained through anther culture were investigated. As sources of explants, 5 to 10 mm long shoot tips or stem segments with a node were used.

Three experiments were carried out and the results are summarized as follows.

(1) *Effects of BA, NAA and IAA on regeneration of plantlets from stem segments with a node.*

Regeneration of plantlets from stem segments with a node was effective on the medium containing 0.05 to 0.1 mg/l NAA or IAA. NAA was superior to IAA in normal root formation and regeneration of plantlets. BA inhibited regeneration of plantlets.

(2) *Effects of BA and NAA on regeneration of plantlets from shoot tips.*

The formation of normal roots was observed on the medium containing 0.01 to 0.1 mg/l NAA alone, 0.01 mg/l BA+0.1 mg/l NAA and 0.1 mg/l BA+0.1 to 1.0 mg/l NAA.

BA at 0.1 mg/l thickened and deformed shoots, so plantlets regenerated on the medium containing 0.1 mg/l BA were abnormal. Regeneration of plantlets from shoot tips was successfully obtained on the medium containing 0.1 mg/l NAA alone.

(3) *Effects of light intensity on regeneration of plantlets from stem segments with a node.*

Among 4 different light intensities (10,000, 4,000, 1,600 and 480 lux) tested, the optimum intensity for root formation from node of stem segment was 1,600 and 4,000 lux, and regeneration of plantlets was observed in these treatments.

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