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Efficient *In vitro* Mass-propagation of Shiode (*Smilax oldhami* Miq.) through Liquid Culture

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Shiode is a wild and edible plant whose flavor is similar to that of asparagus (*Asparagus officinalis* L.). The present experiments were implemented to improve the *in vitro* propagation system of shiode. Internodal explants were cultured to derive protocorm-like bodies (PLBs) on Murashige and Skoog[12] (MS) medium at pH 5.7. The MS medium was supplemented with 30 g/*l* sucrose, 0.1 mg/*l* *a*-naphthaleneacetic acid (NAA) and 0.1 mg/*l* 6-benzylaminopurine (BAP), and solidified with 8 g/*l* agar. The PLBs that were induced on the solid MS medium propagated very rapidly when they were transferred into the 1/2 MS liquid medium at pH 5.7 supplemented with 20 g/*l* sucrose, 1.0 mg/*l* NAA and 0.1 or 1.0 mg/*l* BAP. The liquid MS medium was renewed periodically. The single PLBs were separated from the mother PLB-clusters by a surgical knife, and were used to regenerate the descendant PLB-clusters. The secondary PLBs grew adventitious shoots that formed roots on the solid hormone-free MS medium after subculture for 2 weeks on the solid MS medium supplemented with 0.5, 1.0 and 2.0 mg/*l* NAA. The plantlets were acclimatized following standard procedures.

Introduction

Shiode propagates by seeds and/or tillering in the autogenous areas. However, the rate of seed germination is low (less than 40%) and leaf emergence requires at least two years after germination due to a secondary dormancy during the summer season[13,14]. Furthermore, because of the low rate of growth, it takes more than 5 years after germination to reach harvest.

Generally, defferentiation characteristics of shiode in *in vitro* culture are similar to those of *Cymbidium*[11,14] in relation to the formation of PLBs and little formation of calli. Organs that have been used for *in vitro* propagation of shiode include the shoot, apex, petiole, leaf blade, root, tendril, and anther[7,17-19,21]. However, the formation of PLBs has not been reported, and furthermore the rate of shoot fotmation is still low.

The present experiments were carried out to improve the culture system to increase the rate of *in vitro* propagation of shiode.

Materials and Methods

The original materials, root-stocks of shiode, were collected form Mt. Gassan in Yamagata prefecture. The materials were grown and propagated through tillering in a greenhouse at Yamagata University to decrease various diseases, especially bacteria in the vascular bundles. Internodal explants (*ca.* 3 cm long) obtained from young shoots that developed from axial buds were srerilized with 70% ethanol for 20 sec, 1% sodium hypochlorite for 20 min, and then washed 3 times with sterilized water.

After cutting off both the tips (each about 5 mm long) from the sterilized internodal explants, the

remaining *ca.* 2 cm-long explants were cultured on the MS medium at pH 5.7. The MS medium was supplemented with 30 g/*l* sucrose, 0.1 mg/*l* NAA, and 0.1 mg/*l* BAP, and solidified with 8 g/*l* agar. The PLBs were produced from the internodal explants on the solid MS medium during one month culture.

The first *in vitro* mass-propagation of PLBs in the liquid medium was carried out using the PLBs from the solid MS medium. The liquid 1/2 MS medium was supplemented with 20 g/*l* sucrose, 1.0 mg/*l* NAA and 0.1 or 1.0 mg/*l* BAP. Five PLBs were placed in a 300-m*l* Erlenmeyer flask containing 100 m*l* 1/2 MS medium. The flask was placed on a rotary shaker (model NR-20, TAITEC Co., Ltd., Saitama, Japan) and shaken at the rate of 90 rpm. The liquid MS medium was renewed at intervals of 2 or 3 days to avoid browning of the liquid medium and PLBs, and to relieve a rapid lowering of the pH value of the liquid medium.

The secondary *in vitro* mass-propagation was carried out using PLBs that were cut off with a surgical knife from those in the first liquid culture. The liquid medium compositions were similar to the first liquid culture, and 5 PLBs were incubated in 300-m*l* Erlenmeyer flasks as before. The renewal of the liquid medium was conducted as the same intervals as in the first culture. The PLBs in the secondary liquid culture developed to shoots. They were subjected to subculture on the solid MS medium supplemented with 0.5, 1.0 and 2.0 mg/*l* NAA plus 0.5 or 1.0 mg/*l* BAP in all combinations for 2 weeks. The shoots were then transferred on the hormone-free solid MS medium for induction of roots. After subculture, a part of each shoot was cultured continuously on the solid MS medium supplemented with 0.5 and 1.0 mg/*l* NAA to examine the effects of hormones on the formation and growth of roots.

The plantlets were produced on the hormone-free solid MS medium after subculture on the solid medium containing 0.5, 1.0 and 2.0 mg/*l* NAA for 2 weeks. These plantlets were acclimatized according to standard procedures.

The culture experiments in both the liquid and solid media were conducted at 25°C under a light intensity of 3 klx and 24 hr daylight. All the culture experiments were conducted in three replications.

Results

1. *Browning and changes in pH value of liquid medium*

The solid MS medium was used to derive PLBs, and little browning of the internodal explants and PLBs occurred (**Fig. 1-A**), although the rate of PLB formation was low. In contrast, serious browning of the liquid medium and PLBs occurred in the liquid culture (**Fig. 1-B**). Finally, the browning of the medium and PLBs resulted in the death of the PLBs.

The browning of the liquid medium was accompanied by a lowering of the pH. The pH in the liquid cultures remained below 4.8 until *ca.* 3 weeks after the start of incubation, even though the liquid medium was renewed at intervals of 2 or 3 days. However, the pH value increased and reached the same pH value as that at the start of incubation after 9 weeks. Thereafter, the pH value decreased, and became less than 5.0 (**Fig. 2**). Variations in the pH value between the different concentrations of BAP, *i. e.* 0.1 and 1.0 mg/*l*, were not significant, and the maximum F value was 6.6474 at the degree of freedom (df) of 1 versus 4 among the different incubation stages. Therefore, the pooled data of 0.1 and 1.0 mg/*l* BAP concentration treatments in the first culture are shown in **Fig. 2.** Variations in the pH value with different incubation stages in the secondary culture were similar to those in the first culture (data not shown).

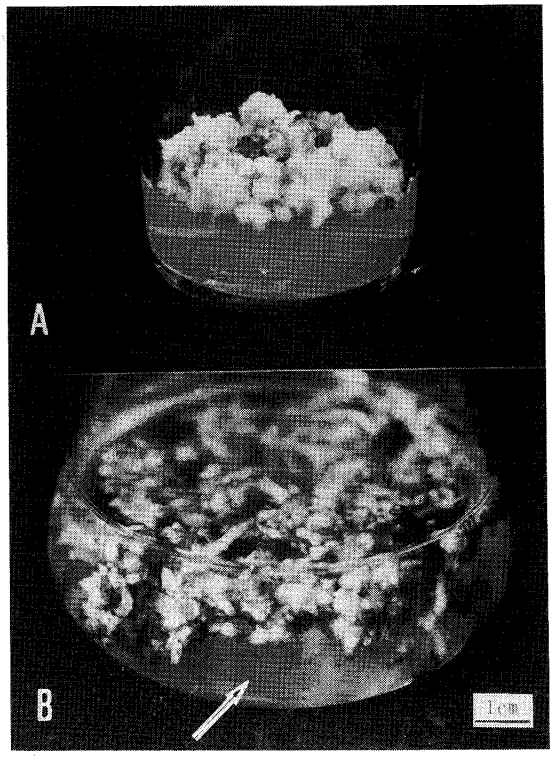

Fig. 1 Formation of protocorm-like bodies on solid(A) and liquid(B) MS medium.
A: PLB-cluster derived from 5 internodal explants on solid medium after 6 months of incubation.
B: PLB-cluster derived from 5 PLBs after 15 weeks of incubation.
Arrow indicates the browning of liquid medium.

2. *First propagation of PLBs in liquid culture*

The number of PLBs formed per PLB from the solid MS medium increased more than three-fold during 15 weeks, and the fresh weight of one PLB-cluster also increased more than forty-fold during 15 weeks in the first culture (**Fig. 3**). The effects of different BAP concentrations, *i. e.* 0. 1 and 1. 0 mg/*l*, on the increase in the number and fresh weight of PLBs also were not significant, and the maximum F value was 2. 7000 and 4. 4805 at the df of 1 versus 4, respectively, among different incubation stages. Therefore, the pooled data of 0. 1 and 1. 0 mg/*l* BAP concentration treatments are shown in **Fig. 3.**

3. *Secondary propagation of PLBs in liquid culture*

The PLBs were re-propagated using the PLBs from the first liquid culture that were cut off with a surgical knife. The PLBs regenerated, and adventitious shoots grew in large numbers (**Figs. 4** and **5**). The propagation rate of PLBs was higher in the secondary than in the first liquid culture. The shoot formation progressed parallel to the differentiation of PLBs. The effects of different BAP concentrations, *i. e.* 0. 1 and 1. 0 mg/*l*, on the increase in the number of PLBs and the rate of growth of adventitious shoots were also not significant, and the maximum F value was 6. 8260 and 5. 9899 at the df of 1 versus 4, respectively, among the different incubation stages. Therefore, the pooled data of 0. 1 and 1. 0 mg/*l* BAP concentration treatments are shown in **Fig. 4.**

4. *Root formation*

The adventitious shoots formed roots at the high rate of almost one hundred percent on the hormone-free solid MS medium after subculture during 14 days on the solid MS medium supple-

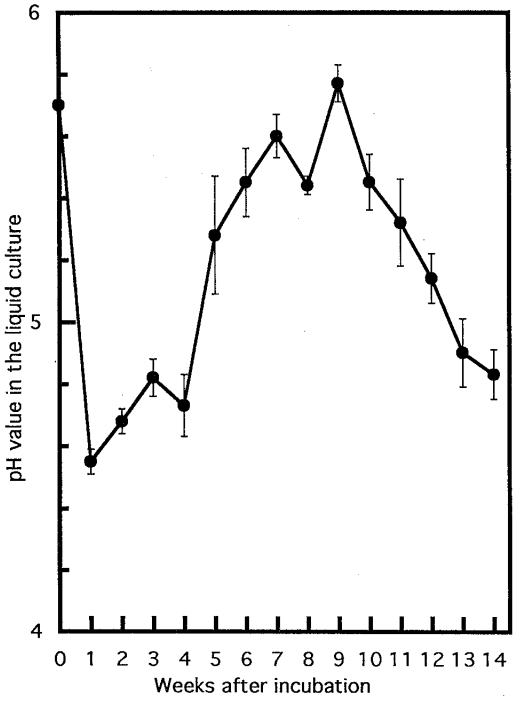

Fig. 2 Changes in the pH of liquid medium containing 5 PLBs with renewal of the liquid medium at intervals of 2 or 3 days.
Vertical bars indicate the standard error based on six replications.

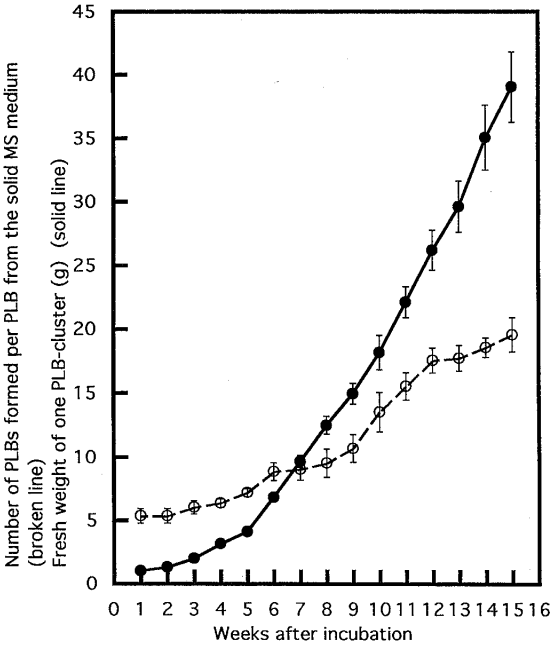

Fig. 3 Changes in the number (broken line) and fresh weight (solid line) of PLBs in the first culture using those derived from the solid MS medium.
Vertical bars indicate the standard error.

mented with 0.5, 1.0 and 2.0 mg/*l* NAA plus 1.0 or 2.0 mg/*l* BAP in all combinations (Plot A in **Table 1**), and almost all the plantlets were acclimatized efficiently (**Fig. 6**).

In contrast, the plantlets were cultured continuously on the solid MS medium supplemented with

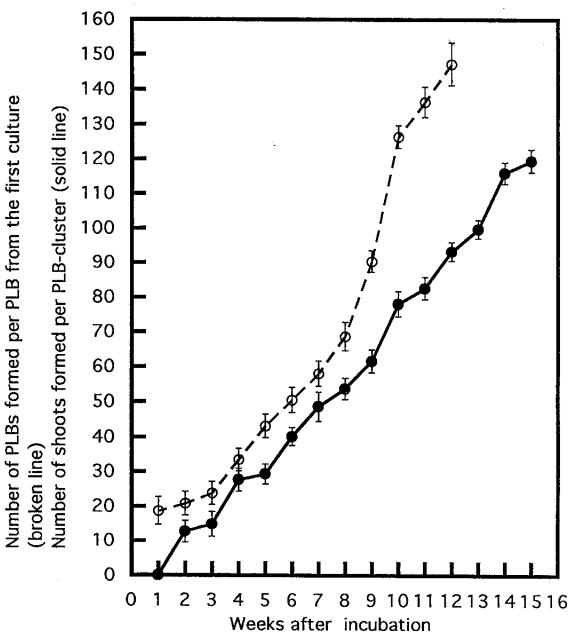

Fig. 4 Changes in the number PLBs (broken line) and the formation rate of shoots (solid line) in the secondary culture using the PLBs derived from the first liquid culture.

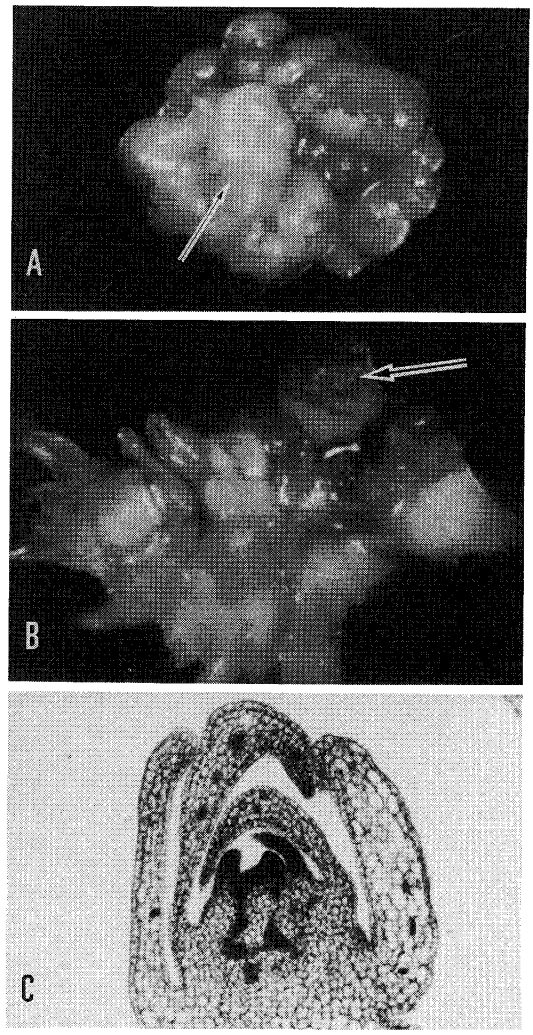

Fig. 5 Differentiation of the PLBs and shoots in the liquid culture.
Arrows indicate the PLBs in A and shoots in B, and C is the cross-section of B.

0.5 and 1.0 mg/*l* NAA plus 1.0 or 2.0 mg/*l* BAP in all combinations. The roots and shoots became brownish and finally died, although the change in the rate of root formation with incubation time (Plot B in **Table 1**) was similar to that in Plot A.

Discussion

It has been reported thet the pH of a culture medium, even in the medium without explants or calli, decreases with time[8]. This is caused by the preferential absorption of either nitrate or ammonium in the culture medium[9]. Furthermore, explants with no calli modified the medium pH value less than explants with calli[9]. However, the PLBs in the liquid culture appeared to suffer from not only the lowering of pH value, but also the browning of the liquid medium. The browning was apparently caused by the secretion of phenol-like substances from the PLBs. However, these deteriorations appeared to be relieved by the periodic renewal of the liquid medium.

The increase in the pH value during the third to ninth weeks of incubation may be due to an equilibrium between the volumes of the increasing PLB-clusters and liquid medium, and the periodic renewal of the liquid medium. However, the volumes of the Erlenmeyer flask and liquid medium after 9 weeks of incubation were not sufficient for the increased PLB-clusters, which resulted in a rapid decrease in the pH value, and hence a crucial browning. Thus, exploitation of a mechanical renewal system would probably promote the *in vitro* mass-propagation of shiode, and hence facilitate the commercial supply of shiode plantlets to farmers.

Explants of shiode produce few calli[8,17,19] in addition to the low formation rate of shoots and

Table 1. Effect of α-naphthaleneacetic acid (NAA) on the rate of root formation and mean number of roots per shoot in Shiode.

Weeks after subculture for 2 weeks	NAA concentration (mg/l) in subculture*1				
	0.5	1.0	2.0	0.5	1.0
	Plot A			Plot B	
	After subculture for 2 weeks, transferred onto solid hormone-free MS medium.			Afrer subculture for 2 weeks, transferred onto solid medium containing 0.5 and 1.0 mg/l NAA.	
	(%)				
3	22.2	41.6	16.7	22.2	33.3
4	33.3	75.0	75.0	44.4	33.3
5	66.7	91.7	91.7	66.7	44.4
	(2.1)*2	(3.8)	(1.0)	(2.1)	(1.2)
6	100.0	91.7	100.0	100.0	66.7
	(3.7)	(4.7)	(2.5)	(3.3)	(7.0)
7	100.0	100.0	100.0	100.0	100.0
	-	(6.0)	(2.7)	(4.5)	(6.1)
8	100.0	100.0	100.0	100.0	100.0
	-	(6.0)	(5.0)	(4.5)	(5.8)
	Formed the thin and nutrient-absorbing roots. The plantlets were efficiently acclimatized.			Formed the thin and nutrient-absorbing roots, but finally became brownish, and the plantlets died.	

*1 1.0 or 2.0 mg/l BAP was supplemented in all combinations, but significant differences were not observed. Therefore, the pooled data of different BAP concentration treatments were shown.

*2 The bold numerals in the parentheses from 5 to 8 weeks after subculture are the mean number of roots per shoot.

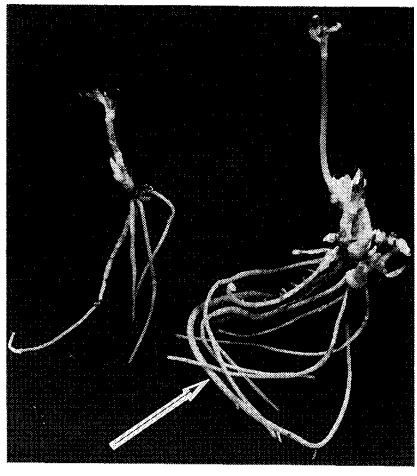

Fig. 6 Roots formed from the shoots following culture on the hormone-free solid medium for 8 weeks after subculture for 2 weeks on NAA- and BAP-containing solid medium. Arrow indicates thin and nutrient-absorbing roots.

plantlets through organogenesis on the solid medium. Thus, the *in vitro* mass-propagation via callus formation could not be adopted for shiode in contrast with other plants[1,2,4-6,15]. Therefore, the present liquid shaking culture method may be practical for *in vitro* mass-propagation of shiode.

In addition, the cyclic propagation of PLBs, followed by plantlet regeneration, appears to increase the efficiency of the liquid culture.

However, the cyclic *in vitro* regeneration of PLBs and their clusters may be accompanied with genetic variations, such as somaclonal variations. Thus, it is important to examine the spectrum of genetic variations in the cyclic *in vitro* culture to ensure the *in vitro* mass-propagation of shiode. Another goal is to utilize the somaclonal variations for enlargement of the genetic variability of shiode.

The effects of the addition of auxins into the culture medium on the induction of roots from shoots in tissue cultures differ among plants[10,16]. The present results showed that the *in vitro* culture-derived shoots of shiode required no hormones for the induction of roots, as is also the case for cucumber, lettuce and cabbage[16]. Although roots were produced on the NAA- and BAP-containing medium at the same rate as on the hormone-free medium, the roots became brownish and finally the plantlets died. In addition, studies on the effects of naturally occurring hormones, such as indole acetic acid, instead of the synthetic auxins, such as NAA and BAP, on the root formation remain to be determined.

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《和文要約》

シオデ(*Smilax oldhami* Miq.)の液体振盪培養による効率的増殖

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本研究では，シオデの節間由来のプロトコーム様体(PLB)を液体培養することによって大量に形成させ，シオデの大量増殖を可能にした．すなわち，NAA を含む液体培地でシオデの PLB を液体振盪培養することによって，1個の PLB から大量の PLB(クラスター)を形成させ，これらが幼芽に生長したのち，一定期間の前培養を行い，ホルモン・フリーの固体培地に移植して発根させ，大量の植物体を順化させた．

さらに，液体培養した PLB-クラスターから人為的に切り取った PLB を再度液体培地に移植した．この二次増殖系では，ホルモン・フリーの固体培地に移植することによってより多くの PLB-クラスターおよび幼芽・幼根が形成された．この手法によって，循環的に PLB-クラスターおよび植物体の再生・増殖が可能となった．

なお，固体培地と異なり，液体培地は褐変し，PLB およびクラスターも褐変し枯死する結果となった．また，褐変とともに液体培地の pH が低下した．この褐変と pH の低下は，液体培地を2ないし3日間隔で新鮮培地で交換することによって緩和することができた．