論　文

オヒルギのカルスおよび多芽体形成

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Callus and multiple shoot formation of *Bruguiera gymnorrhiza*

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SUMMARY : In order to analyze the effects of nutrient conditions of culture media and size of explant on callus and multiple shoot formation of *Bruguiera gymnorrhiza*, tissue culture experiments using hypocotyl as an explant were conducted. Experiments were designed to examine ; (1)callus formation under different nutrient conditions of media, (2)collus and multiple shoot formation from explants of different sizes on sugar free media and (3)root differentiation of cuttings of the multiple shoots. The results obtained were summarized as follows :

(1) Callus was formed only during the first two weeks after explant preparation. The concentration of inorganic nutrients seemed to show no significant effect on the callus formation. The increase of the inorganic nutrient concentration of the media resulted in the decrease of the survivorship of the explant.

(2) Multiple shoots were formed on sugar free media. With increase in the size of explants, the percentage of callus and multiple shoot formation increased and the period required for the formation became shorter. It was observed that the larger the size of explant, the healthier and the larger the shoots developed.

(3) Primordia of roots were observed to have been differentiated at the base of the shoot cuttings by planting on the media containing with IBA and PG.

要　旨：オヒルギ（*Bruguiera gymnorrhiza*）のカルスおよび多芽体の形成に与える培地の栄養条件と外植体の大きさの影響を調べた。胚軸を外植体として，(1)異なった栄養条件の培地によるカルス形成，(2)無糖培地に異なった大きさの胚軸切片の植え付けることによる多芽体の形成の組織培養実験をおこなった。さらに(3)多芽体を切り取り，根の分化を試みたところつぎのような結果を得た。

(1) カルスの形成には最初の2週間が有効であった。培地の無機栄養濃度はカルス形成に有意な影響をあたえなかった。培地の無機栄養濃度が増加すると外植体の生存率は低下した。

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(2) 無糖培地で外植体から多芽体を形成させることができた。外植体の大きさ（生重）が増加するとカルスおよび多芽体の形成率は増加し，形成の時期も早くなった。多芽体は外植体が大きいほど大きく健全であるようにみえた。

(3) 多芽体から切り取ったシュートを培地に植え付けると根の原基が分化することが認められた。

INTRODUCTION

Plant tissue culture is now a proven technology for the *in vitro* production of large numbers of genetically identical plants (Aitken-Christie et al., 1995). The methods have been successfully applied to many tree species, especially in case of gymnosperms and a few angiosperms of temperate regions. The *in vitro* culture on tropical trees in Asia was initiated during the last two decade (Rao and Lee, 1982). Successful results on organogenesis and plantlet development have been reported on the genus of *Acacia, Ailanthus, Citrus, Eucalyptus, Phoenix, Salix, Tectona, Tamarindus,* etc..

As for mangrove tree species, the trials began only a decade ago. Only a few papers on mangrove tissue culture are available compared with those on the terrestrial plants. The most of them still remain the stage of callus formation. A successful technique in tissue culture seems to be a correct combination of explants and media. The purpose of this study is to investigate the effect of nutrient conditions and size of explants on callus and multiple shoot formation and to examine root differentiation from the multiple shoot. This contributes to develop successful tissue culture system for mangrove trees, *Bruguiera gymnorrhiza*.

MATERIALS AND METHODS

Hypocotyls of *Bruguiera gymnorrhiza* were utilized for the experiments. They were collected by Mr. Asao Sunaga during April 1993−June 1994 in the brackish region of Shiira river and Nagura river on Iriomote Ialand and sent to the College of Agriculture, Ehime University within four days. The fully developed, healthy outlook, undamaged, not infected hypocotyls longar than 15 cm were selected.

Sterilization of the hypocotyl was processed as follows：

a) The calyxes were removed, the whole hypocotyls were cleansed with 70% ethanol cotton,

b) The hypocotyls were cut into short pieces of 5−6 cm long with a sharp surgeon knife,

c) Washed with diluted detergent,

d) Soaked with 70% ethanol for 1 min.,

e) Soaked with 10% sodium hypochlorite solution for 10−15min.,

f) Rinsed 3 times with sterile distilled water.

All the experiment was performed at a laboratory of United Graduate School of Agricultural Sciences, Ehime University.

Experiment 1. Callus formation under different nutrient conditions of the media

The experiment was conducted to examine the effect of nutrient concentration and composition on callus formation on the cross sectional cut surface of the hypocotyl of *B. gymnorrhiza*. Murashige and Skoog (MS) media (Murashige and Skoog, 1962) and Woody plant (WP) media (Lloyd and McCown, 1981) supplemented with sugar, hormones and vitamins were used as the culture media. The composition of the media was prescribed in Table 1. The media were adjusted to pH 5.6−5.8 with either 1 N NaOH or HCl. The sterilized hypocotyls were cut into discs of ca. 1 cm thickness and planted on 100, 50, 25, 12.5 and 6.25 % strength of MS and WP media.

Table 1 Recipe for the culture media

	MS media [mg/l]	WP media [mg/l]
Macro elements		
NH_4NO_3	1650	400
KH_2PO_4	170	170
K_2SO_4	-	990
$CaCl_2.2H_2O$	440	-
$Ca(NO_3)_2.4H_2O$	-	556
$MgSO_4.7H_2O$	370	370
Micro elements		
H_3BO_3	6.2	6.2
$MnSO_4.4H_2O$	22.3	22.3
KI	0.83	-
$Na_2MoO_4.2H_2O$	0.25	0.25
$CoCl_2.6H_2O$	0.025	-
Iron		
$FeSO_4.7H_2O$	27.85	27.8
$Na_2.EDTA$	37.85	37.3
Vitamins & Hormones		
Inositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	0.1
Glycine	2.0	2.0
2,4-D	0.442	0.442
Kinetin	4.42	4.42
Others		
Sucrose[g/l]	30	20
Agar[g/l]	10	6

Each treatment included 50 pieces of explants. One piece of explant was put in a test tube (13 cm in height and 4 cm in diameter) and incubated at 28±1℃ with 16 hr day (5700 lux) and 8 hr night (dark) photoperiod. The number of explants which developed callus and survived was counted every two or three days for three months. After two weeks, the explants infected by fungi or bacteria were removed. The explants without the symptom of any infection were transferred to new test tubes with the same formula of the medium.

Experiment 2. Callus and multiple shoot formation from explants of different sizes on sugar free media

The purpose of this experiment is to examine whether size and weight of the explant affect the callus and shoot formation on sugar free media. After the sterilization mentioned above, the

hypocotyls were cut into 3 (A_3), 2 (A_2), 1 (A_1) and 0.5 ($A_{0.5}$) cm thick discs and a quarter of 0.5 ($A_{0.5q}$) cm thick disc. The fresh weight of each explant was 5−3g, 3−2g, 2−1g, 0.5−1g and<0.5g for A_3, A_2, A_1, $A_{0.5}$ and $A_{0.5q}$, respectively. The number of explants used was 35, 55, 72 72 and 77 for A_3, A_2, A_1, $A_{0.5}$ and $A_{0.5q}$, respectively. All explants were incubated on sugar free media in test tubes under the condition of 28 ± 1 ℃ and 5700 lux for five weeks. During the first two weeks the tubes were checked every day for susceptible contamination. The explants contaminated were immediately removed.

Experiment 3. Root differentiation

The shoot developed in the Experiment 2 were cut and planted into 6.25% WP media supplemented with 5mg/l Indolebutyric acid (IBA) and 15mg/l Phloroglucinol (PG). The age of the shoot was one month and the size was 2−3cm in length. The samples were placed in dark condition. After twenty days the shoot was transferred into new media of the same recipe. After forty days the shoots were placed in light condition.

RESULTS

Experiment 1. Callus formation under different nutrient conditions of the media

After one week of incubation, the surface of explant turned dark. In the second week the surface showed split at cortex and pith, and the callus development was observed (Photo 1). The callus looked white−yellow, hard and knobby. Some of the callus in MS media turned brown−black and ceased growing.

The callus developed only within two weeks of incubation. Thereafter, no further development was observed and survival of the explant was declined. It was revealed that the only first two weeks were effective for the callus formation. Figure 1 shows the callus formation of the explants during two weeks of incubation. In total, the callus was formed for 4.0% of the explants on MS media and 3.2% on WP media. There appeared no significant difference in efficiency for the callus formation between MS and WP media. For MS media, the highest percentage of the callus formation was in 12.5% strength while for WP media in 6.25%. Although the highest tend to appeared in lower strength, the concentration of the inorganic nutrients seemed to exhibit no significant effect on the callus formation.

The number of the explants survived after two weeks was shown in Figure 2. The total survival for MS and WP media were 71% and 75%, respectively. Higher survivorship was obtained in media weaker than 50% strength for WP and stronger than 50% strength for MS. The explant survival tended to decrease in higher strength of both MS and WP media.

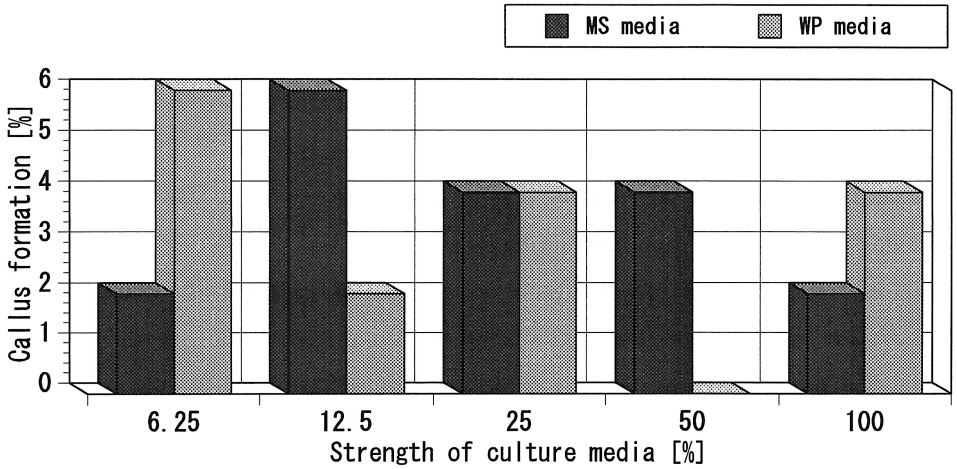

Fig. 1 Callus formation during two weeks of incubation in Experiment 1

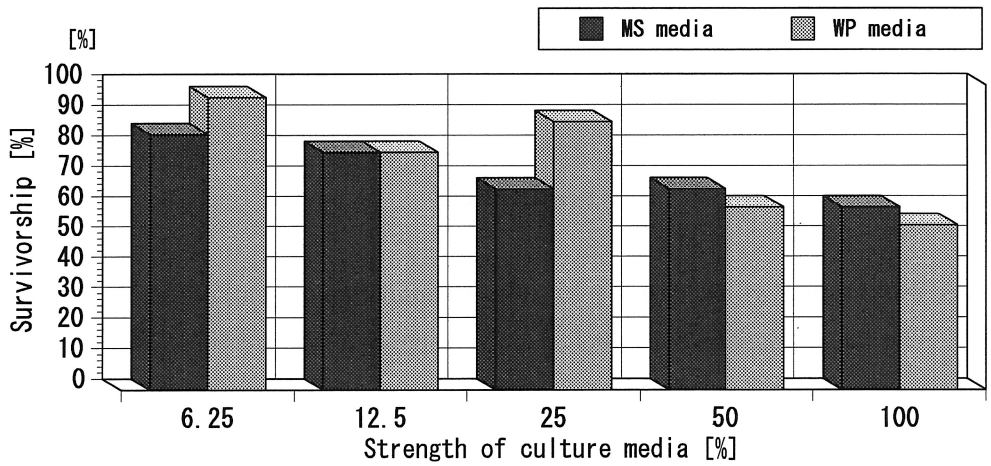

Fig. 2 Survivorship during two weeks of incubation in Experiment 1

Table 2 Callus and shoot formation in Experiment 2

Group	Weight [g]	Callus formed [%]	Callus formed day*	Shoot developed [%]	Shoot developed day*
$A_{0.5q}$	>0.5	18	30	0	-
$A_{0.5}$	0.5-1	33	32	1.3	81
A_1	1-2	50	11	22	44
A_2	2-3	56	11	51	41
A_3	3-5	88.6	11	83	37

*The day indicates the first day of callus formation or shoot development.

Experiment 2. Callus and multiple multiple shoot formation from explants of different sizes on sugar free media

The result of callus and shoot formation was shown in Table 2. Callus formation started on the 11th day for A_1, A_2 and A_3 while on the 32nd day for $A_{0.5}$ and on the 30th day for $A_{0.5q}$. The multiple shoots appeared through the callus (Photo 2) except for $A_{0.5q}$. The bigger the explants the shorter the day required for shoot formation.

The effect of size of explants on the percentage of callus and shoot formation was represented in Figure 3. The percentage increased with increasing the weight of the explants. The shoots from the bigger explants looked healthier (Photo 3). These results indicated that the size of hypocotyl played an important role for the callus and shoot formation.

Experiment 3. Root differentiation

After three weeks of incubation in the dark conditions, the root primordia were apparently observed inside the swollen part at the base of the shoot (Photo 4). But a few days later, browning of the swollen part occurred. After browning, the swollen part did not further develop and the root did not emerged from the epidermis of the shoot.

DISCUSSION

The callus was formed within two weeks after explant preparation and no further formation was observed. The longer period of incubation only resulted in the death of the explants. Two weeks seemed appropriate for the callus formation of *B. gymnorrhiza* hypocotyl. The concentration of inorganic nutrients seems to have no significant relation to the callus formation. The higher concentration of the inorganic nutrients gave less survivorship of the explants.

The activation of microorganisms might cause to the mortality of the explants. Taking the present performance of sterilization into account, the endophytic infection (Isaac, 1992) Should not be denied. The proper period and nutrient concentration should be adopted for the tissue culture.

The multiple shoot formation was succeeded in sugar free culture. The bigger the size of the explants, the larger shoot was formed. The shoot from the bigger explants looked healthier. These results indicate that the shoot differentiation from the explants highly depends upon the nutrient stocks of the hypocotyl.

The callus was seemed to develop from the vascular tissue (Photo 1). The callus should function only to protect the wounded surface. The primordia of multiple shoots were formed underneath the callus, developed through the callus and penetrated into outside (Photo 2). By planting the cuttings of the shoots, the root primordia were differentiated at the base. It was concluded that the hypocotyl of *B. gymnorrhiza* could produce the plantlet through the multiple shoots and root differentiation.

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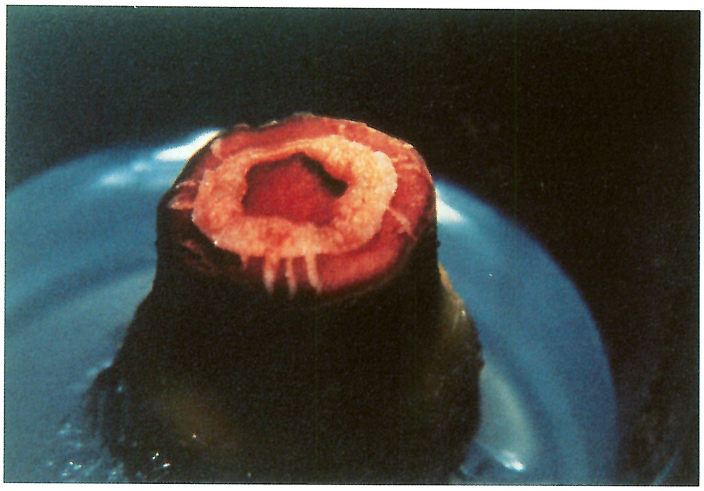

Photo 1　Callus formation in Experiment 1.

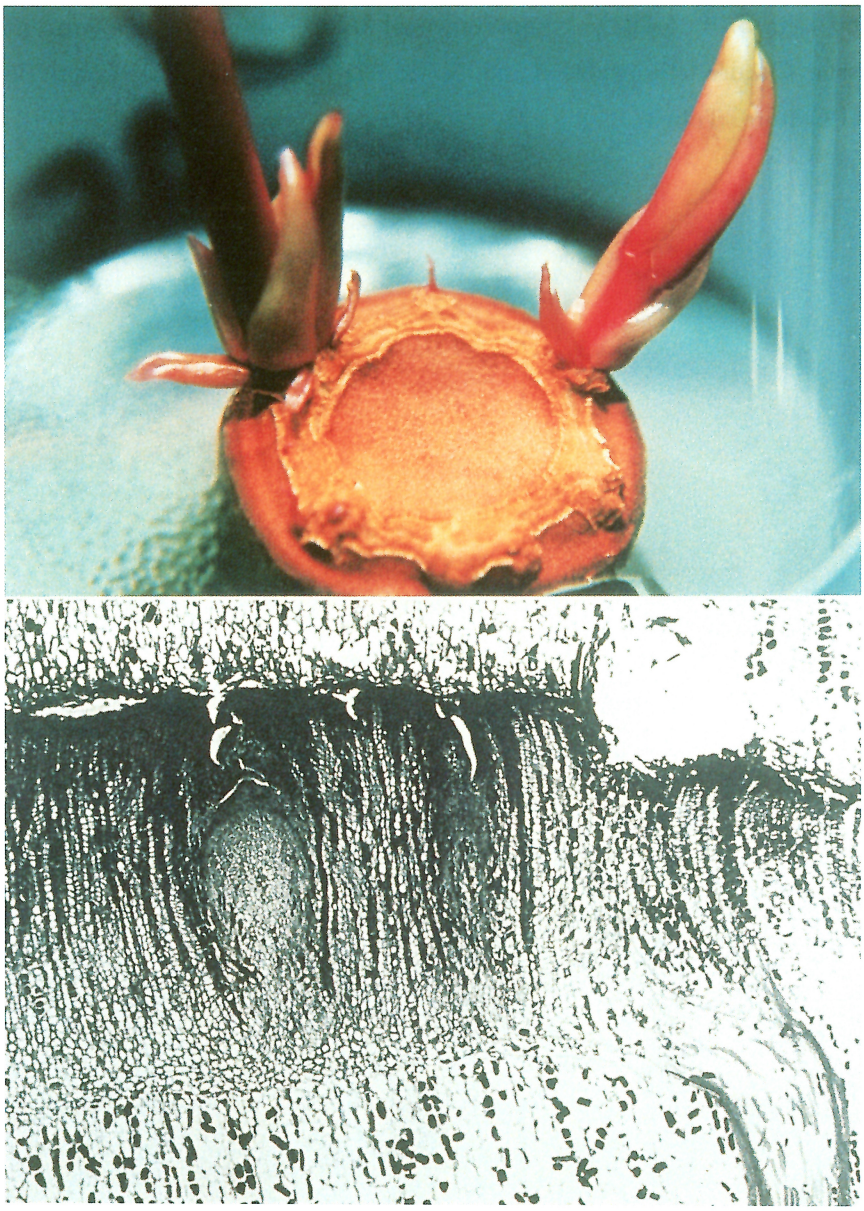

Photo 2　Multiple shoot formation in Experiment 2.

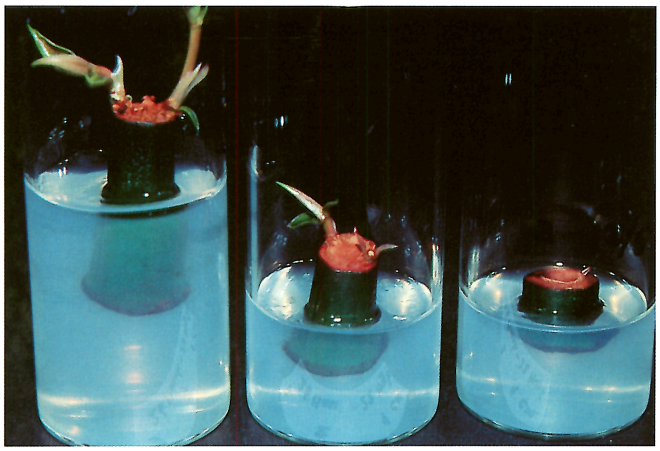

Photo 3 The effect of the size of explants on multiple shoot formation.

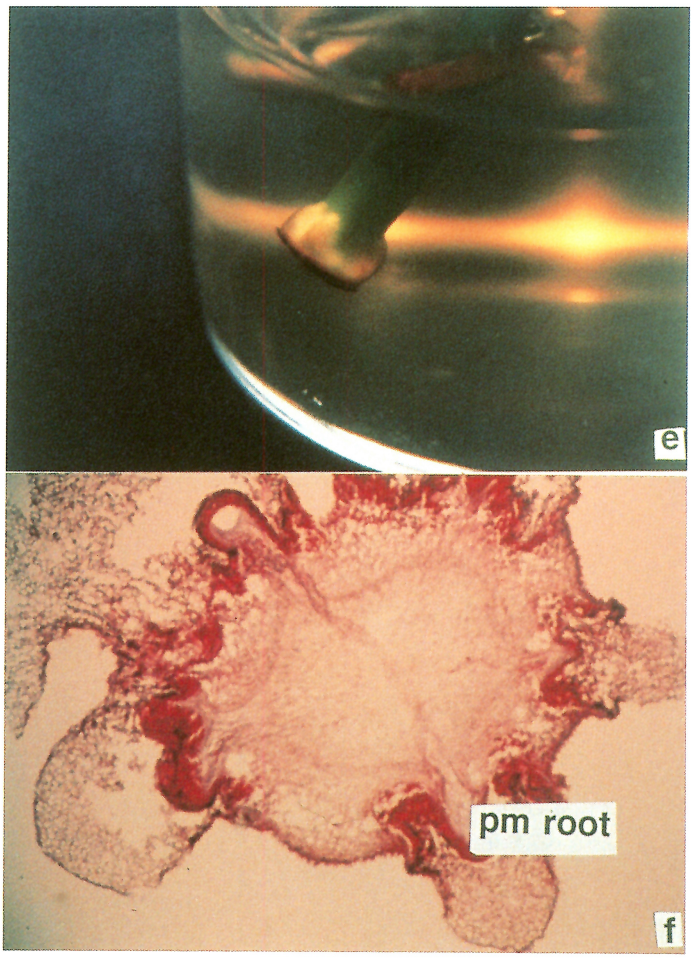

Photo 4 Root primordia differentiation in Experiment 3.