Factors Affecting *in vitro* Plantlet Regeneration from Axillary Buds of *Quercus acutissima* derived from Stump Sprouts.

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Factors affecting plantlet regeneration from axillary buds of *Quercus acutissima* (kunugi) nodal explants, derived from stump sprouts (mature materials), were investigated. Shoot growth varied with cytokinin's types and their concentrations on Sommer *et al*'s medium. BAP stimulated both shoot elongation and bud multiplication. A supplement of 2.0 μM of zeatin was most effective for shoot elongation in all the tested media. The supporting material of the rooting medium affected root initiation and the following acclimatization. Vermiculite was most suitable for rooting and acclimatization among the three tested supporting materials.

Quercus acutissima (kunugi) is one of the important broadleaved forest trees in Japan. And it is vital to propagate its superior clones (plus trees). However it is difficult to propagate it conventional methods. Previously the tissue culture technique has been applied, though, most reports were concerned with young materials.[6)7)11)13)16)] Recently, some authors have reported successful plant regeneration from mature materials.[5)8)9)] They showed the possibility of *in vitro* rapid propagation from selected *Q. acutissima* plus trees. However it remains less than clear about the suitable conditions for *in vitro* plantlet regeneration from mature materials, such as stump sprouts.

The purpose of this paper was to survey the effect of several culture conditions of nodal explants derived from stump sprouts (mature materials).

Materials and Methods

Plant materials - Explants were taken from stump sprouts of 13 - year - old stumps. These stumps were originated from open-pollinated seeds which were collected and sown in Kanto Forest Tree Breeding Institute. They had been cut from ground level every year since 1986. In

1990, they were grown under dark conditions. Dark treatment (etiolation) was carried out by covering stumps with a vinyl tent (about 150 lux per day inside) for reducing contamination of explants.[10] Before sprouting in spring, the stumps were covered with a vinyl tent. New sprouts were collected in June (for experiment 1) and August (for experiment 2).

After removing the leaves, all sprouts were cut into 20 - 25 mm long nodal explants. Explants were first washed in tap-water for 1 hour to remove explant debris. They were then sterilized by 15 minutes immersion in 1 % sodium hypochlorite solution, followed by three washes in sterile distilled water. After cutting 2 mm from both sides of the explants, they were placed vertically in test tubes (18×150 mm) containing 10 ml of the medium. Explants were precultured for three days in a cytokinin free medium of the same nutrient composition to prevent the experimet from being contaminated.

Culture media and culture conditions - The basal medium used for all experiments was SOMMER *et al*'s medium [SBK].[17]

In experiment 1, various types and concentrations of cytokinin as a for growth regulator were added to the media : 6 - benzylaminopurine (BAP), 6 - (4 - hydroxy - 3 - methyl - trans - 2 - butenylamino) purine (zeatin), N - (2 - chloro - 4 - pyridyl) -N' - phenylurea (CPPU), 6 - (r, r - dimethylallylamino) purine (2 iP). Each treatment involved 22 tubes (explants). All media contained 3 % of sucrose and 0.8 % of agar.

In experiment 2, nodal explants were cultured on 8.0 μM of BAP containing medium for bud multiplication. After four weeks of culture, they were transferred to 2.0 μM of zeatin containing medium (40 ml medium in 200 ml size culture bottle) for shoot elongation. These media contained 3 % of sucrose and 0.8 % of agar. After four weeks of cultrure, elongated shoots over 25 mm in length were excised from explants. After dipping the basal end (5 mm) for 25 min-utes in 1.0 mMl^{-1} (about 200 mgl^{-1}) 3 - indolebutyric acid (IBA) solution, they were transferred to the rooting media. The rooting media, in 400 ml size culture bottle, containing three different types of supporting materials : 0.6 % agar [A], 40 g of vermiculite [V], and 20 g of vermiculite+20 g of porous soil "Kanumatsuchi" (V + K). Each medium was supplemented with 60 ml of a half strength SBK medium containing 1 % sucrose. The shoots were transplanted to these media under *in vitro* or *ex vitro* (in laboratory) conditions. Each treatment involved 28 explants (7 bottles). After six weeks of culture, the rooting percentage was in vestigated.

The pH of each medium was adjusted to 5.7 with 0.1 N KOH and HCI, before autoclaving.

Cultures were kept at 25 C with a 16 h light period (3200 lux).

Results

Experiment 1. Effect of cytokinin's types and their concentrations.

The results are summarized in Fig. 1.

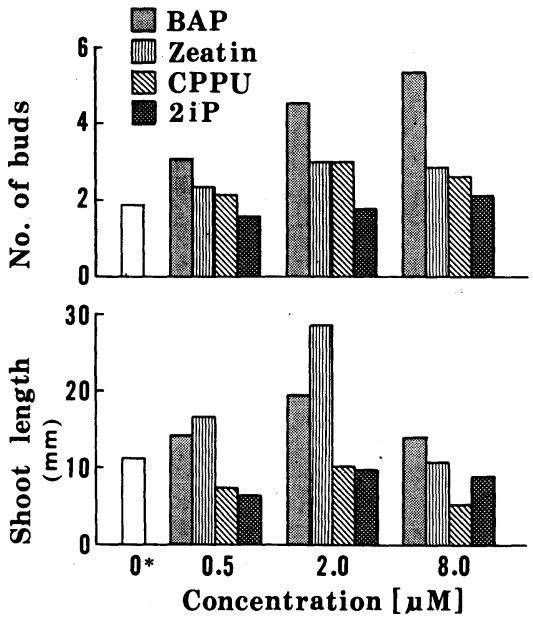

Fig. 1 Effect of interaction of cytokinin's type and its concentration on shoot growth from axillary buds of *Quercus acutissima* (kunugi) cultured *in vitro* for six weeks.

* Cytokinin free medium (control).

Shoot growth varied according to the cytokinin's types and their concentrations. The media containing BAP or zeatin almost gave good results. CPPU induced the largest calli at the basal end of the explants, and on 8.0 μM of CPPU containing media, some shoots showed abnormal growth (vitrification). A supplement of 2 iP did not affect shoot growth.

BAP stimulated bud-breaking, according to the increment of its concentration in the range of 0.5 to 8.0 μM. Multiple bud formation occurred on the media containing 2.0 or 8.0 μM BAP. Zeatin and CPPU also stimulated the bud-breaking, but the effect was inferior to that of BAP.

Compared with cytokinin-free control, shoot elongation was remarkably great with sup-

plementing BAP or zeatin in the range of 0.5 to 2.0 μM. On 8.0 μM of BAP or zeatin containing media, shoot length was decreased, compared with lower concentrations. The supplement of 2.0 μM zeatin was most effective for shoot elongation. A supplement of CPPU inhibited the shoot elongation.

Experiment 2. Effect of culture condition and supporting material for root formation. The results are shown in Table 1. and Fig. 2.

Table 1. Effects of culture condition and media substrates for root formation after six weeks cultures.

Culture codition	Substrate of media *	Number of explants without contamination	No. of explant formed root (%**)		
			Type I #	Type II #	Total
in vitro	A	28	2 (7.1)	6 (21.5)	8 (28.6)
	V	28	7 (25.0)	2 (7.1)	9 (32.1)
	V+K	28	2 (7.1)	2 (7.1)	4 (14.2)
ex vitro	V	22	4 (18.2)	2 (9.1)	6 (27.3)
	V+K	21	2 (9.5)	1 (4.8)	3 (14.3)

* Details are shown in the text.

** (No. of explants root formed / No. of explants without contamination) ×100

\# Root types are shown in Fig. 2

Fig. 2 Root formation on the shoot cultured for six weeks. Left ; root type I. Right ; root type II

Roots were classified into two types (Fig. 2). Type I : Roots were thin and well branched,

and Type Ⅱ : Roots were thick and short.

In vitro condition - On agar medium (A), callus was formed at the shoot base, but on soilbased media (V and V+K), almost no callus was formed at the basal end of the shoot. Both A and V were suitable for rooting compared with V + K. But their main root types were different. Most shoots on medium A formed root type Ⅱ, but on medium V, the main root type was Ⅰ.

Ex vitro condition - Some shoots were damaged by contamination and the total number of root - formed explants were reduced. But the tendency of rooting was the same as the *in vitro* condition. Medium V was superior to V+K.

The rooted plantlets were rinsed gently and transplanted to fresh vermiculite media in pots, and they were moved under greenhouse condition for acclimatization. The plantlets were protected from direct sunlight with a net and periodically fertilized with a liquid solution of 1/10 strength SBK. After four weeks, 23.5 % of the plantlets of root type Ⅰ had survived, but all plantlets of root type Ⅱ had died.

Discussion

Sommer et al's [SBK] medium is modified Gresshoff and Doy's medium[4]. These media were widely used for *in vitro* culture of *Quercus* species.[2)11)12)14)15)18)] In *Q. suber* culture, Manzanera and Pados[12] reported that in the case of juvenile origin, mineral composition of the medium was not important during the establishment of cultures, but in the case of adult materials, Sommer *et al*'s medium was the best.

Little attention has been given to the effect of cytokinin's type to *Q. acutissima* nodal explant cultured *in vitro*. In general, many authors have used BAP, sometimes in combination with α - naphtylacetic acid, as the growth regulator in the tissue culture of the nodal explant of *Q. acutissima*.[5)~9)11)]

Those results showed that a supplement of BAP in the range of 0.1 to 2.0 mgl^{-1} (0.44 to 8.9 $\mu M\,l^{-1}$) gave good results. The results of experiment 1 support them. Compared with the other three cytokinins, BAP was most effective for stimulating both shoot elongation and bud multiplication. Zeatin also advanced shoot growth. Especially, the shoot elongation on 2.0 μM zeatin containing medium was the best of all treatments. San - Jose *et al*[14] reported in *Q. robur* clones that reduction in BAP concentrations relative to that in the multiplication medium, had no effect on shoot elongation. But in one of two clones tested, shoot elongation

from multiple buds had been significantly stimulated when the multiple buds were transferred to zeatin containing media before being transferred to rooting medium. A similar res ult was also reported in *Q. suber*.[15] CPPU (4 PU), one of the pyridyl phenylurea compounds, appear to have strong cytokininlike effect on a wide range of species and on species that respond little to conventional cytokinins.[3] It seems that CPPU was too strong for shoot regeneration from nodal explant. 2 iP had no effect on shoot growth. Similar result was reported in *Q. shumardii*.[1] These observations and the present results may indicate that there is a common tendency among *Quercus* species about cytokinin's type for optimal nodal explant culture *in vitro*.

For practical micropropagation of Q. acutissima from nodal explants, it is important to determine the suitable culture conditions, such as the cytokinin's type and concentration, for each culture stage ; a) inducing multiple buds from initial explant, b) subculturing and multiplicating of buds, c) elongation of shoots from multiple buds, d) rooting from shoots , and e) hardening of plantlets. The data of this report indicates that BAP containing SBK media is suitable for stage a), and zeatin may be useful for stage c).

The physical characteristic (supporting material) of the medium was very important for root initiation. Both agar and vermiculite gave good results for the rooting percentage. But in consideration of the following acclimatization stage, vermiculite was most suitable.

Ex vitro rooting will be useful for reducing culture periods, although the risk of fungal contamination is greater than with *in vitro* rooting. Bennett and Davis[1] succeeded in simultaneous rooting and acclimatization in juvenile materials of *Q. shumardii*. Many authors have repored that there were many factors affecting the rooting stage.[1)9)11)12)13)] Further studies to clarify the most suitable conditions for each stage are needed for practical use of *ex vitro* rooting.

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摘　　要

クヌギ成木萌芽枝の腋芽からの植物体再生に及ぼす要因について検討した。SOMMER らの培地上でのシュートの成長は培地に添加したサイトカイニンの種類と濃度に影響を受けた。ＢＡＰはシュートの伸長および多芽体形成の双方に効果があった。シュートの伸長はゼアチン2.0 μM 添加区が最も良かった。供試した３種の発根培地（寒天，バーミキュライト，バーキュライト＋鹿沼土）のうちでは，バーキュライト培地が発根とその後の順化に最も適していた。