An Efficient Method of Producing Callus and Plantlets by Tassel Culture for Sugarcane Breeding*

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Introduction

After the discovery of organ formation in sugarcane tissue culture by Nickel[10,11] and his suggestion regarding its potential applications in research, Heinz and Mee (1969) developed effective methods of rearing plantlets through artificial culture of leaf, shoot apex and inflorescence tissue of sugarcane using a 2,4-D medium. Since their work, several attempts at applying tissue culture methods in sugarcane improvement[5,6,7,8] have been carried out and it has been shown that genetical trends of somatic cells are changed through dedifferentiation in tissue culture, and many chromosomal or genic variants have been reported.

In Japan, sugarcane is grown as a main crop in subtropical region, and its breeding work has been carried out at Tanegashima (lat. 30°35'N) and Okinawa (lat. 26°30'N). However, flowering of sugarcane varieties is seriously restricted at these sites because of insufficient temperature in the flowering season. The authors have observed that only 39.7% of 131 varieties introduced from different places of the world showed emergent tasseling even in the warm site, Ishigaki (lat. 24°25'N). Moreover, this percentage decreased to 23.3% when observations were limited to 31 varieties of tropical origin. Further, the number of varieties which would produce viable gametes and therefore be usable as parent material for crossing should, in all likelihood, be even more limited. Thus, the production of genetic variation by hybridization of sugarcane in Japan faces a great difficulty.

From a cytological aspect, sugarcane has a great peculiarity both in chromosome constitution and inheritance. It has been considered that sugarcane is a high level polyploid accompanied with wide-ranged aneuploidy having chromosome numbers of about 80 to 140. Past researches have shown that the genome multiplication and production of aneuploidy occur in long evolutionary processes[2,13]. This nature has been demonstrated also through artificial interspecific hybridization[1]. In this way, commercial varieties of sugarcane come to have a great complexity in chromosome constitution[14]. Therefore, manipulating chromosome variation is expected to enlarge the genetical variation in sugarcane breeding [3,9,12,15].

On the basis of foregoing premises, the authors attempted to utilize tissue culture methods in sugarcane improvement. In the present work, the authors have established an efficient tissue culture method for rearing a number of plantlets from the callus using tassel tissue as explant material.

Materials and methods

Young tassels of NCo 310 in the booting stage, still inside the flag leaf sheaths, were used as materials (Fig. 1). These tassels had developed to about 40 cm in length and formed spiklets in different microsporogenetic stages

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from meiotic prophase to the pollen stage. They were taken from the plants for explant material as follows:

The top of the stem with exposed flag leaf node about 5 cm long and with wrapped, well developed, young tassel inside was collected from the plant in the field. After the leaf blades were cut off, leaf sheaths up to the innermost one were removed one by one, while each surface was sterilized with gauze dipped in 70% alcohol before removal. The final leaf sheath was removed under sterile condition (in a clean bench) using the same surface-sterilization and the clean young tassel was detached. The detached tassel was aseptically prepared for explant material in the following way without using sterilants:

The clean tassel was cut crosswise in 10 to 15 sections each about 3 cm long, and each further divided lengthwise into about 10 blocks containing 3 to 5 rachis branches bearing many young spikelets. Each block was transferred in a group to a hormone-enriched nutrient media prepared in 50 ml Erlenmeyer's flasks or test tubes 18 mm in diameter.

For nutrient media, modified Murashige and Skoog's preparation was employed as the basic medium (Table 1). Three hormone treatments were made to examine the effects on induction of callus and organ as follows: α-Naphylacetic acid (NAA), 2,4-Dichlorophenoxy-acetic acid (2,4-D) or 6-Benzyladenine (BA) were added singly to each treatment in the concentration shown in Table 1. The media were adjusted to pH 5.2 with 1N potassium hydrochloride solution and were autoclaved for 25 minutes at 1.2 kg/cm² of pressure. The explants were kept for 35 days in a glass-walled incubator (without artificial illumination) which was adjusted at 28 to 30°C and placed in the laboratory.

Results

Comparison of callus development and organ initiation in the cultures with three hormone treatments are shown in Table 2 and Figs. 2–4. As shown, callus production was 78% with NAA treatment, 100% with 2,4-D treatment and 0% with BA treatment. The calluses showed vigorous growth, and developed to chain-like callus groups surrounding whole explant tissue. Callus induction began at about seven days after explants were transferred to the media. These calluses were first induced on the node of pedicels and of rachis branches

Table 1. Composition of the culture media used

Constituents	Concentration
Inorganic constituents	Murashige and Skoog's preparation
Organic constituents	
Glycine	2.0 mg/l
Nicotinic acid	0.5 mg/l
Pyridoxine-HCl	0.5 mg/l
Thiamine-HCl	0.1 mg/l
Inositol	200 mg/l
Casamino acid	5 g/l
Sucrose	30 g/l
Agar powder	9 g/l
Hormones	
α-Naphtylacetic acid (NAA)	10 mg/l (5.4×10^{-5} mol)
2, 4-Dichlorophenoxy-acetic acid (2, 4-D)	5 mg/l (2.3×10^{-5} 〃)
6-Benzyladenine (BA)	1 mg/l (4.2×10^{-6} 〃)

Note: (1) pH was adjusted to 5.2
(2) Each hormone was used singly, not in combination.

Table 2. Callus induction and organ formation by hormone treatment in tassel culture

Hormone	No. of flasks inoculated	No. of flasks showing callus induction	Callus development						Organ induction	
			Flasks observed	Degree*					Root	Bud
				0	I	II	III	IV		
NAA 10 mg/l	18	14(77.7%)	11			2	8	1	+	+
2,4-D 5 mg/l	20	20(100.0%)	13		1	1	6	5	—	—
BA 1 mg/l	18	0(0.0%)							—	—
Hormone free	20	0(0.0%)							—	—

* Callus development was rated by five degrees as follows: 0; no induction. I; less than 20%, II; 20 to 50%, III; over 50%, and IV; explant tissues showing overall induction.

and at the cut edge of explant tissue. After this first callus induction, new calluses were continuously formed on the glumes and internode tissues. These were apparently clusters of callus cells induced successively on the tissue of the internodes or glumes and not that developed from the first initiated callus, because they were tightly connected with the explant tissue. In contrast, inner organs of inflorescence did not show any dedifferentiational change even under the microscope. Especially the anthers, which had the potential of haploid callus production, were shrivelled and gave no sign of forming callus. Comparing the two effective treatments, 2,4-D-induced callus showed better growth than that induced by NAA, although there were no observable differences in the external morphology.

The 6-Benzyladenine treatment did not show any positive effect on callus or organ initiation, while biological effects including swelling of the ovarian portion of the glumes and exudation of anthocyan from explant tissue were observed.

Following callus initiation, frequent formation of bud and root primordia was observed on NAA-induced calluses. Organ initiation was first observed a few days after callus initiation and continued along with callus development. On the 35th day of final observation new primordia were still initiating among old developed buds on the callus. In the early days of organ formation primordia were formed on thinly developed callue and it was not always clear whether they originated from the callus cell or from intact explant tissue. Further, it was observed that some primordia had bundle tissue connections with the internode and showed apparent germination of dormant explant buds. However, organs initiated later were formed on thickly developed callus and organ bearing callus sections were easily detached from the callus mass with forceps. These sections had no tissue connection with the explant (Fig. 4), indicating that organs initiated later were originated from callus cells. Final observations showed a large portion of callus surface covered with developed plantlets and newly forming organ primordia.

From these cultures an average of 30 plantlets were grown per flask with maximum plantlet development so numerous as to be uncountable (Table 3, Fig. 5). Plantlets thus obtained were transferred to sterilized vermiculite nursery flats and safely reared to seedling size adequate for transplanting to the field.

Table 3. Development of plantlets originated from NAA-induced callus of tassel culture

No. of flasks observed	No. of plantlets/flask				
	0	1–10	11–20	21–30	thickly grown
11	1	2	1	3	4

Note: Observations were made 33 days after inoculation.

2,4-D-treated callus produced no organs at the concentration used even after a one year culture period although callus growth was better than that of the NAA treatment.

Discussion

In addition to the present work, the authors have repeatedly continued to culture tassel tissue for rearing a tissue-cultured population. Rate of success as high as 90% was obtained constantly in callus and plantlet development with more than five hundred test tube cultures. However, from the preliminary culture of leaf, leaf sheath and shoot apex sections in one hundred test tubes using the same media as with tassel tissue, for screening explant materials, callus production was 22.2% and plantlet induction was only 10.7%. From this comparison it would seem that the tassel tissue has a high physiological dedifferentiation potential and that callus cells derived from this organ also have a high differentiability.

In addition, tassel tissue offers considerable advantage for mass culture because of its huge size. The tassel, at booting stage, is about 40 cm in length with a number of rachis branches. Using our explanting methods, about 130 test tubes (18 mm diameter) can be inoculated with material from a single tassel. It is estimated that about 1300 plantlets can be grown from the culture of single tassel because an average of ten plantlets was obtained from each test tube in the above mass culture.

Further, the ease of sterilization in preparing explant materials would be one of the merits for mass culture. The tassels, at booting stage, are kept in a completely sterile status within the leaf sheaths. While complicated sterilizing procedures are needed for other organs, very few of the inoculated tassel explants were contaminated with microorganisms even though only a 70% alcohol leaf sheath surface sterilization technique was used.

The advantages of using explant tassel tissue mentioned above would contribute to the development of a greater sized population of tissue culture derived sugarcane. As genetical change has been obtained practically in past culture of various organs[3,7] tassel tissue-cultured sugarcane would widen the genetical basis for improvement and, through mutagenic treatment of the larger population, the genetical variation could also be enlarged.

In the work reported here only one level of concentration of a single hormone was used. In experiments on hormone concentration now in progress, lower levels of 2,4-D show abundant primordia induction. Also in hormone combination experiment now in progress, callus development and organ induction are much accelerated by combining NAA with Indoleacetic acid. Detailed results will be reported in the next paper.

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Fig. 1. Young tassel material striped at booting stage

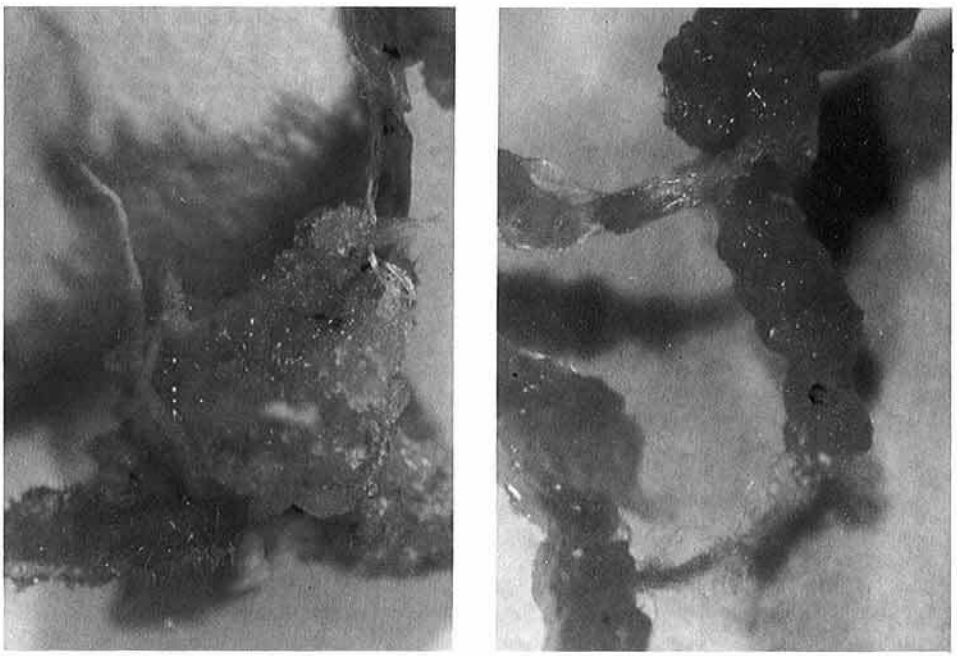

Fig. 2. Left: NAA-induced callus with root elongation from explant and green bud primordia
Right: 2,4-D induced callus developing in chain-like mass

Fig. 3. Early organ formation from the callus developed with NAA treatment

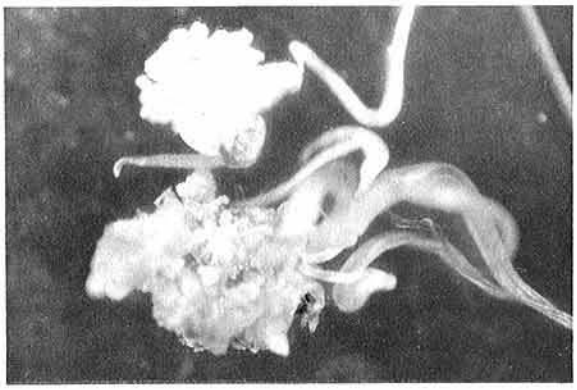

Fig. 4. Callus sections detached from NAA-induced callus mass. Plantlets from callus are shown

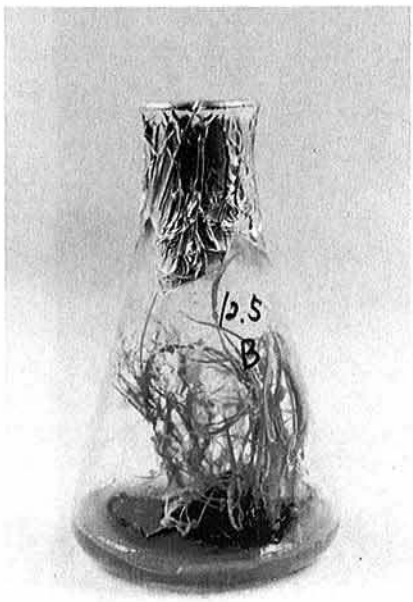

Fig. 5. Plantlets grown from tassel tissue explants with NAA treatment

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