

Effect of Pre-treatment with Excess Sucrose or Mannitol on Plant Regeneration from Rice Callus Cultures

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Abstract : The formation of white and compact rice callus cultures was promoted by preculturing mature seed-derived cells on the medium containing a high concentration of sucrose. The obtained white and compact callus had a high regenerating ability on the regeneration medium containing 5×10^{-5} M kinetin. When mannitol was added to the medium at the same osmotic level as excess sucrose treatment, it offered the intermediate effect between 3% (control) sucrose and 6% (high concentration) sucrose. Scanning electron microscopic examination revealed that the calli which retained the high regenerating ability had a tendency to show a smooth-surfaced appearance.

Key words : Callus culture, Mannitol, *Oryza sativa*, Plant regeneration, Scanning electron microscopy, Sucrose, Water stress.

イネカルス再分化に及ぼす過剰蔗糖あるいはマニトールによる前処理の影響：樋口暢宏・前田英三（名古屋大学農学部）

要 旨：イネ種子胚盤由来のカルスを高濃度の蔗糖を含むMS培地で前培養することにより、カルスの白色・コンパクト化が促進され、得られたカルスは植物体再分化培地に移植することによって効率よく、植物体に分化するのが観察された。またマニトールを添加することによって、培地の浸透ポテンシャルが高濃度蔗糖処理と同じになるように調節し水ストレスをかけたところ、対照カルスより高い植物体再分化効果が得られたが、高濃度蔗糖処理ほどの効果は認められなかった。走査電子顕微鏡を用いた観察によると、高い植物体再分化能をもつカルスは滑面構造を高頻度に有する傾向があった。

キーワード：イネ、カルス培養、植物体再生、蔗糖、走査電子顕微鏡、マニトール、水ストレス。

There is currently considerable interest in the importance of the osmotic role of a culture medium in plant regeneration from callus tissues. At present, sucrose has almost been used as a carbon source in the plant culture medium. Carbohydrate supplied to the medium not only acts as a source of carbon and energy but also as an osmotic agent during organogenesis. Although the role of water potential is not clear, it is known that water stress influences callus growth, colony formation, plant regeneration, somatic embryogenesis and the metabolism of specific compounds such as abscisic acid (ABA), proline, etc. in several culture systems. For instance, Milborrow²⁴⁾ showed that the endogenous level of ABA rises quickly and drastically when intact plants are subjected to water stress, and we know a similar effect even in callus tissue cultures³³⁾.

In the previous report¹¹⁾, we demonstrated the effects of ABA in the preculture medium on rice callus growth and its plant regenerating activity. ABA treatment inhibited callus growth and stimulated the formation of white,

dry and compact regions on the callus. Moreover, this white region of calli showed higher potential to regenerate plantlets on the regeneration medium.

The present report describes the effect of both kind and quantity of carbohydrate in the medium on plant regeneration from rice callus, especially the morphological effects leading to plant regeneration by scanning electron microscope (SEM) observation. And we discuss concurrently the possibility that water stress will offer ABA-like effects on plant regeneration.

Materials and Methods

Husked mature seeds of *Oryza sativa* L. (var. Nipponbare) were cultured as previously described¹¹⁾. The medium designated here as basal medium consisted of MS major and minor salts²⁵⁾, 78.4mg/l FeEDTA, 200mg/l myo-inositol, 1mg/l thiamine-HCl, 3g/l casein hydrolysate, 3% sucrose and 0.65% INA-agar (BA-10). Subcultured calli were inoculated on the preculture medium, basal medium plus 10^{-5} M 2,4-dichlorophenoxy-

acetic acid (2,4-D) and various concentrations of sucrose and/or mannitol as a carbohydrate and/or osmoticum. The osmolarity of each preculture medium was measured with OSMOTRON-5 (Orion Riken Co.) (Table 1). After this preculture, the calli were transferred on the regeneration medium, basal medium plus 10^{-7} M 2,4-D and 5×10^{-5} M kinetin. All cultures were kept in a culture room at 25–27°C under continuous light of about 3,000 lux with fluorescence tubes.

For SEM observation, the materials were fixed with modified Karnovsky's fixative¹⁴⁾, which consisted of 3% glutaraldehyde, 1.5% paraformaldehyde and 0.1M Sørensen's phosphate buffer (pH 7.2), by gently shaking at a room temperature for 5h. The materials were rinsed repeatedly with the above buffer and dehydrated with a graded ethanol series. The dehydrated materials were treated with isoamyl acetate and critical point dried with HITACHI HCP-1. They were coated with gold using ion sputtering equipment, EIKO IB-3 and examined with a SEM, HITACHI S-415.

Table 1. Osmolarity of the preculture medium.

Sucrose (%)	Mannitol (%)	Osmolarity (mOsm)
3	0	190
6	0	280
9	0	376
3	1.63	282
3	3.26	378

Osmolarity of the preculture medium which consisted of basal medium containing 10^{-5} M 2,4-D except that agar was omitted and measured before autoclaving.

Results

We have studied the effect of higher concentrations of sucrose or mannitol in the preculture medium on callus growth, its morphology and its potential to regenerate. As shown in Table 2, the callus growth as measured by fresh and dry weights, decreased with increases in the concentration of sucrose. On the other hand, callus growth was slightly inhibited with the addition of mannitol as compared with excess sucrose.

The reduction of callus growth was also associated with the appearance of particular regions with white, compact and dry characters in the callus about 7 days after transfer onto the medium. However, the frequency of the appearance of such "white regions" differed with each of the treatments. Table 3 demonstrates that higher concentrations of sucrose strongly promoted the formation of the white regions. The white regions were further separated into two types macroscopically: Type 1; The degree of dryness was slight and the color of the callus was pearl white in Fig. 1. Type 2; The degree of dryness was high and the color of the callus was white in Figs. 1 and 2. Especially, mannitol treatment stimulated the formation of the latter type of white regions. These white regions frequently had many starch grains through histochemical investigations (data not shown).

SEM micrographs of such white regions shows mostly smooth-surfaced appearance in Figs. 3 and 5. These structures corresponded to the Type 1 white callus. Superficial appearance in this type of callus was composed of small spherical cells. In addition, we could observe non-membranous structures with several pores at a low frequency in Figs. 4 and 6.

Table 2. Effect of sucrose and mannitol treatment on rice callus growth.

Treatment in preculture		Fresh weight (mg)	Dry weight (mg)	Water content (%)
Sucrose	Mannitol			
3%	0	400.2 ± 78.4	27.3 ± 5.5	93.2
6%	0	293.7 ± 95.0	29.8 ± 8.0	89.9
9%	0	239.8 ± 98.0	30.2 ± 12.8	87.4
3%	1.63%	341.6 ± 103.0	31.9 ± 7.7	90.7
3%	3.26%	341.5 ± 103.0	36.8 ± 10.7	89.2

Data was collected after 25 days culture on the preculture medium. Fresh weight of initial inoculum was approximately 30mg.

Table 3. Effect of sucrose and mannitol treatment in the preculture medium on the formation of white region in rice tissue culteres.

Treatment in preculture		Average number of white regions/callus
Sucrose	Mannitol	
3%	0	0.4
6%	0	3.6
9%	0	3.8
3%	1.63%	1.0
3%	3.26%	1.3

Data was collected after 25 days culture on the preculture medium.

These structures appear to correspond to the Type 2 white callus. Fig. 4 shows that there were some elongated cells on the callus surface. On the other hand, when cultured on the medium containing only 3% sucrose as a carbohydrate, the calli were usually yellow and wet in appearance, and the formation of white regions was rarely observed as presented in Table 3. In this report, this type of calli is referred to as "control callus".

After 25 days of inoculation on the preculture medium, the calli were selected according to differences in color and texture and the white regions predominantly transferred on the regeneration medium in order to obtain plantlets. Since the control calli scarcely had white regions, only the yellow regions of such calli were transferred in this case. As shown in Table 4, the calli precultured on the medium containing 6% sucrose showed the highest percentage of adventitious bud formation. When the concentration of sucrose was raised to 9%, a similar result was obtained with respect to adventitious bud formation. Nevertheless, the viability of plantlets derived from 9% sucrose treatment was low and a large proportion of plantlets were dwarf and/or twisted. The regenerating ability of the white regions formed in the preculture with mannitol was not as good as that precultured in a higher concentration of sucrose. However, this culture gave rise to adventitious roots at a low frequency on the regeneration medium. On the other hand, when the callus remaining yellow on the preculture medium containing excess sucrose or mannitol was transferred on

Table 4. Effect of sucrose and mannitol treatment in the preculture medium on regeneration of rice callus.

Treatment in preculture		% of calli forming adventitious buds	% of greening calli
Sucrose	Mannitol		
3%	0	16.9	37.5
6%	0	76.0	80.0
9%	0	74.8	83.6
3%	1.63%	43.8	50.5
3%	3.26%	57.8	63.2

Data was collected after 25 days culture on the regeneration medium. Each of figures represents mean of three replicates.

the regeneration medium, their plant regenerating activity was less than that of the control callus (data not shown).

As mentioned above, 6% sucrose at the preculture was significantly superior to all other treatments to obtain plantlets. Hence, we have examined the effect of the timing of excess sucrose treatment on regenerating ability. Table 5 demonstrates that the preculture treatment was most suitable for plantlet regeneration, which was cultured on the regeneration medium with or without excess sucrose treatment. When the excess sucrose treatment was carried out at the regeneration stage only, the white regions, which were similar to the case of preculture, formed on the callus about 7 days after inoculation on the regeneration medium and plant regeneration was stimulated.

Discussion

In the present investigation, we focused on the importance of carbohydrates in the medium on rice callus cultures. At present, sucrose has almost been used as a carbon source in the plant culture medium. The roles of sucrose in the medium are thought to be both 1) the energy source for callus growth and plant regeneration and 2) the agent giving the osmotic potential as a constituent factor of water potential. In order to make clear the role of sucrose, we also use mannitol, which is known to be nearly metabolically inert in the rice callus, as an osmoticum in the medium.

Callus growth was inhibited with all treat-

ments, especially excess sucrose treatments. In rice anther culture, higher concentrations of sucrose have been found to stimulate dedifferentiation, that is, callus formation²⁸⁾. However, there is no consistency in the effect of low osmotic potential on callus growth. Wang³²⁾ (*Zea mays*), Chandler and Vasil⁴⁾ (*Pennisetum purpureum*), Ahloowalia and Maretzki¹⁾ (sugarcane) and Brown et al.³⁾ (tobacco) reported the inhibitory effect of higher concentrations of sucrose in several callus cultures. Contrary to these results, Upper et al.³⁰⁾ and Kimball et al.¹⁷⁾ showed the opposite effects in tobacco and soybean calli, respectively.

High levels of sucrose were effective for the formation of the white and compact callus. This observation is consistent with the result of Wang³²⁾. In addition, there are some reports where such treatment has been shown to stimulate the formation of the embryogenic callus^{19,22,23)}. In these reports, these embryogenic calli are found to be white and compact, which is macroscopically similar to that obtained in the present study. Moreover, we found these white and compact regions to have many starch grains through histochemical study. In this respect, a similar effect has been noted in *Picea abies* callus cultures³¹⁾. However, Ho and Vasil¹²⁾ reported that high concentrations of sucrose inhibited the formation of highly regenerative white calli in sugarcane.

Callus growth was slightly inhibited with the addition of mannitol as compared with the excess sucrose treatment as shown in Table 2. These results suggest that the white callus formation in the excess sucrose treatment is partly caused by the low osmotic potential of

the preculture medium. We have known no reports dealing with the effect of the low osmotic potential on the formation of white regions in callus tissues. However, Ozias-Akins and Vasil²⁶⁾ and He et al.¹⁰⁾ showed that the white embryogenic callus formation in *Triticum aestivum* tissue cultures could be increased on a higher level of MS salts media rather than lower level media. Therefore, we cannot exclude the possibility that osmotic/water stress plays a role in the formation of the white callus.

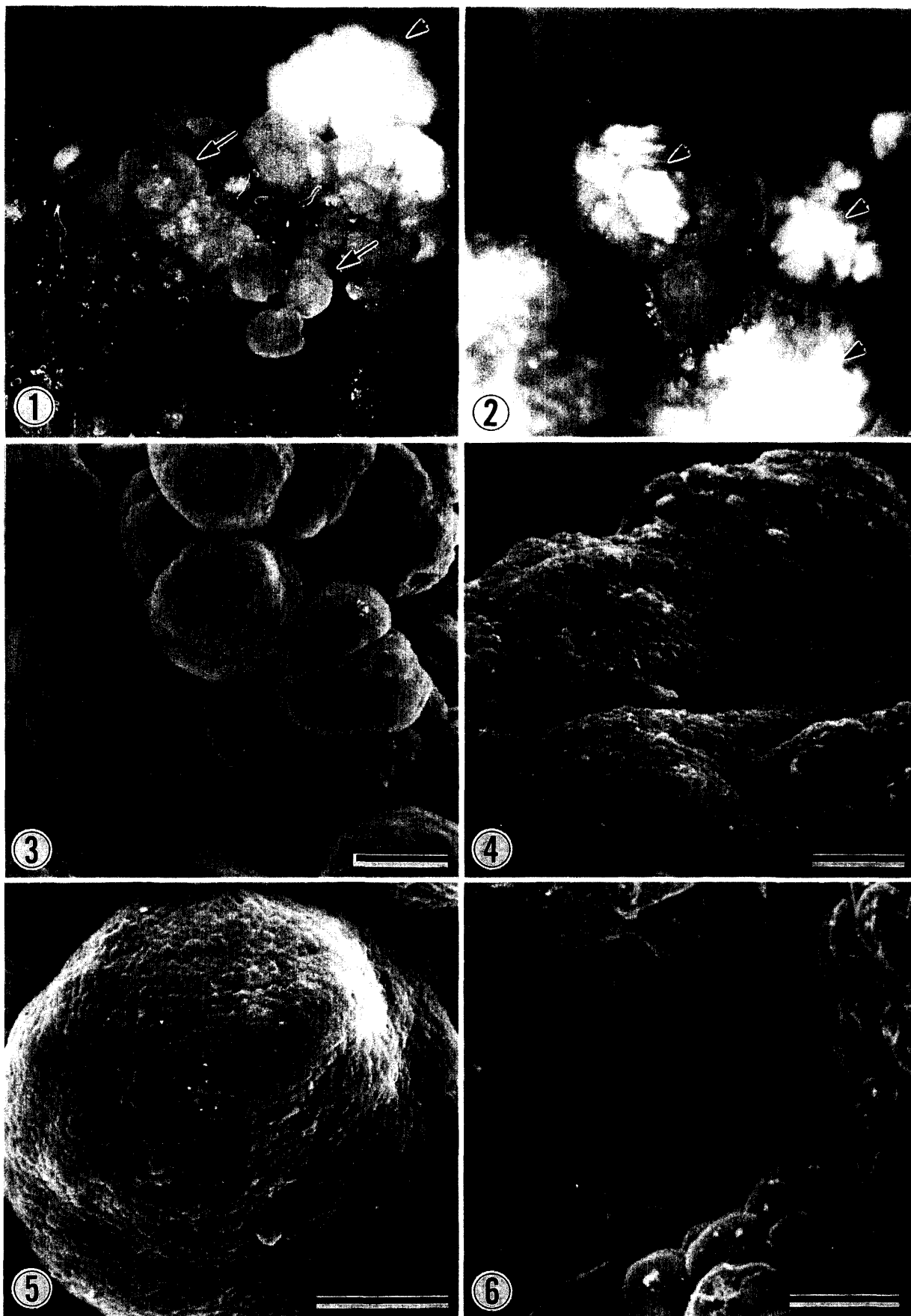
Table 4 demonstrates that the white calli formed with the excess sucrose treatment have the highest potential to regenerate plantlets of all the treatments. However, the yellow calli cultured with the same treatment did not show such high ability (data not shown). Therefore, the advantage of the excess sucrose treatment is thought to be largely due to the accumulation of starch grains during the treatment. This result is in agreement with some other discussions where the accumulation and the subsequent disappearance of starch are closely correlated with organogenesis in several plant culture conditions^{27,29)}.

The preculture treatment with higher sucrose was more suitable for plantlet regeneration than the regeneration treatment, as shown in Table 5. In addition, when the excess sucrose treatment was carried out at the regeneration stage only, the white regions, which were macroscopically similar to the case of preculture, formed on the callus about 7 days after inoculation on the regeneration medium and the stimulatory effect was smaller than that of the preculture treatment. These results also depict the importance of the formation of white regions, that is, the starch grain accumu-

Table 5. Effect of sucrose treatment in the preculture medium and/or the regeneration medium on regeneration of rice callus.

Preculture medium (%)	Regeneration medium (%)	% calli forming adventitious buds	% greening calli
3	3	10.0	32.0
3	6	34.0	58.0
6	3	70.0	86.0
6	6	70.0	76.0

Calli were maintained on the preculture medium each containing a concentration of sucrose as a carbohydrate and/or osmoticum for 25 days, and then transferred on the regeneration medium each containing a concentration of sucrose. Data was collected after 25 days culture on the regeneration medium.



lation at the preculture with high concentrations of sucrose.

However, it was not necessary to transfer callus tissues to the regeneration medium containing high amounts of sucrose at all. With respect to plantlet development, the regeneration treatment showed inhibition rather than stimulation, Hammersley-Straw and Thorpe⁸⁾ and Ahloowalia and Maretzki¹⁾ showed the comparable inhibitory effects on plant development in tobacco and sugarcane, respectively. Several workers have found high levels of sucrose inhibited greening in callus tissues^{2,7,15)}, though our result is not consistent with them.

We have found some reports on the effects of low osmotic potential in plant tissue cultures. Close and Ludeman⁵⁾ reported the genotypic osmotic requirement to callus induction in maize. Lu and Thorpe²¹⁾ showed that lowering the osmotic potential of the medium promoted the maturation of somatic embryos of *Picea glauca*. Imamura and Harada¹³⁾ demonstrated the stimulatory effect on plant regeneration in rice anther culture. Liu and Lai²⁰⁾ also demonstrated that water stress promoted plant regeneration from the *indica* rice callus cultures.

In the present report, water stress at the preculture offers some advantages to plant regeneration from rice callus cultures and this effect is mediated by the formation of white and dry regions. Some reports have shown the increase in abscisic acid level with water stress to plant organ, tissue and tissue culture, that is, the root tips of *Pisum sativum* and *Commelia communis*³⁴⁾, leaf strips of several plants⁹⁾, detached roots of maize¹⁸⁾ and tobacco callus tissue^{6,13)}. We have already shown that the exogenous ABA treatment, which promoted

the formation of white regions like that obtained in the present experience, enhanced the regenerating ability of the rice callus¹¹⁾. We cannot then rule out the possibility that the stimulatory effect with water stress is mediated with the increase in endogenous ABA level. However, Kavi Kishor and Reddy¹⁶⁾ reported that the addition of sorbitol or mannitol to the subculture medium provided long-term regenerating activity in the *indica* rice callus and that such a promotive effect of sorbitol or mannitol could not be reproduced with other osmotic stress by adding various substances such as glucose, fructose, sucrose, sodium chloride or potassium chloride. According to their results, it is necessary to take account of the possibility that other factors play a role in the present results.

Any peculiar structures like a somatic embryo and embryoids were not observed microscopically at the pre-treated calli. Although the preculture with excess sucrose or mannitol allows the callus surface to be relatively smooth (Figs. 3 and 5), the white regions cultured with mannitol were found to have more non-membranous structures with pores than all of other treatments (Figs. 4 and 6). In the preculture with mannitol, the disadvantages of plant regeneration to the excess sucrose treatment might be attributed to these unorganized structures.

On the basis of our present experiment, the optimal response is obtained with the following procedure. After rice callus is treated with 6% sucrose at the preculture for 25 days, the formed white and dry regions are predominantly transferred on the regeneration medium. The superiority of the excess sucrose treatment is thought to be largely a consequence of the formation of starch grains. The

Explanation of Figures

- Figs. 1 and 2. Calli grown on the preculture medium containing 6% sucrose for 25 days. Type 1 white regions (arrows) and Type 2 white regions (arrowheads) are observed on the callus surface.
- Figs. 3 and 5. Surface view of Type 1 white callus grown on the preculture with 6% sucrose. Smooth surface and rather small superficial cells can be confirmed. (Fig. 3. bar 200 μ m and Fig. 5. bar 100 μ m).
- Figs. 4 and 6. Surface view of Type 2 white callus grown on the preculture with 6% sucrose. Rough surface and several pores can be seen. It is composed of relatively long cells. (Fig. 4. bar 200 μ m and Fig. 6. bar 50 μ m).

osmotic effect is also appreciated in the present experiment.

This procedure is superior to some of the different systems, including the two-step culture method with abscisic acid reported previously¹¹⁾. It may be useful to apply this excess sucrose system to other plant tissue culture systems having difficulty in plant regeneration.

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