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Increased Plant Regeneration Frequency in Water-Stressed Rice Tissue Cultures

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Abstract : Plant regeneration ability in cells of rice (*Oryza sativa* L.) was closely correlated with its water status. The water content of the highly regenerating callus of ANT 39 was about 10% less than that of the poorly regenerating callus of TN 5. Plant regeneration frequency of TN 5 callus was apparently increased when its water content was reduced by water stress treatment. Mannitol and higher concentration of agar were used as a water stress inducing agent. Assessment of changes in the proline content of callus suggested the effect of water stress.
Key word : *Oryza sativa*, Plant regeneration, Water stress, Young embryo.

水稲幼胚カルスの水分ストレスが器官再分化の増大におよぼす影響：賴　光　隆・劉　麗　飛（国立台湾大学農芸学系）

要　旨：水稲幼胚起源カルスを異なる濃度の寒天，マニトールで水分ストレス処理を行い，その後で器官再生の誘導を試みた．カルスの水分の様相は上述の処理で著しく異なることがみられたが，器官再生の効率が高い矮南早39号のカルスの水分含有率はその効率が低い台南5号と比べて，常に10% 程低い傾向を示すことも明らかとなった．また台南5号のカルスは，水分ストレスの処理で対照と比べて顕著に器官分化の増大を示すことが認められた．茎葉の水分ストレスで誘起されるプロリン含量の増大もカルスの水分処理で見られたが，このことは，カルスの器官再分化と水分ストレスの関連を考察する際，興味ある現象であると考えられる．

キーワード：器官再分化，水稲，水ストレス，幼胚．

Plant regeneration is a prerequisite for using *in vitro* techniques to study plant improvement. In rice as well as in other plants, genotypes and explant sources were often considered to be important factors in determining plant regeneration after culture[7,8,14,20]. But there is little discussion about what makes the difference of genotypes in regeneration ability. Plant regeneration of callus from young rice embryos has been studied and the variation of plant regeneration among rice cultivars has also been reported[12,13,15]. In previous works, we reported that shoot-forming tissues are always dry and compact when compared with non-shoot-forming tissues[13,15]. The others have indicated also that dry and compact calluses were suitable for shoot regeneration in rice[10,18]. These facts are suggesting that water content of callus may affect its regeneration.

There are several papers reporting that mild osmotic stress increases callus growth of soybean tissue cultures ; it also promotes shoot formation in tobacco callus[3,4]. Culture medium with higher sucrose is also reported to be good for the plant regeneration of corn[17], rye[16] and sugarcane[1] tissue cultures. In addition, enhanced adventive embryogenesis was reported in plasmolysis of cultured wild carrot cells[21]. All these imply the significance of osmotic stress in plant regeneration[6].

This investigation is thus conducted to observe the effect of water stress induction on plant regeneration in rice tissue cultures.

Materials and Methods

Two cultivars of rice (*Oryza sative L.*) were used. One is Ai-nan-tsao 39 (ANT 39), a readily regenerating cultivar, and another is Tainan 5 (TN 5), a poorly regenerating cultivar. Callus was induced from young embryos and subcultured at four week intervals according to an earlier method[12,13]. For induction of calluses, 10 to 12 embryos were cultured in a test tube of 3×15 cm containing 15 ml induction medium. As for the regeneration, two pieces of calluses induced were cultured in each test tube of 2×15 cm containing 8 ml regeneration medium[15]. The basal culture medium contained Murashige and Skoog (MS) salts, 100 mg/l myo-inositol, 1 mg/l thiamin-HCl, 1 mg/l pyridoxin-HCl, 10 mg/l

nicotinic acid, 30 g/l sucrose and 8 g/l agar (Merck). For callus induction and maintenance, the basal medium was supplemented with 5 μM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 10 μM indole-3-acetic acid (IAA).

Plant regeneration was conducted in the medium containing basic MS medium supplemented with 5 μM α-naphthyl acetic acid (NAA) and 10 μM kinetin. At least 40 callus were used in each treatment. Shoot and root regeneration was recorded four weeks after transferring callus to the regeneration medium. Shoot number was expressed as the number of shoot regenerated per callus.

For water stress induction, mannitol (0.4, 0.6M) and/or higher agar (12, 16 g/l) were supplemented into callus induction, maintenance and plant regeneration medium respectively. Supplements were added before autoclaving. All modified media are listed in Table 1.

Water content of callus was determined from $\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}}$ %. Dry weight was obtained by drying the callus in a ventilating oven at 80°C for 24 hrs. Proline content of callus was determined in accordance with Bates et al.[2] Briefly, 100 mg callus were homogenized in 5 ml of 3% aqueous sulfosalicylic acid, and then filtered through filter paper. Two ml of filtrate was reacted with 2 ml acid-ninhydrin and 2 ml glacial acetic acid in a test tube for one hour at 100°C, and terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, stirred for 15 to 20 sec. The absorbance of toluene was read at 520 nm using toluene as blank. Proline concentration was determined from a standard curve and calculated in terms of unit weight.

Results

Effects of mannitol and agar on plant regeneration

In ANT 39, root and shoot regenerations of almost 100% were observed from calluses induced on either control medium (DIA_8) or media supplemented with mannitol and/or agar (DIA_{16}, DIA_8M_6, $DIA_{12}M_4$), then transferred to regeneration medium KNA_8 (Fig. 1-I). A shoot number as high as 7.8 per callus was obtained in treatment DIA_8M_6. After subculturing twice on each medium, callus showed a reduction to as low as 20% shoot regeneration for those subcultured on DIA_8 and DIA_{16}, but only decreased to 47% for those subcultured on DIA_8M_6 (Fig. 1-II).

For TN 5, a remarkable promotion of root and shoot regeneration was achieved using mannitol and agar treatment (Fig. 2). The callus cultured on control medium (DIA_8) and then transferred to KNA_8 medium, showed an extremely low regeneration ability, below 20% for root regeneration and 0% for shoot regeneration. In contrast, the callus from media supplemented with mannitol and/or agar, then after transferred to KNA_8, showed much higher root and shoot regenerations. The increases in shoot number were particularly large. The most conspicuous result was obtained on medium $DIA_{12}M_4$, which showed 68% root regeneration, 44% shoot regeneration and 4.4 shoots per callus (Fig. 2-I).

More prominent improvement in regeneration frequency was obtained when callus was transferred to KNA_{16} medium for regeneration (Fig. 2-II). For DIA_8-induced callus, root regeneration was increased but shoot regeneration was remained very poor. Treatments with mannitol or agar, however, did promote shoot regeneration. In DIA_8M_6-induced callus, regeneration frequencies of 80% for root and 58% for shoot were obtained. However, the shoot number of $DIA_{12}M_4$ was less than that found on KNA_8 medium.

After subculturing on the respective media, callus showed decrease in regeneration ability on both KNA_8 and KNA_{16} media, but this decrease was considerably less on KNA_{16} (Figs. 2-III and 2-IV).

Growth and water content of callus

The results of the callus growth and water content examination are shown in Table 2.

The fresh weight and water content of callus were generally less in ANT 39 than in TN 5, and were reduced by mannitol and/or agar treatment in both cultivars. A comparison of the water contents of controls (DIA_8) showed that it was about 10% lower in ANT 39 than in TN 5. After mannitol treatment, water content of TN 5 callus was reduced by about 20%. Although callus grown on higher agar concentration was dry and compact in outer morphology, the water content in callus was reduced only slightly (Table 2).

Table 1. Media used for induction, maintenance, and plant regeneration of rice callus.

Medium	2,4-D (μM)	IAA (μM)	Kinetin (μM)	NAA (μM)	Agar (g/l)	Mannitol (M)
Induction and Maintenance						
DIA_8	5	10	——	——	8	——
DIA_{16}	5	10	——	——	16	——
DIA_8M_6	5	10	——	——	8	0.6
$DIA_{12}M_4$	5	10	——	——	12	0.4
Regeneration						
KNA_8	——	——	10	5	8	——
KNA_{16}	——	——	10	5	16	——

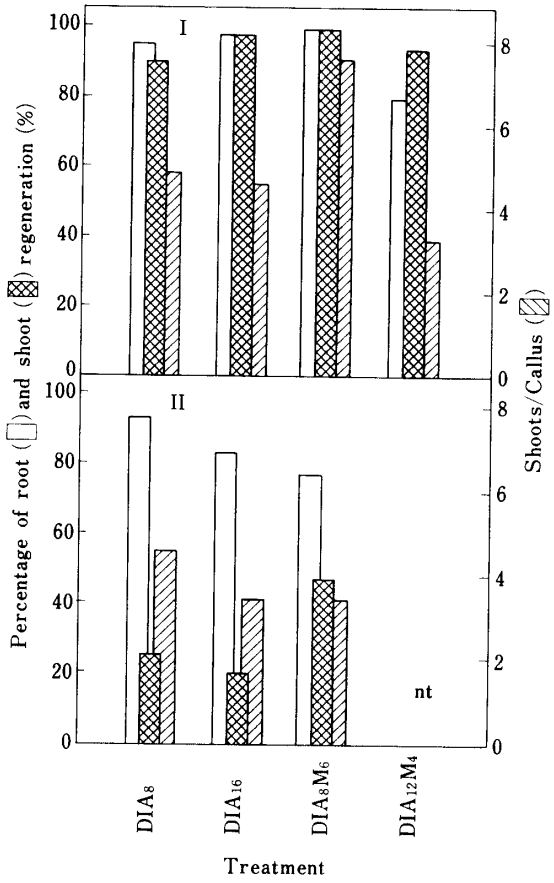

Fig. 1. Effects of mannitol and agar treatments on regeneration of ANT 39 callus. I. First passage, callus induced directly from young embryo. II. Third passage, each callus subcultured twice on its respective medium. Regeneration was conducted on KNA_8. In each treatment, 40 pieces of callus were used. nt: Not test. This experiment has been repeated three times from which similar results were obtained, and only the result from one experiment is shown. It is the same situation in Fig. 2.

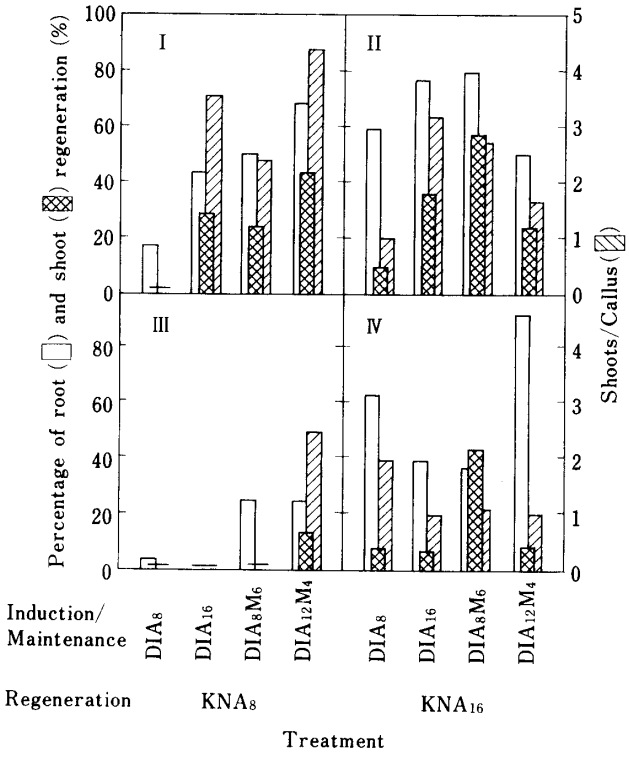

Fig. 2. Effects of mannitol and agar treatments on regeneration of TN 5 callus. I & II, First passage callus, 4 weeks after callus induction from young embryo; III & IV, Second passage callus, 4 weeks after callus being transferred once to each medium; I & III, regenerated on KNA_8 medium; II & IV, regenerated on KNA_{16} medium.

Proline content of callus

Originally, the proline content in ANT 39 was about double that in TN 5. An increase was found for both cultivars after stress treatment; this being especially pronounced after treatment with mannitol (Table 2).

Discussion

This study revealed that the shoot forming

Table 2. Effects of mannitol and agar on growth, water content and proline content of rice callus.

Medium	ANT 39			TN 5		
	Fresh weight (mg)	Water content (%)	Proline content (mole/gfw)	Fresh weight (mg)	Water content (%)	Proline content (mole/gfw)
DIA_8	60.6±50.0	83.1±5.3	1.32	103.0±41.9	93.6±0.9	0.54
DIA_{16}	12.6±10.2	77.2±8.8	2.34	53.0±43.0	89.0±2.2	1.14
DIA_8M_6	5.5± 3.8	63.0±9.4	3.96	21.8±16.3	75.7±3.2	4.80
$DIA_{12}M_4$	4.8± 3.3	60.6±6.3	4.02	19.5±15.8	76.3±6.3	2.46

* In each treatment, 40 pieces of 4-week-old callus induced from young embryo were used.

ability of rice callus cells are closely correlated with their water status. The evidences came from two approaches. First, the water content of the highly regenerating callus of ANT 39 is lower than that of the poorly regenerating callus of TN 5. The difference is approximately 10%. Second , the regeneration frequency of TN 5 callus is apparently increased when its water content is reduced by water stress treatments. A reduction of water about 10% seems suitable for the regeneration. Assessment of changes in the proline content of callus also suggested the effect of water stress.

Osmotic requirement has been reported for shoot formation in tobacco callus, and part of the exogenously supplied sucrose may act in this osmoregulatory role. Bacto-Agar cannot satisfy this osmotic requirement[3]. However, we found both additional mannitol and agar supplemented besides 3% sucrose are effective to promote shoot formation in rice callus. Mannitol neither supports *in vitro* tissue growth[9] nor is metabolized by higher plants[19]. Its osmoregulatory role is clearer than sucrose, which has dual roles as osmotic regulator and energy source. Agar, by virtue of its solidifying effect on the medium, may limit water uptake by tissues and consequently make them drier and more compact. Debergh[5] has noted that with an increasing concentration of Difco Bactor agar, available kinetin is decreased and impurities are introduced. Since we use Merck agar, Debergh's results may not be relevant.

Water stress treatment during either callus growth or regeneration promotes shoot formation. However, further studies are needed to clarify whether the stress is acting at the shoot developmental stage or at the initiation stage.

The use of brief strong plasmolysis as a pretreatment significantly improves the yield of adventive embryogenesis in suspension cultures of wild carrot cells[21]. It is suggested that the effect resulted from disruption of plasmodesmatal interconnections between the pre-embryonic cells. Whether this is also observed in rice cells after water stress will be investigated in the future.

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* In Chinese with English summary.