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Optimization of Rice Regeneration System from Mature Seeds of Five Egyptian Rice Cultivars

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ABSTRACT

Establishment of a suitable system for plant regeneration of rice calli derived form mature embryos is a prerequisite for plant transformation. We report here high frequency plant regeneration from mature seed-derived calli of rice. An experiment with five Egyptian rice cultivars ('Giza 159', 'Giza 171', 'Giza 172', 'Giza 176' and Reiho') showed that callus induction and growth were significantly affected by genotype and medium composition. Murashige and Skoog (MS) medium supplemented with 2 mg/l of 2,4-dicholorophenoxy acetic acid (2,4-D) gave the highest incidence of callus induction for 'Giza 176' and 'Reiho', respectively. The regeneration frequencies were dependent on the genotype, 6-benzylaminopurine (BAP) concentration and callus type. 'Reiho' and 'Giza 176' showed the highest callogenesis ability (100%), when calli from 'Giza 176' and 'Reiho' were induced on 2 and 1.5 mg/l 2,4-D, respectively, and subsequently regenerated on medium supplemented with 0.5 mg/l of 1-naphtaleneacetic acid (NAA) and 3 mg/l BAP. Thus, it is misleading to consider the growth dynamic of callus as the only parameter in optimizing a regeneration protocol. The regeneration ability of the callus type should also be taken into account.

Keywords: callogenesis, *Oryza sativa*, regeneration frequency, tissue culture
Abbreviations: BAP, 6-benzylaminopurine; **2,4-D**, 2,4-dicholorophenoxy acetic acid; **MS**, Murashige and Skoog medium; **NAA**, 1-naphthaleneacetic acid

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops. A vast number of rice cultivars as well as wild species of rice are grown worldwide and their genetic and molecular make-up is under active investigation. Many attempts were made to improve grain quality and quantity, and increase resistance to pests and stresses using biotechnology (Ye *et al.* 2000). The biotechnological approaches depended mainly on a reproducible regeneration system rather than transformation procedure (Jauhar 2006).

Tissue culture *via* somatic embryogenesis is a key step in regeneration and genetic manipulation of rice (Lee *et al.* 2002; Komari *et al*. 2007). In general, two types of organs have commonly been used for the induction of embryogenic callus, mature embryos (Burikam *et al.* 2002) and immature embryos (Grimes and Hodges 1990; Koetje *et al.* 1989). Embryos isolated from mature seeds have several advantages over the immature embryo, e.g., physiological uniformity and availability in sufficient quantities all year round (Azria and Bhalla 2000). During the course of further studies, rice regeneration was found to be affected by constituents of the basal medium and combinations of plant growth regulators (PGRs; Khanna and Raina 1998; Yang *et al.* 1999). There have been many reports concerning embryo culture in rice, including increased frequency of plant regeneration (Maggioni *et al.* 1989; Rance *et al.* 1994), analysis of hereditary characters, variation in regenerated plants (Peng and Hodges, 1989) and genetic transformation (Rainieri *et al.* 1990). Nevertheless, embryos have been cultured in a limited number of rice cultivars; their regeneration frequencies are still low. Since the first report of plant regeneration from embryo-derived calli of rice (Nishi *et al.* 1968), *in vitro* plant regeneration has been attempted in a wide range of rice cultivars (Raina 1989; Croughan and Chu 1991). Many reports revealed that callus induction and regeneration potential in rice was affected by several factors, including genotype, explant, medium composition and culture conditions (Biswas and Zapata 1993; Rance *et al.* 1994; Torbert *et al.* 1998; Lee *et al.* 2002; Hoque and Mansfield 2004; Grewal *et al.* 2005; Carsono and Yoshida 2006).

In this paper, mature embryos of five rice cultivars commonly used in Egypt, were used to test the genotypic variation in callus formation and regeneration capacity. Optimization of the regeneration system and assessing genotype performance *in vitro* are also discussed.

MATERIALS AND METHODS

Plant materials and callus induction

Mature seeds of five Egyptian rice cultivars 'Giza 159', 'Giza 171', 'Giza 172', 'Giza 176' and Reiho' were used for callus induction. Seeds were obtained from the Rice Research and Training Center, Agricultural Research Center (ARC), Ministry of Agriculture, Egypt.

Mature seeds were dehusked (Lee *et al.* 2002), and sterilized with 70% ethanol for 1 min and 40% (v/v) Clorox bleach (5.5% sodium hypochlorite) for 35 min with magnetic stirring, and were rinsed several times thoroughly with sterilized distilled water. Seeds were soaked in sterilized distilled water for 24 h. Then the embryos were aseptically excised from seeds and placed on callus induction media.

Mature embryos were cultured on various callus induction media containing MS salts with vitamins (Murashige and Skoog 1962), 30 g/l sucrose and supplemented with different concentrations and a combination of 2,4-D (0, 1, 1.5 and 2 mg/l) and BAP (0, 0.5, 1.0, 1.5 mg/l) then the media were solidified with 7 g/l Agar (Fluka). Medium pH was adjusted to 5.8 before autoclaving for 20 min at 121°C. The cultures were incubated for callus induction in the dark at 25 ± 2°C for 4 weeks.

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Original Research Paper

Growth dynamics of callus tissue

For fresh weight determination, rice calli were gently pressed on filter paper to remove excess water, and then weighed. The following indicators were determined according to Snedecor and Cochran (1980):

$$GR\ (mg/day) = \frac{FFW - IFW}{T}$$

$$CIF\ (\%) = \frac{No.\ of\ seeds\ producing\ calli}{Total\ No.\ of\ seeds} \times 100$$

$$SIF\ (\%) = \frac{No.\ of\ induced\ shoots}{Total\ No.\ of\ seeds} \times 100$$

where GR = growth rate (mg/day), FFW = final fresh weight (mg), IFW = initial fresh weight (mg), T = time (days) CIF (%) = callus induction frequency and SIF (%) = shoot induction frequency.

Plant regeneration

Calli were transferred onto five MS regeneration media after 4 weeks of culture on callus induction medium. Regeneration media contained full-strength MS medium, 30 g/l sucrose and supplemented with 0.5 mg/l NAA and five different concentrations of BAP, 0.5, 1, 2, 3 and 4 mg/l, and solidified with 7 g/l agar. The pH of the media was adjusted to 5.8 before autoclaving. Afterwards media were autoclaved for 20 min at 121°C. Callus cultures were incubated at 25 ± 2°C and a 16 hr photoperiod (3000 lux) provided by white tubular fluorescent lamps (Philips 40 W LVF 6500 K) for 4 weeks. PGRs used in this work were purchased from Sigma-Aldrich, USA.

The number of shoot primordia appeared at the surface of callus was calculated per callus inoculum after 15 days of culture. The calli with shoot primordia were transferred onto basal MS medium (PGR-free), solidified with 12 g/l agar for shoot and root development and incubated under the same previous conditions. Eventually, the number of plantlets was counted after transfer to PGR-free medium.

Statistics

The results presented are the mean values ± standard errors obtained from at least five replicates. Statistical significance between mean values was assessed using Duncan's multiple range test (Duncan, 1955). Analysis of variance was determined and the value of least significant difference was calculated at the 5% level to compare different treatments.

RESULTS

Callus induction

The intact mature seeds of rice were separated apart to a mature embryo and endosperm. Both explants were cultured onto different concentrations of BAP, 2,4-D and combinations of the two PGRs. Generally, we never obtained callus, plants or any type of development in the endosperm. However, mature embryos were developed to callus or plants in response to the type and concentration of the PGR supplied to the media. In a comparison between the callus induced from the mature embryo and the intact mature seed, there was no observable difference in the quality or quantity of the callus. Therefore, we induced calli using the intact mature seed to save time and effort.

In general, irrespective of the genetic background of the cultivar, all media supplemented with combinations of 2,4-D and BAP were found to promote callus induction. A correlation between the increase in BAP concentration and inhibition of callus induction was observed. However, when 2,4-D was used alone the induction of callus was quantitatively increased (**Fig. 1A**). No callus formation was detected in the absence of 2,4-D although shoots did form. This

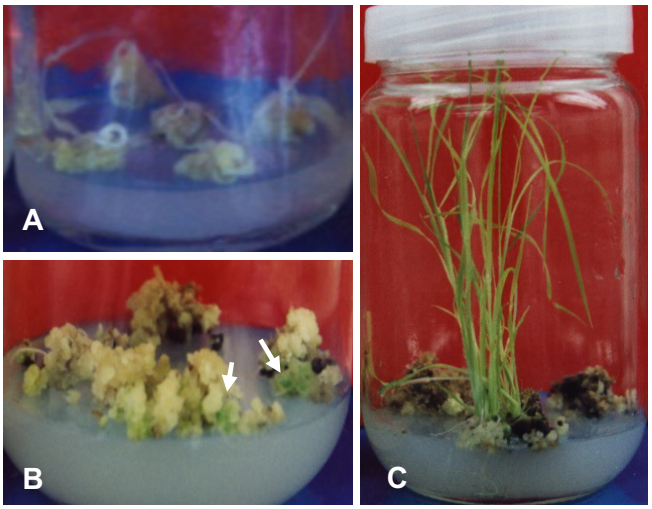

Fig. 1 The different stages of rice regeneration. (**A**) callus induction of mature embryos on MS medium supplemented with 2, 4-D, (**B**) development of somatic embryos as green spots on medium with NAA and BAP and (**C**) plantlets raised on hormone-free medium.

Table 1 The effect of various plant growth regulators on mature embryos of five rice genotypes. (medium: MS; culture period: 4 weeks in dark at 25±1 °C).

Growth regulators (mg/l)		Rice genotypes									
		Giza 159		Giza 171		Giza 172		Giza176		Riho	
2,4-D	BAP	Response	Frequency (%)	Response	Frequency (%)	Response	Frequency (%)	Response	Frequency (%)	Response	Frequency (%)
0.0	0.0	Shoot	75 a	Shoot	67 b	Shoot	100 a	Shoot	90 a	Shoot	83 a
0.0	0.5	Shoot	75 a	Shoot	50 b	Shoot	67 b	Shoot	100 a	Shoot	100 a
0.0	1.0	Shoot	50 b	Shoot	50 b	Shoot	100 a	Shoot	67 b	Shoot	100 a
0.0	1.5	Shoot	90 a	Shoot	43 c	Shoot	83 a	Shoot	80 a	Shoot	87 a
1.0	0.0	C+	100 a	C++	50 b	C++	100 a	C++++	100 a	C++++	100 a
1.0	0.5	C++	83 a	C+	50 b	C++	100 a	C+++	83 a	C+	100 a
1.0	1.0	C+	71 b	C+	71 b	C++	100 a	C++	67 b	C+	97 a
1.0	1.5	C++	57 b	-	0 c	C++	100 a	C++	97 a	C+	97 a
1.5	0.0	C++	60 b	C+	43 c	C++	86 a	C++++	100 a	C++++	100 a
1.5	0.5	C++	75 a	C+	13 c	C++	86 a	C++	92 a	C+++	100 a
1.5	1.0	C++	67 b	C+	43 c	C++	67 b	C+	100 a	C+	94 a
1.5	1.5	C+	33 c	C+	40 c	C+	50 b	C+	92 a	C+	97 a
2.0	0.0	C++	64 b	C+	17 c	C++	67 b	C++++	100 a	C++++	100 a
2.0	0.5	C++	85 a	C+	33 c	C++	67 b	C++	100 a	C+++	97 a
2.0	1.0	C++	75 a	C+	75 a	C++	67 b	C++	97 a	C++	100 a
2.0	1.5	C++	50 b	C+	89 a	C++	50 b	C++	97 a	C+	97 a

Numbers in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 2 Effect of three different concentrations of 2,4-D on fresh weight and growth rate of rice callus cultures (medium: MS; culture period: 4 weeks) .

2,4-D concentration (mg/l) and callus color	Number of Weeks							
	1		2		3		4	
	FW (mg)	GR (mg/day)	FW (mg)	GR (mg/day)	FW (mg)	GR (mg/day)	FW (mg)	GR (mg/day)
Reiho								
1.0 yellow	620 ± 41 a	88.6 ± 5.9 b	1018 ± 89 a	145.4 ± 12.7 b	1102 ± 207 b	157.4 ± 21.5 b	1541 ± 198 a	220.1 ± 28.3 a
1.5 bright yellow	548 ± 43 b	78.3 ± 5.7 b	961 ± 97 a	137.3 ± 13.9 b	1191 ± 95 b	170.1 ± 13.6 a	1348 ± 199 b	192.6 ± 28.4 b
2.0 white yellowish	602 ± 97 a	86 ± 13.9 b	1316 ± 113 a	188 ± 16.1 a	1285 ± 100 a	183.6 ± 14.3 a	1769 ± 177 a	252.7 ± 25.3 a
Giza 176								
1.0 Dull beige	652 ± 98 a	93.1 ± 14 a	742 ± 109 b	106 ± 15.6 a	1001 ± 266 b	143 ± 18 b	1424 ± 249 a	203.4 ± 35.6 a
1.5 beige	787 ± 176 a	112.4 ± 25.1 a	469 ± 62 b	67 ± 8.9 c	1177 ± 153 b	168.1 ± 21.9 a	1551 ± 190 a	221.6 ± 27.1 a
2.0 yellowish brown	567 ± 90 b	81 ± 12.9 b	665 ± 102 b	95 ± 14.6 c	1207 ± 202 a	172.4 ± 28.8 a	1192 ± 211 b	170.2 ± 30.1 b

FW: fresh weight, GR: growth rate

Numbers in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test

Table 3 Effect of various concentrations of BAP on callus cultures of rice. All the regeneration media were supplemented with 0.5 mg/l NAA. Green spots were counted after 15 days of culture, while plantlets were counted 30 days after transferring the cultures with green spots to growth regulators-free medium.

Group	BAP concentration (mg/l)											
	0.0		0.5		1.0		2.0		3.0		4.0	
	SP	P	SP	P	SP	P	SP	P	SP	P	SP	P
Reiho												
A	0	0	4.3 ± 0.5 b	0.14 ± 0.01 b	14.9 ± 2.1 a	0.95 ± 0.1 b	12.9 ± 2.3 a	0.67 ± 0.04 b	12.2 ± 1.5 a	0.56 ± 0.3 b	12 ± 3.1 a	0
B	0	0	12.4 ± 2.5 a	1.8 ± 0.3 b	23.9 ± 2.2 a	2.3 ± 0.3 b	24.7 ± 3.8 a	2.39 ± 0.6 b	44.9 ± 4.1 a	2.9 ± 0.5 b	44.5 ± 4.7 a	2.8 ± 0.5 b
C	0	0	28.1 ± 4.9 a	0.83 ± 0.02 b	33.3 ± 4.2 a	1.5 ± 0.2b	42.1 ± 5.2 a	2.8 ± 0.5 b	37.3 ± 5.1 a	0.33 ± 0.03 b	37.7 ± 7.1 a	0.62 ± 0.1 b
Giza 176												
D	0	0	1.1 ± 0.1 b	0	1.4 ± 0.2 b	0	1.5 ± 0.4 b	0	1.1 ± 0.2 b	0	0.48 ± 0.1 b	0
E	0	0	2.5 ± 1.6 b	0	2.5 ± 1.5 b	0	1.4 ± 0.3 b	0.1 ± 0.02 b	1.0 ± 0.2 b	0.67 ± 0.05 b	0.95 ± 0.12 b	1.6 ± 0.3 b
F	0	0	3.8 ± 2.4 b	1 ± 0.01b	5.2 ± 1.1 b	1.1 ± 0.2 b	4.3 ± 0.4 a	1.4 ± 0.2 b	4.3 ± 0.3 b	2.3 ± 0.4 b	4.11 ± 0.3 b	2.3 ± 0.3 b

SP: number of shoot primordia per callus.

P: number of plantlets after transfer to growth regulators - free medium

Numbers in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test

implies that BAP inhibits callus induction and direct regeneration (**Table 1**). Among the five investigated cultivars, 'Reiho' and 'Giza 176' showed the highest incidence of callus induction at 1.0, 1.5 and 2.0 mg/l 2,4-D (**Table 1**). The calli obtained were hard in texture. Therefore they were chosen to study further the effect of 2,4-D on induction of callogenesis and subsequent plant regeneration.

Growth dynamics of callus tissue

The different concentrations of 2,4-D led to callus induction with distinctive colors. Calli of 'Reiho' induced on 1, 1.5 and 2 mg/l 2, 4-D were yellow (group A), bright yellow (group B) and white yellowish (group C) respectively, while calli induced from 'Giza 176' at the same concentrations were dull beige (group D), beige (group E) and yellowish brown (group F) respectively (**Tables 2, 3**).

The effects of different supplementations of 2,4-D on the growth of two rice cultivars ('Reiho' and 'Giza 176') callus was determined (**Table 2**). In 'Reiho', the growth rate (GR), was increased dramaticaly at the second week of subculture followed by a slower increase in the remaining two weeks, while a gradual increase over time was observed in 'Giza 176'. Regardless of cultivar, the highest GR was reached at the forth week of subculture. However, the highest GR in 'Reiho' was obtained on medium supplemented with 2 mg/l 2,4-D, while the highest value in 'Giza 176' was reached on medium supplemented with 1.5 mg/l 2,4-D. Nevertheless, 'Reiho' mainfested relatively higher GR values than 'Giza 176' (**Table 2**).

Plant regeneration

The different groups of calli were transferred to media supplemented with 0.5 NAA alone or in combination with different concentrations of BAP. Calli transferred to medium supplemented only with NAA formed neither shoot primordia nor plantlets. Generally, Nodular callus was observed two weeks after transfer to regeneration media. Callus browning increased with an increase in BAP concentration. A combination of NAA with different concentrations of BAP led to the development of shoot primordia (**Fig. 1B**), which subsequently developed into plantlets (**Fig. 1C**). The regeneration frequency was dependent on genotype, callus type and BAP concentration.

Data presented in **Table 3** showed that MS-medium supplemented with NAA at 0.5 mg/l and 3 mg/l BAP was most suitable for induction of shoot primordia and plant regeneration from calli types B and F of 'Reiho' and 'Giza 176', respectively. However, calli types A and D showed low regeneration abilities, possibly all media tested were inappropriate for that purpose.

Although media supplemented with 1.5 and 2 mg/l 2,4-D led to the highest GR after 4 weeks of culture on callus induction medium in 'Giza 176' and 'Reiho', respectively (**Table 2**), calli induced on 2 and 1.5 mg/l 2,4-D showed the highest regeneration ability at 3 mg/l BAP in 'Giza 176' and 'Reiho', respectively (**Table 3**). Thus, it is misleading to consider the growth dynamic of callus as the only parameter in optimizing a regeneration protocol. The regeneration ability of the callus type should also be taken into account. Therefore we recommend callus induction on 1.5 and 2 mg/l 2,4-D for 'Reiho' and 'Giza 176', respectively, and induction of regeneration on media containing 0.5 mg/l NAA and 3 mg/l BAP.

DISCUSSION

Under certain conditions, differentiated plant tissues can revert to a dedifferentiated state and through additional cell divisions form unorganized cell aggregates or calli. Calluses can regenerate into whole plants in response to plant hormones that stimulate tissue formation. The competence of whole plant regeneration depends on totipotency, or the genetic potential that makes plant tissue culture possible. In crops, tissue culture and transgenic techniques based on calli have proved to be powerful tools to facilitate the breeding process.

It is possible that all plant species are totipotent, but it is difficult to identify the culture conditions and stimuli required to express totipotency. In this study, a suitable regeneration system from mature seed derived calluses of two Egyptian cultivars showed the maximum proliferation of shoot primordia has been established.

The present investigation revealed that the interaction of both genotype and media composition largely affected callus induction and plant regeneration frequencies (**Table 1**). A comprehensive study of many rice varieties showed that both callus formation and plant regeneration are highly genotype-dependent (Peng and Hodges 1989; Hoque and Mansfield 2004). In this respect, La *et al.* (2003) stated that although genotype was the main restricting factor for regeneration frequency, the alteration of culture media, hormones, or additional supplementing of some chemicals can increase regeneration frequency dramatically. Many authors emphasized the role of 2,4-D in rice tissue culture as an essential element for callus proliferation (Inoue and Maeda 1980). *In vitro* plant regeneration from callus induced from embryos of mature seeds of 4 Australian varieties of rice was studied (Azira and Bhalla 2000). Observations of callus induction on MS and N6 media indicated that MS medium supplemented with 0.5–2 mg/l of 2,4-D is suitable for callus formation from the varieties tested. In this context, Pandey *et al.* (1994) worked on dehusked rice seeds, using different levels of 2,4-D and they concluded that 2,4-D at 2.0 mg/l gave the best response for callus formation. Results of the present experiment also showed that 2,4-D-containing media responded well both for callusing and plant regeneration. On the other hand it was reported that consistent plantlet regeneration from pollen calli in sufficient numbers could enhance the efficiency and use of anther culture technique in rice breeding. Among the factors that influence the differentiation of pollen callus is the ratio of auxin to cytokinin in the regeneration medium (Guiderdoni *et al.* 1991). Pollen calli were induced using N6 induction medium with addition of 2,4-D (2 mg/1), kinetin (Kin; 1 mg/1) and coconut milk (10% v/v). For plant regeneration, MS basal medium supplemented with different concentrations of cytokinins (Kin and BA) was used in a series of step-wise regeneration experiments in which the presence of both Kin and BA was not only important in the regeneration medium but also their ratio was critical in obtaining very high frequency of regeneration. Efficient plant regeneration has been achieved from embryo- and microspore-derived callus in a range of both temperate and tropical *japonica* cultivars of *Oryza sativa* and its African perennial wild relative *Oryza longistaminata* (Heyser *et al.* 1983). Emphasis was placed on understanding the regeneration pathways and differences of regenerability among genotypes using histological analyses. Both somatic and germinal tissue-derived calluses have been used to establish embryogenic cell suspensions from which regenerable protoplasts have been prepared (Guiderdoni *et al.* 1991; Alemanno and Guiderdoni 1994).

The data presented in this work suggested a regeneration scheme for two Egyptian rice cultivars. Stages from callus proliferation till regeneration have been investigated. In our point of view, after the callus induction phase, selecting the most appropriate medium and judging the genotype capacity for callus proliferation and regeneration to facilitate its regenerability are significant for accomplishment of genetic transformation using callus as target material. This also suggests that the callus proliferation was highly affected by medium constituents, especially plant growth regulators, while callus development was independently influenced by genotype and medium (**Tables 1, 3**). In addition, our study showed that callus derived from mature embryos of tested rice cultivars are acquiescent to multiple shoot formation, and could be used for rice transformation studies.

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