Callus induction and plant regeneration of five Egyptian rice genotypes as affected by medium constituents

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ABSTRACT

Callus induction and plant regeneration efficiency of five selected Egyptian rice cultivars, Giza 177, Sakha 101, Sakha 102, Sakha 103, and Sakha 104, were studied using mature embryos as explants. Both plant genotype and medium composition influenced the rate of callus formation. Mature embryo explants of the genotype Sakha 104 grown on N6 medium supplemented with 2 mg l^{-1} 2,4-D and 8.5 mg l^{-1} silver nitrate gave the highest callus induction frequency (95%). Among the five genotypes tested, Giza 177, Sakha 104, and Sakha 103 showed the highest frequency of embryogenic callus formation (48.3%, 48%, and 47.3%, respectively), on N6 medium supplemented with 2 mg l^{-1} dicamba. The highest shoot regeneration efficiency of 75.3 and 70.7%, was observed for Sakha 101 and Sakha 104, respectively, on MS medium supplemented with 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin.

Keywords: *Oryza sativa, Somatic embryogenesis, Shoot organogenesis, Silver nitrate, Dicamba.*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops, which supplies food for more than half of the world's population (Tyagi *et al*., 2004). Despite the success made in the last decades, traditional breeding efforts alone cannot meet the increasing demand of rice consumers in the 21st century. As the world's population continues to grow towards an estimated 10 billion people by 2050, demand for rice will grow faster than that for other crops, because population growth is greatest in the rice-consuming and rice-producing regions of Asia, Africa and the Americas (Dawe, 2007). In recent years, rice stocks have fallen dramatically, such that in 2008 the stock-to-use ratio of rice was at the lowest level in 30 years (FAO, 2008 and Sage and Sage, 2009).

One procedure to increase rice productivity is the introduction of useful traits by genetic transformation methods. Genetic transformation of rice has been an important area of research in the past few years. A number of methods including PEG, electroporation, microprojectile bombardment, and *Agrobacterium* have been used to mediate the actual gene transfer (Ignacimuthu *et al*., 2000). Whatever transformation system was employed, efficient systems for embryogenic callus induction and shoot regeneration were always critical for obtaining adequate numbers of fertile transgenic rice lines. In rice, four different callus types (type I, II, III and IV) can be induced (Visarada *et al*., 2002). In general, immature embryos and meristematic

tissues, having undifferentiated cells, are suitable for callus induction and subsequent plant regeneration (Morrish *et al*., 1987). However, such explants are available only during a very limited period of the rice plant's natural growth cycle (Hoque and Mansfield, 2004). While this limitation does not exist if rice plants are grown in the greenhouse, embryos of mature seeds are very easily available throughout the year and represent an even more convenient source of tissue suitable for somatic embryogenesis (Carsono and Yoshida, 2006). Indeed, embryogenic calluses induced by culture of mature seeds have been effectively used for genetic transformation by particle bombardment (Jiang *et al*., 2000) and *Agrobacterium*-mediated transformation (Kumria *et al*., 2001).

In addition to the genotype and the type of the explant, both callus induction and organogenesis are strongly influenced by the composition of the culture medium including the presence and concentration of plant-growth regulators and by the culture conditions. However, the genotype of the respective species and the type of the explant belong to the most critical factors for successful embryogenic callus induction and regeneration of rice plants (Rueb *et al*., 1994).

In Egypt, rice production has reached a record due to integrated management and traditional breeding programs, but there is further opportunity to increase yields by the use of genetically transformed varieties. Unfortunately, the suitability of Egyptian rice varieties for genetic transformation has not yet been systematically examined, and the development of an efficient regeneration and transformation system is an important task (Saker *et al*., 2006).

In this study, we aim to develop a reproducible and efficient procedure for callus induction and plant regeneration using mature seeds of five economically important rice genotypes from Egypt. We report the effects of genotype and medium composition on callus induction, embryogenic response, and regeneration potential.

MATERIALS AND METHODS

Five elite Egyptian rice (*Oryza sativa* L.) varieties, Giza 177, Sakha 101, Sakha 102, Sakha 103, and Sakha 104 were obtained from the Field Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt (Table 1).

Callus initiation and maintenance

Mature seeds of each variety were dehusked manually and surface-sterilized by immersion in 70% ethanol for 1 min, followed by 30 min shaking in a solution of sodium hypochlorite (with 2% active chlorine) and Tween 20. Seeds were then thoroughly washed 5-6 times with sterilized distilled water and blotted dry on sterile filter paper. For callus induction, we used three different culture media, named Cia (Seraj *et al*., 1997; Khanna and Raina, 1998 and Lee *et al*., 2002), Cib (Bohorova *et al*., 1995), and Cic (Carvalho *et al*., 1997). The compositions of these media, which were also used for callus maintenance, are shown in Table (2). They were made up in distilled water, the pH of the solution was adjusted to 5.8, and sterilized by autoclaving for 20 min at 121°C.

Table (1): Genotypes, parentages and major agronomic features of the used rice cultivars (Moghaieb et al., 2009 and Gaafar, 2010).

Genotype	Origin	Parentage	Features
Giza 177	87.5% Japonica 12.5% Indica	Giza 171 Yomji No.1//Pi No. 4	High blast resistant Amylose content 18%
Sakha 101	Japonica	Giza 176 Milyang 79	High blast resistant Growth duration 140 days Lodging resistant Amylose content 19%
Sakha 102	Japonica	Gz 4096-7-1/Giza 177 Gz 5379-22-2	Growth duration 125 days Amylose content 19% High blast resistant
Sakha 103	Japonica	Giza 177 Suweon 349	Short grain Resistant to blight disease 72% milling
Sakha 104	Japonica	Gz 4096-8-1 Gz 4100-9-1	High resistant for blast, brown spot, and stem borers

Table (2):Composition of media used for callus induction.

Component	Cia	Cib	Cic
Basal medium	MS	N6	N6
Casein hydrolysate	0.3 g l^{-1}	0.3 g l^{-1}	0.3 g l^{-1}
Sucrose	2.5% (w/v)	2.5% (w/v)	2.5% (w/v)
Sorbitol	0.5% (w/v)	0.5% (w/v)	0.5% (w/v)
L-Proline	0.5 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}
L-Glutamine	0.5 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}
L-Alanine	0.45 g l^{-1}	0.45 g l^{-1}	0.45 g l^{-1}
Silver nitrate	-	-	8.5 mg l^{-1}
2,4-D	2.5 mg l^{-1}	-	2 mg l^{-1}
Dicamba	-	2 mg l^{-1}	-
pH	5.8	5.8	5.8

Cia = first callus induction media; Cib = second callus induction media; Cic = third callus induction media.

Sterilized seeds (30 seeds per plate; 10 plates per medium) were placed onto the specified callus induction medium, solidified with 7 g l^{-1} agar, using Petri dishes sealed with parafilm (Figure 1A). They were then incubated at 25±1°C in the dark for two weeks before assessment of callus induction responses (total callus induction frequency, i.e. embryogenic plus non-embryogenic). Callus induction frequency (CIF) is defined as the percentage of calli relative to the number of incubated seeds. Type-I and-II calluses were transferred to fresh callus induction media of the same composition for multiplication and subcultured once every two weeks. Slowly growing and dark calli were excluded from the next subculture. The embryogenic callus frequency is defined as the percentage of embryogenic calli relative to the number of incubated seeds.

Organogenesis

After four weeks on callus induction medium, embryogenic callus pieces were transferred to three different regeneration media (Table 3). To induce shoot development, calli initiated on Cia medium were transferred to regeneration medium Rega, callus cultures on Cib were transferred to Regb, and callus cultures on Cic were transferred to Regc. All regeneration media were based on MS medium basal salts and vitamins, supplemented with 2 g l^{-1} casein hydrolysate, 1.5 g l^{-1} L-proline, 0.5 g l^{-1}

L-glutamine, 0.45 g l^{-1} L-alanine, 3% (w/v) sucrose, and 3% (w/v) sorbitol. In addition, Rega contained 0.02 mg l^{-1} NAA and 2 mg l^{-1} kinetin, Regb contained 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin, and Regc contained 1 mg l^{-1} IAA and 0.05 mg l^{-1} zeatin. Callus cultures were incubated for four weeks at 28°C in a 16/8 h light/dark cycle at 350 µE m^{-2} s^{-1}. Germinated embryoids having small and large shoots were transferred to the rooting medium. The shoots were allowed to develop into plantlets in glass jars. The regeneration frequency was defined as the percentage of regenerated shoots relative to the number of incubated embryogenic calli. Rooting was achieved by subculture on half-strength MS medium containing 3% (w/v) sucrose, and 3g l^{-1} Gelrite.

Acclimatization and plant recovery

The regenerated plantlets were transferred to a translucent box containing modified Hoagland solution according to Johnson *et al.* (1957) and kept in the greenhouse. Rooted plantlets were transferred from Hoagland solution into pots containing a mixture of peat moss and soil (1:1) and grown in a controlled-environment growth room with a 12 hour photoperiod at 28°C/25°C (day/night) and a light intensity of 350 µE m^{-2} s^{-1}. Plantlets were watered using half strength of Hoagland solution as needed.

Table (3): Components of media used for plant regeneration and rooting.

Rega	Regb	Regc	Rooting
MS medium	MS medium	MS medium	MS medium (half strength)
3% (w/v) sucrose	3% (w/v) sucrose	3% (w/v) sucrose	3% (w/v) sucrose
3% (w/v) sorbitol	3% (w/v) sorbitol	3% (w/v) sorbitol	
0.02 mg l^{-1} NAA	1 mg l^{-1} NAA	1 mg l^{-1} IAA	
2 mg l^{-1} kinetin	2 mg l^{-1} kinetin	0.05 mg l^{-1} zeatin	

Rega = first shoot induction media; Regb = second shoot induction media; Regc = third shoot induction media.

Statistical Analysis

Statistical analysis of variance was performed according to Steel and Torrie (1980) using the GenStat computer software (version 12.1) with associated least significant difference (LSD) function.

RESULTS AND DISCUSSION

Callus initiation

Three hundred mature embryos from each genotype were tested for callus induction and regeneration experiments on three culture media, Cia, Cib, and Cic (Table 2). The Cia medium was composed of MS medium (Murashige and Skoog, 1962), basal salts, and vitamins, while Cib and Cic were based on N6 basal medium (Chu *et al.*, 1975) containing the same composition of N6 vitamins.

In addition to some standard additions listed in (Table 2), medium Cia contained 2.5 mg l^{-1} 2,4-D, medium Cib contained 2 mg l^{-1} dicamba, and medium Cic was supplemented with 2 mg l^{-1} 2,4-D and 8.5 mg l^{-1} silver nitrate. Callus induction was observed for all examined genotypes on each medium (Figure 1 B and C), and the callus induction frequencies (CIF) are shown in Figure (2A). These data show that CIF was influenced by the genotype, by the chemical composition of the medium, and by specific interactions between genotype and medium. Highest CIF's were observed with medium Cic, followed by Cib and Cia (mean CIF of 83.5, 75.7, and 64.5%, respectively). Cic is the only medium which contains the anti-ethylene compound $AgNO_3$. Our observation of a significantly higher CIF relative to the other two media corresponds with an earlier report on the enhancement of pollen callusing frequency in indica cultivars from 10.1% to 20.6% by the addition of 10 mg l^{-1} of $AgNO_3$ to the callus induction medium (Lentini *et al.*, 1995).

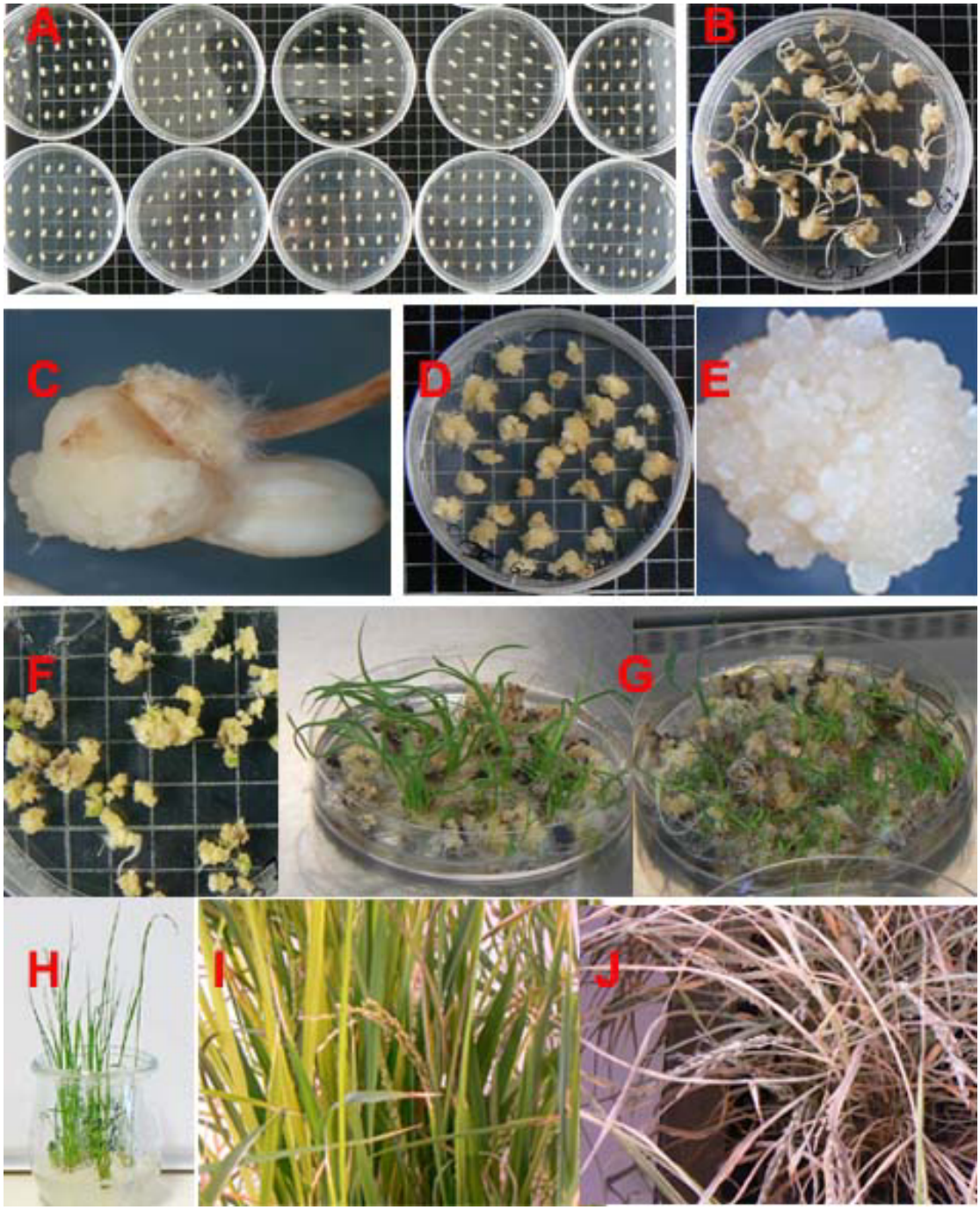

Fig. (1): Plant regeneration from embryogenic calli derived from mature seeds. A: Sterilized seeds planted for callus induction. B and C: Callus induced from mature seed embryos. D and E: Embryogenic callus after 4 weeks. F: Embryogenic callus after 2-4 weeks on regeneration medium. Shoot formation starts from green spots. G: Plants regenerated from embryogenic callus. H: Plants in rooting medium. I and J: Fertile in vitro regenerated and greenhouse-grown rice plants.

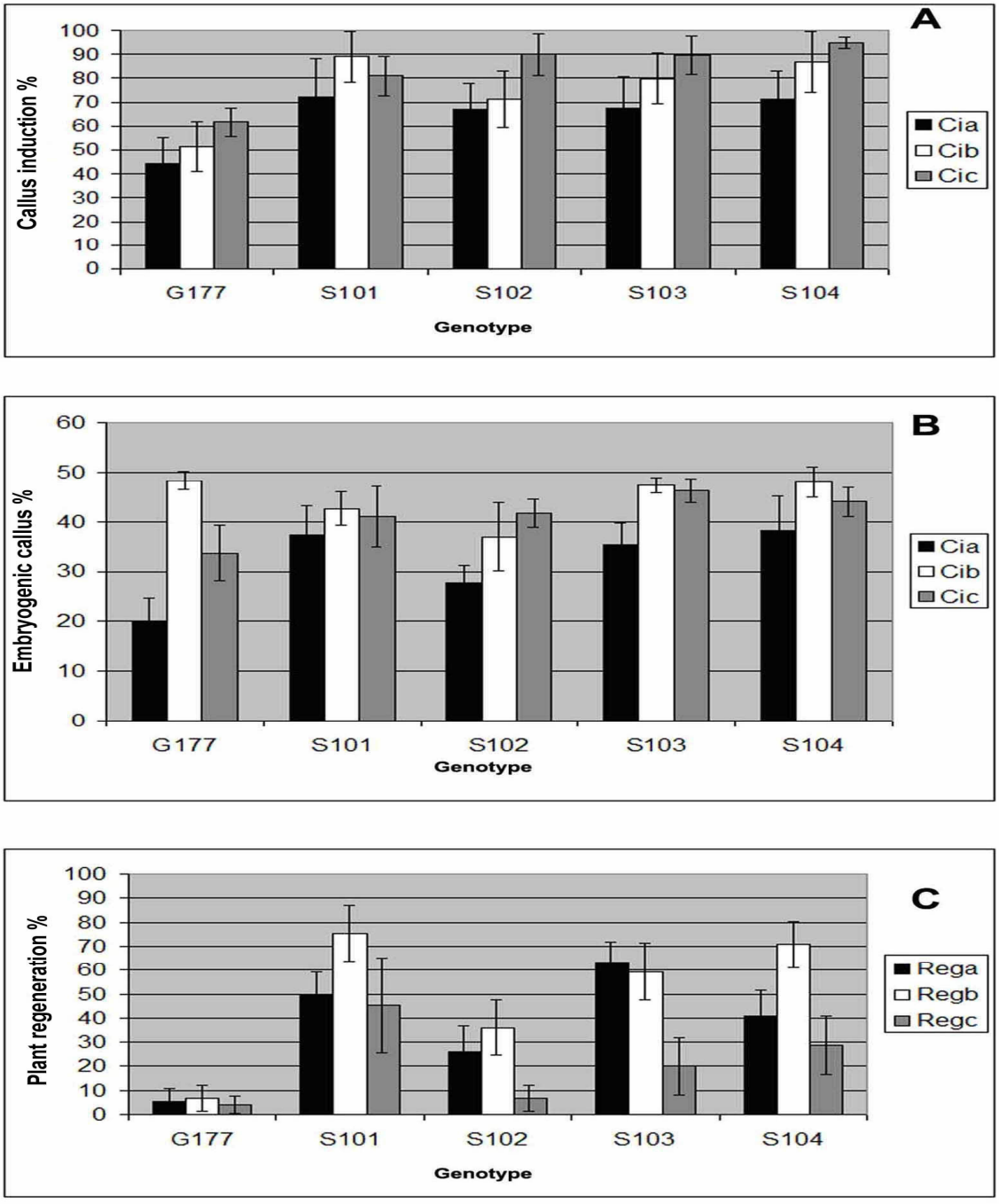

Fig.(2): A: Frequencies of callus induction of the five rice varieties cultured on three different callus induction media. B: Frequencies of embryogenic callus formation of the five rice varieties maintained on three callus induction media. C: Plant regeneration frequencies of five rice varieties on three regeneration media.

Significant differences were also detected between the CIF's of the different genotypes (Figure 2A). The two genotypes Sakha 104, and Sakha 101 showed the highest CIF values (84.4 and 80.7%, respectively), while Giza 177 showed a distinctly lower CIF of 52.4%. Additionally, it appears that significant interactions exist between genotypes and media. The highest CIF was obtained by culturing genotype Sakha 104 on Cic (95.0%), while the lowest CIF (44.3%) resulted from culturing genotype Giza 177 on Cia. This result is different from an earlier study (Moghaieb *et al*., 2009), in which a CIF of more than 80% was observed with Sakha 102 whereas values close to 50% were found with the genotypes Giza 177, Sakha 101, and Sakha 104.

Somatic embryogenesis

Embryogenic calli after 4 weeks are shown in Figure (1 D and E). Frequencies of embryogenic callus formation are shown in Figure (2B). According to Visarada *et al.* (2002), white-, creamy-, and yellow-coloured calluses are embryogenic calluses. They are also associated with at least two other types of calluses, a watery, pastel unorganised callus, which is non-morphogenic or may form only roots, and second, a muddy, soft, mucilaginous callus, which only forms roots.

The inspection of embryogenic callus frequencies (ECF) revealed that the formation of embryogenic callus was influenced both by the genotype and the chemical composition of the medium. The mean ECF observed with the five rice varieties was significantly higher on medium Cib (44.7%) as compared to medium Cic (41.3%) and medium Cia (31.7%). This suggests considerable effects of the constituents of the respective callus induction medium. For most tested varieties, there was a better ECF response on the dicamba-containing medium Cib compared to the media supplemented with 2,4-D and MS salts or 2,4-D, $AgNO_3$, and N6 salts, respectively. This is in agreement with the observations that the N6 medium quite generally shows better performance than the MS medium; for example with japonica rice or in rice anther cultures (Chu, 1975 and Raina, 1989 and 1997), and that the auxin herbicide dicamba increases somatic embryogenesis in some grasses such as maize (Duncan *et al*., 1985 and Bohorova *et al*., 1995) and wheat (Hunsinger and Schauz, 1987).

In the comparison of the rice cultivars examined here across all three media, Sakha 104 (43.4%) and Sakha 103 (43%) showed the highest mean ECF, followed by Sakha 101 (40.3%), while corresponding mean values for Sakha 102 (35.4%) and Giza 177 (34%) were significantly lower. Similar to observations with sorghum, where the production of a high quality callus strongly depends on the genotype (Kaeppler and Pedersen, 1997), our data suggest strong effects of the rice genotype on the ability to induce embryogenic calli. Additionally, we found highest ECFs for the three genotypes Giza 177, Sakha 104, and Sakha 103 in combination with Cib (48.3%, 48.0%, and 47.3%, respectively), again indicating that significant interaction between genotype and medium takes place. The same results were obtained by Lee *et al*. (2002). They found that the number, colour, size, shape and appearance of the embryogenic calluses varied among the rice genotypes depending on the type of basal medium, indicating that induction of high-quality rice callus is influenced by genotype, medium, and the kind of explant as well as by their interactions.

Regeneration of plantlets from somatic embryos

Organogenesis was achieved by transferring the embryogenic calli, obtained on callus induction media Cia, Cib, and Cic, to the plant regeneration media Rega, Regb, and Regc (Table 3). In addition to basic identical constituents for all three regeneration media, Rega contained 0.02 mg l^{-1} NAA and 2 mg l^{-1} kinetin, Regb contained 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin, and medium Regc contained 1 mg l^{-1} IAA and 0.05 mg l^{-1} zeatin. Green shoots were formed within four weeks (Figure 1F and G) and transferred to the rooting medium after reaching 2-3 cm length. After rooting, regenerated plants were hardened and further grown in the greenhouse (Figure 1H and J).

Highest regeneration frequencies for all genotypes were found with medium Regb (containing 2 mg l^{-1} NAA, and 2 mg l^{-1} kinetin) with a total mean frequency of 49.6% (Figure 2C). Comparing the individual genotypes across all three regeneration media, confirmed earlier data by Moghaieb *et al*. (2009), that Sakha 101, Sakha 103, and Sakha 104 (56.7%, 47.5%, and 46.7%, respectively) were the best-performing genotypes. On the other hand, the poor regeneration potential of Giza 177 on all media (mean of 5.3%) is much lower than the regeneration frequency of 35% reported for Giza 177 in the earlier study by Moghaieb *et al.* (2009).

Highest regeneration efficiencies were achieved with Sakha 101 (75.3%) and Sakha 104 (70.7%) on Regb, which is significantly higher than previously achieved efficiencies (about 50% higher as compared to Moghaieb *et al*., 2009). This effect could be possibly correlated to the presence of dicamba during callus induction on Cib medium. At least, it was reported that the use of dicamba yielded a higher plant regeneration efficiency than 2,4-D with a number of African rice genotypes (Brisibe *et al*., 1992) on Rega and Regc; Sakha 101 and Sakha 104 were considerably less efficient.

CONCLUSION

Plant genotype and composition of tissue culture medium are considered the most important factors affecting somatic embryogenesis and organogenesis of rice. In a comparison of five Egyptian rice genotypes, we examined the possibility of callus formation and differentiation using mature embryos. We found distinct genotype-specific differences and clear effects of medium composition, both during the formation of embryogenic calluses and organogenesis. Callus induction medium composed of N6 basal medium supplemented with 2 mg l^{-1} dicamba worked particularly well for the two genotypes Giza 177 and Sakha 104, but gave good results with Sakha 103 and Sakha 101 as well. A remarkably high plant regeneration efficiency of about 75% was achieved with the genotype Sakha 101 on MS medium supplemented with 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin. Future genetic transformation of Egyptian rice varieties will likely benefit from this optimised protocol. Among the examined genotypes, Sakha 101 and Sakha 104 appear as the most suitable genotypes for such experiments.

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ABBREVIATIONS

2,4-D = 2,4-dichlorophenoxyacetic acid; Dicamba = 3,6-dichloro-2-methoxybenzoic acid; MS = Murashige and Skoog's (1962) medium; N6 = Chu *et al*.' s (1975) medium; NAA = 1-naphthalene acetic acid; IAA = Indole-3-acetic acid; Ci = callus induction; CIF = Callus induction frequency; ECF = Embryogenic callus frequency; Cia = first callus induction media; Cib = second callus induction media; Cic = third callus induction media; Rega = first shoot induction media; Regb = second shoot induction media; Regc = third shoot induction media; AGERI = Agricultural Genetic Engineering Research Institute, Giza, Egypt.

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الملخص العربي

استحثاث إنتاج الكالوس وإعادة تكشف النباتات لخمسة تراكيب وراثية من الأرز المصري وتأثرها بمكونات بيئة زراعة الأنسجة

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أجريت هذه الدراسة في معهد العلوم البيولوجية قسم فسيولوجيا النبات جامعة روستوك بمدينة روستوك ألمانيا خلال الفترة من ٢٠٠٩ –٢٠١١. الأهداف الرئيسية هي: (١) تحديد أفضل النظم لإعادة تكشف خمسة أصناف أرز مصرية عن طريق زراعة الأجنة مكتملة النضج والتي تعتبر خطوة أساسيه لإجراء عمليات التحول الوراثي والنقل الجيني. (٢) دراسة أفضل التراكيب الوراثية من حيث الاستجابة لإعادة التكشف. (٣) دراسة تأثير اختلاف مكونات بيئات زراعة الأنسجة المستخدمة لاستحداث وتكشف الكالوس الجنيني. تم زراعة الأجنة مكتملة النضج لخمسة أصناف أرز مصرية وهي جيزة١٧٧، سخا١٠١، سخا١٠٢، سخا١٠٣، سخا١٠٤ على ثلاثة أنواع من البيئات الغذائية الصناعية بهدف إنتاج الكالوس وهى (Cia) و (Cib) و (Cic) البيئة الأولى تتكون من أملاح بيئة MS أما البيئتين الأخيرتين فتحوى أملاح N6 . كل من البيئات الثلاثة تحتوى على ٣٠٠ ملجم / لتر casein hydrolysate ، ٠,٥ جم / لتر من كل من الأحماض الأمينية L-Alanine, L-Glutamine, L-proline بالإضافة إلى ٢٥ جم / لتر سكروز، تحتوى الأولى (Cia) على ٢,٥ ملجم / لتر2,4-D ، بينما تحتوي البيئة الثانية (Cib) علي ٢ ملجم / لتر Dicampa و الثالثة (Cic) تحتوى على ٢,٠ ملجم / لتر 2,4-D بالإضافة إلى ٨,٥ ملجم / لتر $AgNO_3$. وأوضحت النتائج أن أعلي معدل لاستحداث الكالوس سجل بواسطة التركيب الوراثي سخا ١٠٤ بمتوسط ٩٥% يليه سخا ١٠٢ بمتوسط ٩٠% وكان ذلك علي بيئة إنتاج الكالوس (Cic) , بينما أظهر الصنف جيزة ١٧٧ أقل معدل لإنتاج الكالوس بمتوسط ٤٤,٣% وذلك علي البيئة الغذائية لإنتاج الكالوس (Cia) . أوضحت النتائج أن متوسط استحداث الكالوس الجنيني لجميع التراكيب الوراثية على البيئة (Cib) أعلى منه على البيئتين الأخريين بمتوسط عام ٤٤,٧%. أعلى متوسط لاستحداث كالوس جنيني كان للصنف جيزة ١٧٧ بمتوسط ٤٨,٣% يليه سخا ١٠٤ بمتوسط ٤٨% ثم سخا ١٠٣ بمتوسط٤٧,٣%. الأصناف الثلاثة أنتجوا تلك النسب مع بيئة (Cib) المحتوية على ٢ مليجرام / لتر Dicampa. وقد أظهرت جميع التراكيب الوراثية المختبرة كفاءة اعلي في تكوين الكالوس الجنيني عند تنميتها علي البيئة المحتوية علي Dicamba عنها علي البيئة المحتوية علي 2,4-D فقط أو $AgNO_3$ و 2,4-D. هذه النتائج توضح أهمية الأكسين Dicamba في استحداث الكالوس الجنيني على الأقل مع أصناف الأرز المصرية الخمسة المستخدمة في تلك الدراسة. تم نقل الكالوس الجنيني على بيات التكشف (Rega), (Regb), (Regc) كلها تتكون من أملاح MS وتحتوى على ٣% سكروز و٣% سوربيتول الأولى تحوى ٠,٠٢ماجم/ لتر NAA و ٢ملجم/ لتر كينتين أما الثانية فتحوى ١ملجم/ لتر من NAA بالإضافة إلى ٢ملجم/ لتر من الكاينتين أما الثالثة فتحوى ١ملجم/ لتر IAA بالإضافة إلى ٠,٠٥ ملجم/لتر زياتين. أعلى معدل تكشف تم الحصول عليه من الصنف سخا ١٠١ بمتوسط ٧٥,٣% يليه سخا ١٠٤ بمتوسط ٧٠,٧% وذلك على بيئة التكشف (Regb). أوضحت النتائج أن الصنفين سخا ١٠١ وسخا ١٠٤ هما أفضل تركيبين وراثيين استجابة خلال تجارب زراعة الأنسجة باستخدام الأجنة الناضجة كبادئات نباتية. وان استخدام بيئة إنتاج كالوس تتكون من أملاح N6 بالإضافة إلى ٢ملجم/ لتر Dicamba أعطت أعلى معدل لتكون كالوس جنيني. وان بيئة MS المحتوية على ١ملجم/ لتر NAA بالإضافة إلى ٢ ملجم/ لتر كاينتين أعطت أعلى معدل لإعادة التكشف بمتوسط ٧٥,٣% للصنف سخا ١٠١ و ٧٠,٧% للصنف سخا ١٠٤.