High-throughput regeneration from mature embryos of eleven commercial rice (Oryza sativa L.) cultivars through somatic embryogenesis using a novel genotype independent protocol

Ahmed S. Ibrahim and Osama M. El Shihy

Plant Biotechnology Research Laboratories (PBRL), Plant Physiology Division, Faculty of Agriculture, Cairo University, Egypt.

ABSTRACT

In this study, a comprehensive protocol for efficient conditions to enhance the regeneration capacity of rice plants from mature embryos of different Egyptian commercial cultivars through somatic embryogenesis was established. Thus, the obtained results confirmed the establishment of a novel genotype independent protocol for somatic embryogenesis in rice using mature embryos of different genotypes. Therefore, this novel protocol is a milestone and will pave the way for improving the efficiency of genetic transformation protocols of commercial rice cultivars. The developed protocol describes the composition of different media, duration and the sequence of subcultures that have been successfully developed for efficient regeneration of different rice genotypes. Eleven rice cultivars were used and categorized in three groups; a- (Japonica) including (Sakha 101, 102, 103, 104 and 105), b- (Japonica/indica) including Giza 171, 177 and c- (indica/Japonica) including Giza178, Giza 182, Egyptian H1 and Egyptian Yasmine. A short callus induction step (7 days at 32 °C in the dark) on (CIM1) medium containing 2mg/l dicamba followed by three subcultures each 7 days on fresh (DSE1) containing low level of auxin 0.5mg/l dicamba resulted in induction and development of somatic embryos from scutella tissues in all tested genotypes. The developed somatic embryos (in globular and heart stage) were divided into small clusters and transferred to complete the maturity stage for 7 days at 35 °C in the dark on (MSE) containing high concentration of sugars 8% and 4 g/l Gelrite® as a desiccation treatment for maturation of somatic embryos and free of growth regulators. Mature somatic embryos of all genotypes have been successfully germinated and developed green plantlets in 35 days through 5 subcultures each for 7 days on (PDM1) medium containing 1 mg/l 2ip. The developed green plantlets were transferred to magenta boxes for three weeks to complete the development of roots on (RDM) containing 0.1 mg/l 2ip and 0.1 mg/l IBA. Regenerated plants with good root system were transferred to soil in small pots and kept for acclimatization in growth chamber at 25°C with high humidity 80% for three weeks, and then were transferred to big pots and placed in the greenhouse until maturity. The obtained results, recorded a high capacity of regeneration in eleven Egyptian rice cultivars with a main average 78.30% (86.27 % for group a, 75.55% for group b and 69.71% for group c) and proved that, this novel protocol is reproducible and genotype independent system for somatic embryogenesis in rice, and the critical aspects of somatic embryogenesis in rice related to the timing and the concentration of hormones in filter-sterilized media used through the developmental stages of the embryogenesis pathway to provide the required nutrients to the cells in available forms without any probability of complication which could be occurs when the media sterilized by autoclaving.

Key words: Rice (Oryza sativa L.), genotype, commercial cultivars, mature embryo, somatic embryogenesis, regeneration, Dicamba, 2,4-D, BAP, 2ip.

Introduction

The main obstacle of successful establishing of an efficient protocol of rice transformation is the availability of a reliable and reproducible in vitro culture protocol for rice plant capable of providing an appropriate number of regenerated plants per cell line. Hiei and komari, 2008, in their protocol for Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed, reported that from a survey of about 300 papers, randomly chosen from recent issues of leading journals and in which the transformation of rice is described, revealed that 80% of the studies used Agrobacterium as a tool for gene transfer. In most recent studies, either immature embryos or calli induced from mature seeds have been used as the starting material for Agrobacterium-mediated transformation in rice. Immature embryos seem to be most efficiently transformed especially evident when transforming indica rice varieties, which are generally quite recalcitrant to tissue culture and transformation. Although, using of rice immature embryos are...
representing the critical factor of successes in establishing an efficient transformation system due to it needs to be used at the right developmental stage which differs depending on the genotype and the quality of embryos which related to the cultivation conditions of donor plants; that good embryos can be obtained only from healthy plants growing in controlled greenhouse equipped to control temperature, humidity, day length and light intensity for providing properly immature embryos throughout the year to conduct transformation experiments. Thus, to establish a reliable system based on immature embryos there is a need to establish basic infrastructure of this controlled greenhouse, and it is well known that the construction and maintenance of such facility require considerable funds and intensive labor.

Therefore, the use of immature embryos is more expensive than transformation of calli induced from mature seeds and the cheaper alternative may be the method of choice for most labs. But the critical problem using this method is that the response of mature embryos to callus induction is very much dependent on the genotype of rice. For certain japonica cultivars, such as Nipponbare, preparation of a highly transformable calli is straightforward and highly reproducible. Once culture conditions for callus initiation have been established in these cultivars, mature seeds cultured on callus induction media only for 5 d may also be used (Toki et al., 2006; Herve’ and Kayano, 2006). However, many other genotypes, especially indica rice and some high-quality japonica varieties (including Koshihikari) are recalcitrant to callus induction, and painstaking efforts are needed to develop tissue culture techniques (Hiei et al., 2006; Hiei and komari, 2006; Sahoo et al., 2011). Frequently, a culture medium good for one genotype does not work well for others, and thus efforts need to be made on a genotype-by-genotype basis. In addition, callus culture techniques developed in one laboratory often are not reproducible in other laboratories. Thus, many of the recent studies reporting improved protocols for transformation focus on optimization of the preparation and handling of the callus in various genotypes (Martinez-Trujillo et al., 2003; Park, 2003; Rachmawati et al., 2004; Visarada and Sarma, 2004; Hoque et al., 2005; Kumar et al., 2005; Lin and Zhang, 2005; Datta and Datta, 2006; Evangelista et al., 2009; Ghareeb et al., 2009; Mogheib et al., 2009; Syaiful et al., 2009; Youssef et al., 2009; Wanichananan et al., 2010; Zuraida et al., 2010; Sahoo et al., 2011; Zuraida et al., 2012).

To address the solution of the current problem, there is a serious need to establish a reliable protocol for in vitro regeneration via somatic embryogenesis for different rice genotypes using mature rice embryos instead of using immature embryos.

In Egypt, rice production has reached a record due to the use of new high-yield Egyptian rice hybrids which developed recently through successful breeding programs and application of the integrated management programs. Nevertheless, still there are problems confronting the cultivation of rice and these including the limitation in water resources, salinity and abiotic stress conditions. Therefore, there is an immediate need to sustain the high productivity of Egyptian rice cultivars by further development through genetic transformation to introduce specific genes into the genome of these elite Egyptian rice cultivars to develop new cultivars more tolerant to stress conditions such as drought and salt stresses and capable of producing high yield under stress conditions. Unfortunately, the suitability of Egyptian rice varieties for genetic transformation has not yet been systematically established, and several reports have been indicated that the development of an efficient regeneration and transformation system of Egyptian rice varieties is an important task (Saker et al., 2006; Ghaveeb et al., 2009; Mogheib et al., 2009; Youssef et al., 2009). Thus, the current situation strongly emphasize that, there is a need to solve the problem of using mature rice embryos as a source for establishing a reliable and reproducible in vitro culture protocol for rice plant as a reliable shuttle to develop an efficient transformation protocol for elite Egyptian rice cultivars.

The long term goal of this research in our laboratory is to generate salt and drought tolerant rice by genetic transformation. Therefore, the aim of the present study was designed to strategically solve the current issue by developing a novel protocol capable of establishing a highly efficient regeneration system for different rice genotypes via somatic embryogenesis, in total eleven rice cultivars were used and categorized in three groups; a- (Japonica) including (Sakha 101, 102, 103, 104 and 105), b- (Japonica/Indica) including Giza 171, 177 and c- (Indica/Japonica) including Giza178, Giza 182, Egyptian H1 and Egyptian Yasmine.

Materials And Methods

Plant material and explants sterilization:

Mature grains of eleven rice cultivars representing three different genotypes; a- Japonica (including: Sakha 101, 102, 103, 104 and 105), b- Japonica/indica (including: Giza 171, 177) and c- indica/Japonica (including: Giza178, Giza 182, Egyptian H1 and Egyptian Yasmine), were obtained from the Department of Rice, Field Crops Institute, Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. Dehusked rice grains were carefully washed 3 times with sterile de-ionized H2O plus 10% liquid soap, and then washed 5 times with sterile de-ionized H2O. Afterwards, rice grains were sterilized with 100 ml of a 70% Ethanol (v/v) for 30 sec. followed by 100 ml of a 20% solution of commercial Clorox® (Sodium Hypochlorite
5.25% w/v) plus 0.5 ml Tween 20 for 15 min with shaking (250 rpm), followed by soaking of rice grains for 5 min in sterile de-ionized H₂O (pH 3.0), then washing three times in sterile de-ionized H₂O (pH 7.0).

**Callus initiation and induction of somatic embryogenesis:**

The sterilized grains were placed on filter paper in (120 x 20 mm) Petri dish and the dry sterile grains were cultured onto callus induction medium (CIM, Table 1) 50 grains/Petri dish. All media used in this protocol were filter-sterilized using Durapore PVDF 0.22 µm, WHPL 47 mm (Millipore Cat. No. GVWP04700), i.e. double concentrated CIM medium (2x) 500 ml was filter-sterilized then mixed with (2x) 500 ml of Gelrite® 70°C (2x Gelrite® = 6g/l was autoclaved for 20 min at 121°C and 15 psi) and poured 50 ml aliquots into Petri dishes (120 x 20 mm). Rice grains were cultured on CIM media for 7days at 32 °C in the dark, and then the scutellum of mature rice embryo (explant) was dissected from rice grains under a stereo trinocular microscope (Model: SZX-7 OLYMPUS-JAPAN) with OLYMPUS Digital Camera 12.3 Mega pixels Model: E-620.

**Development of rice somatic embryos:**

Dissected scutella tissues were transferred to the second medium for developing of somatic embryos (DSE, Table 1) for 21 days with subculture interval (7 days) as shown in (Fig. 1) and developed somatic embryos were carefully divided into small clusters of embryos to be directly in contact with the medium.

### Table 1: Composition of different media used for induction and development of somatic embryogenesis in different genotypes of rice, callus induction medium (CIM), development of somatic embryos (DSE), maturation of somatic embryos (MSE), plantlets development medium (PDM) and root development medium (RDM).

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<th>CIM2 mg/l</th>
<th>DSE1 mg/l</th>
<th>DSE2 mg/l</th>
<th>MSE mg/l</th>
<th>PDM1 mg/l</th>
<th>PDM2 mg/l</th>
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*MS salts without NH₄NO₃ : (MS medium ;Murashige and Skoog, 1962, Modification No.4: NH₄NO₃ Free - cat. No. 0238, Duchefa).
*MES monohydrate: 2-(N-morpholino)ethanesulfonic acid (cat no. M1501, Duchefa) as a buffering agent.
*AgNO₃ was freshly prepared as silver thiosulfate (STS): 1 mg/l AgNO₃ + 3.716 mg/l sodium thiosulfate anhydrous.

in min at 121°C and 15 psi) and poured 50 ml aliquots into Petri dishes (120 x 20 mm). Rice grains were cultured on CIM media for 7days at 32 °C in the dark, and then the scutellum of mature rice embryo (explant) was dissected from rice grains under a stereo trinocular microscope (Model: SZX-7 OLYMPUS-JAPAN) with OLYMPUS Digital Camera 12.3 Mega pixels Model: E-620.
Maturation of rice somatic embryos (desiccation treatment):

The developed somatic embryos were transferred to new medium with high concentration of sugars (8%) and 4 g/l Gelrite® as a desiccation treatment for maturation of somatic embryos (MSE, Table 1) and kept for 7 days at 35 °C in the dark.

Germination of rice somatic embryos and development of green plantlets:

After 7 days of culture of mature somatic embryos on MSE medium, somatic embryos were capable to germinate vigorously on plantlets development medium (PDM, Table 1) in Petri dishes (120 x 20 mm) and incubated at 28 °C under cool white fluorescent light with 6000 Lux under 16 hr / 8 hr light/dark cycle, after 35 days with subculture interval (7 days) regenerating plantlets were transferred to magenta boxes containing root development medium (RDM, Table 1) for 21 days.

The Regeneration percentage was calculated as follows:

\[ \text{Regeneration} \% = \frac{\text{No of cell lines produced plantlets}}{\text{No of explants produced embryogenic calli}} \times 100 \]

Acclimatization:

After development of a root system, plantlets were transferred to soil mixture peat moss : sand (3:1) respectively, in small pots and covered with plastic pages, and then placed in a controlled growth chamber at 25 °C with high humidity 80 % for 3 weeks, then successfully were transferred to big pots and placed in the greenhouse until maturity.

Statistical Analysis:

Data obtained were recorded and were exposed to the proper statistical analysis of complete randomized design (Snedecor and Cochran, 1969) in three replicates. Means obtained were differentiated using Duncan’s new multiple range test as described by (Duncan, 1955).

Results And Discussion

The obtained results in this study, lead to design the developed protocol as shown in Fig. 1. A speed subculture on fresh filter-sterilized media each 7 days proved to be essential to provide growth requirements to the vigorously growing embryogenic cells derived from the scutellum of mature rice embryos of different genotypes to sustain the development of these cells to further their success to form somatic embryos with high frequencies.

This protocol describes the whole process from the sterilization treatment until the regeneration and acclimatization of rice plants in growth chamber.

The following factors are involved in the development of this protocol:
1- The Effect of sterilization treatment on the activity of the scutellum tissue of mature rice embryo
2- The Effect of media composition and the type and concentration of auxin (Dicamba and 2,4-D) on induction and development of rice somatic embryos
3- The duration of culture on callus induction media (CIM); CIM1 and CIM2 and the duration of culture on development of somatic embryos (DSE) media;DSE1 and DSE2
4- The type and concentration of cytokinin (2ip and BAP)

Finally, the effect of the interaction between the optimal conditions of the above mentioned factors with emphasize on the impact of the duration of culture on callus induction medium (CIM1) and the duration of culture on development of somatic embryos (DSE1) medium (both media containing dicamba), on regeneration frequency of rice plants was examined in a set of experiments of four parallel protocols differs in the duration factor. Thus, five independent experiments were performed for each protocol using 300 explants (mature rice embryos ); 60 explants for each experiment were divided into three replicates (20 embryo/replicate) in total 1200 explants/cultivar were used as shown in (Table 2). The obtained results were evidently confirmed the reliability of the high-throughput regeneration via somatic embryogenesis of this novel protocol (Fig. 1) for the eleven tested rice commercial cultivars representing three different genotypes.
Fig. 1: Schema for regeneration of different genotypes of Egyptian rice cultivars via somatic embryogenesis.

These definite steps of the embryogenesis pathway in rice are critical for conditioning the physiological status of the developed calli with embryo like structures to sustain the development of somatic embryos from a developmental stage to another one through speed subculture on fresh medium with careful dividing of the calli into small pieces (a small cluster of embryos) to be in direct contact with the medium. This lead to an appropriate synchronization for the development of somatic embryos and resulted in successful germination of many embryos/cell line.

1. Effect of sterilization treatment on the activity of the scutellum tissue of mature rice embryo:

In this study, during the initial phase, a set of preliminary experiments for optimizing the sterilization of mature rice grains was carried out (data not shown) and it was noticed that, the sterilization with Clorox® had an inhibitory impact on the tissues of rice mature embryo of different genotypes due to the high pH (high alkalinity). This impact is a crucial and strongly reduced the survival rate of mature rice embryos which can be monitored by the percentage of germination as well as the appearance of browning on the embryo tissues. Moreover, embryos of some cultivars may take a long time to overcome the influence of this high pH which considered as a retardant treatment (a stress condition) for the cells of mature embryo in particular for the epithelial cells of the scutellum (the outer cell layer of the scutellum) which is responsible for producing direct somatic embryos in rice plant. Vega et al. (2009) reported that, histological studies on transverse sections of scutellum tissue of rice mature embryos after three days of culture on callus induction medium containing (2.5 mg/l 2,4-D) showed that, embryogenic regions were formed from the more mitotically active epithelial cells of the scutellum and these cells of the scutellum of rice mature embryos are columnar with a dense cytoplasm and a prominent nucleus and nucleolus, and then mature embryos produced friable yellowish calli derived from the scutellum after two weeks of culture on callus induction medium.

Therefore, in this protocol, to overcome the negative impact of sterilization with Clorox® on tissues of mature rice embryo especially the epithelial cells of the scutellum, rice grains were soaked for 5 min in sterile de-ionized H₂O (pH 3.0) after sterilization with 100 ml of a 20% solution of commercial Clorox®, and this treatment proved to be essential to equilibrate the alkalinity of Clorox®. Finally it was concluded that, this equilibration treatment has an important role to rescue the embryo tissues from the high pH which continue even after washing several times with neutral sterile de-ionized H₂O (pH 7) and this might explain the case of low survival rate with some cultivars after sterilization with Clorox®. This important notice of great importance due to its impact on the activity of the epithelial cells of the scutellum and it can be considered as one of important keys for the success of this novel protocol for high-throughput of somatic embryogenesis in different genotypes of Egyptian rice cultivars which can give this protocol the property of genotype independence.
2. The Effect of media composition, the type and concentration of auxin (Dicamba and 2,4-D) on induction and development of rice somatic embryos:

Somatic embryogenesis is a successive developmental process that involves multiple phases (Arnlod et al., 2002). Therefore, to establish an efficient somatic embryogenesis pathway, it is essential to provide appropriate nutrient supplements and physiological environment from the early events in this process until the germination and development of plantlets. Ge et al. (2006) reported that, the composition of basal media used for callus induction had a significant effect on formation of embryogenic callus and also on callus regeneration. Hence, induction medium has an effect on embryogenic callus formation; i.e. calli initiated from the scutella of the germinating seeds had embryogenic potential, while calli that arose from the radicle tended to be non-embryogenic, and from which parts the calli were initiated was governed greatly by the composition of the induction medium, in addition to embryogenic callus formation, efficient regeneration also poses a major problem for transformation of indica rice. Similar results were found by Khanna and Raina (1998), as well as Visarada et al. (2002), who showed that the regeneration response was also determined by the induction medium. Therefore, extensive research has been conducted to improve the capacity of plantlet regeneration by manipulating the important factors within regeneration medium, such as carbohydrate source (Lee et al. 2002), nitrogen source (Grimes and Hodges, 1990), osmotic stress (Kavi Kishor, 1989; Emons et al., 1993; Etienne et al., 1993), partial desiccation (Tsukahara and Hirosawa, 1992; Chand and Sahrawat, 2001), polyamines (Bajaj and Rajam, 1995, 1996), amino acids, such as proline and tryptophan (Ozawa and Komamine, 1989; Chowdhry et al., 1993), and plant growth regulators (Kavi Kishor, 1987; Peterson and Smith, 1991).

Nevertheless, these great efforts usually contradict with the case in which form we provide the nutrients to the plant cells to grow and develop in particular through the most sensitive and complicated program of gene expression to form the whole organism (somatic embryo) from an individual cell. So, it is ultimate to provide the requirements to the cell with at least minimal side effects to sustain its growth and development. Filter-sterilization of media components proved to be essential for research work especially when we deal with sensitive and active cells such as protoplasts, microspores, and embryogenic cells to develop somatic embryogenesis pathway and this becomes more important to use for recalcitrant plant species.

Here it is notable to mention that, in vitro culture on media sterilized by autoclaving at 121°C for 15 to 20 min is commonly indicated. Although it is well documented that, heat sterilization can induce profound changes in culture media components, including decomposition of heat-labile nutrients, reactions between sugars, and amino acids, pH changes, and formation of toxic compounds (Hsiao and Bornman, 1989; Schenk et al., 1991). Wetzstein et al. (1994) reported that, in all cases, autoclaving resulted in a decrease in pH, ranging from 0.2 to 0.5 pH units. Numerous studies have likewise reported a drop in pH associated with autoclaving (e.g., Owen et al., 1991; Selby et al., 1989; Singh, 1982). Singh (1982) found a decrease in medium pH of 0.5 pH units; pH reduction was less pronounced with increasing agar concentrations. Selby et al. (1989) found that decrease in pH varied depending on the pH range used (0.8 to 0.9 of a pH unit at pH 6 vs. 0.1 to 0.2 of a pH unit at pH 4). One of the main breakdown products of monomeric saccharides, especially hexose is 5-(hydroxymethyl)2-furaldehyde (HMF) which is known to be biologically toxic (Moye, 1964; Weatherhead et al., 1978). Other toxic compounds that are derived from monomeric saccharides are phenolics (Theander and Nelson, 1989; Suortti, 1983). It has been demonstrated that media sterilized by autoclaving may have adverse effects on plant cells and tissues cultured in vitro (Weatherhead et al., 1978; Negrutiu and Jacobs, 1978a&b; Kohlenbach and Wernick, 1976; de Lange, 1988) as well as on microorganisms (Suortti, 1983; Einarsson et al., 1988). The precipitation of minerals may be facilitated by autoclaving. Schneek et al. (1991) demonstrated that the precipitation of minerals during autoclaving was due to the inclusion of Fe-EDTA and KH2PO4. During autoclaving, Fe-EDTA reacted with micronutrients, and KH2PO4 reacted with calcium to form insoluble mineral complexes that were no longer available to the plant for growth and development.

This revised protocol was developed based on a wide survey and intensive investigation on the impact of media composition on the development of somatic embryos in cereals. Moreover, a continuous work with rice in vitro culture which has been carried in our laboratory lead us to set up various experiments to determine the impact of the improvement in media composition on induction and development of embryogenesis pathway in different genotypes of rice plant. Thus, the concentration of ammonium in the media formula was reduced to 1/10 of the concentration in MS medium (Murashige and Skoog, 1962), MS salts without NH4NO3 (MS medium Modification No.4: NH4NO3 Free -cat. No. 0238, Duchefa) was used then 165 mg/l of NH4NO3 was added to obtain the modified MS medium containing 1/10 of NH4NO3 (modified MS) (Eudes et al., 2003; Ibrahim, 2006 and Ibrahim et al., 2010), in addition to the elevated level of CuSO4 .5H2O to be 5 μM which representing about fifty times as the concentration in (MS medium), also 5.8 μM of AgNO3 was freshly prepared as silver thiosulfate (STS): 1 mg/l AgNO3 + 3.716 mg/l Sodium Thiosulfate anhydrous/liter, and then was added to all used media, the anti-ethylene substances such as CoCl2 which presents in MS salts, CuSO4 and AgNO3 have important role in down regulating the biosynthesis of ethylene in plant cells and this role proved essential to obtain in vitro regenerated plants especially with recalcitrant genotypes of different plants (Nirwan and Kothari,
2003; Kothari et al., 2004; Ibrahim et al., 2010; Al-Shafeay et al., 2011). The role of microminerals in the control of regeneration has received little attention, although a few microminerals have been shown to enhance regeneration significantly. For example, nickel and cobalt stimulated morphogenesis in Daucus carota callus cultures (Roustan et al., 1989) and increasing the level of CuSO₄ in the culture medium significantly enhanced shoot regeneration from the calluses of wheat, and triticale, as well as of tobacco leaf discs (Punhauser and Gyulia, 1993). The present media were supplemented with 1mg/l of spermidine which proved to be involved in plant embryogenesis. Their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signaling processes in various signal transduction pathway (Lakshmanan and Taji, 2000), as precursor molecules for certain growth regulators (Ribnicky et al., 1996; Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b) or regulators of DNA synthesis (Kevers et al., 2000; Astarita et al., 2003). Bajaj, and Rajam, 1996, reported that, a significant reduction in morphogenetic potenita occurs in callus cultures of rice (Oryza sativa L. cv. TN-1) (up to 1 year old), and that plant regeneration could be improved in such cultures with spermidine treatment. Additionally, spermidine treatment of long-term cultures caused an increase in cellular spermidine content and a reduction in putrescine content and arginine decarboxylase activity, leading to an adjustment in putrescine to spermidine ratio and the restoration of plant regeneration ability.

The results of the effect of the type and concentration of auxin (Dicamba and 2,4-D) on induction and development of rice somatic embryos revealed that, the presence of dicamba with 2mg/l resulted in the highest values of the percentage of explants producing embryogenic calli for all rice cultivars after 4 weeks of culture on CIM1 in comparison with 2,4-D as shown in (Fig. 2). The obtained results showed that, the cultivar Sakha 105 of (Japonica genotype) recorded the highest values of the percentage of explants producing embryogenic calli (96.33%) with 2mg/l of dicamba and (86.66 %) with 3mg/l of 2,4-D. Giza 171 of (Japonica/Indica genotype) recorded 86.66% with 2mg/l of dicamba and 78.33% with 3mg/l of 2,4-D. Giza 178 of (Indica/Japonica genotype) recorded 80% with 2mg/l of dicamba and 75.66% with 3mg/l of 2,4-D.

![Fig. 2: Effect of type and concentration of auxin (Dicamba and 2,4-D) on percentage of explants producing embryogenic calli form mature embryos of different rice cultivars.](image-url)

3- The Influence of interaction between duration of callus induction and duration of development of somatic embryos on percentage of explants producing embryogenic calli:

The obtained results evidently indicated that, induction of embryogenic callus was observed frequently and recorded the highest values of the percentage of explants producing embryogenic calli when explants cultured for 7 days on callus induction medium (CIM) containing the high concentration of auxin (CIM1) with 2mg/l dicamba and (CIM2) with 3mg/l 2,4-D then transferred to the medium of development of somatic embryos (DSE) which containing the low level of auxin (DSE1) with 0.5mg/l dicamba and (DSE2) with 0.5mg/l 2,4-D for all tested cultivars, as shown in (Fig. 3 and 4). While the lowest values of the percentage of explants producing embryogenic calli were recorded with the treatment of induction of embryogenic callus on CIM containing the high concentration of auxin for 4 weeks with all tested cultivars. Therefore, based on these results dicamba was used in the subsequent experiments in CIM1 medium with 2mg/l for 7 days then in DSE1 medium with 0.5mg/l for induction and development of rice somatic embryos in different cultivars of the three genotypes.

In this study, it was observed that, the physiological status of the explants (scutella of mature rice grains) in vitro is a crucial factor for regeneration of rice plants in particular through somatic embryogenesis. Thus, the transition of the cells of scutella tissues from the initiation and induction of cells division to a specific programmed cell division pattern to form somatic embryos is a critical point to establish an embryogenesis pathway. Here, the results confirmed this observation and two distinct stages were indentified for all tested
genotypes, the first stage 7 days was identified as (the induction phase of somatic embryogenesis; Fig. 5), and the second stage 21 days was identified as (the phase of formation and development of somatic embryos; Fig. 6). The induction of high frequencies of somatic embryos was developed from the scutella tissues cultured on CIM1 with 2mg/l dicamba and the duration of this phase (7 days) is of great importance, that the long duration on a high level of dicamba or 2,4-D resulted in the formation of friable non-embryogenic calli at high frequency. Therefore, the short period of the induction phase with 2mg/l dicamba for 7 days was confirmed and used to develop the embryogenic calli which transferred to the second phase.

![Fig. 3: Percentage of explants producing embryogenic calli of different rice cultivars during two phases of culture on two different media; the first medium containing 2mg/l of dicamba for different durations (7, 14, 21 and 28 days), and the second medium containing 0.5mg/l of dicamba for different durations (7, 14 and 21 days).](image1)

![Fig. 4: Percentage of explants producing embryogenic calli of different rice cultivars during two phases of culture on two different media; the first medium containing 3mg/l of 2,4-D for different durations (7, 14, 21 and 28 days), and the second medium containing 0.5mg/l of 2,4-D for different durations (7, 14 and 21 days).](image2)
with 0.5mg/l dicamba for 21 days through three subcultures (with 7 days interval) to complete the developmental pathway of rice somatic embryos of different genotypes.

Maturation of rice somatic embryos:

The developed somatic embryos after cultivation on DSE media for 21 days with subculture interval 7 days were transferred to new medium of mature somatic embryos (MSE) medium without growth regulators and supplemented with high concentration of sugars (8%) and 4 g/l Gelrite® as a desiccation treatment for maturation of somatic embryos and kept for 7 days at 35 °C in the dark (Fig. 7).

Germination of rice somatic embryos and development of green plantlets:

After 7 days of culture of mature somatic embryos on MSE medium, somatic embryos were transferred to plantlets development medium (PDM) in Petri dishes (120 x 20 mm) and incubated at 28 °C under cool white fluorescent light with 6000 Lux under 16 hr / 8 hr light/ dark cycle. The mature somatic embryos were germinated vigorously on (PDM) and much green plantlets/cell line started to appear after the second subculture on PDM media as shown in (Fig. 8). After 35 days five subcultures were performed with subculture interval (7 days) regenerating plantlets were transferred to magenta boxes containing root development medium (RDM) for 21 days to complete the development of root system of individual plants. An embryogenesis system requires the following steps, which occur in succession: initiation of embryogenic callus from vegetative tissues or cells, maintenance and multiplication of embryogenic cell lines, somatic embryo formation and maturation and finally conversion (germination) of somatic embryos into viable plantlets (Zegzouti et al., 2001). Deo et al. (2010) reported that, maturation is regarded as an essential stage of embryogenesis since the frequency of plant recovery is high from mature embryos. Embryo maturation is a culmination of the accumulation of carbohydrates, lipids and protein reserves, embryo dehydration and a reduction in cellular respiration (Trigiano and Gray, 1996). Thus, maturation is a preparatory stage for embryos for effective germination as Etienne et al. (1993) stated, “maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases”; consequently, bypassing the maturation phase will result in precocious germination of embryos causing a significant reduction in viable plantlets.

Dehydration was hypothesized to be critical for maturation (Etienne et al., 1993). As such, restricting water uptake using osmoticum was studied (Etienne et al., 1993; Attree et al., 1995; Gutmann et al., 1996) for its ability to support development of plant embryos while at the same time suppressing precocious germination. Permeating osmoticum, such as sucrose, is frequently used to reduce the water potential of the culture medium resulting in water stress thereby promoting embryo development during in vitro culture. Finally, it can therefore be concluded that for a somatic embryogenesis system to be practically applied, high frequency embryo formation is of little value unless a large proportion of these embryos are capable of developing into normal plants (Venkatachalam et al., 1999).

4- The effect of type and concentration of cytokinin (2ip and BAP) on regeneration frequencies of eleven rice cultivars of different genotypes:

To study the effect of type and concentration of cytokinin (2ip and BAP) on regeneration frequencies of eleven rice cultivars of different genotypes the developed somatic embryos of each cultivar (which were derived from scutella tissues of mature embryos cultured for 7 days on CIM1 containing 2mg/l dicamba, then for 21 days with three subcultures; 7 days interval, on DSE1 medium containing 0.5mg/l dicamba), were transferred to eight different plant development media (PDM); four PDM media containing different concentration of 2ip (0.5, 1, 2 and 4 mg/l) and four PDM media containing different concentration of BAP (0.5, 1, 2 and 4 mg/l).

The recorded results showed that, the presence of 2ip in PDM media resulted in the highest values of the percentage of cell lines producing green plantlets in comparison with BAP as shown in (Fig.s 9 and 10). The concentration (1 mg/l) of 2ip produced the maximum values of regeneration frequencies for different genotypes; which recorded the highest value 92.5 % with Sakha 105 of (Japonica genotype), Giza 171 of (Japonica / Indica genotype) recorded 76.6 % and Giza 178 of (Indica / Japonica genotype) recorded 72.3%, in comparison with BAP which recorded the highest values with the concentration (2mg/l) which was 71.5 % with Sakha 105 of (Japonica genotype),
Fig. 5: Induction of somatic embryos on scutellum of mature rice embryos of different genotypes after 7 days on CIM medium, just before being transferred to the second stage (Formation and development of somatic embryos). (A) Scutellum of mature rice embryo derived somatic embryos; cv. Egyptian H1. (B) In focus somatic embryos on scutellum with 50x magnification. (C&D) Somatic embryos derived from scutellum of mature rice embryo; cv. Giza 177. (E&F) Somatic embryos derived from scutellum of mature rice embryo; cv. Sakha 103. (G&H) Isolated somatic embryo from scutellum tissue. (I) In focus somatic embryo like structures on scutellum tissue; cv. Egyptian Yasmine.
Fig. 6: Formation and development of somatic embryos derived from scutellum of mature rice embryo of different genotypes after 14 days. (A&B) Clusters of somatic embryos in globular stage on a scutellum of mature rice embryo; cv. Egyptian H1. (C&D) In focus somatic embryos on scutellum with 30x magnification; cv. Egyptian H1. (E&F) Somatic embryos derived from scutellum of mature rice embryo; cv. Giza 177. (G&H) Somatic embryos derived from scutellum of mature rice embryo; cv. Sakha 105.
Fig. 7: Mature somatic embryos derived from scutellum of mature rice embryo of different genotypes. (A&B) somatic embryos in globular and heart stage on a scutellum of mature rice embryo; cv. Egyptian H1 after 21 days. (C&D) In focus somatic embryos in heart stage; cv. Egyptian H1. (E&F) Somatic embryo in torpedo stage derived from scutellum of mature rice embryo; cv. Giza 171. after 28 days. (G&H) Mature Somatic embryos derived from scutellum of mature rice embryo; cv. Sakha 104 after 35 days, just before being transferred to the second stage (Germination of rice somatic embryos and development of the plantlets).
Fig. 8: Germination of rice somatic embryos and development of the plantlets of different genotypes. (A) Germination of somatic embryos and development of the green plantlets on PDM medium; cv. Sakha 101 after 49 days. (B) Development of the green plantlets on PDM medium; cv. Giza 177 after 49 days. (C) Development of the green plantlets; cv. Egyptian H1 after 49 days. (D,E,F) In focus, germination of somatic embryos of different genotypes; D = Sakha 101, E = Giza 177, F = Egyptian H1. (G,H,I) In focus, development of the green plantlets of different genotypes; G = Sakha 103, H = Giza 171, I = Egyptian Yasmine, after 70 days, just before being transferred to RDM in magenta boxes to complete the development of root system. (J,K,L) Development of root system of the green plantlets of different genotypes; J = Sakha 105, K = Giza 177, L = Egyptian H1, after 70 days, just before being transferred to small pots for adaptation stage; 21 days. (M) Successful growth and development of fertile regenerated plants of different genotypes until maturity in the greenhouse.
61.22% with Giza 177 of (Japonica/ Indica genotype) and 60.54% with the cultivar Giza 178 of (Indica/ Japonica genotype). These results strongly indicate that, 6-(γ,γ-Dimethylallylamino)purine (2ip) which is the precursor of zeatin, is the proper cytokinin for germination of the developed rice somatic embryos and development of green plantlets of different genotypes at high frequencies.

Finally, the effect of the interaction between the optimal conditions of the previous factors with emphasize on the impact of the duration of culture on callus induction medium (CIM1) and the duration of culture on development of somatic embryos (DSE1) medium (both media containing dicamba), on regeneration frequency of rice plants was examined in a set of experiments of four parallel protocols differs in the duration factor. Thus, five independent experiments were performed for each protocol using 300 explants (mature rice embryos); 60 explants for each experiment were divided into three replicates (20 embryo/replicate) in total 1200 explants/cultivar were used as shown in (Table 2).

The obtained results were evidently confirmed the reliability of the high-throughput regeneration via somatic embryogenesis of this novel protocol for the eleven tested rice commercial cultivars representing three different genotypes. These results showed that, significant differences were recorded in the rate of regeneration for all genotypes (Table 2) in addition to the rate of No. of plantlets/cell line (each cell line derived from one scutellum) and these results confirmed the success of this protocol to solve the problem of the genotype dependent in rice plant in particular with Indica genotypes. Thus, the obtained results, recorded a high capacity of regeneration in eleven Egyptian rice cultivars with a main average 78.30% (86.27% for group a, 75.55% for group b and 69.71% for group c) and proved that, this novel protocol is genotype independent and the critical aspects of somatic embryogenesis in rice related to the timing and the concentration of hormones in filter-sterilized media used through the developmental stages of the embryogenesis pathway to provide the required
nutrients to the cells in available forms without any probability of complication which could occur when the media are sterilized by autoclaving.

Table 2: Effect of Interaction between callus induction medium (CIM1) containing 2mg/l of dicamba and development of somatic embryos medium (DSE1) containing 0.5mg/l of dicamba and different durations on regeneration frequency and number of regenerated plantlets/cell line cultured on plantlets development medium (PDM1) containing 1mg/l of 2ip.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Average No. of regenerate plantlets/cell line</th>
<th>Regeneration %</th>
<th>Average No. of regenerate plantlets/cell line</th>
<th>Regeneration %</th>
<th>Average No. of regenerate plantlets/cell line</th>
<th>Regeneration %</th>
<th>Average No. of regenerate plantlets/cell line</th>
<th>Regeneration %</th>
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<tr>
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<td>2.11 Dd</td>
<td>62.81 Bb</td>
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<td>2.32 Cc</td>
<td>53.78 Bc</td>
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<td>35.14 Cd</td>
<td>1.22 Cd</td>
<td>34.95 Bd</td>
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<tr>
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<td>2.43 Bc</td>
<td>64.91 Ab</td>
<td>1.41 Cd</td>
<td>47.91 Bd</td>
<td>1.11 Dd</td>
<td>41.66 Ae</td>
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<td>70.53 Ab</td>
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<td>2.75 Ad</td>
<td>73.55 Ab</td>
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<td>55.88 Ac</td>
<td>1.44 Ad</td>
<td>39.53 Ac</td>
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<tr>
<td>Giza 171</td>
<td>76.64 Ca</td>
<td>2.39 Cd</td>
<td>53.65 Bc</td>
<td>1.38 Dd</td>
<td>33.66 Cd</td>
<td>1.25 Cd</td>
<td>31.46 Cd</td>
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<tr>
<td>Giza 177</td>
<td>74.64 Ca</td>
<td>2.46 Bd</td>
<td>43.33 Cc</td>
<td>1.44 Cd</td>
<td>35.29 Cd</td>
<td>1.48 Ad</td>
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<td>2.12 Dd</td>
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<td>39.23 Dc</td>
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<td>30.18 Dd</td>
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</table>

Means followed by different capital letters in columns and those followed by different small letters in rows are significantly different at P = 0.05 according to Duncan’s multiple range test.

Conclusion:

In conclusion, the obtained results in this study confirmed the establishment of a novel genotype independent protocol for somatic embryogenesis in rice using mature embryos of different genotypes. Thus, this novel protocol is a milestone and will pave the way for improving the efficiency of genetic transformation protocols of commercial rice cultivars. The developed protocol describes the composition of different media, duration and the sequence of subcultures that have been successfully developed for efficient regeneration of different rice genotypes; three genotypes were used and categorized in three groups; a- (Japonica) including (Sakha 101, 102, 103, 104 and 105), b- (Japonica/indica) including Giza 171, 177 and c- (indica/Japonica) including Giza178, Giza 182, Egyptian H1 and Egyptian Yasmine.

This reproducible and highly efficient protocol based on the modified media and precise observations day by day for the development of somatic embryos derived from scutella tissues of mature rice embryos on different media. A short callus induction step (7 days at 32 °C in the dark) on (CIM1) medium containing 2mg/l dicamba followed by three subcultures each 7 days on fresh (DSE1) containing low level of auxin 0.5mg/l dicamba resulted in induction and development of somatic embryos from scutella tissues in all tested genotypes. The developed somatic embryos (in globular and heart stage) were divided into small clusters and transferred to complete the maturity stage for 7 days at 35 °C in the dark on (MSE) containing high concentration of sugars 8% and 4 g/l Gelrite® as a desiccation treatment for maturation of somatic embryos and free of growth regulators. Mature somatic embryos of all genotypes have been successfully germinated and developed green plantlets in 35 days through 5 subcultures each for 7 days on (PDM1) medium containing 1 mg/l 2ip. The developed green plantlets were transferred to magenta boxes for three weeks to complete the development of roots on (RDM) containing 0.1 mg/l 2ip and 0.1 mg/l IBA. Regenerated plants with good root system were transferred to soil in small pots and kept for acclimatization in growth chamber at 25°C with high humidity 80% for three weeks, and then were transferred to big pots and placed in the greenhouse until maturity. The obtained results, recorded a high capacity of regeneration in eleven Egyptian rice cultivars with a main average 78.30% (86.27 % for group a, 75.55% for group b and 69.71% for group c) and proved that, this novel protocol is reproducible and genotype independent system for somatic embryogenesis in rice, and the critical aspects of somatic embryogenesis in rice related to the timing and the concentration of hormones in filter-sterilized media used through the developmental stages of the embryogenesis pathway to provide the required nutrients to the cells in available forms without any probability of complication which could be occurs when the media sterilized by autoclaving.

This novel protocol represents an important prerequisite for developing highly efficient transformation protocols for rice plants especially the commercial cultivars of different genotypes. One other benefit of this protocol may be that it could be applicable for many other genotypes worldwide for using of mature rice
embryos as a source for regeneration and transformation that several researchers were studied the response of mature rice in vitro and still there are recalcitrant cultivars especially indica rice and some high-quality japonica varieties as reported by several workers (Park, 2003; Martinez-Trujillo, et al., 2003; Rachmawati, et al., 2004; Visarada, and Sarma, 2004; Hoque, et al., 2005; Kumar, et al., 2005; Lin and Zhang, 2005; Datta, and Datta, 2006; Hiei, et al., 2006, Hiei and komari, 2006; Hiei and komari, 2008; Evangelista, et al., 2009; Ghareeb, et al., 2009; Mogheib, et al., 2009; Syaiful, et al., 2009; Youssef, et al., 2009; Zuraida, et al., 2010; Sahoo, et al., 2011; Wanichananan, et al., 2010; Zuraida, et al., 2012).

Acknowledgment

The authors wish to acknowledge the Science and Technology Development Fund (STDF), that this work was partially supported through a grant for a research project (ID: 2579). Also the authors wish to thank Prof. Dr. Mohamed Khalil, Plant Physiology Division, Faculty of Agriculture, Cairo University, for his valuable suggestions and critical review of this work, and Prof. Dr. Hamdy El Mowafi, Department of Rice, Field Crops Institute, Agricultural Research Center (ARC), for his kind and sincere cooperation and providing the grains of rice cultivars. Heartfelt thanks for team work members at (PBRL).

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