IJABBR- 2014- eISSN: 2322-4827

International Journal of Advanced Biological and Biomedical Research

Journal homepage: www.ijabbr.com

Original Article

Study of Factors Influencing Somatic Embryogenesis in Rice (*Oryza Sativa* L.)

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ARTICLE INFO

Article history:
Received: 06 Dec, 2014
Revised: 29 Jan, 2015
Accepted: 26 Feb, 2015
ePublished: 30 Mar, 2015

Key words:
Rice
Mature embryo
Somatic embryogenesis
PEG
$AgNO_3$
Activated charcoal

ABSTRACT

Objective: n present study effect of various factors including sucrose, agar, PEG, $AgNO_3$, activated charcoal on somatic embryo induction and its subsequent regeneration was investigated using varieties Dom siah and Nemat. **Methods:** 330 explant (Mature embryos) of each varieties were cultured, 33/plate (100 x 15 mm), on callus induction Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 7g/L agar and 2 mg/L 2,4-D. **Results:** Significant difference was observed between two variety in callus induction. Somatic embryogenesis and the plant regeneration were influenced by the genotype as well as composition of the medium used. The callus induction rate and callus morphology were different depend on genotypes. High somatic embryogenesis was obtained in medium (MS medium containing 2 mg/L 2,4-D) supplemented with 3 mg/L $AgNO_3$, 9 gr/L agar and 20 gr/L PEG, respectively. The high frequency plant regeneration was achieved from both varieties in medium supplemented with 9 and 11gr/L agar and 5 mg/L $AgNO_3$.

1.INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's major food crops and staple food for more than half of the world's population (IRRI, 2006; Tyagli et al. 2004). Rice is a good model plant for molecular studies in cereals (Tyagli et al. 2004). Biotechnology is an alternative for conventional breeding methods; however, integration of biotechnology into rice improvement methods through genetic engineering needs an efficient in vitro culture protocols (vega et al. 2009). Among the cereals, rice and maize are largely response to tissue culture and are capable of regeneration in in vitro conditions (Ganeshan et al, 2006). In vitro regeneration occurs in two main way including oraganogenesis and somatic embryogenesis. Somatic embryogenesis is the process of developing bipolar structures that derived from haploid or diploid somatic cells and formed through an embryological stage without fusion of gametes that are not connected to the primary vascular tissues of the mother calli. Somatic embryogenesis is a unique process in plants and have considerable interest for biotechnological application such as clonal propagation, production of synthetic seeds and genetic transformation (Quiroz-Figueroa, 2006). Somatic embryogenesis in integration with classical breeding programs and molecular biology techniques provides a valuable tool to enhance the genetic improvement of crop species (Quiroz-Figueroa, 2006). In rice, somatic embryogenesis is the most common regeneration pathway and has been obtained from roots, leaf bases of young seedlings, mature embryos, immature

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embryos, caryopses, coleoptile, cell suspension, protoplast and young inflorescences (Hoque and Mansfield, 2004; Ramesh et al. 2009; Naqvi et al. 2006; Noouri-Delawar and Arzani, 2001; Gairi and Rashid, 2004; Valedez et al 1996; Akter and AL-Forkan, 2010; Jelodar et al. 2002; Chen et al. 1985; Meneses *et al.* 2005; Ge *et al*. 2006). Several factors including plant growth regulators, explant, culture conditions, plant genotypes, light, charcoal and other biochemical factors such as amino acids affect rice somatic embryogenesis and subsequent plant regeneration (Deo et al. 2010).
The purpose of this study was to study effect of different factor on somatic embryogenesis in two commercially important varieties of rice and optimize culture condition for regeneration plants from mature embryo of rice.

2. MATERIALS AND METHODS

2.1. Explants preparation

The rice (Oryza sativa) varieties (cv. Nemat and cv. Dom siah) were provided from Rice Research Institute of IRAN (Rasht) and dehusked manually. The dehusked healthy seeds were surface-sterilized by immersion in 70% ethanol for 90 sec., followed by 5 minutes shaking in sterile distilled water and then 20 minutes shaking in 50% Clorox (Sodium hypochlorite 2/5%) and finally rinsing three times in sterile distilled water.

2.2. Callus induction

330 explant (Mature embryos) of each varieties were cultured, 33/plate (100 x 15 mm), on callus induction Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 7g/L agar and 2 mg/L 2,4-D (Fig. 1: 1a). The pH of the medium was adjusted to 5.7–5.8 prior to autoclaving. Cultured seeds were maintained in the dark room at 25–27°C under diffuse cool white fluorescent light (30 m mol m^{-2} s^{-1}) and the percentage of callus induction was calculated after 3–4 weeks.

2.3. Somatic embryogenesis and regeneration

After one month calli derived from primary culture sub cultured on same medium supplemented with different concentrations of sucrose, PEG, $AgNO_3$, agar and Activated charcoal (Table 1). After 4 week Somatic embryogenesis of calli were measured for all treatments. To obtain regeneration, embryogenic calli were transferred to same medium containing 4 mg/L BAP + 0/5 mg/L IAA. For experiment analyzing proportion of plants regeneration were calculated as follows = (The number of plantlets regenerated ÷ The number of embryogenic callus) × 100%.

2.4. Statistical analysis

The experiment was carried out in factorial experiment (two factorial, variety × embryogenesis treatments) with a completely randomized design with 3 replications. Differences between means were scored with Duncan's multiple range test. Statistical analysis performed using SPSS for Windows release 16.0 (SPSS, Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Callus induction

Both genotype gave rise callus from the cultured mature embryo on MS medium supplemented with 2 mg/L 2,4-D (Fig. 1: 1b,1c). Two types of callus; non-embryogenic and embryogenic callus were observed in culture. First type was soft, friable, yellow or bright brown in color which defined as non-embryogenic (Fig. 2: 2a). But the embryogenic calli were yellowish white, compact and nodular (Fig. 2: 2b). Frequency of embryogenic callus in Nemat was more than Dom siah. In both varieties the colure of calli were yellowish white but the calli were more compact and with less fragility in Dom siah.
In monocotyledons like rice, auxin is necessary for indirect somatic embryogenesis and 2,4-dichlorephenoxyacetic acid (2,4-D) commonly used to obtain embryogenic calli at concentrations between 10^{-5} M and 10^{-7} M (Meneses, 2005). The difference in 2,4-D concentration that needed for callus proliferation maybe due to genetic variation in interval levels of hormones which affected the callus proliferation (Deo et al. 2010). However, at higher concentration of 2,4-D callus induction in all varieties decreased (Verma et al. 2011). This hormone also promote somatic embryogenesis and have important role in cell division in rice (Panjaitan et al. 2009).
By using t-test significant differences were observed between two varieties in callus induction. The percentage of Callus induction in Nemat variety (77%) was higher in compared to Dom siah variety (62%).
Genotype have been indicated as a main factor in callus induction and regeneration of cereals. Callus induction and regeneration controlled through genetically factors which are in nucleus or cytoplasm. The effect of genotype have been caused response to tissue culture technique be differences among the different species of rice and even between varieties within a species (Kuroda et al. 1998). According with our results Verma et al. (2011) observed significant genotypic difference among different varieties of rice in callus induction on MS medium supplemented with 2,4-D.

3.2. The effect of different treatments on somatic embryogenesis

To study the effect of various factors on somatic embryogenesis 1-month-old calli were further

transferred to the different composition of some materials (MS-A to MS-J). After 4 weeks the percentage of somatic embryogenesis of both varieties were measured. Statistical analysis of variance showed significant genotypic differences in frequency of somatic embryogenesis in different medium culture. The compare means of embryogenic calli percentage with Duncans test showed that ability to form callus is dependent on the genotype. Percentage of somatic embryogenesis in Dom siah (54%) was higher than Nemat (34%).

Previous studies in coffee (Molina et al. 2002) and in sugarcane (Gandonou et al. 2005 b) indicated the ability of embryogenic callus formation dependent on genotype. They report that the genotype effect is a stable trait and the embryogenic capacity of a line could be predicted if the embryogenic rate of its ancestors is known.

There was significantly difference between 11 different medium in somatic embryogenesis, and MS-E (3 mg/L $AgNO_3$), MS-G (9gr/L agar) and MS-C (20 gr/L PEG) gives best results respectively (Fig. 3).

In this experiment $AgNO_3$ were added in two different concentrations (0, 3 and 5 mg/L). The highest embryogenesis response was obtained at 3 mg/L $AgNO_3$ (MS-G) and no significant difference was observed between control and 5 mg/L $AgNO_3$. In media supplemented with $AgNO_3$ highest number of somatic embryogenesis per explant was obtained compared to the other treatments. Browning of callus was not observed in media supplemented with $AgNO_3$. $AgNO_3$ through reduction of ethylene which have inhibitory effect on somatic embryogenesis, increase somatic embryogenesis. In durum wheat (*Triticumdurum* Desf.) addition of 1 mg/L of $AgNO_3$ to culture medium enhanced the induction of direct somatic embryogenesis, both the per cent of embryogenic explants and the number of somatic embryos per explant (Fernandez et al. 1999). Sakhanokho et al. (2009) observed that $AgNO_3$ induced somatic embryogenesis although in high concentrations decrease embryos production.

Effect of three different concentration of agar (7 (control), 9 and 11 g/L) in embryogenesis were tested. Maximum somatic embryogenesis (60%) was observed on medium containing 9 gr/L agar as compared to control (36/66%) and medium containing 11 gr/L agar (46/66%) and no significant difference was observed between control and 11 g/L agar. With increasing concentration of agar the fragility of calli was decreased and callus necrosis postponed. In fact gelling agent with creation osmotic stress and reduction water availability for culture medium affect the endogenous ABA levels (Hammatt and Davey, 1987). Brown et al. (1989) have been reported increasing concentration of gelling agent is effective on somatic embryogenesis in wheat. In sugarcane best results were observed with increases in per cent somatic embryogenesis on medium solidified with 12 gr/L agar as compared to control (7 gr/L) (Manchanda and Gosal, 2012). it assume that with increasing concentration of agar, water is lost from the explant which act as an inducer for somatic embryogenesis but by increasing the concentration of agar, excessive callus dehydration may occur which may have a negative effect on somatic embryogenesis.

For observe effect of water stress on somatic embryogenesis 1-month-old calli were further transferred to MS medium supplemented with PEG (MW 6000) at 20 and 40 gr/L. PEG at 20g/L significantly increased somatic embryo formation compared to the control culture. Increasing PEG concentration to 40 gr/L has no significant effect on somatic embryogenesis in comparison with the control. The color of calli also changed from white to dark yellow with the progress of PEG level. These results were consistent with the experiments conducted on rice by Abdul-Qadir and Al-Ka'aby (2011) and Al-Bahrany (2002) in which, high concentration of PEG decreased somatic embryogenesis development, by contrast low concentration of PEG improvement callus growth and somatic embryos formation. Addition of PEG to culture medium causing cellular dehydration through decreasing water content and cell turgor pressure which consequently reduce callus growth (Joshi et al. 2011; Al-Bahrany, 2002). So the presence of increased concentration of PEG in culture medium during development of primary materials and somatic embryogenesis, prevent further development of somatic embryogenesis (McCoy, 1987).

Effect of sucrose was the another factor that examined in three different concentrations (30 (control), 45 and 60 gr/L). Statistical analysis showed that frequency of somatic embryogenesis was not affected by carbon source. However in medium containing 45 gr/L sucrose somatic embryogenesis was more (45/33%) than control medium (36/66%). It is noteworthy that high concentrations of sucrose accelerates callus necrosis. Grewal et al. (2005) showed that the use of high concentration of sucrose (60 mg/L) caused further callus necrosis and decreased somatic embryos development in rice. Type of carbohydrate and its concentration plays an important role in the various stages of somatic embryogenesis. In almost medium the sucrose is a standard carbon source . It has proven, the type of carbon source and its different concentration have significant effect on somatic embryogenic induction and embryos development (Gerdakaneh et al. 2009). As we known application of high sugars concentration in media affect cell osmolarity and suggested through osmotic stress improved the development of globular somatic embryos (Slesak and Przywara, 2003). But high concentration of carbohydrate caused embryos decline and necrosis. Sucrose leads to callus browning by raising the levels of ethylene production outside the tissue and replacement of sucrose with maltose reduced ethylene production and protected callus tissue (Lentini et al.1995).

Activated charcoal in two concentrations (0/5 and 1) is added to culture medium and its effect on somatic embryogenesis was examined. It was observed those level of activated charcoal had negative effect on

emberyogenesis and somatic embryogenesis decreased compared to control. Activated charcoal is often used to reduce or eliminate undesirable compounds and also improve the morphogenesis response of explants to in vitro tissue culture (Manchanda andGosal, 2012). In the majority of studies which conducted on the effect of active charcoal in the somatic embryogenesis its positive role pointed out. Grewal et al. (2005) indicated that addition of active charcoal to the mature embryo culture medium of rice, prevent of callus browning and improved callus growth and subsequently, somatic embryogenesis. Manchanda and Gosal (2012) also reported when activated charcoal (2 mg/L) was added into the medium, percentage of somatic embryogenesis enhanced from 80.21 to 84.88%. The most important impact of AC is a drastic dip in concentration of plant growth regulators and other organic substances due to absorption of these substances by AC. For example, it was indicated that in liquid medium 99.5% of the added 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D) was adsorbed by 0.025 g/L AC within 5 days of medium preparation (Ebert and Taylor, 1990). However, increased levels of 2,4-D could be obtained by application semisolid medium, decreasing AC levels and adding higher concentrations of 2,4-D (Ebert and Taylor, 1990). In this study, as well as because of low percentage of somatic embryogenesis in the presence of active charcoal can be related to low values of 2,4-D which available in media or high concentrations of active charcoal.

3.3. Regeneration of embryogenic calli

For regeneration, embryogenic calli (Fig. 5: 5a) were transferred to the MS medium supplemented with 4 mg/L BAP and 0/5 mg/L IAA. The regeneration was observed when green spot appears on embryogenic calli within 15 days of transfer to regeneration medium (Fig. 5: 5b) and multiple shoot were formed after one month (Fig. 5: 5c). The effect of 11 different treatments in plant regeneration was investigated.

The ANOVA results indicated that effect of genotype and medium were significant at 1% level. Thus it could be concluded that the two varieties showed significant difference in response to regeneration. Best regeneration (40%) was observed in Nemat in comparison with Dom siah (24%). The different medium used also differed significantly in regeneration of embryos from each other. The best regeneration was obtained in both varieties from medium supplemented with 9 and 11 gr/L agar and also 5 mg/L silver nitrat and lowest response was observed in medium containing activated charcoal (Fig. 4).

Enhancing concentration of agar to 9 and 11 gr/L increased frequency of regeneration. When agar concentration is increased excessive water is lost from explant and created a desiccation state for explant which it provided a trigger regeneration of plant. (Manchanda and Gosal, 2012). Zuraida et al. (2012) found that application of desiccation treatment before or during plant regeneration approved plant regeneration. Kaur and Gosal (2009) indicaed that the desiccation created by using agar caused increasing in regeneration frequency of sugarcane callus.

In media supplemented with 5 mg/L $AgNO_3$ regeneration percent was also enhanced (64/83%) in comparison with control (37%). Addition of ethylene inhibitors such as $AgNO_3$ in culture media which is an inhibitor of ethylene signal transduction has been shown to improve in vitro plant regeneration (Kumar et al. 2007). Plantlet regeneration through somatic embryogenesis in present of $AgNO_3$ have been reported in a number of plant species such as *Cassava* (Zhang et al. 2001), *Ziziphus jujube* Mill (Feng et al. 2010) and *Brassica rapa* L. (cogbill et al. 2010).

PEG (20 and 40 gr/L) did not show significant difference in response to regeneration in comparison with control. PEG in solid culture media by reducing water potential affect cell division and lead to reduced growth of the callus, which consequently affect the regeneration potential (Sakthivelu et al. 2008). Reduction in regeneration potential has been reported by increasing osmotic stress in rice (Wani et al. 2010), wheat (Barakat and Abdel-Latif, 1995) and tomato (Aazami et al. 2010). In the study conducted by Joshi et al. (2011) was found increasing the concentration of PEG, decreases the percentage of regeneration and the normal regeneration of embryonic callus was obtained in the media without PEG. Because the amount of used PEG was low in this experiment so it have not further negative effect on regeneration.

Enhancing concentration of sucrose to 60 mg/L resulted in decrease regeneration frequency. Sucrose is rapidly hydrolysed to glucose and fructose which further increases the osmotic pressure of the medium (Last and Brettell, 1990). Shahnewaz and Bari (2004) have reported higher regeneration obtained in media containing low concentration of sucrose (3% and 4%) in comparison with media supplemented by high concentration of sucrose (5% and 6%).

Both concentration of AC (0/5 and 1 mg/L) that applied in this study did not show significant difference in regeneration in comparison with control. Active charcoal through a change in the ratio of compounds existing in media, affected the regeneration (Dennis Thomas, 2008). Lopez-Perez et al. (2005) showed addition of AC to the somatic embryos germination media of *Vitis vinifera* L. enhanced regeneration frequency. It seems that in our study there was no correlation between the amount of hormone and AC, which reduces somatic embryos frequency.

Table 1.
The embryogenesis medium (MS + 2 mg/L 2-4,D) supplemented with different concentration of some additives

Medium	Treatment
MS-A	45 g/L sucrose
MS-B	60 g/L sucrose
MS-C	20 g/L PEG
MS-D	40 g/L PEG
MS-E	3 mg/L Silver nitrat ($AgNO_3$)
MS-F	5 mg/L Silver nitrat ($AgNO_3$)
MS-G	9 g/L agar
MS-H	11 g/L agar
MS-I	0/5 g/L Activated Charcoal
MS-J	1 g/L Activated Charcoal

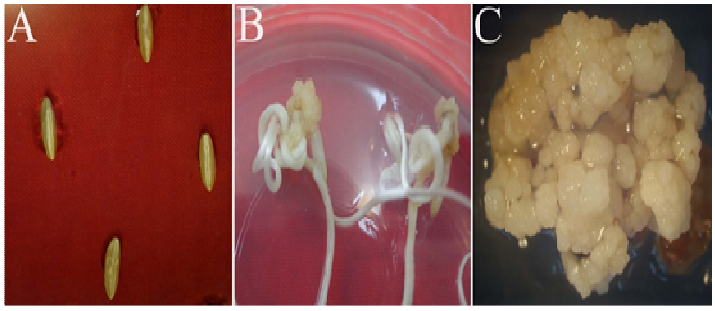

Fig. 1. Callus induction in rice (Oryza sativa). (A) Seeds cultured on Ms medium supplemented with 2,4-D mg/L. (B) Callus induction of explants after 12 days. (C) Growth and development of callus.

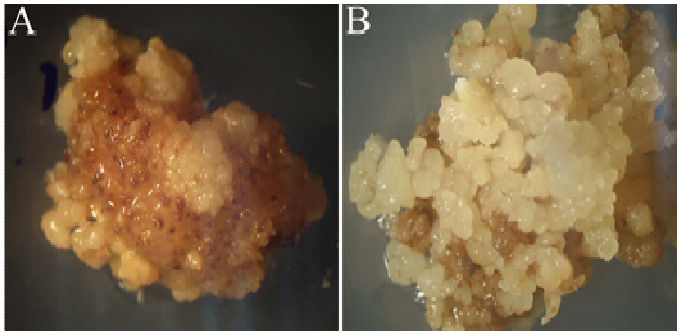

Fig. 2. Morphological aspectof callus of rice(Oryza sativa).(A)Non-embryogenic callus that was soft, friable, yellow or bright brown in color. (B) Embryogenic callus that was yellowish white, compact and nodular**.**

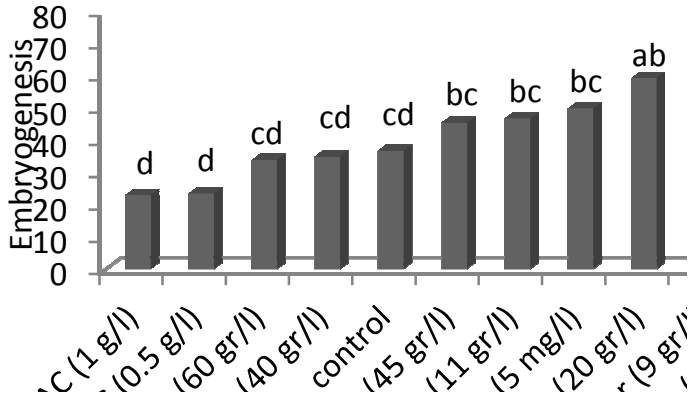

Fig. 3. Effect of different treatments on Rice embryogenesis

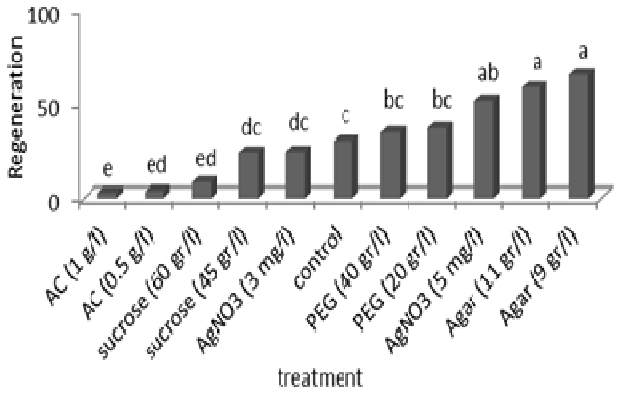

Fig. 4. Effect of different treatments on rice regeneration

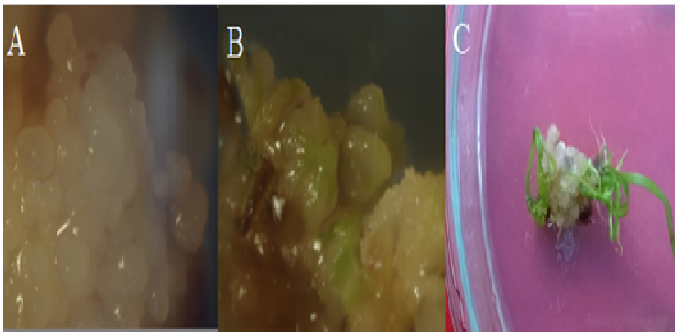

Fig. 5. Embryogenic callus induction and plant regeneration from mature embryo of rice. (A) Initiation of somatic embryos. (B) Appears green spot on embryogenic calli. (C) Multiple shoot formation

CONCLUSION

Production of embryogenic callus with high regeneration potential is critical step for efficient genetic transformation in rice. Here, we report successful somatic embryo formation and as well as regeneration in two rice varieties Nemat and Dom siah. As a result of these studies, a protocol has been developed for

embryogenic callus formation and its subsequent regeneration into plantlets. In this experiment, different media supplemented with different compounds were tested for effect on somatic embryogenic calli induction and plant regeneration of Nemat and Dom siah varieties. Culture medium supplemented with 3 mg/L $AgNO_3$, 9gr/L agar and 20 gr/L PEG was found to be suitable for somatic embryogenesis. For regeneration culture medium containing 9 and 11 gr/L agar and also 5 mg/L $AgNO_3$ was more suitable. Because of explant is important for successful regeneration through somatic embryogenesis, in this work according previous study (Grewalet al.2005) we also reported mature embryo is efficient explant for callus induction, somatic embryogenesis and regeneration.

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