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Callus Induction and Somatic Embryogenesis of Rice (Oryza sativa L.) Improvement with the Addition of Coconut Water

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Abstract
The study was conducted to determine the appropriate concentration of coconut water (CW) that enhance an efficient and cost effective protocol for rice micropropagation through callus induction and somatic embryogenesis.Seeds of a local Iraqi rice (*Oryza sativa* L.) cultivar Amber33, were used as explant which inoculated on basal medium consists of MS inorganic salts, Gamborg vitamins and supplemented with the auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) and the cytokinin Benzyl adenine (BA). In order to evaluate the role of CW, three concentrations (10, 20 and 30 $g.l^{-1}$) were added to MS inorganic salts after the exclusion of growth regulators (2.4-D and BA) and Gamborg vitamins. Results showed that adding CW especially at concentration of $30g.l^{-1}$ have minimized the callus induction period, enhanced callus fresh weight and numbers and germination percentage of somatic embryos when compared with the media enriched with growth regulators only.
Keywords: Rice, callus induction, Somatic embryogenesis, coconut water.

1. Introduction

Rice is a major source of carbohydrates in many regions of the world, especially Asian countries. Feeding more than 3 billion people and providing 50-80% of their calorie intake (Khush, 2005). World population is increasing day by day and by the year 2050, it is expected to reach 9.1 billion. In order to feed this population, food production needs to be increased by 70% of the present production and global agricultural production must be increased by 60-110% (OECD/FAO, 2012). Thus there is a need to improve upon the yield of the local cultivars of rice, because loss in production could lead to hunger and famine, especially in the developing countries. For this an efficient protocol needs to be developed to achieve plant regeneration (Bano *et al*., 2005). Crop improvement through tissue culture techniques is easier and more often in use as compared to conventional plant breeding (Yamada, 1986). It is also faster and can be produced in large numbers in shorter periods, irrespective of the seasons. Plants originate either from pre-existing meristem, without passing through a callus phase, produce uniform plants that are genetically stable traits, which are of great horticultural importance (George, 1993).

Callus is a non-organized and little differentiated tissue. The cells of which are paranchymatous in nature. Callus formation take place under the influence of growth regulators exogenously supplied in the nutrient medium (Bhojoani and Razdan, 1983). Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos. Somatic embryos resemble zygotic embryos morphology. They can either differentiate directly from the explant without any investigating of a callus phase or indirectly after a callus phase (George *et al*., 2008).Therefore beside the mineral formulation, growth regulators are also one of the most component of the *in vitro* culture media and the use of correct types, concentrations or combination of growth regulators, the inclusion of other callus induction and somatic embryo formation factors (such as coconut water) are of great importance to successful and cost effective tissue culture protocol.

Many researchers concluded that rice seeds have more potential for callogenesis and somatic embryogenesis as compared to nodes or tips. Successful callus induction and somatic embryogenesis from rice seeds have been reported by several researchers using different types and concentrations of growth regulators (Navraj *et al*. 1999; Gonalz 2000; Rashid *et al.* 2000; Bano *et al*., 2005. Al-Kaby *et al*. 2010; Abdul-Qadir and Al-Kaaby, 2011).

Coconut water is the colorless liquid endosperm of green coconuts *(Cocos nucifera)*. The liquid endosperm contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals, and other unidentified substances, none of which alone is totally responsible for growth promoting qualities (Neumann *et al*. 2009). The liquid has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis (Thorpe *et al*. 2008). In addition the use of coconut water to substitute an expensive organic compound called zeatin (Nasib *et al*., 2008).

Little is known about the effects of coconut water on callus induction and somatic embryogenesis in rice and there is no study about its role in micropropagation of Iraqi rice, so the objective of this study was to compare the effects of the substitution of growth regulators (2, 4-D and BA) and Gamborg vitamins (Gamborg *et al*., 1968) by different concentrations of coconut water on callus induction and somatic embryos regeneration in the well-known Iraqi rice cultivar Amber33.

2. Materials and Methods

Mature seeds of the local rice (*Oryza sativa* L.) cultivar Amber33, obtained from the rice research station in Mushkhab at Al- Najaf governorate, were used as explants for callus induction and somatic embryogenesis. The study was conducted at plant tissue culture laboratory of Department of Biology, College of Education for Pure Sciences, University of Basrah, Iraq.

The seeds were dehusked manually and surface sterilized with 70% ethanol for 5 min. followed by 5% commercial solution of sodium hypochlorite ($NaOCl_2$) adding few drops of tween-20 for 30min. with mild shacking, and then rinsed thoroughly three times with sterile distilled water.

The basal MS medium (Murashige and Skoog,1962) with Gamborg vitamins (Gamborg *et al.* 1968), brought from Phytotechnology Laboratories company(USA) as prepared powder, was supplemented with 2 $mg.l^{-1}$ benzyl adenine (BA) and $1mg.l^{-1}$ 2,4-Dichlorophenoxyacetic acid (2,4-D), 30 $g.l^{-1}$ sucrose and 5$g.l^{-1}$ agar, represent the control treatment. Culture vessels used were Pyrex test tubes (15×2.5cm) containing 10 ml of the nutrient medium. The pH of all media was adjusted to 5.8 with either 0.1 N HCl or NaOH before autoclave sterilization at 121°C and 0.1 MPa pressure. Coconut water was added at three concentrations (10, 20 and 30 $g.l^{-1}$) to the same previously mentioned nutrient medium with the exclusion of Gamborg vitamins and growth regulators.

Four seeds were inoculated on the nutrient media in each test tube with ten replicates for each treatment and kept in the growth room at $25\pm2°C$ with 16 hr. photoperiod under cool-white fluorescent light of approximately 3000 lux. Subculture was performed every four weeks after inoculation on the medium. After obtaining the embryogenic callus, it was transferred to the same previous medium with the exclusion of BA and 2, 4-D for the regeneration experiments.

2.1 Experiment parameters

The following parameters were estimated:

2.1.1 Callus fresh weight in grams by using Sartorius BL2105 electronic balance.

2.1.2 Germinated somatic embryos numbers were calculated as green spots under low power of Wild Heerbrugg MDG17 dissecting microscope. The photos were taken by Sony digital camera DSC-W360.

2.1.3 Germination percentage of somatic embryos.

2.1.3 Statistical analysis

The experiment was subjected to a proper analysis of variance (ANOVA) and mean values were compared using revised least significant difference test (RLSD) at 0.05 level of significance using GENSTAT statistical program.

3. Results and discussion

3.1 Callus morphogenesis and somatic embryos regeneration:

Callus from the tested media, started to appear after 7-10 days, on the seeds of Amber rice. Two types of callus, primary and embryogenic were detected. The primary callus was white and friable and was much greater in size than the embryogeneic callus, which was nodular and compact white to yellow in color.

In the first subculture after four weeks, about 0.2 g of the weighed embryogeneic callus with the proembryoid cells, were transferred to the growth regulators-free medium. Somatic embryos, which were globular in shape when examined under dissecting microscope, started to appear after 5-7 days from subculturing on media containing 30 $g.l^{-1}$ coconut water and after 10-12 days on control and the rest of coconut water treatments. Their numbers were calculated when green spots appeared on the regeneration media. In the second subculture these green spots where transferred to the new medium with the same constituents and germinated somatic embryos with radicals and plumules were calculated at the end of the experiment.

3.2 The effects of growth regulators and coconut water:

3.2.1 On embryogenic callus fresh weight:

Results in fig1 indicated that CW at concentration of $30g.l^{-1}$ caused a significant increase in embryogenic callus fresh weight when compared to the control and the other CW treatments. The results also showed that there is no significant difference in embryogenic callus fresh weight among growth regulator treatment and CW at concentration of 10 and $2g.l^{-1}$.

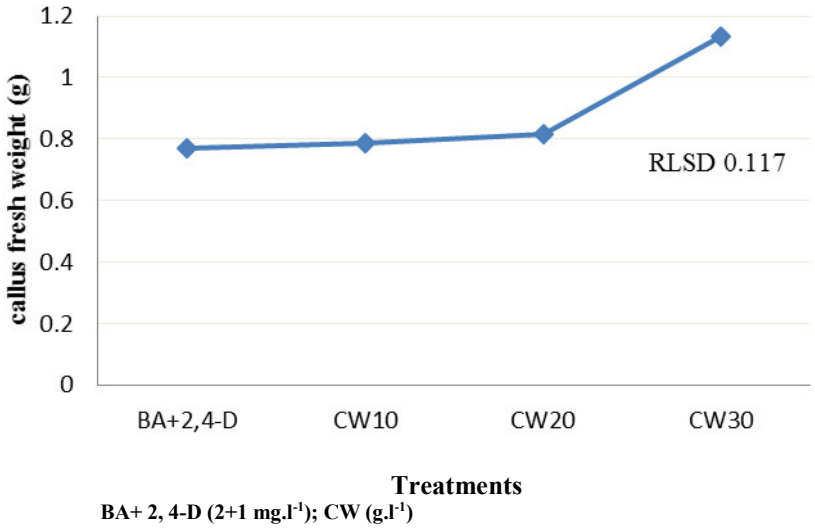

Figure 1. Effect of growth regulators and coconut water on callus fresh weight (g) in amber33

3.2.2 On somatic embryos numbers:

Results in fig 2 showed the gradual and significant increase in somatic embryos number (calculated as green spots) with the increase in CW concentration when compared with the growth regulators treatment and the figure indicated that CW at 30 g.l^{-1} give the highest numbers of somatic embryos.

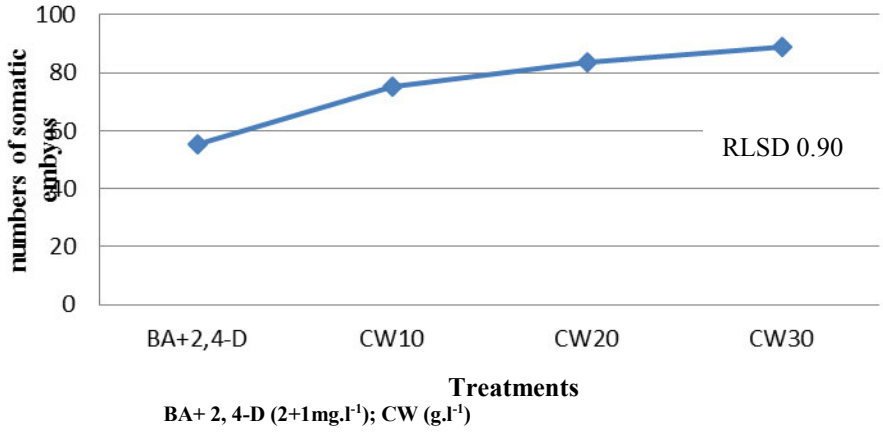

Figure 2. Effect of growth regulators and coconut water on callus number of somatic embryos in amber33

3.2.3 On germination percentage of somatic embryos:

As shown in Fig3 the existence of CW in the nutrient medium caused a significant increase in germination percentage of somatic embryos when compared with the growth regulators treatment especially at concentration of 30 g.l^{-1}. The results showed also that there was no significant increase in germination percent between CW at 10 and 20 g.l^{-1}.

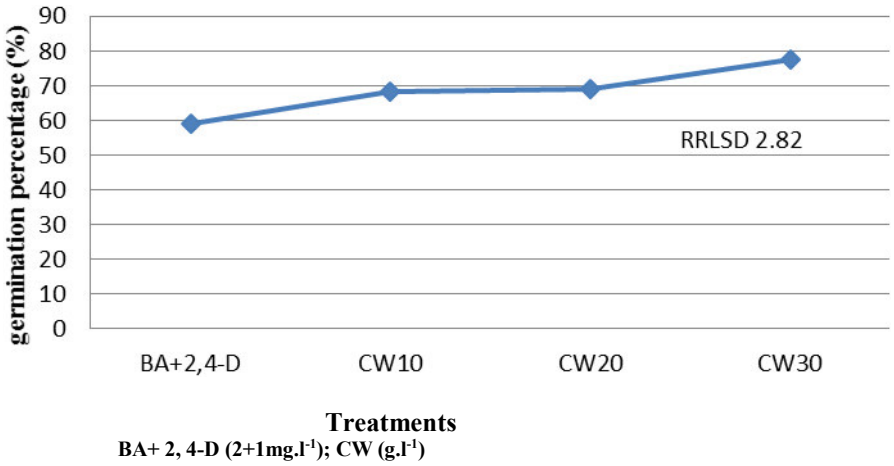

Figure 3. Effect of growth regulators and coconut water on germination percentage of somatic embryos in amber33

4. Discussion

Coconut water is traditionally used as a growth supplement in plant tissue culture. The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones (Jean *et al*., 2009). To stimulate in vitro response, CW is added to the medium at different concentrations ranging from 5 to 30%, however the optimum concentration varies among plant species and must be empirically determined (Al-Khayri, 2010). In watermelon the optimum response occurred at a media supplemented with 10% CW (Krug *et al*., 2005). The same result was obtained in pineapple by Mandal *et al*., (2002). In other plants like Spanish, Al-Khayri *et al*. (1992) observed that the optimum concentration for callus growth and plant regeneration was at 15% CW.

Coconut water is widely used in the plant tissue culture industry, from a scientific viewpoint; the addition of coconut water to the medium is rather unsatisfactory, as it precludes the possibility for investigating the effects of individual components of the medium with any degree of accuracy. The question of which components cause the growth stimulation arose immediately (Jean *et al*., 2009).

Two major effects of coconut water were observed in Kiwifruit. First the addition of coconut water to the media resulted in about 95% increment of overall phosphorus (P) content of the media this effect of coconut water ultimately resulted in the doubling of sub-culturing time from four to eight weeks. The second is enhancing *in vitro* shoot multiplication (Mezetti *et al*., 1991; Nasib *et al*., 2008). In date palm it was noticed by Al-Khayri (2010) that the addition of CW to the nutrient medium have improved callus growth and enhanced somatic embryogenesis and that callus fresh weight and numbers of resultant embryos were directly proportional to CW concentrations. Micropropagation was improved in many plant species with the addition of coconut water (Baskaran *et al*., 2008; Cheong *et al*. 2009; Gnasekaran *et al*., 2010).

5. Conclusions

The results of the study revealed that adding CW to the nutrient medium at $30g.l^{-1}$ enhanced callus induction and somatic embryos formation in Amber rice leading to the formation of healthy seedlings, so we found that it is proper to add it to the nutrient medium for rapid and cost effective protocol of rice micropropagation especially when the question of which components cause the growth stimulation is not under focusing.

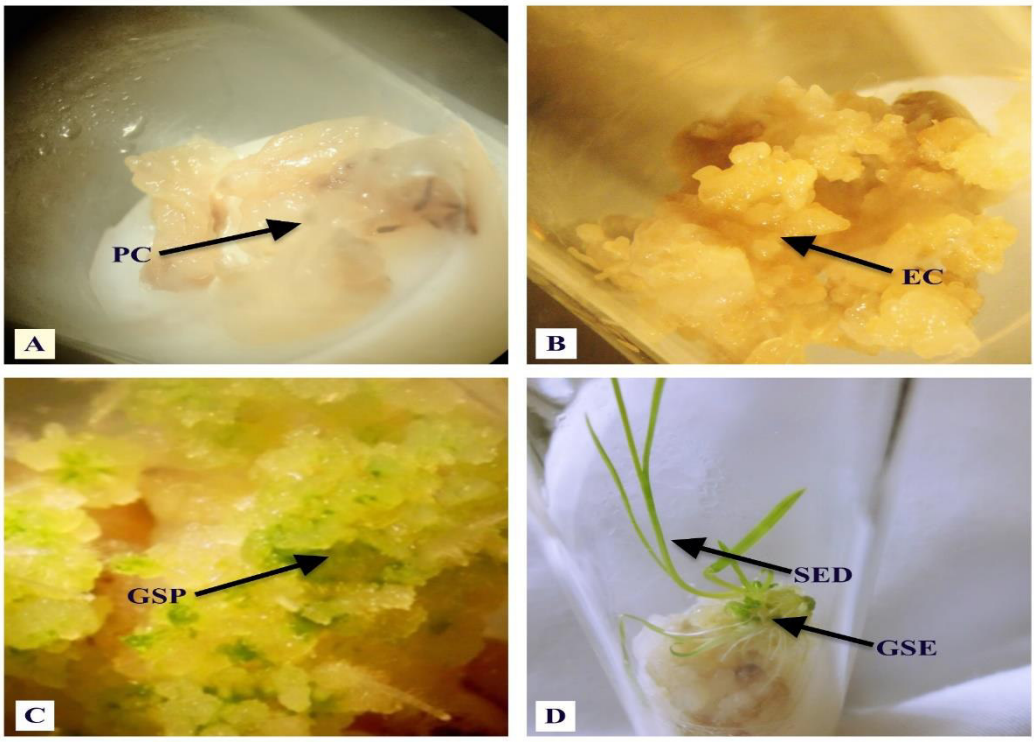

Figure 4. Rice micropropagation stages. A- Primary callus (PC). B-Embryogenic callus (EC). C-Green spots (GSP). D-Germinated somatic embryos (GSE) and Seedlings (SED).

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