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NEW METHOD OF EVALUATION FOR ISOLATION AND FUSION OF THE RICE PROTOPLAST (*ORYZA SATIVA* L.) USING DIFFERENT CONCENTRATIONS OF PEG

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Abstract

Two laboratory experiments were conducted in the college of Agriculture laboratories - Karbala University with the aim of isolating and fusion the protoplast of three types of rice. A factorial experiment according to the Completely Randomized Design (CRD) with three replicates was used in this study, where the first experiment included 3 cultivars (Anber33, Jasmine, and Al-Mushkhab 2) as the first factor, and two methods of isolating protoplast (the enzymatic method and the nano balls method - a new method) which considered as the second factor. The second experiment included three combinations of two protoplast units mixture with a density of $3*10^5$ (protoplast unit /ml) for rice cultivars (Anber and Jasmine), (Anber and Al Mushkhab2), and (Jasmine and Al Mushkhab 2) as the first factor, and treatments by Poly Ethylene Glycol (PEG) with three concentrations 0, 15% and 30% was considered as the second factor. The results showed that the nano balls method to isolate protoplast had a significant superiority by giving the highest protoplast yield of 4.22×10^5 protoplast / ml, while the enzymatic method was superiority by giving the highest percentage of living protoplast of 93.96%. The results also showed that the PEG30% concentration was superiority by giving the highest percentage of binary fusion and number of microcolonies 86.12% and 6038 microcolonies per Petridish, respectively, superiority in the comparison treatment. Within cultivars combinations, the (Anber33+ Jasmine) achieved the highest number of microcolonies with 3562 microcolonies per Petridish, and there was a significant interaction between the concentrations of the fusion factor and the cultivars mixture, where the PEG30%treatment and (Anber33+ Jasmine) was given the highest number of microcolonies, amounted 6,425 microcolonies per Petridish. From the study findings, it can be concluded the possibility of adopting the new method (nano balls) to isolate the rice cultivars protoplast because being low cost and easy to use. The fusion of the local aromatic cultivar Anber33 which characterized by average productivity, high quality and long growth season, with the high-productivity, medium growth season aromatic cultivar Jasmine refers to the opportunity to acquire a new genotype of high-quality (high-aromatic) rice, Palatable, highly productive, average growth period. Therefore, it is a priority to continue with the program of callus production, then the plantlet production to produce this new genotype.

Key words : Fusion of protoplast, PEG, *Oryza sativa*.

Introduction

Somatic hybridization is an alternative technique for traditional breeding methods, which defined as the fusion or integrate of tow protoplast or more isolated from vegetable cells to produce hybrids separated body with desirable qualities (Ephrussi, 2015), that provides a way to get rid of genetic and environment inhibitions that prevents the application of field hybridization (Xia, 2009). Somatic hybridization characterized by the inheritance of the nucleus and cytoplasm genetic material of the parents as the opposite of the hybridization with classical methods in which the site of cytoplasmic traits is inherited from the mother, therefore, it can be used by the increasing of genetic variations to improve the properties of vegetative and fruit growth in quantity and quality (Wang and others, 2013). The protoplast insulation can be done in two methods, either by mechanical method, which depends on using Plasmolytic solutions such as mannitol and then the cutting with a sharp object, or the enzymatic method that depends on incubating the plant part in a mixture of enzymes and it is often a pectinase

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and cellulase enzymes as mentioned by (Al sumaidai, 2017), while most researchers may combine the two methods of mechanically separating the cells and then being followed by the isolation of the enzyme protoplast. Furthermore, (He *et al.*,2016) findings showed that the use of enzymatic solution of (1.5% Cellulase and 0.3% Pectinase) to incubate rice crop leaves has achieved the highest protoplast density with 1.5-2.5 protoplast/ml. (Yang *et al.*, 2014) explained that the combination of mechanical and enzymatic methods achieves the highest protoplast density with $1*10^7$ protoplast/ml, as a result of using mannitol to incubate plant parts for 30 minutes, followed by incubation with an enzymatic solution (1.5% Cellulase, 0.75% Pectinase). After removing the cell wall, there are no barriers that prevent the adhesion of two or more protoplast (Wang *et al.*,2013), there were several methods of protoplast fusion, the most important of which is the physical fusion, this is done by the microfusion device, by the use of microabsorbent, or by using electrical fusion. While in chemical method, the fusion is done with the assistance of different chemical materials, the most famous which is Polyethylene Glycol (PEG), as it stimulates the protoplast clustering with each other and thus achieves the fusion process (Kativat *et al.*,2017) pointed out that, the incubation of protoplast in PEG with a concentration of 20% for 15 minutes achieved the highest rate of binary fusion by 28.9%. Moreover, the results of (Kumar *et al.*,2018) showed that incubation of protoplast in polyethylene glycol (PEG) with a concentration of 50% for 45 minutes achieved the highest rate of binary fusion which reached 21.8%. Finally, this study aims to evaluate a new method of protoplast isolation (the Nano balls method) compared to the enzymatic method which is currently considered as the best method of protoplast isolation, and to detect the best concentration of PEG to achieve the highest protoplast fusion rate for two cultivars.

Materials and Methods

Two laboratory experiments were conducted in the college of Agriculture laboratories of the Karbala University with the aim of isolating and fusion the rice protoplast, the seeds of three rice cultivars (Anber 33, Jasmine, Al-Mushkhab 2) were sterilized by placing them in a dish containing of 75% ethanol for one minute and then washed with distilled water then placed in another dish containing sodium hypochlorite solution for 30 minutes. Finally, it was washed four times with distilled water before being transferred to planting, the sterile cultivars seeds were planted in media Ingredients as shown in table 1, for 10 days at room temperature, under a light cycle (16 hours light: 8 hours dark), so that the leaves are used later in the protoplast isolation.

Table 1: Media Ingredients.

Ingredients	Volume ($gm.L^{-1}$)	Reference
MS	4.4	Puhan and Siddiq (2013)
Agar	8	
Sucrose	30	
The pH has been set to 5.7		

Experiment 1: Rice Protoplast Isolation Experiment

Factorial experiment was carried out according to the Completely Randomized Design (CRD) with three repilcates in order to determine the best method to isolate protoplast of several rice cultivars, the first factor was included 3 cultivars of (Anber33, Jasmine, Al-Mushkhab 2), where the leaves taken without necks and cut in the form of very thin strips, then a 2 grams of them were taken and put it in petri dishes with a diameter of 9 cm and 10 ml added to the plasmolytic solution (mannitol 0.6 M/L) and pH was set at 5.6 for 30 minutes, according to (Zahang *et al.*,2011), (the polymerase solution removed by micro pipette before starting the protoplast isolation).The second factor includes the methods of protoplast isolating (enzymatic method and Nano balls method - new method), as the enzymatic method contained the incubation of leaves in an enzymatic solution (1.5% Cellulase, 0.3% Pectinase) for 4 hours at room temperature with a shaker of 40 cycle /minute, then the enzymatic solution removed. The Nano balls method included the protoplast isolation by a tube containing Nano balls that breaks the cell wall as a result of its collision, where the plant parts are placed in a tube with nano balls, then 2 ml mannitol was added and placed on the vortex device for 5 minutes, and then mannitol removed.

After that, the following steps were applied as illustrated by (He *et al.*,2016):

1. A 20 ml of the purification medium (154mM NaCl, 125Mm $CaCl_2$, 5mM KCl ,, 2Mm MES, 5.7 PH) was added and gently vibrated (80 cycle /minute) for 1 hour to release the protoplast.
2. Sieve of (100 micrometers) was used for filterization and the tissue was washed with 10 ml of the purification media.
3. Protoplast were collected in a 50 ml conical tube.
4. The conical tube were inserted into a centrifugation device of (1000 cycle / 5 minutes), to collect protoplast.
5. The suspended protoplast were gently pulled out.
6. A 10 ml of the purification media was added and re-suspend the protoplast by gently vibrate.
7. The centrifugation procedure was reported by (1000

cycle / 5 minutes) and the protoplast pellet at the top and the sediment supernatant was removed.

8. Re-suspend protoplast (protoplast pellet) with suitable size from the purification media.

The yield protoplast (protoplast unit/ml)was calculated by a Hemicytometer device and while the viability was determined using the Evan dye 0.4% (W/V) by the optical microscopy where the dead cells are colored in blue and the living cell remains without coloring. the ratio of living cells was calculated as follows

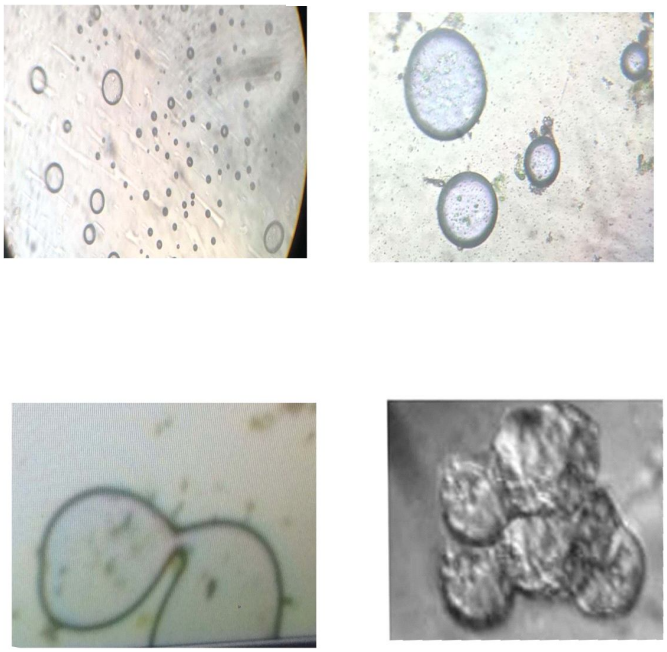
Living protoplast% = (live protoplast number/ total number of protoplast) *100

Experiment 2: Protoplast Fusion Experiment

Factorial experiment was carried out according to the Completely Randomized Design (CRD) with three replicates, in order to determine the best concentration of Poly Ethylene Glycol (PEG) to achieve the highest fusion protoplast ratio of two cultivar. The first factor included: three combinations of two protoplast units with a density of $3*10^5$ protoplast/ml for rice cultivars (Anber+ Jasmine), (Anber+Al-Mushkhab2),(Jasmine + Al-Mushkhab2) while the second factor treated with PEG with three concentrations (0, 15%, 30%), 4% Sucrose, 0.147% $CaCl_2$, where the PEG was sterilized by autoclave for 15 minutes and pH was set at 5.7 before use. The experiment was carried out in a 3mm petri dish, where the protoplast has been mixed with each two cultivars and 300 microliter of fusion solution was added according to the previous factor and incubated for 30 minutes. Finally, 8 ml of the purification media, 0.4M glucose was added to remove the fusion solution (PEG) and subjected to the centrifugal 700 cycle/5 minutes. Fusion process was observed microscopically to estimate the percentage of binary fusions by a cell count slide, the fusion protoplast was planted in an Murashige and Skoog (MS) media with 0.5 mg/l of 2.4-D and 1 mg/l of BA for two weeks to calculate the micro colonies number.

Results and Discussion

Fig. 1 shows the possibility of isolating the protoplast from rice leaves and fusion it down to the micro colonies, where Fig. 1.A explained the acquisition a large amount of protoplast units after removing the cell wall either in the enzymatic or mechanical method, as the solid cellulase wall and the Middle Iamella rich in pectin that prevents the transfer of genetic material. Thus, the use of enzymes such as Cellulase and Pectinase working to break this barrier, by removing the cell wall, there is no obstacle to the fusion of two or more protoplast units. Protoplasts are generally of a negative charge, so the plasma membranes of separated protoplast do not get close enough to each other as a result of incompatibility between them, therefore, when adding the PEG it is working to cover the protoplast units and behaves as a molecular link between the protoplasts that lead to a convergence and adhesion the protoplast as shown in Fig. 1.B. After the convergence of protoplasts, a tight protoplasmic channel forms between protoplasts units to expand later to fusion their contents and become a single cell as shown Fig. 1C, and after fusion it continues to divide till reach the cellular microcolonies as shown in Fig. 1D.



A: Rice Protoplast
B: Protoplast units approached each other to prepare for fusion
C: Starting fusion of two protoplast units
D: The formation of micro colonies after two weeks of fusion

Fig. 1: Stages of isolation and fusion the rice protoplast.

Protoplast Isolation Experiment

(Table 2) showed that the nano balls method for isolating protoplast achieved the highest living protoplast yield of $4.22*10^5$ protoplast/ml, which exceed the enzymatic method that gave an average of $3.74*10^5$ protoplast/ml, this superiority may be attributed to the fact that, the nano balls break the cell wall more than the other. The results of table 2 also indicates that there were no significant differences between the genotype in the yield protoplast, nor was the significant interaction between the methods of isolation and cultivars.

The results of table 3 revealed the superiority of the enzymatic method by giving it the highest percentage of

Table 2: The effect of the isolation method, cultivars, and the interaction between them in the living protoplast yield (protoplast/ml) of the rice crop.

Cultivars	Isolation method		
	Enzematic method	Nano balls method	Average cultivars
Anber33	$3.75 * 10^5$	$4.18 * 10^5$	$3.97 * 10^5$
Jasmine	$3.79 * 10^5$	$4.27 * 10^5$	$4.03 * 10^5$
Al-Mushkhab 2	$3.69 * 10^5$	$4.20 * 10^5$	$3.95 * 10^5$
Significant level	N.S*		N.S*
Average isolasion method	$3.74 * 10^5$	$4.22 * 10^5$	
Significant level	00.095		

Note :- N.S refers to non significant.

Table 3: The effect of the isolation method, cultivars, and interaction between them in the living protoplast percentage (%) of the rice crop.

Cultivars	Isolation method		
	Enzematic method	Nano balls method	Average cultivars
Anber33	93.97	84.17	89.07
Jasmine	95.10	85.80	90.45
Al-Mushkhab 2	92.80	84.66	88.73
Significant level	N.S		N.S
Average isolasion method	93.96	84.88	
Significant level	1.993		

Table 4: The effect of the fusion factor (PEG), the cultivars combinations, and the interaction between them in the binary fusions percentage (%)of the rice crop.

Cultivars	Fusion factor concentrations			Average Composition
	0%	15%	30%	
Anber33 + Jasmine	14.33	66.03	86.90	55.76
Anber33 + Al-Mushkhab 2	15.07	64.13	85.67	54.96
Jasmine + Al-Mushkhab 2	13.73	65.27	85.80	54.93
Significant level	N.S			N.S
Average fusion factor concentration	14.38	64.14	86.12	
Significant level	4.116			

Table 5: The effect of the fusion factor (PEG), the cultivars combinations, and the interaction between them in the growth microcolonies number (microcolonies per Petridish)of the rice crop.

Cultivars	Fusion factor concentrations			Average Composition
	0%	15%	30%	
Anber33 + Jasmine	0	4261	6425	3562
Anber33 + Al-Mushkhab 2	0	4509	5837	3128
Jasmine + Al-Mushkhab 2	0	3444	5815	3086
Significant level	220.6			127.4
Average fusion factor concentration	0	3738	6038	
Significant level	127.4			

living protoplast reached 93.96% superior to the mechanical method, which gave a percentage of 84.88%, this superiority may be attributed to the fact that, protoplast is very fragile, therefore the nano balls collision causes greater damage as a result of cutting. As well as, the poor performance of the protoplast as a result of the substances released from damaged cells, causing the poisoning of the protoplast cells and thus their death (Bhatia *et al.*,2015).

Protoplast Fusion Experience:

(Table 4) results showed that, the two fusion factor treatments (PEG 15% and PEG 30%) were achieved the highest percentage of binary fusions by 64.14%, 86.12%, respectively, superior in the control treatment, which gave a ratio of 14.38%. This is may be due to the fact that PEG acts as a molecular bridge connecting the cells protoplasts as it reduces the negative charge on the surface of the cell protoplasts,which allows its cytoplasmic membranes to approach each other, this resulted in the fusion process (Al-Sumadi, 2017).

The results of table 5 showed that, the PEG treatment, 30% achieved the highest number of small colonies of 6038 microcolonies per Petridish, compared to the comparison treatment (PEG0%), this is due to the fact that the treatment PEG30% achieved the highest number of binary fusions (Table 4). The results also showed that the combination of the two cultivars Anber33 and Jasmine achieved the highest number of colonies by 3,562 microcolonies per Petridish, superior to the two combinations, Anber33 + Al-Mushkhab 2 and Jasmine + Al-Mushkhab 2, which may be attributed to genetic kinship between these two cultivars, or the possibly genetic factors. In addition to, a significant interaction between the fusion factor concentrations (PEG) and the cultivars combinations was observed, where the combination Anber33 + Jasmine treated with a concentration of 30% PEG gave the highest number of colonies at 6425 microcolonies per Petridish.

From the study findings, it can be conclude the possibility of

adopting the new method (nano balls) to isolate the rice cultivars protoplast because being low cost and easy to use. The fusion of the local aromatic cultivar Anber33 which characterized by average productivity, high quality and long growth season with the high-productivity, medium growth season aromatic cultivar Jasmine refers to the opportunity to acquire a new genotype of high-quality (high-aromatic) rice, Palatable, highly productive, average growth period. Therefore, it is a priority to continue with the program of callus production, then the plantlet production to produce this new genotype.

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