Plant Archives Vol. 20 No. 1, 2020 pp. 1014-1018 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

OPTIMAL CONDITIONS FOR ISOLATION AND FUSION OF PROTOPLASTS OF BREAD WHEAT CULTIVARS

Auday Hamid Taha and Radhi Dheyab Abed

College of Agricultural Engineering Sciences, Unversity of Baghdad, Iraq.

Abstract

Two laboratory experiments were conducted at college of agriculture, university of Karbala. The first was designed in completely randomized with factorial arrangement to isolate protoplasts from wheat cultivars . The first factor included three bread wheat cultivars (Al-Iraq, Al-hashmiya and G5) while the second factor had two methods of protoplast isolating (enzymatic and nanoparticles methods). Also, the second experiment was conducted according to compeletly randomized design (CRD) in factorial with two factors. The first factor included three combinations of two units of protoplast mixture at a density was 3×10^5 protoplast units ml^{-1} from wheat cultivars (Al-Iraq, G5) , (Al-Iraq, Al-hashmiya) and (Al-hashmiya, G5) while the second factor was treatment with PEG at three concentrations (0, 35 and 35%). Results showed the nanoparticles methods for protoplast isolation was significantly superior by giving the highest percentage of live protoplast was 91.40% but there was no significant differences between cultivars in protoplast yield and live protoplast percentage. In the second experiment, fusion factor concentration (PEG35%) gave the highest binary fusion and live colonies amounted 12.11% and 5383 colony petri $dish^{-1}$, respectively compared to control treatment. Al-Iraq×G5 gave the highest colonies was 3125. There was significant interaction between fusion factor concentrations and cultivars mixture where the PEG35% treating at Al-Iraq×G5 gave the highest colonies was 5770 colony petri $dish^{-1}$.

Key words: wheat, protoplast, isolation, fusion, nanoparticles.

Introduction

Somatic hybridization is protoplasts fusion separated from vegetative cells to produce a hybrid with desirable traits. This technology offers a promising alternative in eliminating genetic barriers to produce somatic hybridization between close or distant plant species (Shirbash, 2015). This technology has the advantage of carrying important properties including inheritance all the genetic material of the nucleus and cytoplasm, and this genetic information passes through parents contrary to what happens in classical methods of hybridization in which the cytoplasmic traits are inherited only by the mother (Al-Somaidaiy, 2017). Protoplast fusion is a method of molecular genetics that aims to inherit traits away from the use of sexual reproduction. The protoplast fused between two different cells in the chromosome number but they are diploid where a hybrid protoplast is formed in a single cell. If this fused cell is cultured, it produces a plant containing two different groups of chromosomes, one coming from the male and the other from the female (Evan *et al.,* 2013). If two protoplasts of the same plant type fuse, a homokaryon nucleus is formed and surrounded by a cell wall to form a new cell and its culture divides into a whole plant (Sherbash, 2015). This technology can be used to increase genetic variations to produce plants tolerant to environmental stresses and resistance to diseases and insects, as well as improving the properties of vegetative and fruit growth in terms of quantity and quality and thus improving cultivars to increase economic returns (Sowers, 2013). Xia *et al.,* (2009) stated that although somatic hybridization in wheat is a laborious and time-consuming technology, it allows the exploitation of non-nuclear genes from both parents, which is not possible in the sexual reproduction. Most studies have been devoted to cell wall removal and obtaining protoplasts in good quantities and biomaterials, with wide application in plant improvement, especially the production of somatic hybrids (Eriksson *et al.,* 2018). After digestion of the cell wall there is no constrain preventing agglutination of two or more protoplasts and formation of colonies. Jia *et al.,* (2016) obtained a protoplast high vitality amounted 95% isolated from wheat

using the enzymatic method. The fusion occurs during the digestion of the cell wall, where plasmodesmata that connect cells begin to expand and increase in size, allowing cell contents to move to another adjacent cell (Johnson and Veilleux, 2001). Sowers (2013) mentioned the method of fusion using chemical means in which fusion is carried out with different chemicals. The most using of which is polyethylene glycol (PEG). It is the most effective and most commonly used protoplast fusion agent. It has molecular weights ranging from 1800 to 6000, all of which stimulate the clustering of protoplast cells together and thus achieve fusion (Huttly, 1989). Yang *et al.,* (2013) showed that the use of mechanical and enzymatic methods achieved the highest density of protoplast amounted 1×10^7 protoplast ml^{-1}, as a result of using mannitol for incubation of plant parts for 30 minutes, followed by enzymatic solution incubation (1.5% cellulase, 0.75% pectinase). Kativat *et al.,* (2017) indicated that protoplast incubation in PEG at 20% conc. for 15 minutes achieved the highest bilateral fusion ratio of 28.9%. Kumar *et al.,* (2018) confirmed that the incubation of protoplast in PEG at 50% conc. for 45 minutes gave the highest rate of bilateral fusions of 21.8%. Xia *et al.,* (2003) used a chemical method using mannitol and calcium chloride to isolate protoplast cells from wheat and added amounts of PEG to merge these cells with cells of another plant and they confirmed that this compound gave a positive result in the fusion of protoplast. The aim of this study is to determine the best way to isolate cell protoplasts from several cultivars of bread wheat and to determine the best concentration of PEG to achieve the highest fusion rate.

Materials and Methods

Two experiments were conducted in the laboratories of college of agriculture - university of Karbala with the aim of isolating and fusing wheat protoplast. The seeds were sterilized by placing them in a dish containing 75% ethanol for one minute and washed with distilled water and then placed in another dish containing sodium hypochlorite solution for 30 minutes. Then it was washed four times with distilled water before being transferred for planting. Seeds of sterile cultivars were planted in a sterile medium supplemented with nutrients containing (MS salts 4.4 g/L, sucrose 30 g/L, acar 8 g/L) and free of growth regulators while PH was adjusted to 5.7 and incubate for 8 days at room temperature, under a light period (16 hours light and 8 hours darkness) (Jia *et al.,* 2016).

Isolation of wheat protoplast

A factorial experiment with CRD was carried out to determine the best way to isolate protoplasts from several wheat cultivars. The first factor was wheat cultivars (Al-Iraq, Al-hashmiya and G5). Two grams of fresh leaves were cut off in the form of very thin stripes and placed in petri dishes with a diameter of 9 cm and added to it 10 ml of the plasmolysis solution (mannitol 0.6 M/L) and PH was set to 5.6 for 30 minutes (Zhang *et al.,* 2011) (plasmolysis is removed by a pipette before starting isolation of protoplast). The second factor included protoplast isolation methods in two ways (enzymatic method and nanoparticles method). The enzymatic method included incubation of the leaves in an enzymatic solution (1% Cellulase, 0.1% Pectinase) for 4 hours at room temperature with a vibrator of 40 cycles per minute and then enzymatic solution was removed. The method of nanoparticles included the isolation of protoplast where the plant parts are placed in a tube containing nanoparticles and add 2 ml mannitol and then placed on the shaking device for 5 minutes and then remove mannitol.

The following steps are done according to the way which described by Zhong *et al.,* (1992), Thieman and Palladino (2013):

1- Add 15 ml of isolation and washing solution (purification medium) of A (Tris 25Mm and 0.9 Mannitol) and B (25 Mm of MES and 0.9 Mannitol) after mixing, enzymes are added to this solution for digestion. The pH is set at 5.5, incubated at 25°C in the dark and shake gently (50 cycles per min.) for 20 minutes to release the protoplast.

2- Transfer 20 microlitter of the resulting solution onto a slide. Using a microscope, we monitor the cell wall and the released protoplast.

3- Isolated protoplast was washed by removing the digestion solution above using a pipette and filtered through a small sieve with holes (100 micrometer). Rinse again with 5 ml washing solution.

4- The resulting protoplast solution was collected in a 50 ml tube.

5- Put the tube into the centrifuge (500 cycles for 10 minutes), to collect the floating protoplast.

6- Suspended protoplast was withdrawn by pipette quietly.

7- Add 5 ml of purifying solution and resuspend protoplast by gently shaking.

8- Repeat the centrifugation process (500 cycles for 10 minutes) and the protoplast floats on top and the supernatant is removed.

9- Repeat this washing process twice.

10- After the third centrifuge, the protoplast pellet is resuspended with 300 microlitter of purification medium.

11- Transfer 50 microlitter of the solution into a tube and add 5 microlitter of the Ivan blue dye (1% with water) and mix gently.

Twenty microliters were taken to calculate the protoplast yield (a unit of protoplast per millimeter) by a cell counter (Hemicytometer). Determination of vitality by the Ivan blue dye and using a light microscope, where the dead cells are colored blue, the living cell remains uncolored and the proportion of living cells is calculated according to Jia *et al.*, (2016) as follows:

$$\text{Live protoplast \%} = \frac{\text{Number of live protoplast}}{\text{Total number of protoplast)}} \times 100$$

Protoplast fusion

A factorial experiment with CRD was performed to determine the optimal PEG concentration to get the highest ratio of protoplast fusion of two cultivars. The first factor included three combinations of two units mixture of protoplast at 3×10^5 protoplast unit ml^{-1} for cultivars (Al-Iraq and G5), (Al-Iraq and Al-hashmiya) and (Al-hashmiya and G5). The second factor was treatment with PEG at three concentrations (0, 25% and 35%) and 4% sucrose and 0.147% $CaCl_2$. PEG solutions were prepared according to Michel and Kaufmann (1973). The solutions were sterilized by autoclave for 15 minutes and the PH set to 5.7 before use.

The experiment was carried out in a 3mm petri dish. Protoplast of two cultivars was mixed and 300 microlitter of the fusion solution was added according to the treatments and incubated for 30 minutes. eight ml of purification solution and 0.4M glucose were added to remove the PEG solution and centrifuged 700 cycles per min for 5 minutes. Microscopy was observed to estimate the number of bilateral fusions by cell count. The fused protoplast was cultured in a media consisting of MS with 0.5 mg/L of 2,4-D and 1mg/L of benzene adenine (BA) for two weeks to calculate the number of growning colonies (micro colonies) in a petri dish.

Results and Discussion

Fig. 1 shows the possibility of isolating the protoplasts from some cultivars of wheat leaves and fusing them with others to form micro-colonies. Stage A showed that a large quantity of protoplast units were obtained after the cell wall was broken down and digested enzymatically or mechanically. The solid cellulosic wall and the pectin-rich middle lamella preserve the genetic material and prevent its release and transport, therefore the use of lysis enzymes such as cellulase or pectinase break down this barrier, and by removing the cell wall, there was no a

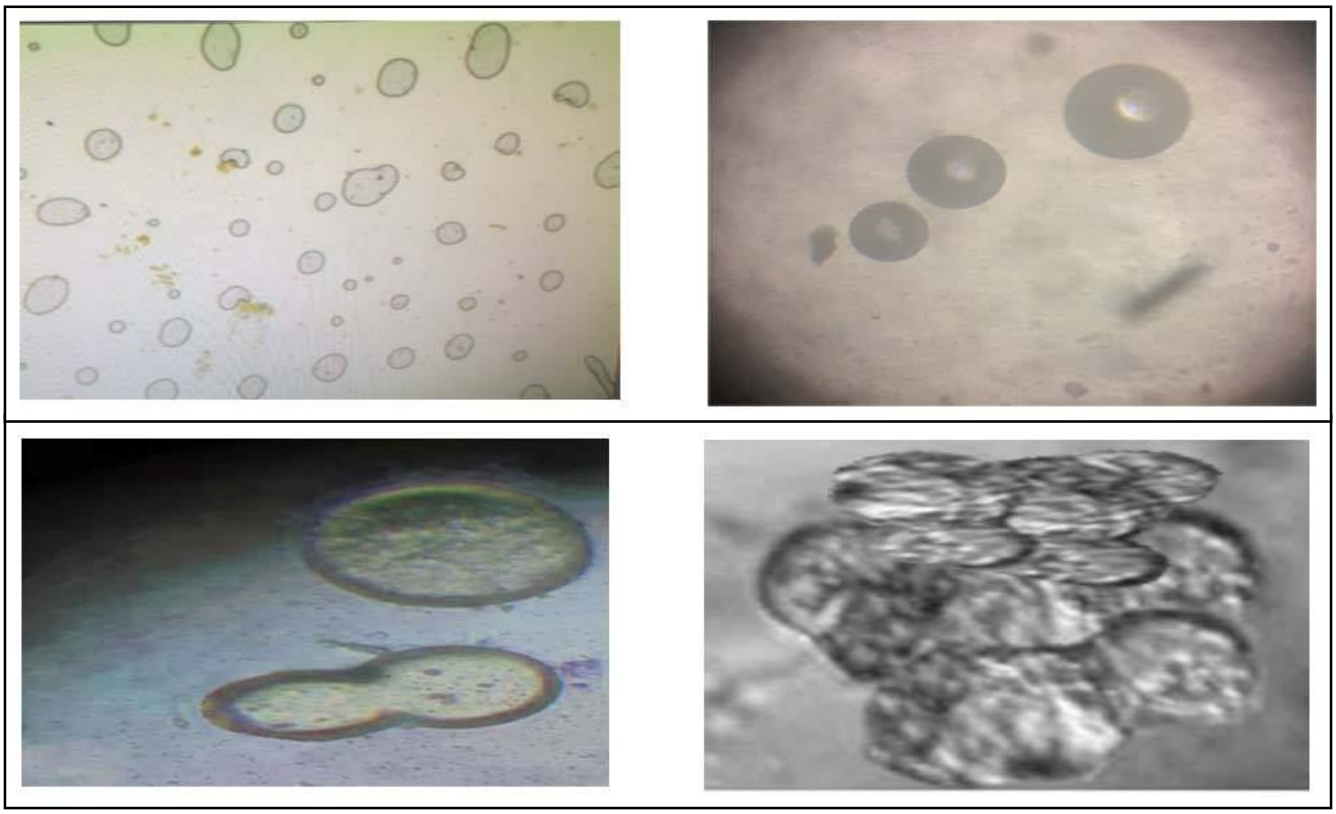

Fig. 1: stages of isolation and fusion of wheat protoplast.

barrier to the fusion of two protoplasts or more. Protoplasts are generally negative, therefore the separated plasma membranes of the protoplast are not sufficiently close to each other due to repulsion between their charges. Therefore when a high molecular weight PEG is added, it encapsulates the protoplast units and behaves as molecular bonds between the protoplasts, thus converging and bonding between the released protoplast cells (stage. 1B). After the agglutination of the protoplasts, narrow protoplasmic channels form between the protoplast units to expand later to merge into their internal contents and become a single cell (stage 1C). After fusion, they continue to divide into small cellular colonies (stage 1D).

Live protoplast yield (protoplast ml^{-1})

The results of table 1 showed significant differences between the methods of isolating protoplast in the live protoplast yield, The nanoparticles method of protoplast isolation gave the highest live protoplast yield of 3.217 × 10^5 protoplast ml^{-1} superior to the enzymatic method that gave an average of 2.743 × 10^5 protoplast ml^{-1}. This superiority may be attributed to the fact that the nanoparticles have further broken down the cell wall, allowing the protoplast cells to release more. There were no significant differences between the cultivars and the interaction in the protoplast yield. Jia *et al.,* (2016) obtained a high protoplast yield when isolating the protoplast from wheat.

Table 1: The effect of the isolation method, cultivars, and their interaction in live protoplast yield (protoplast ml-^{1}) of wheat crop.

Cultivars	Isolation method		
	Enzematic	Nanoparticles	Mean
Al-Iraq	2.747* 10^5	3.184 *10^5	2.965 * 10^5
AL-hashmiya	2.787* 10^5	3.267 * 10^5	3.027 * 10^5
G5	2.694 * 10^5	3.200 * 10^5	2.947 * 10^5
LSD 0.05	N.S		N.S
Mean	2.743 * 10^5	3.217 * 10^5	
LSD 0.05	0.095		

Table 2: effect of the isolation method, cultivars, and their interaction in the live protolast percentage (%) of wheat crop.

Cultivars	Isolation method		
	Enzematic method	Nanoparticles method	Mean
Al-Iraq	91.55	79.58	85.57
Al-hashmiya	92.89	81.66	87.27
G5	89.77	79.99	84.88
LSD 0.05	N.S		N.S
Mean	91.40	80.41	
LSD 0.05	4.41		

Table 4: Effect of fusion factor (PEG), cultivars combinations, and their interaction in the micro colonies number per petri dish of wheat crop.

Cultivars combinations	Fusion factor concentrations			
	0 %	25 %	35 %	Mean
Al-Iraq×G5	0	3605	5770	3125
Al-Iraq×Al-hashmiya	0	2854	5219	2691
Al-hashmiya×G5	0	2789	5160	2650
LSD 0.05	222.8			128.6
Mean	0	3083	5383	
LSD 0.05	128.6			

Percentage of live protoplast (%)

The results in table 2 showed that there were significant differences between the enzymatic method and the mechanical method in the percentage of live protoplast %. The enzymatic method gave the highest percentage of live protoplasts at 91.40% compared to the mechanical method which gave 80.41%. This may be due to the fact that the isolated protoplast has a very thin outer membrane and therefore the nonoparticles have caused greater damage to the protoplast as a result of cutting, scaring and thus causing damage (Sherbash, 2015). Also, some cell damage from the mechanical method caused the release of toxic substances that affected the viability and death of protoplast cells (Bhatia *et al.,* 2015). This finding is consistent with Jia *et al.,* (2016) of obtaining 94% live protoplast when used enzymatic method to isolate wheat protoplast. Results showed no significant differences between cultivars in the percentage of live protoplast %.

Percentage of bilateral fusion (%)

The results of table 3 showed significant differences between the PEG concentrations in the percentage of bilateral fusion between the cultivars. PEG35% gave the highest percentage of bilateral fusion at 83.86%, while the control treatment gave the lowest ratio of bilateral fusion at 12.11%. PEG molecules acted as bridges linking the protoplast cells of the two cultivars and reduced the negative charge of the surfaces of the protoplast cells, which led to the approach of the protoplast cytoplasmic membranes to one another and thus fused between the cultivars (Sherbash, 2015; Sumaidi, 2017). There was no significant differences between the cultivars in the percentage of bilateral fusion %.

Number of small colonies (colony per petri dish)

The results of table 4 showed significant differences between PEG concentrations and cultivars and their interaction in the number of small colonies. The PEG35% treatment gave the highest average of 5383 small colonies per petri dish compared to the PEG0% control treatment

which did not give any colony. This is because PEG35% achieved the highest number of bilateral fusions. The somatic hybridization between the two cultivars (Iraq and G5) had the highest number of colonies with 3125 colonies per petri dish compared to the somatic hybridization between the Al-hashmiya and G5 which gave the lowest number of small colonies amounted to 2650 colonies which did not differ significantly from the hybridization between Al-Iraq and Al-hashmiya, which gave an average number of colonies amounted to 2691 colonies. This superiority may be attributed to the differences of genetic factors between the cultivars or perhaps to the genetic distance between Al-Iraq and G5. The results showed a significant interaction between the concentrations of the PEG and the combinations of the somatic hybridization of the cultivars . The combination (Al-Iraq and G5) which treated with a concentration of 35% PEG gave the highest number of colonies reaching 5770 colonies compared to combinations of somatic hybridization with the control treatment which did not give any colony.

References

Al-somaidai, K.M.I. (2017). Aplications of plant biotechnology. Minsitry of education and higher research. University of Al-Nihrin. Pp:364.

Bhatia, S., K. Sharma, R. Dahiya and T. Bera (2015). Modern applications of plant biotechnology in pharmaceutical sciences. Academic Press.

Eriksson, T.R. (2018). Protoplast isolation and culture. In Plant protoplasts (pp: 1–20). CRC Press.

Evan, D.A., J.E. Bravo and Y.Y. Gleba (2013). Somatic hybridization: fusion methods, recovery of hybrids, and genetic analysis. Plant Protoplasts: *International Review of Cytology*, **16:** 143.

Jia, X., X. Zhang, J. Qu and R. Han (2016). Optimization Conditions of Wheat Mesophyll Protoplast Isolation, 850–858.

Johnson, A. and R. Veilleux (2001). Somatic hybridization and application in plant breeding. In: *Janick J. (ed) Plant Breeding Rev.*, **20:** John Wiley & Sons, New York, pp: 167–225.

Michel, B.E. and M.R. Kaufmann (1973). The osmotic potential of polyethylene glycol 6000. *Plant physiology*, **51(5):** 914-916.

Huttly, A.K. and D.C. Baulcombe (1989). A wheat ca-Amy2 promoter is regulated by gibberellin in transformed oat *aleurone protoplasts*, **8(7):** 1907–1913.

Kativat, C. and P. A. Tantasawat (2017). PEG-Mediated Symmetric and Asymmetric Protoplast Fusion in Sunflower (*Helianthus annuus* L.), **49(5):** 1903–1910.

Kumar, M.K., B.V. Sandeep and P.S. Rao (2018). Development of salt tolerant callus cultures by somatic hybridization between *Oryza sativa* and *mangrove* grass Myriostachya wightiana. *Annals of Agrarian Science*, **16(4):** 396–404.

Shirbash, M.T.M. (2015). Principles of plant tissue culture. House books, Egypt. Pp: 225.

Sowers, A.E. (2013). Cell fusion. Springer Science & Business Media. Libarary of Congress Cataloging in Publication Data. PP: 592.

Thieman, W.J. and M.A. Palladino (2013). Introduction to biotechnology.

Wang, J., J. Jiang and Y. Wang (2013). Protoplast fusion for crop improvement and breeding in China, 131–142.

Xia, G. (2009). Progress of chromosome engineering mediated by asymmetric somatic hybridization. *Journal of Genetics and Genomics*, **36(9):** 547–556.

Xia, G., F. Xiang, A. Zhou, H. Wang and H. Chen (2003). Asymmetric somatic hybridization between wheat (*Triticum aestivum* L.) and Agropyron elongatum (Host) Nevishi. *Theoretical and Applied Genetics*, **107(2):** 299–305.

Zhong-yi, L., X. Guang-min and C. Hui-min (1992). Somatic embryogenesis and plant regeneration from protoplasts isolated from embryogenic cell suspensions of wheat (*Triticum aestivum* L.), 79–85.