Plant Archives Vol. 20, Supplement 2, 2020 pp. 3022-3026 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

IMPACT OF CULTURE MEDIUM AND 2,4-D ON DIRECT SOMATIC EMBRYOGENESIS IN RED CABBAGE (*BRASSICA OLERACEA* VAR. CAPITATA FORMA RUBRA)

Ghassan Muhammed Al- Hardan[1], Magida AL-Shamari[2] and Magid Ali Hanshil[3]

[1]Ministry of Agriculture, Iraq.

[2]Research Department, Horticulture Office, Ministry of Agriculture, Iraq.

[3]Department of Horticulture, College of Agricultural Engineering Sciences, University of Baghdad, Iraq

Abstract

Red cabbage (Brassica oleracea var. capitata forma rubra) is the most important plant in Brassicaceae family as it has the high nutritional and medical value. Because of the cross pollination and self-incompatibility in this crop, the use of biological technique like somatic embryogenesis can be applied to propagate this plant. This paper aimed to find the best medium and concentration of hormones that have used in production of direct somatic embryogenesis. MS medium enriched with four concentrations of 2,4-D (0, 1.5, 2, 2.5 mg/L^{-1}) and B5 medium supplemented with five concentrations of 2,4-D (0, 0.50, 0.55, 0.60, 0.65 mg/L^{-1}) were applied. Three type of explants were used in this work (Cotyledon, Hypocotyl and leaf). Various explants that were cultured on MS medium did not appear any response for somatic embryos formation at all concentrations of 2,4-D. The highest average number of somatic embryos (40) per explant was achieved using cotyledon explants on B5 medium provided with 0.50 mg/L^{-1} of 2,4-D which developed into the all developmental stages of somatic embryo reaching to complete plant after 60 days of culture with 45% percentage of conversion. Somatic embryos that were formed on B5 medium supplemented with other concentrations of 2,4-D (0.55, 0.60 and 0.65 mg/L^{-1}) did not develop to all stages of somatic embryos.

Keywords : In vitro propagation, Red cabbage, Direct somatic embryogenesis, 2,4-D.

Introduction

Red cabbage (*Brassica oleracea* var. capitata forma rubra) is the most important plant in Brassicaceae family which distinguished by red color and high nutrient (Boras *et al*., 2011). The high medical benefit that comes from containing of Anthocyanin pigment that gives the red color to cabbage heads and can be considered as a strong antioxidant as well as have anti-inflammatory properties. Also, red cabbage is a suitable food that can be used to reduce the weight (Al-Rawahy *et al*., 2004). Plants of this family are cross pollinated and self-incompatibility nature (Hassan, 1994). These factors make difficulties in production of hybrid seeds as it needs high cost and effort moreover creates difficulties in the achievement of pure bred lines (Desai, 2004). Thus, the scientist found alternative way for propagation of Brassicaceae plants including somatic embryogenesis (Al-Sumadaee, 2017). Which can be defined as the formation of embryos from somatic cells without gamete fusion and that were produced directly or indirectly pathways (Jain and Gupta, 2018). In direct pathway, the somatic embryos can be derived from various explants without callus tissue formation (Jiménez, 2005). In this way, plant which produce from germinated embryo is mostly like the mother plant and has high genetic stability (Salman, 1988). Many factors can affect the production of somatic embryos like (explants, culture medium, type and concentration of plant growth regulators that added to the culture medium) (Liu *et al*., 1993). The use of 2,4-D is most common for the production of somatic embryos in most plants compared with other auxins, in a study that have reported by Kobayashi *et al*., 2010 on Arabidopsis plant it was found that the addition of 2,4-D at concentration of 4.5 mM per liter to B5 medium led to good produce of somatic embryos which appeared after 14 days of culture. Also, Wójcikowska and Gaj, 2017 have used 2,4-D at concentration of 5 mM per liter and 20 g/L^{-1} of sugar in B5 medium for production of somatic embryos from cotyledon explants in Arabidopsis plant. In another paper, the same previous concentrations were used to produce somatic embryos in Arabidopsis to study the genes variation (Grzybkowska *et al*., 2018). Also, direct production for somatic embryos in cabbage (*Brassica oleracea L. var. acephala*) was achieved by Banjac *et al*., 2019. Few studies have reported the regeneration through direct somatic embryogenesis in red cabbage. Thus, this paper aimed to optimize the best medium type (MS and B5), concentration of 2,4-D as well as the best explants type which were used in this an efficient protocol for direct production of somatic embryogenesis in red cabbage.

Materials and Methods

This experiment was carried out at tissue culture laboratories of Iraqi Ministry of Agriculture from period 7/11/2018 till 15/9/2019. The seed of red cabbage variety Riassa were provided by (DAEHN FELDT) company. This variety needs 85-90 days for growing in the field as well as it has a big heads and external leaves. Surface sterilization for red cabbage seeds was achieved after use for 15 minutes of a 20% solution of sodium hypochloride (6% NaOCl), containing a few drops of Tween-20, followed by a triple rinsing with sterile distilled water (SDW). After that, the seeds were placed on MS medium (Murashige and Skoog, 1962) devoid of growth regulators and provided with 30 g/L^{-1} sucrose and 7g/L^{-1} of agar for germination. The cultures were incubated at 24°C during 16/8 h photoperiod obtained from fluorescent lamps. After seed germination, three type of explants (Cotyledon, Hypocotyl and leaf) were cultured on MS medium supplemented with four concentrations of 2,4-D (0, 1.5, 2, 2.5 mg/L^{-1}) and B5 medium (Gamborg *et al*.,1968**)** provided with five concentrations of 2,4-D (0, 0.50, 0.55, 0.60, 0.65 mg/L^{-1}). These media were supplemented with 20 g/L^{-1} of sucrose as well as 7g/L^{-1} of agar. The PH of the media was adjusted to 6.8 before autoclaving at 1.05 kg/cm²,

121°C for 20 min. The explants were cultured under aseptic conditions inside Laminar air flow cabinet using sterile cutters and forcipes. All cultures were maintained in a growth room under an 8-h dark/16-h light photoperiod and at 24°C. After ten days of culture, the appearance of embryos was classified and counted in each of the 4 stages of development (globular, heart, torpedo and cotyledonary) under a light of microscope.

Statistical analysis

Completely Randomized Design (CRD) was applied with ten replicates for each experiment. Data were analyzed using Genstat software. Test of least significant differences (LSD) at 5% level of probability was used to compare the calculated averages of traits (El-sahookie and Wuhaib,1990).

Results

Effect of 2,4-D concentrations on somatic embryos formation

The results showed that the all concentrations of 2,4-D that were used with MS medium did not appear any response for somatic embryos formation using various type of explants. On the other hand, it was found that hypocotyl and leaf explants did not response for somatic embryos formation when B5 medium used. Thus, only cotyledon explants were applied in this experiment using B5 medium enriched with five concentrations of 2,4-D (0, 0.50, 0.55, 0.60, 0.65 mg/L^{-1}). The results of table (1) showed that the use of 2,4- D at concentration of 0.50 (mg/L^{-1}) was the best for somatic embryos formation as the highest average of somatic embryos (40) was achieved on this medium and differed significantly from all other concentrations (P<0.001). While, the lowest average of somatic embryos (5) was recorded on medium supplemented with 0.55 mg/L^{-1} (Fig.1).

Table 1 : Effect of 2,4-D concentrations that used with B5 medium on somatic embryos production form cotyledons explants .

2,4-D Concentration (mg/L^{-1})	Somatic embryo numbers
Control)(0	0
0.50	40
0.55	5
0.60	7
0.65	11
LSD (0.05)	3.3

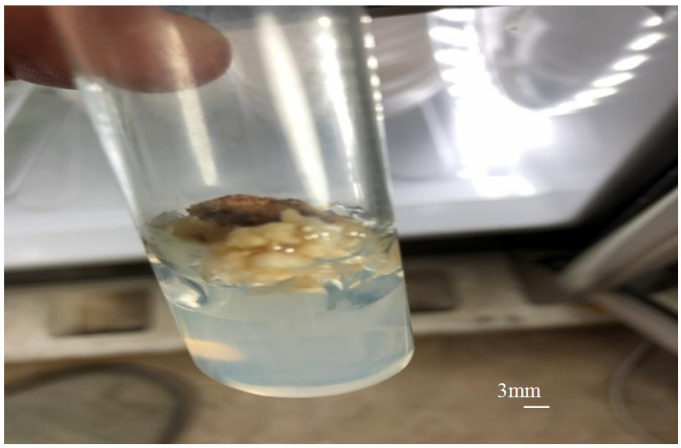

Fig. 1 : Somatic embryos formation from cotyledon explants after 30 days of culture on B5 medium enriched with 0.60 mg/L-1 of 2,4-D.

Effect of 2,4-D concentrations on developmental stages of somatic embryos

The results indicated that somatic embryos at globular stage appeared after 15 days of cotyledons culture on B5 medium provided with 2,4-D at (0.50, 0.55, 0.60, 0.65 mg/L^{-1}). It was noticed that somatic embryos were formed on media containing 0.55, 0.60, 0.65 mg/L^{-1} of 2,4-D did not develop to other developmental stages of somatic embryos, thus the paper focused on results that produced from culture of cotyledon explants on B5 medium supplemented with 0.50 mg/L^{-1} of 2,4-D. All developmental stages reaching to complete plant were observed on this concentration. As shown in figure (2) direct somatic embryos started to appear from cotyledon explants after 15 days of culture. The highest percentage 85% for embryos formation at globular stage was observed while 15% percentage of somatic embryos at heart stage was recorded. After 30 days of culture, it was demonstrated that the percentage of somatic embryos at globular stage decreased to70% percentage also the percentage of somatic embryos at heart stage decreased to 10% with appearance of somatic embryos at torpedo stage with percentage of 20% . After 45 days of culture, globular and heart stages of somatic embryos were demonstrated on explants with percentage 5% (for each stage), also somatic embryos at torpedo shapes with a high percentage 70% were noticed. Cotyledonary shaped somatic embryos were obtained on explants with percentage 20%. After 60 days, somatic embryos at cotyledonary stages were observed with percentage 45% while somatic embryos at torpedo stages were observed with percentage 55% (Fig. 3). These results that were reported in this work can be considered as a good result compared with results that mentioned in other previous papers that published in this field.

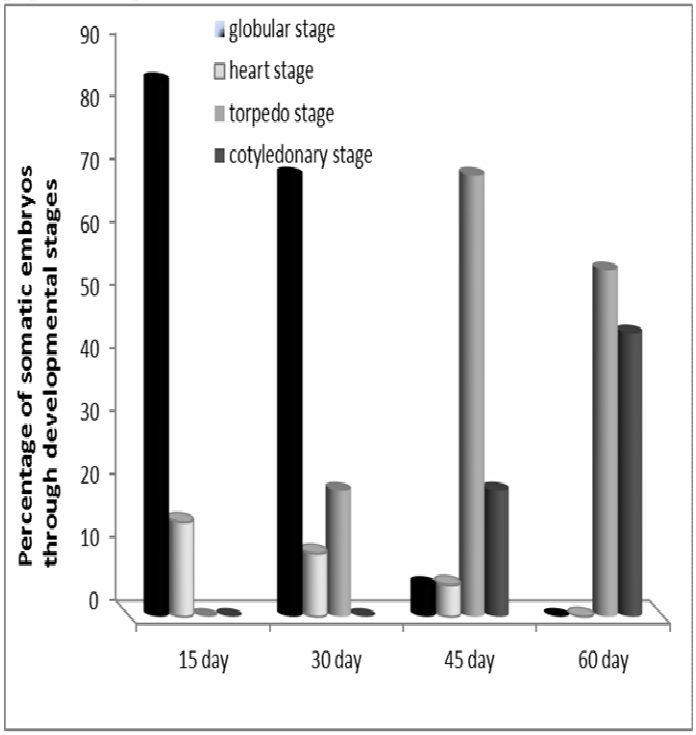

Fig. 2 : Effect of 2,4-D at 0.50 mg/L^{-1} on percentage of somatic embryos through developmental stages. LSD = 3.8 for globular stage, LSD =1.8 for heart stage, LSD =3.4 for torpedo stage and LSD =1.2 for cotyledonary stage.

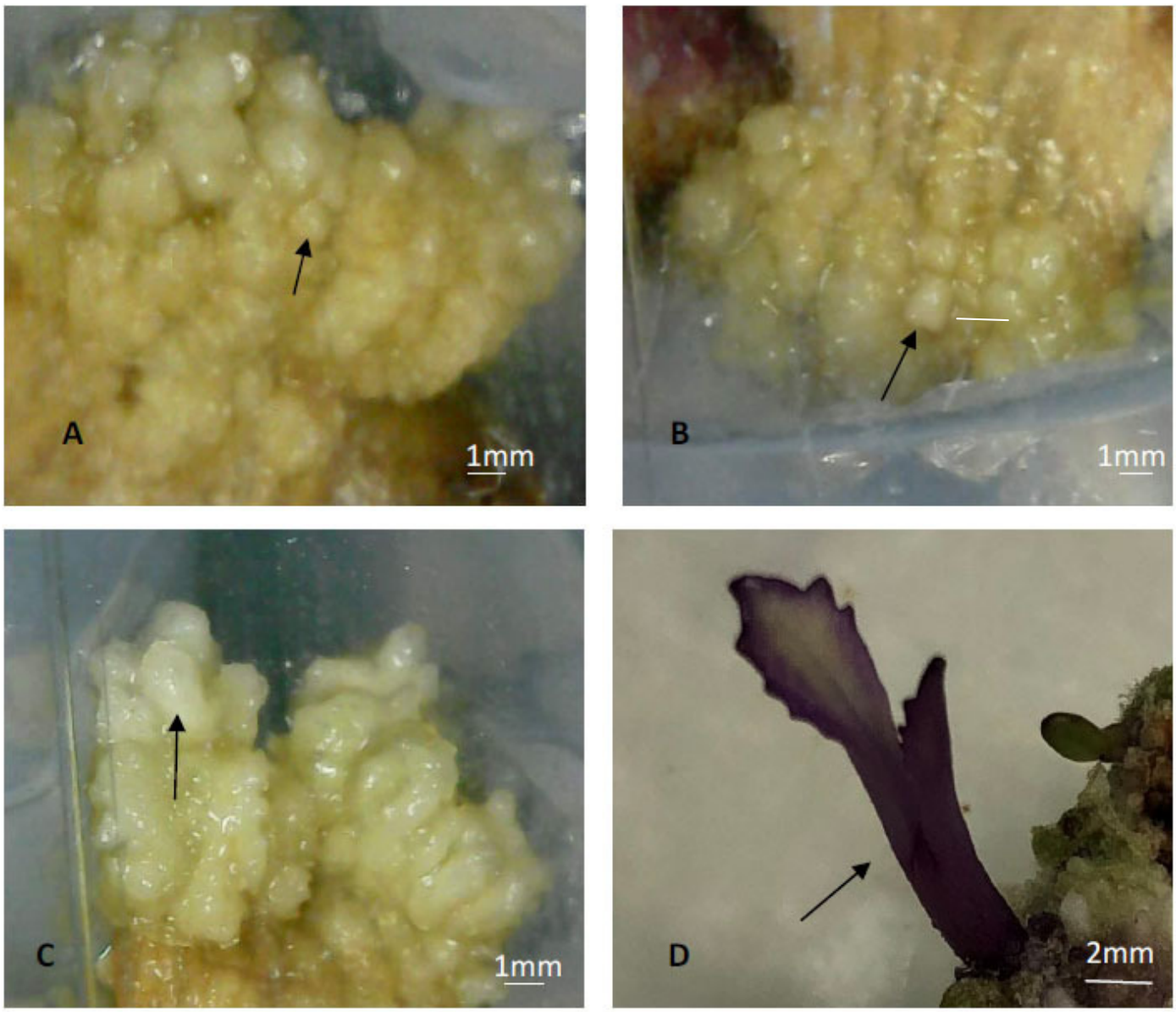

Fig. 3 : Developmental stages of somatic embryos from cotyledon explants on B5 medium with 0.50 mg/L^{-1} of 2,4-D. A) Somatic embryo at globular stage. B) Somatic embryos at heart stage. C) Somatic embryo at torpedo stage. D)Somatic embryo at cotyledonary stage.

Discussion

The results showed that somatic embryos which formed directly from cotyledon explants on B5 medium supplemented with various concentrations of 2,4-D at 0.55, 0.60, 0.65 mg/L^{-1} could not develop to all other stages of somatic embryos. This might due to changes that happened in DNA, many factors could affect on this process like growth regulators, culture medium, the temperature, light type that used inside incubator, source of amino acid and the PH medium (Zavattieri *et al*., 2010). Also, the level of auxin in culture medium could affect on somatic embryogenesis if it was high or low and sometimes after stimulation of cells for somatic embryos formation, no decrease in auxin level might led to inhibit the embryogenic development (Jiménez, 2001). While the development of somatic embryos for all stages from cotyledon explants that were cultured on B5 medium enriched with 2,4-D at 0.50 mg/L^{-1} was recorded. The reason behind this might belong to the using of 2,4-D. This growth regulator can be applied strongly to convert the sexual cell to somatic cell and also led to loss the cell polarity as well as it works with other endogenous auxin to prevent cells approach that led to organogenesis (Karami *et al*., 2009). There are some hypothesis could explain the role of 2,4-D in stimulating of direct and indirect formation of somatic embryos from explants like:-

1) Some authors thought that the effect of 2,4-D due to its role which was found similar to stress responses. Stress-inducible cell signalling compounds, such as H_2O_2 and NO in the target tissue were recognized (Pfeiffer and Höftberger, 2001). In Arabidosis, it was found that stress-related genes to be associated with the induction of somatic embryogenesis (Nowak *et al*., 2015).

2) On the other hand, it was thought that the direct effect for 2,4-D in somatic embryogenesis might be through the gene induction which stimulates the somatic embryos formation or its indirect effect by increasing the endogenous IAA production in plant. In Arabidopsis plant, it was found increasing in endogenous IAA when 2,4-D used in somatic embryos medium (Wójcikowska *et al*., 2013).

B5 medium has positive impact in somatic embryos production, this might due to the reduction of nitrogen source in medium as it contains only KNO3 and plants are appeared different responses to amount and source of nitrogen in a medium (Shirin *et al*., 2015). The other reson behind this efffect is the high contain of (Thiamine) in B5 medium which can be considered as an essential micronutrient for all living organisms (Mangel *et al*., 2017) because it is required as a metabolic cofactor in several enzymatic reactions including, the citric acid cycle, glycolysis, branched-chain amino acid biosynthesis and the cytosolic non-oxidative stage of the pentose phosphate pathway (Goyer, 2010; Rapala- Kozik, 2011). These results are in accordance with other researches who referred that somatic embryos can be produced on MS and B5 medium that enriched with 2,4-D without adding any other hormones (Raghavan, 2004; Kobayashi *et al*., 2010; Wojcikowska and Gaj, 2017). While other studies are in contrast with our results, who reported that using of auxins and cytokinins in a medium are required for somatic embryos formation (Siong *et al*., 2011; Cosic *et al*., 2013; Banjac *et al*., 2019) .

Conclusion

According to our best knowledge, there have been no reports concerning *in vitro* regeneration of this crop by direct somatic embryogenesis technique. So, especially in this time as increasing in requirements around the world for these nutritional and medical crops which can be consumed to increase immunity against diseases, thus many studies about propagation of red cabbage using biological technique are needed.

References

Al-Rawahy, S.A.; Abdul Rahman, H.A. and Mohammed SEIF AL-Kalbani (2004). "Cabbage (*Brassica oleracea L.*) response to soil moisture regime under surface and subsurface point and line applications." Inter. J. of Agric. and Biol 6(6): 1093-1096.

Al-Sumaidaie, Kadhim M.I. (2017). Application of plant Biotechnology, University Printing House.

Banjac, Nevena, Branka Vinterhalter, Dijana Krstić-Milošević, Jelena Milojević, Ljiljana Tubić, Nabil Ghalawenji and Snežana Zdravković-Korać, (2019). "Somatic embryogenesis and shoot organogenesis from the hypocotyl slices and free radical scavenging activity of regenerants of collard greens (*Brassica oleracea L.* var. acephala)." Plant Cell, Tissue and Organ Culture (PCTOC) 137(3): 613-626.

Boras. Mitadi, Bassam Abu Trapp and Ibrahim Al-Basit (2011). Production of vegetables. The theoretical Part. Al-Ajlouni Press. Damascus University. Univ. of Agriculture. Syria.

Ćosić, Tatjana, Branka Vinterhalter, Dragan Vinterhalter, Nevena Mitić, Aleksandar Cingel, Jelena Savić, Borut Bohanec and Slavica Ninković (2013). "*In vitro* plant regeneration from immature zygotic embryos and repetitive somatic embryogenesis in kohlrabi (Brassica oleracea var. gongylodes)." In Vitro Cellular & Developmental Biology-Plant 49(3): 294-303.

Desai, B.B. (2004). Seeds handbook: Processing and storage, CRC Press.

El-sahookie, M.M. and Wuhaib, K.M. (1990). Design and Analysis of experiments. Univ. Of Bagh. Dar al hekma. pp.488

Gamborg, O.L.; Miller, R.A. and Ojima, K. (1968). "Nutrient requirements of suspension cultures of soybean root cells." Experimental cell research 50(1): 151-158.

Goyer, A. (2010). Thiamine in plants: aspects of its metabolism and functions. Phytochemistry, 71: 1615–1624.

Grzybkowska, Daria, Joanna Morończyk, Barbara Wójcikowska and Małgorzata Danuta Gaj, (2018). "Azacitidine (5-AzaC)-treatment and mutations in DNA methylase genes affect embryogenic response and expression of the genes that are involved in somatic embryogenesis in Arabidopsis." Plant Growth Regulation 85(2): 243-256.

Hassan, Ahmed Abdel-Moneim (1994). Production and physiology and accreditation of vegetable seeds. First edition. Cairo: The Arab for Publishing and Distribution.

Jain, S.M. and Pramod, K.G. (2018). Step wise protocols for somatic embryogenesis of important woody plants, Springer.

Jiménez, V.M. (2001). "Regulation of *in vitro* somatic embryogenesis with emphasis on to the role of endogenous hormones." Revista Brasileira de Fisiologia Vegetal, 13(2): 196-223.

Jiménez, V.M. (2005). "Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis." Plant Growth Regulation, 47(2-3): 91-110.

Karami, Omid, Behzad Aghavaisi and Aghil Mahmoudi Pour (2009). "Molecular aspects of somatic-to-embryogenic transition in plants." Journal of chemical biology 2(4): 177-190.

Kobayashi, Toshihiro, Youichi Nagayama, Katsumi Higashi and Masatomo Kobayashi (2010). "Establishment of a tissue culture system for somatic embryogenesis from germinating embryos of Arabidopsis thaliana." Plant Biotechnology 27(4): 359-364.

Liu, C.M.; Xu, Z.H. and Nam-hai Chua (1993). Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. The Plant Cell 5(6): 621-630.

Mangel, Nathalie, Jared B Fudge, Teresa B Fitzpatrick, Wilhelm Gruissem and Hervé Vanderschuren (2017). "Vitamin B1 diversity and characterization of biosynthesis genes in cassava." Journal of experimental botany, 68(13): 3351-3363.

Murashige, T. and Folke, S. (1962). "A revised medium for rapid growth and bio assays with tobacco tissue cultures." Physiologia plantarum 15(3): 473-497.

Nowak, Katarzyna, Barbara Wójcikowska and Małgorzata D Gaj, (2015). ERF022 impacts the induction of somatic embryogenesis in Arabidopsis through the ethylene-related pathway. Planta 241(4): 967-985.

Pfeiffer, W. and Margit, H. (2001). "Oxidative burst in Chenopodium rubrum suspension cells: induction by auxin and osmotic changes." Physiologia Plantarum 111(2): 144-150.

Raghavan, V. (2004). Role of 2, 4 ‐ dichlorophenoxyacetic acid (2, 4 ‐ D) in somatic embryogenesis on cultured zygotic embryos of Arabidopsis: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2, 4 ‐ D." American Journal of Botany 91(11): 1743-1756.

Rapala-Kozik, M. (2011). Vitamin B1(thiamine): a cofactor for enzymes involved in the main metabolic pathways and an environmental stress protectant. Advances in Botanical Research, 58: 37–90.

Salman, Mohammed, Abas, (1988). Basics of plant cell and tissue culture. Mini. of Hig. Edu. and Sci. Res.,.Baghdad Unv., Iraq.

Siong, Poon, Rosna Taha and Fatimah Rahiman, (2011). "Somatic embryogenesis and plant regeneration from hypocotyl and leaf explants of *Brassica oleracea* var. botrytis (cauliflower)." Acta Biologica Cracoviensia Series Botanica, 53(1): 26-31.

Shirin, Fatima, Nitish Singh Parihar and Syed Naseer Shah (2015). Effect of Nutrient Media and KNO_3 on in Vitro Plant Regeneration in *Saraca asoca* (Roxb.) Willd." American Journal of Plant Sciences, 6(19): 3282.

Wójcikowska, B. and Małgorzata, D.G. (2017). "Expression profiling of AUXIN RESPONSE FACTOR genes during somatic embryogenesis induction in Arabidopsis." Plant Cell Reports, 36(6): 843-858.

Wójcikowska, Barbara, Karolina Jaskóła, Przemysław Gąsiorek, Magdalena Meus, Katarzyna Nowak and Małgorzata D Gaj, (2013). "LEAFY COTYLEDON2 (LEC2) promotes embryogenic induction in somatic tissues of Arabidopsis, via YUCCA-mediated auxin biosynthesis." Planta 238(3): 425-440.

Zavattieri, Maria Amely, António Miguel Frederico, Mónica Lima, Rui Sabino and Birgit Arnholdt-Schmitt, (2010). "Induction of somatic embryogenesis as an example of stress-related plant reactions." Electronic Journal of Biotechnology 13(1): 12-13.