EUROPEAN ACADEMIC RESEARCH
Vol. IX, Issue 5/ August 2021

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Sonication Support Proteins and Antioxidants Synthesis in Cell Suspension Cultures Derived-Callus of Chickpea (*Cicer arietinum* L.)

ANWAAR F. AL-TAEE
JAMELLA H. RASHEED
Department of Biology
Education College for Pure Sciences, University of Mosul

Abstract

In this study cell suspension cultures were established from hypocotyls and embryos of Cicer arietinum L. It was observed that Murashige & Skoog medium provided with addition of 1mgl^{-1} NAA Naphthalen acitic acid and 2mgl^{-1} BA Benzyl adenine. The results of cultivating selected densities of cell suspension cultures exposed to ultrasonic waves in agar droplets and supplemented with the supported liquid medium showed the suitability of this technology in the cultivation of cell suspensions, their division and formation of cell colonies and callus primordia, which were successfully transferred to the perpetuation media. Ultrasound resulted in an increase in the density of cell suspensions, and it was recorded at 43×4^{10}, 45×4^{10} cells/ml for each of the cultures of cell suspensions for hypocotyls callus and embryonic. Exposing cell suspensions for 1, 2, 3 minutes at a frequency of 47.6 kHz and implanted in multi-agar droplets encouraged an increased in callus fresh weight, and synthesis of protein and antioxidants. The marker of increasing antioxidant levels was), Hydrogen peroxide (H_2O_2) and Lipid peroxidation (MDA) concentration that record µmol\mg of protein. The most expected effect of ultraviolet due to the pass through of ultrasound wave in medium. The cell suspensions derived from the embryo callus were superior to the cell suspensions derived from the hypocotyls callus.

Keywords: Chickpea (*Cicer arietinum* L.), Cell suspension cultures, Lipid peroxidation, malondialdialdehyde (MDA), Hydrogen peroxide (H2O2), Ultrasound shock

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most economical legumes rich in dietary protein and cultured in more than 55 countries of the world, including Australia (FAO.,2019). and yield has increased rapidly since 1961, however, this is insufficient for protein requirements. Chickpea faces many challenges due to various abiotic environmental condition such as drought and high temperatures (Gaur *et al*., 2019). in addition to its symbiotic ability to fix atmospheric N_2 (Das *et al*., 2020).

Ultrasound has been used for the application of a frequency more than 20 KHz. inaudible to the human ear. many effective means in biotechnology included in cell suspension cultures enhanced the content of secondary metabolites in plant tissue culture, transformation of plant species by direct DNA transfer (sonication facilitated *Agrobacterium* – mediated transformation). Moreover, ultrasound has been used to induce growth of insurgent explants, and to isolate somatic embryo (Gaba *et al*., 2008). Application of ultrasound in biotechnology is one of the effective means that has the ability to improve cellular metabolism, transmit reagents and signals across the membrane, cell wall and enhance chemical reactions (Gaba *et al*., 2008).Inhibiting the enzymes and increasing the effective antioxidants in the plant, and stimulating the growth of stubborn plant calluses, sorting somatic embryos, promoting germination of seeds and subsequent growth of seedlings at low-intensity audio waves (Ordones-Santos *et al*.,2017).

Due to climate change that harms plant growth, and disrupts enzymes associated with different metabolic pathways this lead to change in protein synthesis (Pan *et al*.,2020). Some plants respond to such stresses by reprogramming their proteins industry to ensure a steady state of metabolic processes that help them to survive and function under stress conditions (Wang *et al*.,2020). The genetic basis of chickpea is expected to be an important factor in stress tolerance by restoring cellular homeostasis and its direct effect on cellular and physiological changes (Devasirvatham and Tan, 2018).

The aim of this investigation is to detect the role of sonication in protein content and antioxidants in callus cultures produced from pre-exposed cell suspensions of chickpea.

2. MATERIALS AND METHODS

2.1. Embryos and Hypocotyl Production

Seeds of *Cicer arietinum* L. "Desi variety" were sterilized by immersing in 96% ethyl alcohol for 2 min., then submerging in sodium hypo chlorate solution (NaOCl 5%) for 5 min. then washed four times with sterile distilled water. Sterilized seeds were transferred to the surface of 25 ml of solid basal MS medium free from growth regulators (Murashige and Skoog, 1962). in 100 ml glass containers and incubated in culture room (23°C) under light intensity of 2000 lux at (8/16 light / hour). Sterile seedlings were used as source of embryos and hypocotyls.

2.2. Callus Initiation

Sterile seedlings of 1.5 cm length with 12-15 days old were excised. Embryo and hypocotyls fragment of each seedling were cultured on agar solidified MS medium containing 3% sucrose, provided with 1.0 mgl^{-1} NAA and 2.0 mgl^{-1} BA (Raghavendra, 2018) for callus initiation. The endcation was MS+NAA+BA cultures were kept at culture room condition.

2.3. Establishment of Cell Suspensions from embryos and Hypocotyls Calli:

Twenty five ml of liquid MS medium containing 1.0 mg l^{-1} NAA and 2.0 mgl^{-1} BA in a 100 ml conical flasks in each flask 1.0 g of embryos and hypocotyl callus was placed individually, samples were kept in the shaking incubator (Brunswick, USA) under complete darkness and 28°C rotational speed 150 rpm for 24 hours (Morris and Flower, 1981).

Cultures were filtered by passing through a sterile, fine plastic sieve of 46 µm mesh (Plant Genetic Manipulation Grope, Nott. Univ. U.K.) to remove the cell clumps and collect numerous single cells and complete the original size by adding the necessary amount of the same medium. Cultures were returned to the shaking incubator under the same conditions, follow-up the division of cells and estimate of their densities. Perpetuate the cell suspension cultures by lifting the decanters containing the cultures from the incubator and leaving them inside the cabinet for 3-4 hours by placing the decanters in an

inclined manner resting on the cabinet wall and allowing them to stagnate their cells. Then the old medium was carefully poured to maintain the stable cell and an appropriate volume of the same medium was added to the cells deposited in the flasks. Samples were returned to the incubator under the same conditions (Greshhoff, 1980).

2.4. Estimation of Cell Viability in Cultured Cell Suspensions

Calculated the total number of cell and prepare the live cells to know the percentage of viability of cells, cell viability of cell suspensions was estimated using the standard method (Birkenhead and Willmer, 1986). using Evan blue dye (BDH chemical L td. Poole, England). The dye solution was prepared by dissolving 0.5 g of it in 100 ml. of distilled water and taking 0.1 ml. of the dye solution and mixing it with 0.1 ml. of cell culture. The samples were left for 10 min., then examined with a normal light microscope, where dead cells appear blue, while living cells remain without dye. The following mathematical formula was adopted to calculate the viability ratio: Viability = (Total cells – Number of dead cells / Total cells) ×100 (PauL, 1970).

2.5. Cultivation of Cell Suspensions using Multi-Droplet Agar Technology

One ml of cell suspension culture at the density of the fourth day ranged between 34-45 $\times 10^{4}$ cells / ml was put in a sterile test tube, and added to it 1 ml of pre-sterile 3% liquid agar in a water bath at a temperature of 40°C. Mix them quickly to avoid hardening, and distribute the mixture in uniform sized drops at a rate of 8-12 drops / 9 cm diam. plastic petri dish (Sterilin, UK). The dishes were left open inside the culture cabinet to complete the solidification of the droplets, followed by the addition of 10 ml of liquid MS medium conditions 1.0 mgl^{-1} NAA and 2.0 mgl^{-1} BA to each petri dish. Dishes were closed, then sealed with parafilm. Samples were incubated in conditions of temperature 25±2 ° C, lighting of 700/800 lux in the successive lighting system, Embedded cells were examined by light microscope periodically to observe the initiation of division. Cultures were maintained every four days by removing the old medium from the plates with a sterile pipette and replacing it with the addition of 6 ml

of the same fresh medium until the appearance of the callus-primordia.

2.6. Transfer of callus primordia formed in droplets and formation of callus cultures

After emergence of callus primordia from agar droplets, they were transferred by removing agar drop by sterile spatula and placed on a surface of 25ml of the medium (MS + 1.0 mg l^{-1} NAA + 2.0 mg l^{-1} BA), in 100 ml glass container, at a rate of 3 primordium\ flask. Samples were kept in culture room under the same previous conditions.

2.7. Exposure of Cell Suspension to Ultrasound Wave Shocks

Five samples each of 1.0 ml were taken from cell suspension cultures of hypocotyl and embryos ranged between 34×10^4, 45×10^4 cells / ml, and placed individually in a sterile test tube, and put in the port of the ultrasound device (BRANSONIC 221 Water Bath, 117 V, 1.7 A, USA). These samples were exposed to ultrasound shock, at frequency of 47.6 KHz for with durations of (0, 1, 2, 3, 4, 5) minutes with constant temperature at 23-25 ° C (Lenka *et al*., 2014). The treated, and the non-exposed samples were embedded in agar drops as reported previously.

2.8. Determination of Callus Fresh Weight:

Fresh weight of the growth callus was calculated 30 days after cultivation from the weight difference of the glass bottles containing only nutrient media and their weight after implantation of callus.

2.9. Determination of Total Protein of Callus:

The standard method (Skacterle and Pollack, 1973) was used to quantify protein in callus extract, and BSA was used as the standard solution.

2.10. Determination of Total Chlorophyll Content in Callus:

The method (Arnon,1949) was followed to determine the total chlorophyll content in exposed and non-exposed callus and the total chlorophyll was calculated according to the following formulas expressed in mg / g fresh weight of callus

$$\text{Total chlorophyll (mg\ g)} = (20.2\ A_{645} + 8.02\ A_{663}).$$

2.11. Determination of Lipid Peroxidation:

Lipid peroxidation was quantified by the estimation of malondialdialdehyde content (Nazari *et al*, 2012). and the amount was expressed μ mol/g Fr wt.

2.12. Active Oxygen Species Analysis:

Hydrogen peroxide (H_2O_2) was estimated and determined based on the standard method (Noreen and Ashraf, 2009). The content of H_2O_2 was expressed in μ mol/g Fr wt.

3. RESULTS

3.1. Production of Cell Suspension Cultures:

The result indicated that both types of callus were amenable to create numerous single cells producing homogenized fine cell suspensions. Data showed a subsequent increase of cell densities up to the fourth day of culture age (Table 1)

Table (1) Establishment of cell suspension of embryos and hypocotyl calli of chickpea (*Cicer arietinum.*)

Callus	Densities $\times 10^4$ cell\ ml					
	Days					
	First	Second	Third	Fourth	Fifth	Sixth
Embryos	8	23	36	45	30	28
Hypocotyls	5	8	17	34	23	12

Note: More over viability of cells forming the culture was ranged 90-95%

3.2. Behavior of cell suspension exposed to ultrasound wave

The results indicated that each type of callus suspension cultivated in liquid MS+1.0mg1^{-1} NAA+2.0 mgL^{-1} BA and embedded in agar drop (solid-liquid culture) encourage callus growth. More over exposure of hypocotyl and embryos cell suspension to the frequency 47-6 KHZ for 1,2,3,4,5 min. stimulate cell division. Cell suspension cells exposed to treatments 2 and 3 min. began their first division after the third day of cultivation, compared to the division of cells of the control sample whose first division began on the fifth day of cultivation. These divided cells entered their second and third divisions after forty-eight hours after the first division took place, and divisions continued to form cellular colonies on the tenth day of cultivation, to develop from

them the beginnings of callus after fifteen days of cultures. The continuity of division produced numerous colonies (Fig1-6). Then conversion of these colonies to tiny callus primordial (Fig 1-7), They increased in size cracked the agar and become obvious to naked eyes 30 days after cultivation both those produced from embryos (Fig 2-a) and hypocotyls (Fig 2-b) cell suspension. Later whole drops were transferred to the callus growth medium producing callus cultures.the (2 and 3) min. treatment of cellular suspensions of the embryos Fig2(c-d) and (1 and 2) min. of cellular suspension of the hypocotyl Fig 3(b-c) outperformed the rest of the treatments in stimulating the number of colonies formed and the beginnings of the callus developed from it, and the callus described these beginnings in its yellow color (Fig 1-9).

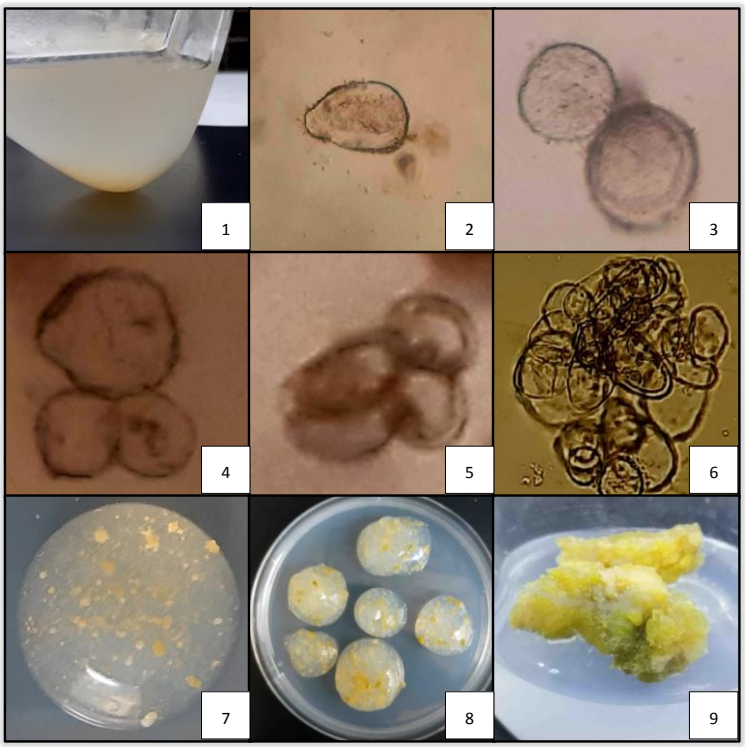


Figure (1): Stages of Chickpea callus formation from cell suspension cultures: (1) Culture of cell suspensions derived from callus embryos and hypocotyls in liquid induction medium MS + 1 mg L^{-1} NAA + 2 mg L^{-1} BA. (2,3) Mitosis of cell and the formation of two nucleated cells. (4,5) Cell divisions of cellular suspensions culture and formation of triple and quadruple cell stages. (6) Initiation of cellular colony (7) emergence of callus primordial from cell colonies. (8) callus primordial developing in the midst of callus development (9) callus output from cell suspension cultures.

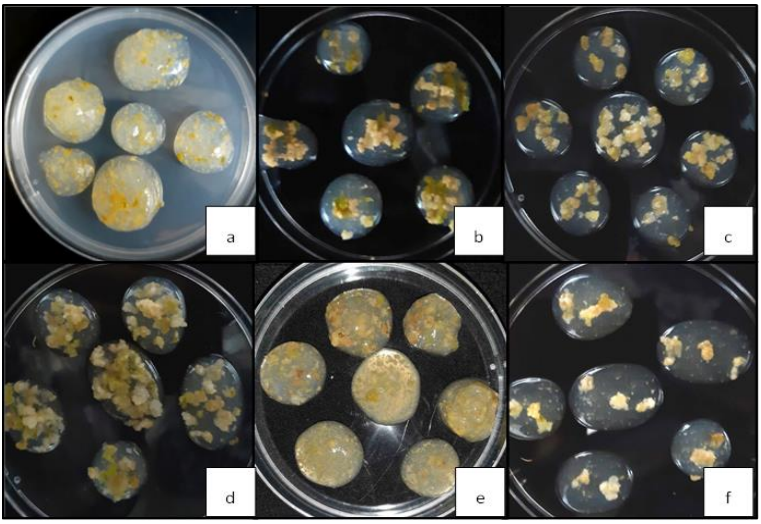


Figure (2) Cultivation of embryos cell suspension of chickpea (*Cicer arietinum L.*) plants: exposed to ultrasound shock for 1, 2, 3, 4, and 5 min. in solid liquid MS medium enriched with NAA & BA after 30 Days.
(a) callus primordial produced from un-exposed cell suspensions 30 days after cultivation (control).
(b,c,d) callus primordia from cell suspension exposed for 1,2 and 3 min. respectively.
(e,f) callus primordial produced from cell suspension exposed for 4 and 5 min.

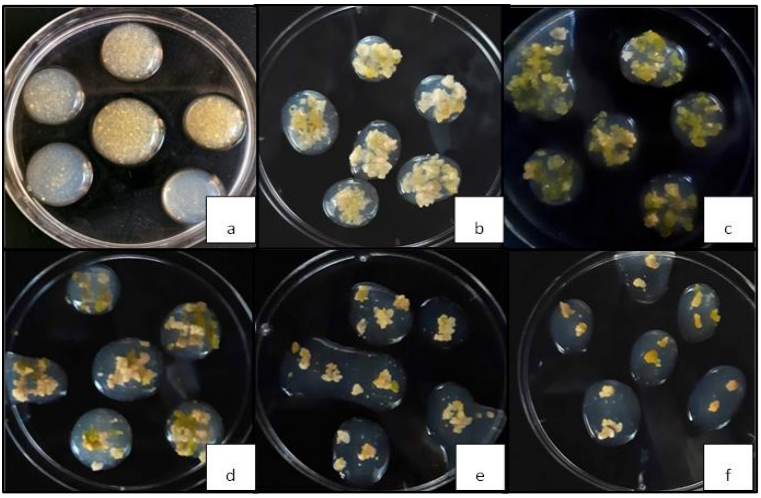


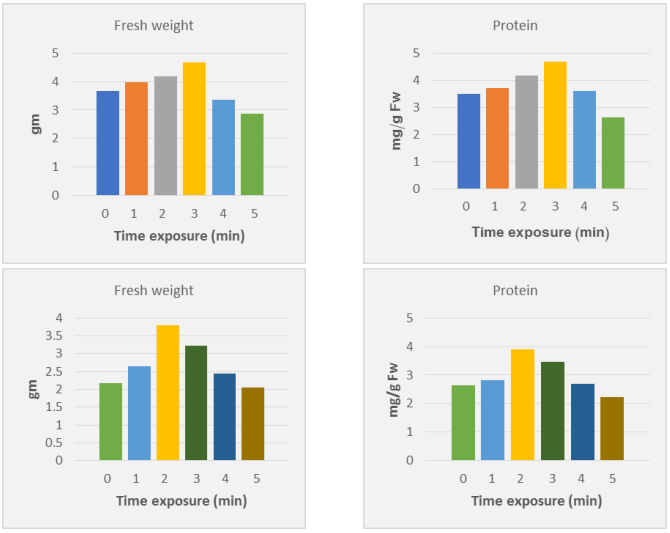
Figure (3) Cultivation of hypocotyl cell suspension of chickpea (*Cicer arietinum L.*) plants exposed to ultrasound shock for 1, 2, 3, 4, and 5 min. in solid liquid MS medium enriched with NAA & BA after 30 Days.
(a) callus primordial produced from un-exposed cellular suspensions 30 days after cultivation (control).
(b,c,d) callus primordial from cell suspension exposed for 1,2 and 3 min. respectively.
(e,f) callus primordial produced from cell suspension exposed for 4 and 5 min.

3.3. Effects of Ultrasound Shock on Callus Fresh Weight and Protein Content

Data indicated Fig (4) the positive role of ultrasound in stimulating the growth of callus when exposed to 1, 2 and 3 min. Also, increased the fresh weights and proteins of callus produced from the cell suspensions of the embryos and hypocotyl grown in MS medium fortified with 1 mg L-1 NAA and 2 mg L-1 BA.

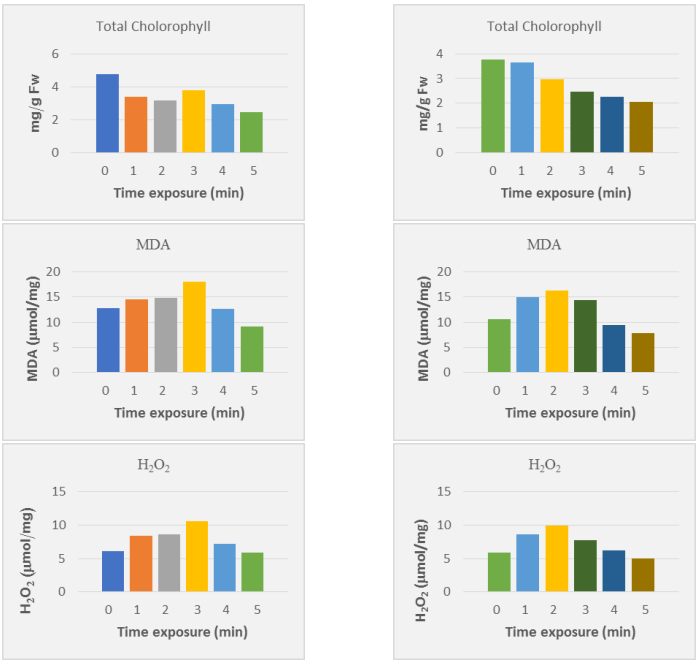
3.4. Effect of Ultrasound Shock on Total Chlorophyll Content, Antioxidants (H2O2 and MDA):

The results showed clear decrease in the photochemical efficiency represented by a decrease in total chlorophyll levels with a prolonged period of exposure to ultrasound shock (46.7 KHz) with a marked increase in antioxidants as exposing the cell suspensions to the is shock for 2, 3 min. Also, this shock stimulating activity and effectiveness of antioxidants equivalent to (1.5-2.0) fold as compared to control Fig (5).



(Fig. 4): The effect of ultrasonic shock on fresh weights and protein content of cell suspension derived callus of Chickpea (*Cicer arietinum* L.)

a) Fresh weight of embryoid callus.
b) Protein content of callus in embryoid.
c) Fresh weight of hypocotyl callus.
d) Protein content of callus in hypocotyl.



(Fig. 5): The effect of ultrasonic shock on chlorophyll content, H_2O_2 content and MDA level in cell suspension-derived callus of Chickpea (*Cicer arietinum* L.)

a) Total chlorophyll of embryoid callus.
b) Total chlorophyll of hypocotyl callus.
c) Lipid peroxidation (MDA)of embryonic callus.
d) Lipid peroxidation (MDA) of hypocotyl callus.
e) Hydrogen peroxide(H_2O_2) of embryonic callus.
f) Hydrogen peroxide(H_2O_2) of hypocotyl callus.

4. DISCUSSION

In this study the successful establishing of cell suspension cultures from hypocotyl and seed embryos calli of chickpea may be attributed to the stable nature of callus and its need for basic requirements available in the culture medium. Efficiency of these suspensions in their establishment pathways and their increasing density Table (1) likely due to the abundance of single cells and the lack of cell clumps (Bharathi and Elavarasi,2012). This encouraged cells to divide and behave in usual pattern in its successive divisions and the formation of callus in the end Fig (1-9). Thus, the cell suspensions of chickpea

plants similar to their counterparts to a number of other plants in leguminous species (Hernandez-Garcia, 2021). In this study, ultrasound had a clear effector the division of cell suspensions of embryoid callus Fig2(c-d). This may due to the viability and efficiency of these cells. The embryos on repeated rapid divisions explanted to the potential energy coupled with the suitability of the genotype. This is encouraged the speed of cell division to stimulated the biosynthesis of auxins and the effect of the parameters on the viability and density of cellular suspensions, which varied with the period of exposure (Loyola-Vargas, 2008).

The increase in the content of biomass and protein Fig (4) was approved by an increase in the specific effectiveness of antioxidants (MDA, H2O2) when using ultrasound waves for different durations (1, 2, 3) minutes, which may be attributed to the effect of ultrasound on the permeability of the plasma membranes of cells, which form small openings gives cells an opportunity to obtain their requirements from the nutrient medium, however stimulating the building of protein in these cells. It has been observed that pre-exposure to ultrasound shock improves the chemical and physical properties of plant tissues especially legumes, as it directly affects the physical properties of protein. Protein molecules with changes in secondary and tertiary structural composition and improvement of plant protein quality due to specific changes of proteins and production of new types of shock proteins HSP70 and HSP90 (Byanju et al., 2020). On the other hand, the results showed that the significant decrease in the biomass and protein content of embryoid callus and hypocotyls resulting from exposure to ultrasound shock for 4 and 5 min. may be due to the mechanical effects of waves and their damage to plant tissue biomolecules and may sometimes lead to cell lysis and death (Salih and Al-Obiedi,2011). Also decrease in the total chlorophyll concentration Fig (5) is due to the sensitivity of the cell suspension cells to ultrasound shock (Meng et al., 2012). This corresponds to the significant reduction of chlorophyll for callus cell suspensions upon exposure to the treatment of ultrasound shock for various durations compared to the non-exposal samples.

The rise in antioxidant levels in callus produced from embryos resulting from exposure to ultrasound shock which led to changes in the oxidation and reduction state of the plant cell represented by

increased levels of hydrogen peroxide and lipid oxidation due to the oxidative damage caused by the ultrasound shock, which in turn had direct effect on the fluidity of the plasma and mitochondria membranes and alter protein shape (JGhanati and Sobannejad,2016). The oxidative damage caused by ultrasound shock causes an increase in cellular membrane integrity, protecting large molecules such as proteins, and maintaining metabolism (Hassanpour, 2017).

5. Acknowledgment

This work was supported by Department of Biology, College of Education for Pure Science. also thanks are due to my supervisor and all colleges in biotechnology lab.

REFERENCES

Arnon DI. 1949. Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris* L. Plant Physiol., 24:1-15.

Bharathi P. Elavarasi N. 2012. Preliminary studies of reactor system designed for cell suspension culture of chickpea (*cicer arietinum* L.) International J. of Chem. Sci. and App.3(1):223-231.

Birkenhead K. Willmer CM. 1986. Some biochemical characteristics of guard cell and mesophyll cell protoplasts from *Commelina communis* L. J, Exp. Bot.,174(37):119-128.

Byanju B. Rahman MM. Hojilla-Evangelista. M P, and Lamsal BP. 2020. Effect of high – power sonication pretreatment on extraction and some physicochemical properties of proteins from chickpea, kidney bean, and soybean. International J. of Biol. Macromolecules, 145(64): 712-721.

Das A. Harika A. Shilpa. Senthi K. Narendra PS. (2020). Symbiotic nitrogen fixation and endophytic bacterial community structure in Bt- transgenic chickpea (*Cicer arietinum* L.) Scientific Repots: 10(1):359-366

Devasirvatham V. Tan D. (2018). Impact of high temperature and drought stresses on chickpea prod. Agron. 8(8): 1-9.

FAOSTAT 2019 Online database at https://www.foa.org/faostat/en/data.

Gaba V. Kathiravan K. Amutha S. Singer S. Xiaodi X. and Ananthakrisbnan G. (2008). The uses of ultrasound in plant tissue culture. Plant. Tiss. Cult. Engin., 417-426.

Gaur PM. Samineni S. Thudi M. 2019. Itegrated breeding approaches for improving drought and heat adaptation in chickpea (*Cicer arietinum* L.) Plant Breed.,138(4): 389-400.

Ghanati F. Sobannejad S. 2016. Effect of low –intensity ultrasound on membrane integrity of suspension culture parsley cells (*Petroselinum Crispum* L.) J. Plant Proc.and Func. 16 (5): 1415-1545.

Golly MK. Ma H. Yuqing D. Wu p. Dabbour M. Sarpong F. Farooq M. 2019. Enzymolysis of walnut (*Juglans regea* L.) meal protein: altrasonication _ assisted

alkaline pretretment impact on kinetics and thermodynamics. J Food Biochem 43(8): 12948.

Gresshoff PM. 1980. Invitro culture of white clover: callus, suspension, protoplast culture, and plant regeneration. Bot. Gazetta, 141(2):157-164.

Hassanpour H. Niknam V. Haddadi D. 2017. High frequency vibration inprove callus growth via antioxidant enzymes induction in (*Hyoscyamus Kurdicus* L.) Plant cell Tiss. and org. culture. 128(5): 231-241.

Hernandez – Garcia C. velazouez – Becerra R. Herrera – Bucio JG. acia – Magana. P. Lopez – Albarran E. 2021. Establishment of callus and cell suspensions cultures of *Dalbergiia congest* flora (Fabaceae) to Mediacarpin production. Asian J. Plant Sci., 20 (1): 109-115.

Lenka T, Jiri T. Helena C. 2014. Effect of ultrasound on the isoflavonoid production *in Genista tinctoria* L. suspension cultures. Pharmacognosy Magazine :38(10): 425-429.

Loyola –Vargas VM. Do- la- peria C. Galaz – Avalos RM. Quimz – figucro FR. 2008. Plant Tissue Culture. In: walker JM. Ripley R (eds) Molecular Bio Methods Handbook. Humana press, Totowa. PP. 875-904.

Meng Q. Zhou Q. Zheng S. Gao Y. 2012. Responses on Photosynthesis and Variable chlorophyll fluorescence of *Fragaria ananassa* L. under sound wave. Energy Procedia. 36(16): 346-352.

Morris P. Flower M.W. 1981. A new method for the production of fine plant cell suspension culture. Plant Cell Tiss. Org. Cult, 38(1):15-24.

Murashige T. Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol. Plant. 15(3): 473-497.

Nazari M. Reza M. Mehraban F. and Khaneghah H, 2012. Change in antioxidaant responses against oxidative damage in black chickpea following cold acclimation. Russian J. of P.P. 59(2):183-189.

Noreen Z. Ashraf M. 2009. Change in antioxidant enzymes and some key metabolites in some genetically diverse cultivars of radish (*Raphanus sativus* L.) Environ. Exp. Bot. 67(2): 395-402.

Ordonez-Santos L E. Martinez - Giron J. Arias-Jaramillo ME. 2017. Effect of ultrasound treatment on visual color, vitamin C, total phenol, and carotenoids content in cape gooseberry juice, Food Chem, 233 (119): 96-100.

Pan Y. Jia X. Li X. Chen L. Pang L. Chen X. 2020. Ultrasound treatment inhibits browning and improves antioxidant capacity of fresh – cut sweet potato during cold storage. J. The Royal Society of chemistry.Rsc.Adv.10(9): 9193-9202.

Paul J., 1970. Cell and Tissue Culture. London.

Raghavendra T, *et al.* ,2018. An efficient in vitro shoot regeneration protocol from embryo explants of chickpea (*Cicer arietinum* L.) Legume Research an International Journal ,3844(2):1-4.

Salih S. Al-Obaedi RF. 2011. Investigation of the effect of some ultrasonic waves and electro treatment in callus initiation and plant regeneration of *Ricinus communis* L. seedlings.Dirasat,Pure Scien. 38(1):61-71.

Skacterle GR. Pollack RL. (1973). A simplified method for the quantitative assay of small amount of protein in biologic materials Anal. Biochem. 51(2): 654-655.

Wang Y. Wanng Y. Li K. BaiY. LiB. Xu W. 2020. Effect of high intensity ultrasound on physicochemical, interfacial and gel properties of chickpea protein isolate, LWT - Food Sci. and Techno.129(7): 109563