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Screening plant secondary metabolites from callus cultures of sunflower

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Key words: EMS: Ethyl Methane Sulphonate, F.C.R: Folin Ciocalteu's Reagent, SF: Sunflower, NAA: Naphthalenic Acetic acid, 2,4-D: 2,4-Diclorophenoxyacetic acid.

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Abstract

Plants produce a diverse group of secondary metabolites to guard themselves against herbivores or to attract pollinating insects. Plant cell biotechnology offers excellent opportunities in order to use such secondary plant metabolites to produce goods with consistent quality and quantity throughout the year, and therefore to act independently from biotic and abiotic environmental factors. This article presents results of an extensive study of plant cell in vitro cultivation. In present investigation callus cultures were used to monitor the effect of mutagenic compounds on the plant secondary metabolites. Sunflower plant was mutagenized using different concentrations of mutagen namely EMS. Callus cultures were maintained using various combinations of phytohormones. Callus were obtained using phytohormones NAA, Thiamin HCL, 2,4-D**.** highest reducing sugars were obtained in 0.2% EMS, and highest reducing power was observed in the callus culture of 0.1% EMS.

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Introduction

The inland sunflower is the universal oil crop that has aptitude for weather alteration adjustment, since it can sustain steady harvests across a diversity of ecological circumstances comprising drought. Induced mutation unmasks novel ways of creating varieties with far better yielding attributes. The historical antecedents, theoretical and practical considerations and the success of induced mutations in crop improvement are reviewed along with how induced mutagenesis underpins plant functional genomics.

This will be the first report of inducing mutagenesis to manipulate antioxidants level and other biochemical components in this plant via *in-vivo* and *in-vitro*. It may also represent a novel approach for getting these all in large quantity if the mutations proved to be beneficial. It is proved by epidemiological studies that foodstuff in addition to providing nutritional support also has some other advantageous effects. In recent studies, investigation in this capacity has engrossed on the revealing of antioxidants in food, since there is an indication that they could show the significant part in the deterrence of quite a lot of illnesses such as cancer and cardiovascular disease as well as in the hindrance of the aging process. Fruits have received specific attention since they encompass prodigious quantities of recognized antioxidants such as polyphenols, vitamin C, vitamin E, ß carotene and lycopene (Kane *et al.,* 2007).

The experiments will be conducted with the objectives of establishing an effective protocol by which the production of natural antioxidants and other medicinally important biochemical components will be altered in sunflower seeds which were formerly exposed to chemical mutagenic agent namely, ethyl methyl sulphonate (EMS). The M1 seeds (first mutant generation) will be grown *in-vitro* and will be compared with results obtained from *in-vivo* studies. This study was carried out to establish tissue culture to obtain callus culture for enhanced production of natural antioxidants.

Materials and methods

The seeds of Sunflower germlines having accessions numbers 017599, 017600, 017601, 017602, 017603, 017604, 017605, 017606, 017607 and 017608 were obtained from Seed Preservation Laboratory & National Gene bank, Plant Genetic Resources Institute, NARC, Islamabad and were given mutagenic treatment of mutagen namely ethyl methyl sulphonate (EMS). Different concentrations of this mutagen were used, starting from 0.1%, 0.2%, 0.3%, 0.4% and 0.5% respectively. Seeds were sown under *in-vivo* conditions. Seeds were made to grow in Randomized Complete Block Design (RCBD). Mature crop was obtained within the period of ninety days. It was followed by the preparation of tissue culture media.

Preparation of aseptic culture media

Aseptic culture is indispensable and major requirement of plant tissue culture. There are different media used for tissue culture of plants but MS medium (Murashige *et al.,* 1962) is mostly used. In the present study, this MS medium was used.

There are three basic components of the media are composed:

a) Macro elements b) Micro elements c) Iron source

Macro elements

All those elements which are taken by plant in larger quantity are termed as macro elements. These elements are required in huge amount for plant growth. These include N, P, K Mg, Ca and S. Usually macro elements make at least 0.1 percent of the dry mass of plant.

Table 1. Macroelements Used in MS medium.

Essential Element	Concentration in 20X Stock solution (mg/L)	Concentration in medium (mg/L)
NH_4NO_3	33000	1650
KNO_3	38000	1900
$CaCl.2H_2O$	8800	440
KH_2PO_4	7400	370
$MgSO_4.7H_2O$	3400	170

Microelementss

The elements needed in traces for the growth & development of plant is called as microelements. They have various functions. These comprise of Mn, I, Cu, Co, Bo, Mb, Fe and Zn. Some formulations may contain Ni & Al.

Table 2. Microelements Used in MS medium.

Micro-elements	Concentration in 20X Stock solution (mg/L)	Concentration in medium (mg/L)
KI	166	0.830
H_3BO_3	1240	6.200
$MnSO_4.4H_2O$	4460	22.300
$ZnSO_4.7H_2O$	1720	8.600
$Na_2MoO_2.4H_2O$	50	0.25
$CuSO_4.5H_2O$	5	0.025
$CoCl_2.6H_2O$	5	0.025

Iron source

Iron is combined in the form of iron sulphate in conjunction with Elthylene-diamine-tetra acetic acid. Iron citrate might also be used. Iron-Ethylenediaminetetraacetic acid is usually used in juxtaposition with the Iron sulphate. The Fe (EDTA) intricate permits the sluggish and uninterrupted discharge of iron into the medium. Free iron is precipitated out as ferric oxide in the medium.

Table 3. Macroelements Used in MS medium.

Iron Source	Concentration in 20X Stock solution (mg/L)	Concentration in medium (mg/L)
$FeSO_4.7H_2O$	5560	27.8
$Na_2EDTA.2H_2O$	7460	37.3

Organic supplements

C-source

One of the most conveniently available, inexpensive, quickly incorporated and pretty steady so is usually used as carbon source. Glucose, maltose, galactose and sorbitol are also used.

Table 4. Carbon Source Used in MS medium.

Carbon Source	Concentration in 20X Stock solution (mg/L)	Concentration in medium (mg/L)
Sucrose	Mixed as a solid	30,000

Phyto hormones

Phyto hormones are one of the significant media ingredients that determine the developmental alleyway of the plant cells. Usually plant hormones or their synthetic analogues are used.

Preparation of growth hormone stock solution	
Compound	Stock preparation
2,4-dichlorophenoxy acetic acid	Dissolve 2mg/L in 10% Ethanol make volume with distilled water.

Table 5. Various Combinations of Phytohormones Used in MS medium.

MS Media 1	NAA 0.01mg/L, Thiamin HCL 0.4mg/L, 2,4-D 1.2mg/L, BAP 1.2 mg/L
MS Media 2	NAA 0.01mg/L, Thiamin HCL 0.4mg/L, 2,4-D 2mg/L, BAP 1.2mg/L
MS Media 3	NAA 0.3mg/L, Thiamin HCL 0.4mg/L, 2,4-D 1mg/L
MS Media 4	NAA 0.01mg/L, Thiamin HCL 0.4mg/L, 2,4-D 1.2mg/L
MS Media 5	BAP 0.5 mg/L, IBA 0.5mg/L, NAA 0.2mg/L.

Media Preparation

Stock solutions using macro, micro and vitamins and Fe (EDTA) were prepared, then appropriate concentrations were taken in 1litre beaker, and other contents like glucose, EDTA, vitamin B5 were also added in that beaker and all were dissolved on the magnetic stirrer, pH was maintained up to 5.8, after adding 1.6 % solidifying agent technical agar to prepare the MS1 medium. Different combinations of phytohormones were prepared to optimize callus cultures. MS3 media gave the desired results.

Sterilization of the explant

Fresh leaflets were separated from fully grown mature plant and were sterilized in 50% bleach (with 5% hypo chloride). Then seeds were washed with sterilized distilled water for three times. Process of sterilization was carried out in laminar airflow in aseptic conditions.

Inoculation of explant on media

After sterilization of the media, it was allowed to cool down (up to 50°C) at RT & transferred in glass bottles, then the leaf disks were inoculated on the medium, media for regeneration was remain in flasks and the leaf disks were directly inoculated in the glass bottles. Then these bottles were incubated in growth chamber at 28± 2°C for the formation of callus.

Sample preparation from callus

After the formation of callus, the sample was prepared from fresh callus using ethanol. Because of the versatility and miscibility of ethanol with water and most of the organic solvents it is used to prepare samples (Franco *et al.,* 2008, Lide. 2000). Ethanol's solvent polarity effects qualitatively as well as quantitatively on the antioxidants of plant extracts.

It is known to give huge quantity of polyphenols from plant extracts (Weinhold *et al.*,1974).

The sample and solvent were mixed on magnetic stirrer for three hours. After the determination of weight, the solvent was added followed by filtration of aqueous extract by using Whatman filter paper of grade number four. The mixing and filtration process was repeated for three times until the extract was completely extracted. The aqueous part was separated by distillation at 78°C from solvent which was stored later at 4°C for future use. Antioxidant's profiling and biochemical analysis were carried out then.

Total Contents of Phenolics

Total phenolics were checked by FCR method of Singleton *et al* (1965). Took 0.1ml sample, mixed with 7.9 ml distilled H_2O, 0.5 ml FCR, and 20% $NaCO_3$. Distilled H_2O was used as blank probe. Absorption was taken after 2 hours at 20°C & 765nm on Spectro-UV-Vis Double PC spectrophotometer, LaboMed, USA 11DV-60Hz or 220V-50Hz Seriol Number 001151. Standard was prepared using Galic acid.

Total Flavonoids Contents

Total Flavonoids were calculated by colorimetric assay of aluminium chloride. 1ml sample was taken & added 5% $NaNO_2$ in the quantity of 0.3ml. Five minutes later, 0.3 ml of 10% $AlCl_3$ was put in it. Six minutes later, 2 milliliter of 1 molar NaOH were mixed & overall amount was raised up to 10 ml using distilled H_2O. After thorough mixing the absorbance was noted in comparison to blank at 510nm. Standard was prepared using quercetin.

Power to reduce

The power to reduce of ethanol extracts of plants holds was checked according to the methodology of Oyaizu (1986). Took 0.5ml sample solution, 2.5 ml P buffer (6.6pH) & 2.5 ml 1% potassium ferricyanide. Kept in water bath at fifty degree centigrade for twenty minutes. After that 2.5ml of 10 percent T.C.A was mixed and spinned at 1000g for ten minutes. Upper layer (2.5ml) was combined with 2.5 milliliter of distilled H_2O & 0.5 milliliter of 0.1 percent of $FeCl_3$. Read absorbance at 700 nm wavelength.

Evaluation of overall Antioxidant's activity

Sum antioxidant's activity was measured by modifying phosphomolybdate method given by Prieto *et al.,* 1999 using α-tocopherol as a standard. A mixture of 0.4ml of plant extracts was pooled with 4milliliter of reagent which contained 0.6 molar H_2SO_4, 28 millimolar $NaH_2PO_4.H_2O$ & 4 millimolar of $(NH_4)_6$ MoO_{24} $4H_2O$. Covered test tubes & kept in boiling H_2O bath at the temperature of 95˚C for 90minutes. Then samples were made to cool at RT & reading was noted at 695nanometer in comparison to blank solution primed in identical situation by substituting the sample with 0.1ml methanol. Total antioxidants activity articulated as µg correspondent of α-tocopherol.

Estimation of total proteins contents (mg/g)

Total proteins contents were estimated by using the method reported by Lowry *et al.*, (1951). According this method 0.5ml of test solution was taken and 2.5ml alkaline copper reagent, shaken thoroughly and allowed to cool down at RT for ten minutes, then 0.25ml of dilute Follin Ciocalteu's reagent (1:1v/v with water) was added. After thirty minutes absorbance was taken in comparison to blank at 750nm on spectrophotometer, blank contained all reagents and water instead of sample. Standard was made using albumin.

Estimation of total carbohydrates (mg/g)

It was estimated by the method reported by Montgomery (1961) for this 0.5ml of test solution, 2.5 concentrated sulphuric acid and 0.05ml 80% phenol (80.0g phenol in 100ml distilled water) solution was taken in test tube. And then reaction mixtures were combined & put at RT for 15 minutes. Finally, absorbance was taken against the blank on spectrophotometer at 485nm. For the preparation of blank distilled H_2O was taken at the place of sample solution. And concentration of reducing sugar was calculated from the standard graph that was prepared in same manner as test sample by using different concentration of the glucose.

Estimation of reducing sugar (mg/g)

Reducing sugar contents were determined by the method reported by Miller (1959).

According to this method, 2.0ml (0.2ml sample + 1.8 Distilled H_2O) of test solution was mixed with 2.0ml of dinitrosalicylic acid in test tube. In boiling water bath the mixture was heated for five minutes. The tubes were made to cool in tap water & color intensity was read against blank at 540nm. Distilled H_2O at the place of test sample was taken for the preparation of blank. And the concentration of reducing sugar was calculated from the standard graph that was prepared in same manner as test sample by using different concentration of the glucose.

Results and discussion

Biochemical analysis

Seeds have great regenerative capacity and are beneficially used in mutagenesis. For both seed and vegetatively propagated species, in vitro techniques can be used. Breeding program is speeded up when tissue culture techniques combines with a mutagenesis treatment. With the given graph it can be easily witnessed that total proteins did not increased in treated plant's callus cultures as compared to the untreated control plant's callus. With each given treatment of EMS, it was decreased to certain level. In present work reducing sugars were seen to be somewhat increased in each treated plant's callus as compared to the control. Likewise, total sugars also elevated in all treated plants callus in contrast to untreated control plant's callus. It is reported with some evidences that not all species in tissue culture are capable of metabolizing sodium azide (Dotson 1986, Wang *et al.*, 1987) which was effective mutant in barley, pea and rice (IATA 1977, Owais *et al.,* 1983, Kleinhofs *et al.,* 1978, Gleason, *et al.,* 1974, Power 1987) but is barely operative in Arabidopsis (Witrezens *et al.,* 1988).

Antioxidants' Profiling

Total Phenolics

The first effort to begin sunflower callus that would discriminate into whole plants were made in 1974 by Gleason, D. F. *et al.,* 1974. The earliest report of positive plant regeneration from sunflower callus was by reported by Sadhu. The solicitation of biotechnological approaches for taming the physiognomies of sunflower is restricted chiefly by the difficulty of regenerating plants in a reproducible and well-organized way. Regenerability of Sunflower by organogenesis is significantly capricious and hinge on genotype, particular media constituents and the nature of the explant (Power 1987, Witrzens *et al.,* 1988, Espinasse *et al.,* 1989, Chraibi *et al.*, 1992, Dhaka *et al.*, 2002, Berrios *et al.*, 1999, Berrios *et al.*, 1999).

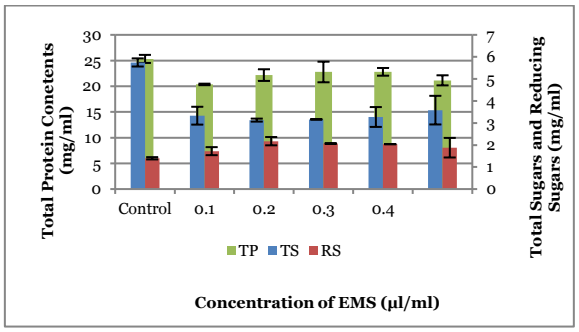

Fig. 1. Effects of different concentrations of EMS on total proteins, total sugars and reducing sugars in callus of sunflower.

The results of given treatment show random results. As compared to control plants all the subsequent treated plants showed reduced phenolic compounds. Among treated plants the highest phenolics compounds were found in plant treated with 0.2% EMS, but it was still lower than the control plant.

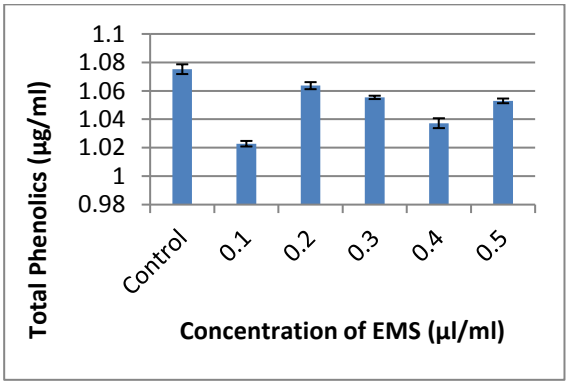

Fig. 2. Effects of different concentrations of EMS on total phenolics (µg/ml) in callus of sunflower.

Total flavonoids

Plant in vitro cultures are well-known to produce and mount up sundry medicinally valuable secondary metabolites (Matkowski 2008). Cultured plant cells produce, accrue and sometimes emanate numerous classes of metabolites. Medicinal complexes are of certain interest and extensive steps has been fervent

to obtaining some of the utmost dynamic and exquisite therapeutics. Several alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been reported from in vitro cultures and reviewed several times have been reviewed (Mulabagal *et al.,* 2004).

In present study the flavonoids contents were reduced after treatment. As compared to control plants the flavonoids contents were reduced in all treated plants.

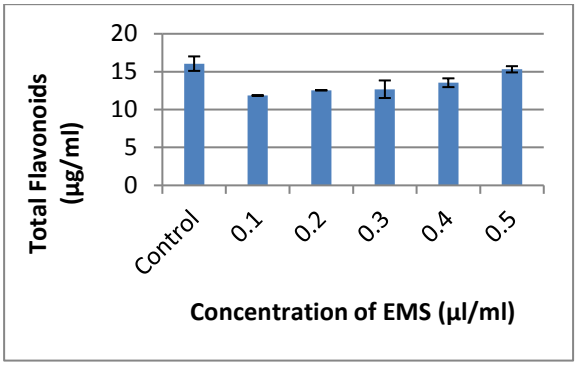

Fig. 3. Effects of different concentrations of EMS on total flavonoids (µg/ml) in callus of sunflower.

Reducing Power

The reducing power of plants treated with EMS does not show any noticeable change. There was slight increment witnessed in plant treated with the 0.1% EMS, where as in consequent treated plants it reduced in comparison to controls.

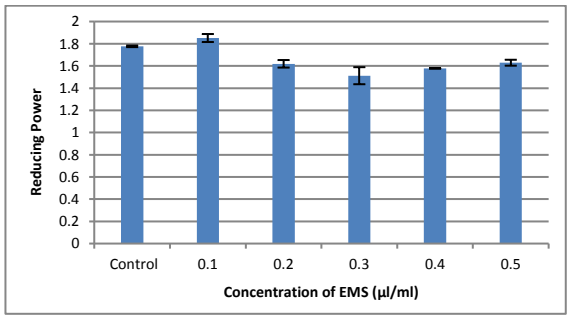

Fig. 4. Effects of different concentrations of EMS on reducing power in callus of sunflower.

Total Antioxidants Activity

Numerous methods have been used to define the antioxidant activity *in vitro* in order to permit quick screening of constituents since constituents that have low antioxidant activity *in vitro*, will possibly display diminutive activity *in vivo* (Tadhani *et al.,* 2007).

In present study the antioxidants activity was decreased after treatment with EMS. The highest antioxidants activity was found to be in control as compare to the treated plants. With the increasing percentage of EMS, the antioxidant potential moved to declivity.

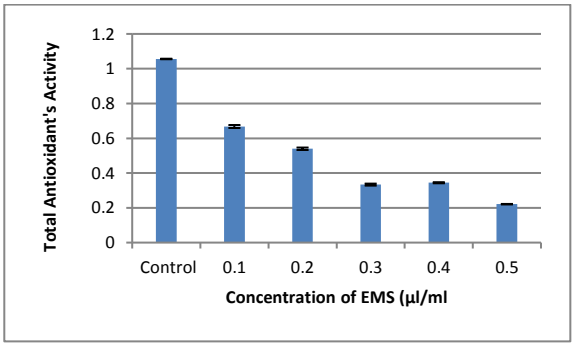

Fig. 5. Effects of different concentrations of EMS on antioxidant's activity (mg/ml) in callus of sunflower.

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