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Response of plant secondary metabolites to mutagenesis in callus cultures of Sunflower plant

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Abstract

Plant in vitro cultures are able to produce and accumulate many medicinally valuable secondary metabolites. Antioxidants are an important group of medicinal preventive compounds as well as being food additives inhibiting detrimental changes of easily oxidizable nutrients. Many different in vitro approaches have been used for increased biosynthesis and the accumulation of antioxidant compounds in plant cells. In the present piece of work some mutagenesis strategies are used to manipulate plant secondary metabolites by means of plant tissue culture. The strategies used for improving the antioxidants in vitro production efficiency are also highlighted via media optimization. This artefact presents results of widespread study of cultivation of plant cells in-vitro. In current research callus cultures were used to screen the upshot of mutagenic amalgams on the plant secondary metabolites. Sunflower plant was mutagenized using mutagen namely Sodium Azide. Callus cultures were sustained using several amalgamations of phytohormones. Callus were attained by means of phytohormones NAA, Thiamin HCL, 2,4-D. At the end screening of primary and secondary metabolites was carried out.

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Introduction

The practice of plant cell and tissue culture methodology for the purpose of producing medicinal metabolites has a extended antiquity (Rout *et al.*, 2000 and Verpoorte *et al.*, 2002). Ever since plant cell and tissue culture arose as a chastisement inside plant biology, scientists have strived to employ plant cell biosynthetic proficiencies for attaining worthwhile products and for reviewing the metabolism (Misawa 1994 and Verpoorte *et al.*, 2002). Cultured plant cells manufacture, amass and sometimes emanate many classes of metabolites. Medicinal compounds are of specific attention and much exertion has been dedicated to gaining some of the most dynamic and valuable therapeutics. Abundant alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been stated from in vitro cultures and appraised numerous times (Misawa 1994, Verpoorte *et al.*, 2002 and Vanisree and Tsay, 2004).

The contemporary paper abridges the consumptions of plant cell and tissue culture technology for the fabrication of secondary metabolites which have significant potential as antioxidants. The induced mutagenesis techniques has been used on antioxidants and the tactics used to increase the in vitro approaches for generating these compounds. The indispensable circumstances that mark using biotechnological approaches for the production of secondary plant metabolites economically sustainable have been determinedly recognized: great commercial worth, inadequate profusion in integral plants, inadequate obtainability from natural sources, and challenging agronomy (Misawa 1994 and Verpoorte *et al.*, 2002). When taking these features into contemplation, in vitro technology deals approximately or all of the succeeding paybacks: simpler abstraction and refining from snooping atmospheres, innovative yields not present in nature, unconventionality from fluctuating weathers, and last but not least, the manipulation of the genetic engineering prospective for eluding lawful limitations against GMO introduction into the natural environment.

Exploration on mutation induction for plant breeding reached its peak a few spans ago, then slackened (Van Harten, 1998; Donini and Sonnino, 1998), after having provided a substantial number of studies on mutagen effects, mutation induction methods, improved traits and their heritability. Contemporary scientific and technical advances can presently provide mutation induction with new promises to subsidize to plant improvement. Spontaneous and induced mutations have previously played an imperative role also in the development of numerous cultivars. Mutations are defined as heritable changes in the DNA sequence that are not derived from genetic segregation or recombination (Van Harten, 1998). Uptill now much work has been done on mutation induction and plant tissue culture separately. The present work focusses on induction of mutation and observation of all its possible effects on plant secondary metabolites.

Materials and methods

The seeds of Sunflower germlines having accessions numbers 017599, 017600, 017601, 017602, 017603, 017604, 017605, 017606, 017607 and 017608 were acquired from Seed Preservation Laboratory & National Gene bank, Plant Genetic Resources Institute, NARC, Islamabad and were given mutagenic treatments mutagen namely sodium azide (SA). Various concentrations of 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 achieved within the period of ninety days and was headed to the preparation to plant tissue culture technique.

Aseptic culture media preparation

Aseptic or sterile culture is obligatory and chief prerequisite of plant tissue culture. Diverse media are usually used for tissue culture of plants among which MS medium remains at top (Murashige *et al.,* 1962). In the current experiment, this MS medium was preferred. MS media is composed of three basic components namely macro elements, which are taken by plant in higher quantities for their growth. Examples include N, P, K Mg, Ca and S. Typically macro elements make at least 0.1 percent of the dry mass of plant.

The second component is of micro elements which are needed in traces for the growth & development of plant. They have innumerable functions. These encompass of Mn, I, Cu, Co, Bo, Mb, Fe and Zn. Some formulations may comprise Ni & Al. Third indispensable component for plant growth is Iron source which is joined in the form of iron sulphate in combination with EDTA. Iron citrate is also sometimes used. Iron-Ethylenediaminet etraacetic acid is frequently used in association with the Iron sulphate. The Fe (EDTA) intricate allows the slow-moving and unremitting liberation of iron into the medium. Free iron is precipitated out as ferric oxide in the medium. Apart from these organic supplements were also used such as carbon source. One of the most accessibly obtainable, low-cost, quickly assimilated and pretty sturdy so is usually used as carbon source. Glucose, maltose, galactose and sorbitol are also used. Phyto hormones are one of the substantial media constituents that control the evolving passageway of the plant cells. Generally, plant hormones or their synthetic equivalents are used.

Table 1. 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.

Preparation and Optimization of Media

Stock solutions using macro, micro and vitamins and Fe (EDTA) were prepared, then suitable quantities were taken in 1litre beaker, and other substances like glucose, EDTA, vitamin B5 were also added in that beaker and all were dissolved on the magnetic stirrer. Before the addition of 1.6% solidifying agent technical agar, pH was maintained up to 5.8. Different amalgamations of phytohormones were primed to optimize callus cultures. MS3 media gave the desired results.

Explant Sterilization

When plant was fully grown and matured, its fresh leaves were separated and were made to sterilize in 50% bleach (with 5% hypo chloride), followed by three times washing with sterilized distilled water out in laminar airflow under highly aseptic conditions.

Explant Inoculation

After sterilization of the media, it was allowed to cool down (up to 50°C) at RT & shifted 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

Once the callus was obtained, the sample was made from it using ethanol as an organic solvent. Because of the flexibility and changeability of ethanol with water and furthermost of the organic solvents it is used to formulate 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 amount of polyphenols from plant abstracts (Weinhold *et al.,* 1974).

For three hours sample and solvent were mixed using magnetic stirrer. The solvent was added after the determination of weight which was then followed by filtration of aqueous extract by using Whatman filter paper of grade number four. Until the extracts was completely extracted from mixture, the mixing and filtration process was repeated for three times. 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). Standard was prepared using Galic acid.

Total Flavonoids Contents

Total Flavonoids were calculated by colorimetric assay of aluminium chloride. 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).

Total 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. 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). Standard was made using albumin.

Estimation of total carbohydrates (mg/g)

It was estimated by the method reported by Montgomery (1961). 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 sugars were determined by the method reported by Miller (1959).

Results and discussion

Biochemical analysis

In mutagenesis seeds are frequently used because of their capability to regenerate. In-vitro techniques can be used for both seed and vegetatively propagated varieties. When mutagenesis treatment collaborates with plant tissue culture technique, the methods for breeding programs are boosted up and plants primary as well secondary metabolites are enhanced. In present investigation, highest total proteins were noted in the callus of plant treated with 0.2% sodium azide which was higher than the control plant's callus. At each given concentration, the total proteins were augmented as compared to control which had the lowest protein contents. Reducing sugars showed some fluctuating results. Highest were seen in the callus of plant treated with 0.1% sodium azide. Whereas it showed almost equal amounts in the callus of 0.3% and 0.4% treated plant, 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).

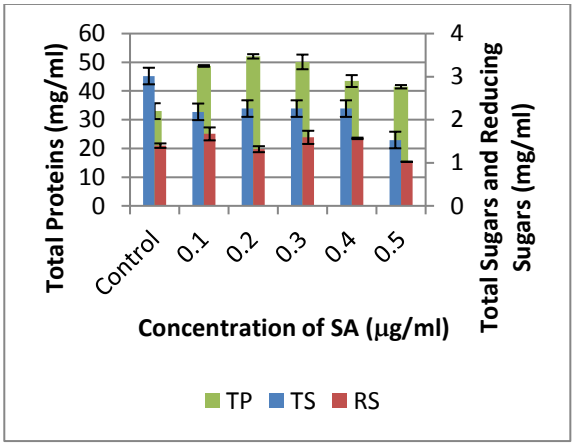

Fig. 1. Effects of different concentrations of SA on total proteins, total sugars and reducing sugars in callus of sunflower.

Antioxidants' Profiling

Total Phenolics

The first effort to begin sunflower callus that would discriminate into whole plants were made in 1974 by Gleason *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).

The methanol abstract unveiled the uppermost total phenolics content, whereas the contents gained with remaining aqueous portion were much lesser that is in agreement with other reports *(C. Ao et al., 2008)*. The total phenolics compounds increased after respective treatments as compared to control plants. The highest phenolics compounds were noted in plant treated with 0.5% SA, which was highest among all plants. Phenolic compounds of plants are also very essential for their hydroxyl groups' converse rummaging capacity (*X. P. Nunes et al., 2012)* . It is also reported previously that not all species in tissue culture are capable to metabolize sodium azide to the mutagenic agent (Dotson 1986, Wang *et al.*, 1987) which is powerful mutant in barley, pea and rice (Awan *et al.,* 1980, Prina *et al.,* 1983) but is scarcely operational in Arabidopsis (Gichner *et al.*, 1994).

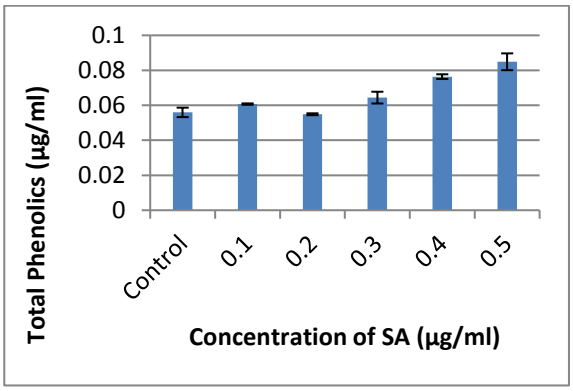

Fig. 2. Effects of different concentrations of SA on total phenolics (µg/ml) in callus of sunflower.

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 (Misawa 1994, Mulabagal *et al.*, 2004).

The results found after sodium azide treatment were quite interesting. The flavonoids contents were enhanced after treatment. As compared to control plants the flavonoids contents were found to be higher in plant treated with 0.5% SA. Cakir *et al.,* in 2003 also reported that plant extracts having rich flavonoids exhibit higher antioxidant's activity.

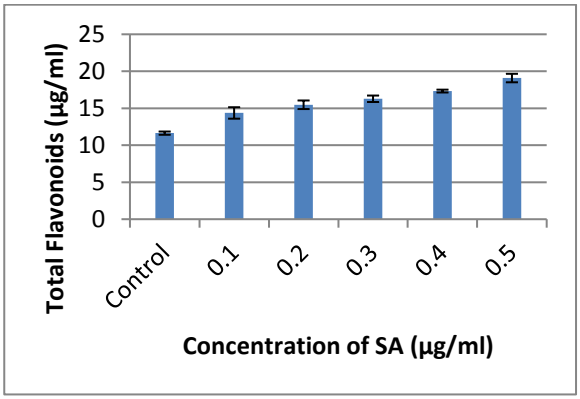

Fig. 3. Effects of different concentrations of SA on total flavonoids (µg/ml) in callus of sunflower.

Reducing Power

The reducing power of plants treated with SA show elevated effect. In comparison to control plant the highest reducing power was noticed in plant treated with 0.5% which was much higher than the control plant.

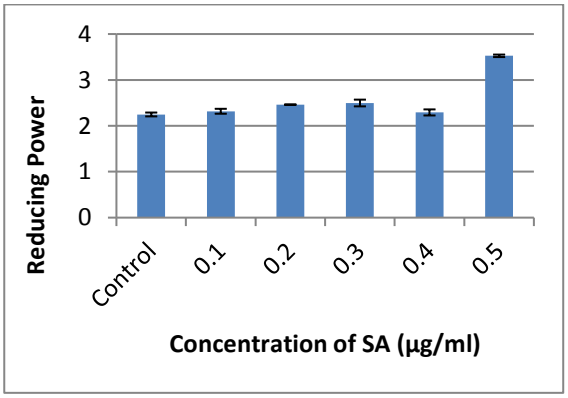

Fig. 4. Effects of different concentrations of SA on reducing power in callus of sunflower.

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 2007)*. In herbal sweetener Stevia rebaudiana callus extracted with water and methanol had greater antioxidant activity than the leaves of field-grown plants, which was allied

with the greater levels of flavonoids and total polyphenols. In current analysis compared to control plants the treated ones showed varied results. Highest antioxidants activity was found to be in the plant treated with 0.1% and 0.3% SA.

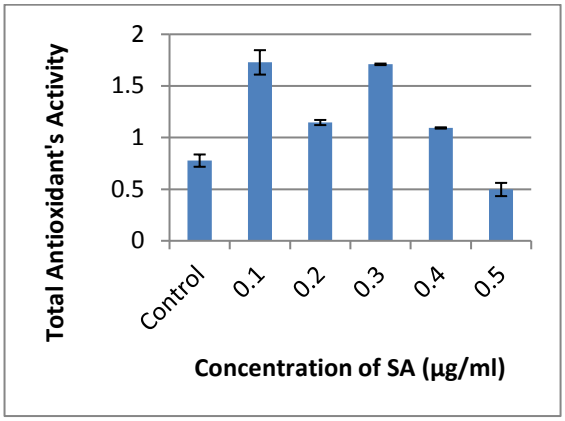

Fig. 5. Effects of different concentrations of SA on antioxidant's activity (mg/ml) in callus of sunflower.

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