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Full Length Research Paper

Antioxidant activity influenced by *in vivo* and *in vitro* mutagenesis in sugarcane (*Saccharum officinarum* L.)

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The antioxidant potential (1,1-diphenyl-2-picrylhydrazyl (DPPHº)-scavenging activity) of *in vitro* regenerated and induced mutant sugarcane (*Saccharum officinarum* L.) was investigated. Efficient callus induction and shoot regeneration were induced in bud explants when incubated on Murashige and Skoog (MS) medium supplemented with different plant growth regulators (PGRs). Best callogenesis was observed on MS-medium supplemented with 3 mg L^{-1} 2,4 dichlorophenoxyacetic acid (2,4 D) and on ½ MS medium with 2 mg L^{-1} 2,4 D after 30-days of culture. Almost 85% shoot organogenesis was observed on MS-medium supplemented with 2 mg L^{-1} 6-benzyladenine (BA) and 0.5 mg L^{-1} gibberellic acid (GA_3) within 30 days. Optimum percentage rooting (89%), were obtained for 2 mg L^{-1} of BA alone. Mother plant setts were irradiated with ^{60}Co mutagen source. Assay of antioxidant activity of *in vitro* and *in vivo* grown tissues was evaluated as gross parameter of medicinal efficacy. Significantly higher antioxidant activity (60%) in *in vitro* regenerated sugarcane was observed as compared to induced mutant (57%) and mother plant (53%).

Key words: *Saccharum officinarum*, *in vitro* regeneration, induced mutation, antioxidant.

INTRODUCTION

Sugarcane is an important food crop cultivated in about 74 countries of the tropic and sub tropical regions (Anon, 1998). Sugarcane, popularly known as noble cane, due to its high sucrose content and low fiber content is one of the important industrial crops in the world. Besides sugar production, both the roots and stems of sugarcane are used in Ayurvedic medicine to treat skin and urinary tract infections, as well as for bronchitis, heart conditions, loss of milk production, cough, anemia, constipation as well as general debility. Some reports advise its use for jaundice and low blood pressure (Kadam et al., 2007).

The active ingredients of a plant are mainly its secondary metabolites, among which the phenolics are very important with respect to their antioxidant activity (Khanavi et al., 2009; Huda-Faujan et al., 2009). These ingredients are naturally produced during a plant's growth metabolic process, in the active substances with antioxidant function such as scavenging reactive oxygen species (ROS), free radicals (hydroxyl radicals, ·OH and superoxide anion radicals, $\cdot O^{2-}$) or non-free radical ROS (peroxide, H_2O_2) production from body metabolism (Ramarathnam et al., 1995). Studies of natural anti-oxidants in plants have become a major area of scientific research (Demo et al., 1998; Sanchez-Moreno et al., 1999). Natural antioxidants are known to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory activities. In fact, a fundamental property important for life is antioxidant activity and this property may give rise to anti-carcinogenicity, anti-mutagenicity and anti-aging activity, among others (Cook and Samman, 1996; Liyana-Pathirana and Shahidi, 2006). In this study, *in vitro* system for production of *Saccharum officinarum* L. was established from bud explants of mother plants and it induced mutation through ^{60}Co

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Table 1. Approximate characteristics of *in vitro* regenerated induced mutant and mother plant of *S. officinarum*.

Tissue	Brix	Thickness	Height	Stalk	Node	Internodes	Internodes length	Color
In vitro regenerated plant	20	14.6	149	11	22	23	7	Red
Induced mutant plant	18.3	17	88	5	15	14	5.4	Red
Mother plant	19.8	19	198	5	24	23	10	Green

Values are means of 9 replicates.

gamma irradiation. Furthermore, 1,1-diphenyl-2-picryl-hydrazyl (DPPHº)-based antioxidant assays were conducted to evaluate antioxidant activity of the main secondary metabolites in different *in vitro* derived and *in vivo* plant tissues. The objective of this study was to investigate the antioxidant activity of *in vitro* regenerated, induced mutant and its parent.

MATERIALS AND METHODS

In vitro regeneration

Lateral buds were collected from mother plants of *S. officinarum* maintained inside the field at NIFA, Peshawar, Pakistan. Explants were sterilized according to the method of Abbasi et al. (2010a). Briefly, buds were immersed in 70% (v/v) ethanol for 60 s, 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for ~1 min, and rinsed three times with sterile distilled water. These surface sterilized explants were placed on Murashige and Skoog (1962) medium containing 30 g l^{-1} sucrose, and solidified with 8 g l^{-1} agar (Agar Technical LP0013, Oxoid, Hampshire, England). Different plant growth regulators (PGRs) including 2,4-D, BA, GA_3, IBA, IAA and NAA alone and in combination were used for percent callus induction, percent shooting and percent rooting of *S. officinarum*. The pH of the medium was adjusted to 5.6 to 5.8. All media were autoclaved at 121°C for 20 min. All cultures were maintained in a growth room at temperature of 25 ± 1°C under a 16 h photoperiod with a light intensity range of ~40 to 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent tube lights.

Mutation induction

The calli and 2 to 3 setts of mother plant was irradiated by gamma rays using ^{60}Co source with different doses and transferred to MS regeneration medium for further growth. The buds containing setts were directly sown in field. After one month of cultivation, these green buds were examined for different growth responses. Surviving shoots were raised to maturity. A huge population of callus derived plants after acclimatization was grown in the field under regular spacing of 35 cm between plants in rows with 1 m between each row in field. First, M_1V_1 populations were raised. The $M_1 V_2$ population raised from plants of $M_1 V_1$ was transplanted to the field in 1 m spacing. About 100 plants from each treatment along with their control were planted. The data on plant height, nodal length and stem diameter was recorded. One plant was selected for antioxidant activity with change in morphological characteristics (Table 1). Similarly, setts containing 2 to 3 buds/setts of mother plant were irradiated for induced mutagenesis through ^{60}Co source with different doses of gamma rays (Figure 6). The irradiated mother plant setts were grown in the field as M_1 generation. After harvesting the M_1 generation, M_2 generation was raised. Out of 100 M_2 plants, one plant was selected for antioxidant activity after full maturity.

Extract preparation

Juice from *in vitro* regenerated plant, induced mutant and mother plant was extracted by juice extractor. Ethanol extract of the three plants was obtained by taking 10 g of juice of each plant in separate containers. With this, 50 ml of ethanol was added and kept for 1 week with periodic stirring after every 18 h using a sterilized glass rod, filtered and the filtrate was collected. This procedure was repeated three times with fresh volume of ethanol. The filtrates were pooled. The final extracts were passed through Whatman filter paper No. 1 (Whatman Ltd., England). The pooled ethanolic extracts were concentrated separately by rotary vacuum evaporator at 40°C till dryness and stored at 4°C in air tight bottle. Extracts (10 mg) obtained from each plant were dissolved in 20 ml ethanol independently to get stock solutions.

Antioxidant activity

The antioxidant (free radical scavenging) activity of *in vitro* regenerated, induced mutant and mother plant was measured in terms of hydrogen donating or radical scavenging ability using the stable radical (1,1-diphenyl-2-picrylhydrazyl (DPPH)). The test extracts were prepared in ethanol therefore the DPPH was also prepared in ethanol. DPPH (3.96 mg) was dissolved in 20 ml of ethanol to get stock solution. With 0.5 ml volume of each sample solution, 1 ml of DPPH solution was added separately. These solution mixtures were incubated in the dark for 30 min at room temperature. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All tests were carried out in triplicate. Finally the radical scavenging activity was calculated as percentage of DPPH discoloration using the formula:

Scavenging DPPH free radical (%) = 100 × (1-AE/AD)

Where AE is absorbance of the solution mixed with DPPH and AD is the absorbance of the DPPH solution (blank, without extract).

RESULTS AND DISCUSSION

The effects of various PGRs such as BA, GA_3, 2,4-D alone or BA in combination with 0.5 to 1.0 mg L^{-1} GA_3 on indirect organogenesis were evaluated. The bud explants of *S. officinarum* used in the present study responded to all PGRs used (Figure 1). Best callus induction was recorded on MS medium supplemented with 3.0 mg L^{-1} 2,4-D (85%; Figure 1). Callus induction recorded for 1 and 4 mg L^{-1} 2,4-D were 56 and 60%,

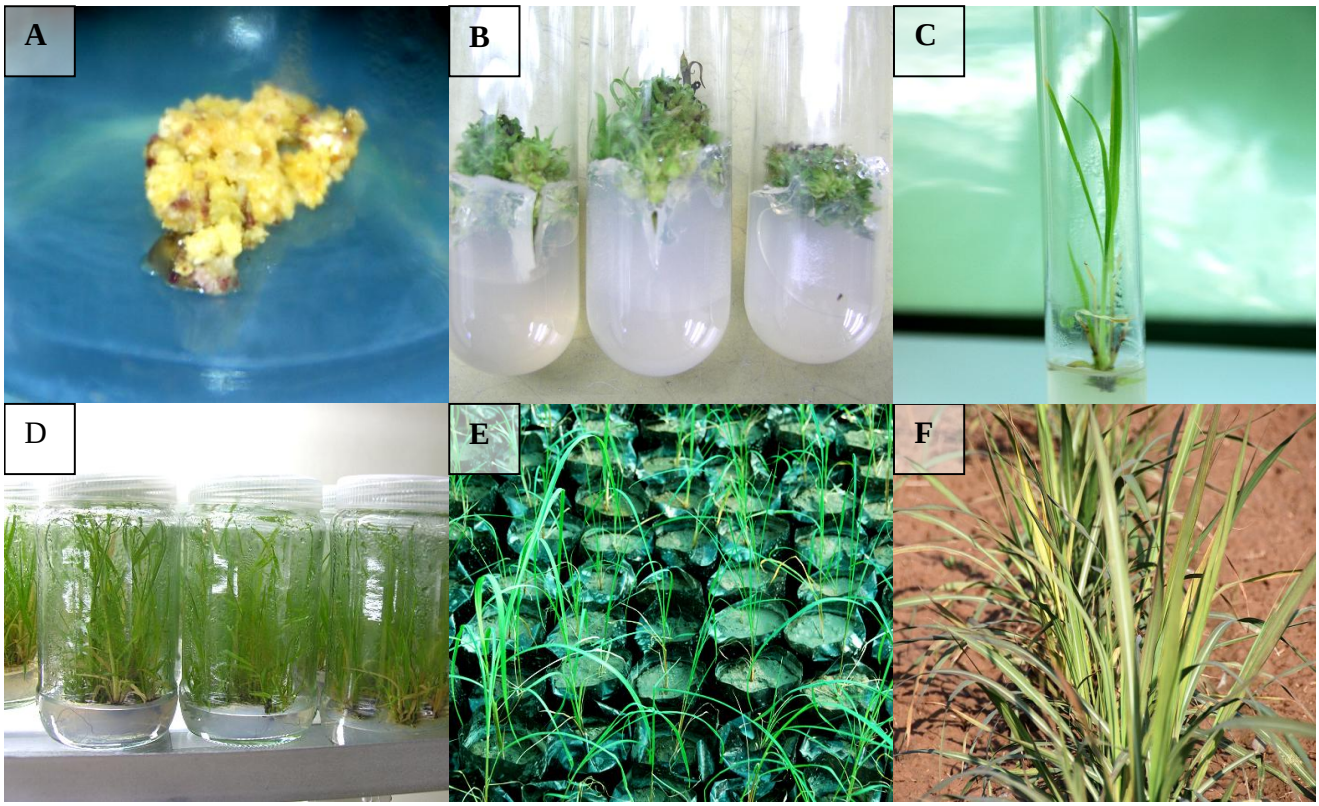

Figure 1. Plant regeneration in *S. officinarum* L. (A) Callus; (B) regenerated shoots (C); shoot elongation; (D) rooting (E); acclimatization and regenerated plantlets; (F) field transfer. C for callus, S for shoots and R for roots.

whereas 70% callus was observed on 2 mg L^{-1} 2,4-D, respectively. 1 mg L^{-1} 2,4-D (56%) was significantly lower than other PGRs, and no callus was observed on MS0 medium. However, addition of BA to the medium containing 2,4-D reduced callus induction (33%). Data on organogenesis was determined after 5 weeks of sub-culture and the best percentage shooting was recorded for combination of 2.0 mg L^{-1} BA and 0.5 mg L^{-1} GA_3 (85%). However, 2.0 mg L^{-1} BA alone produced 92% shooting which was higher than in combination (Figure 2). Khalil et al. (2011) also observed that BA in the medium enhances number of shoot per explant. Contrarily, addition of 2,4-D to medium incorporated with BA inhibited percentage shooting significantly. Similar reports are available on *Capsicum* species (Rubluo and Barroso, 1992). However, in another study on *Silybum*, Abbasi et al. (2010a) made different observations regarding incorporation of auxin in cytokinin containing medium. It was concluded that the overall response of medicinal plant species to BA is positive (Kelkar and Krishnamurthy, 1998; Liu et al., 2003). Shoots grown on shoot organogenesis medium were transferred to MS0 and MS medium incorporated with different concentrations of IBA, IAA, NAA and BA for rooting (Figure 1). Optimum percentage rooting (89%), were obtained for 2 mg L^{-1} of BA. In our experiments, we also obtained healthy rooting on 0.5 mg L^{-1} IAA (87%) and NAA (80%), respectively. Lal and Singh (1999) was also reported *in vitro* morphogenesis from leaf explants of sugarcane. White roots appeared after 15 days of culture which became brown afterwards. Rooted plantlets were successfully transferred to pots for further growth and were maintained in controlled conditions. Potted plants were successfully transferred to field.

The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary disease and antioxidant constituents of plant material are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress. Regenerated plantlets can accumulate secondary metabolites similar to those found in mother plant. Antioxidant potential of regenerated, induced mutant and mother plant of *S. officinarum* was determined by using DPPH^{o}-free radical (Figures 3 to 5). *In vitro* regenerated plant transferred to field had significantly higher antioxidant potential than other plants. Abbasi et al. (2010a) also observed the same results in *Silybum marianum*. Regenerated plant had significantly higher capacity to detoxify DPPH free radicals (60%) than other plants (Figure 3). The antioxidant activity was determined in time dependent manner, the results were taken with consecutive 3 min gap to evaluate and

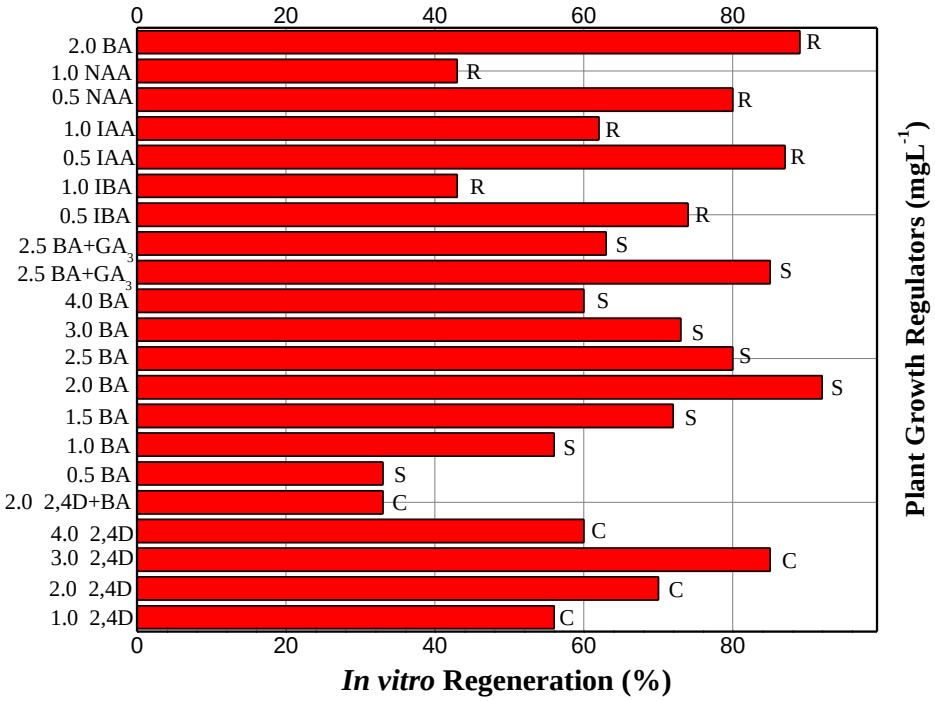

Figure 2. Effects of various concentrations and combinations of 2,4-D, BA, GA_3, IBA,IAA and NAA on percent callus induction, percent shooting and percent rooting of *S. officinarum*. Data were collected after 4 weeks of culture. Values are means of 5 replicates. Columns are not significantly different at P<0.05.

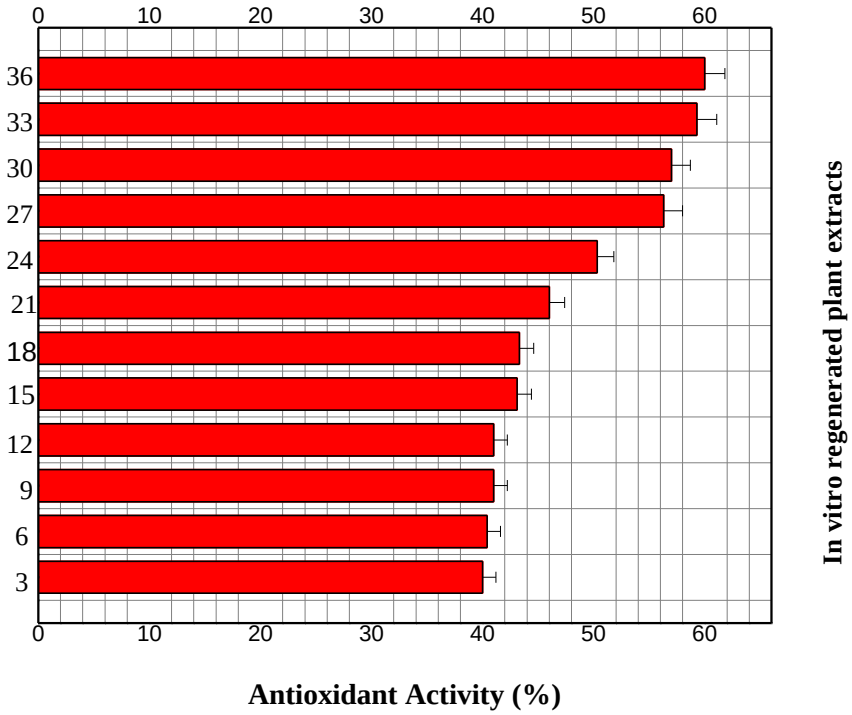

Figure 3. Time dependent antioxidant (free radical scavenging) activity of *in vitro* regenerated plants of *S. officinarum*. Values are means of 3 replicates. Values in each column are not significantly different at p < 0.05.

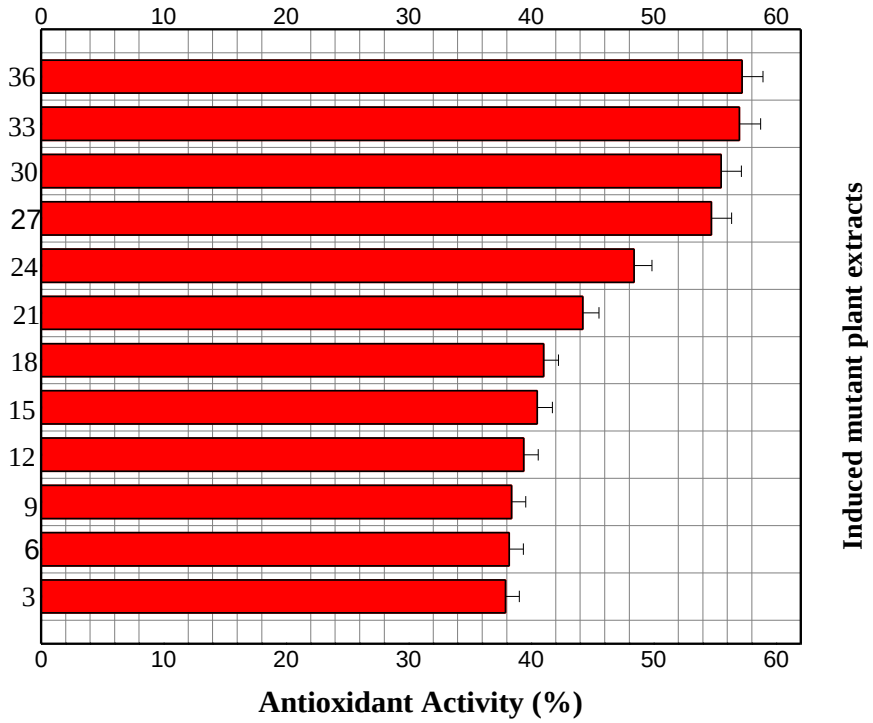

Figure 4. Time dependent antioxidant (free radical scavenging) activity of induced mutant plants of *S. officinarum*. Values are means of 3 replicates. Values in each column are not significantly different at $p < 0.05$.

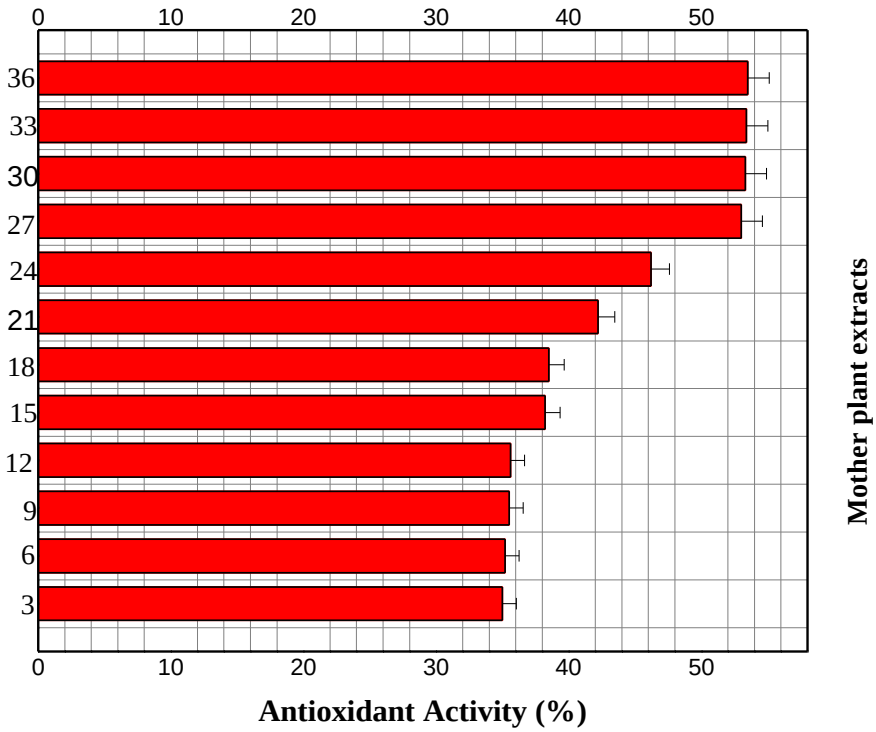

Figure 5. Time dependent antioxidant (free radical scavenging) activity of mother plant of *S. officinarum*. Values are means of 3 replicates. Values in each column are not significantly different at $p < 0.05$.

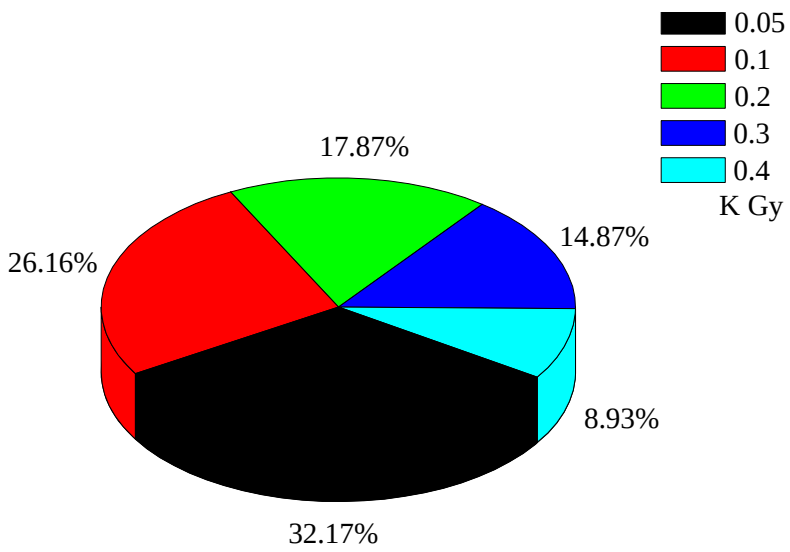

Figure 6. Effect of different doses of gamma rays on the percent germination of setts in mother plant of *S. officinarum*.

compare maximum activity. Ahmad et al. (2010) also observed higher antioxidant activity in regenerated tissues of *Piper nigrum* L. Sugarcane extract displayed a wide range of biological effects including immuno-stimulation (El-Abasy et al., 2002), anti-thrombosis activity, anti-inflammatory activity, vaccine adjuvant, modulation of acetylcholine release (Barocci et al., 1999) and anti-stress effects. Sugarcane juice has broad biological effects in raising innate immunity to infections (Lo et al., 2005).

The present study reports that sugarcane juice has potent antioxidant activity under various experimental conditions. The data was collected after 3 min, it was concluded that at 30 min, the antioxidant activity was high. Significantly lower antioxidant activity was noted in all three plants extract after 3 min, but when the time intervals increased, the plants extract scavenged more free radicals of DPPH. The phenolics compound of sugarcane was also assessed to have antioxidant activity (Duarte-Almeida et al., 2006). Positive linear correlation between antioxidant activity and total phenolic content for alcoholic extracts of *S. officinarum* was reported by Tawaha et al. (2007). Several studies suggested that the phenolics compounds contributed significantly to the antioxidant capacity of the 112 wild grown Chinese herbs (Cai et al., 2004). The present data are consistent with the findings of many research groups who reported such positive correlation between total phenolics content and antioxidant activity (Cai et al., 2004; Zheng and Wang, 2001). When the explants were inoculated on the medium to induce callus production, the medium were supplemented with different chemicals. These chemicals including plant growth regulators slightly change the metabolic pathways of plant. Therefore, during indirect organogenesis, the plant metabolites change which leads to increase in antioxidant activity. Increase antioxidant activity of bound phenolics of sugarcane was also reported (Nayaka et al., 2008).

Out of 100 M_2 plants, one plant was selected for antioxidant activity after full maturity. The antioxidant activity of induced mutant plant juice was checked (Figure 4). Maximum activity was observed after 30 min at 517 nm spectrophotometrically. Maximum activity of 57% was recorded for induced mutant plant which is lower than *in vitro* regenerated plant (60%). However, after 3 min, the activity was 37.9% but as the time interval increased, the activity also increased and highest activity was observed in 30 min because plant extracts scavenge more free radicals. Patade and Suprasanna (2008) also used *in vitro* mutagenesis for sugarcane improvement. Nevertheless, the "DPPH-test" is a commonly employed assay in antioxidant studies and offers a rapid technique to screen the radical scavenging activity (RSA) of pure synthetic compounds, isolated natural compounds, crude plant extracts and foods (Kahkonen et al., 1999). It is also important to note that the DPPH test only recognizes free-radical scavenging effects and not pro-oxidant activity (Iuri Bezerra et al., 2010). Patade and Suprasanna (2009) also showed *in vitro* mutagenesis for salinity and tolerance stress in sugarcane.

As compared to the *in vitro* regenerated and induced mutant sugarcane, parent plant exhibit lower antioxidant

activity of 53% (Figure 5). Activity of 3 min was very low (35%) but as the time increases and detoxify more free radicals, the percent activity reached 53% which is lower than both *in vitro* regenerated and induced mutant plant. Regenerated plantlets can accumulate secondary metabolites similar to those found in mother plants (Shilpa et al., 2010). Damaging effects of irradiation and steam on antioxidant activity of piperine were reported (Waje et al., 2008). Andarwulan and Shetty (1999) found higher antioxidant activity and total phenolic contents in differentiated tissues of *Pimpinella anisum*. This variation in data shows accumulation of different components during different phases of growth and mutation.

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