International Journal Of
Recent Scientific
Research
ISSN: 0976-3031
Volume: 7(6) June -2016

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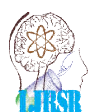

THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 6, pp. 11775-11780, June, 2016

International Journal of Recent Scientific Research

Research Article

OCIMUM SANCTUM AND ITS CALLUS CULTURE – A POTENTIAL SOURCE FOR ANTIOXIDANT ENZYMES

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ARTICLE INFO

Article History:

Received 29th March, 2016
Received in revised form 19th April, 2016
Accepted 25th May, 2016
Published online 28th June, 2016

Key Words:

in vitro; *Ocimum sanctum*; morphotype; antioxidative enzymes.

ABSTRACT

In vitro plant cultures are potential sources for obtaining natural products on larger scale. Callus and cell culture could not only be an alternate continuous source of proteins, but could also be a useful model system to study their regulation and biosynthesis. The leaf derived callus cultures of green and purple morphotype of *O. sanctum,* maintained on MS medium + 2,4-D, NAA and KN and the leaves from their respective mother explants were evaluated for the activity of antioxidant enzymes. The specific activity of APOX, CAT, GPX, GST and SOD was evaluated employing standard biochemical protocols. On comparing the leaves of purple and the green morphotypes of *Ocimum sanctum* Linn. for antioxidative enzymes, the purple morphotype was found to be a rich source. When their leaves were cultured and their callus maintained on MS medium, the expression of these enzymes increased significantly *in vitro* for both the plants. The increase in the activity may be due to abiotic stress faced by the callus under artificial conditions. The *in vitro* raised callus of both the plants show higher expression of APOX, GPX and SOD, while the activity of CAT and GST was reported to be higher in the *in vivo* leaves. To overcome oxidative stress related disorders, *Ocimum sanctum* particularly the purple morphotype can be used as an alternative to the commonly used green morphotype in the herbal drug industry. Besides, the callus cultures of both the plants can be exploited for commercial production of these enzymes on large scale.

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INTRODUCTION

Plants are a vast repository of products also called secondary metabolites that are economically important as medicinal drugs, dyes and pigments, flavor and fragrances, food additives and pesticides (Hussain *et al*. 2012). The evolving commercial importance of these products and their ever increasing demand has aroused interest in alternative methods of production such as tissue culture technology. Plant tissue culture holds great promise for controlled production of myriad of useful secondary metabolites on demand. Also, plant cell and callus cultures can be exploited for antioxidant production, particularly antioxidative enzymes (Agarwal and Patwardhan 1993). With an ever increasing demand in the food and other commercial industries and the safety legislative guidelines, plant derived products are gaining importance worldwide. Literature suggests that calli obtained from different plants such as radish (Yamada *et al*. 1987), cow pea (Oscar *et al.* 1990), citrus (Agrawal and Patwardhan, 1993) and carrot (Kim and Yoo 1996; Xu *et al*. 1998) are possible sources for the production of enzymes particularly peroxidases. Besides, *in vitro* cultures also serve as models for studying the molecular and biochemical responses of plants at cellular level in a uniformly controlled environment.

Ocimum sanctum Linn. commonly called Tulsi or holy basil, is the most common herb used since time immemorial for treatment of plethora of diseases. In nature two distinct morphotypes of *O. sanctum* are observed. The most common morphotype is the green leaved and the other is the purple leaved. The medicinal value of both the morphotypes has been well documented in ancient Indian texts and virtually every part of the plant is used in the traditional, Ayurvedic and Siddha systems of medicine. In Ayurveda, Tulsi is known as the “elixir of life”. The plant is stimulant, diaphoretic and expectorant. A decoction of roots is given in malarial fever. The juice of the leaves is applied to treat ringworm and other cutaneous diseases and is taken in cases of gastric troubles. The leaves are chewed or their infusion is taken in cold, cough and bronchial infections. Seeds are demulcent and given in the disorders of genito-urinary system. The plant is also used in snake bite and scorpion sting. In recent the plant has been studied extensively for its anti-cancerous (Kim *et al*. 2010), immunostimulatory (Nahak and Sahu 2014), anti-diabetic (Dineshkumar *et al*. 2010), neuroprotective (Raghavendra *et al.*

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2009), antimicrobial (Amber *et al*. 2010), immunomodulatory (Mukherjee *et al*. 2005), radioprotective (Bhartiya *et al*. 2006) and anti-inflammatory (Choudhury *et al*. 2014) activities.

Oxygen is the most indispensable element for life. The biochemical reactions taking place in the cells and the organelles are the driving forces that sustain life. When cells use oxygen to generate energy, free radicals are produced by the mitochondria (Koksal *et al*. 2004). Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. The commonly formed free radicals in the body that are of physiological importance are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). These by-products are generally referred to as reactive oxygen species (ROS). For normal physiological processes such as cellular growth, gene expression and defense against infection, a low concentration of ROS is essential. In normal cells, a balance is constantly being maintained between formation and removal of these species. When this balance shifts towards formation of more ROS, oxidative stress occurs. Under such conditions these species are detrimental since they target DNA and RNA molecules, proteins, sugars and lipids (Lu *et al*. 2010; Craft *et al*. 2012). To control the levels of ROS for cell protection under stress conditions, plants produce several enzymes involved in their scavenging such as catalase, ascorbate peroxidase, guaicol peroxidase, superoxide dismutase, glutathione S-transferase etc. (Bartels and Sunkar 2005). The enzyme Catalase plays an important role in the adaptive response of cells to oxidative stress (Abassi *et al*. 1998). Peroxidase decomposes hydrogen peroxide by oxidation of co-substrates and leads to the formation of bioactive phenolic compounds (Mika *et al*. 2004; Mayer 2006). Superoxide dismutase is responsible for converting superoxide ion to hydrogen peroxide and molecular oxygen thus playing important role in defense mechanisms (Harinasut *et al*. 2003).

MATERIALS AND METHODS

Establishment of callus cultures

The leaves of both the morphotypes of *O. sanctum* were used in the present study. For callus induction the leaves were thoroughly washed with tween-20 for 20 min and rinsed with water. On surface sterilization with 0.1% mercuric chloride the explants were placed on medium containing MS salts and vitamins supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid, 30 g/l sucrose and 8 g/l agar for callus induction. Cultures were incubated at 25±2°C and 16 h light and 8 h dark period. The callus thus obtained was maintained on same media along with addition of kinetin (KN) and used for the present study.

Protein and enzyme extraction

To estimate the protein content and the activity of the antioxidant enzymes, leaf and callus extracts of both the plant types were prepared by homogenizing 1g of the material (leaf/callus) in chilled 3 ml 50 mM phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonylfluoride (PMSF), 0.5% (v/v) Triton X-100 and 2% (w/v) PVP-30, using a pre-chilled mortar and pestle. In case of analysis of ascorbate peroxidase, 0.5 mM ascorbate was added to the extraction buffer. The homogenates were centrifuged at 12,000×g for 20 min at 4°C. The supernatant (enzyme extract) was separated and used for biochemical analysis of proteins and antioxidant enzymes.

Protein quantification

Total protein content of different samples green leaves (GLe), green callus (GCe), purple leaves (PLe) and purple callus (PCe) was determined following the method of Lowrey *et al* (1951) using bovine serum albumin as a standard.

Enzyme assays

To estimate the antioxidative enzyme activity of different sample (PLe, GLe, PCe, and GCe) following methods were followed:

Ascorbate peroxidase assay

Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was determined following the method described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 1.0 mM H_2O_2 and 100 µl enzyme extract. The H_2O_2 dependent oxidation of ascorbate was monitored spectrophotometrically as decrease in absorbance at 290 nm. The enzyme activity was calculated using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$. The reaction was carried out for 3 min at 25°C. One unit of APOX activity is defined as the amount of enzyme that can oxidize 1 µmol of ascorbate per minute.

Catalase assay

Catalase (CAT, EC 1.11.1.6) activity was determined following the method of Aebi (1984) by measuring the rate of disappearance of H_2O_2. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 µl enzyme extract. The decrease in H_2O_2 was followed as decline in optical density at 240 nm for 30s at 25°C. The enzyme activity was calculated using an extinction coefficient 39.4 mM^{-1} cm^{-1} for H_2O_2.

Guaiacol peroxidase assay

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was determined following the method of Sànchez *et al* (1995) with slight modifications. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 12.3 mM H_2O_2 and 100 µl enzyme extract. The enzyme activity was recorded at 436 nm and calculated using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$. One unit of GPX activity represents the ability of the enzyme to catalyze the oxidation of 1 µmol of guaiacol in 1 min.

Glutathione-S-transferase assay

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was determined following the method of Habig *et al* (1974) by measuring the increase in absorbance at 340 nm for 2 min resulting from the formation of the conjugate between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH). The reaction mixture consisted of 0.1mM phosphate buffer (ph 7.5), 1mM GSH, 1 mM CDNB and 70µl enzyme extract. The enzyme activity was calculated using an extinction coefficient 9.6 $mM^{-1}cm^{-1}$ for GSH.

Superoxide dismutase assay

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined following the method of Kono (1978) by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The reaction mixture consisted of 50 mM sodium carbonate (pH 10.2), 24 µM NBT, 0.1 mM EDTA, 1 mM hydroxylamine, 0.03% (v/v) Triton X-100 and 70 µl enzyme extract. The absorbance was recorded at 560 nm for 2 min. One unit of SOD activity was defined as the amount of enzyme required for 50% NBT reduction at 25°C.

Statistical analysis

Results of three replicates for each sample were observed and recorded as mean ± standard error. Data was subjected to one way analysis of variance (ANOVA) between plant *in vivo* and *in vitro* for each morphotype to determine statistical significant difference in the production of antioxidative enzymes.

RESULTS

Table 1 and Table 2 depict the specific activity of different enzymes in the leaves and callus of *O. sanctum*.

Ascorbate peroxidase activity

Amongst the two plants, higher content of APOX was recorded in the green leaved Tulsi (GLe) *in vivo*. During *in vitro* conditions, the expression of the enzyme increased in the callus obtained from both the plants, with GC expressing the highest activity (304.88±4.11 U/mg protein). Both the callus cultures were able to express approximate 2.5 times higher activity compared to their respective plants. The order of APOX expression was recorded as GCe > PCe > GLe > PLe

Catalase activity

The concentrations of CAT enzyme were very low when compared to the activities of other enzymes. Also reduced catalase activity was observed for *in vitro* raised callus cultures. Maximum CAT activity was observed for PLe (5.42 U/mg protein) followed by GLe (2.37 U/mg protein). On comparing the callus raised *in vitro*, CAT activity was found to be higher for the purple morphotype. The order of increased expression of enzyme was recorded as: PLe>GLe>PCe>GCe.

Glutathione-S-transferase activity

Maximum GST enzyme expression was recorded in PLe (10.81±0.94 U/mg protein) from amongst all the samples. PLe was a better source of GST compared to GLe when compared *in vivo*. The increased expression of the enzyme was recorded in the order: PCe>PLe>GLe>GCe.

Guaicol peroxidase activity

Purple Tulsi was found to exhibit higher content of GPX compared to the green one. During *in vitro* conditions there was a significant increase in the expression of the enzyme for both the morphotypes. PCe expressed maximum POD activity (152.48±3.77 U/mg protein) followed by GCe. The order of increased enzyme expression was PCe > GCe > PLe > GLe.

Superoxide dismutase activity

Of amongst all the enzymes tested during the study, high SOD enzyme expression was recorded both *in vivo* and *in vitro*. Of the two plants *in vivo*, PLe showed higher activity of SOD (958.07±41.20 U/mg protein). Again the expression of the enzyme enhanced during in vitro culture conditions with PC expressing (2256.92±128.8 U/mg protein). SOD expression was in the order PCe > GCe > PLe > GLe.

The results as shown in Table 1 and Table 2 revealed that there is a statistically significant difference in the expression of all enzymes between the i*n vitro* grown callus and the leaves of purple morphotype of *O. sanctum* respective explants at 5% level of significance. In case of the green morphotype significant difference was observed in the expression of APOX, GPX and SOD in the leaves and the callus. Thus, from the above data it is clear that callus culture of both the morhpotypes of *O.sanctum* express much higher content of antioxidative enzymes. Also, the purple Tulsi is a better option compared to the green one for overcoming oxidative stress related disorders.

Table 1 One-way ANOVA analysis of enzyme activity between green leaves and green leaf derived callus of *Ocimum sanctum*

ENZYME	Green leaves (GLe) (Mean±SE) (U/mg protein)	Green callus (GCe) (Mean±SE) (U/mg protein)	F-ratio
Ascorbate peroxidase	125.58±5.49	304.88±4.12	683.07*
Catalase	2.37±0.59	0.87±2.40	6.19
Glutathione-s-transferase	7.43±0.61	5.50±0.96	2.68
Guaicol peroxidase	11.77±1.42	102.60±11.10	191.73*
Superoxide dismutase	180.04±48.29	1330.62±25.84	441.24*

*represents significance at p≤0.05

Table 2 One-way ANOVA analysis of enzyme activity between purple leaves and purple leaf derived callus of *Ocimum sanctum*

ENZYME	Purple leaves (PLe) (Mean±SE) (U/mg protein)	Purple callus (PCe) (Mean±SE) (U/mg protein)	F-ratio
Ascorbate peroxidase	79.26±7.69	220.94±3.7	275.94*
Catalase	5.42±0.73	1.62±1.20	26..65*
Glutathione-s-transferase	10.81±0.94	32.84±1.29	13.84*
Guaicol peroxidase	16.79±3.78	152.48±1.19	1157.57*
Superoxide dismutase	958.06±23.79	2265.92±128.85	99.71*

*represents significance at p≤0.05

DISCUSSION

Oxidative stress results from overproduction of ROS during metabolic reactions that utilize oxygen and shift the oxidant-antioxidant equilibrium in the favor of the oxidants. Besides, environmental factors such as air pollutants, ionizing radiations or cigarette smoke are also responsible for ROS generation. ROS are highly reactive molecules because of unpaired electrons in their structure and react with several biological macromolecules in cell, such as carbohydrates, nucleic acids, lipids, and proteins thus altering their biological functions. ROS also affects the expression of several genes by upregulation of redox-sensitive transcription factors and chromatin remodeling via alteration in histone

acetylation/deacetylation (Birben *et al* 2012). Regulation of redox state is critical for cell viability, activation, proliferation and organ function. A highly sophisticated antioxidant protection system has evolved in humans. A variety of endogenous and exogenous components interact synergistically to neutralize ROS. These components include antioxidant enzymes, non-enzymatic antioxidants, metal binding proteins and numerous other phytonutrients (Percival 1998). For treatment of disorders related to oxidative stress, antioxidants (synthetic and natural) are supplemented in the diet. To replace synthetic antioxidants, to reduce the risk of chronic ailments and to improve the stability and quality of food active research is carried out for identifying plants as a rich source of natural antioxidants. Oral supplementation of medicinal plants has been shown to combat disorders related to oxidative stress. Therefore, to obtain enzymes that help overcome oxidative stress it is necessary to search plants, callus and cell cultures as potential sources for their commercial production.

Peroxidases are a group of oxidoreductase enzymes that catalyse oxidative reactions using hydrogen peroxide and a reductant. Ascorbate peroxidase is a haem containing enzyme that uses ascorbate as a substrate whereas guaicol peroxidase uses guaicol. Peroxidases are widely employed in microanalysis. About 80% of immune-enzymatic kits use peroxidase as a labeling enzyme. Recently, peroxidases have been used for biotransformation of organic molecules. These enzymes also find their application in waste water treatment (Caza *et al* 1999) to eliminate aromatic and phenolic compounds, resin production by polymerization and soil detoxification. Horseradish roots present traditional source of peroxidase enzyme but other sources as turnip (Musthapa *et al* 2004, Saboora *et al* 2012) and soyabean (Hailu *et al* 2010) are also explored and exploited. From the data obtained for Tulsi in our study, green morphotype was a better source of APOX both *in vitro* (304.88±4.12 U/mg protein) and *in vivo* (125.58±5.49 U/mg protein) while the purple morphotype expressed highest GPX activity 152.47±3.78 U/mg protein in callus culture and 16.79±3.78 U/mg protein in the native plant. The specific activity of peroxidases in *Ocimum sanctum* is comparable to the commercial source (horseradish).

The enzyme catalase decomposes H_2O_2 produced during metabolic reactions into O_2 and H_2O. CAT finds it commercial application in medicine as an antioxidant, food and textile industry for sterilisation and bleaching. CAT deficiency is also reported to be associated in hypocatalasemic patients with diabetes mellitus (Góth 2008). Commercially, this enzyme is obtained from animal liver and microorganisms (fungi).

Glutathione-S-transferase belong to a group of intracellular xenobiotic-elimitating antioxidant enzymes involved in conjugation of various electrophilic compounds, toxic endogenous metabolites and various environmental contaminants, with tripeptide glutathione. GST deficiency or alterations in the gene encoding the enzyme, results in lung cancer (Mohr *et al.* 2003). The enzyme is currently being explored as targets for anti-cancer drugs due to its role in drug resistance. In this study both types of Tulsi were found to be a good source of GST with the purple morphotype exhibiting twice the activity (10.81±0.94 U/mg protein) of the enzyme *in vivo* compared to the green one(7.44±0.61 U/mg protein).

Superoxide dismutase protects the cell from damage caused by superoxide radicals (Fridovich 1978). According to Vucic and Kiernan (2009), SOD deficiency leads to neurodegenerative disease called amyotrphic lateral syndrome characterized by paralysis and subsequent death. At present bovine liver is the commercial source for SOD production. But this enzyme from plant sources has been shown to express twice the specific activity as SOD from mammalian sources (Sawada *et al*. 1972). SOD is used for the treatment of anti-inflammatory disorders and also finds application in cosmetic industry. Our study reveals that the purple leaved Tulsi expressed 5.32% more SOD compared to the green Tulsi *in vivo,* 958.06±23.79 U/mg protein and 180.04±48.29 U/mg protein, respectively. A marked increase in the production of this enzyme was noted in callus culture. For green Tulsi this increase was almost 7 times whereas for the purple one the increase was about 2 times. From amongst all the samples assessed, purple Tulsi callus produced the maximum enzyme (2265.92±128.85 U/mg protein). In the present study it was observed that both the plants are a good source of antioxidative enzymes (APOX, CAT, GST, GPX and SOD) and the expression of these enzymes increased during *in vitro* conditions. It is inferred that artificial culture conditions impose a challenge on the growing tissue resulting in oxidative/abiotic stress and generation of ROS (Tian *et al*. 2003). Generation of ROS is a part of signaling cascade that results in the protection from the stress. As a general strategy adopted by plants cells, activation of antioxidative enzymes help them overcome oxidative stress. The antioxidant enzymes APOX, CAT, GST, GPX, and SOD functions are quenchers for ROS. Catalases and peroxidases are scavengers of H_2O_2. CAT and SOD together scavenge the superoxide radical and hydrogen peroxide and converts them to water and molecular oxygen thus preventing cellular damage under unfavourable conditions (Chaitanya *et al*. 2002). GST on the other hand helps in the removal of harmful electrophillic compounds.

CONCLUSION

Present study suggests that callus derived from leaves of *O. sanctum* (green and purple morphotype) and grown under controlled artificial conditions produced antioxidative enzymes (APOX, GPX and SOD) at significantly higher levels compared to their native plants. The experimentation results also reveal the higher antioxidative potential of purple leaved Tulsi in comparison to the green leaved plant. Thus purple Tulsi can be preferred over the green one and supplemented in diet and herbal drugs to overcome disorders related to oxidative stress. The callus culture of *O. sanctum* particularly of the purple morphotype is the richest source of antioxidative enzymes from amongst all the samples. Thus, the callus culture of *O. sanctum* can be exploited as an alternative and potential source for commercial production of these useful enzymes.

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How to cite this article:

Harsimran Kaur and Gurdeep Singh Virk.2016, Ocimum Sanctum and Its Callus Culture – A Potential Source For Antioxidant Enzymes. *Int J Recent Sci Res.* 7(6), pp. 11775-11780.

ISSN 0976-3031
9 770976 303009 >