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Role of Hydrogen Peroxide on Viability, Morphology and Antioxidant Enzyme Activity in Callus Cells of *Catharanthus roseus* L.

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Abstract. Hydrogen peroxide (H_2O_2) is a representative reactive oxygen species (ROS), molecules that readily damage biological molecules including DNA and proteins, and which can eventually lead to apoptotic or necrotic cell death. In this experiment, the influence of H_2O_2 on viability, morphology and antioxidant enzyme activity in cell suspension culture of *Catharanthus roseus* was investigated. For callus induction, leaves of *Catharanthus roseus* seedlings were incubated on Murashige and Skoog (MS) medium. Following callus culture, the liquid medium was supplemented with sucrose, vitamins such as thiamin, pyridoxine, nicotine and macro and micro elements and hormones including 2-4-D, Kin, IAA. In addition, the calluses were treated with different concentrations of H_2O_2 (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µm) for 1, 3 or 6 days. The viability of these cells was investigated by a method using trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Additions of 1, 5 or 10 µm H_2O_2at day 6 were selected as the treatments most suitable for further research. Callus morphology was studied using coloring Hoechst and acridine orange. Furthermore, antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were measured. Results indicated that H_2O_2 leads to a significant decrease in viability of treated *Catharanthus roseus* calluses compared to a control. Activity of the antioxidant enzymes SOD, POD and CAT showed a significant increase in treated cells when compared to the control and were related to H_2O_2concentration. Furthermore, oxidative stresses caused generation of H_2O_2 and resulted in significant enhancement of antioxidant enzyme activity and intensified intracellular H_2O_2content as well.

Keywords: *Catharanthus roseus*, H_2O_2, ROS, Suspension culture, Cell viability, Antioxidant enzymes.

INTRODUCTION

Catharanthus roseus is a prominent medicinal plant in which a great deal of attention is paid to secondary compounds such as alkaloids, vincristine and vinblastine, which are used as cancer remedies. Hydrogen peroxide (H_2O_2) is produced in plants by an electron transport chain in chloroplasts and mitochondria, involving peroxidase and NADPH oxidase enzymes, and appears to be the main signaling molecule for growth and differentiation (Mittler, 2002). In some reactions H_2O_2 is produced directly and in some other cases it is generated through mediocrity reactions involving, for example, oxygen and superoxide (Desikan *et al*., 2004). When oxygen receives one electron, it is converted to a superoxide radical, that is a very transient molecule which spontaneously or via a superoxide dismutase (SOD) enzyme reaction converts to a proton and is then converted to hydrogen peroxide (Sutherland, 1991). Finally, generation of reactive oxygen species (ROS) leads to oxidative damage to intact cells and organs. Plants have defense systems which include the antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) or non-enzymatic antioxidants for scavenging reactive oxygen species (ROS) free radicals.

Hamburger and Hostettmann (1991) reported that the content of H_2O_2 and alkaloids were increased in leaf tissue of plants exposed to different levels of H_2O_2. Also mentioned is the fact that *in vitro* synthesis of a valuable alkaloid, vinblastine, depended on H_2O_2 content and was regulated by POD enzyme activity (Kumar and Gupta, 2008). Moreover, the levels of ROS and antioxidant systems activity was altered by the addition of H_2O_2 and subsequent synthesis of pharmacologically active compounds was induced by oxidative stress in reference plants (Ros-Barcelo, 1998). Further, it is known that intracellular levels of H_2O_2 has a direct correlation with

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generation of superoxide radicals such as hydroxyl radicals, and also has a major role in regulation of gene expression and plant cell death (Lamb and Dixon, 1997).

Therefore, the aims of this study were to investigate the effects of different levels of H_2O_2on viability of cells, morphological changes in nuclei, intracellular levels of H_2O_2 and on the activity of antioxidant enzymes including SOD, POD and CAT in cell suspension culture of *Catharanthus roseus* L.

MATERIALS AND METHODS

Plant materials and medium conditions. Seeds of *Catharanthus roseus* were provided by the Pakan seed Nursery Co., Esfehan, Iran. Seeds were soaked in distilled water for 24 hours and then put in two layers of tissue in a dark room for 48 hours before seeds were cultured in plastic pots containing perlite. The pots were kept in a greenhouse under ambient temperatures (25±2ºC). Leaves of *Catharanthus roseus* were used for callus induction. Young leaves were detached from the head of the plant and sterilized in 5% (v/v) sodium hypochlorite solution for 15 minutes then washed with distilled water and sterilized in ethanol 70% for 10 minutes, followed by three washes with sterile distilled water. The sterilized leaves were excised into 1 cm pieces and cultured on solid Murashige and Skoog (MS) medium containing 30 g l^{-1} sucrose, 8 g l^{-1} agar, vitamins including 0.1 mg l^{-1} thiamin, 0.5 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotine, macro- and micro elements and hormones such as 0.5 mg l-1 kinetin and 1.5 mg l^{-1} 2-4-D. After that glasses were transferred to a culture room set to the temperature of 25±2°C. Calluses were sub-cultured after 3 weeks and a second callus sub-culture applied as suspension culture (Figure 1).

Suspension culture. Twenty ml of suspension solution (liquid media MS) was placed into an Erlenmeyer flask and callus explants developed and floated in it. The suspensions were maintained at 25°C with shaking at 70 rpm. After 3 weeks they were centrifuged and used for morphological studies (Saifullah and Khan, 2012).

Viability of cells tested by the trypan blue method. In all H_2O_2 treatments, regardless of the concentration (0-10 µm), 500 µl was used per 24 plates, and 50 µl of suspension containing 80,000 cells was added to it before plates were put to be shaken at 70 rpm at 25°C. After 1, 3 or 6 days, samples were transferred to micro-tubes for centrifugation and then 50 µl PBS buffer with 10 µl trypan blue 4% was added. Cells were left to rest for 2 minutes, and then cells were counted using the Neo bar lam method (Meimoun *et al.*, 2009). Blue cells were considered to be dead and colorless cells alive. Finally, the percentage of viability of cells was determined using the following equation:

$$\% \text{ Viability} = \frac{\text{Number of alive cells}}{\text{Total cell}} \times 100$$

Assay by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Plates of cells were prepared exactly as for the trypan blue method described above, and plates left to be shaken. After 1, 3 or 6 days all samples were centrifuged and then 50 µl PBS buffer was added to each. For each 100 µl of cell suspension, 10 µl MTT solution was added and then suspensions put in the shaker for 2 hours. Thereafter, the solution was centrifuged at 5000 rpm for 10 minutes and 100 µl DMSO was added to each sample, before they were

Figure 1. Illustrations of steps of callus culture of *Catharanthus roseus*. (A) Seed culture of *Catharanthus roseus* in plastic pots to obtain plant stocks for *in vitro* assays. **(B)** Seedling growth, the stage that plant leaves were taken and used for callus induction *in vitro*. **(C)** Sample of callus culture grown in MS medium.

transferred to a dark room for 30 minutes. All samples were centrifuged again for 10 minutes as 5000 rpm and supernatant transferred to 96 plates. The absorbance was read at a 505 nm wavelength by an Elisa-reader (Medical SCO GmbH, Germany).

Morphological change assays using fluorescent coloring methods.

Nuclear staining using fluorescent Hoechst color. Explants, along with the 50 µl cell suspensions, were transferred to micro-tubes to which were added 10 µl Hoechst color (1 mg ml^{-1} PBS buffer). After 5 minutes, the nuclei were examined for morphological changes and photographs were taken by fluorescence microscope (Olympus Px41 Japan equipped camera model DP71) (Gorpenchenko *et al*., 2012).

Coloring of the cytoplasm using fluorescent acridine orange. For morphological study of the cytoplasm, explant suspensions containing treated and control cells, were colored with 0.01 g mg^{-1} acridine orange for 2 minutes. For each 50 µl of cell suspension 10 µl of acridine orange was added and then the cytoplasms of cells were examined for morphological changes and photographs were taken by fluorescence microscope (Olympus Px41 Japan equipped camera model DP71).

Assay of intracellular hydrogen peroxide (H_2O_2) concentration. To assay the intracellular H_2O_2 content, 0.5 g of callus tissues was ground and homogenated with 1 ml 0.1 % TCA and then centrifuged at 12000 rpm for 15 minutes at 4 ºC. After that, 700 µl supernatant was added to 700 µl of 10 mM pH=7 phosphate potassium buffer and 700 µl 1M potassium iodide. Subsequently, absorbance was read at 390 nm. H_2O_2content was determined by the methods of Ghanati *et al*., (2002) and expressed as µmol g fw^{-1}.

Antioxidant enzyme assays.

Half a gram of *Catharanthus roseus* calluses were homogenized in a mortar chilled to 4 ºC using 100 mM potassium phosphate buffer (pH 7.0). The homogenate was the centrifuged at 12,000 rpm for 15 minutes at 4 ºC and used for enzymatic assays as described below.

Superoxide dismutase (SOD) (EC 1.15.1.1) assay. Total activity of the SOD enzyme was measured using the method of Giannopolitis *et al*. (1997), which is based on monitoring the inhibition of the photochemical reduction of nitro-blue tetrazolium (NBT). One unit of SOD activity is defined as the amount of enzyme needed to cause 50% inhibition of NBT reduction as read at 560 nm by a spectrophotometer (T80=PG instrument UV/Vis).

Peroxidase (POD) (EC 1.11.1.7) assay. Peroxidase activity was measured in a reaction medium containing 100 mM potassium phosphate buffer (pH 7.0), 18 mM guaiacol, and 0.37% H_2O_2 according to the method of Polle *et al*. (1994). The absorbance was read at 436 nm over 2 minutes. The enzyme activity was calculated using the extinction coefficient (26.6 mM^{-1} cm-1).

Catalase (CAT) (EC 1.11.1.11) assay Total CAT activity was assayed according to the method of Cakmak and Marchner (1992). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 25 mM H_2O_2 and 50 µl enzyme extract. Absorbance was read after 20 minutes at 240 nm.

Statistical Analysis. All data were analyzed by one-way analysis of variance (ANOVA), and the mean differences were compared by the Duncan multiple range test. All data was reported as the average of three replicates (n = 3); treatments with $P \leq 0.05$ were considered to be significantly different.

RESULTS

Viability of callus cells. Data from trypan blue and MTT testing indicated that 1, 3 or 6 days after treatment a significant ($P \leq 0.05$) difference observed between plants treated with different concentrations of hydrogen peroxide. Although the viability of cells in some concentrations did not differ significantly, all doses showed significant differences when averaged between 3 replicates when compared to the controls (Table 1). Statistical analysis of data obtained from MTT analysis was confirmed by the results of coloring with trypan blue (Table 2). For the proceeding research, 3 levels of H_2O_2 (1, 5 and 10 µm) were selected because 25, 50 and 70 % mortality was observed in cells treated with LD 25, LD 50 and LD 70 levels, respectively. According to our results, control cells

Table 1. Comparison of mean callus cell viability in *Catharanthus roseus* after 1, 3 and 6 days treatment with different concentration of H_2O_2 according to the trypan blue method.

H_2O_2 (µm)	Time (day)		
	1	2	3
0	93.68 ±1.8[a]	90.68 ±1.8[a]	86.26 ±5.23[a]
1	82.17±2.02[b]	74.29±0.55[b]	70.55±5.65[b]
2	77.17 ±5.26[b]	66.19 ±0.55[b]	64.30 ±4.02[bc]
3	67.42±2.42[c]	58.14±0.57[d]	56.47±4.00[bc]
4	58.05 ±4.09[d]	56.76 ±0.31[d]	55.25 ±0.67[cd]
5	54.23±0.56[d]	53.35±0.59[d]	48.14±0.57[de]
6	52.28 ±0.48[d]	52.70 ±0.77[d]	47.1 ±0.61[de]
7	43.40±0.42[e]	46.35±0.28[e]	45.11±0.66[def]
8	42.06 ±0.49[e]	38.14 ±0.57[e]	43.07 ±0.40[ef]
9	40.29±0.71[e]	36.83±0.34[f]	34.70±0.62[fg]
10	33.26 ±0.70[f]	30.14 ±5.46[g]	28.17 ±4.36[g]

Values expressed are means ± SD. Mean values in each column with the same letter did not differ significantly at P ≤ 0.05 according to the Duncan multiple range test.

Table 2. Mean comparison of the number of live callus cells of *Catharanthus roseus* after 1, 3 or 6 days treatment with different concentrations of H_2O_2 according to the MTT method.

H_2O_2 (µm)	Time (day)		
	1	2	3
0	61.14±0.5[a]	57.12 ±0.05[a]	53.08 ±0.04[a]
1	54.17±0.03[b]	51.67±0.14[b]	50.17±0.03[b]
2	54.08 ±0.05[b]	50.18 ±0.06[b]	49.79 ±0.06[b]
3	53.38±0.18[b]	49.85±0.03[d]	49.35±0.11[b]
4	42.97 ±0.45[c]	47.74 ±0.03[d]	46.85 ±1.39[c]
5	42.80±0.33[c]	46.9±0.034[d]	37.17±0.60[d]
6	40.22 ±0.05[d]	46.61 ±0.05[d]	36.76 ±0.08[d]
7	40.13±0.04[d]	38.89±0.02[e]	36.22±0.06[d]
8	37.25±0.03[e]	37.55 ±0.05[e]	3.046 ±0.04[e]
9	37.1±0.10[e]	36.02±0.14[e]	30.11±0.03[e]
10	34.86±0.07[f]	32.3 ±0.03[f]	28.5 ±0.22[f]

Values expressed are means ± SD. Mean values in each column with the same letter did not differ significantly at P ≤ 0.05 according to the Duncan multiple range test.

had a visible cytoplasm with a flat border and regular shape, but treated cells had a crimped cytoplasm with an irregular shape. Furthermore, morphological alteration was apparent in the globular shape of nuclei in control cells compared to the nuclei in treated cells which didn't have a regular shape (Figure 2).

The staining with fluorescent Hoechst of control cells and those treated with different concentrations of H_2O_2 (1, 5 and 10 µm) for 6 days showed lower nucleus size and nucleus density in the treated cells, most prominent in cells treated with 10 µm H_2O_2 (Figure 3). Coloring of cells with fluorescent acridine orange indicated a change in the cytoplasm and the condition of nuclei in cytoplasm in treated cells (Figure 4).

Effect of H_2O_2 on intracellular level of H_2O_2 in the calluses of Catharanthus roseus. Data obtained from measuring intracellular concentration of H_2O_2 in the treated cells of *Catharanthus roseus* calluses was significantly (P

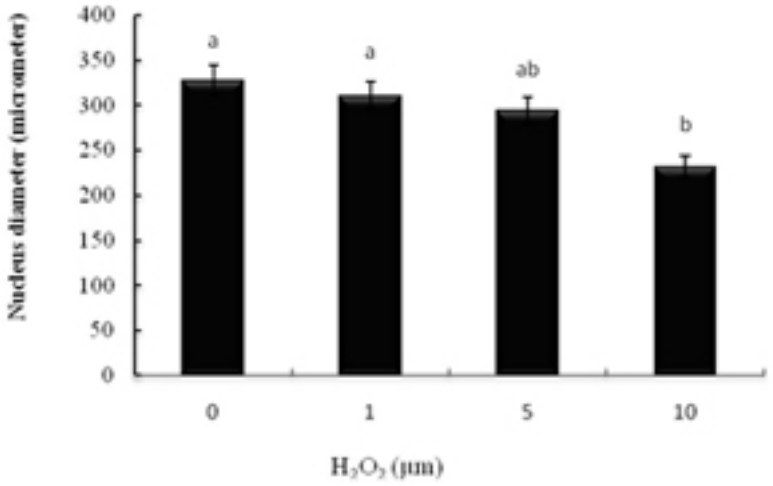

Figure 2. Mean comparison of nucleus diameter (micro meter) of *Catharanthus calli*, 6 days after treatment with H_2O_2 (1, 5 and 10 µm) and control. Bars indicate standard deviation values. Values with the same letter did not differ significantly at P ≤ 0.05 according to the Duncan multiple range test.

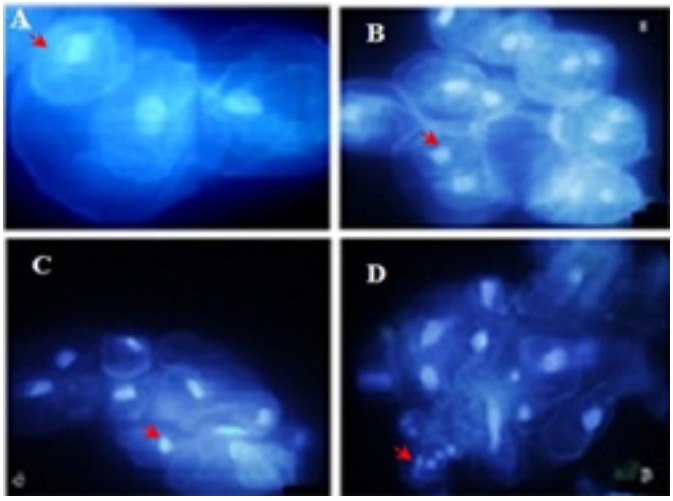

Figure 3. Coloring of *Catharanthus roseus* callus cell according to the Hoechst method. (A) Control, **(B)** Cell treated with 1µm H_2O_2 during 6 days, **(C)** Cell treated with 5µm H_2O_2 during 6 days, **(D)** Cell treated with 10µm H_2O_2 after 6 days. Arrows indicate position of the nuclei (40x).

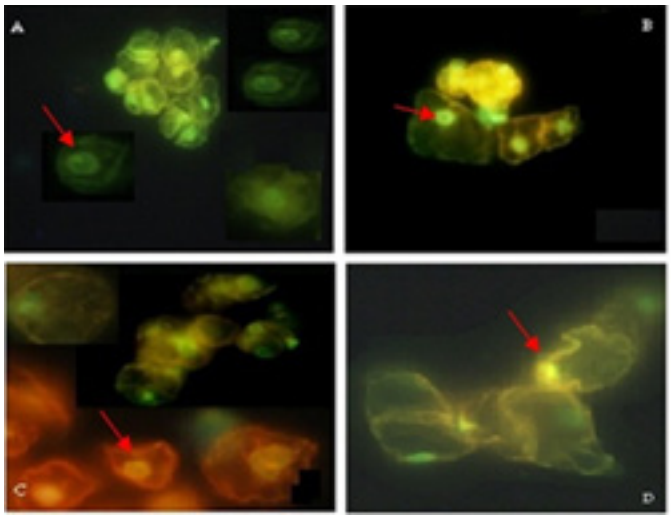

Figure 4. Coloring of *Catharanthus roseus* callus cells using fluorescent acridine orange. (A) Control cells, **(B) C**ells treated with 1µm H_2O_2 for 6 days, **(C)** Cells treated with 5µm H_2O_2 for 6 days, **(D) C**ells treated with 10µm H_2O_2 for 6 days. Arrows indicate position of the nuclei (40x).

≤ 0.05) different compared to control cells (Figure 5). H_2O_2 content in calluses treated with 1, 5 or 10 µm had a significant increase compared to control cells: 31.43, 69.13 and 44.82 times the level, respectively. However, the content of H_2O_2 in the 10 µm treatment was less than that in the 5 µm tretament.

Effect of H_2O_2 on activity of antioxidant enzymes (SOD, POD and CAT). Statistical analysis of SOD activity in callus cells of treated and control *Catharanthus roseus* indicated that the effect of H_2O_2 on this enzyme's activity was significant (Table 3). Treating cells with different concentrations of H_2O_2 led to a prominent increase in SOD activity compared to control cells. According to our results, SOD enzyme activity is dependent on dosage as it showed the highest level of activity in cells treated with 10 µm H_2O_2. The increase in enzyme activity in cells treated with 1, 5 and 10 µm H_2O_2, was 3.87, 19 and 30.5 %, respectively, compared to control cells. Activity of POD was significantly increased in treated cells as well, 23.31, 56.44 and 41.10% higher than control cells when treated with 1, 5 or 10 µm H_2O_2, respectively. However, the prominent difference ($P \geq 0.05$) in activity between the 5 or 10 µm H_2O_2 treatments wasn't observed with POD. The effect of H_2O_2 on CAT activity was significant (Table 3); calluses treated with 5 or 10 µm H_2O_2 showed a notable increase (36.76 and 103.67%, respectively) in CAT activity compared to control cells. However, CAT activity wasn't significantly altered between the 1 µm H_2O_2 treatment and control cells.

DISCUSSION

Among reactive oxygen species (ROS), H_2O_2 seems best suited to play the role of signaling molecule due to its high stability and long half-life, but for it to really be a specific signaling molecule, a mechanism must exist in cells to detect elevated concentrations. H_2O_2 can interact with cysteine residues within proteins, and this redox modulation could potentially alter their conformation, affecting activity, and therefore initiating subsequent cellular responses. The redox state of the cells and its alteration through the oxidative burst is an important regulatory element of this defense response. H_2O_2 signaling pathways are indeed an important part of signal transduction, influencing the entire signal transduction framework (Boka and Orban, 2007).

In this research, dead cells could be identified by staining a blue color because they were permeable to trypan blue. In the MTT method, the sucsinate dehydrogenase enzyme in mitochondria has the ability to reduce the yellow color of de methyl thiazol de phenyl tetrazialom to a yellow crystal of de methyl thiazol de phenyl tetrazialom and to purple crystals and insoluble formazan (Yamamoto *et al.*, 2004). Results obtained from these combined methods showed a dose-dependent response to H_2O_2. Treatment with 1, 5 or 10 µm of H_2O_2 correlated with a lethal dose (LD) 25, 50 and 75 that led to the death of about 25, 50 and 75 % of treated cells, respectively. One of the main roles of H_2O_2, induction of programmed cell death (apoptosis) in

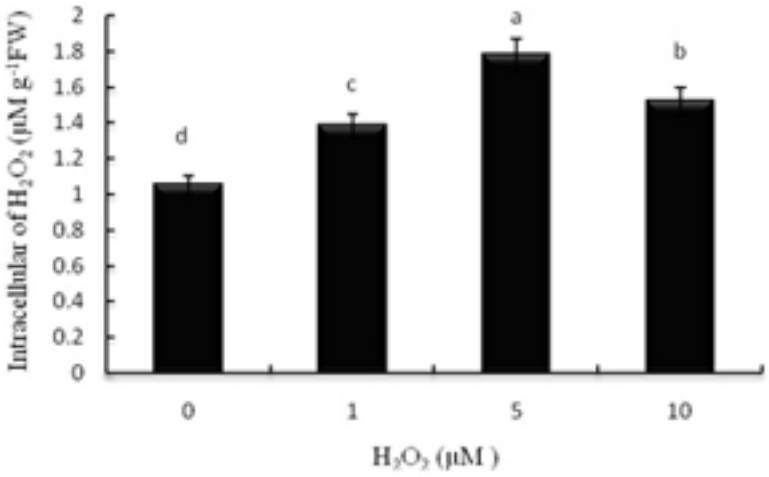

Figure 5. Comparison of mean intracellular H_2O_2 content in callus cell of *Catharanthus roseus* after 6 days treated with 1, 5 and 10μm H_2O_2. Bars indicate standard deviation values. Mean values with the same letter did not differ significantly at P ≤ 0.05 according to the Duncan multiple range test.

plant cells (Desikan and Hancock, 2002), explains the morphological changes in treated compared to control cells through the induction of apoptosis by H_2O_2. In this way, Roger *et al*. (1996) reported that treatment of soybean cells with H_2O_2leads to apoptosis.

Hydrogen peroxide, as well as inducing apoptosis, has the ability to produce other ROS and can cause the activation of antioxidant enzymes (Mittler, 2002); in conditions of oxidative stress antioxidant enzymes are an important part of the defense mechanisms of plants (Neill *et al*., 2002). Lipid peroxidation due to H_2O_2 leads to intensified activity of antioxidant enzymes (Mittler, 2002), and in this study it appeared that activation of the SOD enzyme increased in response to the presence of H_2O_2 in calluses of *Catharanthus roseus*. Hydrogen peroxide acts as a secondary messenger in cells and increases some antioxidant enzymes' activity, including SOD, and/or their expression (Lamb and Dixon, 1997). Increased SOD activity acts as a defense system for the removal of excess ROS and this fact helps cells to tolerate stress conditions better. Consistent with these results, Tian *et al.* (2003) reported that in strawberry calluses hydrogen peroxide heightened SOD activity. According to Yang *et al*. (2012) H_2O_2 can increase the activity of the POD enzyme in calluses of *Nitraria tangutorum*. In the current investigation, hydrogen peroxide also increased POD activity in calluses of *Catharanthus roseus.* POD has a role in plant defense systems by destroying H_2O_2 during abiotic stress. Activity of this enzyme depends on plant species and the type of stress (Gill and Tuteja, 2010). Therefore, the lack of dosage-dependent increase in activity of this enzyme on treatment with 10 μm H_2O_2 may be due to a higher tolerance of *Catharanthus roseus.*

CONCLUSION

CAT is one of the most important enzymes that is activated in conditions of oxidative stress and enables scavenging of H_2O_2 (Gill and Tuteja, 2010), which thus significantly increased activity of CAT in treated *Catharanthus roseus* calluses compared to control cells, dependent on dose. It is reported that this enzyme didn't cause activation in low ROS concentrations, but only in high concentrations, indicating the importance of the

Table 3. Comparison of mean antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity in calluses obtained from *Catharanthus roseus* after 6 days treatment with different concentrations of H_2O_2.

H_2O_2 (μm)	SOD (U $min^{-1}mg^{-1}$ protein)	POD (U $min^{-1}mg^{-1}$ protein)	CAT (U $min^{-1}mg^{-1}$ protein)
0	$53.44±0.22^{d}$	$1.63±0.21^{c}$	$1.36±0.01^{c}$
1	$55.51±0.25^{c}$	$2.01±0.07^{b}$	$1.46±0.02^{c}$
5	$63.62±0.25^{b}$	$2.55±0.12^{a}$	$1.86±0.10^{b}$
10	$69.74±0.17^{a}$	$2.30±0.10^{ab}$	$2.77±0.19^{a}$
Values expressed are means ± SD. Mean values in each column with the same letter did not differ significantly at P ≤ 0.05 according to the Duncan multiple range test.			

intensity of oxidative stress (Gill and Tuteja, 2010). Therefore, activity of this enzyme was not significantly different in our experiment on treatment with only 1µm H_2O_2compared to the controls. In addition to this, H_2O_2 is a substrate of CAT and POD enzymes, so it was assumed that increasing CAT and POD activities may be caused by an increased concentration of H_2O_2 in calluses treated with H_2O_2compared to control calluses. In our current study, the significant effects of different levels of H_2O_2 on examined traits were consistent with others. For example, Tang *et al*. (2009) showed that treatment with 100 mM H_2O_2 led to a heightened concentration of H_2O_2 found inside *Catharanthus roseus* calluses when compared to controls. This finding was similar to the result of Tian and Zhu (2003) who examined calluses of strawberries treated with 50µm H_2O_2. However, Yu *et al.* (2003) reported that treatment of *Vigna radiate* with 200 mM H_2O_2 diminished the inner content of H_2O_2. These differences represent species-specific differences in responses to oxidative stress.

CONCLUSION

From the results of this study, it can be concluded that treatment of calluses of *Catharanthus roseus* with exogenous hydrogen peroxide activates their defense systems through altering the activity of antioxidant enzymes. Expression of these enzymes acts as a defensive mechanisms for removal of reactive oxygen species (ROS) such as H_2O_2 and help callus cells to better tolerate oxidative stress conditions.

REFERENCES

Boka, K. and Orban, N. 2007. New aspect of H_2O_2 signaling. *Plant Signaling and Behavior* 2: 498–500.

Cakmak, I. and Marschner, H. 1992. Magnesium-deficiency and high-light intensity enhance activities of superoxide-dismutase, ascorbate peroxidase and glutathione-reductase in been leaves. *Plant Physiology* 98:1222-1227.

Desikan, N.R. and Hancock, J. 2002. Hydrogen peroxide signaling, *Current Opinions in Plant Biology* 5: 388-395.

Desikan, R., Hancock, J.T. and Neill, S.J. 2004. Oxidative stress signaling. *Topics in Current Genetics* 4: 121-149

Ghanati, F., Morita, A., and Yokota, H. 2002. Induction of suberin and increase of liginin content by exess Boron in Tabacco cell. *Soil Sci Plant Nutrition* 48: 357-364.

Giannopolitis, C.N. and Ries, S.K. 1997. *Superoxide dismutases* I. Occurrence in higher plants. *Plant Physiology* 59: 309-314.

Gill, S.S. and Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48: 909-930.

Gorpenchenko, T.Y., Aminin, D.L., Vereshchagina, Y.V., Shkryl, Y.S.H., Veremeichik, G.N., Tchernoded, G.K. and Bulgakov, V.P. 2012. Can plant oncogenes inhibit programmed cell death? The rolB oncogene reduces apoptosis-like symptoms in transformed plant cells. *Plant Signaling and Behavio*r 7: 1058-1061.

Hamburger, M. and Hostettmann, K. 1991. Bioactivity in plants: the links between phytochemistry and medicine. *Phytochemistry* 12: 3864-3874.

Kumar, J. and Gupta, P.K. 2008. Molecular approaches for improvement of medicinal and aromatic plants. *Plant Biotechnology Reports* 2: 93-112.

Lamb, C. and Dixon, R.A. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 251-275.

Meimoun, P., Tran, D., Baz, M., Errakhi, R., Dauphin, A., Lehner, A., Briand, J., Biligui, B., Madiona, K., Beaulieu, C. and Bouteau, F. 2009. Two different signaling pathways for thaxtomin A-induced cell death in Arabidopsis and tobacco BY2. *Plant Signaling and Behavior* 4: 142-144.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7: 405-410.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D. and Hancock, J.T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany* 53:1237-1247.

Polle, A., Otter, T. and Seifert, F.A. 1994. Apoplastic Peroxidases And Lignification In Needles Of Norway Spruce (*Picea Abies* L.). *Plant Physiology* 106: 53-56.

Roger, A.L., Maria, I.P., Robert-Palmer, E.A. and Lamb, C. 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Current Biology* 6: 427- 437.

Ros-Barcelo, A. 1998. Hydrogen peroxide production is a general property of the lignifying xylem from vascular plants. *Annals of Botany* 82: 97-103.

Saifullah and Khan, S. 2012. Callus induction and cell suspension culture production of *Catharanthus roseus* for biotransformation studies of (-)- caryophyllene oxide. *Pakistan Journal of Botany* 43: 467-473.

Sutherland, M.W. 1991. The generation of oxygen radicals during host plant responses to infection. *Physiological and Molecular Plant Pathology* 3: 79-93.

Tang, Z., Yang, L., Zu, Y. and Guo, X. 2009. Variations of vinblastine accumulation and redox state affected by exogenous H_2O_2 in *Catharanthus roseus* (L.). *Plant Growth Regulation* 57: 15-20.

Tian, M., Gu, Q. and Zhu, M. 2003. The involvement of hydrogen peroxide and antioxidant enzymes in the process of organogenesis of strawberry callus. *Plant Science* 165: 701-707.

Yamamoto, T., Ewis, J., Wataha, J., Dickinson, D., Singh, B., Bollag, W., Ueta, E., Osaki, T., Athar, M., Schuster, G. and Hsu, S. 2004. Roles of Catalase and Hydrogen Peroxide in Green Tea Polyphenol-Induced Chemopreventive Effects. *Journal of Pharmacology and Exprimental Therapeutics* 308: 317-323.

Yang, Y., Zhang, Y.Y., Lu, J., Zhang, H., Liu, Y., Iang, J. and Shi, X. 2012. Exogenous H_2O_2 increased catalase and peroxidase activities and proline content in *Nitraria tangutorum* callus. *Biologia Plantarum* 56: 330-336.

Yu, C.W., Murphy, T.M. and Lin, C.H. 2003. Hydrogen peroxideinduced chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. *Functional Plant Biology* 30: 955-963.

Zhao, J. and Verpoorte, R. 2007. Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering, *Phytochemical Reviews* 6: 435-457.