EFFECT OF PARACETAMOL ON ANTIOXIDANT SYSTEM AND OSMOPROTECTANTS OF MUNG BEAN (*VIGNA RADIATA)*

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Abstract. There is no report on panadol mediated changes in antioxidant system and osmoprotectants of *Vigna radiata* plants. An experiment was carried out to dissect the response of activities of antioxidant enzymes, stress biomarkers and proline accumulation in the presence of panadol in *Vigna radiata* plants. 10 days after germination, a range of panadol concentration (0.0005, 0.01, 0.1 or 1.0 ppm) was administered to the foliage of *Vigna radiata* plants, at the end of germination process (30 days from the beginning of germination) the plants were sampled to evaluate stress biomarkers, leaf water potential, antioxidant enzymes and proline content. The results demonstrate that the highest concentration of panadol was 1.0 ppm that induced oxidative stress that was noticeable via increasing levels of lipid peroxidation, and H_2O_2 accumulation in *Vigna radiata* plants. On the other hand, 0.1 ppm of PD treatment modified the antioxidant system and the range of osmolytes to make the plants tolerate unfavorable conditions and helped to increase gross production through increasing photosynthetic activity.
Keywords: *panadol, proline, mung bean, biomarker, stress physiology*

Introduction

In the recent years, researchers have dissected out the role of pharmaceutical drugs and how they can affect target organs in the human body and how to make the drugs insistent so to maintain their chemical structure long enough to do their remedial work (Heberer, 2002; Kümmerer, 2004). However, the possible physiological and ecological influence of those compounds on non-target species or the environment are neglected (Hilton and Thomas, 2003). Therefore, the release of pharmaceuticals compounds in the environment is going to emerge as a great concern for both the ecosystem and human health (Kümmerer, 2004; Picó et al., 2017). Many prescribed drugs for humans are not completely absorbed by patients' body following intake, and approximately more than fifty percent of these are excreted as human waste to sewage systems in a non-biodegradable form or degraded to only a certain extent (Breton and Boxall, 2003; Hilton and Thomas, 2003).

Panadol (Paracetamol/Acetaminophen; PD) which represents one of the most widespread pharmaceuticals in surface waters and sediments even after wastewater treatment in the world, has negative effects on humans, animals, microbes, and plants (Kümmerer, 2004; An et al., 2009). Kolpin et al. (2002) found that 10 USA streams are contaminated with PD at maximum concentrations up to 10 ppm. In Madinah City-Saudi Arabia alone, tons of PD had been dispensed by public hospitals and medical clinics in the year of 2009 (Shraim et al., 2017). Even though wastewater treatment plant may remove 80% of PD in the water, they are far from the negligible amount (Ternes, 1998, 2000; Heberer, 2002). Increased human population in Saudi Arabia also may increase the consumed and unused PD, which lead to steady continual increase of PD in the wastewater. Continual use of sewage sludge and reclaimed waters in irrigation may lead to increased concentration of Panadol in agricultural lands (Kümmerer, 2004; Alyemeni et al., 2014).

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Mung bean, *Vigna radiata*, Fabaceae, *Vigna* genus include about 150 species. One of the most important crop plants among these species is *Vigna radiata* with green seeds. Mung bean contains about 23.9% protein; rich in lysine which is generally low or deficient in cereals. Mature seeds are rich in proteins. The tender pods of mung bean are also consumed as vegetable (Agrawal et al., 2006). Its fast growth and sensitivity to abiotic stress make it suitable as a test subject to understood the phytotoxic effect of PD toward crop plant (Fariduddin et al., 2014).

In our previous study, we have found that varied concentrations of Panadol influences the photosynthetic efficiency of *Vigna radiata* plants in a concentration dependent manner (Al-Muwayhi, 2018; Almohisen, 2018). Therefore, it is essential to dissect out the behavior of antioxidant enzymes and osmoprotectants to unravel the panadol mediated changes in antioxidant systems and osmoprotectants.

Materials and Methods

Growth conditions and analysis of various parameters

Seeds source of *Vigna radiata* was from National Seed Corporation Ltd., New Delhi, India. Healthy seeds were decontaminated with 1% Chlorox (NaOCl) for ten minutes, and then washed twice with distilled water. Panadol was used as the source of Paracetamol. It was acquired from Sigma Aldrich India Ltd. The stock solution of PD (10 ppm) was obtained by dissolving the required amount (0.0000005, 0.00001, 0.0001 and 1 mg) of paracetamol in distilled water in a volumetric flask and the final volume was made up to the required volume. The required concentrations (0.0005, 0.01, 0.1 or 1.0 ppm) of PD were prepared by diluting stock solution and the concentrations of PD was based on the screening of various concentrations (Data unpublished).The seeds were sown in washed sand media, and allowed to germinate in growth chamber. The sand media kept moist using deionized water. When germination completed, the seedlings were irrigated with a solution of nutrients (Hewitt, 1966) every other day during the experimental period. At day 10^{th}, the seedlings sprayed with deionized water (control), and 0.0005, 0.01, 0.1 or 1.0 ppm of PD for 10 days. Each seedling was speckled. The sprayer nozzle was adjusted to push out approximately 1 mL in one spray. Thus, each seedling received 3 mL of deionized water or PD treatement. The plants were grown under controlled conditions that is the average temperature ranged between 28/22°C day/night, maximum 75% relative humidity (RH) at day and minimum RH was 65% at night, and the photoperiod was 14 hour. Irrigation was applied with deionized water and nutrient solution on every other day. Harvesting of plants began at 30 days stage to estimate the chlorophyll content, net photosynthetic rate, maximum quantum yield of PSII and related attributes. The experimental design was completely randomized block design (CRB). Total cups were 25, cup size was 350 mL, each cup contained three plant and replicated five times. The replication of treatment was five times. The leaf water potential (LWP) was measured in the fully expanded leaves of the plant using Psypro Water Potential System (Wescor, Inc. 370 West 1700 South Logan, Utah 84321, USA).

Estimation of Lipid peroxidation rates was performed with the measurement of the malondialdehyde equivalents as described by Hodges et al. (1999). 80% ethanol was used to homogenize 0.5 g of the leaf. Then the homogeneous sample was centrifuged at 3000g for 10 minutes under 4°C temperatures. The pellet was extracted twice with the

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same solvent. 1 mL of supernatant was added to a test tube containing the same volume of a solution of 20% trichloroacetic acid, 0.01% butylated hydroxy toluene and 0.65% thiobarbutyric acid.

Samples were heated for 25 min at 95°C, and then cooled down to 24±1°C. Wavelengths of 440, 532 and 600 nm were used to measure absorbance of the samples. The formula of Hodges et al. (1999) was used to calculate Lipid peroxidation rates equivalent (nano mole malondialdehyde mL^{-1}).

The accumulation of hydrogen peroxide was estimated according to the method explained by Jana and Choudhuri (1982). 500 mg from samples was homogenized in 3.0 mL of phosphate buffer (50 mM and pH 6.8). Then the homogeneous sample was centrifuged for 25 minutes at 6000g.

3.0 mL from the extract was mixed with 0.1% titanium chloride in 20% (v/v) sulphuric acid, the mixture was centrifuged at 6000g for 15 minutes. A spectrophotometer was used for the measurement of color absorbance at 410 nm, and the color absorbance was compared with that of the calibration curve. The standard curve of known concentration of H_2O_2 was used to compute H_2O_2 content on fresh mass basis. 0.5 g from leaf was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone, for the assessment of antioxidant enzymes. The homogeneous sample was centrifuged for 10 minutes at 27600 g under a temperature of 4°C, the supernatant was used as the source of catalase, peroxidase and superoxide dismutase enzymes. The procedure described by Chance and Maehly (1955) was followed to assay Peroxidase and catalase. Catalase was measured by titrating the reaction mixture consisting of phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract and 2% H_2SO_4, against solution of 0.1 N potassium permagnate. The reaction mixture for peroxidase consisted of pyragallol, phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract. Change in absorbance caused by catalytic conversion of pyragallol to purpurogallin, was noticed at an interval time of 20 second and 2 minutes, at wavelength 420 nm on a spectrophotometer. Control set was prepared by using distilled water as an alternative of enzyme extract. The activity of superoxide dismutase was computed by estimating its capability to inhibit the photochemical reduction of nitroblue tetrazolium by following Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0–50 mL enzyme extract and was situated under 15W fluorescent lamp. The reaction began by switch on the light and run for 10 minutes. The reaction was stopped by switching off the light. 50% inhibition by light was considered as one enzyme unit. The estimation of proline content in fresh leaves samples was carried out according to Bates et al. (1973). Sulphosalicylic acid was used for sample extraction. Equivalent volume of glacial acetic acid and ninhydrin solutions were added to the extracted sample. The mixture was boiled at 100°C after adding 5 mL of toluene. The toluene layer absorption was recorded at 528 nm wavelength on a spectrophotometer.

Statistical analysis

Data were analyzed by using SPSS, 17.0 for Windows (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was achieved to examine vitiations between treatments at $P<0.05$, least significant difference (LSD) was used for comparison of the means.

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Results

Lipid peroxidation (LPO), H_2O_2 content and leaf water potential

It is evident from *Table 1* that LPO and H_2O_2 accumulation in plant leaves significantly increased with increasing of PD concentration. Plants treated by 1.0 ppm of PD accumulated 27.96 and 24.95% LPO and H_2O_2, respectively, as compared to control plants. However, plants treated by 1.0 ppm of PD accumulated higher content of LPO than H_2O_2. The plants treated by 0.005, 0.01, 0.1 or 1.0 ppm of PD had varied leaf water potential compared with control plants in a concentration dependent manner and maximum increase was noted at 0.1 ppm of PD (*Table 1*) whereas the minimum increase was noted at 1.0 ppm of PD.

Table 1. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on lipid peroxidation, H_2O_2 content and leaf water potential of Vigna radiata 30 days after sowing*

Treatment	Lipid peroxidation (n mol g^{-1} FM)	H_2O_2 content (n mol g^{-1} FM)	Leaf water potential (-MPa)
Control	8.01 ± 0.11 c	5.01 ± 0.05 c	- 0.84 ± 0.01 b
PD (0.005)	8.15 ± 0.10 c	5.12 ± 0.06 c	- 0.77 ± 0.01 c
PD (0.01)	8.97 ± 0.14 b	5.81 ± 0.05 b	- 0.69 ± 0.02 d
PD (0.1)	9.21 ± 0.15 b	5.61 ± 0.06 b	- 0.63 ± 0.01 e
PD (1.00)	10.25 ± 0.09 a	6.26 ± 0.05 a	- 0.92 ± 0.02 a
LSD	0.42	0.27	0.03
Significannce level	***	***	**
F- value	117.13	119.99	137.77

** P <0.01
*** P < 0.001

Proline and sugar content

The leaf proline and sugar content significantly increased with increasing concentrations of PD (0.005, 0.01, 0.1 or 1.0 ppm) but 1.0 ppm showed the highest increase of proline and sugar content by 29.95% and 25.96%, respectively, in comparison to control plant contents. Also catalase activity was increased by 31.87% and 24.49% in plants treated by 0.1 and 1.0 ppm PD, respectively, as compared to control plants (*Table 2*).

Table 2. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on proline content, sugar content and catalase activity of Vigna radiata 30 days after sowing*

Treatment	Proline content (µ mol g^{-1} FM)	Sugar content (mg g^{-1} FM)
Control	8.98 ± 0.10 e	25.11 ± 0.04 d
PD (0.005)	9.96 ± 0.14 d	28.12 ± 0.03 c
PD (0.01)	10.68 ± 0.15 c	29.12 ± 0.05 bc
PD (0.1)	11.67 ± 0.07 a	31.63 ± 0.04 a
PD (1.00)	11.13 ± 0.08 b	30.13 ± 0.02 b
LSD	0.52	1.04
Significance level	***	**
F- value	132.65	154.12

** P <0.01
*** P < 0.001

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Activities of antioxidants enzymes CAT, POX, and SOD

PD significantly affect the antioxidant enzyme activities (catalase/CAT, peroxidase/POX, and super oxide dismutase/SOD) in all plants treated by the PD, and the three enzyme activities increased with the increasing level of PD. The highest enzyme activities were recorded in plants treated by 0.1 ppm PD (*Table 3*). The highest activity rate for CAT, POX, and SOD were recorded at 0.1 ppm of PD in *Vigna radiata* plants demonstrating increases of 24.94%, 21.92%, and 17.51%, respectively, in comparison to the control plants.

Table 3. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on catalase, peroxidase activity, and superoxide dismutase activity of Vigna radiata 30 days after sowing*

Treatment	Catalase activity (m mol L^{-1} H_2O_2 decomposed g^{-1} FM)	Peroxidase activity	Superoxide dismutase activity
Control	298 ± 1.10 d	10.90 ± 0.10 e	177 ± 1.75 d
PD (0.005)	327 ± 2.10 c	12.20 ± 0.12 d	196 ± 1.95c
PD (0.01)	357 ± 2.22 b	12.82 ± 0.11c	205 ± 1.92 bc
PD (0.1)	393 ± 2.59 a	13.95 ± 0.13 a	221 ± 2.00 a
PD (1.00)	371 ± 3.09 b	13.29 ± 0.14 b	208 ± 1.99 b
LSD	11.9	0.46	9.09
Significance level	***	***	**
F- value	111.11	124.76	121.21

** P <0.01
*** P < 0.001

Discussion

Lipid peroxidation and H_2O_2 accumulation are considered biochemical markers for the free radical attributed injury under different environmental cues and external stimuli (Verma and Dubey, 2003). Furthermore, these biochemical processes are commonly defined by increasing formation of reactive oxygen species (ROS) (Foryer and Noctor, 2000; Gill and Tuteja, 2010). The results of this study, confirmed and verified this phenomenon, as our results showed an increasing accumulation level of lipid peroxidation, and H_2O_2 at the highest concentration (1 ppm) of PD (*Table 1*).

Therefore, it could be assumed that higher concentration would be toxic to *Vigna radiata* plants and lead to excess production of reactive oxygen species (ROS). ROS in plant tissues are regulated by plant antioxidant systems or antioxidant enzymes such as Catalase, Peroxidase, and Superoxide dismutase (Schutzendubel, 2002) as well as osmoprotectants i.e. proline (Szabados and Savouré, 2010). The present study revealed that treatment of plants with 0.1 ppm of PD boosted the activity of antioxidant enzymes (CAT, POX and SOD; *Tables 2 and 3*) along with the level of proline accumulation (*Table 2*). In the similar line with An et al. (2009) who found that the activities of peroxidase and superoxide dismutase significantly increased in the wheat plant when exposed to paracetamol in a duration dependent manner. Proline increase plant stress tolerance by maintaining NADPH/$NADP^+$ balance, GSH levels, and during infection, drives the oxidative rupture of the hypersensitive response (Miller et al., 2009; Ben Rejeb et al., 2014). As Szabados and Savouré (2010) confirmed proline as a signaling molecule to modest mitochondrial functions, and it activates specific gene expression that could be

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necessary for plant recovery in stress conditions. The findings of this study are in consistence with these previous studies, as this study showed that presence of PD significantly increased the proline accumulation and sugar content (*Table 2*). With cumulative effort of enhanced antioxidant system and proline accumulation under exogenously sourced PD i.e. paracetamol enhanced tolerance capacity of *Vigna radiata* plants to withstand various environment cues and helped in increasing their gross production.

In this study, we determined that the limit of PD in rivers and agricultural irrigation systems should be less than 1 ppm to decrease the negative effect of PD on mung bean plantation. Environmental Protection Agency and Saudi Government may use this limit as one of the allowable pharmaceutical water quality standard for the protection of the plant.

Conclusions

The highest concentration of panadol that induced oxidative stress was 1.0 ppm that was obvious through increased levels of lipid peroxidation, and H_2O_2 accumulation in *Vigna radiata* plants. On the other hand, 0.1 ppm of PD treatment improved the antioxidant system and the level of osmolytes to make the plants tolerate unfavorable conditions and helped in the increase of gross production through increasing the photosynthetic activity.

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