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Antioxidant enzyme activities and markers of oxidative stress in the life cycle of different Earthworm species

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Earthworms play a crucial role in soil fertility through decomposition, nutrient mineralization and water infiltration, and they are used as a standard organism in ecotoxicological testing. However, to use them for evaluations of environmental pollution, knowledge on the age-related variations in antioxidant enzymes within this species as they undergo different environmental conditions such as treatment to heavy metal insecticides, herbicides, salinity and polluted soil, vermifiltration etc. that cause stress. Due to oxidative stress, the growth and reproductive potential of earthworms are affected because of an imbalance between antioxidant enzymes and Reactive oxygen species (ROS). This study provides a fundamental understanding of the antioxidant enzyme activity and oxidative stress in three earthworm species (*Eisenia fetida*, *Eudrilus eugeniae* and *Pheretima posthuma*) at various stages of their lives. Before studying their usage as potential biomarkers, it is necessary to explore the age-related variations in antioxidant enzymes within these species which serve as the quintessential terrestrial invertebrates in evaluations of environmental pollution. The superoxide dismutase (SOD), catalase (CAT), ascorbic peroxidase (APX), and peroxidase (POD), as well as hydrogen peroxide (H_2O_2) as an oxidative stress marker, were measured in the total body of juvenile, sub-adult, and adult earthworms. SOD and CAT activities respectively declined and increased with age in all earthworm species and maximum SOD activity (4.86 U/g FW) and CAT activity (12.33 U/g FW) were found in respectively juvenile (EF- J) and adult (EF- A) stages of *E. fetida*. In *P. posthuma*, APX and POD activities rose with age. APX activity was significantly maximum (6.18 U/g FW) in *P. posthuma* adult stage (PP-A) whereas significant maximum POD activity (0.46±0.01ΔOD/min) in EF- J stage. H_2O_2, an oxidative stress marker, increased with age in all earthworm species. *P. posthuma* adult (PP-A) had the maximum activity (4.06 µmole/g FW), and EF- J life stage had the lowest activity (1.35 µmole/g FW). In response to increased oxidative stress (H_2O_2), the antioxidant enzymes (SOD, CAT, APX, and POD) work together. Overall, *Eisenia fetida* performed better than *E. eugeniae* and *P. posthuma.* This study fills the gaps in antioxidant enzyme activities at the different age stages of earthworms.

Keywords: *Eisenia fetida*, *Eudrilus eugeniae*, Oxidative stress marker, *Pheretima posthuma*, Reactive oxygen species (ROS)

The reactive oxygen species (ROS) and antioxidant defence systems must balance a cell's appropriate physiological functioning. ROS production is necessary for the cell's normal function; however, oxidative damage to proteins, nucleic acids, and lipids can occur under oxidative stress. An imbalance of oxidants and antioxidants causes oxidative stress. Hydrogen peroxide, superoxide radicals, hydroxyl radicals, and singlet molecular oxygen are ROS[1]. The oxidation of amino acid side chains and oxidatively damaged (carbonylated, cross-linked, or aggregated) proteins can result from an imbalance between antioxidant and pro-oxidant systems caused by an excess of ROS or their derivatives in the cell[2]. The antioxidant defence system is divided into water-soluble reductants like GSH and enzymes such as POD, SOD and CAT. A range of abiotic stressors can induce direct or indirect molecular damage by forming ROS[3].

Earthworms are the most prolific soil invertebrates and are used as a standard organism in eco-toxicological testing. They play a crucial role in soil fertility through decomposition, nutrient mineralization, and water infiltration so earthworms are known as ecosystem engineer[4]. Before studying their usage as potential biomarkers, it is necessary to explore the age-related variations in antioxidant enzymes within this species which serve as the quintessential terrestrial invertebrate in evaluations of environmental pollution[2,5]. Earthworms undergo

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different environmental conditions (like treatment to heavy metal insecticides, herbicides, salinity and polluted soil, vermifiltration, etc.) that cause stress. When earthworms were used as vermifiltration, they undergo oxidative stress and affect antioxidant enzyme (CAT, GSH and SOD) activities and ROS was enhanced[6]. Even due to oxidative stress, the growth and reproductive potential of earthworms were affected because of an imbalance between antioxidant enzymes and ROS[7,8]. After the exposure to flupyradifurone (insecticide), oxidative damage was observed in the earthworm with the change in antioxidant activities resulting in altered pathway of cell metabolic processes, immune system, environmental responses and ultimately DNA damage[9]. A significantly dose-dependent effect of formesafen (herbicide) was observed on the *E. fetida* antioxidant enzyme activities[10].

Many researchers used the different earthworm species to understand the ecotoxicological effect on the earthworm by considering the antioxidant activities as biomarkers. But these activities also vary in different earthworm species at various life stages. However, there are few investigations on changes in antioxidant enzyme activity (SOD, CAT, POD & APX) and oxidative stress (H_2O_2) over the life cycle of various earthworm species (*E. fetida*, *E. eugeniae* and *P. posthuma*). In this study, we have made an attempt to provide the reference value regarding antioxidant enzyme activities across different age stages of three earthworm species mentioned above.

Materials and Methods

Procurement of Earthworms

Identified five grams of each studied life stage of *E. fetida*, *E. eugeniae* and *P. Posthuma* were collected by hand sorting method from the vermicompost unit of the Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Hisar. To minimize pre-exposure or lingering impacts of any form of chemical, the earthworm culture was kept to employ third-generation earthworms in the departmental vermicompost unit.

Chemicals and Reagents

About 0.1 M cold phosphate buffer (pH 7.0 and 7.2); 0.2% O-dianisidine; 0.5%, t 0.2 M, and 35 mM H_2O_2; 1.2 mM and 3.0 mM EDTA; 1.80 mM NBT; 15 mM L- ascorbate;420 mM L-methionine; 5% potassium dichromate; 50 mM phosphate buffer (pH 6.5); 60 mMTris-HCl (pH 7.8); 90 mM riboflavin; dichromate reagent; glacial acetic acid

Preparation of earthworm tissue homogenates.

Three earthworm species (*E. fetida*, *E. eugeniae* and *P. posthuma*) were studied for antioxidant activity and oxidative stress at different life stages (juvenile, semi-adult, and adult). To facilitate the depuration of their gut contents, different earthworm species were collected and placed on wet filter paper in Petri plates for 12-24 h. One gram of gut-cleared earthworms was homogenized in a pre-chilled mortar and pestle in 5 mL of 0.1 M cold phosphate buffer (pH 7.2) under ice-cold conditions and centrifuged (Model-NT2178GK, Remi Elektrotechnik Limited, Vasai, India) at 10,000 g for 20 min at 4°C. For further investigation, the supernatant was kept at −60 °C.

Estimation of superoxide dismutase (SOD) activity

SOD was measured using a modified version of Beauchamp and Fridovich's method[11]. Add 2.5 mL of 60 mMTris-HCl (pH 7.8), 0.1 mL of 420 mM L-methionine, 0.1 mL of 90 mM riboflavin, 0.1 mL of 1.80 mM NBT, 0.1 mL of 3.0 mM EDTA, and 0.1 mL enzyme extract to make an assay mixture (3.0 mL). The tubes were set 30 cm below the three 20W fluorescent light sources. The reaction began by turning on the light and ended by turning it off after 40 min of incubation. The tubes were covered with black cloth to protect them from light when the reaction was completed. At 560 nm, the absorbance was measured. The amount of enzyme that inhibits the nitroblue tetrazolium photoreduction by 50% was defined as one enzyme unit. The activity of enzymes was measured in units g^{-1} FW.

Estimation of catalase (CAT) activity

Catalase was measured using a modified version of Sinha's[12] method. Add 0.55 mL of 0.1 M assay buffer (potassium phosphate buffer, pH 7.0), 0.4 mL of 0.2 M H_2O_2, and 50 µL of enzyme extract to make a 4.0 mL assay mixture. The reaction was stopped by adding 3 mL dichromate reagent (5 percent potassium dichromate and glacial acetic acid in a 1:3 ratio) and then placing the test tubes in a boiling water bath for 10 min. The test tubes were then cooled in water and the absorbance was measured at 570 nm. One unit of CAT activity was defined as the enzyme that catalyzed the oxidation of 1 µM H_2O_2 min^{-1}.

Estimation of Ascorbic peroxidase (APX) activity

The APX was measured using a modified version of the Nakano & Asada[13] method. Add 2.5 mL of 0.1 M potassium phosphate buffer with pH 7.0, 0.25 mL of 1.2 mM EDTA, 0.1 mL of 15 mM L- ascorbate, 0.1 mL of enzyme extract, and 0.05 mL of 35 mM hydrogen peroxide to the assay mixture (3.0 mL). At 290 nm, the absorbance was measured in a decreasing pattern. The enzyme activity was calculated using a molar extinction value of 2.8 mM^{-1} cm^{-1}. The amount of enzyme required to oxidize one nmole of ascorbic acid per minute at 290 is defined as one enzyme unit.

Estimation of peroxidase activity (POD)

POD was measured using a modified version of Shannon *et al.*[14] method. Add 50 mM phosphate buffer, pH 6.5 (2.75 mL), 0.5 percent H_2O_2 (0.1 mL), 0.2 percent O-dianisidine (0.1 mL), and enzyme extract (0.05 mL) to make the assay mixture. The reaction began with 0.1 mL of H_2O_2, and the assay blank was a mixture lacking H_2O_2. At 430 nm, the absorbance was measured for 3 minutes. A change in 0.01 absorbance/minute/mg protein was used to determine peroxidase activity.

Estimation of H_2O_2

The H_2O_2 was measured using Sinha[12] method. About 3 mL mixture of 5% (w/v) potassium dichromate and glacial acetic acid (1:3, v/v) was added to 0.4 mL of extract and 0.6 ml of 0.1 M phosphate buffer (pH 7.0). After 10 minutes in a boiling water bath, the tube was cooled. The absorbance at 570 nm was measured against a reagent blank. The amount of H_2O_2 was calculated using the H_2O_2 standard curve (0-160 μmole).

Results

In the same as well as in different species of earthworm, antioxidant enzyme levels were actively modulated with age.

Analysis of superoxide dismutase (SOD)

SOD activity declined with age in all earthworm species, with the highest levels found at the juvenile stage. *E. fetida* had the maximum activity followed by *E. eugeniae* and *P. posthuma*. *E. fetida* had the maximum activity (4.86 U/g FW) in the juvenile stage (EF-J), whereas *P. posthuma* had the lowest activity (2.00 U/g FW) at the semi-adult stage (PP-SA) as shown in Fig. 1A.

Analysis of catalase (CAT)

CAT activity increased with age in all earthworm species, peaking at adulthood. *E. fetida* exhibited maximum activity followed by *E. eugeniae* and

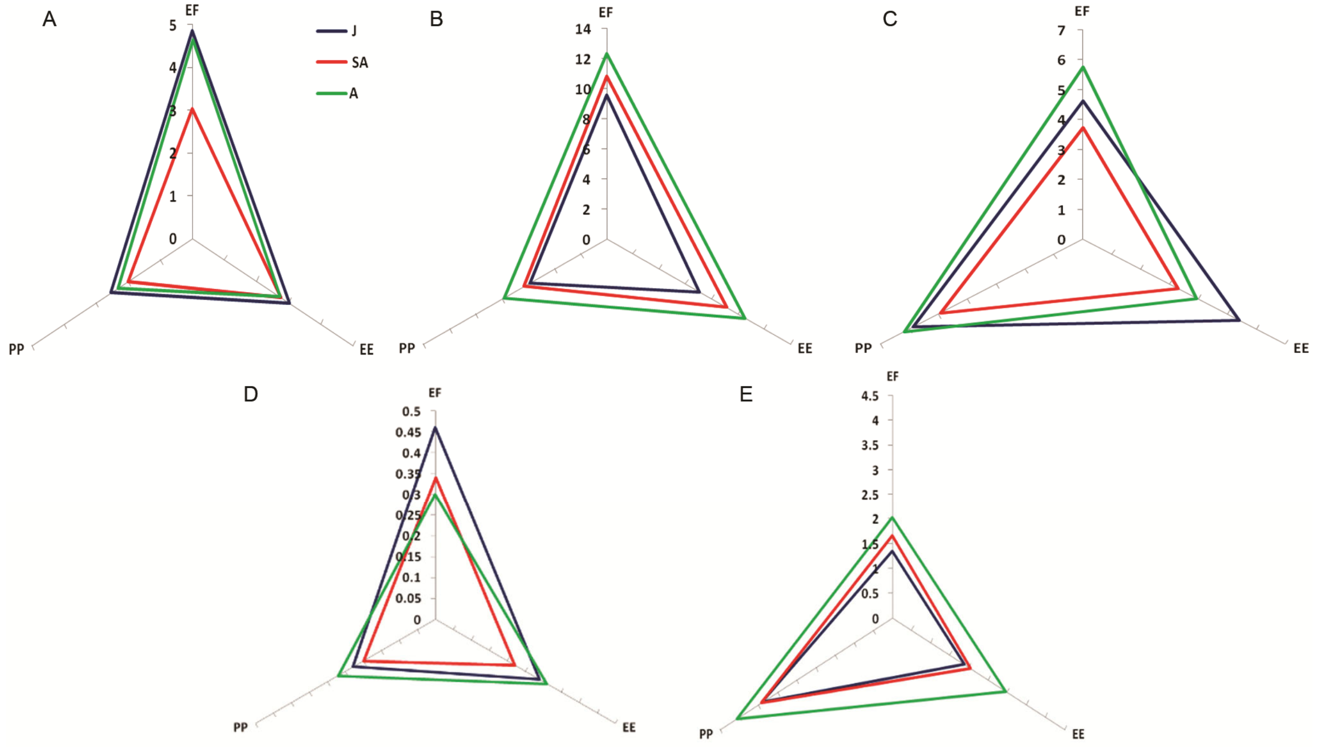

Fig. 1 — (A) SOD; (B) CAT; (C) APX; (D) POD; and (E) H_2O_2 activity (oxidative stress) at different life cycle stages of different earthworms. The values are mean ± SE of one gram of earthworms per age group. [EF, *Eisenia fetida*; EE, *Eudrilus eugeniae*; PP, *Pheretima posthuma*; J, Juveline; SA, Semi Adult; A, Adult]

P. posthuma. *E. fetida* had the maximum activity (12.33 U/g FW) in the adult (EF-A) stage, while *P. posthuma* had the lowest activity (5.84 U/g FW) at the juvenile stage (PP-J) as shown in Fig. 1B.

Analysis of ascorbic peroxidase (APX)

In *E. fetida* and *P. posthuma*, APX activity rose with age, whereas in *E. eugeniae*, it decreased. *P. posthuma* displayed the maximum activity among the earthworm species at the adult stage, followed by *E. fetida* and *E. eugeniae*. APX activity was significantly higher (6.18 U/g FW) in *P. posthuma* adult stage (PP-A) than in the other earthworm life stages as shown in Fig. 1C.

Analysis of peroxidase (POD)

In *E. eugeniae* and *P. posthuma*, POD activity rose with age, whereas in *E. fetida*, it decreased. The highest activity (0.46±0.01ΔOD/min) was found in the EF-J life stage, while the lowest activity (0.20 ΔOD/min) was found in the PP-SA life stage as shown in Fig. 1D.

Analysis of hydrogen peroxide (H_2O_2)

H_2O_2, an oxidative stress marker, increased with age in all earthworm species. *P. posthuma* had the maximum content followed by *E. eugeniae* and *E. fetida*. The PP-A life stage had the highest activity (4.06 µmole/g FW), whereas the EF- J life stage had the lowest activity (1.35 µmole/g FW) as shown in Fig. 1E.

Comparison between antioxidant enzymes of different earthworm species

Comparison between the different antioxidant enzymes of one species was calculated at $P < 0.01$ and $P < 0.05$ through Pearson Correlation. *E. fetida* APX activity has a significant positive correlation with SOD (0.90) and CAT (0.77). SOD had a positive non-significant correlation with CAT (0.435) and POD (0.064) as shown in Table 1. In *E. eugeniae* SOD had a significant positive correlation (0.801) with APX activity whereas CAT activity have a significant negative correlation with SOD (-0.726) and APX (-0.712) activities as shown in Table 1. In *P. posthuma* APX activity have a significant positive correlation with SOD (0.766) and POD (0.818) as shown in Table 1. Overall, H_2O_2 had a non-significant negative correlation with all the antioxidant enzymes.

Discussion

The free radical hypothesis of ageing describes the harmful effects of free radicals in the ageing process. Free radicals induce stress in the cell by causing non-specific damage to macromolecules like DNA, lipids, and proteins. According to the notion, a cell's ability to resist or prevent oxidative stress and have an antioxidant mechanism to counteract it is a significant predictor of animal longevity[15].

Table 1 — Pearson correlation between the different antioxidant enzymes in different earthworm specis

Enzymes	SOD	CAT	APX	POD	H_2O_2
Eisenia fetida					
SOD	1				
CAT	0.435	1			
APX	0.900**	0.770*.	1		
POD	0.064	-0.628	-0.316	1	
H_2O_2	-0.604	-0.017	-0.472	-0.019	1
Eudrilus eugenie					
SOD	1				
CAT	-0.726*	1			
APX	0.801**	-0.712*	1		
POD	0.176	0.088	0.493	1	
H_2O_2	-0.195	0.015	-0.573	-0.928**	1
Pheretima posthuma					
SOD	1				
CAT	-0.105	1			
APX	0.766*	0.387	1		
POD	0.366	0.520	0.818**	1	
H_2O_2	-0.618	-0.012	-0.581	-0.634	1

[**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed)]

Oxidative stress (H_2O_2) levels rise with age, indicating that adults are more stressed than the other two life stages (Juvenile and semi-adult). Our findings on SOD and CAT activities are consistent with those of Kiran & Aruna[1]. They found that SOD activity declined dramatically from juvenile to adulthood, although CAT activity increased with age. The results of CAT activity resemble those of Saint-Denis *et al.*[16], who explored how CAT activity increased linearly to prevent oxidative stress by H_2O_2 in the earthworm *E. fetida*. POD activity was also elevated in *E. eugeniae* and *P. posthuma* in response to H_2O_2 since POD uses H_2O_2 as a substrate and shields the cell from oxidative damage[3]. APX enzyme activity increased with age in *E. fetida* and *P. posthuma* to counteract oxidative damage caused by H_2O_2. Wang *et al.*[2] found a similar result when oxidative stress was generated by increasing ciprofloxacin concentration.

To prevent oxidative stress, different earthworm species try to balance different antioxidant systems. To counter oxidative stress and preserve proper physiological functioning of the cell, the activity of some enzymes decreases with age, while the activity of others increases[1]. Similarly, Liu *et al.*[3] discovered antioxidant enzyme cooperation in response to oxidative stress. The antioxidant enzymes SOD, CAT, APX and POD have a mutual synergistic impact in scavenging free radicals created by normal metabolism or environmental stress to ensure the normal physiological metabolism of living organisms[17,18]. Zhao *et al.*[19] also discovered that in response to oxidative stress generated by Perfluorooctanoic acid, a balance was maintained between different enzyme (SOD, CAT and POD) activities in *E. fetida* by rising and reducing their value with days. Within three days, Liu *et al*.[3] detected variations in the activities of SOD (16.10 to 22.75 U/mg protein), CAT (70 to 95 U/mg protein), and POD (39 to 53 U/mg protein) in *E. fetida* (control). These data revealed that antioxidant capabilities vary with age in earthworm species under control. Song *et al.*[17]; Kiran & Aruna[1] also showed that antioxidant activity in different earthworm species changed with time.

The SOD, CAT, APX and POD antioxidant enzymes were engaged in antioxidant defence against reactive oxygen species (ROS) caused by stress[20]. SOD catalyzes the dismutation of O_2^- into H_2O_2 and O_2[21,22]. SOD absorbs two molecules of superoxide, then removes the excess electron from one and transfers it to the other. As a result, one has one less electron and forms O_2, while the other has one extra electron. CAT then picks up the additional electron and deactivates H_2O_2, preventing macromolecules like lipids, proteins, and DNA[23,24]. POD can also degrade H_2O_2 by oxidising ascorbate and guaiacol[25]. As a result, antioxidant enzyme activities are strong chemical markers of oxidative stress.

All of the earthworm species studied had higher levels of H_2O_2, indicating that adults are vulnerable to oxidative stress. The examined antioxidant enzymes work together to cope with oxidative stress, and the amounts of antioxidant enzymes differed between species. *Eisenia.* fetida exhibits higher antioxidant enzyme activity than the other earthworm species tested (*E. eugeniae* and *P. posthuma*).

Different earthworm species show a significant Pearson correlation between the studied antioxidant enzyme activities (Table 1). A positive value of Pearson correlation of antioxidant enzyme activities signifies a positive linear correlation whereas a negative value shows a negative linear correlation[26]. Stress is increased in the form of H_2O_2 with age which signifies the scavenging ability of antioxidant enzymes decreased with age. Our findings match with the findings of Kiran & Aruna[1]. In the biological system, ecotoxicological effect can be measured by antioxidant enzyme activities in the altered pathway of cell metabolic processes, immune system, environmental responses and DNA[9]. A negative correlation was observed between POD and alkaline phosphatase activities which signified that phenanthrene modified the biological characteristics by affecting the antioxidant and digestive system of *E. fetida* cast[27]. Earthworm behaviour is also associated with antioxidant enzyme activities. CAT, GSH, MDA and ROS significantly correlated with the daily burrowing length of vermifilter of *E. fetida*[6].

Conclusion

All of the earthworm species studied had higher levels of H_2O_2, indicating that adults are vulnerable to oxidative stress and maximum (4.06 µmole/g FW) and minimum (1.35 µmole/g FW), respectively were reported in PP- A and EF- J stages. The examined antioxidant enzymes work together to cope with oxidative stress. The results suggest as earthworms grow older, oxidative damage increases which justifies the ageing process of an organism. The amounts of antioxidant enzymes differed between species, with maximum SOD activity (4.86 U/g FW), CAT activity (12.33 U/g FW) and POD activity (0.46±0.01ΔOD/min) found in *E. fetida* whereas maximum APX activity was reported in *P. posthuma*. The study also suggests antioxidant enzyme activities work in a correlated manner to maintain the body function.

Conflict of Interest

Authors declare no competing interests.

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