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ORIGINAL PAPER

Antioxidant response of *Cassia angustifolia* Vahl. to oxidative stress caused by Mancozeb, a pyrethroid fungicide

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Abstract Seeds of *Cassia angustifolia* Vahl., treated with various concentrations (0, 0.1, 0.15, 0.2 and 0.25 %) of Mancozeb, a broad-spectrum contact fungicide, were sown in field conditions to study the effect of the treatments on lipid peroxidation, proline accumulation and modulation of antioxidant system of seedlings obtained. Significant increase over the control was observed in treated plants for thiobarbituric acid-reactive substances content (up to 207 %), proline content (96 %) and total glutathione content (144 %), whereas the total ascorbate content decreased by 44 %. Increased enzymatic activity was recorded for ascorbate peroxidase (63 %), glutathione reductase (154 %) and superoxide dismutase (109 %), whereas catalase activity decreased by 58 % with 0.25 % Mancozeb treatment. The changes observed were dose-dependent, showing a strong correlation with the level of treatment.

Keywords Antioxidant defence system · Enzyme activity · Lipid peroxidation · Senna plant · Stress management

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DAS	Days after sowing
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
EBDC	Ethylenebisdithiocarbamate
ETU	Ethylene thiourea
GSH	Reduced glutathione
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
MDAR	Mono-dehydroascorbate reductase
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances

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Introduction

Plants subjected to environmental stress often suffer from oxidative damage, because the balance between the production of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical, and the quenching activity of antioxidants is upset (Scandalios 1993). Pesticides are known to induce oxidative stress in plants (Asada and Takahashi 1987; Halliwell 1987; Bashir et al. 2007), either by overproduction of free radicals or by alteration in antioxidant enzymes. Plants have evolved mechanisms to protect the cellular and subcellular systems from the effects of ROS through modulation of enzymatic (like APX, GR, SOD, CAT) and non-enzymatic (like ascorbate and glutathione) compounds. Changes in these antioxidant compounds are indicative of oxidative stress caused by different stressors including fungicides (Wauchope et al. 1992).

Use of fungicides against diseases of agricultural crops is common all over the world. Although fungicide application quickly controls the disease, it also imparts adverse

effects on plants. Mancozeb, showing activity of ethylenebisdithiocarbamate (EBDC), is a broad-spectrum contact fungicide of the chemical family Pyrethroids and has empirical formula $C_{23}H_{19}Cl\ F_3NO_3$. It is commonly used to control a variety of diseases such as early and late blights, rusts, black rots, downy mildews, black spots, gummy stem blights and leaf molds, and applied to a wide range of food/feed crops and sod farms. Mancozeb rapidly and spontaneously degrades to ethylene thiourea (ETU), which may persist for long (Wauchope et al. 1992). The present study was undertaken with *Cassia angustifolia* Vahl. to examine the effects of Mancozeb, a frequently used contact fungicide, in terms of oxidative stress figured in plant cells and the consequent modulation of enzymatic and non-enzymatic components of antioxidant defence system of the species.

Materials and methods

Certified healthy seeds of Indian Senna (*C. angustifolia* Vahl.), procured from Indian Agricultural Research Institute, New Delhi, were sown in the Kharif season (July–October) at the experimental field of Hamdard University. Each plot (4 m × 1.5 m) carried five rows of seedlings maintaining a 15-cm distance between rows and 10-cm distance between plants of a row. The experiment was conducted in three replications. Prior to sowing, seeds were treated with five (0–0.25 %) Mancozeb concentrations prepared in double distilled water, which were designated as Control (0 %), T_1 (0.10 %), T_2 (0.15 %), T_3 (0.20 %) and T_4 (0.25 %). The control as well as the treated plants were maintained at a uniform water supply. Sampling was done at three stages of plant developmental, i.e., at pre-flowering (45 DAS = days after sowing), flowering (75 DAS) and post-flowering (90 DAS) stages, from six plants of each treatment raised in three sets. The samples were either used immediately or stored in deep freezer (at −80 °C) for use in future.

Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS), considered as "oxidative damage products", were determined in leaf samples by the method of Heath and Packer (1968). The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ and expressed as nmol TBARS g^{-1} fresh weight.

Proline content

Proline content in leaf samples was estimated by the method of Bates et al. (1973). The corresponding concentration of proline was determined against the standard curve of L-proline.

Enzyme assays

Activity of ascorbate peroxidase (EC 1.11.1.11) was determined by the method of Nakano and Asada (1981), on the basis of decrease in absorbance of ascorbate in supernatant due to its enzymatic breakdown, as observed at 290 nm. The activity was calculated using the coefficient of absorbance 2.8 $mM^{-1}\ cm^{-1}$. One unit of enzyme indicates the amount of enzyme necessary to decompose 1 μmol of ascorbate per min.

Activity of glutathione reductase (EC 1.6.4.2) was determined by the method of Foyer and Halliwell (1976) modified by Rao (1992). It was assayed through glutathione-dependent oxidation of NADPH at 340 nm, and calculated using the coefficient of absorbance of 6.2 $mM^{-1}\ cm^{-1}$. One unit of enzyme means the amount of enzyme necessary to decompose 1 μmol of NADPH per min.

Activity of superoxide dismutase (EC 1.15.1.1) was determined by the method of Dhindsa et al. (1981). The activity was assayed by the ability of SOD to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The amount of enzyme required to bring about fifty percent reduction of NBT was considered as one unit of enzyme activity.

Activity of catalase (EC 1.11.1.6) was determined by the method of Aebi (1984) by monitoring the disappearance of hydrogen peroxide (H_2O_2) in supernatant and measuring the decrease in absorbance at 240 nm. The activity was calculated using the coefficient of absorbance 0.036 $mM^{-1}\ cm^{-1}$. One unit of enzyme is the amount of enzyme necessary to decompose 1 μmol of H_2O_2 per min.

Ascorbate content

Ascorbate content was determined by the method of Law et al. (1983). The ascorbate (Asc) and total ascorbate (Asc + DHA) were assayed separately and the absorbance was recorded at 525 nm. A standard curve in the range of 0–100 nmol L^{-1} of Asc was used for calibration. Values in both cases were corrected for the absorbance by eliminating the supernatant in the blank prepared separately for ascorbate and total ascorbate. The difference of the total ascrbate and the ascorbate gave the amount of dehydroascorbate (DHA).

Glutathione content

Glutathione content was estimated following the method of Anderson (1985), using acid ninhydrin, glacial acetic acid and toluene as chemical agents to be added to the supernatant of the fresh leaf homogenate. Toluene layer was

Table 1 TBARS content of leaves (nmol g^{-1} fr. wt.), as recorded at various stages of growth of *Cassia angustifolia* plants raised from the control and Mancozeb-treated seeds

Treatments (%)	Plant growth stages		
	Pre-flowering	Flowering	Post-flowering
Control	9.25 ± 0.015 (0.0)	10.34 ± 0.21 (0.0)	25.19 ± 0.003 (0.0)
0.10 %	10.24 ± 0.027[a] (10.7)	13.44 ± 0.021[a] (29.98)	25.64 ± 0.024[a] (1.78)
0.15 %	16.32 ± 0.015[a,b] (76.43)	20.64 ± 0.018[a,b] (99.61)	30.23 ± 0.014[a,b] (20.01)
0.20 %	21.94 ± 0.012[a,b,c] (137.22)	27.23 ± 0.011[a,b,c] (163.34)	37.44 ± 0.014[a,b,c] (48.63)
0.25 %	22.04 ± 0.015[a,b,c,d*] (138.34)	31.76 ± 0.011[a,b,c,d] (207.15)	40.14 ± 0.024[a,b,c,d] (59.35)

Values indicate mean ± SE. Parentheses include percent variation

CD at 5 %: plant growth stages: 0.021, treatments: 0.027, treatment × growth stages: 0.047

[a] $P < 0.001$ vs control, [b] $P < 0.001$ vs 0.10 %, [c] $P < 0.001$ vs 0.15 %, [d] $P < 0.001$ vs 0.20 %, [d*] $P < 0.05$ vs 0.20 %

Table 2 Variation in proline content of leaves (μg g^{-1} fr. wt.), as recorded at various stages of growth of *Cassia angustifolia* plants, raised from the control and Mancozeb-treated seeds

Treatments (%)	Developmental stages		
	Pre-flowering	Flowering	Post-flowering
Control	23.22 ± 0.913 (0.0)	76.41 ± 0.19 (0.0)	121.53 ± 0.332 (0.0)
0.10 %	36.19 ± 0.631[a] (55.81)	77.43 ± 0.165[a*] (1.34)	127.29 ± 0.761[a] (4.73)
0.15 %	40.29 ± 0.093[a,b*] (73.47)	78.47 ± 0.167[a,b**] (2.69)	144.47 ± 0.347[a,b] (18.87)
0.20 %	43.64 ± 0.168[a,b,c*] (87.91)	80.44 ± 0.193[a,b,c] (5.28)	152.50 ± 0.439[a,b,c] (25.48)
0.25 %	45.50 ± 0.725[a,b,c,d*] (95.89)	86.31 ± 0.19[a,b,c,d] (12.94)	161.37 ± 0.853[a,b,c,d] (32.77)

Values indicate mean ± SE. Parentheses include percent variation

CD at 5 %: plant growth stages: 0.613, treatments: 0.792, treatment × growth stages: 1.37

[a] $P < 0.001$ vs control, [a*] $P < 0.05$ vs Control, [b] $P < 0.001$ vs 0.10 %, [b*] $P < 0.01$ vs 0.10 %, [b**] $P < 0.05$ vs 0.10 %, [c] $P < 0.001$ vs 0.15 %, [c*] $P < 0.05$ vs 0.15 %, [d] $P < 0.001$ vs 0.20 %, [d*] $P < 0.05$ vs 0.20 %

separated from the mixture and the absorbance recorded at 520 nm on a Beckman's spectrophotometer, using toluene as blank.

Detailed procedures of all these techniques have been described by Arshi et al. (2012) and Umar et al. (2011).

Statistical analysis

The data obtained were put to statistical test using the software package SPSS version 14 (SPSS Inc, Chicago, USA). One-way analysis of variance (ANOVA) was conducted with a Tukey–Kramer multiple comparison post-test. Values presented for each treatment and each developmental stage are mean ± SE of three samples each with three replicates.

Results

The content of TBARS increased with age of the plant. It was enhanced significantly in the Mancozeb-treated plants, showing a concentration-dependent response to the treatment. The TBARS accumulated maximally with 0.25 % Mancozeb concentration (T4), showing up to 207 % increase over the control (Table 1).

Proline content also showed similar trend of variation, increasing significantly with plant age as well as with increase in Mancozeb concentration. The maximum effect, up to nearly 96 %, appeared with 0.25 % mancozeb application, as compared with the control (Table 2).

Activity of antioxidant enzymes, like ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) initially increased in *C. Angustifolia* with growing age of the plant, attained the maximum at flowering stage, and then declined sharply. Application of Mancozeb significantly enhanced the activity of APX, GR and SOD over the control, corresponding to the dose applied. Percent variation from the control was the maximum (by 63 % for APX, 154 % for GR and 109 % for SOD) at pre-flowering stage with T4 treatment, although the highest activity (1.88 μmol mg^{-1} protein min^{-1} for APX, 0.824 μmol mg^{-1} protein min^{-1}

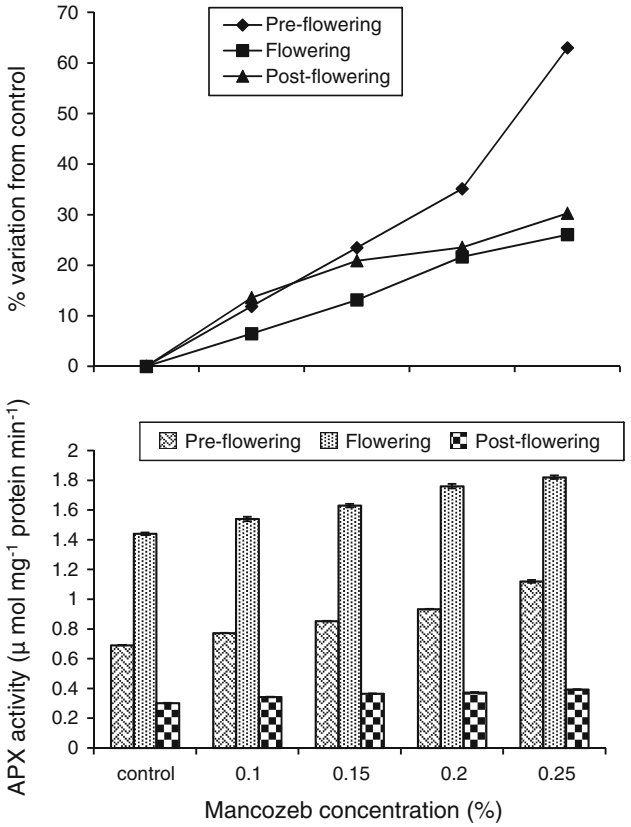

Fig. 1 Ascorbate peroxidase activity as observed in the control as well as Mancozeb-treated plants of *Cassia angustifolia* sampled at pre-flowering, flowering and post-flowering stages. *Bar diagrams* show the level (mean ± SE) of enzyme activity, while *graphs* depict the percent variation from the control

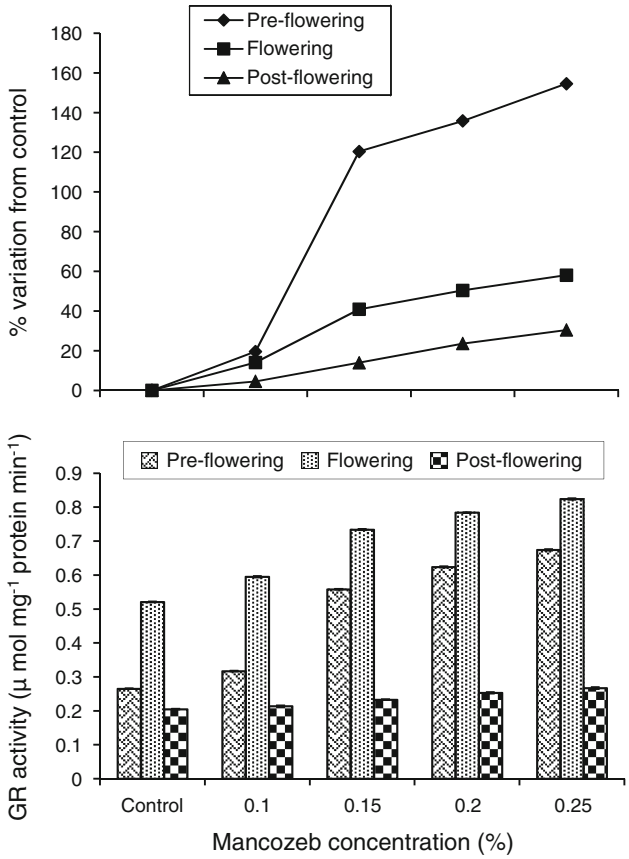

Fig. 2 Glutathione reductase activity as observed in the control as well as Mancozeb-treated plants of *Cassia angustifolia* sampled at pre-flowering, flowering and post-flowering stages

for GR and 14.00 EU mg^{-1} protein min^{-1} for SOD) was recorded at the flowering stage (Figs. 1, 2, and 3).

Catalase activity also exhibited similar trend of variation with reference to plant age, i.e., a rise up to flowering stage and then a sharp decline in post-flowering stage. However, it showed a dose-dependent decrease under Mancozeb stress; the maximum decline coming with 0.25 % concentration (T4). In this case, percent variation attained its maximum (58 %) during post-flowering stage (Fig. 4).

The ascorbate and glutathione contents (non-enzymatic antioxidants) exhibited similar variation trend with respect to age of the plant, i.e., initial increase up to the flowering stage followed by a sharp decline in the post-flowering phase of the plant life. However, the two compounds differed in their response to Mancozeb treatment. The ascorbate content decreased, whereas the glutathione content increased over the control under Mancozeb stress. The maximum decline (over 44 %) in ascorbate content was observed with 0.25 % Mancozeb treatment (T4) at the post-flowering stage, followed by the flowering, and thus leaving the minimum for pre-flowering stages (Table 3). Likewise, the dose-dependent increase in response to the Mancozeb stress also touched its vertex (nearly 144 %) with T4 at the post-flowering stage. However, the minimum variation was observed here at the flowering stage (Table 4).

Discussion

Herbicides are known to generate activated oxygen species, which possibly contribute to the toxic effects of these herbicides (Asada and Takahashi 1987; Halliwell 1987). Increase in lipid peroxidation rate is regarded as a general response to many stresses like heavy metals (Ansari et al. 2009; Khan et al. 2009; Diwan et al. 2010a, b), high salinity (Hernandez et al. 2000; Arshi et al. 2010), and low temperature (Rodionov et al. 1973). Lipid peroxidation, due to increased production of toxic oxygen-free radicals is used as a marker of oxidative stress (Huggett et al. 1992; Chagas et al. 2008). TBARS, the cytotoxic products of lipid peroxidation, are the major TBA-reacting compounds that indicate the magnitude of oxidative stress (Qadir et al. 2004; Qureshi et al. 2005, 2007; Bashir et al. 2007).

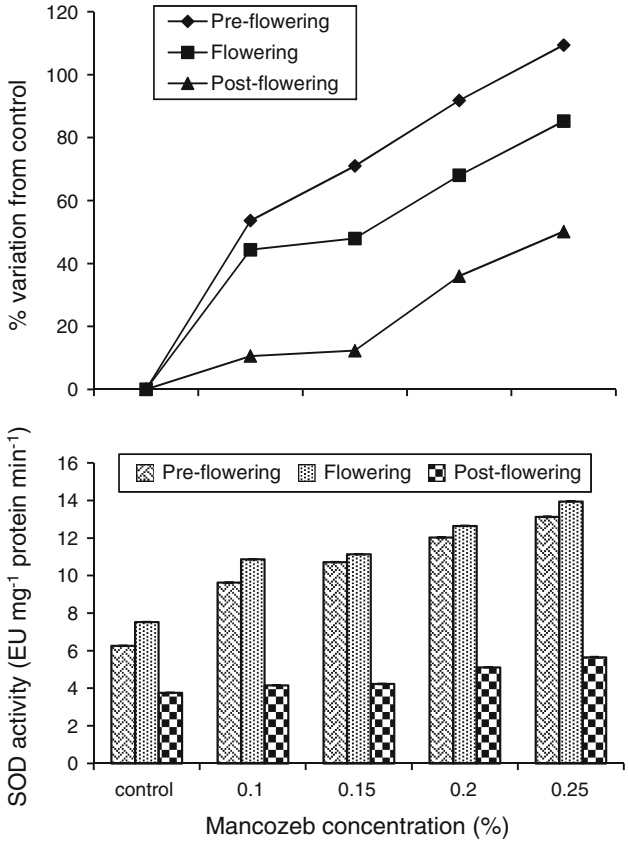

Fig. 3 Superoxide dismutase activity as observed in the control as well as Mancozeb-treated plants of *Cassia angustifolia* sampled at pre-flowering, flowering and post-flowering stages

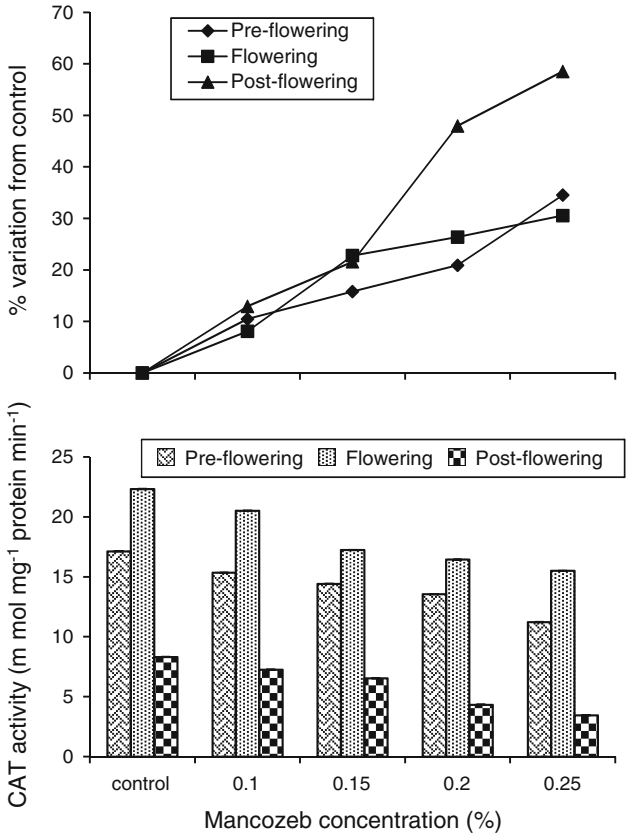

Fig. 4 Catalase activity as observed in the control as well as Mancozeb-treated plants of *Cassia angustifolia* sampled at pre-flowering, flowering and post-flowering stages. *Bar diagrams* show the level (mean ± SE) of enzyme activity, while *graphs* depict the percent variation from the control

Increased concentration of TBARS in *C. angustifolia* due to Mancozeb treatment confirms the occurrence of intracellular oxidative stress.

One of the diverse roles of proline in plants is to protect cellular functions by scavenging reactive oxygen species (Delauney and Verma 1993). Since plants respond to a variety of environmental stress by accumulating specific metabolites such as proline (Babber and Varghese 1996; Arshi et al. 2010), increased proline accumulation in pesticide-treated plants indicates that pesticides cause stress situations (Bashir et al. 2007), as do environmental aberrations. Proline may affect solubility of various proteins and protect them against denaturation under stressful conditions (Alia et al. 1994). Increase in proline content may be linked to stimulated proline oxidation or impaired protein synthesis (Delauney and Verma 1993).

Ascorbate peroxidase protects the cell from oxidative damage by detoxifying the toxic H_2O_2 (Morimura et al. 1996). The increase in APX activity due to Mancozeb treatment is suggestive of its role in detoxification of H_2O_2 under pesticide-induced oxidative stress. GR usually operates in cycle with APX; increased GR activity with increase in concentration of Mancozeb possibly shows that either (1) the Asc–Glu cycle was operative at a high rate to detoxify the ROS, or (2) the reduced glutathione pool was maintained at a high level to ensure synthesis of phytochelatins (Cobbett 2000; Stolt et al. 2003) and inactivate pesticides by conjugate formation. Our observations on GR activity confirm some earlier reports of GR upregulation during oxidative stress (Reddy et al. 2005). Superoxide dismutase (SOD) also plays a significant role in mitigating and repairing the damage caused by the ROS (Bowler et al. 1992). The increased SOD activity in Mancozeb-treated plants could be because of a de novo synthesis of enzymatic protein (Slooten et al. 1995; Allen et al. 1997). Many other stressors have a similar effect on SOD (Anjum et al. 2008, 2011, 2012; Khan et al. 2009; Diwan et al. 2010a, b; Hameed et al. 2011).

Catalase metabolizes peroxides liberated in peroxisomes on conversion of glycolate during photorespiration. It catalyzes conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Chelikani et al. 2004). The significant dose-dependent decrease in CAT activity at different stages of plant development

Table 3 Variation in total ascorbate content (m mol g^{-1} fr. wt.), as recorded at various stages of growth of *Cassia angustifolia* plants raised from the control and Mancozeb-treated seeds

Treatments (%)	Developmental stages		
	Pre-flowering	Flowering	Post-flowering
Control	8.09 ± 0.009 (0.0)	10.87 ± 0.006 (0.0)	5.34 ± 0.003 (0.0)
0.10 %	7.30 ± 0.015[a] (9.83)	8.32 ± 0.013[a] (23.42)	4.09 ± 0.01[a] (23.45)
0.15 %	7.25 ± 0.027[a] (10.37)	8.03 ± 0.015[a,b] (26.03)	3.80 ± 0.006[a,b] (28.69)
0.20 %	6.58 ± 0.032[a,b,c] (18.65)	7.42 ± 0.013[a,b,c] (31.7)	2.99 ± 0.01[a,b,c] (43.85)
0.25 %	6.13 ± 0.021[a,b,c,d] (24.16)	6.88 ± 0.02[a,b,c,d] (36.67)	2.96 ± 0.054[a,b,c] (44.56)

Values indicate mean ± SE. Parentheses include percent variation

CD at 5 %: plant growth stages: 0.027, treatments: 0.035, treatment × growth stages: 0.062

[a] $P < 0.001$ vs control, [b] $P < 0.001$ vs 0.10 %, [c] $P < 0.001$ vs 0.15 %, [d] $P < 0.001$ vs 0.20 %

Table 4 Variation in total glutathione content (nmol g^{-1} fr. wt.), as recorded at various stages of growth of *Cassia angustifolia* plants raised from the control and Mancozeb-treated seeds

Treatments (%)	Developmental stages		
	Pre-flowering	Flowering	Post-flowering
Control	648.33 ± 1.66 (0.0)	836.32 ± 2.4 (0.0)	428.66 ± 0.88 (0.0)
0.10 %	771.99 ± 2.00[a] (19.07)	1,194.33 ± 2.02[a] (42.8)	575.66 ± 2.96[a] (34.52)
0.15 %	889.66 ± 1.76[a,b] (37.22)	1,271.00 ± 1.15[a,b] (51.97)	616.66 ± 3.18[a,b] (43.85)
0.20 %	1,195.66 ± 2.9[a,b,c] (84.42)	1,538.66 ± 0.33[a,b,c] (83.97)	877.33 ± 1.45[a,b,c] (104.66)
0.25 %	1,474.32 ± 0.67[a,b,c,d] (127.4)	1,597.66 ± 3.18[a,b,c,d] (91.03)	1,045.32 ± 3.18[a,b,c,d] (143.85)

Values indicate mean ± SE. Parentheses include percent variation

CD at 5 %: plant growth stages: 2.62, treatments: 3.38, treatment × growth stages: 5.86

[a] $P < 0.001$ vs control, [b] $P < 0.001$ vs 0.10 %, [c] $P < 0.001$ vs 0.15 %, [d] $P < 0.001$ vs 0.20 %

could be due to disturbed enzyme synthesis or assembly of enzyme subunits (MacRae and Fergusam 1985; Somashekaraiah et al. 1992). Alternatively, a flux of superoxide radicals could inhibit CAT activity (Kono and Fridovich 1982). In any case, the situation confirms that pesticides cause ROS formation (Scandalios 1992, 1993; Bashir et al. 2007). Intensity of stress, time of assay after stress, and induction of new isozymes may affect CAT activity (Shim et al. 2003).

Concentration of non-enzymatic cellular antioxidants, such as ascorbate and glutathione, changes under oxidative stress. Causes of depletion or decline of ascorbate content include Mancozeb-caused disturbance with glutathione (GSH)-independent DHAR, structural integrity of MDAR or activity of PS-I. On the other hand, enhanced GSH level under Mancozeb stress is suggestive of an active GSH participation in detoxification of oxygen species and free radicals. On the basis of similar observations, Nair et al. (2012) have concluded that Triadimefon, a triazole fungicide, increases the antioxidant capacity of *Ocimum tenuiflorum* by enhancing the response of various components of antioxidant defence system.

Conclusion

Our results suggest that a prolonged plant exposure to pesticides enhances lipid peroxidation rate, indicating degradation of cell membrane system, which would disturb the key metabolic processes in plants and enhance ROS production. The induced oxidative stress in *C. angustifolia* showed positive correlation with Mancozeb concentration applied, as was evident from the modulation of some enzymatic and non-enzymatic components of antioxidant defence system, to protect the plant from oxidative damage by ROS. The decline in CAT activity was indicative of disturbance in the synthesis or assembly of subunits of the enzyme.

Author contribution All authors have made equal contribution. U Majid and Mahmooduzzafar have been concerned mainly with the experimental work and artwork (drawing of Figures). TO Siddiqi and M Iqbal have planned and supervised the experiment, applied statistical treatment and developed the paper manuscript for submission to the journal.

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Conflict of interest All the four authors have contributed equally towards the development of this manuscript. We declare that we have no clash of interest.

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