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Research article

ROS management is mediated by ascorbate-glutathione-α-tocopherol triad in co-ordination with secondary metabolic pathway under cadmium stress in *Withania somnifera*

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ABSTRACT

Being static, plants are frequently exposed to various essential and non-essential heavy metals from the surroundings. This exposure results in considerable ROS generation leading to oxidative stress, the primary response of the plants under heavy metal stress. *Withania somnifera* is a reputed Indian medicinal plant in Ayurveda, having various pharmacological activities due to the presence of withanolides. The present study deals with the understanding endurance of oxidative stress caused by heavy metal exposure and its management through antioxidant partners in synchronization with secondary metabolites in *W. somnifera*. The quantitative assessment of enzymatic/non-enzymatic antioxidants revealed significant participation of ascorbate-glutathione-α-tocopherol triad in ROS management. Higher activities of glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) resulted in glutathione and ascorbate accumulation. In addition, superoxide dismutase (SOD), glutathione peroxidase (GPX) and peroxidase (POD) were contributed considerably in ROS homeostasis maintenance. *In-situ* localization and assays related to ROS generation/scavenging revealed key management of ROS status under Cd stress. Higher antioxidative and reducing power activity attributed to the tolerance capability to the plant. Increased expression of withanolide biosynthetic pathway genes such as *WsHMGR, WsDXS, WsDXR* and *WsCAS* correlated with enhanced withanolides. The present study indicated the crucial role of the ascorbate-glutathione-α-tocopherol triad in co-ordination with withanolide biosynthesis in affording the oxidative stress, possibly through a cross-talk between the antioxidant machinery and secondary metabolite biosynthesis. The knowledge may be useful in providing the guidelines for developing abiotic stress resistance in plants using conventional and molecular approaches.

1. Introduction

Cadmium is a non-essential heavy metal and exhibits severe toxic effects on plant as well as human health (Flores-cáceres et al., 2015). Cd is taken up by the root system of the plants from their surroundings and majorly accumulated in roots and further transported to aerial parts (Bari et al., 2019). The growth and metabolism of the *Withania somnifera* were reported to adversely affected by Cd exposure (Mishra et al., 2014; Mishra and Sangwan, 2019). The generation of the excessive amount of reactive oxygen species (ROS) against heavy metal stress exhibited negative and toxic effects on various macromolecules including protein, chlorophyll, lipid and nucleic acids (Foyer and Noctor, 2005; Andresen and Küpper, 2013; Mishra et al., 2014). ROS are constantly produced as a by-product of aerobic metabolism in

Abbreviations: APX, ascorbate peroxidases; ASA, ascorbate; CAT, catalase; CAS, cycloartenol synthase; Cd, cadmium; DAB, 3,3′-diaminobenzidine; DCFH, 2′,7′-dichlorofluorescin; DCF, 2′,7′dichlorofluorescein; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DXS, deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GSH, glutathione; G-POD, guaiacol peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase; HMGR, hydroxymethylglutaryl-CoA reductase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBRAS, 2-thiobarbituric acid reactive substances

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various cellular organelles such as mitochondria, chloroplasts and peroxisomes under optimal physiological conditions. Under stress conditions, the equilibrium between ROS production and elimination get disturbed resulted in oxidative stress to plants. This oxidative stress further leads to the toxic effect on plant growth and development. For the ROS homeostasis maintenance, two crucial interlocked protective mechanisms such as enzymatic and non-enzymatic antioxidants have been reported in plants (Schopfer et al., 2001). Glutathione (GSH) and ascorbate (AsA) are considered as foremost soluble antioxidants, which are involved in the ROS management *via* well recognized of ascorbate-glutathione cycle. Tocopherol, a low molecular weight antioxidant, involved in antioxidant mechanism synergistically with AsA and GSH under abiotic stress to maintain ROS homeostasis. This triad is reported to be involved in the tolerance mechanism of plants under oxidative stress conditions (Foyer and Noctor, 2005). Ascorbate specially detoxifies the H_2O_2 by acting as an electron donor in AsA-GSH cycle. The reduced glutathione (GSH) reported as the second most abundant non-enzymatic antioxidant. Under natural conditions, plant maintains the ratio of GSH and oxidized glutathione (GSSG) in the direction of reduced GSH manner of 20:1 ratio (Szarka et al., 2012). During AsA-GSH cycle, these two low molecular weight and soluble antioxidants interact to each other (Fig. 1). Tocopherol is an important part of the ascorbate-glutathione-α-tocopherol triad (Munne, 2005; Szarka et al., 2012). Tocopherol reported to be involved in the quenching of singlet molecular oxygen ($^1O_2$) and total 120 molecules of $^1O_2$ were reported to be scavenged by only one molecule of α-tocopherol (Munne, 2005). In the ascorbate-glutathione-α-tocopherol triad pathway (Fig. 1), tocopherols quenched the lipid peroxy radical and converted into tocopheroxyl radicals which further reduced by AsA (Munne, 2005; Szarka et al., 2012). The APX enzyme detoxified the H_2O_2 using AsA as a substrate and synthesized two molecules of monodehydroascorbate which further oxidized to dehydroascorbate (DHA) by the action of monodehydroascorbate reductase (MDHAR) enzyme (Munne, 2005). Due to highly unstable nature, DHA rapidly converted into AsA (reduced form) *via* dehydroascorbate reductase (DHAR) enzyme. DHAR used GSH as a substrate and converted the GSH into oxidized form of glutathione i.e. GSSG. The GSSG further reduced into the reduced GSH in the presence of glutathione reductase (GR) enzyme at the expense of NADPH molecules (Munne, 2005; Szarka et al., 2012) (Fig. 1). These components were first described in chloroplast and often called as Foyer–Halliwell–Asada cycle (Foyer and Halliwell, 1976; Foyer and Noctor, 2005) and later reported in mitochondria (Chew et al., 2003) and cytosol (Foyer and Noctor, 2011). Besides, various enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and guaiacol peroxidase (G-POD) reported for their crucial role in the plants to scavenge the reactive oxygen species (Sabir et al., 2012; Mishra et al., 2014). Primary step of ROS scavenging involves dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 by SOD followed by the scavenging of produced H_2O_2 through various peroxidases such as CAT, POD, APX, GPX and G-POD into their respective products and water molecules (Mishra et al., 2014). These components and enzymes were reported to be involved in defense mechanism against stress and also influenced the plant growth and development by modulating the various cellular processes such as mitosis, cell elongation to senescence and cell death. *W. somnifera* (Ashwagandha) is one of the most reputed Indian medicinal plants in Ayurveda, gaining lots of attention because of its unique secondary metabolite such as withanolides, having wide range of pharmacological properties (Misra et al., 2008; Sangwan et al., 2004, 2017; Sangwan and Sangwan, 2014). Our previous reports on *W. somnifera* have shown various aspects related to the growth, development, biosynthesis of secondary metabolites, elucidation of biosynthetic pathway through metabolic and transcriptomic resources of our superior varieties (Sangwan et al., 2004; Sabir et al., 2008, 2012; 2013; Mishra et al., 2014; Tripathi et al., 2017; Mishra and Sangwan, 2019). Plants secondary metabolite biosynthesis found to be affected by biotic and abiotic stress signals (Zheng and Wu, 2004; Rai et al., 2005; Ch et al., 2012). Secondary metabolites such as withanolides, have been reported to get influenced by various biotic and abiotic conditions (Jacob et al., 2014; Mir et al., 2015). Our previous study reported the effect of Cd under *in vitro* conditions in *W. somnifera* (Mishra et al., 2014). Recently, the involvement of primary and secondary metabolic pathways in Cd stress management were suggested by our group (Mishra and Sangwan, 2019). The present study is aimed at defining and understanding various biochemical and metabolic alterations affording the ability of plant to survive under stress conditions. The major emphasis of the study is on important components of the ascorbate-glutathione-tocopherol triad pathway in ROS homeostasis and stress management in *W. somnifera* under the exogenous supply of Cd. The findings of the study will be useful in providing guidelines for developing plants for various stressful environments.

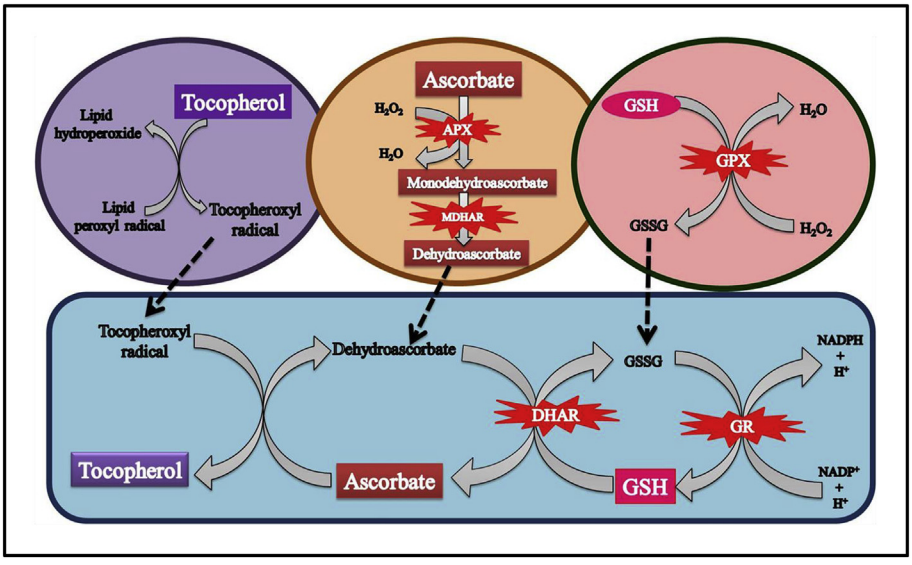

Fig. 1. The interlinked network of ascorbate-glutathione-α-tocopherol triad.

2. Materials and methods

2.1. Plant materials

The seeds of the high yielding variety of Ashwagandha NMITLI-118 developed by our group were sown at CSIR-CIMAP experimental farm. The four-leaf stage plants were treated with different concentrations of cadmium sulphate (5–300 μM). Control plants were grown and maintained under similar conditions and received no Cd treatment to the soil.

2.2. Cd treatment and growth conditions

The plants were treated weekly with half strength of MS basal media for control or media containing different concentrations of cadmium sulphate (5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 150 μM, 200 μM and 300 μM). All the treatments were carried out on twelve plants in each treatment. Uniformly surviving treatment (with at least six plants) was proceeded further for the analysis, after 45 days of the respective treatment.

2.3. Estimation of physiological parameters

The shoots length was measured after 7th, 14th, 21st, 28th, 35th and 45th days of the treatment. The freshweight of stem, root and leaf tissues were measured after 45 days of the treatment.

2.4. Analysis of Cd content

For the Cd content analysis, dried samples of control as well as Cd-treated plants were ground and digested with $HNO_3/HClO_4$ (3:2). The digested samples were filtered and subjected to ICP–atomic absorption spectrometry (ICP-AAS; Perkin–Elmer) for Cd content analysis (Mishra et al., 2014).

2.5. Estimation of lipid peroxidation rate

Lipid peroxidation rate was determined by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). For MDA analysis, tissue was homogenized with 0.1% TCA and the absorbance of the supernatant was observed at 532 nm after adding 0.5% TBA and 20% TCA as described earlier (Heath and Packer, 1968; Mishra et al., 2014).

2.6. Estimation of tocopherol, AsA and GSH content

For the tocopherol estimation, tissue was homogenized in methanol and absorbance was taken at 522 nm after adding dipyridyl (Kıvçak and Akay, 2005). Total ascorbate (AsA + DHA), ascorbate (AsA) and dehydroascorbate (DHA) was measured using L-AsA as standard. For total ascorbate (AsA + DHA) analysis, tissue was grounded in TCA (5% w/v) and suitable aliquots of supernatant was added to 0.05 M potassium phosphate buffer (pH 7.4) containing 3.0 mM EDTA and 1.0 mM DTT. NEM (0.1 ml), 0.61 M TCA, 0.8 M orthophosphoric acid and α,α′-bipyridyl were added after 10 min incubation °. The assay mixture was incubated for 1 h at 40 °C and absorbance was recorded at 525 nm after addition of $FeCl_3$ (Tzure-Meng et al., 2009).

2.7. Mining of antioxidants related genes and in-silico expression analysis

SOD, CAT, POD, APX and GPX transcripts were mined out from the leaf transcriptome data (SRA053485), from NCBI data resource (https://www.ncbi.nlm.nih.gov/). *In-silico* expression analysis was carried out to determine their abundance in *W. somnifera*.

2.8. Primer designing

Oligonucleotide primers were designed from the conserved regions from the coding sequence of enzymatic antioxidants (SOD, CAT, APX, GR, GPX and PPO) as well as withanolide biosynthetic pathway related genes such as hydroxymethylglutaryl-CoA reductase (*WsHMGR*), deoxylulosephosphate reductoisomerase (*WsDXR*), deoxylulosephosphate synthase (*WsDXS*) and cycloartenol synthase (*WsCAS*) for relative quantitative expression analysis. Primers were designed using online software Oligo Analyser tool 3.1- Integrated DNA Technology (IDT) (https://www.idtdna.com/calc/analyzer) by keeping various parameters in consideration such as % of GC content (40–60%), Tm (50–60 °C), self-dimer, hairpin structure formation and heterodimer formation etc (Tripathi et al., 2017).

2.9. Assay of enzymes involved in the ascorbate-glutathione-tocopherol triad

For the enzymatic assays related to ascorbate-glutathione-tocopherol triad enzymes such as APX (EC 1. 11. 1. 11), GR (EC 1.6.4.2), GPX (EC 1.11.1.9), MDHAR (EC 1.6.5.4) and DHAR (EC 2.5.1.18) were measured by methods as described earlier (Khatun et al., 2008; Mishra et al., 2014). The protein content was determined according to Bradford (1976) method using bovine serum albumin as a standard.

2.10. Activity assay for enzymatic antioxidants

SOD (EC 1.15.1.1), CAT (EC 1. 11. 1. 6), GPX (EC 1.11.1.9) and POD (EC 1. 11. 1. 7) enzymes activities were assayed for the control as well as Cd-treated plants. The SOD activity was analysed by monitoring the inhibition in the reduction of NBT at 560 nm (Sabir et al., 2012). The CAT activity was measured by monitoring the reduction of H_2O_2 after adding the enzyme extract in 50 mM KPB (pH 7.0) containing 30 mM H_2O_2 (Sabir et al., 2012). For the GPX activity analysis, 0.56 M KPB (pH 7) was used containing enzyme extract, 0.5 M EDTA and 0.2 mM NADPH. The change in absorbance was measured at 340 nm (Khatun et al., 2008; Sabir et al., 2012). The POD activity was assayed using reaction mixture consisting of 0.1 M KPB (pH 6.8), 5 mM H_2O_2, 0.01 M pyrogallol and enzyme extract (Khatun et al., 2008).

2.11. Expression analysis of enzymatic antioxidants

Total RNA was isolated by Trizol method from fresh leaf tissues of Cd-treated as well as control plants. The first-strand cDNA was synthesized using RevertAid Premium first strand cDNA synthesis kit (Fermentas). The qRT-PCR was performed for SOD, CAT, APX, GPX and POD with their specific primers. The constitutively expressed β-actin gene was used as endogenous control. The real-time PCR was performed in a 20 μl reaction volume, using 100 ng of the respective cDNA, 10 μl of SYBR Green master mix (Applied Biosystems) and 5 pmol of each primer. All the reactions were performed in triplicates. The PCR conditions were as 95 °C for 10 s, 95 °C for 15 s and 55 °C for 1 min for 40 cycles and melting curve 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The relative gene expression levels were expressed as quantification (RQ) values.

2.12. Analysis of isozyme via native polyacrylamide gel electrophoresis

For isozymic profiling of POD, SOD, CAT, GPX and APX proteins, 20 μg extracted protein from each sample tissue was subjected to native PAGE followed by incubation in their respective staining solutions. For visualization of POD isozymes, 0.2 M acetate buffer (pH 5.5) was used containing 0.02 mM H_2O_2 and 1 mM o-dianisidine. The SOD isozymes were visualized by using reaction mixture consisting of KPB (pH 7.5, 0.1 M), methionine (20 mM), EDTA (1 mM), NBT (250 μM) and riboflavin (28 μM). CAT isozymes were visualized using staining solution

containing KPB (50 mM, pH 7.0) buffer, horseradish peroxidase (50 μg ml^{-1}) and H_2O_2 (5 mM). The incubation of GPX was performed in KPB (0.1 M, pH 6.5), benzidine (1.7 mM), guaiacol (12.5 mM) and H_2O_2 (12 mM). For the isozymic analyses of APX, the gel was incubated in staining solution as described earlier (Mishra et al., 2014).

2.13. *In situ* localization of oxidative burst (ROS), H_2O_2 and O_2—• radicals

The oxidative burst, generated due to Cd exposure, was localized using reactive oxygen intermediates (ROI) probe, 2′,7′-dichlorofluorescin (DCFH) which get oxidized to 2′,7'dichlorofluorescein (DCF) after incubating the leaf tissues in incubation medium containing 50 mm DCFH-diacetate in 20 mM KPB (pH 6.0) (Schopfer et al., 2001). For *in situ* histochemical analysis of H_2O_2, leaves of Cd-treated and control plants were treated with 3,3′-diaminobenzidine (DAB). Visible brown spots were appeared due to the oxidation of DAB *via* H_2O_2 and images were captured by Leica microscope (LEICA EZ4D) (Romero-Puertas et al., 2004). For *in situ* localization of $O_2^{\cdot-}$, leaves from control and Cd-treated plants were incubated in 0.1% NBT solution and illuminated until the appearance of dark blue/purple spots (Romero-Puertas et al., 2004).

2.14. Determination of ROS content and scavenging activity

Total ROS content, H_2O_2, $O_2^{\cdot-}$ and OH˚ were measured in control as well Cd-treated plants. For the total ROS quantitation, tissues were incubated in KPB (50 mM) aided with 0.01 mM DCFH-DA for 30 min and processed as reported earlier (Schopfer et al., 2001). For $O_2^{\cdot-}$ and OH˚ radicals estimation, tissue was incubated in 20 mM KPB (pH 6.0) contained XTT (500 μM) and 2-deoxy-D-ribose (20 mM) respectively and processed as reported earlier (Schopfer et al., 2001). For H_2O_2 analysis, tissues were dipped in solution of KPB (pH 6.0, 20 mM), scopoletin (5 μM) and horseradish peroxidase (1 U ml^{-1}) (Schopfer et al., 2001). The scavenging activities were determined using previously reported methods (Schopfer et al., 2001; Mishra et al., 2014).

2.15. Extraction and quantitation of withanolide content

For withanolide extraction, fresh tissues were frozen in liquid nitrogen and ground to a fine powder. Homogenates were mixed with 50% methanol and further processed for withanolide extraction as described earlier and extracted withanolides were dissolved in HPLC-grade methanol & subjected to HPLC (Sangwan et al., 2006; Chaurasiya et al., 2008).

2.16. Quantitative expression analysis of withanolide biosynthetic genes

Withanolide biosynthetic pathway genes such as *WsHMGR*, *WsDXR*, *WsDXS* and *WsCAS* were quantitatively analysed in Cd-treated plants as well as in control plants using 100 ng of the respective cDNAs as template and reactions were carried out as earlier reported method using actin as endogenous control (Srivastava et al., 2015).

2.17. Statistical analysis

All the experiments were repeated for three times and each treatment had twelve individual plants. Results were compared with the standard deviation from the mean value at the level of significance ($p < 0.005$).

3. Results

3.1. Cd uptake and its physiological effect

ICP analysis exhibited uptake of Cd in concentration dependent manner (Fig. 2A). No visual toxic symptoms appeared in the plants after 45 days of the Cd treatment. Moderate changes were observed in FW of the different tissues (leaf, stem and root) and length of shoots (Figs. S1A and B).

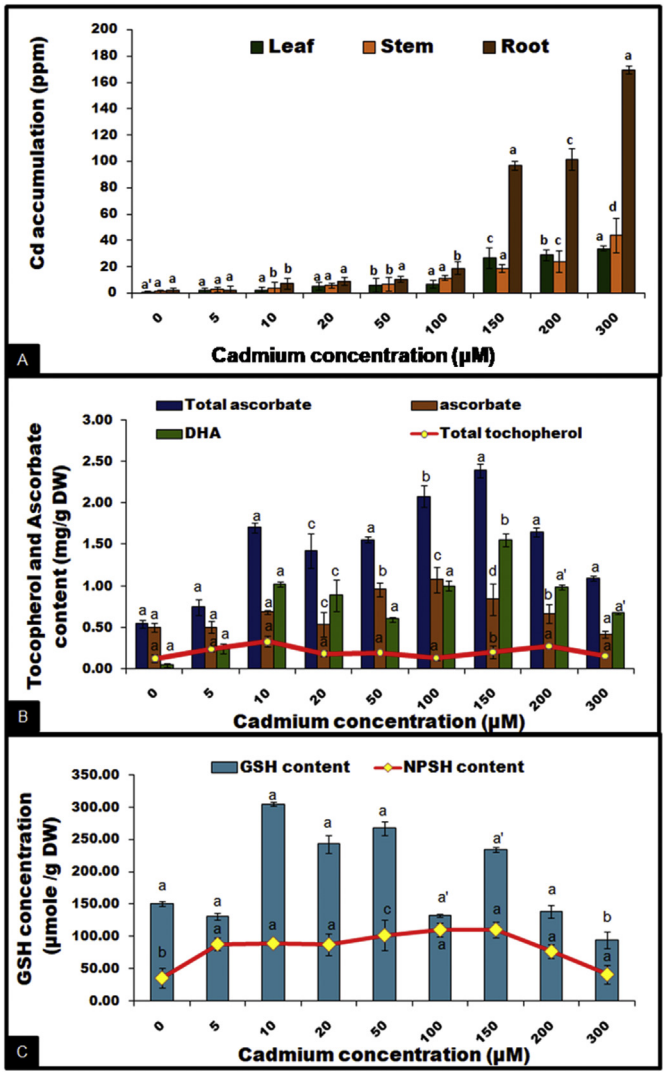

Fig. 2. (A) Cd accumulation in different tissues of *W. somnifera* grown under normal and Cd supplemented soil after 45 days of treatment. (B, C) Effect of Cd supplementation on ascorbate-glutathione-α-tocopherol triad including tocopherol, total ascorbate (AsA + DHA), ASA & DHA and GSH & NPSH content of *W. somnifera* under *in vivo* condition after 45 days of treatment. Data were taken after 45 days of Cd treatment and represent mean value of triplicate with ± standard deviation.

3.2. Lipid peroxidation rate

Lipid peroxidation rate was found to be enhanced at lower concentrations of Cd and maximum MDA content was found at 10 μM (by 2.4 folds in comparison to control) while at higher concentration of Cd, moderate changes were observed (Fig. S1C).

3.3. Modulation of the ascorbate-glutathione-tocopherol triad

The low molecular weight antioxidants such as AsA, GSH and tocopherol were accumulated upon Cd exposure. Tocopherol content was maximum at 10 μM by 2.75 folds (Fig. 2B). GSH and NPSH were increased by 2.02 and 3.07 folds in Cd-treated plants in comparison to control plants (Fig. 2C). Heat map of transcripts represented the overall participation of enzymatic antioxidants under the Cd stress (Fig. 3). The

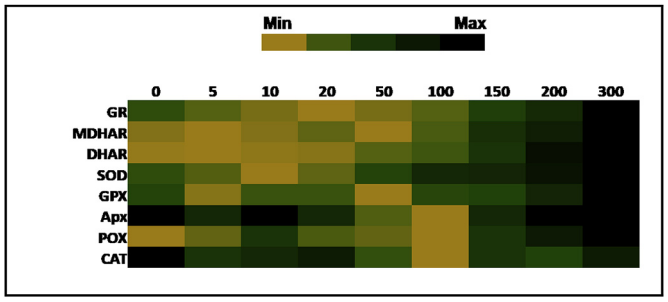

Fig. 3. Cumulative representation of enzymatic antioxidants after 45 days of the Cd treatment in *W.somnifera*.

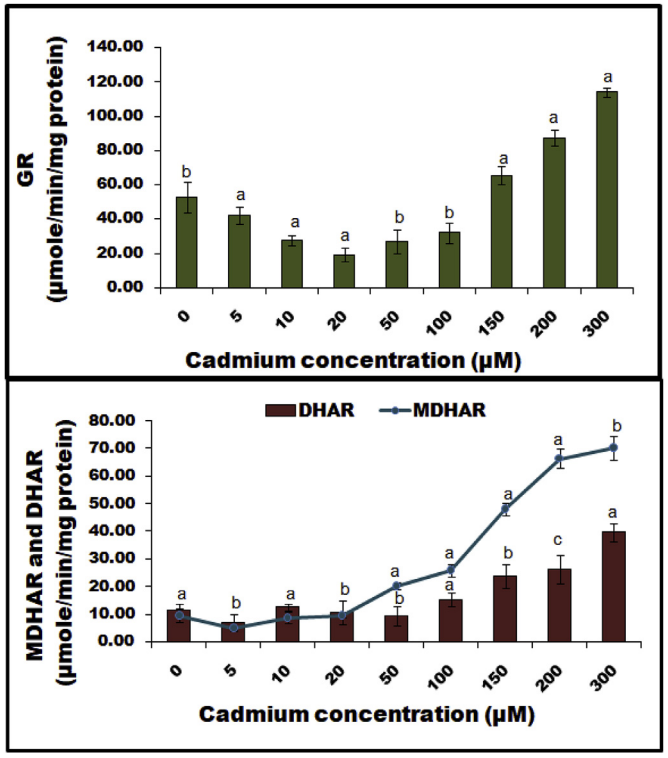

Fig. 4. Effect of Cd supplementation on activities of enzymes involved in ascorbate-glutathione-α-tocopherol triad such as GR, MDHAR and DHAR of *W. somnifera* under *in vivo* condition after 45 days of treatment. Data were taken after 45 days of Cd treatment and represent mean value of triplicate with ± standard deviation.

activities of GR and GPX were upregulated in Cd-treated plants (Figs. 4 and 5). The ascorbate content including total ascorbate, AsA and DHA were also enhanced by 4.46, 2.16 and 38.75 folds in comparison to control, respectively (Fig. 2C). The increment in the activities of enzymes involved in AsA-GSH pool such as MDHAR and DHAR activities were higher by 7.29 and 3.47 folds upon Cd exposure (Fig. 4).

3.4. Role of enzymatic antioxidants

In-silico expression analysis of various enzymatic antioxidants revealed that the most abundant transcripts were SOD, CAT, GPX, APX & POD (Fig. 5) and affected by Cd supplementation. Peroxidases such as GPX and POD activities were enhanced by 1.68 and 2.5 folds and their mRNA transcripts levels were higher by 5.2 and 4.5 folds in Cd-treated plants, respectively (Fig. 5 A, B). Other peroxidases *viz* CAT and APX activities were downregulated at 100 µM and their mRNA transcript levels were also decreased in comparison to control plants (Fig. 5 A, B). Similarly, increment in SOD activity and transcripts were observed in Cd-treated plants in comparison to control plants (Fig. 5 A, B).

3.5. Native polyacrylamide gel electrophoresis

The effect of Cd stress was shown on the isozymic pattern of plants grown under glass house conditions (Fig. 5C). Three SOD isozymes (SOD 1, SOD 2 and SOD 3) and GPX isozymes (GPX 1, GPX 2 and GPX 3) were found and their band intensities were increased upon increasing Cd concentrations. Only one isozyme of CAT was observed but its intensity was higher in Cd-treated plants. The two isozymes of APX (APX 1 and APX 2) and POD isozymes (POD 2 and POD 3) were detected and intensity was higher at higher dose of Cd. POD 1 was recorded as a new appearance in Cd-treated plants (Fig. 5C).

3.6. In situ localization of ROS

The strong green fluorescence of oxidized DCF indicated that the more ROS production occurred in tissues under Cd stress (Fig. S2A). In case of H_2O_2 localization, DAB reacted with H_2O_2 and developed visible brownish spots (Fig. S2B). The dark blue regions were found more prominent in Cd-treated tissues which indicated more $O_2^{\bullet-}$ generation in Cd-treated plants in comparison to control (Fig. S2C).

3.7. ROS modulation under Cd stress

The reactive oxygen species including total ROS, H_2O_2, $O_2^{\bullet-}$ and •OH radicals were accumulated under Cd stress. Total ROS, H_2O_2, $O_2^{\bullet-}$ and •OH radicals were maximum at 100 µM (except $O_2^{\bullet-}$ radical) by about 2.1–3.0 folds in comparison to control (Fig. S3). The scavenging activity of $O_2^{\bullet-}$ was increased by 1.4 folds while H_2O_2 scavenging activity decreased by about 3.0 folds upon Cd exposure. No significant changes were observed for •OH radical scavenging activity (Fig. S4).

3.8. Effect on withanolide metabolism

Quantitative HPLC analysis of Cd-treated plants showed ~2.0 folds higher withanolide content with about 2.0–3.0 folds higher mRNA expression of withanolide biosynthetic genes i.e. *WsHMGR, WsDXS, WsDXR* and *WsCAS* in comparison to control plants (Fig. 6).

4. Discussion

4.1. Cadmium accumulation and physiological effect

Cadmium is a non-essential heavy metal and highly toxic environmental pollutant, causes detrimental effects and problems in agriculture due to the high mobility in the plant-soil system (Shanmugaraj et al., 2019). The effect of Cd supplementation under *in vitro* shoot cultures of *W. somnifera* as well as on full plant showed differential responses. This differential response may be attributed to the restricted growing environment and different organizational level of both the tissues (Mishra et al., 2014). Analogous findings were also reported earlier in plants such as in Lupin (Vazquez et al., 2007), *Thlaspi caerulescens* (Xie et al., 2009) and Lactuca spp (Ramos and Jose, 2002). Furthermore, various extrinsic factors including environmental factors (soil type, pH, texture, temperature, humidity, redox potential, cation exchange capacity) and plant-associated factors (root exudates and root rhizosphere) affect the Cd uptake by plants *via* roots. Recently, it was reported that Sonarbangla (rice) retained excess Cd in roots *via* vacuolar sequestration without interfering any cellular functions (Bari et al., 2019). Whereas *in vitro* shoot cultures of *W. somnifera* grown under sterile conditions, exhibited restricted environment devoid of all the above interaction (Mishra et al., 2014; Sabir et al., 2012). No visual toxic symptoms such as yellowing and necrosis were observed in Cd-treated leaves. Low growth responses were also observed in *E. andevalensis* and *Iris lactea* (Márquez-García et al., 2012; Guo et al., 2017) whereas retarded

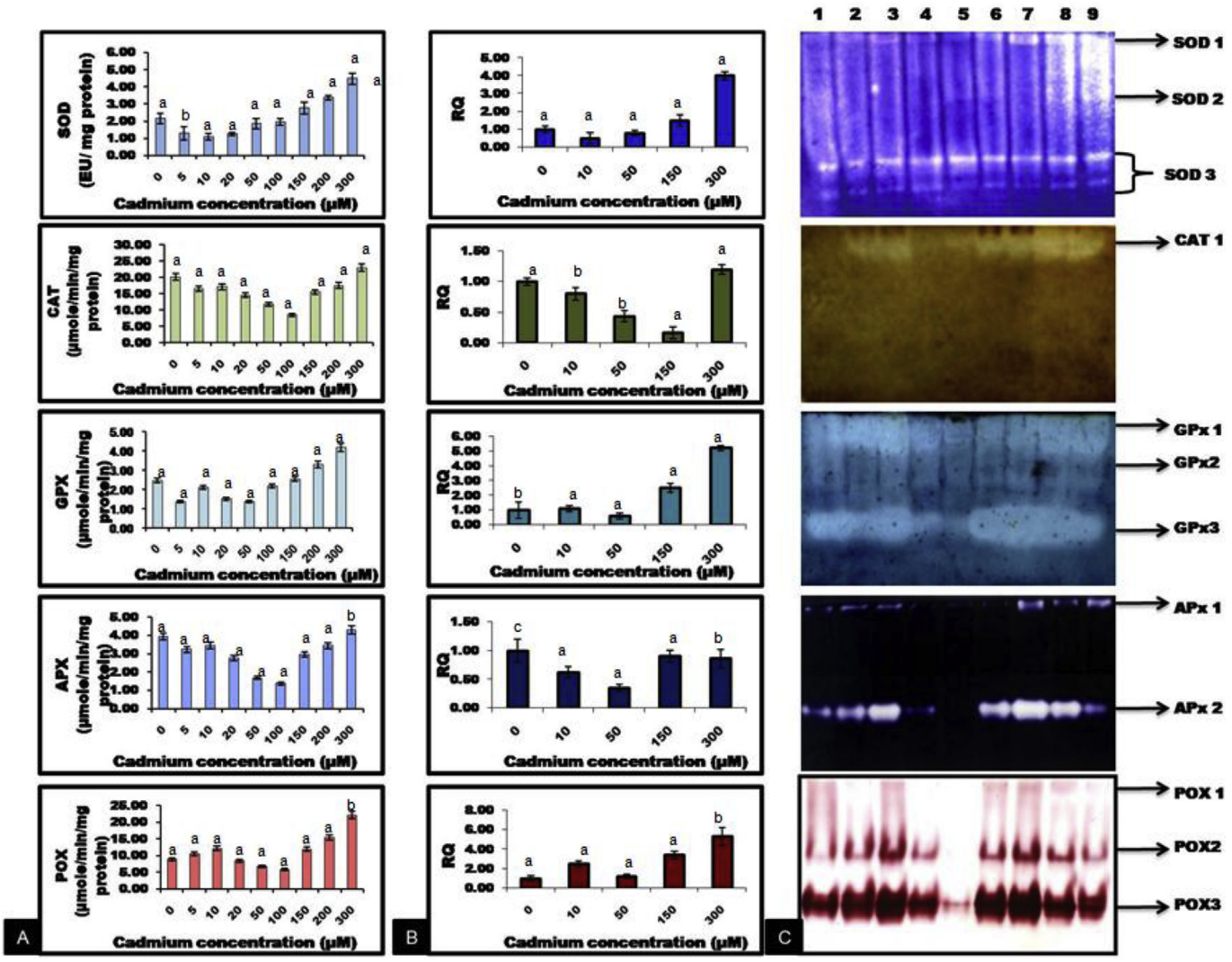

Fig. 5. Effect of Cd supplementation on enzymatic antioxidants (SOD, GPX, POD, CAT and APX) of *W.somnifera* after 45 days of treatment under *in vivo* condition. (A) Spectrophotometric enzyme activity assays, (B) q-RTPCR analysis (Data represent mean value of triplicate with standard deviation), (C) Native –PAGE analysis Lane 1: control; Lane 2: 5 µM; Lane 3: 10 µM; Lane 4: 20 µM; Lane 5: 50 µM; Lane 6: 100 µM; Lane 7: 150 µM; Lane 8: 200 µM; Lane 9: 300 µM.

growth was recorded in alfalfa (Flores-cáceres et al., 2015).

The induction of the lipid peroxidation considered as one of the instant response of plants under stress conditions (Gonçalves et al., 2007). The moderate changes in MDA content at higher Cd dose revealed no severe lipid peroxidation occurred in *W. somnifera* Cd stress. This might be due to the enhanced antioxidant adaptive mechanism of the plant (Fig. S1C). Similar results were also found earlier (Bačkor and Loppi, 2009; Guo et al., 2017) whereas other reports showed increased lipid peroxidation leading to phytotoxicity under Cd stress (Dong et al., 2006; Singh et al., 2006; Singh and Shah, 2014; Dobrikova et al., 2017).

4.2. Involvement of GSH-AsA-tocopherol antioxidant triad

Accumulation of tocopherol, GSH, NPSH, total ascorbate (AsA + DHA), AsA and DHA under Cd stress suggested their active participation in Cd tolerance and detoxification mechanism by scavenging of excessive ROS production. The AsA and GSH have been considered as the center of redox hub (Foyer and Noctor, 2011). The ascorbate-glutathione-tocopherol triad showed significant contribution in the maintenance of the ROS homeostasis in plants abiotic stress (Fig. 1). These metabolites interplay in ROS scavenging mechanism under abiotic stress conditions (Foyer and Noctor, 2011; Szarka et al., 2012). Tocopherol accumulation at a lower Cd concentration revealed its active participation in ROS homeostasis maintenance against Cd stress as it posses strong antioxidant activity because of the presence of three methyl groups in its molecular structure (Gill et al., 2011). Decreased tocopherol at higher Cd concentration might be a result of inhibition of the synthesis of tocopherol or activation of tocopherol degrading enzymes. Tocopherol also involved in the maintenance of the reduced form of ASC-GSH through ascorbate-glutathione-tocopherol triad cycle (Szarka et al., 2012). It is also reported to be involved in affording membrane stability, maintenance of the photosynthetic system and quenching of the excess of light (Gill et al., 2011; Szarka et al., 2012). Previous reports confirmed the accumulation of tocopherol under Cd stress and its involvement in ROS scavenging mechanism (Gill et al., 2011; Hédiji et al., 2015; Li et al., 2015; Ahmad et al., 2016).

Ascorbate and glutathione (the major soluble antioxidants; and main component of the ascorbate-glutathione cycle) were found upregulated in *W. somnifera* under Cd stress. Among these antioxidants, ascorbate participation in Cd detoxification and acclimatization was found relatively more prominent under Cd stress. Enhanced AsA content might be due to the enhanced activities of MDHAR and DHAR, which maintain the AsA level in the plant cells. Upregulated MDHAR, DHAR and GR activities were also found in wheat, which reflected more AsA accumulation *via* increment in AsA-GSH cycle (Paradiso et al.,

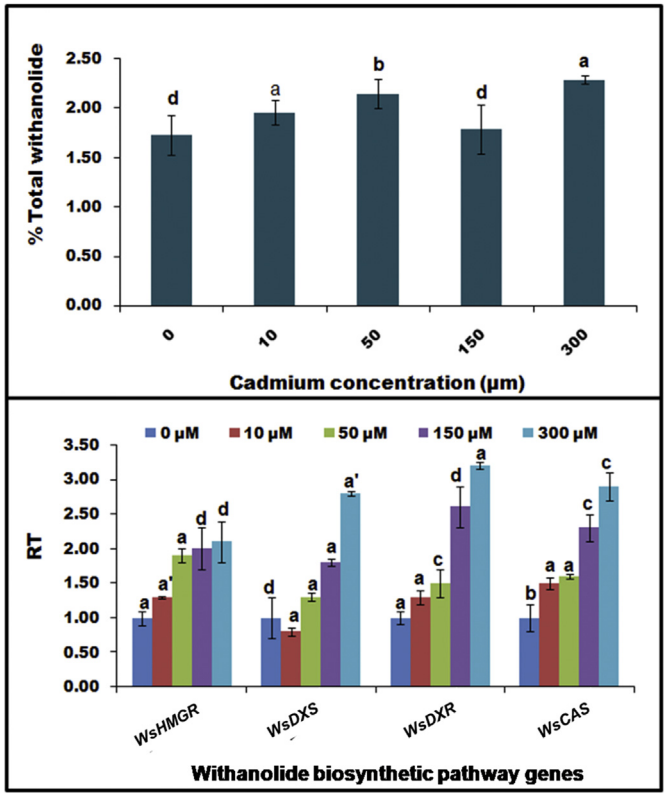

Fig. 6. Effect of Cd on withanolide content and transcripts expression of withanolide biosynthetic pathway genes viz *WsHMGR, WsDXS, WsDXR and WsCAS*. Data were taken after 45 days of Cd treatment and represent mean value of triplicate with ± standard deviation.

2008). Similar results were also reported in *Triticum durum* cv Cres (Paradiso et al., 2008), mung bean (Nahar et al., 2016), *Trigonella foenumgraecum* (Bashri and Prasad, 2016) and in multiple shoot cultures of *W. somnifera* against heavy metal stress (Khatun et al., 2008; Mishra et al., 2014). Similar findings were also found in cucumber seedling, *S. densiflora*, *E. andevalensis* and in *B. juncea* (Ahmad et al., 2016; Gonçalves et al., 2007; Márquez-García et al., 2012; Martínez Domínguez et al., 2010). Ascorbate plays an important role in the scavenging of H_2O_2, maintenance of α-tocopherol in reduced form, maintenance of chloroplast against photo-oxidative damage and in many other physiological processes such as regulation of growth and minimization of the oxidative damage against metal stress *via* synergic function with other antioxidants (Foyer and Noctor, 2011).

Accumulation of GSH and NPSH under Cd stress revealed their active participation under oxidative stress in *W. somnifera* which minimized the damaging effect of Cd. AsA-GSH cycle was reported to get induced under Cd stress in *T. aestivum* and *Vigna radiata* L. (Paradiso et al., 2008) while declined in *Hordeum vulgare* L. (Wu et al., 2005). The involvement of GSH was comparatively higher than tocopherol and AsA, as their accumulation was found greater than tocopherol and AsA contents. GSH generally considered as a marker of oxidative stress to the plants. Higher thiol content under Cd exposure reflected the competition for GSH between Cd chelation and ROS detoxification (Jozefczak et al., 2014). Increased GSH content under Cd stress suggested its involvement in Cd tolerance mechanism by maintaining and scavenging of ROS levels in *W. somnifera* and by regenerating of another potential water-soluble antioxidant like AsA *via* the AsA-GSH cycle (Khatun et al., 2008). Elevated level of GSH content was also reported in *Medicago sativa* L., *S. densiflora*, *E. andevalensis*, *A. thaliana* and *Phaseolus vulgaris* under Cd exposure (Jozefczak et al., 2014; Márquez-García et al., 2012; Martínez Domínguez et al., 2010; Nahar et al., 2016; Sobrino-Plata et al., 2009). GSH is also involved in metal chelation by providing the precursor for phytochelatin (PC; important metal chelator). Besides, GSH plays a central role in various physiological processes such as signal transduction, regulation of sulphate transport, detoxification of xenobiotics and protection of photosynthetic apparatus and conjugation of metabolites (Mishra et al., 2014; Xiang et al., 2001). GSH and non-protein thiols (NPSH) accumulation revealed its active contribution in the Cd detoxification and ROS scavenging mechanism against oxidative stress. NPSH accumulation also found in *B. monnieri*, *C. demersum* L., *A. thaliana, P. vulgaris* and in *D. viscosa* against Cd stress (Fernández et al., 2014; Jozefczak et al., 2014; Nahar et al., 2016; Singh et al., 2006) while in *Oryza sativa* and *A. atacamensis* it was declined under metal stress (Bhoomika et al., 2014). NPSH also play an important role in the maintenance of the "S" levels by upregulating genes expression involved in the "S" assimilation (Zeng et al., 2009). The present study also revealed that the antioxidative defense process is fundamentally correlated with the use and recycling of AsA and GSH with the involvement of SOD, CAT, MDHAR, DHAR and other enzymes. Several other indispensable antioxidants used them as a substrate for rejuvenation of other essential antioxidants, which implicated in neutralization and regulation of ROS (Jozefczak et al., 2015). Increment in tocopherol, AsA and GSH accumulation under Cd stress revealed their interlinked function in ROS scavenging mechanism. Thus, the main constituents of GSH-AsA-tocopherol antioxidant triad pathway found to be very crucial to maintain their levels through the recycling process using electron transfer mechanisms. The pathway also reported to provide continuously these low molecular antioxidants to the plants as per their requirements specially under stress conditions (Szarka et al., 2012).

4.3. Involvement of other enzymatic antioxidants under Cd stress

The spectrophotometric assay, qRT-PCR expression and native-PAGE analyses of SOD, CAT, APX, GPX and POD revealed differential response of Cd on enzymatic antioxidants under *in vitro* as well as *in vivo* conditions in *W. somnifera* (Fig. 6). The enhanced activities of the enzymes specially SOD, GPX and POD revealed their active participation in Cd detoxification and ROS homeostasis maintenance. The metalloenzyme SOD dismutated $O_2^{-}\bullet$ into H_2O_2, resulting into decreased $O_2^{-}\bullet$ and increased H_2O_2 content. In addition, SOD also minimized the •OH radical formation *via* the metal catalyzed Haber–Weiss reaction (Gill and Tuteja, 2010; Mittler et al., 2004; Sharma et al., 2012). Reduced •OH radical in *W. somnifera* revealed that SOD may inhibit the •OH formation at higher concentration of Cd. Increased SOD activity was recorded under stress in *Hordeum vulgare* (Wu et al., 2004), *Cicer arietinum* (Hasan et al., 2008) and *T. aestivum* (Hsu and Kao, 2004; Khan et al., 2007). Similar results were also found in qRT-PCR expression analysis of enzymatic antioxidants, which confirmed its involvement in Cd detoxification and ROS scavenging mechanism. Increased isoforms number and intensity of protein bands of APX, POD, GPX and CAT isozymes suggested an inducing response in their activities which reflected the strong antioxidative response of *W. somnifera* against Cd stress. APX was reported to have higher affinity for H_2O_2 than CAT & POD and scavenged H_2O_2 through water-water and AsA-GSH cycles by utilizing AsA as electron donor (Gill and Tuteja, 2010). In *V. mungo* (Gill et al., 2008), *T. aestivum* (Khan et al., 2007) and *B. juncea* (Gill et al., 2011), APX activity was upregulated upon Cd exposure. The GPX used GSH as substrate and scavenged the H_2O_2 radicals, organic and lipid hydroperoxides to minimize Cd induced oxidative stress. The noteworthy enrichment in CAT activity was also found in *O. sativa, T. aestivum, B. juncea* and in *C. arietinum* while significant reduction obtained in *Glycine max*, *A. thaliana* against Cd toxicity (Cho and Seo, 2005; Gill and Tuteja, 2010; Khan et al., 2007). Increased GR activity attributed the Cd tolerance, detoxification and ROS homeostasis maintenance property to plants under Cd stress as also found in *A. thaliana* (Cho and Seo, 2005) and *T. aestivum* (Gill and Tuteja, 2010). Recently, network of ABA, ROS and antioxidants was reported to minimize the oxidative

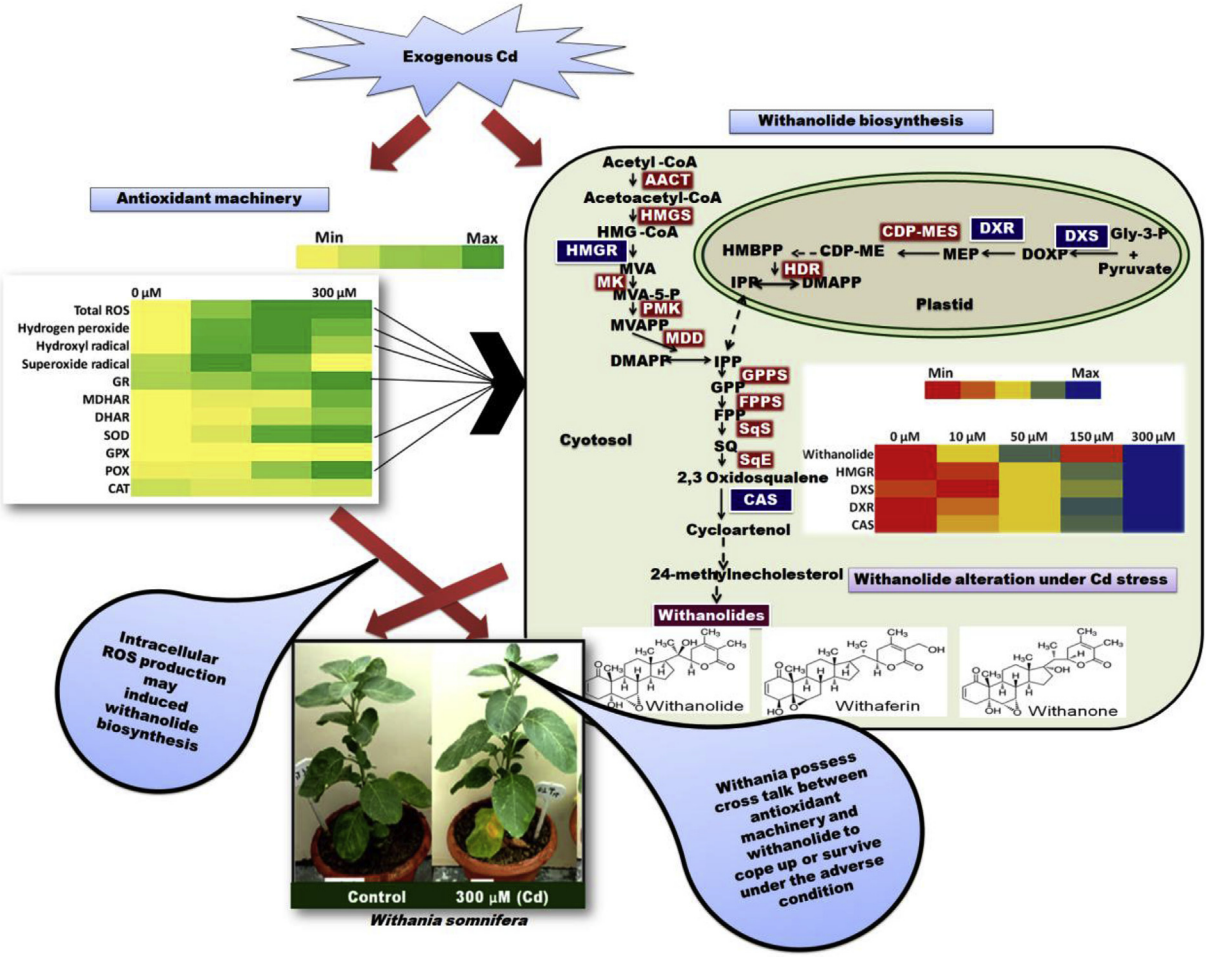

Fig. 7. Proposed cross-talk between the antioxidant machinery and withanolide metabolism in *W. somnifera* under Cd stress.

stress in mung bean seedlings (Sahu and Kar, 2018).

4.4. ROS status under Cd stress

ROS play a dual regulatory role in plants; amendments in ROS levels that are part of natural functioning of the plant should not surpass the threshold frontier between cytotoxic or cytostatic levels and redox potentials (Saini et al., 2018). ROS related assays suggested that H_2O_2, O_2^{-}• and •OH radicals decreased at higher Cd concentration due to increased scavenging activities (Fig. S 2, 3, 4). Enhanced H_2O_2 accumulation was also reported for *P. sativum* (Romero-Puertas et al., 2004), and in *B. juncea* (Ahmad et al., 2016) under Cd stress. An earlier report suggested that during Cd stress, H_2O_2 production increased and accumulated in various cellular organelles such as plasma membrane, cell wall, mitochondria, tonoplast and chloroplast (Lomaglio et al., 2015). Increased total antioxidant activity and reducing power activity in *W. somnifera* under Cd stress might be a result of induced AsA-GSH-tocopherol triad along with various enzymatic antioxidants. Similar results were also obtained in *Spartina densiflora* (Martínez Domínguez et al., 2010) and *E. andevalensis* (Márquez-García et al., 2012). Enhanced antioxidative activity attributed the reduced effect of Cd stress in black cumin (Espanany et al., 2016) and *I. lacteal* against Cd stress (Guo et al., 2017).

4.5. Withanolide content and possible cross-talk with intracellular ROS

Secondary metabolites are reported to get modulated under various stress conditions (Sabir et al., 2012). Increased withanolide content under stress might be a result of higher expression of *WsHMGR, WsDXS, WsDXR* and *WsCAS* (Fig. 6). Accumulation of withanolide content suggested withanolides might be involved in the maintenance of the ROS homeostasis in plant to survive against the adverse conditions as withanolides itself possess antioxidative property. Previous studies reported accumulation of withanolides under adverse or moderate conditions such as drought stress, low light stress (Jacob et al., 2014) and cold stress (Mir et al., 2015). Alkaloids were also enhanced *via* plant growth promoting rhizobacteria (PGPR) like *Azotobacter, Pseudomonas, Azospirillum and Bacillus* (Rajasekar and Elango, 2011). Accumulation of secondary metabolites was also reported in *C. roseus*, *P. amarus* Schum. and Thonn, *G. sylvestre* under Cd stress (Ch et al., 2012; Rai et al., 2005; Zheng and Wu, 2004) while decreased in *H. perforatum* L. (pseudohypericin and hypericin) (Murch et al., 2003). The results of antioxidant system as well as withanolide biosynthetic pathway *via* heat map and transcript profiling indicated that apparently there was cross-talk between antioxidant and withanolide metabolism (Figs. 6 and 7). Higher ROS, H_2O_2 and $OH^{\bullet}$ i.e. involved in H_2O_2 production and lower superoxide radicals reflecting higher H_2O_2 and comparatively lower superoxide dismutase and H_2O_2 detoxifying enzymes such as GPX/CAT may result in upregulation of withanolide metabolism. Earlier reports confirmed the interlink between the intracellular ROS and secondary metabolite production (Beites et al., 2011; Kanth et al., 2011; Kwon and Kim, 1998). Results also revealed the possibility of involvement of plastidial pathway (MEP) due to the increase in ROS in chloroplast. Plastids are considered as the primary site for ROS generation. In earlier report, enhanced H_2O_2 and CAT activity was co-related with production of pimaricin (Beites et al., 2011). Our study revealed that lower SOD

activity resulted in higher H_2O_2 production which might reflect the signaling for withanolide accumulation. In *S. natalensis,*SOD and H_2O_2-detoxifying enzymes defective mutant showed higher production of secondary metabolite (Beites et al., 2011), while heterologous expression of SOD led to overproduction of secondary metabolites (Kanth et al., 2011; Kwon and Kim, 1998), thus suggesting its role in secondary metabolism.

In conclusion, AsA-GSH-α tocopherol triad pathway was found to be the key mechanism for the ROS management along with other major enzymatic antioxidants under Cd stress. Intracellular H_2O_2 production and decreased SOD reflected the overproduction of withanolides under Cd stress. This is indicative of cross-talk between antioxidant machinery and withanolide biosynthesis in *W. somnifera* to cope up to adverse conditions of heavy metal stress. Further, *in-depth* studies are needed to establish the entire mechanism and knowledge generated may help to improve and develop superior tolerant varieties of the plants.

Author contributions

N.S.S. planned the study and experiments and supervised. B. M. performed the experiments, compiled data and prepared the manuscript draft. NSS, BM wrote and edited MS. S.C. helped in ICP instrument operation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.040.

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