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Research article

Regulation of cadmium toxicity in roots of tomato by indole acetic acid with special emphasis on reactive oxygen species production and their scavenging

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A B S T R A C T

Toxic impact of cadmium (Cd) on plants is well known which affects their productivity. To mitigate toxic impact of metals such as Cd, exogenous application of phytohormones like indole acetic acid (IAA) has been well recognized in the recent past. But, mechanisms related to the IAA-mediated mitigation of metal toxicity remain elusive. Therefore, in this study, effect of IAA on growth and photosynthetic attributes, nitric oxide, cell viability, reactive oxygen species (ROS) and ascorbate-glutathione cycle (AsA-GSH cycle) was investigated in tomato roots exposed to Cd stress. Cd declined growth and photosynthetic attributes which were accompanied by the excess accumulation of Cd and decreased level of nitric oxide (NO). Among photosynthetic attributes, quantum yield parameters were more sensitive to Cd and these results were in parallel of photosynthetic pigments. However, exogenously applied IAA together with Cd significantly improved level of NO, growth and photosynthetic attributes together with reduced accumulation of Cd. Cd enhanced level of superoxide radical and hydrogen peroxide leading to severe damage to lipids and membranes as indicated by increased level of lipid peroxidation and electrolyte leakage which collectively reduced cell viability of roots. Moreover, components of the AsA-GSH cycle i.e. enzymes (ascorbate peroxidase, monodehydroascorbate reducatse, dehydroascorbate reducatse and glutathione reductase) and metabolites (ascorbate and glutathione) were declined by the Cd. However, addition of IAA with Cd had up-regulated components of the AsA-GSH cycle. Interestingly, application of 2,4,6-triiodobenzoic acid (TIBA, a polar auxin transport inhibitor) diminished growth attributes and its combination with Cd worsened its toxicity and these events were in parallel with decline in NO content and enhancement in Cd accumulation. The results also showed that IAA was also able in mitigating Cd toxicity in tomato roots even in the presence of TIBA. Overall results show the essentiality of IAA in mitigating Cd stress in tomato roots through NO that up-regulates components of the AsA-GSH cycle for balancing ROS and their associated damages and hence much improved growth and photosynthetic attributes were noticed.

1. Introduction

Heavy metals including metals or metalloids have atomic density greater than 4 g/cm^3 or 5 times, or more, than the density of water (1 gm/cm^3) and include around 38 elements. Some heavy metals (Fe, Mn, Zn, Cu, Mo, Ni) are essential micronutrients and required at optimal concentration for the growth of plant. Whereas other heavy metals like Cd, Pb, Hg are non-essentials and cause severe toxic effects at concentrations higher than the tolerance limit of plants (Nagajyoti et al., 2010; Singh et al., 2017). Heavy metal toxicity results in reduced

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growth of root, shoot and number of lateral roots (Dias et al., 2013). Reduction in the leaf surface area and chlorosis which ultimately results in the decreased photosynthesis and plant metabolism, are also the effects of heavy metal toxicity (Parmar et al., 2013; Nazar et al., 2012). Necrotic spots or leave necrosis can be observed in highly susceptible plants or at highly toxic level of metal contamination (Fodor, 2002). Cadmium is a toxic metal that causes deleterious effect on plants (Benavides et al., 2005; Tripathi et al., 2012; Andersen et al., 2013; Ismael et al., 2019; Singh et al., 2019). Accumulation of contaminants such as Cd in soil involves natural or anthropogenic processes. Cd significantly affects the parameters like F_v/F_o, F_v/F_m, ψ_o, ϕE_o, PI_{ABS} and F_o/F_v, ABS/RC, TR_o/RC, ET_o/RC, DI_o/RC, etc. of chlorophyll *a* fluorescence and thus gives insight of its effect on the photochemical efficiency and energy fluxes per reaction centre (Strasser et al., 2000).

Cd presents in the rhizosphere and competes with cations in cell wall to inhibit normal functioning of embedded enzymes and produces reactive oxygen species-ROS (Pinto et. al. 2017). ROS produced in the cell cause lipid peroxidation (MDA), membrane leakage and inhibition of enzymes by the oxidation of their thiol groups (Shaw et al., 2004). Cd is transported across the plasma membrane using the same channel which is responsible for the transportation of Fe^{2+}, Ca^{2+} and Zn^{2+} (Lux et al., 2010). In response to the stress imparted by the environmental Cd, plants have also evolved with strategies like enhanced production of cysteine rich chelators i.e. phytochelatins and metallothioneins and thus making metabolically less active Cd in the cellular compartments (Di Toppi and Gabbrielli, 1999). Enzymatic and non-enzymatic antioxidizing pathways are also stimulated to overcome the excess production of Cd mediated ROS in the cell (Ahmad et al., 2009; Edokpolor and Ikhajiagbe, 2018). Ascorbate-glutathione cycle helps in most effective scavenging of ROS particularly H_2O_2 (Hydrogen peroxide) produced in the chloroplast (Singh et al., 2018). Moreover, it is also witnessed that changes in the pattern of phytohormones like auxin, cytokinins, gibberellins, brassinosteroids, salicylic acid, jasmonic acid, ethylene help to counter Cd toxicity (Ghorbanli et al., 2000; Hasan et al., 2011; Masood et al., 2012; Ha et al., 2012; Agami and Mohamed, 2013; Ali et al., 2018). Studies also showed that metal toxicity coincides with altered levels of endogenous levels of phytohormones so the exogenously applied phytohormones could compensate for their decreased levels and thus may impart metal tolerance (Agami and Mohamed, 2013; Bücker-Neto et al., 2017). This strategy has attracted wider scientific attention for inducing metal tolerance capability in plants in the last few years. However, mechanisms through which exogenously applied phytohormones impart metal tolerance in plants remain elusive.

Tomato is an economically important crop and its worldwide production comes second after potato. It belongs to the family of solanaceae and is also widely used for physiological, cellular, biochemical and molecular studies due to its ease of cultivation. Further, tomato is also grown under Cd contaminated soil which could have negative impact on its productivity. Besides this, absorbed Cd may enter food chain and could be harmful for human being when fruits are consumed. Indole acetic acid (IAA) is a phytohormone which plays a prominent role in the growth and development of a plant (Kukavica et al., 2007). Shoot apex and young leaves are the major sources of IAA in a plant (Vernoux et al., 2010). IAA is transported, either in acropetal fashion through tissues in the stele or basipetally through cell-to-cell movement, to the target (Tanimoto, 2005). Resistance to abiotic stress has been shown to be imparted by IAA (Korver et al., 2018). But studies related to IAA-mediated mitigation of Cd toxicity in tomato are still less known. Moreover, mechanisms related with IAA-governed amelioration of Cd toxicity in plants remain elusive. Therefore, in the present study role of IAA in mitigation of Cd stress in tomato roots was investigated with an emphasis on ROS production and their scavenging by the AsA-GSH pathway. To further ascertain whether Cd toxicity mitigation is associated with IAA, we used 2,4,6-triiodobenzoic acid (TIBA), an inhibitor of IAA efflux transporter (Tucker, 1978).

2. Materials and methods

2.1. Plant material and growth conditions

Tomato (*Lycopersicum esculantum*) seeds of cv. Navoday were acquired from a registered retailer in Alopibagh, Prayagraj, Uttar Pradesh, India. Surface sterilization of seeds was performed with 2% sodium hypochlorite solution for 10 min and rinsed with distilled water three times. Later, seeds were immersed in distilled water for 12 h under dark conditions. Afterwards, sterilized muslin cloth wetted using Hoagland nutrient solution (Hoagland and Arnon, 1950). of half strength was used to wrap seeds in dark. After 4 days, germinated seeds were taken and cultured using semi hydroponic culture system with sand as an inert porous medium. The sand was pre-sterilized with acid treatment and sieved to obtain uniform sized particles. Photosynthetically active radiation (PAR) for growth condition was maintained at 300 μmol photons $m^{-2}s^{-1}$ and light-dark regime of 16:8 h with relative humidity of 60–70% at 26 ± 1 °C during growth of the seedlings for 23 days.

2.2. Treatment with Cd, IAA and TIBA

After 23 days, seedlings with mature secondary leaves were uprooted from the sand and transferred into half strength Hoagland solution filled in 50 mL pots for 7 days to allow their acclimatisation into the hydroponic culture system. The seedlings were then screened for uniformly sized plants. Uniformed sized seedlings were given the treatments of Cd, IAA and TIBA. Following combinations were made- control (only half strength Hoagland solution), IAA (5 μM), Cd (100 μM), TIBA (25 μM), Cd + IAA (100 μM + 5 μM), Cd + TBA (100 μM + 25 μM), Cd + TIBA + IAA (100 μM + 25 μM + 5 μM) and three replicates were arranged for each combination. Same growth conditions which were used for raising seedlings were used after treatment. After 7 days of treatment, seedlings were harvested for analyses of various parameters.

2.3. Growth parameters

Effect of Cd and IAA on the growth of tomato seedlings was measured by observing the changes in the length of root and shoot using centimetre scale. Root and shoot of the seedlings were also weighed separately using a digital weighing balance of accuracy 1 mg to estimate the change in their fresh weight.

2.4. Estimation of Cd content

To estimate the accumulation of Cd in plant tissues of root and shoot, method described by Allen et al. (1986) was followed. The harvested dried plant material was digested with a mixture of HNO_3, H_2SO_4 and $HClO_4$, taken in a ratio of 5:1:1 (v/v), to obtain a clear solution at 80 °C. The cooled filtrate obtained after passing the solution through Whatman No 42 filter paper, was diluted to make a total volume of 15 ml. Diluted sample was used for the analysis of Cd content using atomic absorption spectrometer (iCE 3000 Series, Thermo scientific, UK).

2.5. Assessment of photosynthetic pigments

Leaves (20 mg) were taken from treated and untreated seedlings and chopped into small pieces to extract the photosynthetic pigments using acetone (80%, v/v). Method described by Lichtenthaler (1987) was used to estimate and quantify photosynthetic pigments-chlorophyll *a*, chlorophyll *b* and carotenoids from the absorption values of extract measured at 663.2, 646.5 and 470, respectively using UV–visible spectrophotometer.

2.6. Chlorophyll a fluorescence analysis

To evaluate the performance of photosystem II under various treatments, chlorophyll *a* fluorescence parameters were measured in the dark adapted leaves with the help of portable fluorometer (FluorPen FP 100, Photon System Instruments, Czech Republic). Parameters of JIP test (F_v/F_o, F_v/F_m, ψ_o, ϕE_o, PI_{ABS}, F_o/F_v, ABS/RC, TR_o/RC, ET_o/RC and DI_o/RC) were calculated as discussed by Strasser et al. (2000).

2.7. Nitric oxide detection using DAF-2DA

To visualize the production of NO in the cells of root tips, plasma membrane permeable 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) was used. DAF-2 DA is hydrolysed into 4,5-diaminofluorescein-2 by cellular esterases, which reacts with the intracellular NO to produce fluorescent DAF-2 triazole. Approximately 3 mm long root tip sections were taken and stained with 10 μM DAF-2 DA (in 10 mM Tris-HCl, pH 7.4) for 10 min in dark. Samples were later washed 2 times for 10 min in the same buffer (10 mM Tris-HCl, pH 7.4). Images were taken using Olympus BX51 Florescent microscope at the excitation wavelength of 495 and emission at 515 nm.

2.8. In vitro estimation $O_2^{\bullet-}$, H_2O_2, lipid peroxidation and electrolyte leakage

Superoxide radicals (SOR, $O_2^{\bullet-}$) estimation was performed in sample seedling by following Elstner and Heupel (1976). Hydrogen peroxide (H_2O_2) content was determined in root extract obtained from 40 mg root tissue using trichloroacetic acid (0.1% w/v) after centrifuging at 10,000 g for 15 min and 4 °C. Extract was used according to the protocol given by Velikova et al. (2000) for H_2O_2 determination. Methodology described by Hodges et al. (1999) was followed to quantify malondialdehyde (MDA) content produced as a result of peroxidation of membrane lipids. Electrolyte leakage from the root cells was also evaluated using method explained by Gong et al. (1998).

2.9. $O_2^{\bullet-}$, H_2O_2 and cell viability visualization with fluorescence microscopy

$O_2^{\bullet-}$ were detected in the root tips using dihydroethidium (DHE) as explained by Sandalio et al. (2008). DHE is a non-fluorescent reduced form of ethidium bromide and can easily diffuse across the membrane of live cells. Inside cells, DHE is oxidised by $O_2^{\bullet-}$ to a fluorescent product, oxyethidium, which is quite stable and intercalates into the cellular DNA. To observe the $O_2^{\bullet-}$ in the root tips of tomato, staining with 20 μM DHE, obtained after diluting DMSO stock of the dye, for 15 min was performed. Later samples were washed 3 times for 10 min and observed using Olympus BX51 fluorescent microscope with excitation at 488 nm and emission at 520 nm.

H_2O_2 localization was estimated using 2,7-dichlorodihydrofluorescin diacetate (DCFDA) following the method described by Morina et al. (2010). At setting of 485 nm (excitation wavelength) and 535 nm (emission wavelength) images were taken using Olympus BX-01 spectroflurometer (USA). Cell viability was observed in root tips stained with propidium iodide (Ubeda-Tomás et al., 2009) using fluorescent microscope.

2.10. Enzyme assay for AsA-GSH cycle

Root samples were prepared for assay of AsA-GSH cycle enzynmes as described in Singh et al. (2015). Ascorbate peroxidase (APX; EC 1.11.1.11) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was determined by following the method of Nakano and Asada (1981). One unit of APX activity oxidises 1 nmol ascorbate min^{-1} whereas 1 unit of DHAR activity represents reduction of 1 nmol DHA min^{-1}. The assay to measure activity of monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) was conducted using the method of Hossain et al. (1984). One unit of MDHAR activity represents oxidation of 1 nmol NADPH min^{-1}. Glutathione reductase (GR, EC 1.6.4.2) activity was estimated according to the method described by Schaedle and Bassham (1977). One unit of GR activity is responsible for 1 nmol NADPH oxidised min^{-1}.

2.11. Estimation of ascorbate and glutathione contents

The total ascorbate and total glutathione estimation was done following the methods of Gossett et al. (1994) and Brehe and Burch (1976) using standard curves of ascorbate and glutathione, respectively.

2.12. Statistical analysis

All the exhibited values are obtained from the mean of three independently conducted experiments and each experiments having two replicates (n = 6). All the values were analysed by one-way analysis of variance to determine their significance. Multiple comparison of mean of result in control and treatments was performed using the statistical hypothesis of Duncan's multiple range test with $p < 0.05$.

3. Results

3.1. Growth and biomass

Observations about the effect of different treatments on the growth parameters i.e, shoot length, root length and their fresh weight are shown in Fig. 1 (A-D). Treatment with Cd caused a significant decrease of 29.3%and 25.8% in the root and shoot length, respectively with reference to control (Fig. 1A and B). Cd supplemented with TIBA further enhanced Cd toxicity and caused a reduction of 43.1% and 38.7% in root and shoot length, respectively. However, exogenously supplied IAA alleviated toxic effect of Cd by increasing the root and shoot length by 29.2% and 23.9% respectively in comparison to Cd alone treated seedlings. In comparison to Cd treated plants, treatment of Cd + TIBA + IAA led to 17.3% increase in shoot length and 29.2% in root length.

Comparison of fresh weight of root and shoot of differently treated samples with control showed that root and shoot fresh weight was reduced by 16.4% and 18.3% in Cd treated samples and by 38.2%and 26.4%in Cd + TIBA treated seedlings, respectively (Fig. 1 C, D). While, addition of IAA with Cd increased root and shoot fresh weight by 16.3% and 11.2%, respectively as compared to Cd alone treated seedlings. For Cd + TIBA + IAA treatment the pattern of root fresh weight also showed slight stimulation.

3.2. Content of photosynthetic pigments

The results related to chlorophyll *a, b* and carotenoids are shown in Table 1. Cadmium caused a remarkable reduction in chlorophyll *a, b* and carotenoids contents, when compared with control samples. Chlorophyll *a, b* and carotenoids contents were declined by 26.3%, 20.2% and 11.9% under Cd stress and by 38.3%, 30.1% and 18.8% in Cd + TIBA treated samples, respectively. In contrast, contents of Chl *a*, Chl *b* and carotenoids increased by 14.3%, 14.2% and 8.1%, respectively in seedlings treated with the combination of Cd and IAA with reference to Cd alone treated seedlings. In Cd + TIBA + IAA treated plants Chl *a*, Chl *b* and carotenoids contents showed increase of 11.2%, 12.1% and 6.5% respectively in comparison to Cd treated plants.

3.3. Cd accumulation

Treatment of seedlings with Cd resulted in 3.8 times more accumulation of Cd in tissues of root than in shoot (Fig. 2A and B). Addition of TIBA with Cd further increased Cd accumulation. However, exogenously added IAA reduced Cd accumulation by 29.99% in root and by 17.59% in shoot. Combination of Cd + TIBA + IAA also showed

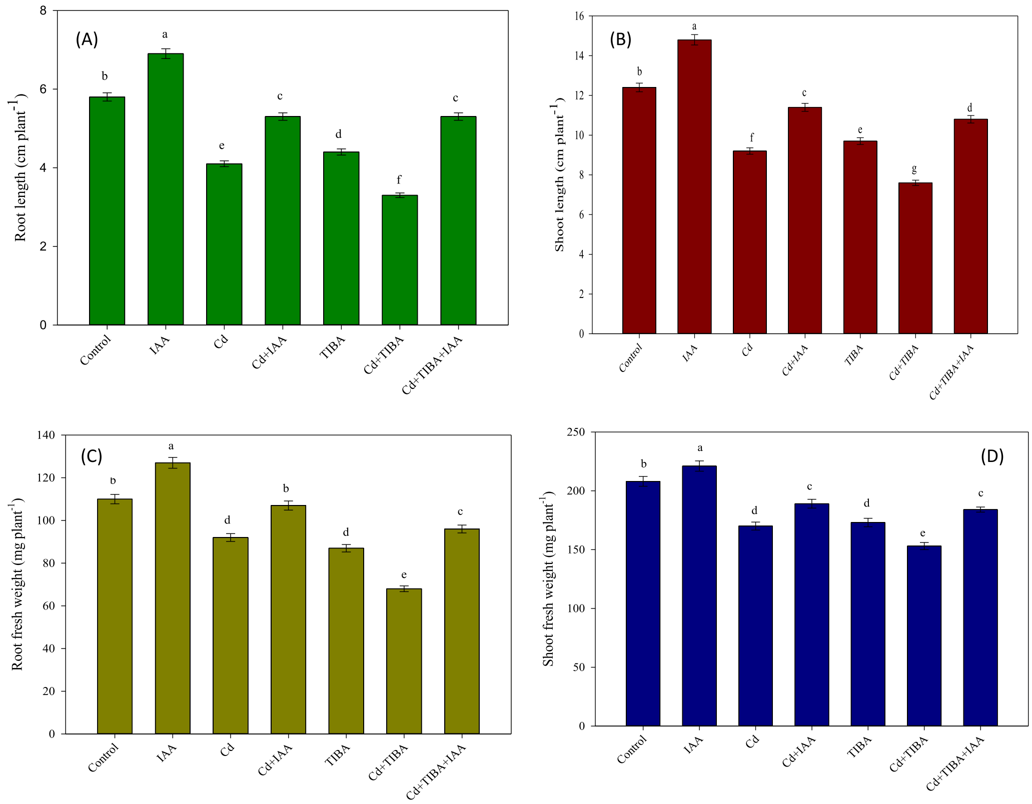

Fig. 1. Effect of IAA on growth attributes of tomato seedlings under Cd stress. Data are means ± standard error of three replicates. Bars followed by the different letters are different at $P < 0.05$ according to the DMRT.

reduction in Cd accumulation by 20.21% in root and by 13.58% in shoot with respect to only Cd treated seedlings.

3.4. NO accumulation

Imaging of NO accumulation, which was performed using DAF-2DA, showed that NO accumulation was decreased by Cd in roots (Fig. 3). TIBA also decreased the level of NO relative to the control. However, exogenously supplied IAA with Cd increased NO level in roots. Moreover, the fluorescent intensity resulted from the combination of Cd + TIBA + IAA was higher than the Cd + TIBA treated roots but lower than the Cd + IAA treated roots.

3.5. Chlorophyll *a* fluorescence using JIP test

Analysis of chlorophyll *a* fluorescence showed that F_v/F_o, F_v/F_m, ψ_o, ϕE_o and PI_{ABS} decreased by 29.7%, 12.4%, 15.6%, 17%, 27%, respectively after treatment with Cd whereas they were decreased by 29.7%, 16.9%, 17.2, 19.1%, 35% respectively due to TIBA treatment (Table 2). Under Cd stress, values of TR0/RC and ET0/RC were decreased by 11% and 13 %, respectively while F0/Fv, ABS/RC, and DI0/RC were enhanced. But application of exogenous IAA with Cd increase yield parameters and performance index and maintained energy flux parameters when compared to Cd treatment alone (Table 2).

3.6. Indicators of oxidative stress

Measurement of SOR and H_2O_2, MDA, accumulation and percentage leakage in electrolyte indicates the severity of oxidative stress in Cd and TIBA treated samples. Accumulation of SOR and H_2O_2 in roots was increased by 60.5 %and 73.6%, respectively in Cd treated seedlings in comparison to the control seedlings (Fig. 4A–D). TIBA treated seedlings also showed an increase of 76.3%in SOR and 82.1% in H_2O_2 (Fig. 4A and B). Combination of Cd and TIBA showed even higher level of SOR and H_2O_2 accumulation which was 94.7%and 132.1%, respectively in comparison to Cd alone treated seedlings. Exogenous application of IAA with Cd showed reduction in the accumulation of SOR and H_2O_2 by 31.9%and 27.2%, respectively when compared with Cd alone treated seedlings. Cd + TIBA + IAA treated seedlings had lower SOR and H_2O_2 contents with respect to Cd treated, but slight higher than the Cd + IAA treated seedlings. MDA accumulation was increased by73.3% by Cd while it was enhanced by 51.1% in TIBA treated seedlings (Fig. 4C). Combination of exogenous IAA with Cd, again showed a reduction in the level of MDA by 37.17% relative to the Cd alone treatment (Fig. 4C). Combination of TIBA and Cd further increased MDA level by 17.9%in comparison to Cd alone treated seedlings. Electrolyte leakage,

Table 1
Effect of IAA on photosynthetic pigments in tomato seedlings under Cd stress. Data are means ± standard error of three replicates. Values within the same row followed by the different letters are different at $P < 0.05$ according to the DMRT.

Parameters	Treatments						
	Control	IAA	Cd	Cd + IAA	TIBA	Cd + TIBA	Cd + TIBA + IAA
Chl *a* (mg g^{-1} FW)	1.33 ± 0.021b	1.43 ± 0.020a	0.98 ± 0.010d	1.12 ± 0.022c	0.99 ± 0.019d	0.82 ± 0.016e	1.09 ± 0.022c
Chl *b* (mg g^{-1} FW)	0.352 ± 0.020a	0.392 ± 0.007a	0.281 ± 0.005d	0.321 ± 0.006c	0.292 ± 0.005d	0.246 ± 0.004e	0.315 ± 0.006c
Car (mg g^{-1} FW)	0.421 ± 0.008b	0.486 ± 0.009a	0.371 ± 0.007d	0.401 ± 0.008c	0.387 ± 0.008d	0.342 ± 0.006e	0.395 ± 0.007c

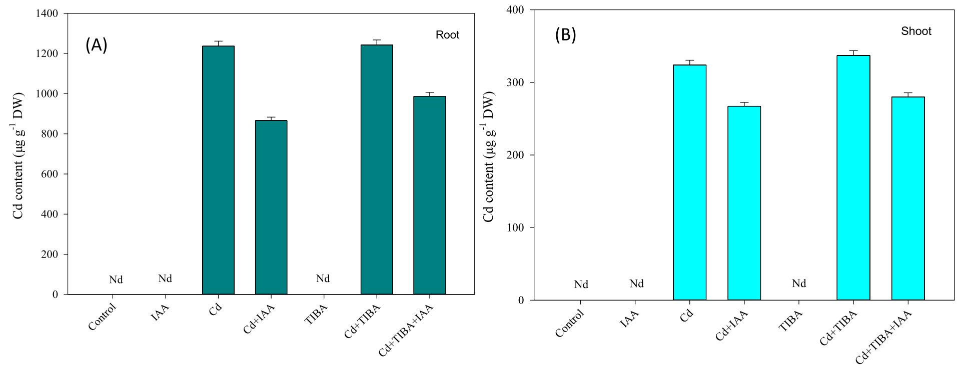

Fig. 2. Effect of IAA on Cd accumulation in tomato seedlings exposed to Cd stress. Data are means ± standard error of three replicates. Bars followed by the different letters are different at P < 0.05 according to the DMRT.

another indicator of oxidative damage, was appeared to rise by 157.1% in Cd treated seedlings while comparing to the control (Fig. 4D). But IAA mitigates this effect of Cd by reducing the electrolyte leakage up to 52.77% relative to Cd alone treated seedlings. Cd + TIBA treated seedlings had 16.3% higher electrolyte leakage than the Cd alone treated seedling.

Detection of $O_2^{\bullet-}$ using DHE and H_2O_2 using DCF-DA fluorescent probe showed that their accumulation was maximum in Cd treatment followed by TIBA with reference to control (Fig. 5a-c). There was not any significant difference in their fluorescence in IAA and control seedlings. Application of exogenous IAA with Cd showed significant reduction in their accumulation in comparison to Cd alone treated seedlings. Combination of Cd with TIBA further increased fluorescent intensity in respect to Cd alone treated seedlings while combination of all three (Cd + TIBA + IAA) showed lower fluorescence.

3.7. Response of AsA-GSH cycle enzymes

The assays of APX, MDHAR, DHAR and GR activity showed reduction in their activity by 11.4%, 31.8%, 21.3%and 30.11%, respectively after exposure to Cd stress (Fig. 6A–D). Exogenous IAA with Cd showed significant increase of 27.79, 81.52%, 88.85% and 100% in the activity of APX, MDHAR, DHAR and GR, respectively with respect to Cd alone treated seedlings. Furthermore, the combination of Cd + TIBA caused the maximum decrease in the activity of all the AsA-GSH cycle enzymes. There was a decrease of 24.9% in APX, 38.63% in MDHAR, 45.74% in DHAR and 21% in GR activity due to addition of TIBA with Cd. The combination of Cd + TIBA + IAA led to increase in activity of APX, MDHAR, DHAR and GR by 27.1%, 69.68%, 68.7% and 88.7% respectively.

3.8. Response of ascorbate and glutathione contents

Effect of Cd on non-enzymatic components of AsA-GSH cycle was estimated by the content of ascorbate and glutathione. Cadmium resulted in 22% decrease of total ascorbate pool and 23% reduction in total GSH pool in comparison to the control seedlings (Table 3). TIBA caused decline of 20% and 32%, but TIBA and Cd together contributed to a decrease of 38% and 45%, in total ascorbate and total GSH level, respectively. Exogenously supplied IAA showed stimulation of total ascorbate and total GSH level by 55% and 63%, respectively, under stress of Cd. Even, Cd + TIBA + IAA treated seedlings have higher level of AsA and GSH content in their root cells than the control seedlings (Table 3).

4. Discussion

In the present study, the Cd caused significant negative effect on plant growth as indicated by growth attributes (Fig. 1). Similar trends were seen in previous finding showing negative impact of Cd in tomato, grown in soilless media (Moral et al., 1994). However, exogenous application of IAA prevented Cd-mediated negative impact on growth (Fig. 1). Similarly, exogenous application of IAA was shown to reduce Cd mediated toxicity in eggplant seedlings (Singh and Prasad, 2015) but clear mechanisms remain elusive. Our finding showed that when exogenous IAA was supplemented with Cd it reduced Cd toxicity in tomato seedlings by inducing NO accumulation and restricting Cd accumulation. The results showed that increased accumulation of Cd in tomato seedlings coincided with decreased level of NO (Figs. 2 and 3). However, addition of IAA with Cd resulted in significant reduction of Cd accumulation in both root and shoot together with higher level of NO (Figs. 2 and 3). Under similar condition, growth attributes were higher than Cd alone treated seedlings suggesting that IAA had a role in mitigating Cd stress by involving NO (Figs. 1–3).

Further, results show that TIBA alone was able in causing toxicity in tomato seedlings, and together with Cd, it further increased Cd toxicity along with a drastic decline in NO (Figs. 1–3), suggesting that homeostasis of IAA, which requires NO, is essential for stress tolerance and plant survival under Cd stress. Since negative impact of TIBA was nullified by exogenous IAA along with higher level of NO, suggesting that it might have compensated reduced level of IAA in roots which occurred due to disturbances in basipetal transport of IAA. Our

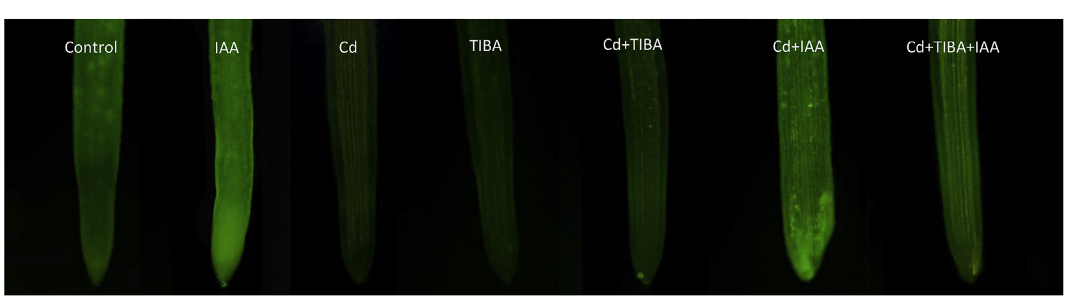

Fig. 3. Fluorescence histochemical staining of roots for nitric oxide (NO) using DAF-2 DA in tomato. Experiments were repeated three times. Scale bar = 500 μm.

Table 2
Effect of IAA on chlorophyll *a* fluorescence in tomato seedlings under Cd stress. Data are means ± standard error of three replicates. Values within the same row followed by the different letters are different at P < 0.05 according to the DMRT.

Chl *a* Fluorescence Parameters	Treatments						
	Control	IAA	Cd	Cd + IAA	TIBA	Cd + TIBA	Cd + TIBA + IAA
F_v/F_o	3.7 ± 0.074b	3.9 ± 0.078a	2.6 ± 0.052d	3.1 ± 0.062c	2.6 ± 0.051d	2.3 ± 0.046e	3 ± 0.06c
F_o/F_v	0.27 ± 0.005d	0.25 ± 0.005de	0.32 ± 0.006b	0.29 ± 0.005bc	0.31 ± 0.006b	0.34 ± 0.006a	0.29 ± 0.006bc
F_v/F_m	0.785 ± 0.015b	0.821 ± 0.015a	0.687 ± 0.014e	0.765 ± 0.015c	0.65 ± 0.014f	0.621 ± 0.013g	0.735 ± 0.014d
Ψ_o	0.64 ± 0.012a	0.65 ± 0.013a	0.54 ± 0.010c	0.62 ± 0.012 ab	0.53 ± 0.010c	0.49 ± 0.009d	0.59 ± 0.011bc
ϕE_o	0.47 ± 0.009 ab	0.49 ± 0.009a	0.39 ± 0.007d	0.44 ± 0.008c	0.38 ± 0.007d	0.33 ± 0.006e	0.42 ± 0.007c
PI_{ABS}	2.23 ± 0.044 ab	2.25 ± 0.045a	1.62 ± 0.037e	2.09 ± 0.041c	1.45 ± 0.040f	1.32 ± 0.033 fg	1.92 ± 0.038d
ABS/RC	0.27 ± 0.005cd	0.26 ± 0.005cd	0.35 ± 0.007b	0.29 ± 0.005c	0.35 ± 0.007b	0.39 ± 0.007a	0.34 ± 0.006b
TR_o/RC	2.1 ± 0.042bc	2.2 ± 0.044 ab	1.87 ± 0.03d	2.3 ± 0.046a	1.68 ± 0.048e	1.54 ± 0.030f	2.2 ± 0.044ab
ET_o/RC	1.39 ± 0.027ab	1.41 ± 0.028a	1.21 ± 0.024d	1.36 ± 0.027bc	1.21 ± 0.024d	1.15 ± 0.023e	1.37 ± 0.027ab
DI_o/RC	0.69 ± 0.013e	0.70 ± 0.014e	0.79 ± 0.015bc	0.71 ± 0.014e	0.81 ± 0.016b	0.87 ± 0.017a	0.75 ± 0.015d

hypothesis is justifiable as it is noticed that shoot-synthesized IAA and its re-distribution leads to maximum auxin level in roots for development (Armengot et al., 2016). Similar to our results, TIBA has been shown to reduce maize growth by reducing polar transport of auxin (Bronsema et al., 2001).

Previous studies showed that Cd remarkably affected photosynthetic pigments and photosynthesis in *Cajanus cajan, Brassica napus, Gracilaria domingensis* (Khudsar and Iqbal, 2001; Baryla et al., 2001; dos Santos et al., 2012). The photosynthetic pigment Chl *a* and Chl *b* contents were adversely affected by Cd treatment. Reduction in photosynthetic pigments under Cd stress can be related with their oxidative damage as evidenced from higher level of oxidative stress markers (Fig. 4). Besides this, Cd is also reported that it causes interference in their biosynthesis (López-Millán et al., 2009). However, exogenous addition of IAA with Cd maintained higher level of photosynthetic pigments (Table 1).

Chlorophyll *a* fluorescence is fast and non-destructive technique to assess photosynthetic performance of stressed plants. Past findings have shown that Cd affects net photosynthesis and other photosynthetic pigments along with chlorophylls in tomato (Baszyńki et al., 1980; Chen et al., 2011; Wang et al., 2014). Lack of active reaction centres in PS II was indicated by decreased value of F_v/F_o due to reduction in the available Q_A. Decreased value of ϕE_o and ψ_o suggested disturbed electron flow between the photosystems which ultimately reduced the performance index (PI_{ABS}) of the seedlings. Moreover, increase in F_o/F_v shows about degrading effect of Cd on the oxygen evolving complex that might have occurred due to ability of Cd to replace similar ions like Mn^{2+} and Ca^{2+} from enzymatic complexes. Increase in the values of specific energy flux parameters such as DI_o/RC suggested about dissipation of excess light energy under Cd stress. Exogenous IAA showed its alleviating effects on quantum yields, size and number of active reaction centres and water splitting complex which was signified by

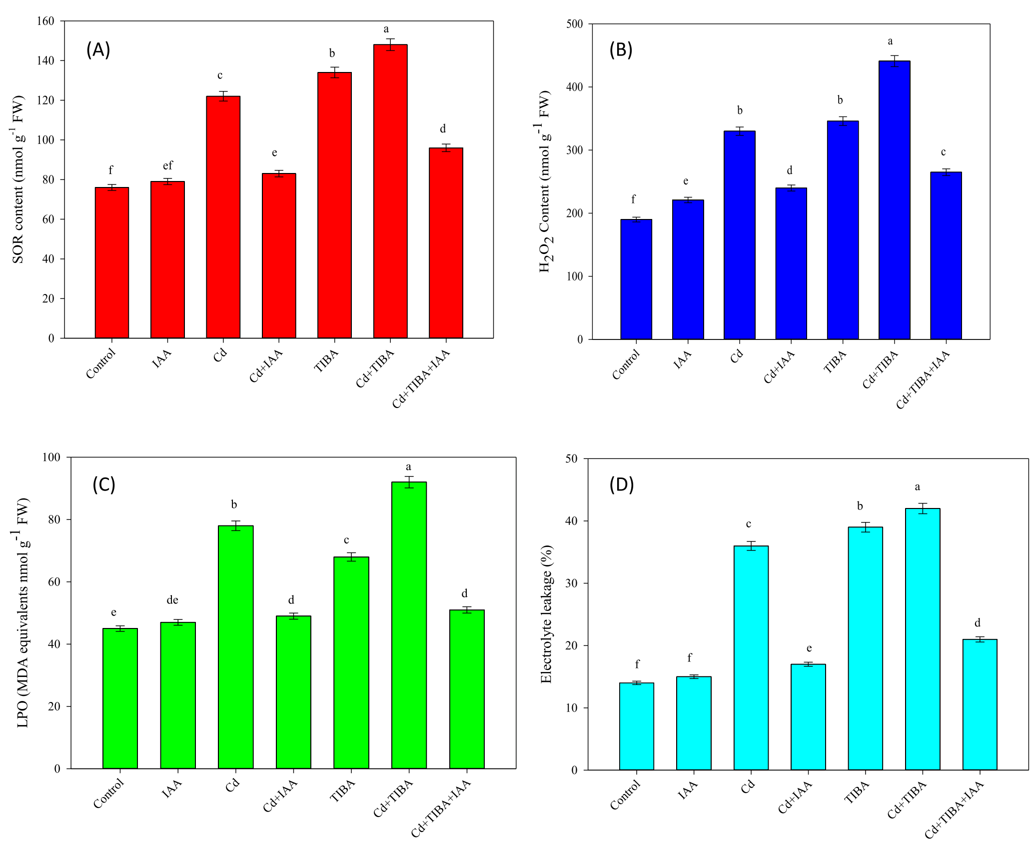

Fig. 4. Effect of IAA on superoxide radical (SOR, $O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), lipid peroxidation (LPO) and electrolyte leakage in tomato seedlings exposed to Cd stress. Data are means ± standard error of three replicates. Bars followed by the different letters are different at P < 0.05 according to the DMRT.

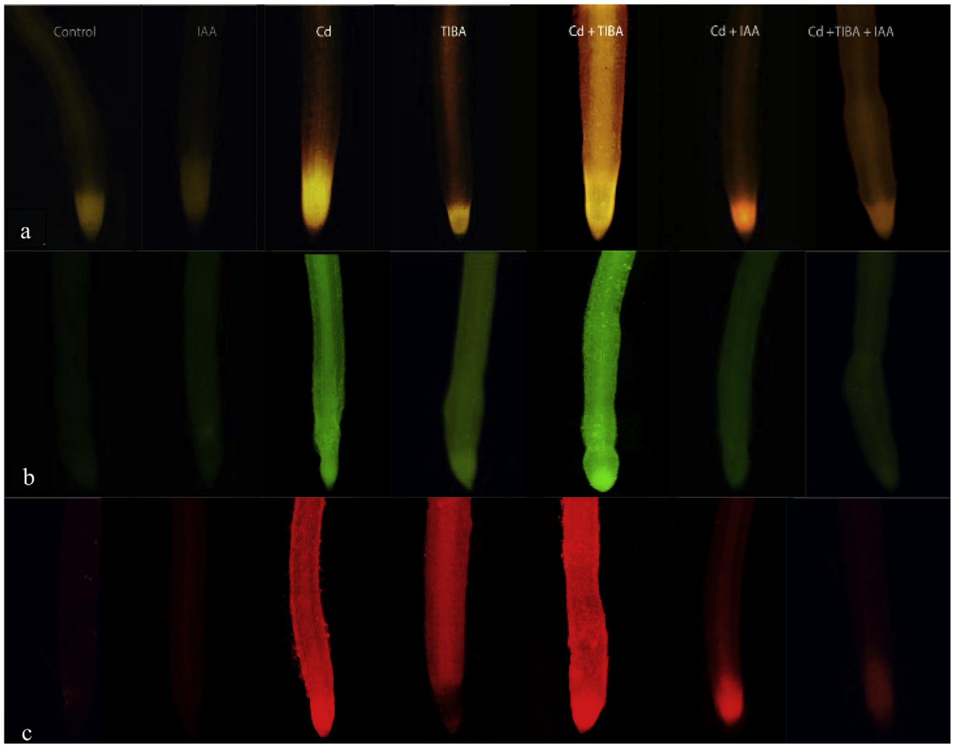

Fig. 5. Fluorescence histochemical staining of roots for superoxide radical (a), hydrogen peroxide (b) and cell viability (c) using dihydroethidium (DHE), 2′, 7′-dichlorofluorescein diacetate (DCFDA) and propidium iodide (PI), respectively. Scale bar = 500 µm.

considerable increase in F_v/F_o, ϕE_o, ψ_o and PI_{ABS}. These enhanced parameters can be the result of reduced Cd uptake. Specific energy flux parameters also reduced in response to the exogenous IAA while TIBA with Cd worsened the damage on the PS II.

Cadmium leads to deleterious effect on plants by inducing ROS formation (Romero-Puertas et al., 2004; Kumar et al., 2008; Andersen et al., 2013). Significant increase in $O_2^{\cdot-}$ and $H_2O_2$was observed after exposure to Cd stress due to damage occurred in photosynthetic

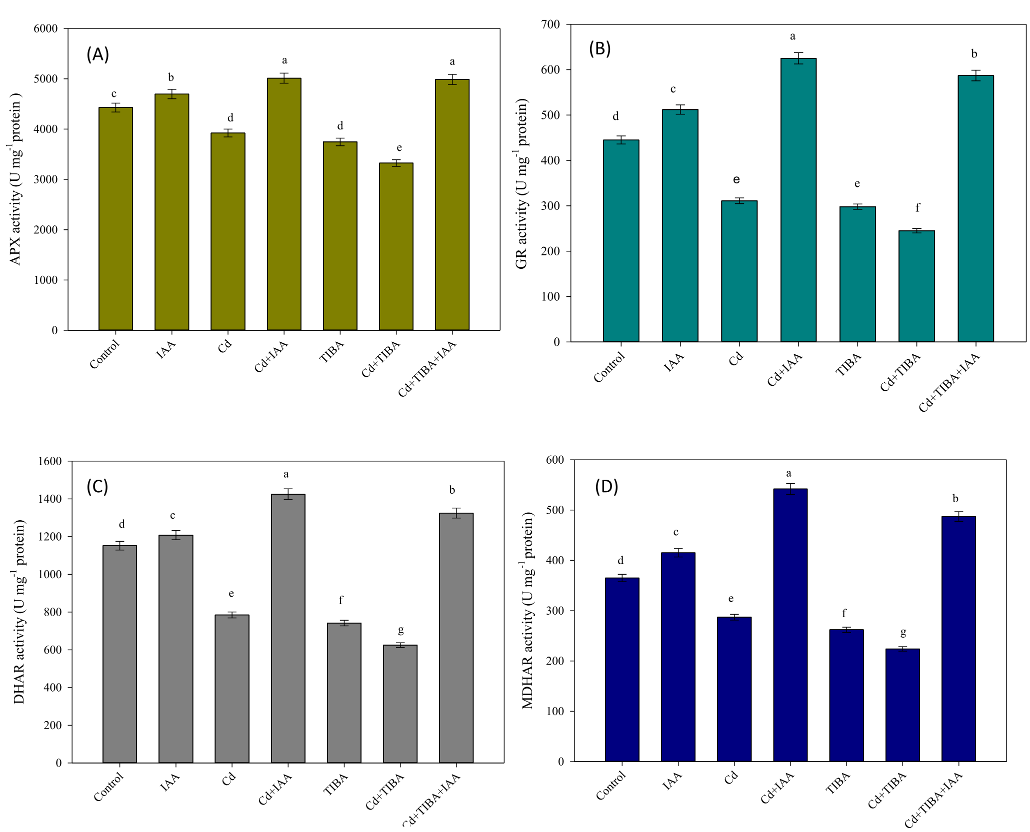

Fig. 6. Effect of IAA on ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) in tomato seedlings exposed to Cd stress. Data are means ± standard error of three replicates. Bars followed by the different letters are different at P < 0.05 according to the DMRT.

Table 3
Effect of IAA on total ascorbate (AsA + DHA) and total glutathione (GSH + GSSG) contents in tomato seedlings under Cd stress. Data are means ± standard error of three replicates. Values within the same row followed by the different letters are different at P < 0.05 according to the DMRT.

	Treatments						
	Control	IAA	Cd	Cd + IAA	TIBA	Cd + TIBA	Cd + TIBA + IAA
AsA + DHA (μmol g^{-1} FM)	3.45 ± 0.06c	4.25 ± 0.07b	2.69 ± 0.04	5.36 ± 0.09a	2.75 ± 0.04d	2.13 ± 0.03e	4.89 ± 0.08b
GSH + GSSG (μmol g^{-1} FM)	2.15 ± 0.04d	2.69 ± 0.05c	1.65 ± 0.03	3.52 ± 0.07a	1.45 ± 0.02e	1.18 ± 0.02ef	3.25 ± 0.06b

machinery and leakage of electron to the oxygen. TIBA also induced $O_2^{\bullet-}$ and H_2O_2 production and together with Cd it further enhanced their production in the seedlings. Under similar conditions, higher level of ROS was also confirmed by fluorescent microscopy of root tips using probes for $O_2^{\bullet-}$ (DHE) and H_2O_2 (DCFDA). This increased level of ROS consequently increased the level of lipid peroxidation, membrane damage and leakage of electrolyte. However, exogenous addition of IAA with Cd significantly lowered accumulation of ROS and their associated damage to lipids and membranes.

Cadmium has been shown to alter level of enzymes and metabolites associated with AsA-GSH cycle in mung, eggplant and cucumber (Zhang et al., 2002; Anjum et al., 2011; Singh and Prasad, 2015). Enzymes i.e. APX, DHAR, MDHAR and GR that are part of AsA-GSH cycle showed alteration in their activities in the presence of Cd. Similarly, total ascorbate and glutathione contents were also declined by Cd (Singh et al., 2018). Oxidative stress triggered the activation of plants defence mechanism in the cells which can regulate the activity of AsA-GSH cycle enzymes. Cd decreased the activity of APX, MDHAR, DHAR and GR which coincided with declined level of ascorbate and glutathione leading to build-up of cellular ROS and their associated damage as indicated by the *in vivo* imaging of $O_2^{\bullet-}$and $H_2O_2$and higher oxidative stress markers. A component of AsA-GSH cycle further declined by TIBA suggesting that homeostasis of IAA is essential for proper growth as well as survival of plants under metal stress. Application of exogenous IAA showed increase in the activity of antioxidant enzymes along with significant decrease in the oxidative stress markers. It indicates the regulatory role of IAA in AsA-GSH cycle which collectively works to alleviate Cd toxicity.

Reduction in the content of AsA-GSH cycle metabolites, ascorbate and GSH under Cd stress indicates either their higher consumption or decreased regeneration due to Cd stress. Under Cd stress, their reduced pools also limit the activity of AsA-GSH cycle enzymes which may contribute to the ineffective ROS scavenging. However, exogenous IAA stimulates the level of ascorbate and GSH which enhanced the ability of tomato seedlings to counter the Cd induced oxidative stress. Bashri and Prasad (2016) also observed the stimulatory role of IAA on ascorbate and GSH level in *Trigonella foenum-graecum* L. under Cd stress.

5. Conclusions

This work shows that exposure of Cd to the tomato seedlings causes significant decrease in the root length, shoot length and their fresh weight. These effects occurred due to the higher accumulation of Cd which leads induction of oxidative stress markers due decline in NO level and components of AsA-GSH cycle and thus collectively reduced growth attributes due to altered photosynthesis. However, exogenous application of IAA together with Cd considerably alleviates Cd toxicity by reducing Cd and oxidative stress markers accumulation, inducing NO level and AsA-GSH cycle which protect photosynthetic pigments and photosynthesis and thus improved growth was noticed. TIBA alone as well as with Cd reduced tomato growth along with reduced level of NO suggesting that homeostasis of IAA is essential for stress tolerance and plant survival and requires NO. Since negative impact of TIBA was nullified by exogenous IAA along with higher level of NO suggesting that it might have compensated reduced level of IAA in roots which occurred due to disturbances in basipetal transport of IAA. All together results showed that IAA was able in significantly reducing Cd stress and for this task requires NO.

Conflicts of interest

No conflicts of interest from authors.

Contribution

MYK, DKT, SS designed experiments. MYK, VY, VP and DKT performed experiments. MYK, VP, VPS, DKT, SS and VPS analysed data and wrote the manuscript. SS, SMP, DKC, NR, VPS, DKT and SS critically evaluated the manuscript.

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