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Differential responses of indole-3-acetic acid on *Trigonella* seedlings exposed to cadmium stress: Impact on nitrate and ammonium assimilating enzymes

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Abstract: **Cadmium (Cd) is a hazardous environmental pollutant because of its high solubility and mobility in soil, which alters various physiological processes and reduces plant productivity. Thus, there is a need to develop the techniques to counteract the adverse effects of heavy metals on medicinally important *Trigonella foenum-graecum* L., a widely used vegetable all over the world. Considering the above point, the present work was conducted to investigate whether exogenous application of indole-3-acetic acid [low (IAA_L, 10 µM), middle (IAA_M, 50 µM) and high (IAA_H,100 µM)] can alleviate cadmium (Cd_1, 3 mg Cd kg^{-1} and Cd_2, 9 mg Cd kg^{-1}) induced damaging effect in *Trigonella* seedlings. Cadmium at both doses declined the growth, total chlorophyll, net photosynthesis, nitrate and nitrite contents and activity of nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase. Exogenous application of IAA at IAA_L and IAA_M doses attenuated the Cd-induced decline in growth and enzymes of nitrogen metabolism, and these effects were more pronounced in IAA_L treated seedlings. However, the IAA_H dose resulted in a significant decrease in the above parameters compared to the control. Overall results suggest that IAA behaved differentially under Cd stress in *Trigonella* seedlings and produced alleviating effect at a low dose, i.e., 10 µM.**

Keywords: **Cadmium, indole-3-acetic acid, nitrogen metabolism, *Trigonella* seedlings.**

1. INTRODUCTION

Industrial activities have generated many pollutants which contaminate soil, air and water and create a global problem. Heavy metals like cadmium (Cd) are among the most common soil contaminants. The global scale of concentration in surface soils of various heavy metals ranges from 00.07 to 1.1 mg kg-1 soil, with values above 0.5 mg kg-1 indicating anthropogenic Cd inputs (WHO, 2007). Due to the overuse of phosphate fertilisers and municipal sewage sludge in agriculture for soil amendment, the threshold level of Cd in the soil is consistently rising (Gill et al., 2012). Cadmium, in particular, is a major problem since plants may quickly absorb it and contribute to bio-concentration. Plants are the most common source of Cd intake by humans. Cd, on the other hand, is a non-essential element for plants, but it influences nutrient uptake and limits root and shoot growth because it is readily taken up by roots and transmitted through the xylem to the vegetative and reproductive organs (Sanita'diToppi and Gabbrielli, 1999; Metwally et al., 2005). Exposure to

excessive amounts of Cd causes chlorosis, slows photosynthesis, restricts development, and lowers water and nutrient intake, ultimately killing plants (Sanita'diToppi and Gabbrielli, 1999).

Nitrogen metabolism is also important for plant growth and development, and nitrate and ammonia absorbing enzyme activity is dramatically reduced under abiotic stress (Debouba et al., 2006). Though soil contains several forms of inorganic nitrogen, plants prefer nitrate as a nitrogen source. Once the root absorbs nitrate, it assimilates to ammonium (NH_4^+) through regulated action of nitrate reductase (NR) and nitrite reductase (NiR) activities which are found to decrease under Cd stress, leading to reduced nitrate assimilation by plants. NH_4^+ Enters into the carbon skeleton via the primary route of ammonia assimilation, which involves two enzymes, i.e., glutamine synthetase (GS) and glutamate synthase (GOGAT). Furthermore, the aof minating role GDH activity provides an alternate pathway o,f NH_4^+ assimilation, which generally functions condition stress condition. The glutamine synthetase–glutamate synthetase (GS/GOGAT) cycle, which involves ammonium incorporation into carbon skeletons, is likewise influenced by Cd stress (Ouariti et al., 1997, Gouia et al., 2000). Under Cd stress, however, glutamate dehydrogenase (NADH-GDH) activity rose significantly (Boussama et al., 1999). All of these investigations demonstrate that Cd can have a deleterious impact on nitrogen metabolism and reduce plant production.

Plant growth regulators (PGRs) regulate nutrient intake, stomatal movement, photosynthesis, and the activity of numerous enzymes under a variety of stress conditions (Hayat and Ahmad, 2007). PGRs have recently been used to increase plant tolerance to stress (Leul and Zhou, 1999; El-Tayeb, 2005; Singh and Prasad, 2015). Auxin is a plant hormone that affects a variety of physiological and developmental processes (Zeevaart and Creelman, 1988; Seo and Koshiba, 2002). As a signal hormone in higher plants, IAA governs plant growth and development and plays an important role in stress tolerance. Several studies have found that exogenously administered IAA can stimulate growth while also alleviating the harmful effects of abiotic stressors in several plant species (Egamberdieva, 2009, Singh and Prasad, 2015). As a result, it is possible that applying IAA to agricultural plants will protect them against increased Cd levels.

Trigonella foenum-graecum L. (fenugreek or methi) is a Fabaceae annual crop. Trigonella leaves and seeds have long been used to make extracts and powders for medical purposes such as anti-diabetic, anti-fertility, anti-cancer, anti-microbial, anti-parasitic, and hypocholesterolemic properties (Al-Habori and Raman, 2002; Basch et al., 2003). Findings have demonstrated that the growth and physiology of the *Trigonella* seedlings are affected adversely under metal stress (Elleuch *et al.,* 2013; Patel and Patel, 2013; Zayneb *et al.,* 2015); mainly prevalent in the catchment area of highly populated cities; however, detail mechanism related to the protection against Cd under foliar application of IAA is not yet known. Thus, our present study aimed to examine the mechanisms of IAA-induced regulation of leaf nitrogen metabolism under cadmium stress in *Trigonella foenum-graecum* L.

2. MATERIALS AND METHODS

Plant material and growth conditions

Trigonella seeds were sterilised for 15 minutes in a sodium hypochlorite solution (2 percent, v/v) before being soaked in distilled water for 4 hours. Wet seeds were covered in muslin fabric and kept at 25±2 °C in the dark to germinate. After 24 hours, the germinated seedlings were transplanted into plastic pots containing 150 g sandy loam soil and 9:1 w/w farmyard manure. Cd_1:3 mg Cd kg^{-1} dirt (below the allowed limit, Awasthi, 2000) and Cd_2: 9 mg Cd kg^{-1} soil were blended with soil before sowing germinated seedlings (beyond the permissible limit). The pots were placed in an open field with photosynthetic active radiation of 900±100 mol m^{-2} sec^{-1}, 65 % humidity, and a temperature of 20±5 °C. The seedlings were watered with distilled water on a regular basis, and after 15 days of development, three doses of IAA were administered exogenously: low (IAA_L, 10 µM), middle (IAA_M, 50 µM), and high (IAA_H, 100 µM) . The experimental design includes twelve combinations i.e. control (without treatment), Cd_1, Cd_2, IAA_L, Cd_1+IAA_L, Cd_2+IAA_L, IAA_M, Cd_1+IAA_M, Cd_2+IAA_M, IAA_H, Cd_1+IAA_H, Cd_2+AA_H. The different parameters were analyzed in leaves of the *Trigonella* seedlings after 15 days of IAA treatment.

Estimation of growth

The leaves were harvested from the different treatments, and fresh weights were recorded using a digital balance. After that, the leaf area of the same leaves was taken per plant using a leaf area meter.

Estimation of total chlorophyll and net photosynthesis

Total chlorophyll content was estimated according to the method of Lichtenthaler (1987). Net photosynthesis was estimated in terms of oxygen evolution from leaf discs by the methods of Kurra-Hotta *et al.* (1987).

Estimation of nitrate, nitrite and ammonium contents

Estimation of nitrate content was performed by the method of Cataldo *et al.* (1975). Nitrite content was estimated using the method of Snell and Snell (1949). Ammonium content was estimated by the method of Molins-Legua *et al.* (2006) using Nessler reagent.

Estimation of nitrogen assimilation enzymes

Robin (1979) techniques were used to measure nitrate reductase activity (NR, EC 1.7.99.4), which was expressed as mol NO_2^-formed g^{-1} FW h^{-1}. Losada and Paneque (1971) used the Losada and Paneque technique to measure nitrite reductase (NiR, EC 1.7.2.1), which was represented as mol NO_2^- reduced g^{-1} FW h^{-1}. The activity of glutamine synthetase (GS; EC 6.3.1.2) was determined using Lillo's (1984) technique, which was expressed as A_{540} nm g^{-1} FW h^{-1}. The activity of glutamine-2-oxoglutarate aminotransferase, commonly known as glutamate synthase (NADH-GOGAT; EC 1.4.1.14) assayed by the method of Singh and Srivastava (1986) and represented with one unit equaling 1 nmol NADH oxidised h^{-1}. The activity of glutamate dehydrogenase (GDH; EC 1.4.1.2) was measured using Singh and Srivastava's (1983) technique and represented in terms of nmol NADH oxidised h^{-1}.

Statistical analysis

ANOVA (analysis of variance) was used to examine the data, and DMRT (Duncan's Multiple Range Test) was used to look for significant differences between treatments at $P<0.05$ levels. The provided findings are the averages of three separate trials (n=3).

3. RESULTS

Growth

Growth was assessed in terms of leaf fresh weight and area, which decreased substantially ($P<0.05$) by 8 and 15%, and 6 and 12%, respectively, under both cadmium (Cd) dosages (Cd_1; 3 mg Cd kg^{-1} soil and Cd_2; 9 mg Cd kg^{-1} soil) in Trigonella seedlings. Foliar applications of IAA at 10 µM (IAA_L), 50 µM (IAA_M), and 100 µM (IAA_H) resulted in distinct growth responses in Cd-treated and untreated seedlings. Exogenous IAA considerably reduced the repressive effects of Cd on development, notably at IAA_L (10 µM IAA), demonstrating 100% relief under Cd1 stress and just an 8% loss in leaf fresh weight under Cd2 stress. *Trigonell*a seedlings showed smaller increase than IAA_L at 50 µM dosage of IAA and improved growth metrics under Cd stress. However, at a greater dosage of IAA (IAA_H, 100 µM), Cd toxicity was exacerbated in Trigonella seedlings, with a decrease in leaf fresh weight of 24 and 38 percent and a decrease in leaf area of 21 and 34 percent under Cd_1 and Cd_2 stress, respectively, compared to control (Fig. 1 a-b).

Total chlorophyll and net photosynthesis

Cadmium at both dosages significantly ($P<0.05$) reduced chlorophyll content in Trigonella seedlings by 8 and 16 percent, respectively. The application of IAAL and IAAM to Cd-exposed seedlings resulted in considerable reductions in chlorophyll content, and the reduction was even more pronounced in the case of Cd1 with IAAL treated seedlings. In contrast, the use of IAAH under Cd stress reduced the chlorophyll concentration even more (Fig. 1 c). Net photosynthesis was assessed as oxygen evolution in Trigonella seedlings grown under Cd1 and Cd2 conditions and exhibited substantial ($P<0.05$) suppression of 22 and 35%, respectively, above the control value. Cd-induced inhibitory impact was greatly reduced with IAAL and IAAM dosages; however, with IAAH dose, photosynthetic activity was further hindered, with 33 and 36 percent reductions under Cd1 and Cd2, respectively (Fig. 1 d).

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Nitrogen contents

The results shown in Fig.2 a-b revealed that Cd treatments decreased NO_3^- and NO_2^- contents in leaves of *Trigonella* seedlings; however, the decline in contents was more prominent at higher concentrations of Cd, i.e., Cd_2 stress. The decrease was 10 and 25 % in NO_3^- content and 5 and 18 % in NO_2^- content under Cd_1 and Cd_2 stress, respectively. When seedlings were sprayed with low and middle doses of IAA, the adverse effects of both doses of Cd on nitrate and nitrite content were significantly alleviated. On the other hand, a high IAA dose further decreased the NO_3^- NO_2^- contents under Cd_1 and Cd_2 stress (Figs. 2 a-b). The result of ammonium content revealed that Cd treatments increased the NH_4^+ content in leaves of *Trigonella* seedlings by 6 and 15 % under Cd_1 and Cd_2 stress, respectively. The application of low and middle doses of IAA ameliorated the Cd-induced content enhancement NH_4^+. Contrary to this, high doses of IAA further increased the NH_4^+ content under Cd_1 and Cd_2 stress (Figs. 2c).

Nitrate and nitrite reductase activities

Figure 3 shows the findings of nitrate reductase (NR) and nitrite reductase (NiR) activities. Cadmium at Cd_1 and Cd_2 dosages reduced NR activity by 20% and 38%, respectively, and NiR activity by 16 and 26%, compared to control. The inhibitory effect generated by both the dosages of Cd on NR and NiR activities was ameliorated by foliar application of IAA at low and middle doses, and this effect was more noticeable in Cd1 stress with IAA_L and treatments. In contrast, IAA_H treatment reduced the activity of NR and NiR under Cd stress even further (Figs. 3 a-b).

Ammonium assimilating enzymes

In comparison to control, the activity of GS and GOGAT was reduced by 30 and 44 percent, and 18 and 35 percent, respectively, under Cd_1 and Cd_2 stress. Exogenous IAA at low (IAA_L) and moderate (IAA_M) levels significantly reduced Cd-induced reduction in GS and GOGAT activity, which was especially noticeable in Cd_1 stress. In comparison, the IAAH treatment reduced GS and GOGAT activity under Cd stress even more (Figs. 4 a-b). In comparison to control, the aminating activity of GDH in *Trigonella* seedlings subjected to Cd_1 and Cd_2 dosages increased by 80 and 90 percent, respectively. Application of IAA at low (IAA_L) and middle (IAA_M) doses resulted in a further rise in the activity of GDH in Cd_1 and Cd_2 treated seedlings. Upon exposure of seedlings to the higher dose of tested IAA (IAA_H), there was an increase in GDH activity, but less than that of low and middle doses of IAA (Fig. 4c).

4. DISCUSSION

The goal of this study is to see how exogenous indole acetic acid (IAA, an auxin) affects the growth of *Trigonella foenum-graecum* L. seedlings that have been exposed to two different cadmium dosages (Cd_1 and Cd_2) of cadmium (Cd). Furthermore, by assessing light-harvesting pigments, net photosynthetic activity (photosynthetic oxygen output), and nitrogen metabolism, efforts have been made to link the growth performance of test seedlings under the above conditions. Cadmium treatments (Cd_1 and Cd_2) impeded *Trigonella* seedling growth, resulting in reduced leaf area and fresh weight (Fig. 1 a-b). This decrease in growth could be correlated with a considerable accumulation of Cd in *Trigonella* seedlings, enhanced level of reactive oxygen species (ROS) (Bashri and Prasad, 2016), decreased light-harvesting components (Fig. 1 c), nutrient uptake (Guo *et al.*, 2007), net photosynthetic activity (Fig. 1 d) and activity of nitrogen assimilating enzymes (Figs. 3-4). These results are in agreement with earlier findings where Cd-induced suppression in the growth of *Vigna radiata* L. (Anjum *et al.*, 2011) was also correlated with the damaging effect on light-harvesting pigments and photosynthetic activity. Besides this, Chaoui and El Ferjani (2005) also correlated the Cd-induced suppression in the growth of pea seedlings with the sharp decline in growth promoting hormone, i.e., auxin, due to enhanced activity of auxin degrading enzyme, i.e., IAA oxidase. Recently, Xu *et al.* (2010) and Hu *et al.* (2013) have supported this view that endogenous auxin levels declined rapidly under Cd stress in *Medicago truncatula* and *Arabidopsis thaliana* seedlings, respectively. Similarly, Cd-induced growth inhibition was also reported in *Solanum melongena* seedlings (Singh and Prasad,

2015). Further, the outcome of this study suggests that IAA at tested doses differentially modulated Cd toxicity on growth in *Trigonella* seedlings by regulating photosynthesis and nitrogen metabolism. Significant amelioration in Cd-induced toxicity on the growth of test seedlings by low (IAA_L) and middle (IAA_M) doses of IAA might have been explained based on significant improvement in chlorophyll content (Fig. 1 c), photosynthetic performance (Fig. 1 d), and activity of nitrogen assimilating enzymes (Figs. 3-4). The IAA has also been reported to cause alleviating effect on Cd-induced toxicity on growth in *Solanum melongena* seedlings (Singh and Prasad,2015). In contrast, IAA at a higher dose (IAA_H) resulted in a further reduction in growth under Cd stress, which could be due to decreased uptake of essential mineral nutrients, as seen in maize plants treated with a high dose (500 µM) of IAA (Wang et al. 2007), and excess production of ethylene, which causes leaf senescence (Arteca and Arteca, 2008).

Total chlorophyll content showed a significant decline at both doses of Cd, and these results were consistent with the earlier report where Cd inhibited the chlorophyll biosynthesis (Qian et al., 2009). The considerable rise in Chl content over the respective values of these pigments observed in only Cd treated samples by the application of IAA at low (IAA_L), and middle (IAA_M) doses might have alleviated the growth performance seedlings under Cd stress. IAA was also found to be involved in the substantial preservation of Chl contents in a plant cultivated under Cu stress, according to Ouzounidou and Ilias (2005). In contrast, additional chlorophyll breakdown in test seedlings with higher dosages (IAA_H) of IAA could be explained by an excess of ROS formation (Bashri and Prasad, 2016), which likely accelerated the degradation process under Cd stress (Fig. 1 c).

Because photosynthetic oxygen yield during light reaction was shown to be directly related to photo-fixation of CO_2 during the Calvin cycle, a Cd-induced decrease in O_2 yield in *Trigonella* seedlings implied a slowing down in the rate of CO_2 fixation. Cadmium inhibits CO2 fixation directly by lowering the activity of Calvin cycle enzymes, notably Rubisco, and causes a change in the oxygen-evolving center's redox cycling (Popova et al., 2009). As a result, Cd treatment may slow photosynthesis (Popova et al. 2009), resulting in reduced plant development, as seen in the current study (Fig.1). The use of IAA_L and IAA_M dosages resulted in a significant reduction in Cd levels in Trigonella tissues (Bashri and Prasad, 2016), indicating that Cd has a photosynthesis-improving impact. Exogenous treatment of IAA boosted hill activity in radish seedlings, according to Buschmann and Lichtenthaler (1977), perhaps due to major improvements in photosynthetic apparatus at structural and functional levels.

Nitrogen is considered one of the most vital macronutrients for the organism's growth. The current investigation found that Cd treatments had a deleterious impact on nitrogen assimilation in test seedlings. Cd treatments reduced nitrate level in leaves considerably (P0.05), especially when Cd2 was used (Fig. 2 a). This might be owing to Cd-induced ROS overproduction in root cells (Bashri and Prasad, 2016), which increased membrane permeability and reduced root nitrate absorption. As a result, the decrease in nitrate absorption by roots might be explained by Cd-induced damage to the plasma membrane. In this study, the lower NR activity caused by Cd stress resulted in less nitrite production than in the control group. The decrease in NR and NiR activity after Cd exposure might be attributed to an increase in ROS generation, which can modify the active site of enzymes and/or cause de novo synthesis (Bashri and Prasad, 2016). (Balakumar and Paliwal, 1998). Similarly, Cd stress was found to reduce leaf nitrate concentration and limit NR activity in rice seedlings (Huang and Xiong, 2009). Under Cd stress, nitrate content was enhanced in IAAL and IAAM treated seedlings, which may be connected with the increased rate of transpiration by IAA as observed in barley plant (Ashraf et al., 2012), which enabled nutrient absorption and translocation to leaf. Less inhibitory effect on NR and NiR activities in low and middle-dose treated IAA Cd stressed seedlings might be attributed to lower ROS generation (Bashri and Prasad, 2016). Furthermore, decreased influence on NR activity may be associated with enhanced nitrate content in low and middle-dose treated IAA seedlings, since NR is a substrate-inducible enzyme, and increased nitrate content under IAA treatment stimulates NR activity.

In the conversion of ammonium ions to organic nitrogen, the GS/GOGAT cycle and the GDH alternate pathway are crucial (Crawford and Glass, 1998). Cadmium also inhibited the glutamine synthetase/glutamate synthetase (GS/GOGAT) cycle, which involves ammonium incorporation into carbon molecules (Ouariti et al., 1997; Gouia et al., 2000).

Glutamate dehydrogenase (NADH-GDH) activity, on the other hand, rose dramatically in response to Cd stress (Boussama et al., 1999). The inhibition of GS and GOGAT activities under Cd stress resulted in a considerable increase in ammonium

content in leaf tissue (Fig. 4 c). Oxidative alterations may have contributed to a significant decline in GS and GOGAT activity as a result of increased ROS generated by Cd stress (Bashri and Prasad, 2016). Under Cd stress, IAA seedlings with low and intermediate dosages had a lesser inhibitory effect on GS and GOGAT activity, which could be linked to decreased ROS generation (Bashri and Prasad, 2016), resulting in a modest change in the active site of these enzymes. Despite this, increased ammonium accumulation in leaf tissue under Cd stress activated the enzyme responsible for the alternative route of ammonium incorporation; hence, enhanced GDH activity (with a high Km) may have protected the tissue against ammonium toxicity. When cyanobacteria were subjected to UV-B stress, Srivastava et al. (2014) observed an increase in GDH activity. Furthermore, Kanamoriet al. (1972) observed that when rice plants were cultivated in liquid medium and provided with varying doses of an ammonium salt, GDH activity increased owing to de novo synthesis. According to studies, an increase in GDH activity under stress circumstances may reduce the strain of accumulating hazardous amounts and supply glutamate for protein synthesis (Gouia et al., 2000). Application of IAA also increased the GDH activity that may help in relieving the pressure of ammonium toxicity; thereby, growth was improving in test seedling (Fig. 1).

5. CONCLUSION

In conclusion, the present study showed that Cd treatments decline the growth of *Trigonella* seedlings by inhibiting the photosynthetic oxygen yield and nitrogen assimilation. However, exogenous application of low (IAA_L, 10 µM) and middle dose (IAA_M, 50 µM) of IAA alleviated the Cd-induced reduction in growth by improving the photosynthetic oxygen yield and nitrogen assimilation. In contrast, a high dose of IAA further aggravates the Cd-induced toxicity. Overall results suggest that IAA at low dose successfully ameliorated Cd-induced toxicity and improved the productivity of vegetables in general and *Trigonella foenum—graecum*L. in particular grown in Cd contaminated soil.

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International Journal of Recent Research in Physics and Chemical Sciences (IJRRPCS)
Vol. 13, Issue 1, pp: (8-19), Month: April 2026 – September 2026, Available at: www.paperpublications.org

LIST OF FIGURES

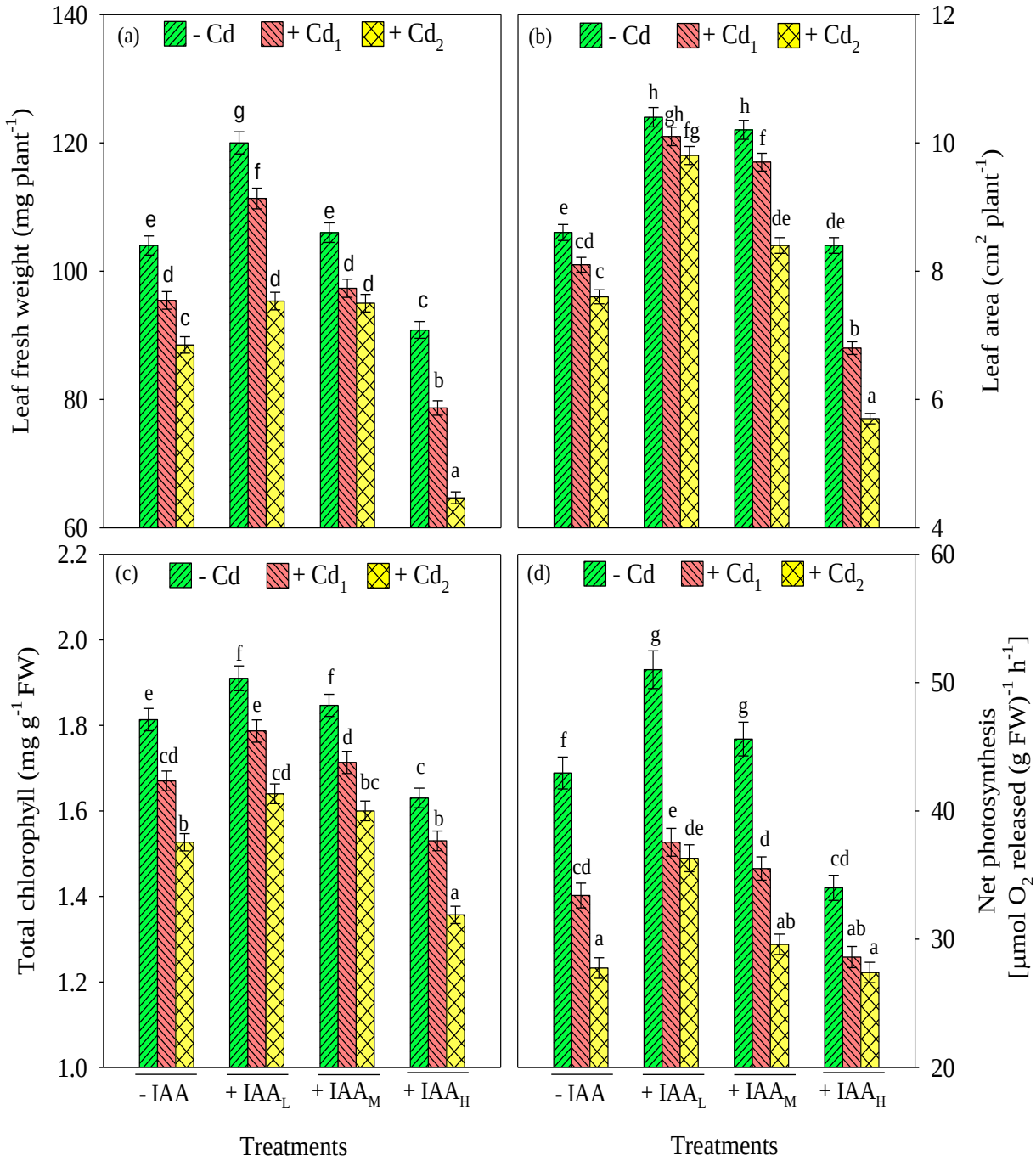

Fig.1: Impact of foliar application of IAA on leaf fresh weight and area, total chlorophyll content and net photosynthesis in *Trigonellafoenum—graecum*L. seedlings exposed to Cd stress. Data represent the mean value ± standard error from three replicates (n=3). Values followed by the different letters at each bar differ at P<0.05 among treatments by the DMRT.

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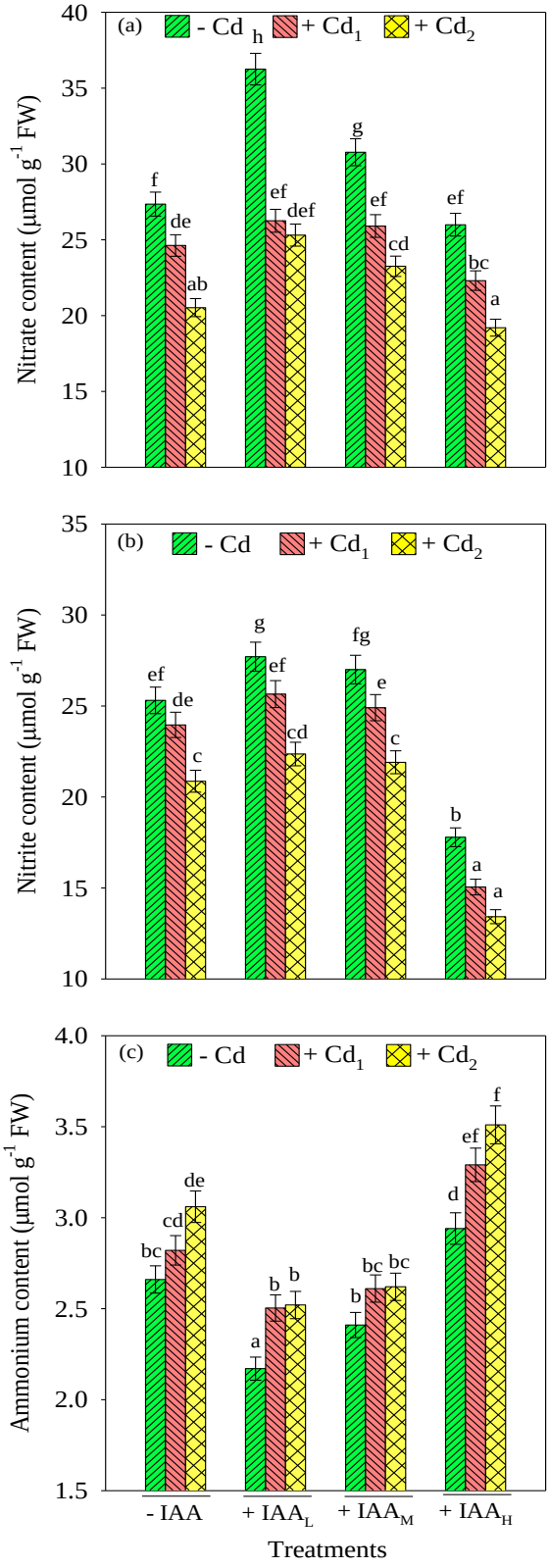

Fig.2: Impact of foliar application of IAA on nitrate, nitrite and ammonium contents in *Trigonellafoenum—graecum*L. seedlings exposed to Cd stress. Data represent the mean value ± standard error from three replicates (n=3). Values followed by the different letters at each bar differ at P<0.05 among treatments by the DMRT.

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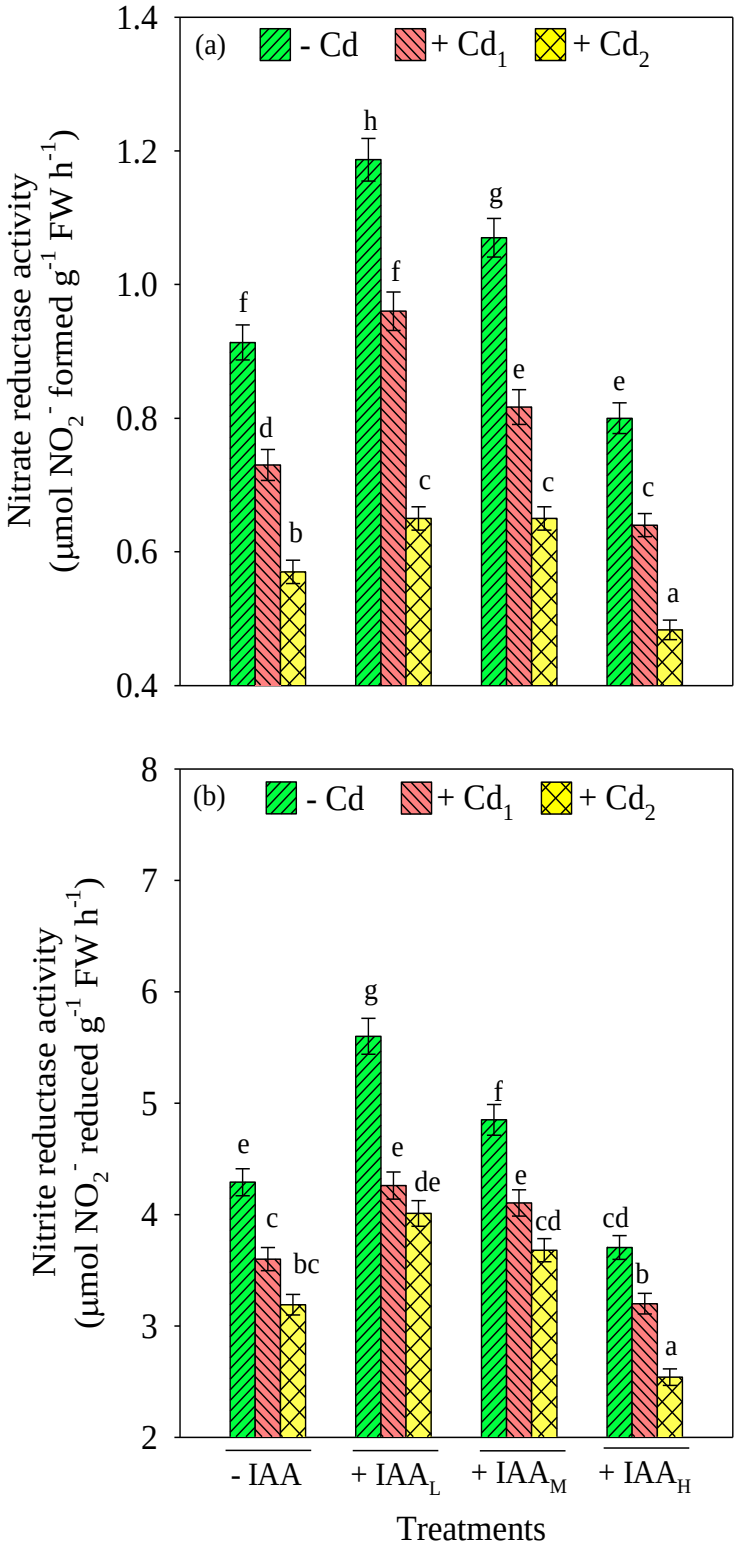

Fig.3: Impact of foliar application of IAA on nitrate and nitrite reductase activities in *Trigonellafoenum—graecum*L. seedlings exposed to Cd stress. Data represent the mean value ± standard error from three replicates (n=3). Values followed by the different letters at each bar differ at P<0.05 among treatments by the DMRT.

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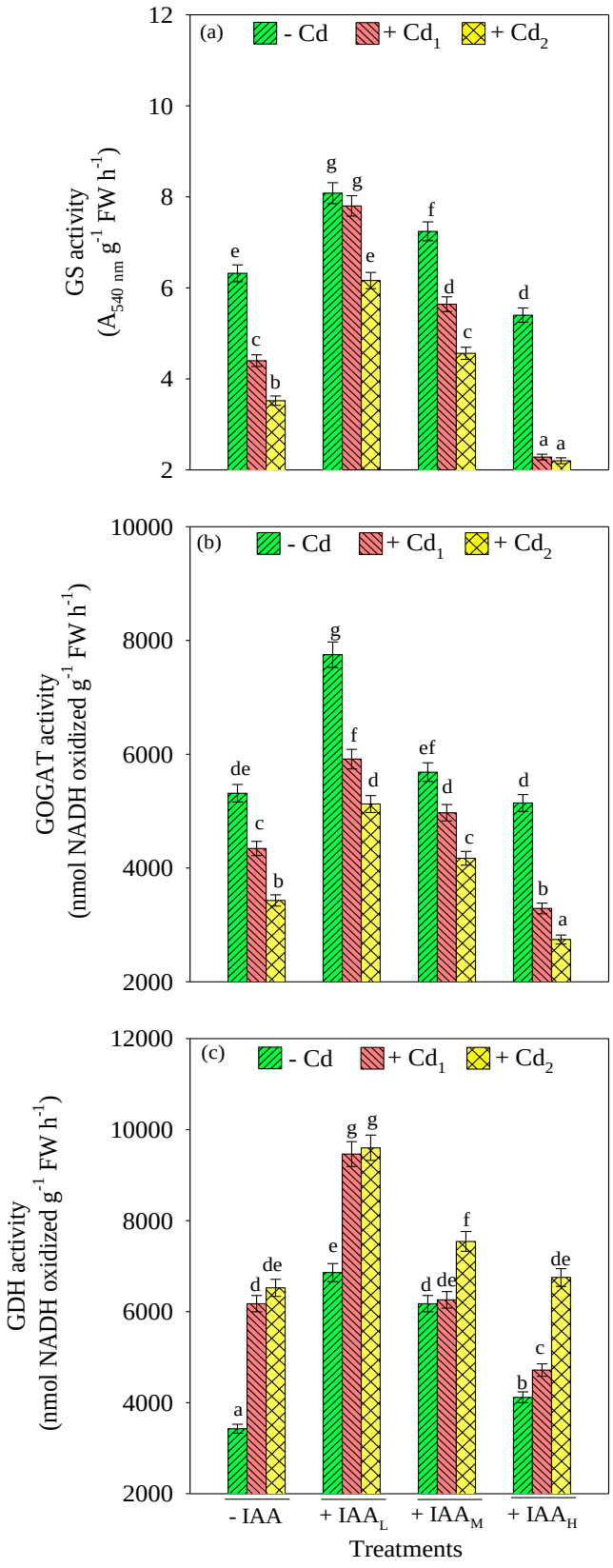

Fig.4: Impact of foliar application of IAA and KN on GS, GOGAT and GDH activities in *Trigonellafoenum—graecum*L. seedlings exposed to Cd stress. Data represent the mean value ± standard error from three replicates (n=3). Values followed by the different letters at each bar differ at P<0.05 among treatments by the DMRT.