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RESEARCH ARTICLE

Recuperative performance of 4-Cl-IAA and IAA in the fixation and assimilation of nitrogen in *Vigna radiata*

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Abstract Auxin analogues indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) were assessed for their comparative impact on nitrogenase, glutamine synthetase (GS), glutamate synthase (GOGAT), glutamine dehydrogenase (GDH), nitrate reductase (NR), and nitrite reductase (NiR) in mung bean cv T-44. The plants were grown in earthen pots and the seedlings were percolated with 10^{-8} or 10^{-6}M of IAA or 4-Cl-IAA at 7 and 14 days after sowing (DAS) through roots. Leaf/nodule samples were collected at 30 days stage of growth to study different parameters pertaining to root, nodules and leaves. The treated plants exhibited an increase in different parameters; GS, GOGAT, GDH, NR, and NiR, both in nodules/roots and leaves when treated with different concentrations of IAA or 4-Cl-IAA. Leghemoglobin concentration, nitrogen and protein contents also showed a pattern synchronous with that of the enzymes of GS-GOGAT and NR-NiR systems. Overall 4-Cl-IAA was more effective then IAA. However, 10^{-6}M concentration IAA had an effect comparable to that of 10^{-8}M of 4-Cl-IAA. Moreover, 4-Cl-IAA at 10^{-6}M concentration was more effective than 10^{-8}M dose of the analogue.

Keywords: Auxin, 4-Cl-IAA, Glutamine synthetase, Nitrate reductase, Nitrogenase

Introduction

Plant growth and development is highly complex and well regulated meshwork of diverse metabolic responses. The cumulative impact of these responses facilitates the overall growth and development of the plant. Among the endogenous regulatory factors, phytohormones play a pivotal role in the plant growth and development. They are represented by six groups, auxins being the first and classic one (Taiz and Zeiger 2010). Auxins are represented by the natural forms namely IAA, 4-Cl-IAA, phenyl acetic acid and indole-3-butyric acid (IBA) (Davies 2004). Among them 4-Cl-IAA is exclusively found in the members of Fabaceae family, (Reinecke 1999; Lam *et al*. 2015). Furthermore, 4-Cl-IAA is the most active analogue that has been proved in a number of experiments (Reinecke 1999). Karcz and Burdach (2002) have attributed the high biological efficiency of the 4-chloroindole-IAA to its structural stability and its efficacy to the signal transduction receptors.

Various physiological and metabolic processes influenced by auxins include cell elongation, lateral root formation, apical dominance, ethylene biosynthesis, phototropism, geotropism, vascular differentiation, embryogenesis, fruit setting, fruit ripening and senescence (Davies 2004, Taiz and Zeiger 2010). Besides this, some other important physiological processes such as growth phenotype of plants (Walter *et al*. 2020), seed germination (Hirasawa 1989, Ahmad and Hayat 1999), enzyme activities and photosynthesis (Ahmad *et al*. 2001a, b), stress modulation (Mazid *et al*. 2010) and nitrogen fixation (Ali *et al*. 2008) are also affected by the exogenous application of IAA and 4-Cl-IAA.

Nitrogen (N), a macronutrient is another important factor determining the growth and development of the plant. It is an indispensable component of amino acids, proteins/enzymes, nucleic acids, phytohormones, cellular components and all other primary and secondary metabolites (Hopkins 1995). Leguminous plants have the

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potential to fix the atmospheric N, as they possess the nitrogen fixing enzyme nitrogenase in their nodules. Other plants depend on soil nitrate for N source. Both these forms are converted to ammonia once fixed/absorbed by the plants. This ammonia is subsequently incorporated into the amino acids and proteins by the collaborated action of GS-GOGAT system and GDH (Kishorekumar *et al*. 2020). These amino acids and proteins are transported to the sink/seeds and stored there as a reserve food material (Taiz and Zeiger 2010), thus contribute significantly in the yield of a crop.

It is evident from the above that the enzymes of N metabolism viz. GS, GOGAT, GDH, NR, and NiR, play a key role in the N metabolism making sufficient amount of N available for the storage in sink/seeds. Moreover, auxin analogue 4-Cl-IAA shows very high auxin-like activity. Therefore, this study was designed with an objective to explore the effect of 4-chloroindole-IAA on the activities of the enzymes of N metabolism that could boost the yield of the crop. Moreover, the hypothesis tested is that 4-Cl-IAA can favourably affect these enzymes that could be beneficial in improving the productivity of the crop.

Materials and methods

The healthy seeds of mung bean (*Vigna radiata* L. Wilczek) cv. T-44 were dipped in sodium hypochlorite solution (5%) for five minutes for surface sterilization. The surface sterilized seeds were kept under running tap water to remove the adhering solution. These washed seeds were sown in pots filled with sandy loam soil mixed with farmyard manure in a ratio of 9:1, after coating them with specific *Rhizobium sp*. The resulting seedlings were drenched/percolated with water (control) or 10^{-8}M or 10^{-6}M of IAA or 4-Cl-IAA (25 ml each) at one and two week stage of growth, through roots. The pots containing plants were kept under natural conditions of temperature, light and humidity in a net house. Root, nodule and leaf sampling were collected at 30 days stage and were immediately transfers to a deep freezer (-18°C) and were immediately used for the assessment of nitrogenase activity, leghemoglobin content, GS, GOGAT, GDH NR, and NiR, activities. Ten pots were maintained for each treatment.

The nitrogenase (E.C. 1.7.99.2) activity was assayed by the method described by Hardy *et al*. (1968) by using gas liquid chromatography (GLC). The reaction mixture used for the extraction of GOGAT and GDH contained Tris HCl, sucrose and γ-mercaptethanol. For GDH assay, the enzyme extract (1 ml) was added to Tris-HCl (pH 7.5), 2-oxoglutarate and NADH. Similarly, for GOGAT assay, the enzyme extract was added to NH_4Cl or L-glutamine. Enzyme activity was calculated by the method proposed by Thimmaiah (1999). Enzyme GS was extracted from nodules and leaves by using potassium phosphate buffer (pH 7.8) containing sucrose, dithiothreitol, KCl, $MgCl_2$ and EDTA. The absorbance of the reaction mixture containing enzyme extract was read at 540 nm and the enzyme activity was calculated by using a standard curve plotted by using γ-glutamylhydroxamate.

The method proposed by Jaworski (1971) was followed for NR assessment. The enzyme was extracted from the plant source by using phosphate buffer (pH 7.5), KNO_3 and isopropanol in the reaction medium. The enzyme extract was added to the reaction mixture containing sulphanilamide and N-1-napthylethylenediamine hydrochloride and absorbance of the colour so developed was read spectrophotometrically at 540 nm. The enzyme NiR was extracted by grinding the plant material in Tris-HCL buffer to which were added sodium dithionite, glutamic acid, $NaNO_2$ and Nesselers reagent. The absorbance of the colour developed during the reaction was read by using a spectrophotometer (Srivastava *et al.* 1979).

The leghemoglobin was estimated by method presented by Sadasivum and Manickam (1992). The procedure proposed by Lindner (1944) was followed for the estimation of N content using a spectrophotometer. Digestion of the plant material was done with sulphuric acid and the extract was added with H_2O_2, NaOH, sodium silicate and Nessler's reagent. The colour developed was read by using a spectrophotometer. Protein content was assessed according to procedure proposed by Lowry *et al*. (1951). Protein content was calculated by plotting a standard curve from the absorbance of graded concentrations of egg albumin.

The statistical analysis of the data was performed by the formulae presented by Gomez

and Gomez (1984). Random sampling was done for sample collection. Each treatment was replicated five times.

Results

The activity of nitrogenase (Fig 1A) exhibited a concomitant increase with the increase in the concentration of auxin analogues. The higher concentration of IAA enhanced the enzyme activity similar to that of the lower concentration of 4-Cl-IAA. However, the effects generated, both by these treatments were significantly higher than the untreated plants (control). The higher concentration of 4-Cl-IAA (10^{-6}M) used generated a better response, which increased the activity of the enzyme by 16%, compared to the control.

The GS and GOGAT activities, irrespective of the treatments, were higher in nodules than the leaves (Fig 1B, C). All the treatments significantly elevated the activities of the enzymes, compared to the control. There was a proportionate elevation in the enzyme activities with the increase in the concentrations of the hormones used. Comparing the concentrations of IAA used, the higher (10^{-6}M) concentration generated a better response than the lower (10^{-8}M) one, both in the leaves and the nodules. Similar pattern of the response was observed with respect to the concentrations of 4-Cl-IAA used in the treatments. Overall, 4-Cl-IAA (10^{-6}M) was the best treatment that enhanced the leaf GS by 14%, nodule GS by 13%, leaf GOGAT by 38% and nodule GOGAT by 30%, compared to the respective controls.

The activity of GDH (Fig. 1D), both in the leaves and nodules exhibited a significant increase in response to the treatments. Treating the plants with the auxin analogues (IAA or 4-Cl-IAA) caused a significant elevation in the enzyme activity. Comparing the response generated by (10^{-6}M) of IAA and (10^{-8}M) of 4-Cl-IAA the values were almost similar (at P=0.05). Moreover, 10^{-6}M of 4-Cl-IAA increased the activity of the enzyme by 55% and 52% in the leaves and nodule, respectively.

The activities of NR and NiR (Fig 1E, F) in the leaves and roots resulted in a significant improvement in response to different treatments. However, the enzyme activity was higher in the roots compared to the leaves. The pattern of the response to IAA and 4-Cl-IAA was more or less similar, both in the roots and the leaves. Out of the treatments, IAA (10^{-6}M) and 4-Cl-IAA (10^{-8}M) generated a response, which was almost similar, with respect to NR and NiR. 4-Cl-IAA (10^{-6}M) treatment enhanced the activity of these enzymes to the highest level.

Leghemoglobin concentration was significantly elevated by the treatments (Fig 2A). 4-Cl-IAA at 10^{-6}M concentration increased the level of the leghemoglobin by 74%, compared to the control that was the maximum response generated by any treatment. Similarly, nitrogen content in the leaves and nodules increased proportionately with the increasing concentrations of IAA and 4-Cl-IAA (Fig. 2B). 10^{-6}M concentration of either of the auxin analogues showed a better response compared to their respective lower concentrations. Among the treatments, 4-Cl-IAA (10^{-6}M) generated the best response. Overall, the level of N was higher in the nodules then the leaves. The content of protein in the roots and the leaves followed a trend similar to that of the nitrogen, in response to the treatments (Fig. 2C). Comparing the two plant parts, the content of the protein was higher in leaf then the root. Likewise, the seed yield of the plants treated with IAA and 4-Cl-IAA exhibited a significant increase in response to the treatments. Moreover, the response was proportionate to the concentrations used. Compared to the control, 4-Cl-IAA (10^{-6}M) significantly enhanced the yield of the plants upto 48% that proved to be the best treatment (Fig 2D).

Discussion

The present investigation revealed an increase in the enzyme activities related to the fixation and assimilation of nitrogen in mung bean plants (Fig 1A-F). Nitrogen fixation is a unique phenomenon exhibited by different leguminous plants, which is facilitated by the nitrogenase enzyme occurring in their nodules. The nitrogenase enzyme is very sensitive to the oxygen, whose activity is maintained by restricting the supply of oxygen facilitated by leghemoglobin (Hopkins 1995). Therefore, the activity of nitrogenase and the quantity of the fixed nitrogen is determined by the number, size and metabolic state of the nodule (Ali

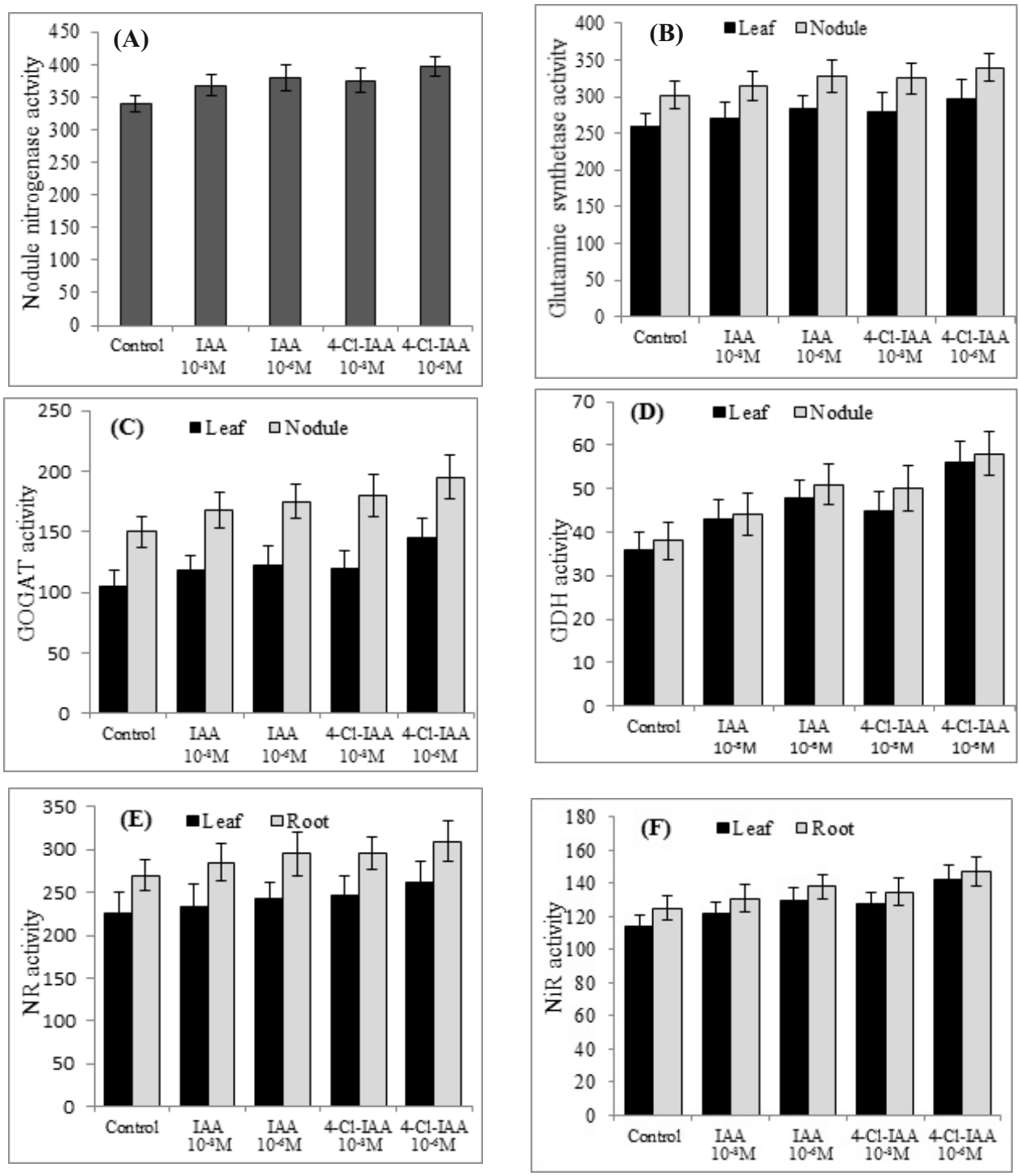

Figure 1. Effect of root percolation treatment of IAA or 4-Cl-IAA on (A) nitrogenase activity [n M C_2H_4 g^{-1} (F.W.) h^{-1}], (B) Glutamine synthetase (GS; Units g^{-1} F.W.) activity, (C) glutamate synthase (GOGAT; Units g^{-1} F.W.), (D) glutamate dehydrognase (GDH) activity (Units g^{-1} F.W.), (E) nitrate reductase (NR) activity (n mol NO_2 $h^{-1}g^{-1}$ F.W) and (F) nitrite reductase (NiR) activity (m Mol NH_3 $h^{-1}g^{-1}$ F.W) activity in *Vigna radiata* (L.) Wilczek at 30 days after sowing (DAS).

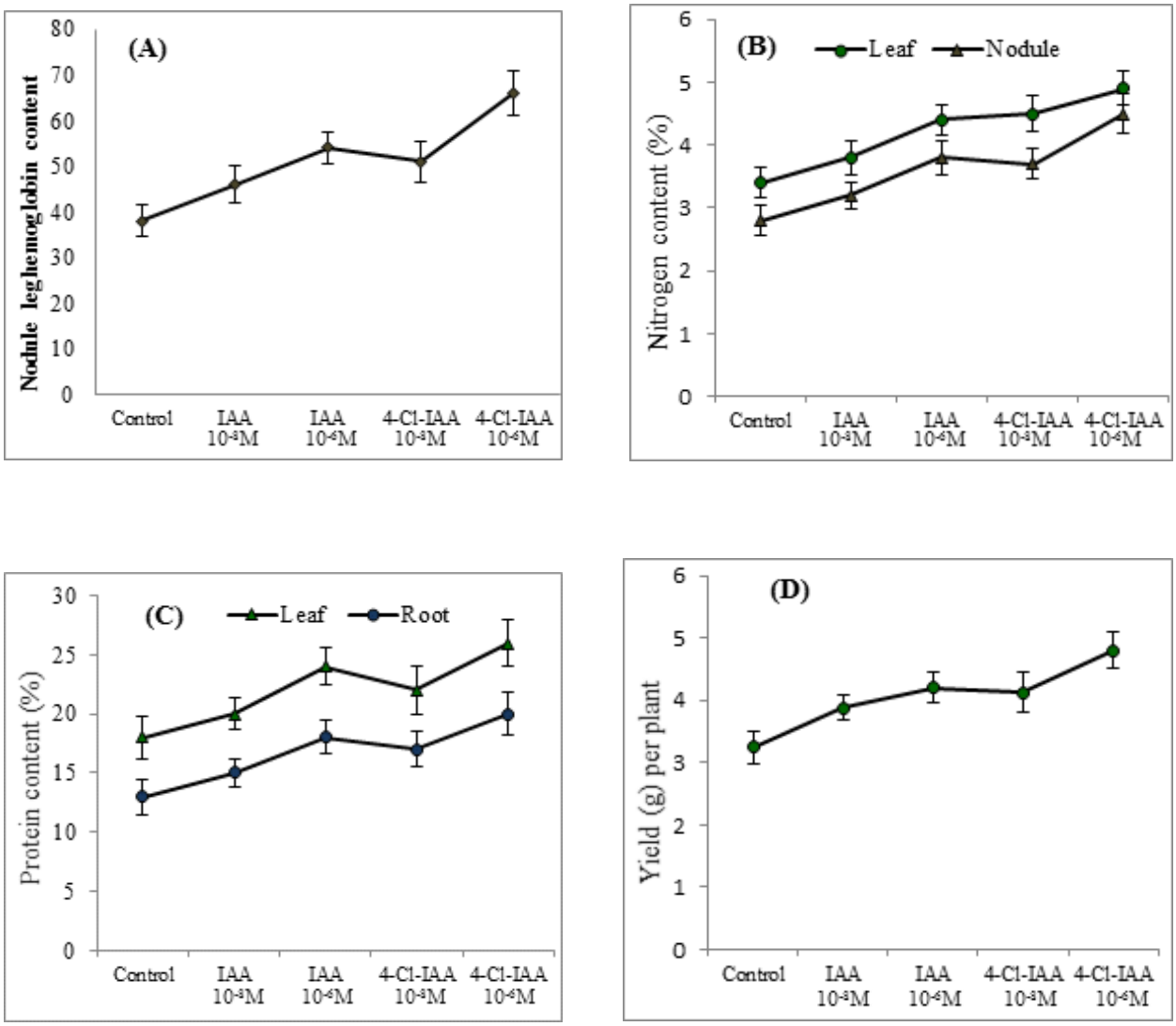

Figure 2. Effect of root percolation treatment of IAA or 4-Cl-IAA on (A) leghemoglobin content [mg g^{-1} fresh weight, (B) nitrogen content (%) in leaf and nodule (C) protein content (%) in leaf and root at 30 days after sowing (DAS) and (D) seed yield per plant at harvest in *Vigna radiata* (L.) Wilczek.

et al. 2008). Auxin analogues used in the present study also stimulated the accumulation of leghemoglobin (Fig. 2A) which could maintain the appropriate supply of oxygen and maintain the activity of the enzyme. In addition to this, phytohormones exhibit an inherent property to influence the nodulation process (Desbrosses and Stougaard 2011). Among the phytohormones, auxins influence rhizobial infection (Breakspear *et al*. 2014), cell cycle control (Kondorosi *et al*. 2005) and nodule vascular tissue differentiation (Takanashi *et al*. 2011), besides acting as important signalling molecule in the nodulation process (Kohlen *et al*. 2018). These auxin-induced cascades facilitate the healthy nodulation (Mathesius 2020) and consequently the enhanced activity of nitrogenase (Fig. 1A). The nitrogen fixed by nitrogenase is immediately converted into ammonia and subsequently to organic form to prevent the possible damage which could take place due to ammonia accumulation (Hopkins 1995). The conversion of ammonia into organic form and subsequently the incorporation into different amino acids is facilitated by the enzymes GS, GOGAT and GDH (Kishorekumar *et al*. 2020). The activities of these enzymes show a pattern of response similar that of nitrogenase (Fig 1A-D). The increase in the activities of these enzymes can also be attributed to the availability of sufficient amount of the substrate

(ammonia) due to the enhanced nitrogenase activity (Hayat *et al*. 2009, Fig 1A), healthy state of the nodules (Ali *et al*. 2008, Wang *et al*. 2014) and auxin regulated expression of the specific genes (Woodward and Bartel 2005). Moreover, Ali *et al*. (2008) observed increase in the activity of nitrogenase after supplementing the auxin analogues via the pre-sowing seed soaking.

Nitrate reductase-nitrite reductase is another enzymatic system exhibited by plants including the legumes, to assimilate the nitrogen from the soil present in nitrate form (Taiz and Zeiger 2010). Both these enzyme showed a positive response to the auxin treatments (Fig. 1E-F). The enhancement in the enzyme activities can be attributed to increase in the auxin-induced NO_3 uptake (Hayat *et al*. 2005, 2009), an important regulator of NR activity (Campbell 1999), solute uptake mediated via the influence on the cellular turgor (Macdonald 1997) and the inherent involvement of auxins in the regulation of protein/enzyme synthesis (Woodward and Bartel 2005). The enhancement in both these enzymatic systems, GS-GOGAT and NR-NiR resulted in an overall improvement in the level of nitrogen in the plants (Fig. 2B) to be assimilated into different amino acids and subsequently into the proteins (Fig. 2C). Thus, seed yield of the plants exhibited an increase in response to different auxin treatments (Fig 2D). Auxins also favour the enhancement of the plant biomass (Wang *et al*. 2019) in presence of increased level of nitrogen and protein in the plants (Fig 2C, D) indicating a healthy state of the plants. Biomass accumulation has a great correlation with plant productivity. The increased potential of N fixation and assimilation is an important reason behind the increase in the seed yield of the plants in response to the auxin treatments (Fig 2D). Similarly, 4-Cl-IAA treatment also enhanced the yield of black cumin (*Nigella sativa*) (Shah 2011). However, the auxin analogues showed significant difference with respect to different the parameters including the seed yield. Comparing the effect of auxin analogues, 4-Cl-IAA was more efficient with respect to all the parameters studied. Karcz and Burdach (2002) attributed the high efficiency of 4-Cl-IAA to the resistance of 4-Cl-IAA to metabolism and to its efficacy with the receptors of signal transduction pathway. Furthermore, a recent study has demonstrated that a strong auxin like activity of 4-Cl-IAA is due to the modification of auxin receptor populations of the TIR/AFB (Transport inhibitor response/Auxin signalling F-Box) family, which degrades auxin/IAA protein and subsequently leads to an auxin-specific tissue response (Jayasinghege *et al*. 2019). These authors also attributed the differential effects of 4-Cl-IAA and IAA to the differential catabolism of these auxins.

Conclusion

The data presented in this study revealed that the auxin treatments significantly favoured different parameters including leghemoglobin concentration and nitrogenase. Moreover, the key enzymes associated with N metabolism namely GS, GOGAT, GDS, NR, and NiR were also favourably affected by both the auxin analogues. The recuperated N fixation and assimilation was synchronous with the seed yield of the plants at harvest. The chloro substituted auxin was more efficient and effective than IAA. Furthermore, among all the treatments, 10^{-6}M dose of 4-Cl-IAA generated the best response.

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