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RESEARCH ARTICLE

Effects of imazethapyr herbicide and *Rhizobium* inoculation on growth, nodulation, and certain biochemical parameters in cowpea (*Vigna unguiculata*) cultivar

Richa Chaudhari[1*], Shivani Sangal[2] and Amita Sharma[1]

Abstract

The present study examines the impact of Imazethapyr herbicide and *Rhizobium* inoculation on growth parameters, nodulation, and biochemical responses of cowpea (*Vigna unguiculata*). There were 8 treatments such as different levels of Imazethapyr with and without *Rhizobium* inoculation. The outcome suggests that Imazethapyr application had significantly influenced seed germination, shoot and root length, fresh and dry weight of shoot and root, nodulation, and biochemical parameters including protein content, proline level, and nitrogen fixation efficiency. This experiment demonstrated the interaction between Imazethapyr application and *Rhizobium* inoculation on cowpea plant's overall growth and health, but the relationship is complex. This research contributes to understanding the impact of herbicides on leguminous crops and emphasizes the role of beneficial bacteria in reducing herbicide stress.

Keywords: Imazethapyr, *Rhizobium*, Cowpea, *Vigna unguiculata*, Herbicide, Biochemical responses.

Introduction

Cowpea (*Vigna unguiculata*) is an important leguminous crop and one of the most widely cultivated legumes in many parts of the world because it is rich in protein and enhances soil fertility through nitrogen fixation. It is significant for food security and economic stability in several regions, especially Africa and Asia. This is a good food security crop as it blends well with other recipes (Muoneke *et al.*). While cowpea is resilient to many internal and external factors, it is still challenged by the competition of weeds which can lead to severe yield losses (Norsworthy *et al.* 2012). Several weed species are reducing the yield by competing with the crop from seedling to maturity (Patel *et al.* 2003), and this yield loss of cowpea by weeds varied from 12.7% - 60.0% (Li *et al.* 2004). This way, herbicides such as Imazethapyr are used to control weed populations.

Cowpea was selected as the model crop for this study due to its global significance as a protein-rich legume and its critical role in enhancing soil fertility through biological nitrogen fixation (Dakora and Keya 1997; Giller 2001). This crop is widely cultivated in tropical and subtropical regions, where it serves as an essential food security crop and an economic resource for smallholder farmers (Muoneke *et al.* 2012). However, cowpeas are highly vulnerable to weed competition, which necessitates herbicides such as Imazethapyr to control weed populations. Investigating the effects of herbicides on cowpea and exploring mitigation strategies like *Rhizobium* inoculation is vital to ensuring its sustainable production in herbicide-intensive farming systems.

Imazethapyr is an imidazolinone herbicide that inhibits the acetolactate synthase enzyme disrupting amino acids synthesis in susceptible plants. Although it is an effective herbicide, its use can harm all non-target biosystems including leguminous crops. Studies have confirmed that Imazethapyr negatively affected seed germination, root development, and overall plant vigor (Beckie *et al.* 2012). In addition, the application method, time of year applied and concentration of Imazethapyr can influence growth parameters (Bhowmik 1999).

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Rhizobium has a well-documented impact on plant health, especially in leguminous crops. Symbiotic rhizobia established an effective association with legume plants, which augments nitrogen fixation and nutrient uptake. Such a symbiosis can be very positive under stressful circumstances including herbicide (Bashan *et al.* 2012). Nevertheless, the interactive effects of herbicides such as Imazethapyr and *Rhizobium* inoculation on cowpeas have received little attention.

The objective of this work was to assess the Imazethapyr effects on growth parameters, nodulation, and biochemical aspects of cowpea cultivars with and without *Rhizobium* inoculation. The hypothesis is that when used together with Imazethapyr *Rhizobium* will not reduce the harm of herbicides, thereby increasing plant health and productivity.

Materials And Methods

The investigation studied the cumulative effects of herbicide Imazethapyr and *Rhizobium* Inoculation on different physicochemical properties of cowpea (*Vigna unguiculata*) cultivars to monitor plant response under the influence of these factors.

Geographical location of the site of the experiment

The experiment was conducted at the Department of Botany, Chaudhary Charan Singh University, Meerut (U.P.), India, located between 28°44′ and 29°18′ north latitude and 77°08′ and 78°47′ east longitude with an elevation of about 228 meters above sea level. The region has a summer mean temperature of 32°C – 42°C and a winter mean temperature of 10°C – 25°C, with an annual approximate rainfall of 740 mm.

Materials used during study

The seeds of cowpea (*Vigna unguiculata*) used for the experiment were procured from IARI New Delhi ensuring high quality and uniform size. The *Rhizobium* inoculum was specific to the cowpeas and obtained from IARI New Delhi. This strain was chosen for compatibility with cowpeas and effectively encourages faster nitrogen fixation. The Imazethapyr herbicide was purchased from a credible agrochemical company that guaranteed the prescription standard concentration of these respective chemicals for their accurate application in this study.

Experimental Design

The experiment was performed over three trials, each with three replications for every treatment (data are presented as the means of these three trials). The experiment was performed using a completely randomized design (CRD) with 8 treatments, replicated 3 times in 24 pots. The treatments were as follows: control (C), *Rhizobium* inoculation (R), and Imazethapyr with and without *Rhizobium*. Imazethapyr (10%SL) was applied at rates of 400mL/acre (I1 + R, I1), 500mL/acre (I2 + R, I2), and 600mL/acre (I3+R, I3). As per the area of the pots (213.82 cm^2), spaced volumes of herbicide were applied to the pots i.e., 21.1 µL, 26.4 µL, and 31.6 µL for Imazethapyr (at target field conditions) Only seed germination percentage was measured in Petri plates, and all other parameters (growth and biochemical assays) are from plants grown in pots.

Methods

Seed germination percentage

Cowpea healthy seeds were sterilized with 0.1% sodium hypochlorite solution and rinsed twice with distilled water for germination study. In germination tests, ten seeds were put on moistened Whatman No. 1 filter paper in Petri dishes and held at 25°C with a 12-hour light/dark cycle. We checked for moisture daily to make sure it was getting enough water. Germination was noted daily for 10 days based on the emergence of the radicle (≤ 2 mm), and counts were converted to percent germination. Germination percentage was calculated as:

$$\text{Germination Percentage} = \frac{\text{Number of Germinated Seeds}}{\text{Total Number of Seeds}} \times 100$$

Shoot length and root length

Measurements were taken in cm with two measurements per plant averaged for the final lengths.

Fresh weight of shoot and root

At maximum vegetative growth, plants were harvested, and fresh weights of separated shoot and root samples were recorded using an electronic balance Each treatment included three replicates to ensure statistical reliability.

Dry weight of shoot and root

Plant samples were oven-dried at 70°C for 72 hours until constant weight was achieved. Dry weights were measured with the same electronic balance. This procedure was repeated to keep consistency for each treatment.

Nodule numbers

The number of nodules per plant was counted visually at the highest growth of the plant. The average was calculated based on three replicates per treatment.

Nodule volume

The water displacement method was used to measure the volume of nodules. Nodules from three plants per treatment were washed, blotted dry, and submerged in a graduated cylinder filled with water; volume displacement was recorded in ml. The average volume per nodule was calculated based on three replicates.

Fresh weight of nodules

Nodules were harvested (at peak growth of plants), separated from roots, and weighed immediately using an electronic balance. Fresh weight was recorded and averaged from nodules of three plants per treatment.

Dry weight of nodules

Nodules per plant were oven-dried at 70°C for 48 hours until constant weight was achieved. The dry weights were measured with the same electronic balance, and the average dry weight per nodule was calculated based on three replicates.

Protein

The concentration of protein was estimated according to the Bradford method (1976), which is extremely sensitive. Fresh leaf tissue (100 mg) was pulverized with a mortar and pestle in 5 ml Tris-EDTA buffer, centrifuged at 5000 rpm for 10 minutes, and the supernatant was collected. Protein concentration was estimated based on Coomassie Brilliant blue (CBB) dye-binding capacity (1 ml supernatant in 5 ml CBB dye, incubation at 5 min). Absorbance at 595 nm was assayed in a spectrophotometer, and the protein concentration of the sample was estimated based on a casein standard curve. All results are presented as mg/g fresh weight with three assays performed for reproducibility.

Proline

Proline contents in leaves of plants were determined by the method of Bates *et al.* (1973) reaction of proline and ninhydrin to produce a colored complex. Homogenization of fresh leaf samples (100 mg) was carried out in 10 ml of 3% sulphosalicylic acid and the homogenate was filtered. After centrifugation, the supernatant of each was taken and mixed with glacial acetic acid and ninhydrin working solution. The preparation was incubated for 1 hour in a water bath and cooled down in an ice bath. To separate the phases, toluene was poured and the absorbance of the upper toluene layer at 520 nm was measured. The quantity of proline was measured and represented as µg/g fresh weight according to standard curves. The assay was carried out in triplicates.

Nitrogen

The nitrogen content in plant species was determined based on the Snell and Snell (1967) method. The sample (25 mg) was first homogenized using concentrated H_2SO_4, followed by the addition of 30% H_2O_2 and heated on a sand bath. This process was then repeated after the addition of a catalyst (K_2SO_4 + $CuSO_4$), and digestion was continued till a clear or pale yellow solution suggested complete digestion. After cooling, samples were diluted in distilled water, and 1 ml of supernatant was combined with Nessler reagent. Absorbance at 425 nm was recorded against the blank, and nitrogen concentration was measured using a standard curve. Triplicates of the assay were performed for accuracy.

Leghemoglobin

Leghaemoglobin is an important protein that occurs in the root nodules of legumes, where it helps control oxygen supply for nitrogen fixation (the conversion of atmospheric nitrogen into a biologically usable form). For the quantification of leghaemoglobin, we used the Bergersen and Turner (1980) method. To maintain structural integrity, fresh root nodules (200 mg) were frozen in liquid nitrogen and then homogenized using 6 ml of ice-cold phosphate buffer (pH 7.4). The suspension was then filtered using cheesecloth and the resulting filtrate was centrifuged at 10,000 rpm for 10–30 minutes at 4°C to give a clear supernatant that contained leghaemoglobin. In the assay, 3 ml supernatant was mixed with 3 ml of alkaline pyridine reagent. Sodium dithionite and potassium hexacyanoferrate were respectively added, and the optical density (O.D.) was quantified at 556 nm for sodium dithionite and then quantitated at 539 nm for potassium hexacyanoferrate by triplicate. Leghaemoglobin (mM) was determined according to the following equation:

$$\text{Leghaemoglobin concentration (mM)} = \frac{A556 - A539 \times 2D \times 100}{23.4}$$

Where A_{556} and A_{539} are the absorbance readings, and D is the initial dilution.

Nitrate reductase activity

The enzyme nitrate reductase (NR) is a key component in the nitrogen cycle and plays an important role in plant nitrogen assimilation through the reduction of nitrate (NO3-) to nitrite (NO2-). The Hageman *et al.* (1980) method was adapted to estimate the activity of nitrate reductase in the leaves of cowpeas. An extraction buffer containing 1 mM EDTA, 1-25 mM cysteine, and 25 mM potassium phosphate buffer (pH 8.8) was used to homogenize fresh leaf material (1 g). The homogenate was filtered through cheesecloth and the filtrate, after centrifugation at 30,000 rpm for 15 min at 5 °C, was used in the assay as a clear supernatant. The reaction mixture was made up of 0.5 ml phosphate buffer (pH 7.5), 0.2 ml potassium nitrate solution, 0.4 ml NADH solution, and 0.7 ml water, and the reaction was initiated by adding to the mixture 0.2 ml of enzyme extract Then, a control was prepared to replace the enzyme extract by water. The component combinations were incubated at 30°C for 15 minutes and stopped by adding 1 ml of sulphanilamide solution and 1 ml of naphthyl ethylenediamine reagent. After 90 min, absorbance was measured at 540 nm. By using sodium nitrite, a standard curve was prepared, and nitrate reductase activity is expressed as µ mol of nitrite produced min per gram of fresh leaf tissue. Samples were analyzed in triplicate to ensure accurate and reliable results.

Hydrogenase activity

The hydrogenase activity in cowpea root nodules was determined using Turner *et al.* (1980) method. Harvested fresh nodules were immediately frozen in liquid nitrogen to prevent the loss of enzymatic activity. About 0.5 g of frozen nodules were ground in extraction buffer (50 mM potassium phosphate, pH 7.0; 5 mM dithiothreitol; 1

mM EDTA; 10% glycerol) on ice. Afterward, the obtained homogenate was centrifuged at 10,000 rpm at 4°C for 15 min and the supernatant of this suspension was used as enzyme extract. The effect of methylene blue on hydrogenase activity was determined by measuring the reduction of the dye, methylene blue, at 670 nm, after mixing appropriate volumes of the enzyme extract with an assay mixture containing potassium phosphate buffer, methylene blue, and sodium dithionite. This reaction mixture was then incubated at 30°C for 10 min, and quenching by the same volume of 5% trichloroacetic acid. Decreased absorbance at 670 nm was monitored by a UV-Vis spectrophotometer, and enzyme activity is expressed as micromoles of hydrogen consumed per minute per gram of fresh weight (μmol H_2 min^{-1} g^{-1} FW). Samples were processed in triplicate to ensure accuracy and reliability.

Results And Discussion

Seed Germination and Growth Parameters

The application of Imazethapyr exhibited significant effects on growth parameters in cowpea cultivars. The following data were observed for different treatments:

Seed Germination Percentage

Rhizobium inoculation and Imazethapyr treatments significantly affected the percentage of seed germination. Control (C) germination percentage was 80.129 %, which increased to 93.576 % when combined with *Rhizobium* (R) in the present study (Table 1). The conjunctive treatment of Imazethapyr + *Rhizobium* (I1 + R, I2 + R, I3 + R) showed moderate reductions with emergence percentages of 83.362, 81.171 and 85.816. The treatments with Imazethapyr (I1, I2, I3) have also shown germination rates of 85.484 %, 88.161 %, and 83.881 %, respectively. Kumar *et al.* (2017) also indicated that *Rhizobium* inoculation decreases the harmful effects of herbicides on seed germination. Jha, P. (2017) also found similar results and noted that herbicide application may affect the germination and seedling vigor of many crops.

Shoot and Root Length (cm)

The shoot and root length of the cowpea were affected especially due to Imazethapyr and *Rhizobium* treatments. The shoot length (67.83 cm) and root length (12.46 cm) were in the control (C). *Rhizobium* (R) alone improved both parameters where a significant effect was recorded with shoot length (110.16 cm) and root length (16.13 cm) (Table 1). Imazethapyr, with or without a combination with *Rhizobium* (I1 + R, I2 + R, I3 + R), had variable effects on shoot and root lengths. I3 + R resulted in the shortest shoot length (66.13 cm) and root length (9.73 cm), while the maximum shoot length (79.53 cm) and root length (11.56 cm) were recorded with I1+R among all the herbicide treatments. Meanwhile, as herbicide concentration increased, shoot and root length decreased. These findings imply that *Rhizobium* inoculation minimizes the negative impact of Imazethapyr on shoot and root development, corroborating the observations of previous reports displaying improved development and root growth with *Rhizobium* treatment (Abdallah *et al.* 2021, Adesemove *et al.* 2008, and Giller *et al.* 2001, and Kumar *et al.* 2017).

Fresh and Dry Weight of Shoots and Roots (g)

Rhizobium inoculation and Imazethapyr treatments significantly influenced shoots' and roots' fresh and dry weights. For control (C), the fresh weight of shoots and roots reached 19.722 g and 0.980 g, dry weight of shoots and roots was respectively 1.711 g and 0.205 g. The treatment with *Rhizobium* (R) and *Rhizobium* with Imazethapyr herbicide resulted in an average response of 25.055 g of fresh shoot weight, 1.291 g of fresh root weight, 2.801 g of dry shoot weight, and 0.318 g of dry root weight (Table 1). It was reported in earlier studies that *Rhizobium* enhances biomass production under stress (Hayat *et al.* 2012 and Tran, 2015). The effects of I + R (I1 + R, I2 + R, I3 + R) on fresh and dry

Table 1: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on seed germination percentage (after 10 days of seeding), shoot and root length (cm), fresh and dry weight of shoot and root (g) (after 45 days of seeding) of cowpea (*Vigna unguiculata*). Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500 mL/acre, I3: Imazethapyr @600 mL/acre.

Treatments	*Seed Germination %*	*Shoot Length (cm)*	*Root Length (cm)*	*Fresh Weight of Shoot (g)*	*Fresh Weight of Root (g)*	*Dry Weight of Shoot (g)*	*Dry Weight of Root (g)*
C	80.129 ± 2.226	67.833 ± 3.600	12.466 ± 0.986	19.722 ± 1.509	0.980 ± 0.025	1.711 ± 0.025	0.205 ± 0.001
R	93.576 ± 1.288	110.166 ± 1.960	16.133 ± 0.152	25.055 ± 2.467	1.291 ± 0.025	2.801 ± 0.082	0.318 ± 0.004
I 1 + R	83.362 ± 0.738	79.533 ± 1.847	11.566 ± 2.218	20.833 ± 2.113	0.935 ± 0.111	2.232 ± 0.031	0.263 ± 0.008
I 2 + R	81.171 ± 0.188	76.566 ± 1.960	9.966 ± 1.222	17.096 ± 1.546	0.786 ± 0.071	2.087 ± 0.025	0.213 ± 0.001
I 3 + R	85.816 ± 1.273	83.933 ± 1.616	8.733 ± 0.251	18.463 ± 0.020	0.831 ± 0.017	2.106 ± 0.049	0.206 ± 0.007
I 1	85.484 ± 2.250	71.033 ± 1.553	11.066 ± 0.251	16.644 ± 1.007	0.875 ± 0.022	1.871 ± 0.029	0.174 ± 0.006
I 2	88.161 ± 2.236	68.066 ± 1.550	10.533 ± 0.251	15.885 ± 1.155	0.839 ± 0.011	1.726 ± 0.025	0.153 ± 0.010

Table 2: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on nodule numbers, nodule volume (ml), and fresh and dry weight of nodules (mg) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500 mL/acre, I3: Imazethapyr @600 mL/acre.

Treatments	*Nodule Numbers*	*Nodule Volume (ml)*	*Fresh Weight of Nodules (mg)*	*Dry Weight of Nodules (mg)*
C	55.666 ± 1.527	1.766 ± 0.152	191.776 ± 1.628	23.733 ± 0.197
R	70.666 ± 1.154	2.400 ± 0.100	261.113 ± 2.144	39.506 ± 2.896
I 1 + R	60.000 ± 2.645	2.000 ± 0.100	224.763 ± 0.680	35.596 ± 2.010
I 2 + R	56.666 ± 1.527	2.033 ± 0.057	209.646 ± 1.739	33.610 ± 2.430
I 3 + R	50.666 ± 2.081	1.766 ± 0.057	198.560 ± 0.816	30.960 ± 1.090
I 1	31.666 ± 1.527	1.266 ± 0.057	144.546 ± 0.886	25.773 ± 1.113
I 2	29.666 ± 2.081	1.366 ± 0.057	138.026 ± 0.372	25.443 ± 2.062
I 3	25.666 ± 0.577	0.766 ± 0.057	129.213 ± 0.193	24.436 ± 1.181

weights differed. The highest fresh shoot weight was 20.833 g and the roots were 0.935 g and dry weight shoot and root was 2.232 and 0.263 g from I1 + R, and the lowest fresh and dry weight for shoot and root from Imazethapyr only treatment (I1, I2, I3), in which the fresh weight of shoot 13.323 g, fresh root weight was 0.771 g, 1.631 g dry shoot, and 0.139 g dry root weight was observed in I3; this indicates that Imazethapyr negatively affected growth and the application of *Rhizobium* increased this adverse effect on growth, consistent with other findings that *Rhizobium* with herbicide improved stressed (Zhao *et al.* 2019) and biomass.

Nodule Development

Nodulation was notably influenced by both Imazethapyr and *Rhizobium* inoculation. The data show that nodule numbers decreased with increasing concentrations of Imazethapyr. For instance:

Nodule Numbers

Nodule numbers were significantly affected by *Rhizobium* inoculation and Imazethapyr treatments. Control (C) exhibited lower nodule numbers (55.667), compared to *Rhizobium* alone (R) which showed the highest nodule formation (70.667) (Table 2), confirming previous findings by Dakora and Keya (1997), which described the favorable role of *Rhizobium* in legume nodulation. The number of nodules decreased in a concentration-dependent manner when Imazethapyr and *Rhizobium* were combined, where the treatments I1 + R (60.00) showed higher values than I2 + R (56.667) and I3 + R (50.667). I1 (31.667), I2 (29.667), and I3 (25.667) were observed in a similar study conducted by Bhatt (2012) who reported the same impact of herbicides on the legume nodulation. Co-application of *Rhizobium* mitigated these herbicide-associated reductions, highlighting the protective action of the organism. This concurs with Kosslak and Bohlool (1984) finding that herbicides inhibited legume nodule development. Anderson *et al.* (2004) and Khan *et al.* (2006) show in their studies that the reduction in total and effective nodules and nodule dry weight plant^{-1} due to herbicide application may be because of the adverse impact of herbicides on cowpea plant nodulation.

Nodule Volume

Nodule volume was significantly affected by *Rhizobium* inoculation and Imazethapyr treatments. The nodule volume (1.767 ml) was observed for the control (C), and the highest for *Rhizobium* alone (R) (Table 2); (2.4 ml) which was also confirmed by Dakora and Keya (1997). *Rhizobium* and Imazethapyr combination treatments showed a concentration-dependent decrease in nodule volume, where I1 + R (2 ml) showed higher values as compared to I2 + R (2.034 ml) and I3 + R (1.767 ml). Only Imazethapyr had caused relevant reductions with 1.267 ml (I1), 1.367 ml (I2), and 0.767 ml (I3), confirming the herbicide's injuries which was also confirmed by Bhatt (2012). *Rhizobium* co-application alleviated these impairments, highlighting its protective effects. These findings underscore the importance of careful use of herbicides to maintain nodulation in cowpeas.

Fresh and Dry Weight of Nodules

The lower nodule weights as compared to *Rhizobium* alone were in the control (191.776 mg fresh, 23.734 mg dry) and the highest was with *Rhizobium* alone (261.113 mg fresh, 39.507 mg dry) (Table 2) which agrees with Dakora and Keya (1997). Imazethapyr at *Rhizobium* reduced the weights in a dose-dependent manner, similar to that reported by Zahir *et al.* (2000). Results showed that reductions are greatest with Imazethapyr alone (I3: 129.213 mg fresh, 24.437 mg dry), indicating consistent results with prior findings by Bhatt (2012). These results show that *Rhizobium* has a role in reducing herbicide stress.

Biochemical Analysis

Biochemical analyses revealed that Imazethapyr application led to increased proline accumulation, indicative of stress response mechanisms in plants. The data indicated:

Protein Content

Protein content is a key indicator of nitrogen assimilation and overall plant metabolic activity. *Rhizobium* inoculation significantly increased protein levels compared to the control (134.952 ± 5.044 mg/g) (Figure 1), likely due to enhanced nitrogen fixation, which promotes amino acid synthesis and protein accumulation. Rhizobium-only treatments show the highest protein content (266.615 ± 1.014 mg/g). Herbicide application led to a concentration-dependent reduction in protein content, suggesting that herbicide-induced stress disrupted nitrogen metabolism and protein biosynthesis. Higher herbicide concentrations resulted in more pronounced declines, indicating a potential inhibition of enzymatic activities involved in nitrogen assimilation.

When *Rhizobium* was combined with herbicide treatments, protein content remained higher than in herbicide-alone treatments but lower than in *Rhizobium*-only plants. The higher protein content as a result of *Rhizobium* inoculation in the presence of higher concentrations of Imazethapyr can be interpreted as an ameliorative effect of *Rhizobium* overcame the toxic stress induced by the herbicide, resulting in the higher amount of protein synthesized in the plant, consistent with the findings of previous researchers such as Glick (2012) and Zaidi *et al.* (2005).

Proline Content

Rhizobium and Imazethapyr had a combined significant effect on proline. Proline content (6.560 ± 0.588 mg/g) in the control (C), while proline content for the *Rhizobium* inoculation treatment alone (R) was reduced to 5.371 ± 0.038 mg/g (Figure 2), indicating that *Rhizobium* could alleviate stress. Among all the Imazethapyr with *Rhizobium* treatments, the higher proline content was registered for the combination I3 + R as compared to I2 + R and I1 + R, showing a reduction in this component, evidencing that at higher concentrations, herbicides contribute to the accumulation of proline in stressed plant systems. Imazethapyr alone (I1, I2, I3) exhibited comparatively higher proline content. This is consistent with the findings of Ashraf and Foolad (2007), who noted that proline accumulation acts as a protective mechanism under abiotic stress.

Nitrogen Content

The nitrogen content in cowpeas was significantly affected by both *Rhizobium* inoculation and treatments of Imazethapyr. Control (C) plants showed a nitrogen content of 47.845 ± 0.073 mg/g (Figure 3), while the increase of nitrogen content to 72.703 ± 0.591 mg/g for *Rhizobium* alone (R) is significant, indicating modification in nitrogen metabolism by *Rhizobium*. *Rhizobium* and Imazethapyr had a concentration-dependent interaction concerning nitrogen content because I1 + R had 65.167 ± 1.726 mg/g, and it decreased as the concentrations of Imazethapyr increased, which suggests that although *Rhizobium* was capable of alleviating some of the negative effect of Imazethapyr on the nitrogen content, it still inhibited it. In comparison, Imazethapyr only (I1, I2, and I3) was associated with the lowest nitrogen contents among all the treatments, suggesting that the herbicide can affect nitrogen fixation or assimilation pathways (Elamari *et al.* 2022, Zaidi *et al.* (2005).

Leghaemoglobin Content

The *Rhizobium* inoculation and Imazethapyr treatments significantly affected leghaemoglobin content in cowpeas. Control (C) had 0.553 ± 0.020 mM and increased in *Rhizobium* (R) to mM 1.100 ± 0.018 (Figure 4). Imazethapyr with *Rhizobium* combined treatments decreased the rate in a concentration-dependent manner concerning Imazethapyr alone. The lowest content of leghaemoglobin was obtained

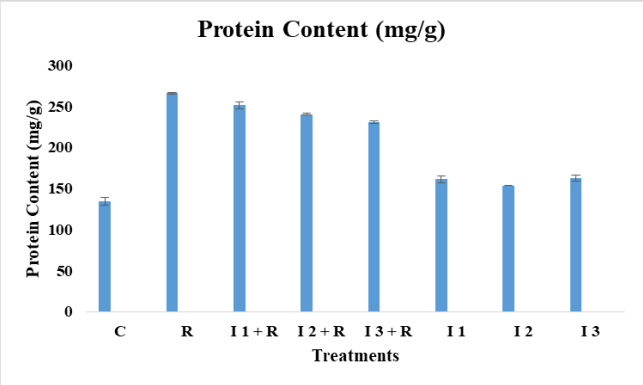

Figure 1: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on protein content (mg/g) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

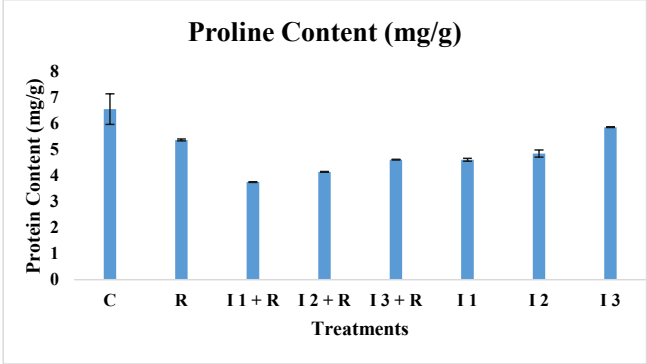

Figure 2: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on proline content (mg/g) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

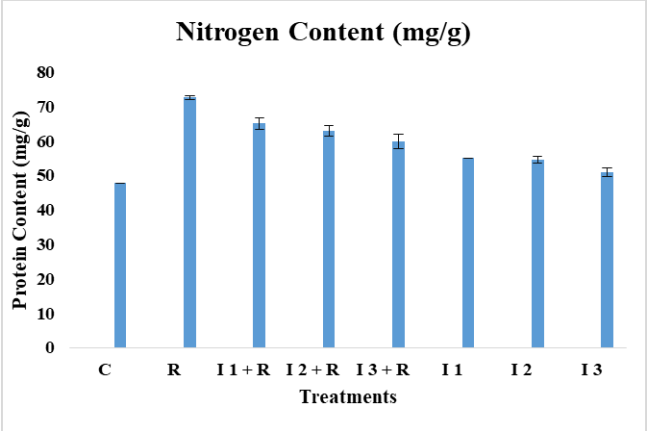

Figure 3: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on nitrogen content (mg/g) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

in Imazethapyr-alone treatments. The results obtained were per other studies that had reported the detrimental effects of herbicides on leghaemoglobin production and nitrogen fixation in legumes, a finding that was alleviated upon *Rhizobium* inoculation (Kumar *et al.* 2020; Ott *et al.* 2005 and Chikoye *et al.* 2014). These findings emphasize the need for the management of herbicide applications to protect the nitrogen fixation of cowpeas.

Enzyme Activities

Nitrate reductase activity

Nitrate reductase is a key enzyme in nitrogen metabolism, playing a crucial role in nitrate assimilation and plant growth. Nitrate reductase activity was higher in *Rhizobium*-treated plants (0.858 ± 0.029 µmol/g/h) and the lower

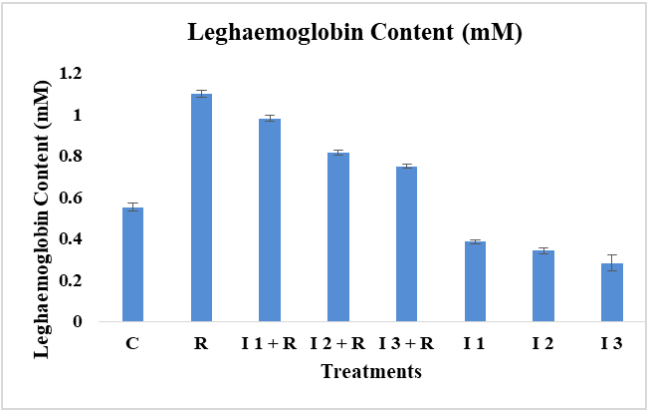

Figure 4: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on leghaemoglobin content (mM) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

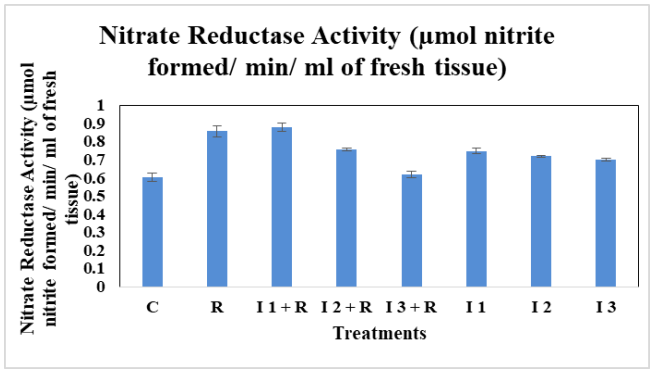

Figure 5: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on Nitrate Reductase Activity (µmol nitrite formed/ min/ ml of fresh tissue) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

concentration of Imazethapyr with *Rhizobium* (0.859 ± 0.028 µmol/g/h) compared to untreated controls (0.604 ± 0.022 µmol/g/h) (Figure 5), reflecting enhanced nitrogen utilization. *Rhizobium* and Imazethapyr treatments significantly influenced nitrate reductase activity (NRA), For physicochemical characterization, NRA results of I1 + R, I2 + R, and I3 + R were observed in a concentration-dependent decrease. NRA values were lowest for treatments with Imazethapyr alone (I1, I2, I3). The fact that *Rhizobium* increases the activity of nitrate reductase and Imazethapyr reduces it, confirms the results of several other studies regarding the province of herbicides reducing the nitrate reductase activity in plants (Prasad and Shivay 2008).

Hydrogenase activity

Hydrogenase activity plays a critical role in nitrogen fixation by recycling hydrogen produced during nitrogenase activity, thereby improving overall nitrogen assimilation

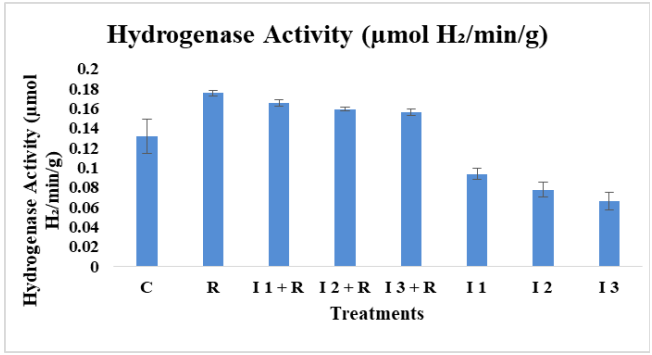

Figure 6: Effects of herbicide Imazethapyr and *Rhizobium* bacteria on Hydrogenase Activity (µmol H_2/min/g) of cowpea (*Vigna unguiculata*) after 45 days of seeding. Values represent the arithmetic mean ± S.D. (n=3). C: Control, R: *Rhizobium*, I1: Imazethapyr @400 mL/acre, I2: Imazethapyr @500mL/acre, I3: Imazethapyr @600mL/acre.

efficiency. In this study, *Rhizobium* inoculation significantly enhanced hydrogenase activity (0.175 ± 0.002 µmol H_2/min/g) compared to the control (0.131 ± 0.017 µmol H_2/min/g), indicating improved nitrogen-fixing efficiency and better symbiotic performance (Figure 6). Herbicide application led to a notable reduction in hydrogenase activity, with higher herbicide concentrations showing more pronounced declines. This suggests that herbicide-induced stress negatively affected nitrogenase function, leading to decreased hydrogen metabolism in root nodules. The reduction in hydrogenase activity may be attributed to oxidative stress, enzyme inhibition, or impaired nodule function due to herbicide toxicity.

In treatments where *Rhizobium* was applied alongside herbicides, hydrogenase activity remained higher than in herbicide-alone treatments but lower than in *Rhizobium*-only plants. This indicates that *Rhizobium* inoculation partially counteracted the inhibitory effects of herbicides on nitrogenase-associated hydrogen metabolism. However, at higher herbicide concentrations, the protective effect was diminished, suggesting that excessive herbicide exposure severely limits nitrogen-fixing efficiency despite *Rhizobium* presence.

These findings underscore the importance of *Rhizobium* inoculation in sustaining nitrogen fixation under herbicide stress and suggest that careful management of herbicide application is necessary to maintain efficient nitrogen metabolism in cowpea cultivars.

Conclusion

The present study highlights a more pronounced significant influence of the Imazethapyr herbicide on the growth, physiological, and biochemical parameters of cowpeas, where the increase in herbicide concentrations also led to decreases in shoot and root length, shoot and root fresh and dry biomass, nodule formation and activity of N uptake stimulating enzymes. On the other hand, inoculation with *Rhizobium* alleviated the effect of herbicide, especially in low concentrations (I1 + R), as it significantly increased growth parameters (height, shoot weight, and root weight), number of nodules and biochemical parameters (protein, proline, and nitrogen).

The findings of this study highlight the critical role of *Rhizobium* inoculation in mitigating the adverse effects of herbicide application on cowpea growth and biochemical responses. By alleviating herbicide-induced stress, *Rhizobium* enhances plant health and productivity, contributing to the development of eco-friendly farming practices. These results underline the importance of integrated weed management strategies that combine herbicide use with biological interventions to promote sustainability in legume-based cropping systems. This combined method offers a sustainable solution for cowpea production in herbicide-contaminated habitats. Further studies should consider the universal dosages of *Rhizobium* on various cultivars in different agroclimatic regions to define the optimized management strategies.

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