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RESEARCH ARTICLE

Rhizobium Inoculation and Herbicide Stress: Interactive Effects on Cowpea (*Vigna unguiculata* L. Walp.)

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

Abstract

The effect of *Rhizobium* inoculation and the application of two herbicides (Pendimethalin and Glyphosate) on the growth, physiological, and biochemical parameters of *Vigna unguiculata* was evaluated in this study. *Rhizobium* inoculation significantly improved seed germination, shoot, root development, and nodule formation, suggesting its symbiotic role in enhancing plant resilience and productivity. Application of herbicides induced a dose-dependent response with low rates of Pendimethalin and Glyphosate, causing minimal damage to growth and nodulation in combination with *Rhizobium*. However, root length, nodule number, and nitrogen fixation were adversely affected at elevated concentrations of Glyphosate. Importantly, *Rhizobium* increased the protein content and nitrogen assimilation, but the herbicide treatments negatively affected these parameters at elevated concentrations. Higher concentrations of Glyphosate also produced higher proline levels, a widely used plant stress indicator, and were detrimental in all cases. The activity of some important enzymes, such as nitrate reductase and hydrogenase, was reduced by increasing doses of the herbicide; however, *Rhizobium* inoculation significantly reversed the noted impacts. In conclusion, the findings highlight the significance of *Rhizobium* inoculation in improving the physiological performance and stress endurance of cowpeas under herbicide-induced circumstances and provide further evidence for the rational use of *Rhizobium* for the sustainable cultivation of legumes in herbicide-treated environments.

Keywords: *Vigna unguiculata*, *Rhizobium*, Herbicides, Cowpea, Inoculation, Symbiosis, Stress Endurance.

Introduction

Vigna unguiculata L. Walp., commonly known as cowpea is an important legume crop cultivated across tropical and sub-tropical climates due to its high nutrition value and growth in low fertility soils, especially with a high protein content. Cowpea, with its high protein and essential nutrient composition, is identified as an important food security crop contributing towards sustainable agricultural practices (Singh et al. 2003; Abebe and Alemayehu 2022). Cowpea also has a symbiosis with *Rhizobium* bacteria (Adesemoye and Kloepper 2009), which increases biological nitrogen fixation (BNF), turning the soil fertile and eventually facilitating other crops' productivity.

[1]Department of Botany, R.G.(P.G.) College, Meerut

[2]Botany Department, CCS University, Campus, Meerut

***Corresponding Author:** Shivani Sangal, Botany Department, CCS University, Campus, Meerut, E-Mail: geetsangal181@gmail.com

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Nonetheless, the weed competition had been reported to potentially entail a great challenge for cowpea production since weeds could compete with the crop for essential resources like light, water, and nutrients that could finally reduce yields (Bhowmik 1999). Therefore, effective weed management is necessary for optimal growth and productivity of cowpeas. (Ugbe et al. 2016). Application of Pendimethalin during pre-emergence and post-emergence respectively, has shown favorable results in Greengram (Singh et al. 2015).

Glyphosate inhibits root and shoots development via the disruption of cell division and is active on a broad range of annual grasses and broadleaf weeds (Duke and Powles 2008; Matloob et al. 2017). Glyphosate affects microorganisms responsible for nitrogen fixation in legumes (Zobiole et al. 2010; Rose et al. 2016) Glyphosate is a systemic post-emergence herbicide that inhibits the shikimic acid pathway, impeding the biosynthesis of aromatic amino acids, hence making it a broad-spectrum herbicide employed for weed control (Bruggen et al. 2018; Zobiole et al. 2010). Both these herbicides would effectively control the weeds but could also adversely affect the physiology of crop species such as photosynthesis, nitrogen fixation, and protein synthesis

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(Vishwakarma et al. 2023).

Due to the importance of *Rhizobium*-legume symbiosis in biological nitrogen fixation, the interaction between herbicides and *Rhizobium* inoculation is becoming a new focus. Conversely, this symbiosis significantly enhances nitrogen uptake in plants, thereby boosting their growth and yield (Vessey 2003; Oyatokun et al. 2023). The use of *Rhizobium* as an inoculant to tard more nodulation and Nitrogen assimilation has been demonstrated to enhance the growth of cowpea (Kyei-Boahen et al. 2017; Rebika and Nongmaithem 2019). *Rhizobium* can be very effective in restoring agricultural soil and can help farmers improve their practices, improve soil quality, and ultimately result in better plant growth (Chaudhary et al. 2022). On the other hand, Glyphosate and other herbicides may also affect the *Rhizobium* population negatively, impairing the nodule formation and consequently compromising the inoculation benefits (Zobiole et al. 2012; Corral et al. 2022)

Although these interactions have a significant role, there is a dearth of literature investigating the effect of herbicide treatments and *Rhizobium* inoculation on cowpeas' growth and biochemical characteristics. This study aims to evaluate the influence of Pendimethalin, Glyphosate, and *Rhizobium* inoculation on cowpea development, nodulation, and biochemical properties such as protein and nitrogen fixation. These conclusions should be useful to maximize herbicide usage for cowpea production avoiding damage to soil health and crop yield.

Materials and Methods

Cowpea and three different herbicides in association with *Rhizobium* inoculation, alone or combined, were used in this study to assess if different physicochemical parameters of the cultivars would be impacted.

Geographical Location of the experimental site

The current experiment was performed at the Department of Botany, Chaudhary Charan Singh University, Meerut, U.P., India, situated between 28°44' and 29°18' north latitude and 77°08' and 78°47' east longitude at an altitude of 228 meters from sea level. It has a mean summer temperature between 32°C and 42°C, a mean winter temperature between 10°C and 25°C, and an annual rainfall of about 740 mm.

Materials used during study

The cowpea seeds used in the experiment purchased from IARI New Delhi have uniform high-quality seeds. The cowpea-specific *Rhizobium* inoculum was also obtained from IARI New Delhi. We chose this strain for compatibility with cowpeas and its efficient enhancement of nitrogen fixation. Pendimethalin (30% EC) and Glyphosate (41% SL) were purchased from a well-known agrochemical company to replicate basal chemical conditions.

Experimental Design

The experiment was conducted in three trials, with three replicates per treatment (data shown correspond to the mean values of the trials), which is considered sufficient for reproducible results. The experimental design employed a completely randomized design (CRD) with 14 treatments replicated three times (for a total of 42 pots). The treatments consisted of a control (C), *Rhizobium* inoculation (R), and Pendimethalin and Glyphosate (with and without *Rhizobium*). Pendimethalin (30% EC) was applied at the rate of 1L/acre (P1 + R, P1), 1.25L/acre (P2 + R, P2), and 1.5L/acre (P3 + R, P3), and Glyphosate (41% SL) at the rate of 1L/acre (G1 + R, G1), 1.5L/acre (G2 + R, G2), and 2L/acre (G3 + R, G3). The volumes of herbicide applied per pot were based on the area of the pot (213.82 cm^2), which corresponded to 52.8 µL, 79.2 µL, and 105.7 µL per pot for Glyphosate and 52.8 µL, 66.0 µL, and 79.2 µL per pot for Pendimethalin, representing true to field conditions.

Methods

Seed Germination Percentage

In the germination study of cowpea, healthy seeds were sterilized in a 0.1% sodium hypochlorite solution and rinsed with distilled water. Ten seeds were placed in sterile Petri dishes with moistened Whatman No. 1 filter paper and incubated at 25°C with a 12-hour light/dark cycle. Daily moisture checks ensured adequate hydration. Germination was defined as a radicle emergence of at least 2 mm, with counts recorded daily for 10 days. Germination percentage was calculated as:

$$\text{Germination Percentage}\,(\%) = \left(\frac{\text{Number of Germinated Seeds}}{\text{Total Number of Seeds}}\right) \times 100$$

Shoot Length and Root Length

The shoot and root lengths were measured in cm and the final length per plant was determined by averaging two measurements per treatment.

Fresh Weight of Shoot and Root

During the peak period of vegetative growth, the aboveground parts of plants were cut off from the roots and weighed on an electronic balance after being separated to obtain the fresh weight for each system, with all treatments represented in three replications for statistical power.

Dry Weight of Shoot and Root

When the plant samples reached a constant weight, the samples were oven-dried at 70°C for 72 hours, and then weighed again with the same electronic balance. This process was done for every treatment to keep it consistent.

Nodule Numbers

The visually estimated number of nodules per plant was recorded at the maximal growth of the plant. Replicates per

treatment were averaged.

Nodule Volume

The volume of the nodules was determined by the water displacement method. Nodules were washed and blotted dry from 3 plants per treatment and submerged in a graduated cylinder containing water; the volume displacement in ml was recorded. The volume per nodule was averaged and calculated over the 3 replicates.

Fresh Weight of Nodules

Nodules were harvested at peak growth of plants, separated from roots, and immediately weighed with the electronic balance. Nodules from 3 plants per treatment were weighed and averaged.

Dry Weight of Nodules

The nodule samples were oven-dried for 48 h at 70 °C until constant weight was attained. The dry weights were determined using the same electronic balance, and the average dry weight of nodule per sample (3 replicates) was presented.

Protein estimation (Bradford method 1976)

Fresh leaf tissue (100 mg) was homogenized in 5 ml of Tris-EDTA buffer and was centrifuged at 5000 rpm for 10 minutes, collecting the supernatant. The protein estimation was based on the quantitative binding of Coomassie Brilliant Blue (CBB) dye, where 1 ml of supernatant was mixed with 5 ml of CBB dye and kept for 5 min. The absorbance was taken at 595 nm using a spectrophotometer, and the protein concentration was determined relative to a standard curve for casein. The results were reported as mg/g fresh weight, and each assay was performed three times to ensure accuracy.

Proline

Analysis of proline content in leaves of plants was measured according to the method developed by Bates et al. (1973), that is, a colored complex from the reaction of proline with ninhydrin. For sulfur estimation, 100 mg of fresh leaf samples were homogenized in 10 ml of 3% sulphosalicylic acid and filtered. The supernatant was separated by centrifugation and added with glacial acetic acid and ninhydrin solution. The resulting solution was incubated in a water bath for 1 h and cooled in an ice bath. Phases were separated by the addition of toluene and the absorbance of the top toluene layer was measured at 520 nm. The proline level was determined from a standard curve and expressed as µg/g fresh weight. To verify the accuracy, the assay was performed in triplicate.

Nitrogen

Nitrogen content of plant tissues was determined using a method of Snell and Snell (1967). Homogenization of 25 mg of plant sample was performed in concentrated H_2SO_4, 30% H_2O_2, and was placed into the sand bath. Then catalyst (K_2SO_4 + $CuSO_4$) was added and digestion continued until the solution was scattered clear or pale yellow, indicating digestion was complete. After cooling down the samples were diluted with distilled water, and 1 ml of supernatant was mixed with Nessler reagent. Absorbance was measured at 425 nm using a blank reference, and nitrogen concentration was obtained based on the standard curve. The assay was conducted in triplicate for accuracy.

Leghaemoglobin

A method of quantifying leghaemoglobin was used, as provided by Bergersen and Turner (1980). To recover fresh root nodules, 200 mg were plunged into liquid nitrogen for immediate freezing to prevent degradation before they were homogenized in 6 ml of ice-cold phosphate buffer with a pH of 7.4. The mixture was filtered through a cheesecloth and the resulting filtrate was centrifuged at 10,000 rpm for 10–30 min at 4°C to yield a clear supernatant containing leghaemoglobin. Three mL of the supernatant was then added to 3 mL of alkaline pyridine reagent for the assay. Sodium dithionite was added, and the optical density (O.D.) at 556 nm was measured in triplicate. Potassium hexacyanoferrate was added, and the O.D. at 539 nm was measured in triplicate. Leghaemoglobin concentrations (mM) were calculated using the following formula:

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

(Where; A_{556} and A_{539} are the absorbances at 556 nm and 539 nm respectively, and D is the dilution factor.)

Nitrate Reductase

Nitrate Reductase (NR) is one of the most important enzymes in the nitrogen cycle, which catalyzes the reduction of nitrate (NO3-) to nitrite (NO2-); and is the first step in nitrogen assimilation in plants. Nitrate reductase activity was estimated in cowpea leaves according to the method; described by Hageman et al. (1980). 1 g of leaf material was homogenized with an extraction buffer (1 mM EDTA, 1-25 mM cysteine and 25 mM potassium phosphate buffer, pH 8.8). The homogenate was filtered through cheesecloth, and the supernatant was prepared by centrifuging the filtrate at 30,000 rpm for 15 min (5° C), and the obtained supernatant was used for the assay. The following constitutions were used as reaction mixture: 0.5 ml phosphate buffer (pH 7.5), 0.2 ml potassium nitrate solution, 0.4 ml NADH solution, and 0.7 ml water (total of 1.8 ml), and to begin the reaction, 0.2 ml of the enzyme extract was added. A control was prepared by substituting the enzyme extract with distilled water. The reaction was stopped after 15 min of further incubation at 30°C with solubilization of the dye by adding 1 ml sulphanilamide solution and 1 ml naphthyl ethylenediamine reagent. The absorbance was measured after 90 m in 540

nm. Sodium nitrite was used to create a standard curve, and nitrate reductase activity was expressed as µmol of nitrite produced per minute per gram of fresh leaf tissue. Every sample was analyzed in triplicate to ensure accuracy and reliability.

Hydrogenase

The hydrogenase activity of cowpea root nodules was determined by the method of Turner et al. (1980). New nodules were collected and snap-frozen in liquid nitrogen to preserve enzyme activity. Frozen nodules (0.5 g) were ground in ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0; 5 mM dithiothreitol; 1 mM EDTA; 10% glycerol). Centrifugation was performed for 15 min at 10 000 rpm at a temperature of 4°C for homogenate of tissues and supernatant was used as enzyme extract. Hydrogenase activity was measured by mixing enzyme extract with an assay mixture; consisting of potassium phosphate buffer, methylene blue, and sodium dithionite, and monitoring a decrease in absorbance at 670 nm. Incubation was at 30°C for 10 minutes followed by termination of the reaction mix with 5% trichloroacetic acid. The absorption reduction of 670 nm was determined with a UV-Vis spectrophotometer, and enzyme activity was reported as micromoles of consumed hydrogen per minute and gram of fresh weight (µmol H_2 min^{-1} g^{-1} FW). The analysis of each sample was performed in triplicates to get accurate and reproducible results.

Results and Discussion

This study was conducted to evaluate the effects of Pendimethalin and Glyphosate on various health and biochemical parameters of cowpeas which have justified the impact of both the herbicides on the physiology and development of the plants. Their results highlighted the differences in effectiveness and phytotoxicity of the different control methods and the advantages of *Rhizobium* inoculation in providing benefits to the plants.

Seed Germination and Growth Parameters

Seed Germination Percentage

Rhizobium increased cowpea seed germination to 92.58%, compared to control (76.46%), indicating its effectiveness in seed viability. The germination rate was also high (90.83%) of the G1 + R treatment; which suggests that Glyphosate at this level does not affect germination with this *Rhizobium* (Table 1). Even other combinations such as P1 + R (84.65%) and G2 + R (88.72%) seem preferable, indicating that herbicides can be used safely in *Rhizobium*. On the other hand, proliferation treatments from P2 and P3 alone showed lower germination; highlighting the potential negative effects of herbicides without *Rhizobium*. These findings emphasize the importance of careful treatment decisions for improving cowpea germination.

Shoot and Root Length (cm)

Shoot and root lengths of cowpea differed significantly among treatments. *Rhizobium* treatment showed the maximum growth in the shoot (108.83 cm) and root (16.13cm) compared with control (62.4 cm shoots, 13.8 cm roots). Both P1 + R and G3 + R contributed to robust growth (99.67 cm shoots, 16.27 cm roots); however, G3 + R produced the shortest roots (10.53 cm), demonstrating a detrimental effect (Table 1). Treatments P2 + R and G2 + R also contributed to good root growth, which suggests that some herbicides are compatible with *Rhizobium*. This demonstrates the growth promotion role of *Rhizobium* and the need for careful selection of compatible herbicides.

Fresh and Dry Weight of Shoots and Roots (g)

Rhizobium treatment increased the growth parameters significantly which was confirmed by fresh weights of Shoots (25.39 g) and Roots (1.29 g) compared to control (16.39 g for shoots, 0.98 g for roots) (Table 1). Thereafter, P1 + R exhibited high shoot fresh weight (22.09 g), which suggests a higher interaction with the herbicide. On the other hand, G3 + R negatively affected root fresh weight (0.94 g). Similar trends were also observed in dry weight patterns, with *Rhizobium* leading to the highest dry weights, while G3 treatments had the lowest dry weight. *Rhizobium* has an important role in utilization, and the correct treatment was selected for optimum growth of cowpea as inferred from these results. Rhizobium inoculation increased shoot dry weight and other crop growth variables (Onduru et al. 2008; Nyoki and Ndakidemi 2013).

Nodule Development and Biochemical Responses

Nodule Numbers and Volume

The results show significant differences in nodule numbers and volumes in cowpeas across treatments. The highest number (70.33) and volume (2.73 ml) of nodules were observed in *rhizobium*-treated plants which indicates that effective nodulation and nitrogen fixation occurred in this treatment (Bergersen et al. 1980). *Rhizobium* inoculation increased nodulation and other crop growth variables (Onduru et al. 2008; Nyoki and Ndakidemi 2013). The nodule counts and volume of control (C) were lower than the ones of treated (T) (51.67 vs 0.83 ml) (Table 2). Notably, moderate improvement (52.67 nodules, 1.47 ml volume) was observed in the P1 + R combination, suggesting a synergistic interaction between these two compounds. The lowest values (37.33 nodules, 0.77 ml) among the treatments were verified for herbicides, with G3 + R being the treatment with the most significantly negative influence on nodule formation. These results highlight the importance of cautious treatment decisions to achieve enhanced nodulation in cowpeas.

Fresh and Dry Weight of Nodules

The fresh and dry weights of cowpea nodules increased

Table 1: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on seed germination percentage, shoot, and root length (cm), fresh and dry weight of shoot and root(g) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

Treatments	*Seed Germination Percentage*	*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	76.461 ± 0.280	62.400 ± 0.347	13.800 ± 0.200	16.389 ± 0.020	0.980 ± 0.026	1.712 ± 0.026	0.205 ± 0.001
R	92.576 ± 0.245	108.834 ± 0.643	16.134 ± 0.153	25.389 ± 0.052	1.291 ± 0.026	3.867 ± 0.035	0.319 ± 0.005
P 1 + R	84.649 ± 0.206	99.667 ± 0.379	16.267 ± 0.252	22.093 ± 3.849	0.845 ± 0.021	2.420 ± 0.089	0.130 ± 0.002
P 2 + R	81.648 ± 0.384	93.567 ± 0.643	14.267 ± 0.209	19.621 ± 0.022	0.801 ± 0.014	2.281 ± 0.039	0.072 ± 0.002
P 3 + R	83.992 ± 0.385	97.534 ± 1.002	14.700 ± 0.100	19.656 ± 0.048	0.967 ± 0.023	2.195 ± 0.211	0.099 ± 0.002
G 1 + R	90.833 ± 0.259	70.667 ± 0.405	12.900 ± 0.300	23.442 ± 0.023	0.639 ± 0.014	2.357 ± 0.032	0.224 ± 0.004
G 2 + R	88.716 ± 0.479	98.367 ± 0.352	16.767 ± 0.252	15.533 ± 0.005	0.544 ± 0.014	1.763 ± 0.031	0.204 ± 0.001
G 3 + R	89.740 ± 0.255	91.234 ± 0.851	10.534 ± 0.252	18.483 ± 0.020	0.943 ± 0.015	2.178 ± 0.115	0.185 ± 0.003
P 1	82.459 ± 0.175	74.600 ± 0.625	9.467 ± 0.306	18.429 ± 0.063	0.0777 ± 0.010	1.930 ± 0.010	0.083 ± 0.003
P 2	79.748 ± 0.593	77.467 ± 0.666	12.7 ± 0.300	18.123 ± 0.021	0.686 ± 0.010	1.555 ± 0.074	0.077 ± 0.002
P 3	76.414 ± 0.251	68.767 ± 0.586	9.667 ± 0.153	17.064 ± 0.027	0.622 ± 0.011	1.553 ± 0.095	0.067 ± 0.001
G 1	91.138 ± 0.691	66.667 ± 0.116	9.867 ± 0.252	20.323 ± 0.018	0.728 ± 0.011	2.418 ± 0.053	0.118 ± 0.003
G 2	90.571 ± 0.403	60.700 ± 0.265	13.034 ± 0.252	18.551 ± 0.017	0.841 ± 0.019	2.201 ± 0.204	0.087 ± 0.002
G3	88.569 ± 0.182	62.200 ± 0.872	10.900 ± 0.300	18.112 ± 0.029	0.706 ± 0.013	1.717 ± 0.107	0.072 ± 0.002

Table 2: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on nodule numbers, nodule volume (ml), and fresh and dry weight of nodules(mg) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

Treatments	*Nodule Numbers*	*Nodule Volume (ml)*	*Fresh Weight of Nodules (mg)*	*Dry Weight of Nodules (mg)*
C	51.667 ± 6.807	0.834 ± 0.116	123.11 ± 1.536	23.734 ± 0.198
R	70.334 ± 1.528	2.734 ± 0.058	461.114 ± 2.115	96.84 ± 0.186
P 1 + R	52.667 ± 3.215	1.467 ± 0.058	157.91 ± 1.646	29.837 ± 0.316
P 2 + R	51.334 ± 3.056	1.267 ± 0.058	147.537 ± 1.002	31.237 ± 0.255
P 3 + R	46.667 ± 2.082	1.300 ± 0.265	142.65 ± 0.258	34.897 ± 0.134
G 1 + R	50.667 ± 1.528	1.867 ± 0.058	193.184 ± 1.497	27.737 ± 0.298
G 2 + R	45.667 ± 2.082	2.034 ± 0.058	238.374 ± 0.502	50.657 ± 0.152
G 3 + R	37.334 ± 0.578	0.767 ± 0.058	123.284 ± 0.558	48.17 ± 0.053
P 1	42.000 ± 4.359	0.867 ± 0.058	131.08 ± 0.637	38.077 ± 0.103
P 2	30.667 ± 7.235	1.234 ± 0.153	139.26 ± 0.666	30.544 ± 0.196
P 3	25.334 ± 3.215	0.867 ± 0.058	136.44 ± 0.201	26.154 ± 0.103
G 1	18.667 ± 1.115	0.734 ± 0.209	130.534 ± 0.397	28.534 ± 0.107
G 2	26.334 ± 5.034	0.0667±0.153	128.06 ± 0.414	26.337 ± 0.282
G 3	16.667 ± 5.508	0.667 ± 0.058	119.42 ± 0.715	22.594 ± 0.065

significantly due to *Rhizobium* inoculation compared to the control and herbicide-only treatments. Among treatments, the highest amount of nodule biomass in the crop was obtained with *Rhizobium* alone, indicating a strong effect of this microorganism on nodule growth (Table 2). When applied alone, herbicides inhibited nodule formation; however, in combination with *Rhizobium*, there was an increase in nodule weight, considering G2 + R, which showed the greatest fresh and dry weight values among all combinations. Thus, *Rhizobium* inoculation may mitigate the negative effects of herbicides on nodulation and may be a suitable complementary treatment to promote legume growth under herbicide stress.

Biochemical Analysis

Protein Content

It is essential for the evaluation of plant health and nitrogen metabolism. Control plants (C) showed high levels of protein (135.286 mg/g), suggesting healthy nitrogen metabolism (no herbicidal stress). Nonetheless, *Rhizobium* inoculation

(R) still showed lower protein content (101.616 mg/g) than the control, indicating that it is still under moderate stress although it alleviated stress (Figure 1). On the other hand, the combinations of those with *Rhizobium* (P1 + R, P2 + R, P3 + R) increased the protein levels (162.091 mg/g for P1 + R) while the Glyphosate treatments (G1, G2, and G3) significantly reduced the protein content (G3 + R (74.119 mg/g)), showing differences with the higher concentrations harming the synthesis of this biomolecule. Significantly, an increase in the levels of the protein when compared to the control was found in all Pendimethalin treated groups (P1, P2, P3) with the least interference of Pendimethalin with nitrogen metabolism was in P1 as reflective from higher protein value (232.532 mg/g). These results were consistent with reports on the effects of herbicides on legumes (Zaidi et al. 2005).

Proline Accumulation

Accumulation of Proline plays a key role in osmoregulation as well as protecting plant cells against the damage caused by stress (pharmacological and environmental). Plant physiologists commonly use Proline quantification to gauge how well plants can tolerate stress and assess their antioxidant capabilities in challenging conditions (Spormann et al. 2023). The content of free proline was another effective measure under treatment variance due to the combination of herbicides and *Rhizobium* inoculation in this study. The control plants (C) had the highest level of proline (7.894 mg/g), which indicates that the untreated plants naturally have a higher accumulation of this amino acid under stress. Proline content (5.371 mg/g) was lowest with *Rhizobium* inoculation (R), which may also alleviate stress from improved nitrogen fixation (Figure 1). In the case of plants treated with herbicide, the lowest level of proline (4.329mg/g) was found in the combined treatment of Pendimethalin along with *Rhizobium* (P1 + R) due to the beneficial effect of *Rhizobium*. Glyphosate + resistant variety (G2 + R) and Glyphosate + resistant variety (G3 + R) again displayed the highest levels of proline (47 mg/g) on the plants treated with Glyphosate herbicide. Our findings match reports from other researchers (Bajpai and Srivastava 2015; Hosseinifard et al. 2022) who concluded that proline accumulation is affected by symbiotic bacteria and herbicides, affirming the value of proline as a biochemical marker for studying plant herbicide stress responses.

Nitrogen Content

The nitrogen content in cowpea nodules varied significantly between treatments, demonstrating the impact of inoculation with *Rhizobium* in combination with herbicide application on nitrogen assimilation. The control group (C) had the highest nitrogen content (47.846 mg/g), which indicated that the plant could provide better utilization of the nitrogen. Conversely, plants given *Rhizobium* (R) showed a lower nitrogen value of 24.037 mg/g, which suggests some stress or grazing distribution that impacts nitrogen fixation efficiency there (Figiure 1). For Pendimethalin treatments, the levels further decreased, and the lowest nitrogen content was recorded with the P3 + R treatment, evidenced by 12.014 mg/g of nitrogen, which indicates that higher concentrations of Pendimethalin might disturb the symbiotic relationship that is necessary to perform nitrogen fixation and allow nothing to settle in the roots. For Glyphosate treatments, it was also evident that the nitrogen contents were different from each other; G2 + R showed the highest nitrogen content value of 26.674 mg/g (the more diluted Glyphosates) and G3 + R showed the

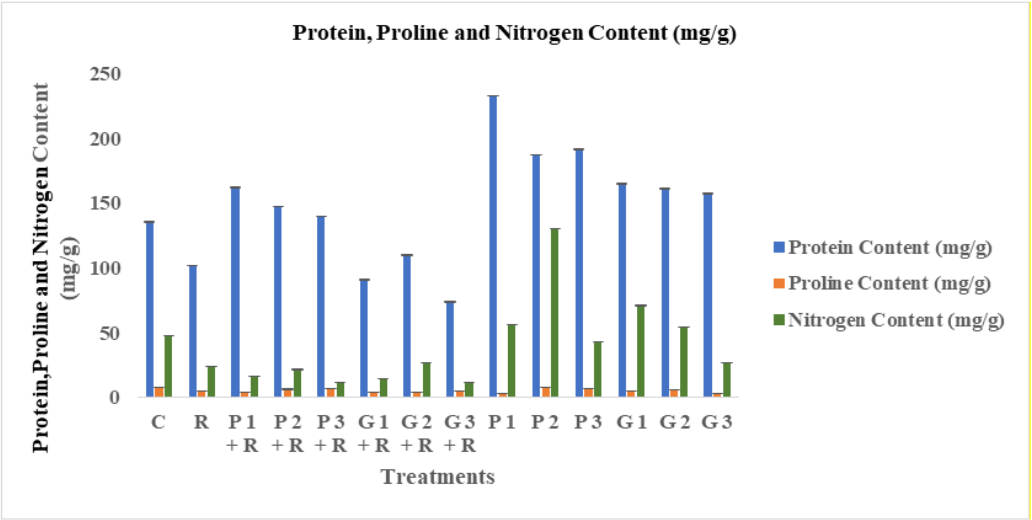

Figure 1: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on protein, proline, and nitrogen content (mg/g) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

lowest content value of 11.502 mg/g (the most concentrated Glyphosates), indicating that lower amounts of Glyphosate can be less harmful for nitrogen accumulation. This suggests that herbicide application must be managed carefully to ensure nitrogen comes out of the pool, thereby impacting overall plant health.

Leghaemoglobin Content

The levels of leghaemoglobin in the cowpea nodules treated with *Rhizobium* inoculations and herbicide applications showed great variation. Leghaemoglobin was significantly higher (1.355 mM) in the *Rhizobium* inoculated (R) when compared to the control (C) leghaemoglobin content (0.551 mM) (Figure 2). This emphasizes the contribution of the symbiotic relationship in improving nitrogen fixation in legumes. This is consistent with earlier studies documenting that leghaemoglobin is crucial for establishing the microaerobic environment required for optimal nitrogenase function (Ott et al. 2005). The highest amount (0.973 mM) of leghaemoglobin extract was recorded in P1 + R. Still the leghaemoglobin extract decreased with increasing concentrations of Pendimethalin, as P3 + R indicated a leghaemoglobin extract of 0.878 mM, which may mean that the application of high doses of this herbicide can harm lead to a further drop in the synthesis of leghaemoglobin in the root nodules, reducing the efficiency of these structures in the process of nitrogen fixation. In a similar pattern, with Glyphosate-treated plants, G1 + R recorded 0.854 mM but the higher concentration, G3 + R recorded a leghaemoglobin content of 0.659 mM which also confirms detrimental effects on both the nodule health and *Rhizobium* populations. In conclusion, the R treatment produced the highest leghaemoglobin content, evidencing the importance of *Rhizobium* in improving the health and yield of legumes. Such results highlight the need for delicately managing herbicides in agriculture as they may have deleterious effects on nitrogen-fixing symbiosis in legumes.

Enzyme Activities

Nitrate Reductase Activity

The analysis for nitrate reductase activity showed highly significant differences between treatments with *Rhizobium* inoculation and herbicide applications. *Rhizobium* inoculation (R) increased nitrate reductase activity to 0.859 µmol nitrite formed/min/ml of fresh tissue as opposed to the control (C), which had an activity of 0.604 µmol/min/ml (Figure 3). In the case of Pendimethalin (P1 + R (0.859 µmol/min/ml)), P2 + R, and P4 + R showed stable activity; however, with P3 + R (0.690 µmol/min/ml) the enzyme function decreased due to the negative impact of higher doses. Likewise, Glyphosate treatments were associated with a decline in nitrate reductase activity, and all treated groups showed lower activities than the control, which is complementary to the highest concentration in G3 + R (0.417 µmol/min/ml), where a more pronounced decline of this enzyme was observed, signaling herbicide stress disrupting the nitrogen metabolism (Burul et al. 2022). Higher concentrations of herbicides decrease this enzyme's activity (El-Saht et al. 1994). In fact, between treatments, the G1 + R treatment had the most nitrate reductase activity (0.876 µmol/min/ml); this suggests that *Rhizobium* may be supported by lower Glyphosate levels, resulting in increased activity. These findings highlight the need for prudent herbicide use to maintain the positive effects of *Rhizobium* on nitrogen assimilation in legumes.

Hydrogenase Activity

Hydrogenase activity analysis indicated that *Rhizobium* inoculation significantly improved the enzymatic activity in

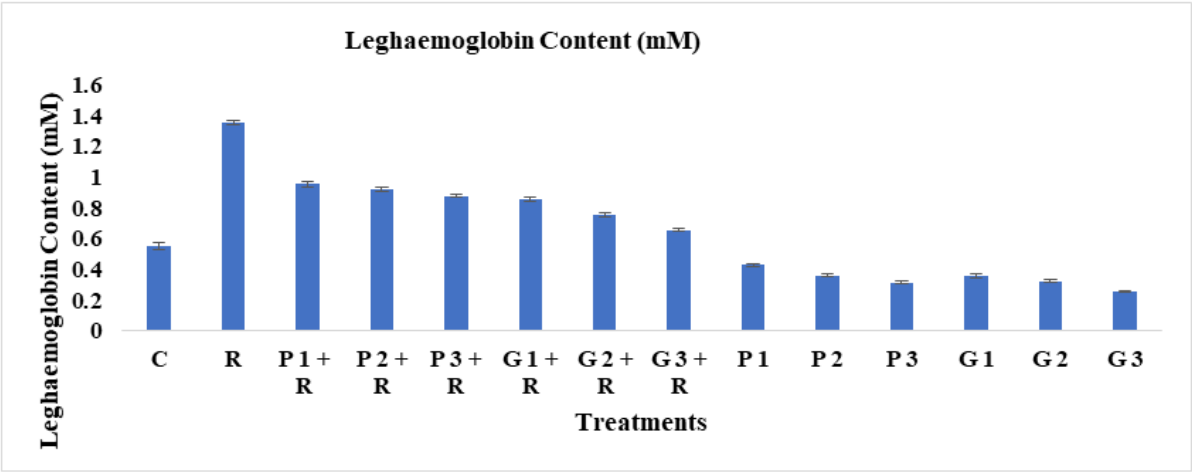

Figure 2: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on leghaemoglobin content (mM) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

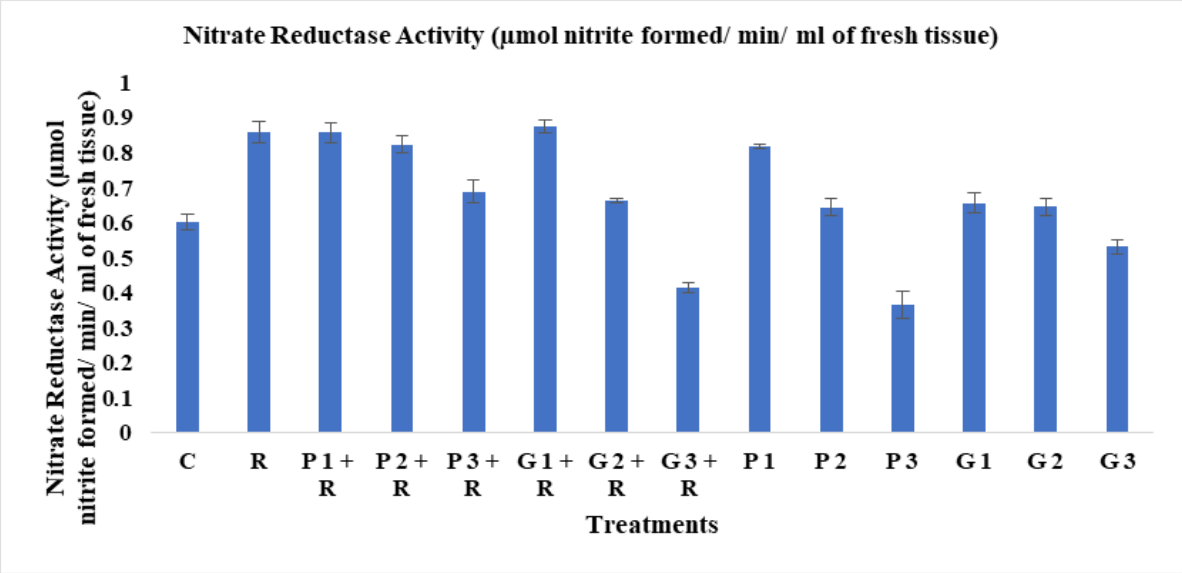

Figure 3: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on Nitrate Reductase Activity (µmol nitrite formed/ min/ ml of fresh tissue) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

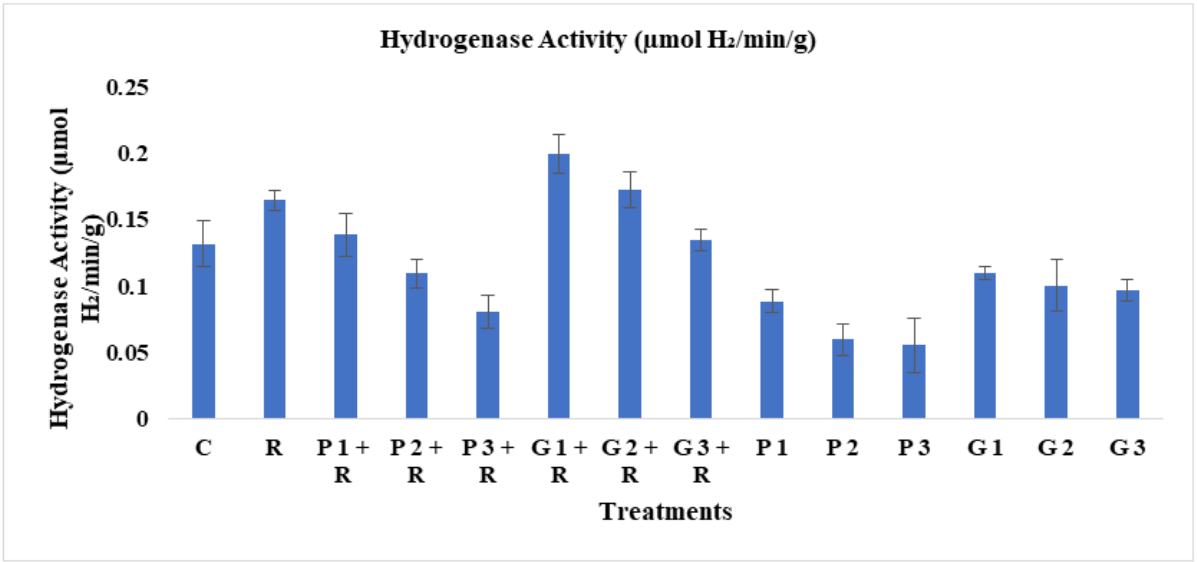

Figure 4: Effects of two herbicides (Pendimethalin and Glyphosate) and *Rhizobium* bacteria on Hydrogenase Activity (µmol H_2/min/g) of *Vigna unguiculata*. C: Control, R: *Rhizobium*, P1: Pendimethalin @1 litre/acre, P2: Pendimethalin @1.25 litre/acre, P3: Pendimethalin @1.5 litre/acre, G1: Glyphosate @1 litre/acre, G2: Glyphosate @1.5 litre/acre, and G3: Glyphosate @2 litre/acre.

contrast to the control (C), with results from 0.132 µmol H_2/min/g to 0.165 µmol H2/min/g, emphasizing the essential role of the *Rhizobium* in nitrogen metabolism priorities (Figure 4). In contrast, hydrogenase activity decreased with increasing concentrations of Pendimethalin and Glyphosate in a dose-dependent manner; the lowest activity was observed in P3 + R (0.081 µmol H_2/min/g) and G3 + R (0.135 µmol H_2/min/g), showing the toxic effects of herbicide toxicity and characterized inhibitory effects (Burul et al. 2022). The enzyme activity was significantly further compromised under herbicide treatments without *Rhizobium* (P3: 0.056 µmol H_2/min/g), indicating the negative effect of herbicides and the important function of the *Rhizobium* in the hydrogen metabolism. These results suggest that the regulation of microbial symbiosis in the rhizosphere by herbicide applications is essential for optimal hydrogenase

activity. Therefore, *Rhizobium* inoculation enhanced enzyme efficiency and was characterized by activity of 0.165 µmol H_2/min/g compared to the control (0.132 µmol H_2/min/g). This highlights the importance of *Rhizobium* in nitrogen metabolism. Additionally, with increasing concentrations of Pendimethalin and Glyphosate concentration, the activity of hydrogenase reduced to the lowest value in P3 + R (0.081 µmol H_2/min/g) and G3 + R (0.135 µmol H_2/min/g) indicating that reduced activity was caused due to herbicide toxicity (Burul et al. 2022). Enzyme activity in herbicide treatments without the presence of *Rhizobium* was reduced again (P3: 0.056 µmol H_2/min/g), indicating the negative impact of herbicide on hydrogenase activity and the significant role of *Rhizobium* for hydrogen functions. These results suggest that balanced herbicide use, to microbial symbiosis, would promote increased legume health and productivity.

Conclusion

The interaction of herbicides with *Rhizobium* shows how the use of these bacteria can positively influence the development of the cowpea plant, with significant increases in terms of germination, shoots, roots, and nodules of the plant. Although herbicides, especially at higher concentrations harmed plant growth, nodulation, and nitrogen fixation, inoculation with *Rhizobium* offset some of the negative effects, indicating that inoculated organisms could play a potential role in this regard. Pendimethalin was the least phytotoxic herbicide of those studied; Glyphosate at higher concentrations decreased root length, nodule number, nitrogen content, and key enzyme activity (nitrate reductase and hydrogenase) to a far greater extent. The exacerbated abiotic stress in the plants was reflected in increased proline accumulation under Glyphosate stress. In summary, this study demonstrates the importance of properly managing herbicide doses in cowpea-plant development, demonstrating that *Rhizobium* inoculation in cowpea may improve the tolerance of plants to herbicide, contributing to sustainable agriculture.

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