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Research Article

COWPEA NODULATION AND NITROGEN FIXATION UNDER POTYVIRUS AND RHIZOBIUM COINCIDENCE

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ABSTRACT

The present study aimed to assess the impact of seed Rhizobia treatment and potyvirus inoculation on bacterial nodulation and nitrogen fixation in cowpeas. The plants were infected with the virus two weeks post-germination. Nodules were present on the roots of plants treated with Rhizobia; however, almost no nodules were detected on untreated plants. The average number of nodules per plant on virus-inoculated plants was significantly lower than the average number per non-inoculated plant. The virus caused a substantial decrease in the weight of nodules also. The study revealed that the presence of Rhizobia resulted in a significant rise in nitrogen content in the foliage. Specifically, the nitrogen percentage increased from 1.29% in plants not treated with Rhizobia and not inoculated by the virus to 2.502% in Rhizobia-treated plants that were inoculated by the virus and to 2.550% in Rhizobia-treated plants that were not inoculated by the virus. This difference was statistically significant at a p-value less than 0.05. The virus resulted in a rise in nitrogen content in plants that were not inoculated with Rhizobia. The Rhizobia resulted in a significant augmentation in the number of pods per plant, seed count per plant, and seed dry weight per plant compared to plants that were not treated with Rhizobia. The application of Rhizobia to virus-inoculated plants resulted in a notable enhancement of these parameters, except for the nitrogen content in seeds.

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INTRODUCTION

Plant viruses cause significant losses to agriculture in Iraq (Adhab et al., 2021; Adhab and Alkuwaiti, 2022; Khalaf et al., 2023a). Numerous studies have been conducted to characterize and understand the impact of viruses on cultivated plants using surveys and different detection methods (Mohammed et al., 2021; Nasir and Adhab, 2021; Schoelz et al., 2021) and to understand their relationship with natural vectors in the field (Adhab and Schoelz, 2015; Adhab et al., 2019a; Adhab, 2021; Khalaf et al., 2023b). Other studies focused on plant virus management by inducing systemic resistance in plants (Al-Ani et al., 2011a,b; Al-Ani et al., 2013) or using genetic means (Adhab et al., 2018; 2019b; 2023; Adhab, 2021). Like other plant hosts, legumes are targets of plant viruses, and several trials have been conducted to decrease or eliminate the losses caused by such infections (Al-Ani and Adhab, 2013; Chatzivassiliou, 2021). Among the safest methods of plant disease control is using microbes to improve plant health and compete with pathogens in the fields.

Biological nitrogen fixation is a crucial process in agriculture as it enables the generation of nitrogen through the symbiotic relationship between legumes and rhizobia. This process leads to increased nitrogen levels in the soil, promoting improved plant growth (Moawad et al., 2005). Plant Growth Promoting Rhizobacteria (PGPR) enhances plant development and immunity, making plants more resilient to external stressors and diseases. Rhizobial bacteria, of the genus Rhizobium, are widely recognized as a highly significant bacterial population in the soil. They establish a mutually beneficial connection with the roots of leguminous and non-leguminous plants in a system that converts atmospheric nitrogen into a usable form (Fahde et al., 2023).

These microorganisms have a broader scope of influence beyond nitrogen fixation. Several species have antibacterial properties against harmful microorganisms and promote the production of certain plant development factors. The bacteria in question are categorized as PGPR (Alemneh et al., 2020; Vocciante et al., 2022).

Leguminous plants are frequently affected by numerous viruses, which can substantially impact the nitrogen fixation process carried out by rhizobial bacteria. Multiple sources indicate that the majority of these viruses lead to a decrease in both the quantity and mass of bacterial nodules. For instance, research has demonstrated that the infection of soybean plants with Soybean mosaic virus (SMV) decreases the quantity and mass of root nodules, ultimately leading to a decline in crop productivity (Liu et al., 2021).

Furthermore, a correlation was identified between virus infection and the chemical compound called leghemoglobin, present in bacterial nodules and crucial for nitrogen fixation. It was observed that the concentration of leghemoglobin in nodules of healthy plants is higher compared to nodules of plants infected with a virus. In their study, Ahmed and Abdulbaqi (1986) found that the presence of Peanut stunting virus (PSV) in bean plants resulted in a significant decrease of 78.4% in the number of bacterial nodules on the roots of the beans. Additionally, there was a substantial reduction of 78.6% in the overall weight of the roots. The results were detected one week after the mechanical inoculation of the seven-day-old plants, following their development under a controlled greenhouse environment.

A study investigating the interaction between Rhizobium and SMV at various phases of soybean plant growth revealed that the virus inhibited the growth and production of root nodules. Additionally, the virus led to a decrease in the leghemoglobin ratio (Andreola et al., 2019). Fava bean plants infected with Pea necrotic yellow dwarf virus (PNYDV) were found to inhibit the formation of new bacterial nodules in the infected plants (Seeger et al., 2022).

Infection of bean plants with Bean mosaic virus led to a reduction in the number of nodules, their shedding, and premature decay before their complete formation (van't Hof et al., 2023). Cheng et al. (2020) found that infection of alfalfa with Clover mosaic virus resulted in stunted plant growth, decreased number and weight of bacterial nodules, reduced nodule size, and high concentrations of the virus within the nodules. Moreover, infection of fava bean plants with Bean mosaic virus and Pea enation mosaic virus caused a decrease in the rate of nodular formation, and infection of bean plants with various viruses led to varying reductions in nodule formation rates depending on the virus type (Lu et al., 2022).

Improving soil fertility is crucial for enhancing agricultural productivity to meet the rising food requirements of the expanding global population. The utilization of rhizobia, which forms a highly effective symbiotic relationship, presents a promising and sustainable approach to boost cowpea production in semi-arid regions, as cowpea heavily relies on biological nitrogen fixation (BNF) for its nutritional needs (Pule-Meulenberg et al., 2010). We hypothesize that the natural rhizobia found on farms in the semi-arid regions of Iraq had varying abilities to fix nitrogen and that inoculating cowpeas with these rhizobia would enhance the development and production of cowpeas. Hence, the primary aim of this study was to assess the significant consequences of potyvirus on the quantity and mass of bacterial nodules and to investigate the influence of Rhizobium inoculation on infected plants, as well as their contribution to plant resilience against viral infection.

MATERIALS AND METHODS

Virus

A strain of potyvirus was isolated from infected cowpea (*Vigna unguiculata* L.) plants in a cowpea field in the Abu Ghraib region (west of Baghdad), Iraq, following the protocol outlined by Al-Ani and Adhab (2013). The infection was confirmed using the ELISA technique with a PathoScreen potyvirus-specific antigen-coated plate (ACP) enzyme-linked immunosorbent assay (ELISA) kit (Agdia, USA). Subsequently, the virus was cultured on cowpea plants placed in cages covered with cheesecloth or muslin within a greenhouse. Plants exhibiting virus symptoms served as a source for viral inoculum. Additionally, potyvirus-free cowpeas were cultivated as a healthy control, as confirmed by ELISA.

Bacteria

Rhizobium was isolated from the roots of cowpea plants obtained from a cowpea field in the Abu Ghraib region (west of Baghdad). After a two-minute surface sterilization using 1% sodium hypochlorite (free chlorine), the nodules underwent several rinses with sterile distilled water. The nodules were crushed in Petri dishes, and a portion of the extract was transferred using a sterilized inoculating needle to the culture medium: yeast extract - mannitol agar (1 g K_2HPO_4, 1 g KH_2PO_4, 0.2 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.1 g $CaSO_4.2H_2O$, 0.08 g $FeCl_2.2H_2O$, 1 g KNO_3, 1 g yeast extract, 10 g mannitol, and 15 g agar per liter of distilled water) (Osa-Afiana and Alexander, 1979) in Petri dishes. The dishes were incubated upside down in an incubator at 28°C for 4 days. Well-isolated individual colonies were transferred to the liquid yeast extract - mannitol culture medium in 250 ml conical flasks and incubated at 28°C for 4 days.

The concentration of viable bacterial cells in the medium was determined using the plate count method. This involved preparing a series of dilutions (10^{-1} to 10^{-8}) and transferring 1 ml of each dilution to a sterilized Petri dish, followed by adding 20 ml of solid nutrient agar to each dish. The dishes were incubated upside down in an incubator at 28°C for 4 days, and the number of colonies in each dish was recorded. Three dishes were used for each dilution. The average number of viable bacterial cells per colony was calculated by multiplying the number of colonies by the reciprocal of the dilution factor. The average density of viable bacterial cells per cubic centimeter of the nodules was determined to be (2.193×10^7 cells/cm^3).

The efficiency of bacteria in nodule formation on cowpea plants was tested by sterilizing healthy and uniform-sized cowpea seeds superficially with 95% ethanol for one minute, followed by several rinses with sterile distilled water. The seeds were mixed well with the rhizobial inoculum loaded onto the carrier in a sterilized metal container and left in the open air for two hours to allow attachment of the inoculum to the seeds. The seeds were then planted in pots at a rate of 4 seeds per pot. After germination, 3 seedlings were left in each pot. For comparison, untreated seeds were planted using the same method.

Seed treatment with bacteria

The bacteria were grown in a liquid medium on river mud containing 0.5% K_2HPO_4, 1% mannitol, and 1% powdered Arabic gum added to help it stick together (500 ml of liquid culture to 300 g of soil). The carrier was sterilized before use by autoclaving at a pressure of 1.5 kg/cm^2 and a temperature of 121°C for 2 hours. Healthy and uniform-sized local cowpea seeds, in terms of shape and size, were washed with water and surface sterilized with sodium hypochlorite (1% free chlorine). The seeds were then rinsed multiple times with sterile distilled water. Subsequently, they were mixed with the preloaded and prepared vaccine in a sterilized metallic container. The mixture was left uncovered for 2 hours and then planted in pots containing mixed soil at a rate of 4 seeds per pot. The following treatments were applied:

1. Half of the seeds were inoculated with Rhizobium (RI), with half of them being infected with the virus (VI) and the other half kept non-infected (V0).
2. Half of the seeds were not inoculated with Rhizobium (R0), with half of them being infected with the virus (VI) and the other half non-infected (V0).

Three seedlings were left in each pot after germination. Each treatment was repeated 5 times. The pots were watered with a nitrogen-free nutrient solution composed of 1 gram of the powder mixture (31.7g KCl, 18g $Ca_3(PO_4)2$, 13.7g $CaSO_4.2H_2O$, 5.5g $MgSO_4.7H_2O$, 2.7g $Fe_2(SO_4)3$, 0.5g $CuSO_4.5H_2O$, 0.6g $MnSO_4.H_2O$, 0.5g H_3Bo_3, and 26.8g K_2HPO_4) dissolved in one liter of sterile distilled water (Burton et al., 1972) every week until the end of the experiment.

Plant infection with the virus was carried out by crushing leaves from virus-infected cowpea plants in a warm neutral phosphate buffer solution of a 0.01 molar concentration. The extract was filtered through 2 layers of cheesecloth or muslin, and the prepared cowpea plant leaves were wiped with the filtered extract at a two-week age.

Data collection

All treatments were arranged in a completely randomized design with three replications. Data were collected three months after cultivation. The number of root nodules formed per plant and their dry weight were determined. Furthermore, the pods formed on the plants were collected, and the pod/plant ratio and the seed/pod ratio were determined from the beginning of pod formation until the end of the experiment. Percentage values were arcsine transformed before analysis. The treatment means were compared using Fisher's least significant difference (LSD) test at P=0.05.

Estimation of nitrogen content

Samples of seeds and above-ground biomass were dried and ground using an electric grinder. A 0.2 g portion of each sample was placed in a 250 ml conical flask. To the flask, 1 g of selenium, 8 ml of sulfuric acid, and 3 ml of hydrogen peroxide (H_2O_2) were added. The mixture was placed in a sand bath at 100°C for a full day, followed by transfer to an incubator at 30°C for an hour and 15 minutes. The mixture was then cooled, transferred to a 250 ml volumetric flask, and made up to 100 ml. The sample was analyzed using an Auto-analyzer to determine the total nitrogen content (Wiltshire and Laubscher, 1988).

RESULTS AND DISCUSSION

Effect of Rhizobium inoculation and virus infection on the number and weight of bacterial nodules

The results presented in Figure 1 indicate that all plants inoculated with Rhizobium formed bacterial nodules, with the lowest number of nodules 100 days post-inoculation being 39 nodules per plant in virus-infected plants, and the highest being 86.6 nodules in non-infected plants. Only one to two small nodules were observed on non-inoculated plants (Figure 1). This explains the previously observed effect of Rhizobium inoculation on the wet and dry weight of the above-ground and root biomass. Aserse et al. (2020) mentioned that rhizobia improve the biomass of the roots of common beans and soybean plants.

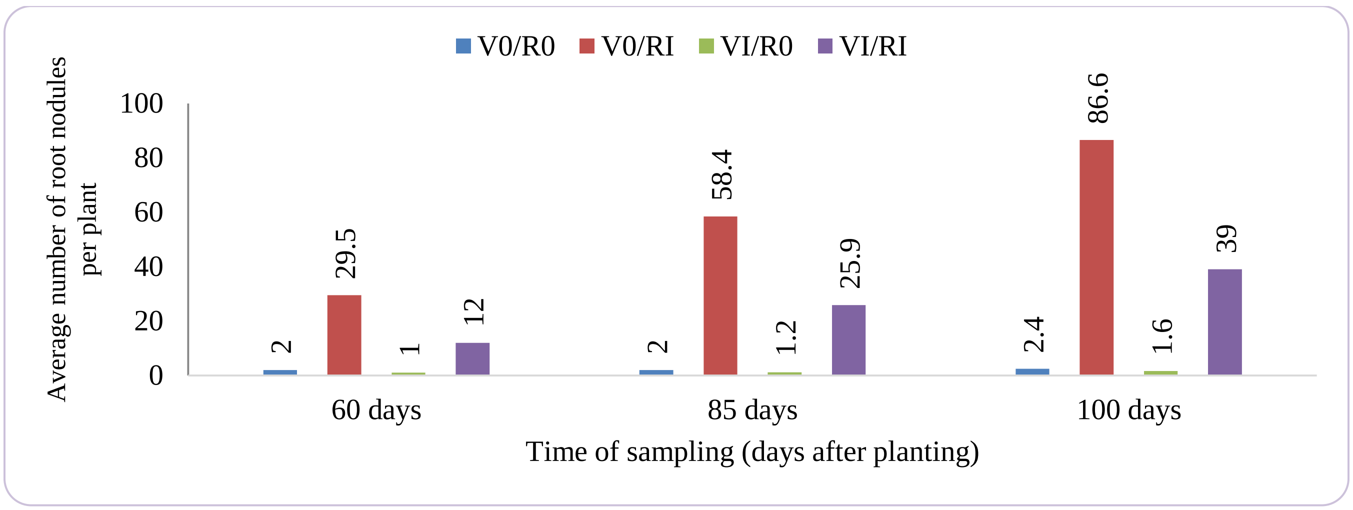

Figure 1. Effect of treating cowpea plants with rhizobium and potyvirus on the development of an average number of root nodules per plant across time.

It is noteworthy that virus infection led to a significant reduction in the number of root nodules, indicating that the virus primarily affects nodule formation. It has been reported that infection of soybean plants with Soybean mosaic virus (SMV) and Bean pod mosaic virus (BPMV), either individually, in combination, or with Tomato ringspot virus (TRSV), resulted in a significant decrease in the number of bacterial nodules formed on their roots (Andreola et al., 2019; Liu et al., 2021). Similarly, infection of bean plants with Bean yellow mosaic virus (BYMV) led to a similar decrease in the number of nodules (Al-Ani and Adhab, 2013). Other researchers have also indicated that viral infection affects the number of bacterial nodules on the roots of various legume crops (van't Hof et al., 2023).The impact of virus infection was not limited to the number of bacterial nodules but also extended to nodule weight. After 100 days of inoculation, the average weight of bacterial nodules was 3.08 g in Rhizobium-inoculated plants without virus presence, compared to 1.00 g in virus-infected plants inoculated with Rhizobium (Figure 2).

This may be attributed to the fact that virus infection resulted in physiological changes in the nodules, such as a decrease in the number of bacteroid-filled symbiosomes within the nodule, which hindered the transfer of materials from the cytoplasm to the bacteria (Rhizobium), ultimately leading to weakened nodule formation (Basu et al., 2021).

Virus infection may lead to a narrowing of this space, resulting in a reduction in the amount of leghemoglobin. Leghemoglobin plays a crucial role in nitrogen fixation by regulating the oxygen requirements of the nitrogenase enzyme under anaerobic conditions. The virus may also hinder the production of this enzyme, thereby inhibiting the process of atmospheric nitrogen fixation (Andreola et al., 2019).

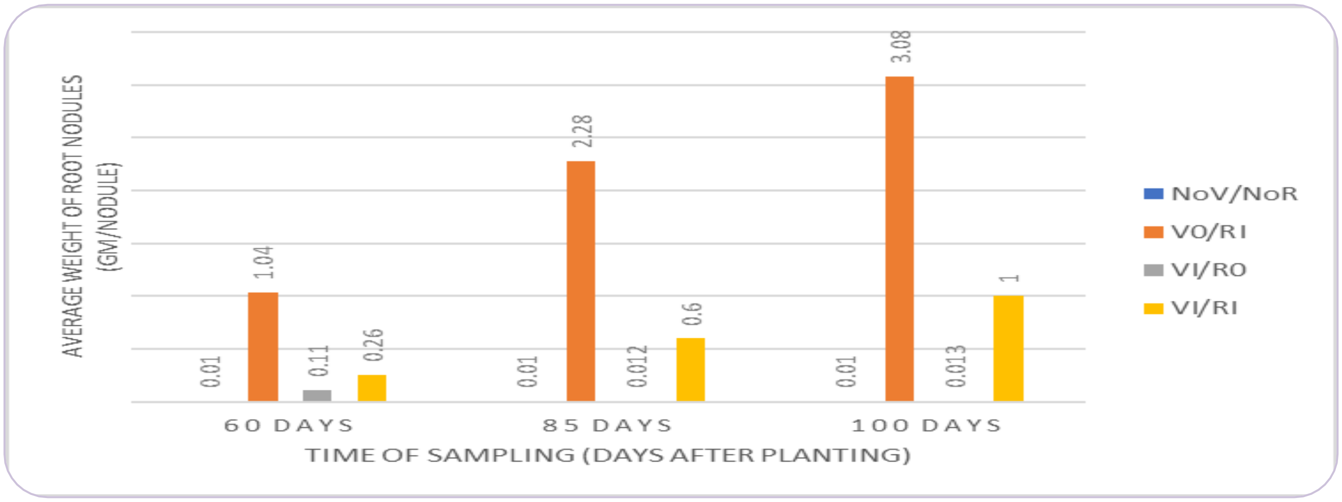

Figure 2. Effect of treating cowpea plants with rhizobium and potyvirus on the average weight of root nodules (gram per nodule) across time.

Moreno-Delafuente et al. (2020) proposed that viral infection disrupts the carbon-to-nitrogen ratio (C:N). It is known that viral infection reduces photosynthesis and increases respiration, leading to a decrease in carbohydrate production by plants. Consequently, the C:N ratio becomes abnormal, which inhibits the process of atmospheric nitrogen fixation. The virus's impact may extend to protein synthesis in the infected nodules, resulting in the inhibition and impairment of the nitrogenase enzyme's role in nitrogen fixation.

The effect of Rhizobium inoculation and virus infection on nitrogen content in the above-ground biomass was examined. Rhizobium inoculation (Figure 3) significantly increased the average nitrogen content ratio, reaching 1.78 in non-inoculated and non-virus-infected plants after 100 days of inoculation, and increasing to 2.91 with Rhizobium inoculation. Virus infection led to a significant increase in the nitrogen content ratio in the green parts at a 0.05 significance level, reaching 2.12 compared to 1.78 in non-virus-infected plants. These results indicate the importance of Rhizobium inoculation in increasing the nitrogen content ratio. Moreover, virus infection did not have a negative effect on the nitrogen content ratio; on the contrary, it significantly increased this ratio.

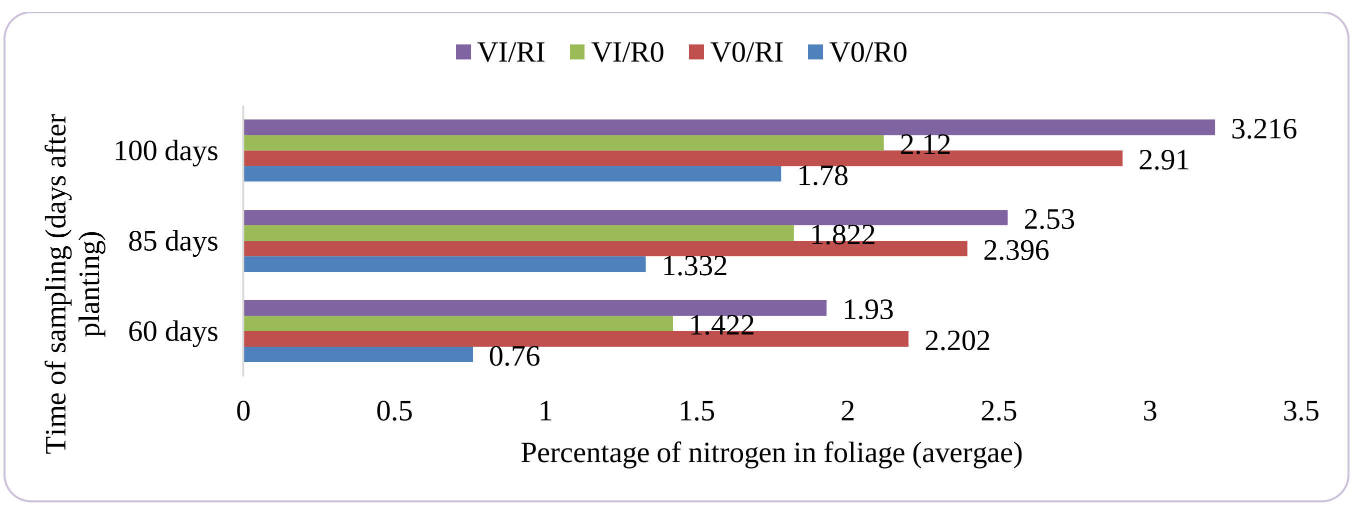

Figure 3. Effect of treating cowpea plants with rhizobium and potyvirus on the average nitrogen content in the aboveground biomass (%) across time.

Rhizobium inoculation in virus-infected plants led to an increase in nitrogen content, resulting in a statistically insignificant difference between Rhizobium-inoculated, non-inoculated, and virus-infected plants (2.91 and 3.216, respectively) after 100 days of inoculation. These findings align with previous research indicating that viral infection increases nitrogen content (Andreola et al., 2019). This increase may be attributed to the virus utilizing nitrogen for protein and nucleic acid synthesis, consequently leading to elevated nitrogen content in infected plants (Trebicki, 2020).

The effect of Rhizobium inoculation and virus infection on yield components was examined. The results of this experiment (Figures 4, 5, 6, 7) demonstrated that Rhizobium inoculation resulted in increased pod/plant ratio, seed/plant ratio, dry seed weight (g/plant), and nitrogen content ratio in seeds. These values reached 25.2, 144, 32.4, and 4.7, respectively, compared to 12.8, 70.6, 18.7, and 3.8 in non-inoculated plants. The virus significantly reduced these parameters, with values of 5.8, 30.0, 7.9, and 2.7, respectively, as shown in Figures 4, 5, 6, 7. Rhizobium inoculation in the presence of the virus increased these parameters, although the increase was not statistically significant at the 0.01 level, except for the nitrogen content ratio in seeds, where the increase was significant. The parameters are valued at 16.4, 84.6, 20.4, and 4.3, respectively. These results confirm the significance of Rhizobium inoculation in enhancing plant growth and may increase resistance to the virus or at least reduce the negative effect of the virus infection (Trebicki, 2020).

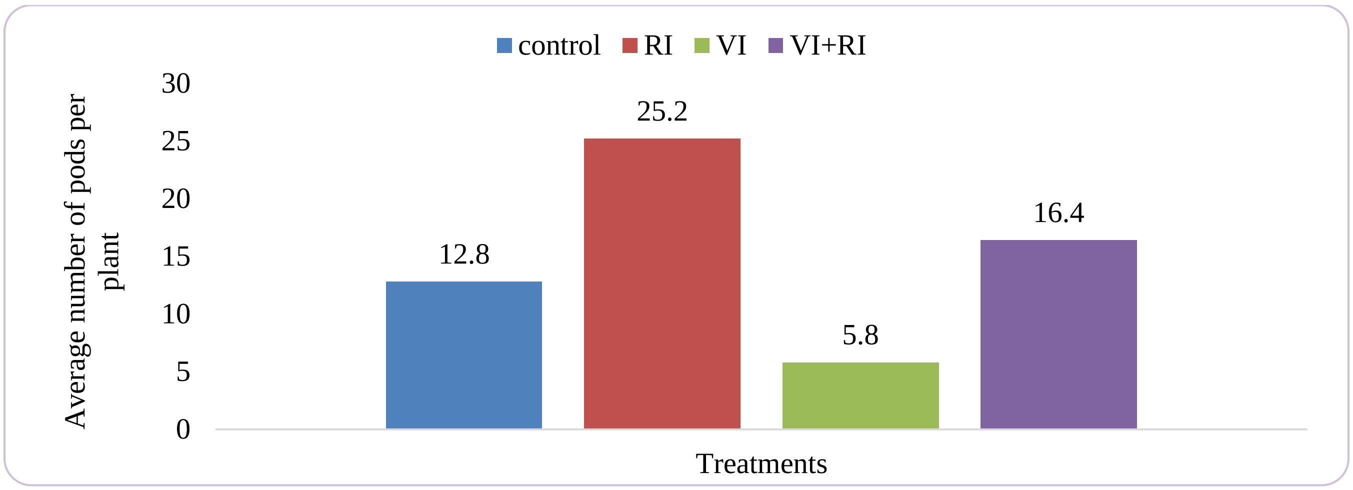

Figure 4. Effect of treating cowpea plants with rhizobium and potyvirus on the average number of pods per plant across time. Data was collected three months after planting.

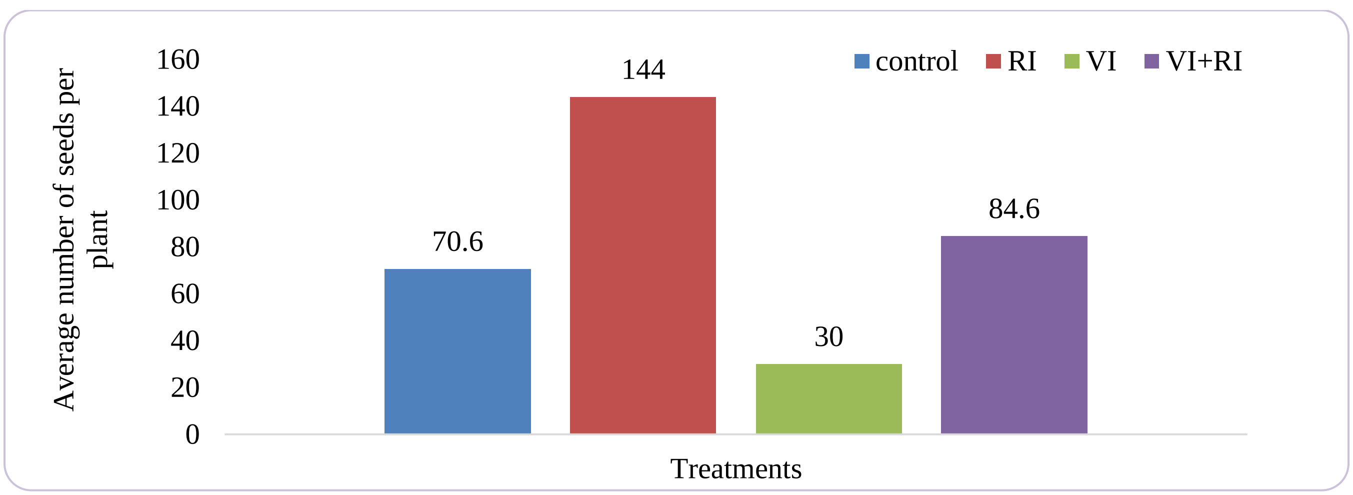

Figure 5. Effect of treating cowpea plants with rhizobium and potyvirus on the average number of seeds per plant. Data was collected three months after planting.

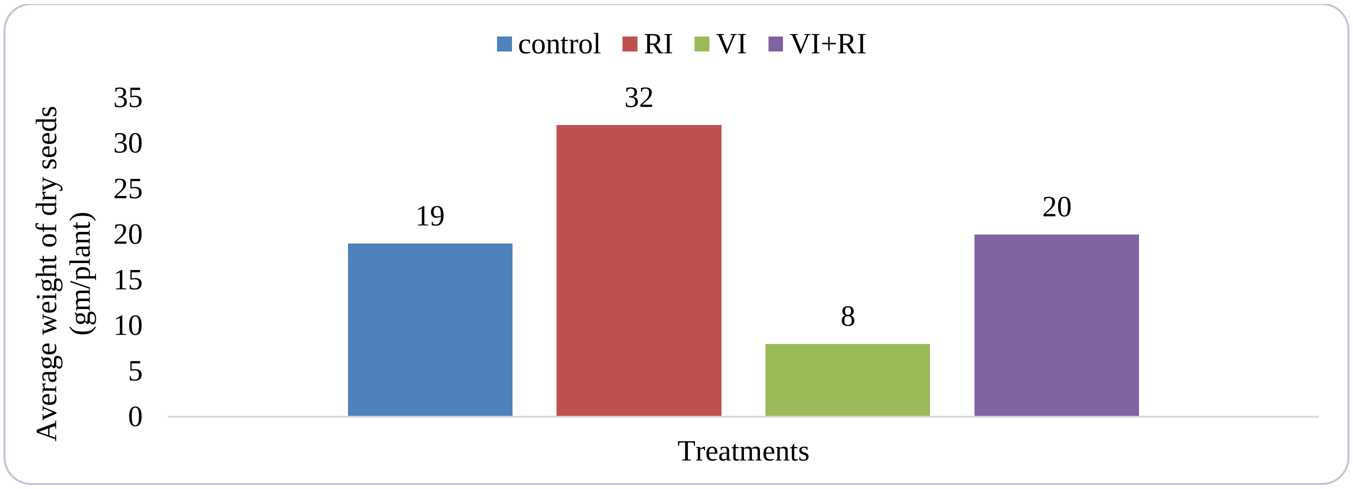

Figure 6. Effect of treating cowpea plants with rhizobium and potyvirus on the average weight of dry seeds (gram per plant). Data was collected three months after planting.

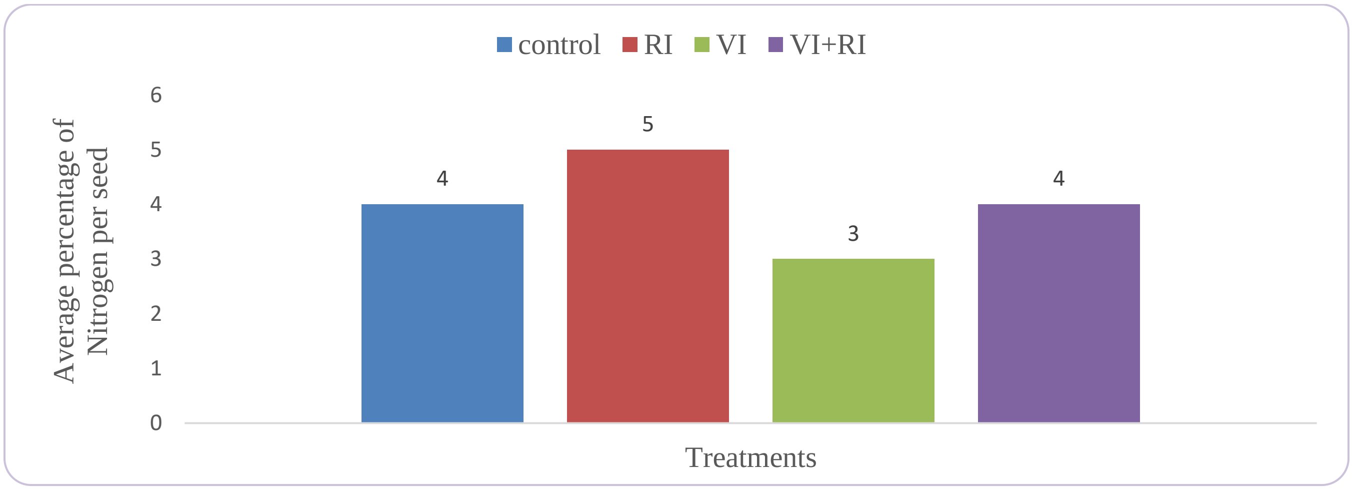

Figure 7. Effect of treating cowpea plants with rhizobium and potyvirus on the average percentage of per seed (%).Data was collected three months after planting.

CONCLUSIONS

The results obtained in this study indicate that viral infection reduces nitrogen content in seeds, possibly because the virus does not attack the seeds directly, leading to a decrease in their nitrogen content. Rhizobium inoculation has been proven to improve plant productivity and mitigate the harmful effects of the virus across all studied parameters. Rhizobium may play a role in stimulating innate resistance in plants by inducing protein synthesis that interferes with viral replication and prevents its formation. Generally, inoculation with rhizobia leads to improved nodulation rates and increases the nitrogen-fixing process in cowpeas under the coincidence of potyvirus and rhizobium.

Our work has demonstrated the presence of efficient indigenous rhizobia isolates with significant potential to enhance the growth and productivity of cowpea crops in the face of climate change. Additional research should prioritize thorough field tests on the local isolates to determine the most promising candidates for the creation of affordable and efficient biofertilizers. These biofertilizers will enhance the sustainable cultivation of cowpeas in small-scale agricultural systems.

AUTHOR'S CONTRIBUTIONS

The author designed, formulated and laid out the study; the author also conducted the experiments, collected, analyzed the data, wrote and proofread the the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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