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SOIL AMENDMENT WITH DRY LEAF BIOMASS OF RED CLOVER (*TRIFOLIUM PRATENSE L.*) ALTERS PHYSIOCHEMICAL AND GROWTH PARAMETERS OF CHICKPEA PLANTS GROWN UNDER COLLAR ROT DISEASE INFLUENCE

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ABSTRACT

The present study investigated the effects of soil amendment with dry leaf biomass of red clover (*Trifolium pratense* L.) on physiochemical and growth parameters of Chickpea plants (*Cicer arietinum)* under the influence of collar rot disease. Three different concentrations of dry leaf biomass (1%, 2%, and 3%) were utilized, and the results were compared to the effects of the standard drug chloramphenicol. Various parameters such as plant height, shoot and root dry weight, total phenolics, flavonoids, chlorophyll contents, and ascorbic acid were measured, alongside disease incidence and mortality rates. The findings of this study revealed significant improvements in the growth and physiological characteristics of Chickpea plants upon soil amendment with red clover dry leaf biomass. Plant height, shoot and root dry weight, total phenolics, flavonoids, chlorophyll contents, and ascorbic acid levels exhibited considerable increases compared to the control group. Moreover, the incidence and mortality rates of collar rot disease were significantly reduced in the Chickpea plants treated with red clover biomass. The results also demonstrated a dose-dependent response, with higher concentrations (2% and 3%) of dry leaf biomass exhibiting more pronounced effects on the measured parameters. However, even the lowest concentration (1%) of red clover biomass still showed significant improvements over the control group. In comparison to the standard drug chloramphenicol, the effects of red clover dry leaf biomass were found to be comparable or even superior in some cases. This suggests the potential of red clover biomass as a natural and sustainable alternative for managing collar rot disease and promoting the growth of Chickpea plants. These findings contribute to the development of eco-friendly and effective strategies for plant disease management and agricultural sustainability.

Keywords: Soil amendment, dry leaf biomass, red clover, *Trifolium pratense L.,* physiochemical parameters, growth parameters, Chickpea plants.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a top legume crop that provides protein, dietary fibre, minerals and vitamins to millions of people around the world. It is also a valuable source of income for smallholder farmers in developing countries (Jukanti *et al*., 2012). However, chickpea production is constrained by various biotic and abiotic stresses, among which soil-borne diseases are a major threat. Collar rot, caused by the fungus *Sclerotium rolfsii*, is one of the most destructive soil-borne diseases of chickpea that can cause up to 95% seedling mortality and 10–30% yield loss. The fungus has a wide host range and can infect more than 500 plant species belonging to different families. It produces sclerotia that can survive in the soil for several years and germinate under favourable conditions to infect the host plants. The disease symptoms include wilting, yellowing and necrosis of leaves, dark brown lesions at the stem base, white mycelial growth and sclerotia formation on the infected tissues (Hussain *et al*., 2006).

The management of collar rot disease in chickpea is challenging due to the persistence and ubiquity of the pathogen in the soil. Chemical fungicides are often used to control the disease, but they have several drawbacks such as high cost, environmental pollution, health hazards and development of resistance by the pathogen (Tewari *et al*., 2003). Therefore, there is a need to explore alternative and eco-friendly strategies to manage this disease and improve chickpea productivity. One such strategy is the use of dry leaf biomass of red clover (*Trifolium pratense* L.) as a soil amendment. Red clover is a forage legume that has been widely used as a cover crop for its high nitrogen fixation rate and soil fertility-building properties. It can enhance soil organic matter, water holding capacity, nutrient availability and microbial activity. It has also been reported to have allelopathic effects on some weeds and pathogens by releasing phenolic compounds and other secondary metabolites into the soil (Boller *et al*., 2010).

The objective of this study was to evaluate the effect of dry leaf biomass of red clover on the incidence and severity of collar rot disease in chickpea and the possible mechanisms involved in disease suppression. The hypothesis was that dry leaf biomass of red clover can reduce the inoculum potential of *S. rolfsii* in the soil and induce resistance in chickpea plants against the pathogen. The specific objectives were: (i) to determine the optimal dose of dry leaf biomass of red clover for soil amendment; (ii) to measure the effect of dry leaf biomass of red clover on collar rot disease incidence and severity in chickpea; (iv) to analyse the effect of dry leaf biomass of red clover on growth parameters of chickpea; and (v) to evaluate the effect of dry leaf biomass of red clover on some important metabolites and phytochemical.

MATERIALS AND METHODS

Pot trial

The pot trial was conducted in a greenhouse at the Department of Botany, Mirpur University of Science and Technology, Mirpur, Pakistan. The soil used was sandy loam with a pH of 7.2 and an electrical conductivity of 0.8 dS/m. The soil was sterilized by autoclaving at 121°C for 15 min. The pots (20 cm diameter) were filled with 2 kg of sterilized soil and watered to field capacity.

The seeds of chickpea were surface-sterilized with 0.1% mercuric chloride for 2 min and rinsed thoroughly with sterile distilled water. The seeds were sown at a rate of two seeds per pot and thinned to one plant per pot after germination. The plants were watered regularly and maintained under natural light and temperature conditions.

The experiment was laid out in a completely randomized design with three replications. The treatments consisted of three levels of dry leaf biomass of red clover as soil amendment (1%, 2%, and 3% w/w) and one level of chloramphenicol (50 mg/kg soil) as a treatment control. A negative control without any amendment was also included and a positive control without any treatment was followed by growing the plants in pathogen inoculated soil. The dry leaf biomass of red clover was obtained from a local farm and ground to a fine powder. The amendments were mixed thoroughly with the soil before sowing the seeds.

Inoculation with collar rot pathogen

The collar rot pathogen, *Sclerotium rolfsii*., was isolated from naturally infected chickpea plants and cultured on potato dextrose agar (PDA) plates at 28°C for 7 days. The sclerotia were harvested by scraping the surface of the plates and stored in sterile glass bottles at 4°C until use (Khan *et al*., 2020).

The inoculation was done at the time of sowing by placing five sclerotia around each seed at a depth of 2 cm. The inoculum density was approximately 2×10^4 sclerotia/kg soil.

Measured parameters

The following parameters were measured at 30 days after sowing:

Plant height

The height of each plant was measured from the base to the tip of the main stem using a ruler.

Root and shoot dry weight

The plants underwent a meticulous process of uprooting and subsequent cleansing with tap water to eliminate any soil particles. Following this, the roots and shoots were separated and subjected to a drying period of 48 hours at a temperature of 70°C within an oven. The electronic balance was utilized to accurately measure and document the dry weight of each sample.

Total chlorophyll

The estimation of the total chlorophyll content followed the method established by Arnon (1949). A fresh leaf sample weighing 0.5 grams was homogenized in 10 milliliters of 80% acetone and subsequently centrifuged at 3000 revolutions per minute (rpm) for a duration of 10 minutes. The resulting supernatant was then subjected to absorbance measurements at wavelengths of 645 nm and 663 nm using a spectrophotometer. The total chlorophyll content was determined using the following formula.

Total chlorophyll (mg/g) = $(20.2 \times A645 + 8.02 \times A663) / W$

Total phenolics and flavonoids

The determination of the total phenolic and flavonoid contents followed the method described by Singleton *et al*. (1999). A fresh leaf sample weighing 0.5 grams was extracted using 10 milliliters of methanol at room temperature for a period of 24 hours. The resulting extract was then filtered through Whatman No.1 filter paper and subsequently diluted to a final volume of 25 milliliters using methanol.

To determine the phenolic content, 0.5 milliliters of the extract was mixed with 2.5 milliliters of Folin-Ciocalteu reagent, which had been diluted ten times with distilled water. The mixture was incubated for 5 minutes at room temperature. Following this, 2 milliliters of sodium carbonate solution (75 grams per liter) were added and the mixture was further incubated for 90 minutes at room temperature. The absorbance of the resulting solution was measured at 760 nanometers using a spectrophotometer. A standard curve was constructed using gallic acid as the reference compound, and the total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight.

For the determination of the flavonoid content, 0.5 milliliters of the extract were mixed with 1.5 milliliters of distilled water and 0.15 milliliters of sodium nitrite solution (5%). After a 6-minute incubation period, 0.15 milliliters of aluminum chloride solution (10%) were added, followed by another 6-minute incubation. Subsequently, 1 milliliter of sodium hydroxide solution (1 M) was added, and the mixture was diluted to a final volume of 5 milliliters using distilled water. The absorbance of the resulting solution was measured at 510 nanometers using a spectrophotometer. A standard curve was prepared using quercetin as the reference compound, and the total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of dry weight.

Ascorbic acid

The measurement of ascorbic acid content followed the method outlined by Omaye *et al*. (1979). A fresh leaf sample weighing 0.5 grams was extracted using a solution of 3% metaphosphoric acid-acetic acid (in a 4:1 volume-to-volume ratio) for a duration of 30 minutes while kept on ice. The resulting extract was then filtered through Whatman No.1 filter paper and diluted to a final volume of 10 milliliters using the same solution.

To determine the ascorbic acid content, 0.5 milliliters of the extract was mixed with 0.5 milliliters of dinitrophenylhydrazine reagent (2% in sulfuric acid) and incubated in darkness at room temperature for 3 hours. Subsequently, the mixture was neutralized by adding 2 milliliters of thiourea solution (10% in sulfuric acid) and diluted to a final volume of 10 milliliters using distilled water. The absorbance of the resulting solution was measured at 520 nanometers using a spectrophotometer. A standard curve was generated using ascorbic acid as the reference compound to quantify the ascorbic acid content.

Statistical analysis

The mean values were analysed statistically and interpreted to describe the results.

RESULTS AND DISCUSSION

Effect on disease parameters

The efficacy of 3% dry leaf biomass of red clover (*Trifolium pratense*) as a soil amendment for the control of collar rot disease caused by Sclerotium rolfsii in chickpea (*Cicer arietinum*) was evaluated in pot experiments. The disease incidence and mortality of chickpea seedlings were recorded after 30 days of sowing. The results were compared with the standard drug chloramphenicol, which was used as a positive control. The negative control was infected soil without amendment. The results showed that 3% dry leaf biomass of red clover significantly reduced the disease incidence and mortality compared to the negative control. The disease incidence and mortality were similar to those of the chloramphenicol-treated soil as shown in figure 1.

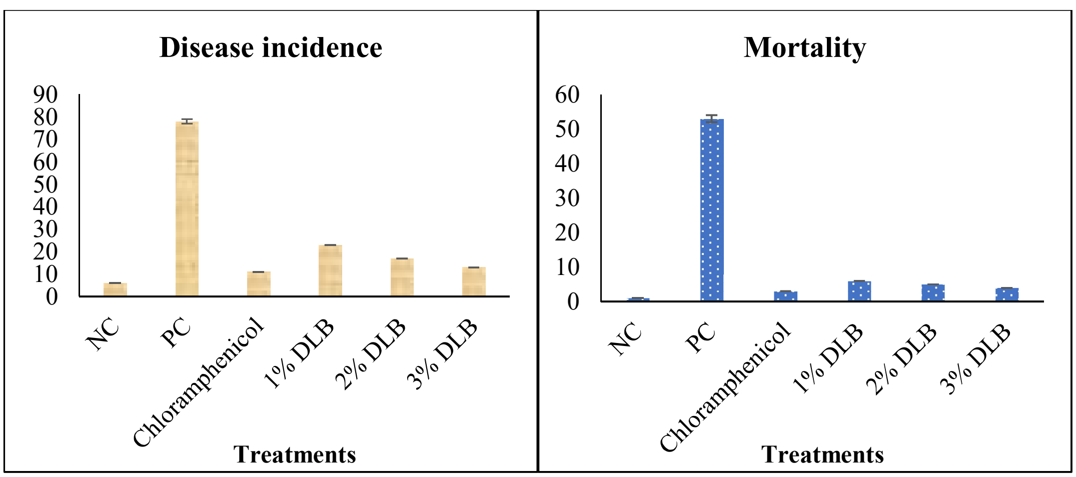

Figure 1. Disease incidence and mortality percentage of chickpea under collar rot disease influence

Red clover is a leguminous plant that has been reported to have various bioactive compounds, phenolic acids, coumarins, and terpenoids that can inhibit the growth of pathogens (Khan *et al*., 2019). Red clover also has other benefits as a cover crop, such as improving soil fertility, structure, and water retention, suppressing weeds, and providing forage for livestock. The mechanism of action of red clover leaf biomass against Sclerotium rolfsii could be due to the direct antifungal effect of its bioactive compounds or the indirect effect of improving soil health and plant resistance (Saviranta *et al*., 2008).

Effect on biomass accumulation

The results of this study showed that soil amendment with dry leaf biomass of red clover at different concentrations (1%, 2% and 3%) significantly increased the plant height of chickpea under collar rot stress caused by S. Rolfsii (Figure 2). The highest increase in plant height was observed at 3% concentration of dry leaf biomass of red clover, which was comparable to the antibiotic chloramphenicol treatment. These results suggest that dry leaf biomass of red clover can improve the growth and vigour of chickpea plants by reducing the negative impact of the pathogen on the root system and enhancing the nutrient and water uptake by the plants.

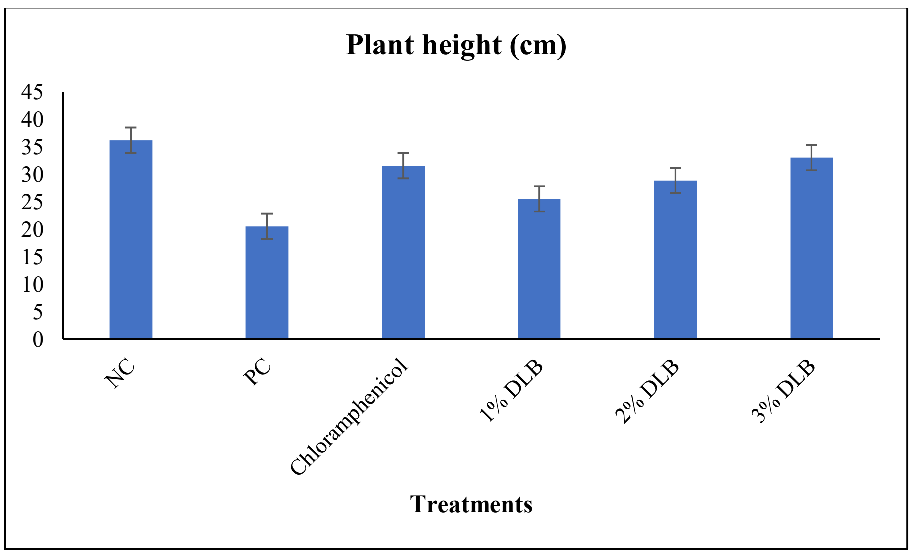

Figure 2. Impact of dry leaf biomass (DLB) of red clover on the height of chickpea plants grown under collar rot stress.

The mechanism by which dry leaf biomass of red clover affects the plant height of chickpea under collar rot stress could be explained by its allelopathic and biofertilizer effects. Previous study has reported that red clover releases phenolic compounds and other secondary metabolites into the soil, which can inhibit the growth and germination of some weeds and pathogens (Cesco *et al*., 2012). It is possible that these compounds also suppress the growth and sclerotia formation of S. rolfsii in the soil, thereby reducing its inoculum potential and disease incidence. Moreover, red clover is a legume that can fix atmospheric nitrogen and increase the soil organic matter, nitrogen availability and microbial activity. These effects can improve the soil fertility and health.

The beneficial effects of soil amendment with different plant materials on plant height and other growth parameters of various crops under biotic or abiotic stress conditions. For instance, Ali *et al*. (2020) found that soil amendment with dry biomass of Chenopodium album, a weed species, increased the plant height, shoot and root biomass, and grain yield of chickpea under collar rot stress. Similarly, Sharf *et al*. (2021) reported that soil amendment with dry biomass of Anagallis arvensis, another weed species, enhanced the plant height, shoot and root biomass, chlorophyll and carotenoid contents, and catalase, peroxidase and polyphenol peroxidase activities of chili under southern blight stress. Furthermore, Jing *et al*. (2021) showed that plant height and biomass density of red clover populations were influenced by their leaf economic strategy, wood density and height at maturation traits.

The soil amendment with dry leaf biomass of red clover at different concentrations (1%, 2% and 3%) significantly increased the root and shoot dry weights of chickpea under collar rot stress caused by *S. rolfsii* (Figure 3-4). The highest increase in root and shoot dry weights was observed at 3% concentration of dry leaf biomass of red clover, which was comparable to the antibiotic chloramphenicol treatment. These results indicate that dry leaf biomass of red clover can enhance the biomass production and yield potential of chickpea plants by stimulating their photosynthesis and carbon allocation. The mechanism by which dry leaf biomass of red clover affects the root and shoot dry weights of chickpea under collar rot stress could be related to its biofertilizer and bio stimulant effects. Previous studies have reported that red clover can increase the soil nitrogen availability and microbial activity by fixing atmospheric nitrogen and decomposing its organic matter. This can improve the soil nutrient status and provide a favourable environment for the survival of chickpea plants (Tucak *et al*., 2021).

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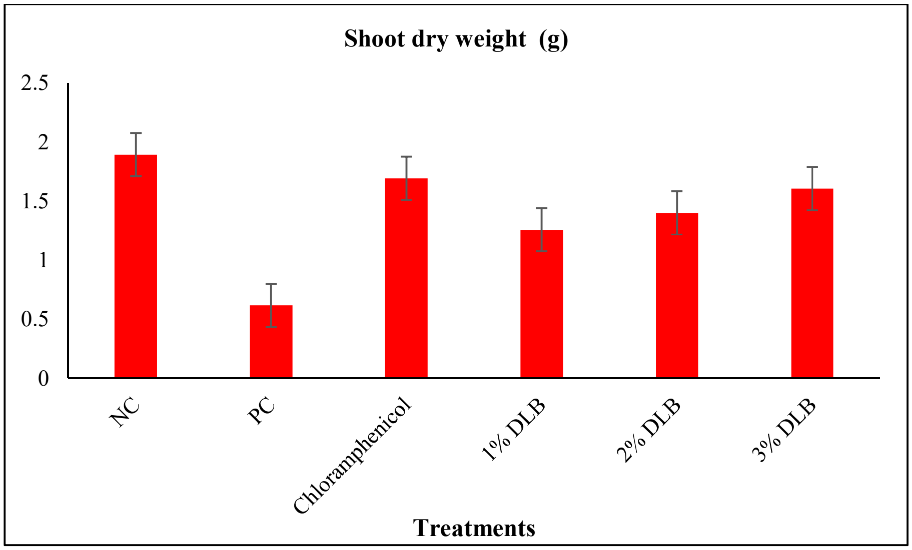

Figure 3. impact of dry leaf biomass (DLB) of red clover on the shoot dry weight of chickpea plants grown under collar rot disease stress.

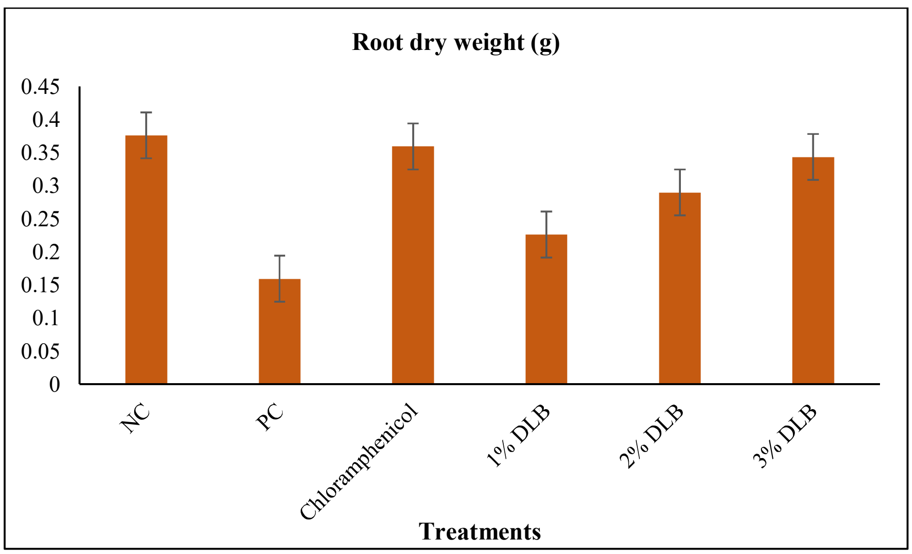

Figure 4. Impact of dry leaf biomass (DLB) of red clover on the root dry weight of chickpea plants grown under collar rot disease stress.

Moreover, red clover can also act as a bio stimulant by releasing plant growth regulators such as auxins, cytokinin and gibberellins into the soil, which can modulate the plant hormonal balance and enhance their physiological processes. The beneficial effects of soil amendment with different plant materials on root and shoot dry weights and other biomass parameters of various crops under biotic or abiotic stress conditions. For example, Ali *et al*.

(2020) found that soil amendment with dry biomass of *Chenopodium album*, a weed species, increased the root and shoot dry weights, root length density and root volume of chickpea under collar rot stress. Similarly, Sharf *et al*. (2021) reported that soil amendment with dry biomass of Anagallis arvensis, another weed species, enhanced the root and shoot dry weights, root length density and root volume of chili under southern blight stress. Furthermore, Jing *et al*. (2021) showed that root and shoot dry weights of red clover populations were influenced by their leaf economic strategy, wood density and height at maturation traits.

Effect on total phenolics and flavonoids production

The total phenolics and flavonoids content in chickpea leaves was determined. The results showed that 3% dry leaf biomass of red clover significantly increased the production of total phenolics and flavonoids in chickpea leaves compared to the positive control. The total phenolics and flavonoids content in chickpea leaves treated with 3% dry leaf biomass of red clover was similar to that of the chloramphenicol-treated leaves. The results suggest that 3% dry leaf biomass of red clover not only reduced the disease incidence and mortality caused by *Sclerotium rolfsii*, but also enhanced the accumulation of phenolic compounds and flavonoids in chickpea leaves (Figure 5-6).

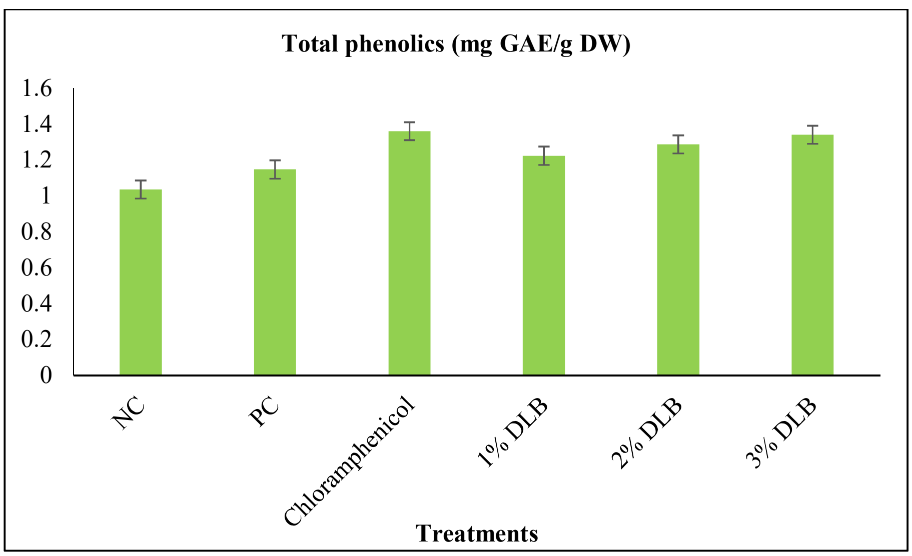

Figure 5. Impact of dry leaf biomass (DLB) of red clover on the total phenolics contents of chickpea plants grown under biotic stress.

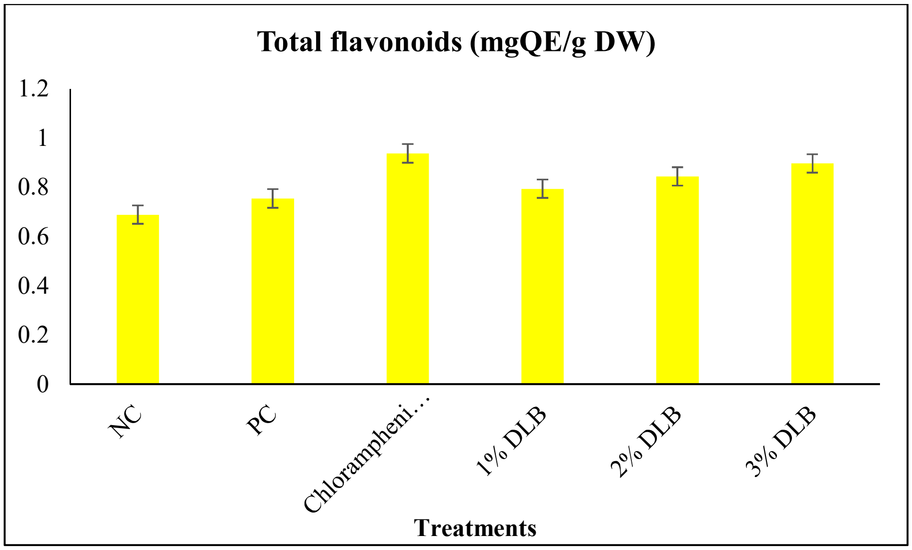

Figure 6. Impact of dry leaf biomass (DLB) of red clover on the total phenolics contents of chickpea plants grown under biotic stress.

Phenolic compounds and flavonoids are important secondary metabolites in plants that play important role in modulating enzyme activities. Phenolic compounds and flavonoids can also act as phytoalexins, crucial to negotiate biotic or abiotic stress. Red clover is a leguminous plant that has high concentrations of phenolic compounds and flavonoids, especially isoflavones, such as biochanin A and formononetin. Isoflavones have been reported to have antifungal activity against various plant pathogens, including *Sclerotium rolfsii*. Therefore, it is possible that the application of 3% dry leaf biomass of red clover to the soil increased the availability of phenolic compounds and flavonoids to the chickpea plants, which in turn enhanced their resistance to the fungal infection and increased their antioxidant capacity (Srikhumsuk *et al*., 2023).

Effect on total chlorophyll contents and ascorbic acid values

The soil amendment with dry leaf biomass of red clover at different concentrations (1%, 2% and 3%) significantly increased the total chlorophyll (Figure 7) and ascorbic acid contents (Figure 8) of chickpea leaves under collar rot stress caused by S. rolfsii. The highest increase in total chlorophyll and ascorbic acid contents was observed at 3% concentration of dry leaf biomass of red clover, which was comparable to the antibiotic chloramphenicol treatment.

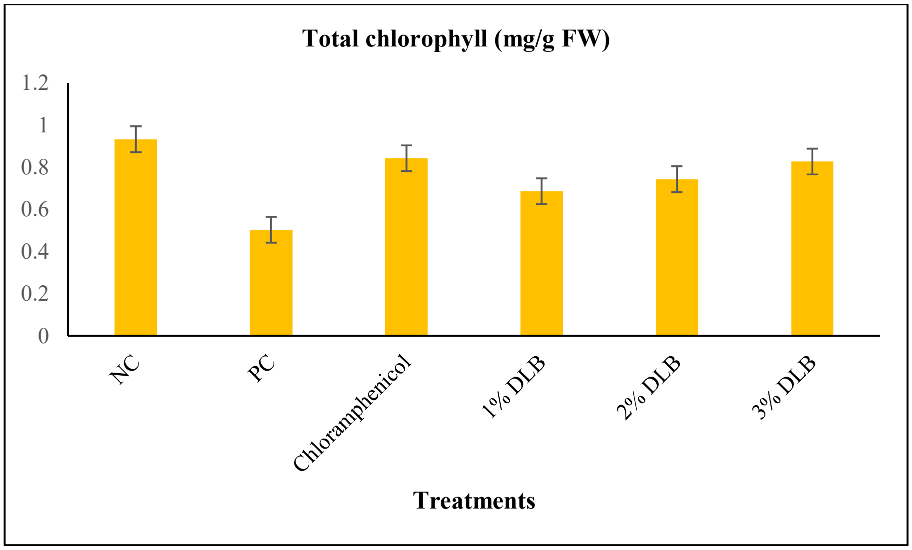

Figure 7. Impact of dry leaf biomass (DLB) of red clover on the total chlorophyll contents of chickpea plants grown under biotic stress.

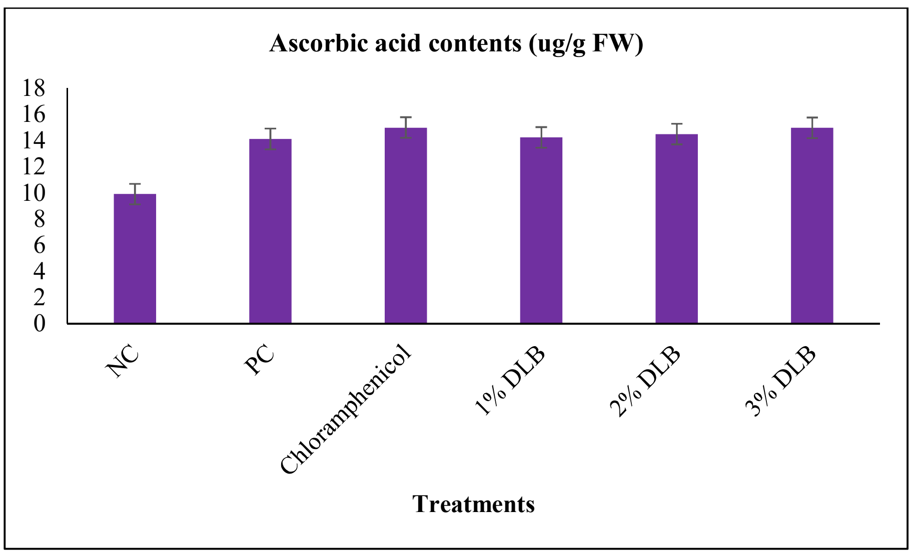

Figure 8. Impact of dry leaf biomass (DLB) of red clover on the ascorbic acid contents of chickpea plants grown under biotic stress.

These results indicate that dry leaf biomass of red clover can improve the photosynthetic efficiency and antioxidant capacity of chickpea plants by increasing their pigment and vitamin synthesis. The mechanism by which dry leaf biomass of red clover affects the total chlorophyll and ascorbic acid contents of chickpea leaves under collar rot stress could be related to its bio stimulant and allelopathic effects. Previous studies have reported that red clover can release plant growth regulators such as auxins, cytokinin and gibberellins into the soil, which can modulate the plant hormonal balance and enhance their physiological processes. These hormones can stimulate the biosynthesis of chlorophyll and ascorbic acid in plant leaves, which are essential for photosynthesis and antioxidant defence. Moreover, red clover can also release phenolic compounds and other secondary metabolites into the soil, which can inhibit the growth and germination of some weeds and pathogens. It is possible that these compounds also suppress the growth and sclerotia formation of S. rolfsii in the soil, thereby reducing its inoculum potential and disease incidence. This can alleviate the biotic stress of the pathogen on the plant leaves, which can otherwise cause chlorosis, necrosis and oxidative damage. Spearman corelation matrix has been presented in table 1 showing positive and significant correlation among the variables.

Table 1. Spearman correlation matrix for the variables under study

Variables	SDW	RDW	PH	TC	TP	TF	ASA
SDW		0.9411	0.9224	0.9664	0.1395	0.1146	0.0962
RDW	0.9411		0.9462	0.9395	0.1774	0.2087	0.1802
PH	0.9224	0.9462		0.9581	0.0978	0.1303	0.1358
TC	0.9664	0.9395	0.9581		0.1432	0.1177	0.0921
TP	0.1395	0.1774	0.0978	0.1432		0.9659	0.9347
TF	0.1146	0.2087	0.1303	0.1177	0.9659		0.9684
ASA	0.0962	0.1802	0.1358	0.0921	0.9347	0.9684	

Values in bold are significantly different from zero at alpha 0.05.

The beneficial effects of soil amendment with different plant materials on total chlorophyll and ascorbic acid contents and other photosynthetic and antioxidant parameters of various crops under biotic or abiotic stress conditions has been reported on several instances. For example, Ali *et al*. (2020) found that soil amendment with dry biomass of *Chenopodium album*, a weed species, increased the total chlorophyll and ascorbic acid contents, chlorophyll fluorescence parameters and antioxidant enzyme activities of chickpea leaves under collar rot stress. Similarly, Sharf *et al*. (2021) reported that soil amendment with dry biomass of Anagallis arvensis, another weed species, enhanced the total chlorophyll and ascorbic acid contents, chlorophyll fluorescence parameters and antioxidant enzyme activities of chili leaves under southern blight stress. Furthermore, Wang *et al*. (2023) showed that total chlorophyll content in natural forests was influenced by plant functional groups, latitude and elevation.

Utilizing dry leaf biomass as a strategy for crop improvement offers several advantages. One of the key benefits is nutrient recycling. Dry leaf biomass contains vital nutrients, including nitrogen, phosphorus, potassium, and micronutrients. By incorporating the biomass back into the soil through practices like mulching or composting, these nutrients are returned to the soil, replenishing its fertility and ensuring a sustainable source of nutrition for future crops.

In addition to nutrient recycling, the inclusion of dry leaf biomass adds organic matter to the soil. Organic matter plays a crucial role in enhancing soil structure, moisture retention, and nutrient-holding capacity. By increasing the organic content of the soil, the biomass improves its overall fertility and creates a favourable environment for microbial activity. This, in turn, supports nutrient cycling and promotes the growth of beneficial microorganisms, leading to improved soil health and subsequent crop productivity.

Another important aspect of utilizing dry leaf biomass is its contribution to carbon sequestration. By diverting biomass from burning or decomposition, the carbon present in dry leaves can be stored in the soil. This helps mitigate the release of carbon dioxide into the atmosphere and reduces the impact of greenhouse gas emissions, thus contributing to climate change mitigation efforts.

Furthermore, the utilization of dry leaf biomass can serve as a sustainable source of energy. Through processes like biomass combustion or biogas production, the energy content of dry leaves can be harnessed, reducing reliance on non-renewable fossil fuels. This promotes a more sustainable and environmentally friendly approach to energy generation.

Overall, incorporating dry leaf biomass into agricultural practices presents a multifaceted strategy for crop improvement. It provides nutrient recycling, enhances soil fertility and structure, contributes to carbon sequestration, and offers a renewable energy source. By adopting these practices, farmers can improve soil health, increase crop yields, and contribute to sustainable agricultural systems.

CONCLUSION

In conclusion, this study demonstrated that soil amendment with dry leaf biomass of red clover can increase the total chlorophyll and ascorbic acid contents of chickpea leaves under collar rot stress by stimulating their pigment and vitamin synthesis and by alleviating the biotic stress of *S. rolfsii.* This strategy can be a cost-effective and eco-friendly alternative to chemical pesticides for the improvement of photosynthetic efficiency and antioxidant capacity of chickpea plants. However, further studies are needed to optimize the dose and timing of dry leaf biomass application, to evaluate its effect on other photosynthetic and antioxidant parameters such as chlorophyll fluorescence, carotenoids and phenolics, to identify the plant growth regulators and phenolic compounds responsible for its bio stimulant and allelopathic effects, and to assess its impact on crop quality and human health.

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