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The dual inoculation with *Rhizobium* sp. and cyanobacterial extracts enhances the common bean (*Phaseolus vulgaris* L.) responses to white rot disease caused by *Sclerotinia sclerotiorum*

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ABSTRACT

In this study, laboratory and greenhouse experiments were conducted to investigate the suppression effect of two biocontrol agents caynobacterial (*Spirulina* sp., *Nostoc linckia* and *Anabaena variabilis*) extracts, along with *Rhizobium leguminosarum* biovar *phaseoli*, against *Sclerotinia sclerotiorum* in common bean plants. Four isolates of *S. sclerotiorum* were tested for pathogenicity in bean plants, and all isolated fungi proved to be pathogenic and caused white rot symptoms. Results of *in vitro* studies showed that algal extracts significantly inhibited the mycelial growth of the pathogen when compared to the untreated control. *N. linckia* gave the highest reduction (56.29%), followed by *A. variabilis* (51.85%) and *Spirulina* sp. (45.93%), respectively compared to control (0%). In greenhouse experiments, the combined effect of *Rhizobium* sp. and cyanobacterial extracts significantly reduced disease incidence and severity under artificial infection with *S. sclerotiorum*. The treatments showed the maximum effects for controlling disease incidence and severity caused by *S. sclerotiorum*, which were in the range of 13.33 to 26.67 % and 1.24 to 1.82, compared to 73.33 and 4.50 % in infested control, respectively. In addition, these treatments increased number of nodules, plant height, root length, fresh and dry weight of shoots, N_2 % and total nitrogen compared to control. The effects were similar to those of the fungicide Vitavax, which reduced the disease incidence and severity but adversely affected *Rhizobium* sp. and the symbiotic N_2 fixing parameters. Considerable increases in activity of oxidative reductive enzymes (peroxidase and polyphenol oxidase) were recorded in plants grown from treated bean seeds.

***Keywords*:** Biological Control, Cyanobacteria, *Spirulina* sp., *Nostoc* sp., *Anabaena* sp., *Rhizobium* sp., *Sclerotinia sclerotiorum*, Root Rot, Common Bean (*Phaseolus vulgaris*).

Introduction

The common bean (*Phaseolus vulgaris* L.; Family: Fabaceae) is one of the major leguminous crops that cultivated for human consumption, not only in Egypt but in many countries worldwide. The common bean is a highly variable and economical species for its edible dry seeds or unripe fruit due to its high protein content, balanced amino acid composition, and good digestibility (Broughton *et al*., 2003). Phytopathogens are a vital factor that often limits the bean yield and cultivation. Common bean could be infected by numerous plant diseases that can affect the roots, leaves, pods, and/or stems. The root rots, particularly white root rot, are the most widely distributed disease of common beans.

White rot disease is caused by the ascomycetous fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Class: Leotiomycetes, Family: Sclerotiniaceae). *S. sclerotiorum* has a wide range of host plants, where it can infect many herbaceous, succulent plants, particularly flowers and vegetables causing substantial losses in crop yield. The fungus infects a wide range of vegetable crops including carrots, cabbage, lettuce, tomato, and severely affects the common bean. White rot can attack their host plants at any growth stage including seedlings, mature plants, and harvested products. The most characteristic sign of *S. sclerotiorum* is its ability to produce sclerotia (black resting structures that survive environmental extremes) and forming white/cottony fuzzy growths of fungal mycelium on the infected tissues of host

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plants, which allow the fungus to effectively colonize ecologically different niches (Hatamleh *et al.,* 2013).

White rot disease is mainly controlled by fungicide-based management strategies, which is costly, environmentally harmful, and can lead to the development of resistant strains of the pathogen with repeated use (Vinale *et al*., 2008). Based on the current situation of *S. sclerotiorum* management, more sustainable methods are required to improve novel, eco-friendly alternative control strategies to reduce the usage of pesticide entirely or partially by combining with safe and environment-friendly methods such as using of biocontrol agents, which help in production of safety food and reduction of the environment pollution (Barakat and Al-Masri, 2005). Biological control depends on the utilization of beneficial microorganisms themselves or their extracellular metabolites. Microbial extracellular metabolites (MEMs) are produced extracellularly in the early stationary growth phase of some microbes in their culture fluid, and usually have significant anti-microbial activity against a broad spectrum of microorganisms and may serve as promising alternatives to conventional control methods.

Although *Rhizobium* species (Family: Rhizobiaceae) are well-known for their beneficial roles as diazotrophic alpha-proteobacteria that are associated with the nitrogen fixation via the formation of root nodules on legumes, however, their role in inducing/activating the plant resistance has been reported recently (Khalequzzaman and Hossain, 2008). For instance, Six *Rhizobium* strains and three biofertilizers reduced seed, foot, and root rots of Bush bean caused by *S. sclerotiorum*, but increased germination, plant growth parameters (Khalequzzaman and Hossain, 2008). Furthermore, *R. leguminosarum* biovar *phaseoli* (*Rlp*) and/or arbuscular mycorrhizal fungi (AMF) such as *Glomus mosseae* or *G. fasciculatum* were able to decrease the percentage of disease severity but increased the morphological parameters and total contents of phosphor (P) and nitrogen (N) in of common bean plants in the presence of white rot pathogen (Aysan and Demir, 2009). Additionally, *Rhizobium* spp. reduced the disease severity of several soil-borne phytopathogens such as *Fusarium* spp., *Macrophomina phaseolina, Pythium* spp., *Rhizoctonia* sp., and *Sclerotium rolfsii* on some legume crops (Baraka *et al*., 2009). Likewise, the dual inoculation with *R. leguminosarium* and *Trichoderma harzianum* reduced the harmful effects of damping-off and root rot diseases caused by *F. oxysporum*, *F. solani*, *M. phaseolina*, *R. solani*, and *Sclerotium rolfsii* on some legumes field crop such as faba bean (*Vicia faba*), chickpea (*Cicer arietinum*), lupine (*Lupines terms*) under greenhouse conditions (Shaban and El-Bramawy, 2011).

Cyanobacteria (also known as blue-green algae or cyanophyta) is major group of photosynthetic bacteria that could be found in almost every terrestrial and aquatic habitat and might have potential biological roles for controlling several phytopathogens, particularly soil-borne fungi (Kim 2006; Abo-Shady *et al*. 2007; Maqubela *et al.* 2009; Osman *et al.* 2011; Alwathnani and Perveen 2012). Numerous cyanobacteria strains can produce several metabolites intracellularly and/or extracellularly with diverse antimicrobial roles against phytopathogenic microbes such as fungi, bacteria, and viruses (Noama *et al.*, 2004). Regardless of the production of toxic blooms in aquatic habitats by few cyanobacteria, cyanobacteria can grow heavily in mass culture and eco-friendly manner (Osman *et al.*, 2011). There are several reasons behind the antimicrobial role of cyanobacteria that mainly depend on bioactive metabolic compounds such as phenolic compounds, saponins, and alkaloids which act as natural defense mechanisms against several phytopathogens and pests (El-Mahmood and Ameh, 2007).

Cyanobacterial extracellular metabolites (CEMs) extracted from terrestrial cyanobacteria have been previously showed significant biological activity in both *in vivo* and *in vitro* conditions (Reisser, 2000). Several culture filtrates or cell extracts from cyanobacteria and algae have been previously used for biological control against numerous phytopathogenic fungi and bacteria (reviewed by Kulik, 1995). For example, the CEMs from *Nostoc muscorum* and the methanolic extracts from its biomass significantly inhibited the fungal growth of *R. solani*, the causal agent of damping-off of soybean (De Caire *et al.*, 1990). Likewise, the ethanolic extracts from *Anabaena laxa* were cytotoxic and inhibited the mycelial growth of *Aspergillus oryzae* and *Penicillum notatum* due to the presence of some antibiotics and toxins which subsequently isolated, purified, and identified as Laxaphycins (A, B, C, D and E) (Frankmolle *et al.,* 1992a and Frankmolle *et al*., 1992b). Moreover, the caynobacterial filtrates of *A. subcylindrica*, *N. muscorum,* and *Oscillatoria angusta* inhibited the growth and reduced the fungal dry weight of several phytopathogenic fungi of faba bean in dose-dependent manner (Abo-Shady *et al.*, 2007). In lupine, four cyanobacteria species including *A. sphaerica*, *N. muscorum*, *Oscillatoria agardhii*, and *Spirulina platensis* had significant inhibitory effects on soil-borne fungi such as *F. solani,*

R. solani, and *M. phaseolina*, the causal agent of lupine damping-off and root rot diseases (Abdel-Monaim *et al.,* 2016). Recently, the chloroform extraction of the red algae *Gracilaria confervoides* was the most efficient against *R. solani* on cucumber plants, followed by ethyl acetate extraction (Soliman *et al*. 2018).

Dual inoculation of *Rhizobium* sp. and cyanobacteria increased plant growth, the number of nodules, and yield of chickpea and stimulated the elicitation of defense and pathogenesis-related enzymes under greenhouse conditions (Prasanna *et al*., 2005). Herein, the main purpose of this study is to examine the potential role(s) of inoculation with *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) individually or combined with some cyanobacteria extracts of *Anabaena variabilis* (Family: Nostocaceae), *Nostoc linckia* (Family: Nostocaceae), and *Spirulina* sp. (Family: Spirulinaceae) as biological control agents against the white rot disease of common bean caused by *S. sclerotiorum* in both *in vitro* and *in vivo* conditions. In addition, to determine their effects on some plant growth parameters, nitrogen fixation, some enzymatic activities. We believe that the *Rhizobium*-cyanobacteria combination might minimize the fungicide use, thus contributing to the development of sustainable control strategies for white rot disease and soil-borne disease in general.

Materials and Methods

Isolation and identification of the causal organism

Diseased common bean samples, showing typical white rot symptoms, were collected from different locations at Kafr El-Sheikh Governorate, Egypt. Infected tissues had watery-brown soft rot that combined with white-cottony mold. Mycelial growth of *S. sclerotiorum* was induced on infected tissues by incubation in a damp chamber in Petri dishes for 48 h. at 25±2^{o}C and ~100 % relative humidity under continuous fluorescent light. Hyphal tip of each isolate from the surface growing fungus was transferred on to potato dextrose agar (PDA) medium. After incubation for 2-3 days at 20^{o}C, the isolates were purified by the hyphal-tip method according to Dhingra and Sinclair (1985). The formal identification of purified isolates was identified according to their morphological (direct observation of the symptom on bean plants) and microscopical characteristics produced by the fungus as described by Kora (2003). Identification was confirmed by the specialists at the Laboratory of Mycology, Plant Pathology Institute, Agriculture Research Center, Giza, Egypt. Stock cultures were maintained on PDA slants and kept at 5 ^{o}C.

Pathogenicity tests

The purified *S. sclerotiorum* isolates secured from diseased common bean plants were tested for their pathogenicity. Sterilized clay pots (40 cm diameter) filled with 7 Kg autoclaved soil were planted with common bean seeds (*Phaseolus vulgaris* L.) cv. Bronco (obtained from Vegetable Crops Research Department Agricultural Research Centre, Giza, Egypt) at the rate of seven seeds per pot. The pathogenicity tests carried out in a completely randomized design with five replications (five pots each) for each isolate. The four obtained isolates were grown separately on barley grain medium in conical flasks for ten days to be used as a source of inoculum. The soil was infested with pathogenic fungi at a rate of five g kg^{-1} soil in different pots 7 days before planting the common bean seeds. Pots having only sterile soil were used as control. The disease incidence of bean plants was recorded after 30 days using the formula of Ajmal *et al.* (2001) as follow:

$$Disease\ Incidence\ (\%) = \frac{Total\ number\ of\ infected\ plants}{Total\ number\ of\ plants} \times 100$$

In addition, disease severity was estimated using a 0-5 scale according to (Zhang and Xue, 2010) where: 0 = No lesions/disease; 1= few lesions on leaves (total diseased area [TDA] of < 5%); 2 = several lesions on leaves (TDA of 5-10%); 3 = many lesions on leaves and stem tops (TDA of 11-30%); 4 = large lesions on leaves and stem top rotted (TDA of 31-50%); and 5 = lesions on leaves and rot on stem top progressing downward or plant dying (TDA of ≥ 50%).

Cyanobacteria extracts

Three cyanobacterial extracts of *A. variabilis, N.,* and *Spirulina* sp. were kindly obtained from the biological nitrogen fixation unit, Sakha Agric. Res. Station, Kafr El-Sheikh, Egypt. The cyanobacterial extracts were prepared according to (Madkour *et al*., 2019) by soaking 50 g of dry cyanobacterial powder in 1000 ml methanol in a conical flask for 48 h. After the two-days soaking, the supernatants were filtered through Whatman filter paper and then concentrated using rotary evaporator at room temperature (30±2°C) and the crude methanolic extract kept deep-frozen for further assays.

Rhizobial culture

Rhizobium leguminosarum pv. *phaseoli* strain was obtained from the biological nitrogen fixation unit, Sakha Agric. Res. Station, Kafr El-Sheikh, Egypt. One loopful from purified isolate was recultured on 250 ml yeast extract mannitol broth (YM) medium in a 500-ml flask. The cultures were incubated at 28-30ºC and 150 rpm from 3-5 days. Subsequently, the number of the bacterial cells of each culture was counted as (cell/ml), using hemocytometer.

In vitro* antifungal activity of cyanobacterial extracts against *S. sclerotiorum

The antifungal activity of cyanobacterial extracts against aggressive isolates of *S. sclerotiorum* was determined using the disc diffusion method (Thornberry, 1950). Briefly, 15 mL of PDA medium was poured into a 9-cm Petri dishes and allowed to solidify for approximately 5-10 min. Subsequently, each plate was divided into two equal halves and each half was inoculated individually. The first half was inoculated with a 5 mm-disk of a 7-day old culture of *S. sclerotiorum* grown on PDA, whereas, the second half was inoculated with a disk (0.5 cm in diameter) saturated with 250 µg ml^{-1} of each cyanobacterial extract (Nair *et al.,* 2005). Three plates were used as replicates for each cyanobacterial extract and the control (disc impregnated with sterilized distilled water). All inoculated plates were incubated at 20±2°C until mycelial growth of *S. sclerotiorum* covered the surface of medium in control treatment. All plates were then examined and the radial growth of *S. sclerotiorum* was measured. The inhibition of *S. sclerotiorum* (%) was calculated according to Soliman *et al*. (2018) using the following formula:

$$Growth\ inhibition\ (\%) = \frac{G1\text{-}G2}{G1} \times 100$$

Where: G1: the radial growth (cm) of *S. sclerotiorum* fungus in control plates; and G2: the corresponding radial growth (cm) of *S. sclerotiorum* fungus in dual plates with cyanobacterial extract treatments.

Pot experiments

To evaluate the antifungal activity of different cyanobacterial extracts against *S. sclerotiorum*, an artificially infested pot-experiment was carried out at the Dept. of Agric. Botany, Faculty of Agriculture, Kafr El-Sheikh University during October 2018. Briefly, three seeds were cultivated in a 30-cm pot, filled with 5 kg of clay soil. The experiment was carried out in a completely randomized design with five replications (five pots) for each treatment. Six treatments have been tested in this study including 1) control (soil infested with *S. sclerotiorum* only); 2) *R. leguminosarum* pv. *Phaseoli* (*Rlp*); 3) *Rlp* + *Spirulina* sp.; 4) *Rlp* + *N. linckia*; 5) *Rlp* + *A. variabilis*; 6) *Rlp* + Vitavax (a fungicide) as seed coating before sowing at the rate of 3g kg^{-1} seeds.

For *S. sclerotiorum* infestation, all pots were artificially infested with *S. sclerotiorum* fungus grown on barley grain medium at a rate of 20 g kg^{-1} soil one week before planting. Pots were irrigated weekly till the field capacity. For cyanobacterial extracts treatments, the seeds were dipped before planting for 12 hours in previously prepared cyanobacterial extracts as described by Abdel-Monaim *et al*., (2016) at the same concentration (250 µg ml^{-1}). Subsequently, bean plants were foliar sprayed with 10 ml of the cyanobacterial extracts four times with two weeks interval and the first foliar treatment was done three weeks after transplanting as described by Tassara *et al.,* (2008). For the rhizobial treatments, seedlings were thinned to three per pot then all treatment, except control, were inoculated with liquid cultures of *Rlp* isolate (5 ml×10^8 cell ml^{-1} $plant^{-1}$), in addition to *Rlp*-free liquid medium as control (El-Nady and Belal, 2005).

For all treatments, disease incidence was recorded after 30 days. In addition, disease severity, plant height, root length, fresh and dry weight, the number of nodules per plant, nitrogen (N) %, and nitrogen content were estimated after 60 days of sowing. The dry weight of both control and treated plants was estimated (g plant^{-1}) after drying till a constant weight in an oven at 70^{o}C. N% was determined in shoot tissues using Kjeldahl methods (AOAC, 1990). Total N content was calculated using the formula = N% × dry weight (Black *et al.*, 1965).

Enzymatic activity

Fresh samples were collected from both control (untreated) and treated bean plants to assay the biochemical change associated with tested treatments. Briefly, the enzymatic activities of both peroxidase and polyphenol oxidase (PPO) were determined using colorimetric methods according to Allam and Hollis (1972) and Snell and Snell (1953), respectively.

Statistical analysis

All experiments were designed in a completely randomized design. Data were statistically analyzed according to the analysis of variance technique (one-way ANOVA). Post hoc pairwise comparisons between the studied treatments were performed with the Tukey-Kramer honestly significant difference test (Tukey HSD) using JMP statistical analysis software, version 12, and statistical significance was established as $p \leq 0.05$. In addition, principal component analysis (PCA) was performed using normalized data and its associated loading-plot was generated using PAST 3.24 software. Furthermore, all studied parameters were presented as a heat map combined with two-ways hierarchical cluster analysis (HCA) using standardized means of all studied treatments. Distance and linkage were done using Ward's minimum variance method (Ward 1963) with 95% confidence.

Results and Discussion

Isolation and Identification of the Causal Organisms

Four isolates were isolated from diseased common bean plants showing typical white rot symptoms on the stem, collected from different locations at Kafr El-Sheikh Governorate, Egypt. Isolates were purified and identified according to their morphological and microscopical characteristics as *Sclerotinia sclerotiorum*. This fungus was previously reported to be associated with bean white rot diseases (Khalequzzaman and Hossain, 2008; Aysan and Demir, 2009 and Elsheshtawi *et al*., 2017).

Pathogenicity tests

The pathogenicity of obtained isolates was tested using bean cv. Bronco in pots under greenhouse conditions. Results presented in figure 1 reveal that all tested isolates could infect the roots of common bean plants, causing white rot symptoms and reduced the plant survival but in different degrees. Isolate SC4 of *S. sclerotiorum* was the most aggressive isolate, which recorded the highest disease incidence (79.89±4.91 %) followed by isolate SC1 (71.43±3.09%), and isolate SC3 (48.57±1.86 %), while isolate SC2 had the lowest disease incidence percentage (42.86±1.66 %), compared to control, which is disease-free (Figure 1A). These results are in agreement with those obtained by (Figueirêdo *et al.*, 2018 and Prova *et al*., 2018).

Likewise, the disease severity was positively correlated with the disease incidence (Figure 1). The disease severity was using a 0-5 scale. The *S. sclerotiorum* isolate SC4 was most effective in reducing disease severity (4.60±0.14) followed by isolate SC1 (4.30±0.12) which came together in the same category (category 4). Both SC1 and SC4 showed large lesions on leaves and stem top rotted with the total diseased area between 31-50%. On the other hand, both isolates SC2 and SC3 were not significantly different from each other (3.33±0.18 and 3.56±0.13, respectively) (Figure 1B) and came together in category 3. Both isolates SC2 and SC3 showed many lesions on leaves and stem tops with total diseased area between 11-30%). These findings were in agreement with (Aysan & Demir, 2009 and Zhang & Xue, 2010)

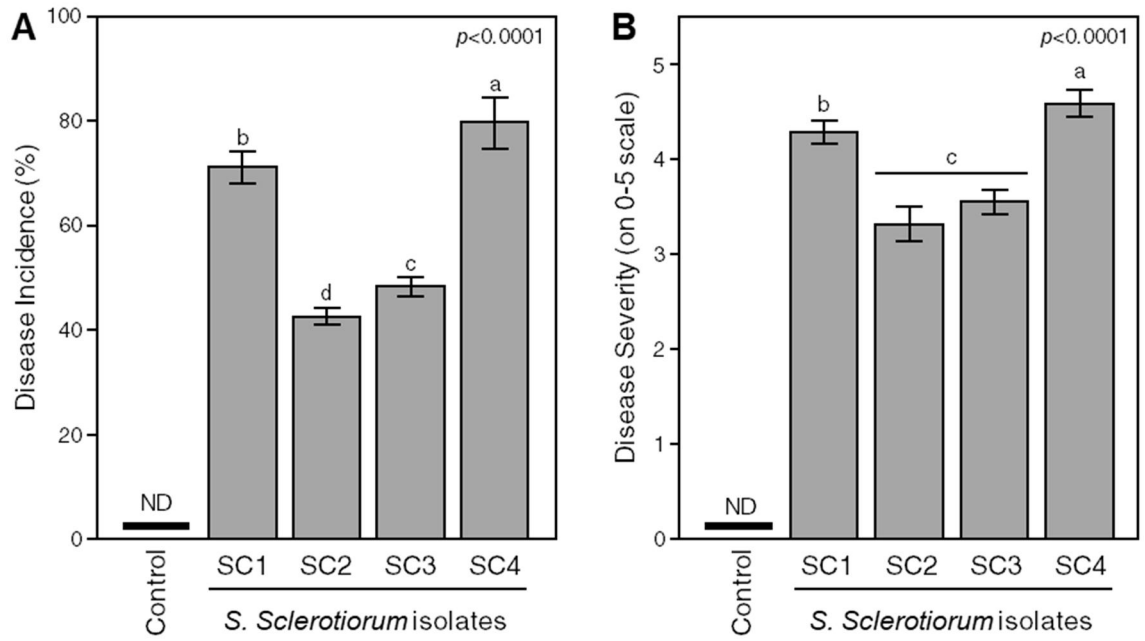


Fig. 1: Pathogenicity test of different *S. Sclerotiorum* isolates under greenhouse conditions during 2017/18 season. (A) Disease Incidence (%) and **(B)** Disease Severity on a 0-5 scale**.** The bar-graph and error bars represent means and standard deviation (SD), respectively ($n = 5$). Different letters indicate statistically significant differences between treatments ($p < 0.05$).

In vitro* antifungal activity of cyanobacterial extracts against *S. sclerotiorum

Results in figure 2 show that all the tested cyanobacterial extracts significantly reduced the radial mycelial growth of *S. sclerotiorum* compared with the control plates. The antifungal effects of cyanobacterial extracts against the pathogenic fungus were in the range of 45.93-56.29 %. *Nostoc linckia* negatively affected the radial mycelial growth (3.93±0.06 cm) (Figure 2A), which means the highest growth inhibition against *S. sclerotiorum* (56.29±0.64 %) (Figure 2B), followed by *Anabaena variabilis* (51.85±1.70 %), while the least inhibition effect was recorded by *Spirulina sp* (45.93±1.28%). These results were almost similar to those obtained by Soliman *et al*. (2009). They found that all the tested algal filtrates decreased the mycelial growth of *S. sclerotiorum* as compared with control. It may be used to inducible plant defenses which provides protection against a broad spectrum of diseases causing organisms.

This activity might indicate the ability of cyanobacteria to produce bioactive secondary metabolites, secreted into the surrounding medium. These bioactive ingredients seem to hinder the growth of the isolates of the tested. As well as, they might have the ability to produce variety of lethal toxins (Surakka *et al.*, 2005).

Pot experiments

1- Dual inoculation of *Rlp* and some cyanobacterial extracts reduced white rot incidence on common bean plants.

Generally, all tested treatments significantly reduced white rot incidence and severity on common bean plants compared to untreated control under greenhouse conditions (Figure 3). The treatment with the fungicide (Vitavax) showed the best results in reducing disease incidence and severity (6.67±1.18 and 0.88±0.08, respectively). Similar results were obtained by Elsheshtawi *et al.* (2017) who reported that the "Topsin" fungicide significantly reduced the disease incidence caused by *S. sclerotiorum* by 90% and 95% survival plants, respectively.

In addition, dual inoculation of *Rlp* and some cyanobacterial extracts also significantly reduced the white rot incidence (Figure 3A) and severity (Figure 3B) on common bean plants.

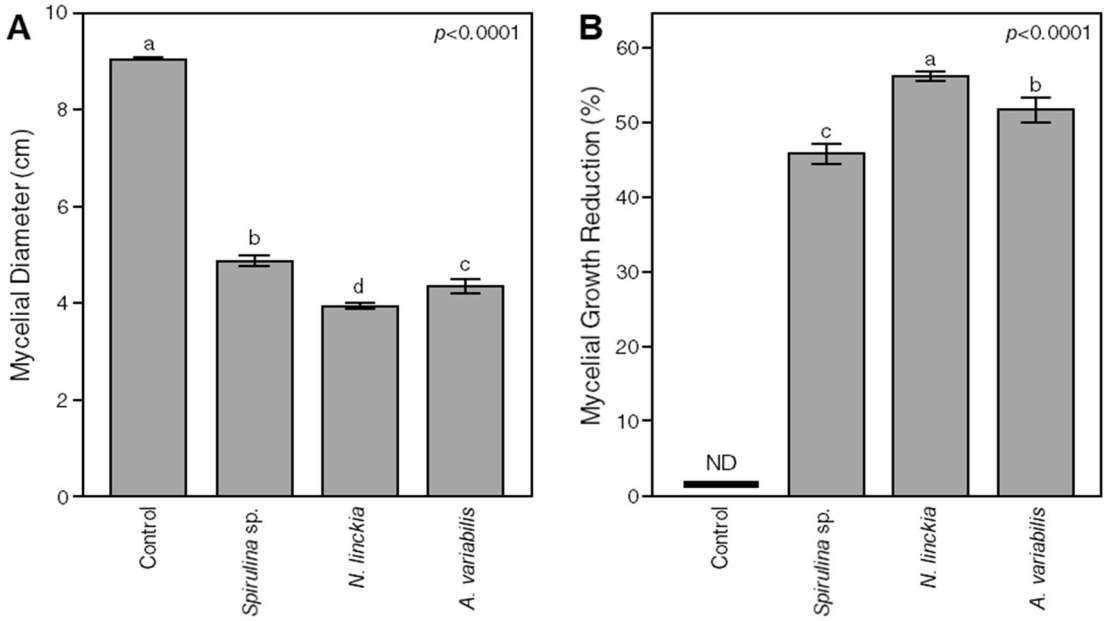


Fig. 2: Effect of some cyanobacterial extracts on the radial mycelial growth of *S. sclerotiorum* on PDA medium. (A) mycelial diameter (cm) and **(B)** mycelial growth reduction (%)**.** The bar-graph and error bars represent means and standard deviation (SD), respectively (n = 5). Different letters indicate statistically significant differences between treatments (p <0.05).

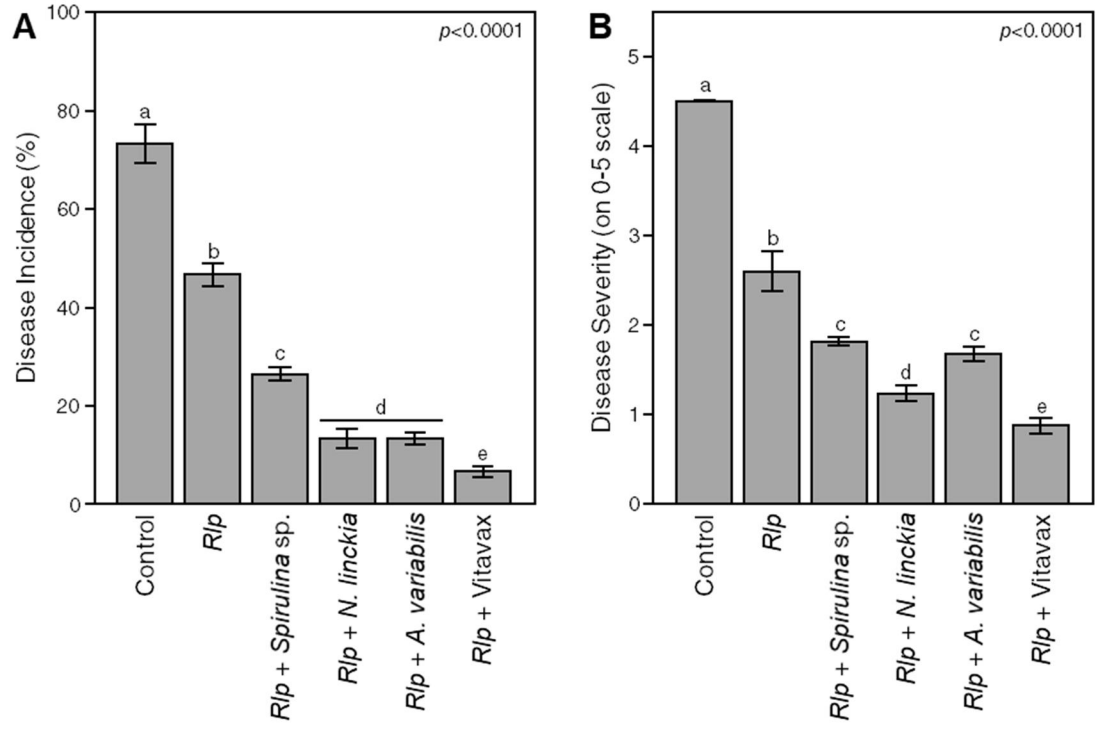


Fig. 3: Antifungal activity of *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) individually or in dual inoculation with some cyanobacteria extracts on disease incidence and disease severity of white rot disease caused by *S. sclerotiorum* on common bean plants under greenhouse conditions in 2018/19 season. (A) Disease Incidence (%) and **(B)** Disease Severity on a 0-5 scale. The bar graph and error bars represent means and standard deviation (SD), respectively (n = 5). Different letters indicate statistically significant differences between treatments (p <0.05).

The combined effect of rhizobia and cyanobacteria extracts showed the maximum effects for controlling disease incidence (ranged from 13.33±1.23 to 26.67±1.39 %) and severity (ranged from 1.24±0.09 to 1.82±0.04) caused by *S. sclerotiorum*, compared to 73.34±3.92 % and 4.50±0.00 in infested control, respectively. These findings were in accordance with Abdel-Kader and El-Mougy (2013) and Prasanna *et al.* (2017).

The reduction of white rot incidence and severity caused by the pathogenic fungus was mainly depending on the cyanobacteria species. *N. linckia* recorded the highest reduction in both disease incidence (13.33±1.96 %) and severity (1.24±0.09), followed by *A. variabilis* (13.33±1.23 % and 1.68±0.08, respectively) and *Spirulina* sp. (26.67±1.39 % and 1.82±0.04). These were in agreement with those of Tassara *et al*. (2008), who indicated that *N. muscorum* had a remarkable antifungal activity against soil-borne phytopathogenic fungi. Furthermore, *Nostoc* ATCC 53789 showed cytotoxic antagonistic effects against phytopathogenic fungi, insects, and nematodes due to the production of cryptophycin and being used as a source of natural biopesticides (Biondi *et al*., 2004).

The effectiveness of soaking seeds in cyanobacterial filtrates/extracts might be due to the absorption of bioactive substances, which prevented infection and disease development (EL-sayed and Mousa, 2015). Additionally, the efficacy of cyanobacterial filtrates/extracts as a treatment for irrigated soil might be due to the capability of antifungal-like substances to penetrate into the fungal cell, consequently causing alterations in fungal metabolism (Biondi *et al*., 2004).

2- Dual inoculation of *Rlp* and some cyanobacterial extracts enhanced the symbiotic N_2 fixation of common bean plants.

In general, all applied treatments significantly induced the symbiotic N_2 fixation in bean plants compared to *S. sclerotiorum*-infested control (Figure 4). These results were in agreement with other reports demonstrating the efficient red algae extracts as a unique antiprotozoal and anti-mycobacterial agents plus increasing plant growth parameters (Jimenez, *et al*., 2011 and Sultana, *et al.,* 2011).

Although *Rlp* treatment significantly increased the number of nodules being 7.99±0.26 nodules per plant compared to un-inoculated control, which did not have any nodules at all, however, dual inoculation of Rlp and some cyanobacterial extracts significantly enhanced the number of nodules of common bean plants (Figure 4A). Likewise, *Rlp* treatment increased N_2% (Figure 4B) and N-content (Figure 4C), being 2.13±0.03 % and 112.35±1.47 mg/plant comparing with un-inoculated control (1.47±0.00 % -33.91±2.19 mg /plant, respectively). In this respect, Arfaoui *et al.* (2005) reported that different mechanisms might be involved in the biological control of fungal diseases by *Rhizobium*. These mechanisms including competition for nutrient or iron, production of antibiotics, promotion of the plant growth and induced or enhanced resistance within the host.

Although the seed dressing with the fungicide Vitavax significantly reduced the disease incidence and severity, its harmful effects on number of nodules and N_2 fixing parameters were significantly remarkable (Figure 4). These results were approved with Singh and Wright (2002) who showed that the negative effects of several herbicides including terbutryn/terbuthylazine, trietazine/simazine, prometryn and bentazone on the plant growth, number of nodules and N fixation parameters of pea (*R. leguminosarum* bv.*viceae)*.

It is noticeable that the application of the fungicide Vitavax negatively affected the *Rhizobium* population and deprives the legumes of any beneficial effects of Rhizobial inoculation. The number of nodules, N_2 (%), and total N_2 was increased in all treatments with bio-agents (*Rlp* individually or in combination with cyanobacteria extracts) and was significantly higher than the unprotected plants (control) and protected plants by Vitavax (Figure 4). This result was confirmed by (El khateeb, 2014).

3- Dual inoculation of *Rlp* and some cyanobacterial extracts stimulate the plant growth of common bean plants.

Generally, combined inoculation of *Rlp* and cyanobacteria extracts showed a significant increase in plant height (cm), root length (cm), fresh and dry weight (g plant^{-1}) compared to inoculation with *Rhizobium* individually (Figure 5). The same results were observed by Abdel-Monaim *et al.* (2016) who showed that lupine seeds treated with cyanobacteria extracts recorded the highest protection against infection with soil-borne pathogens and significantly improved plant growth and yield parameters under field conditions.

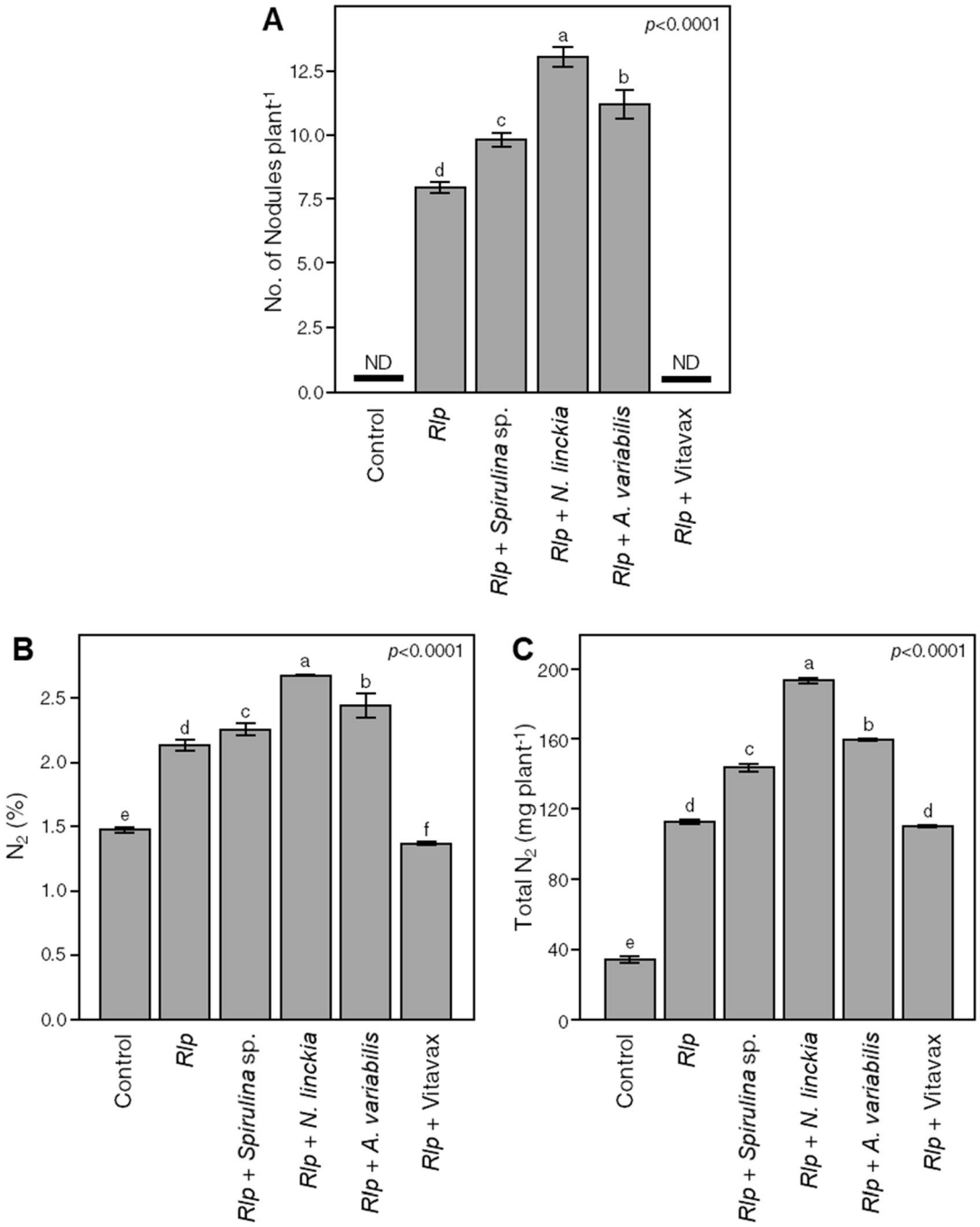


Fig. 4: Effect of *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) inoculated individually or in dual inoculation with some cyanobacteria extracts on symbiotic N_2 fixation of common bean plants infected with white rot disease caused by *S. sclerotiorum* under greenhouse conditions in 2018/19 season. (A) No. of nodules plant^{-1}, **(B)** N_2 (%) and **(C)** total N_2 (mg plant^{-1}). The bar-graph and error bars represent means and standard deviation (SD), respectively (n = 5). Different letters indicate statistically significant differences between treatments (p <0.05).

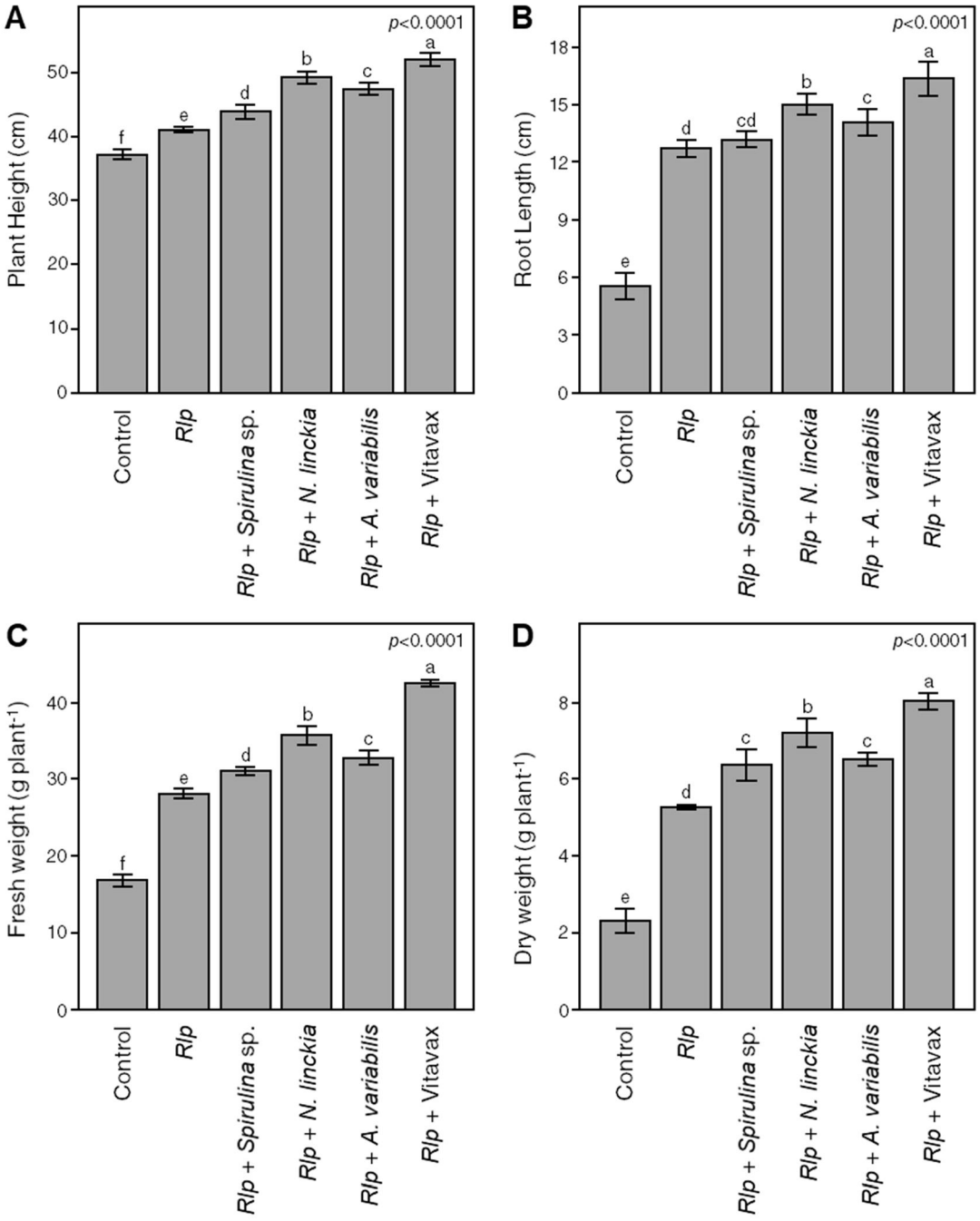


Fig. 5: Effect of *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) inoculated individually or in dual inoculation with some cyanobacteria extracts on some plant growth parameters of common bean plants infected with white rot disease caused by *S. sclerotiorum* under greenhouse conditions in 2018/19 season. (A) plant height (cm), **(B)** root length (cm), **(C)** fresh weight (g plant^{-1}), and **(D)** dry weight (g plant^{-1}). The bar-graph and error bars represent means and standard deviation (SD), respectively (n = 5). Different letters indicate statistically significant differences between treatments (p <0.05).

Plant height in treated bean plants with *Rlp* and cyanobacteria was in the range of 41.09±0.34 to 49.22±0.71 (cm), while in untreated plants it was 37.23±0.53 cm (Figure 5A). Likewise, root length had the same trend as the plant height (Figure 5B). The dual treatment of *Rlp*+Vitavax recorded the highest root length (42.54±0.34 cm), followed by the *Rlp*+ *N. linckia* treatment (35.82±0.89 cm) and the *Rlp*+ *A. variabilis* treatment (32.78±0.71). Likewise, both fresh and dry weight had the same trend

(Figure 5C and 5D, respectively). The dual treatment of *Rlp*+Vitavax recorded the highest fresh and dry weight (42.54±0.34 and 8.05±0.16 g plant^{-1}, respectively), followed by the *Rlp*+ *N. linckia* treatment (35.82±0.89 and 7.19±0.26 g plant^{-1}, respectively) (Figure 5C and 5D). These findings were in agreement with those recorded by Abdel-Monaim *et al.* (2016) and Prasanna *et al.* (2017).

4- Dual inoculation of *Rlp* and some cyanobacterial extracts enhanced the enzymatic activities of common bean plants.

Data presented in figure 6 indicate that bean plants grown from seeds soaked in some cyanobacteria extracts resulted in an increase of peroxidase and polyphenol oxidase (PPO) activity compared to the untreated control plants. Peroxidase activity was induced to its highest level when common bean plants were treated with *Rlp*+ *N. linckia* (1.13±0.01; approximately 195.03% increase) followed by the *Rlp*+ *A. variabilis* treatment (0.94±0.01; around 146.21% increase) compared to the untreated control plants (0.38±0.00) (Figure 6A). Whereas, the treatment with Rlp only had lower peroxidase activity (0.76±0.01) compared to mixed inoculation (*Rlp*+cyanobacteria) and Vitavax fungicide treatment (0.68±0.00).

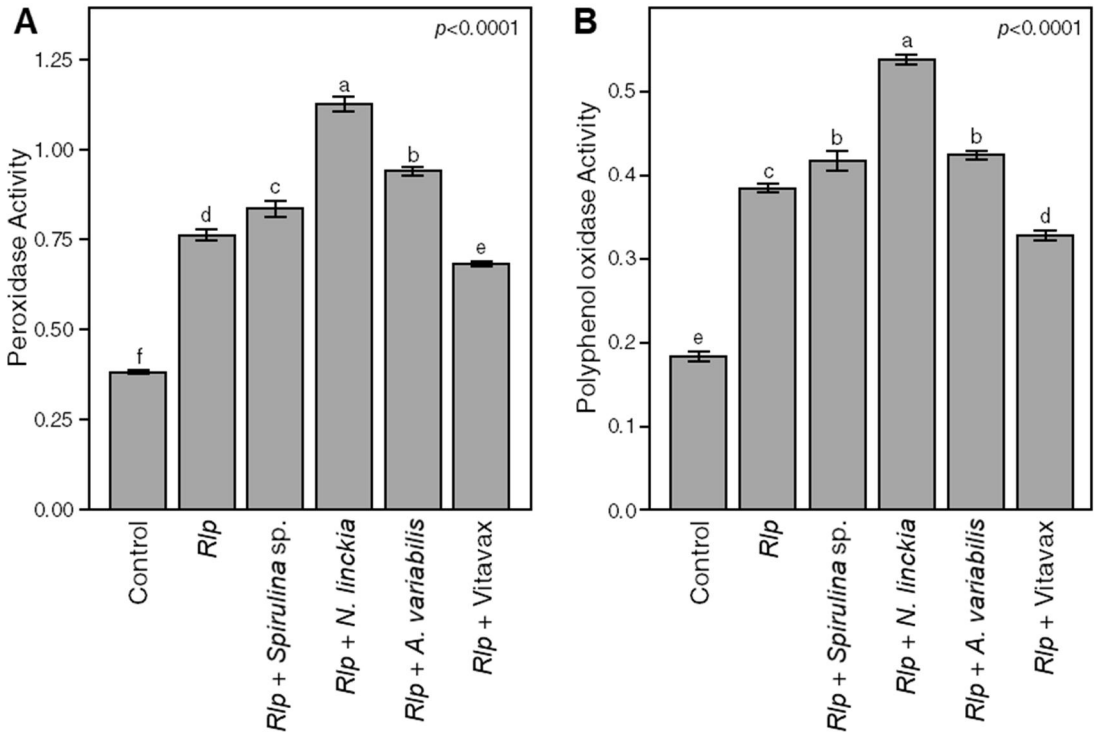


Fig. 6: Effect of *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) inoculated individually or in dual inoculation with some cyanobacteria extracts on some enzymatic activities of common bean plants infected with white rot disease caused by *S. sclerotiorum* under greenhouse conditions in 2018/19 season. (A) peroxidase activity, and **(B)** polyphenol oxidase (PPO) activity. The bar-graph and error bars represent means and standard deviation (SD), respectively (n = 5). Different letters indicate statistically significant differences between treatments (p <0.05).

Likewise, PPO had almost the same trend as peroxidase enzymatic activity (Figure 6B). The optical density of polyphenol oxidase was ranged from 0.33±0.00 to 0.54±0.00 in treated plants, compared to control common bean plants (0.18±0.00). Our findings showed that the application of *Rlp* and cyanobacteria extracts enhanced the activity of PPO enzyme in common bean plants from 98.91 to 192.89 % (Figure 6B). Furthermore, mixed inoculation of *Rlp* and *N. linckia* increased the PPO activity (0.54±0.00; approximately 192.89%) followed by *Rlp*+*A. variabilis* and *Rlp*+*Spirolina* sp. treatments without significant differences between them (0.42±0.01 and 0.42±0.00, respectively).

Our findings showed that the use of *Rlp* and cyanobacteria as biocontrol agents enhanced the enzymatic activities of some enzymes such as peroxidase and PPO in treated bean plants more than the untreated control ones. These enzymes play a key role in plant defense responses against different phytopathogenic microorganisms. The positive correlation between peroxidase enzymatic activities and the resistance development in infected plants has been reported previously (Nawar and Kuti, 2003) and it might play a defensive role against phytopathogen infections (Caruso *et al*., 2001). It has been shown previously that treatments with cyanobacterial extracts significantly reduced the root rot incidence of faba bean. The reduction of disease incidence was combined with higher enzymatic activities of peroxidase and PPO enzymes in plants grown from treated seed compared to the untreated control plants (EL-sayed and Mousa, 2015).Treatments with *Rlp* and cyanobacterial extracts provided high protection for common bean seedlings against white rot disease caused by *S. sclerotiorum*. This protective role might be related to the ability of cyanobacteria to stimulate the enzymatic activities of peroxidase and PPO in common bean plants associated with plant defense responses and the protection against phytopathogenic fungi.

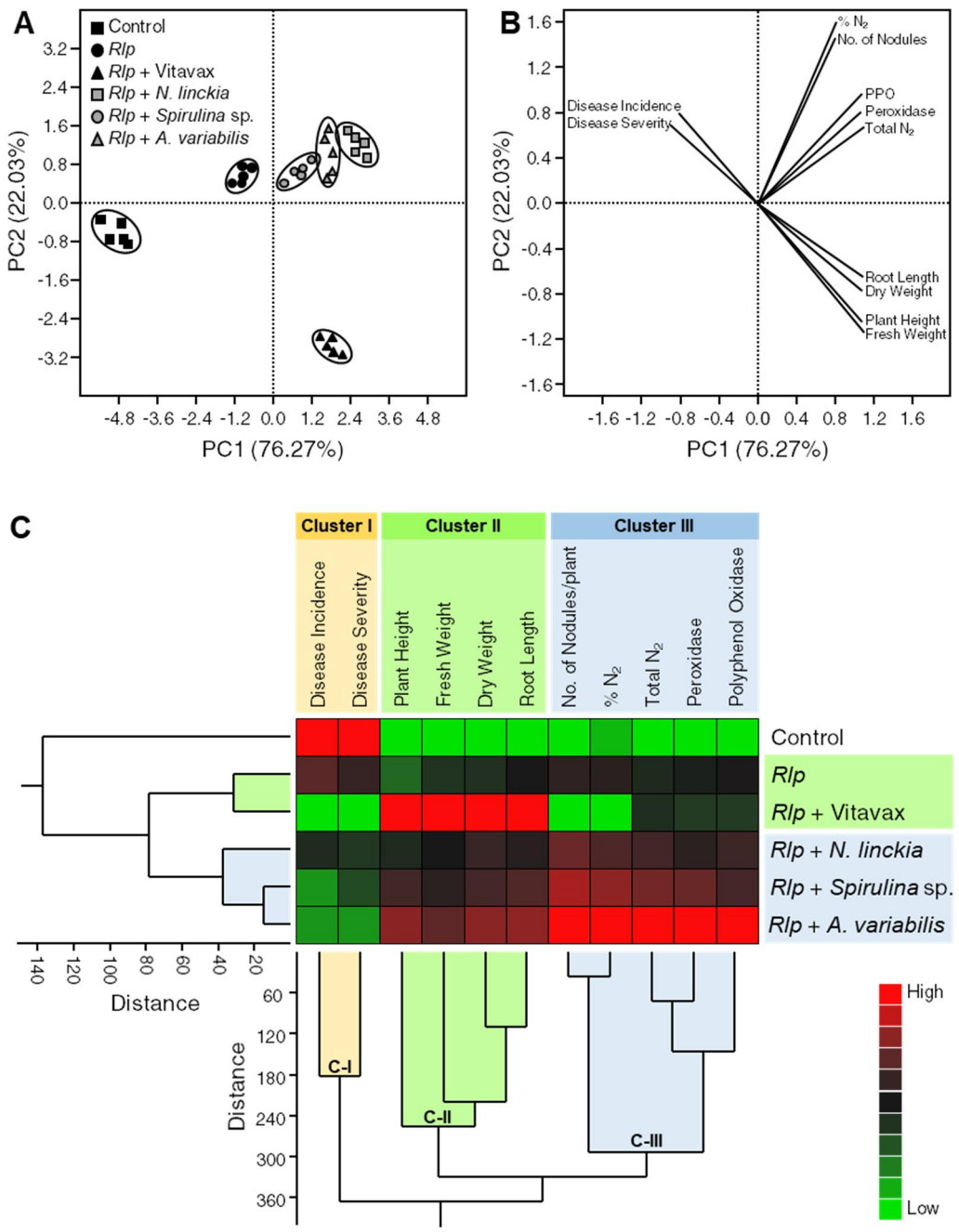
5- PCA and HCA analyses revealed the differences among treatments.

The scatter plot (Figure 7A) obtained from principal components analysis (PCA) showed a clear separation among the six studies treatments. Generally, the dual inoculation with *Rlp* and *N. linckia*, *A. variabilis* or *Spirolina* sp. (as overlapping groups) were clustered together in the positive quart of the graph with respect to PC1 (26.27%) and PC2 (22.03%) (Figure 7A). Furthermore, the loading-plot (Figure 7B) showed that the symbiotic N_2 fixation parameters (number of nodules, N_2 (%), and N-content) and enzymatic activities (peroxidase and PPO activities) were positively correlated with the dual inoculation with *Rlp* and *N. linckia*, *A. variabilis,* or *Spirolina* sp. (Figure 7B). Whereas, plant growth parameters (plant height, root length, fresh and dry weight) were positively correlated with Vitavax treatment.

In addition, the two-way hierarchical cluster analysis (HCA) and heatmap were performed using all studied parameters. The total profiles of the dual inoculation with *Rlp* and *N. linckia*, *A. variabilis,* or *Spirolina* sp. (Figure 7C) were more similar to each other (approximately 40) than other treatments. In addition, both *Rlp* and *Rlp*+Vitavax treatments were clustered together and separately from the control. For the cluster between studied parameters, the 11 studied parameters were split into three clearly separated clusters. Cluster I (C-I) included the white rot disease parameters (disease incidence and disease severity) which were higher in *S. sclerotiorum*-infested (control) plants (Figure 7C). Cluster II (C-II) included the plant growth parameters (plant height, root length, fresh and dry weight) which were higher in *Rlp*+Vitavax treatment. Finally, cluster III (C-III) included the symbiotic N_2 fixation parameters (number of nodules, N_2 (%), and N-content) and enzymatic activities (peroxidase and PPO activities) which were associated with the dual inoculation treatments (*Rlp+N. linckia*, *Rlp+A. variabilis,* and *Rlp+Spirolina* sp.) (Figure 7B).

Conclusion

In conclusion, our findings indicated that the dual inoculation with *R. leguminosarum* pv. *Phaseoli* and cyanobacterial extracts *N. linckia*, *A. variabilis,* or *Spirolina* sp. were efficient in protection of common bean plants against white rot disease caused by *S. sclerotiorum* without affecting the symbiotic N_2 fixation. Furthermore, the dual inoculation enhanced the plant growth parameters (plant height, root length, fresh and dry weight), symbiotic N_2 fixation parameters (number of nodules, N_2 (%), and N-content), and the enzymatic activities of peroxidase and PPO enzymes. It may be concluded that application of cyanobacterial extracts might be promising, eco-friendly, and cost-efficient method for controlling soil-borne disease. However, more investigations are required to chemically analyze these cyanobacterial extracts to purify and identify the bioactive compounds/substance which may lead to the production of cyanobacteria-based biopesticides in the future.



Figu. 7: Principal components (PCA) and two-ways hierarchical cluster analysis (HCA) of *Rhizobium leguminosarum* pv. *phaseoli* (*Rlp*) inoculated individually or in dual inoculation with some cyanobacteria extracts on some disease parameters, plant growth parameters, and enzymatic activities of common bean plants infected with white rot disease caused by *S. sclerotiorum* under greenhouse conditions in 2018/19 season. (A) PCA-scatter-blot, **(B)** PCA-loading-plot, and **(C)** Heat map combined with two-ways HCA using the standardized means of all studied parameters for the six studied treatments. Rows represent the individual treatments, while columns represent the studied parameters.

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