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INDUCTION OF ANTIOXIDANT RESPONSE WITH COMPATIBLE COMBINATION OF *MESORHIZOBIUM* SP. AND *PSEUDOMONAS* SP. AGAINST *FUSARIUM OXYSPORUM* SP. *CICERIS* IN CHICKPEA

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Abstract

Chickpea (*Cicer arietinum* L.) is severely affected by *Fusarium oxysporum* sp. *ciceris* causing vascular wilt disease accounting about 10% of yield losses. The present study was conducted to assess the potential of compatible dual inoculants of *Mesorhizobium* sp. and PGPR (rhizospheric and endophytic) in initiating and boosting the antioxidant response towards the phytopathogen *in vivo* under wilt sick conditions in chickpea. Among the different dual inoculated treatments, LGR191+NE8 (*Mesorhizobium* sp. + nodule endophyte) showed maximum augmentation in the antioxidants *viz.,* catalase (15.16 U/min/g fresh weight of root), guaiacol peroxidase (2.492mmoles/min/g fresh weight of root) and total phenolics (110.25 tannic acid equivalents/g fresh weight of root) over recommended fungicide (captan). Percent wilt control was highest with LGR191+NE8 (95.77%) followed by LGR1+LPGPR-1 (94.17%) after 120 days of sowing. The results suggested an expanded antioxidant state of the wilt infected plants with the dual inoculation of *Mesorhizobium* sp. with PGPR (rhizospheric and endophytic) decreasing fungal proliferation sustainably.

Introduction

Plants interact with the microbes in the vicinity of the rhizosphere synergistically and provide valuable niche to soil microbes where in return the microbes contribute to plant growth promotion and mitigate various biotic and abiotic stresses. *Fusarium oxysporum* sp. *ciceris* is known to affect chickpea production worldwide and annual chickpea yield losses due to the pathogen vary from 10 to 15%, but can result in total loss of the crop under specific conditions (Singh *et al*. 2013). The fungal pathogen is known to perpetuate in seed and soil, and hence is difficult to manage by the use of chemicals. In current years substantial awareness has been specified to the rhizosphere microbes in mediating induced systemic resistance (ISR) (Kumar and Jagadeesh 2016). It is a state of improved defensive competence where plant's natural resistance is potentiated in opposition to consequent biotic stress, in addition to their antagonism. Plants enhance their defensive capacity by mobilizing apt cellular defense responses before or upon pathogen attack (Palmieri *et al.* 2016). It has been documented that diverse protections are elicited after microbial infection such as activation of the phenylpropanoid pathway leading to deposition of lignin and phenolics beyond the infection sites and initiation of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POx) and catalase (Nagpal *et al.* 2020). Many of these defenses are known to be amplified by plant growth promoting rhizobacteria (PGPR)-mediated ISR that guard the plants against diverse pathogens (Kumar *et al.* 2017, Nagpal *et al*. 2019). Antagonistic rhizospheric bacteria can elicit the phenyl-propanoid pathway in a variety of crop plants that may result in reduced infection by their respective pathogens (Palmieri *et al.* 2016).It has been advocated that the accomplishment of a plant in warding off an invading pathogen relies chiefly on its capability

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to put up a swift stroke of defense. The use of chemical fungicides poses many threats to sustainable agriculture. Thus, there is an ever-growing interest towards exploiting potential antagonistic microbes for effective control of plant diseases. Thus the present investigation was carried out for examining the amelioration in host defense mainly the antioxidant responses mediated by the dual inoculation of *Mesorhizobium* sp. and PGPR in suppressing the vascular wilt disease in chickpea.

Materials and Methods

Identified and characterized *Mesorhizobium* sp. (LGR1 and LGR 191) and endophytic isolates (LPGPR-1 [*Pseudomonas argentinensis,* Accession no. JX239745.1] and NE8) selected on the basis of multifarious plant growth promotional traits and tested for their antagonism against *Fusarium oxysporum* sp. *ciceris in vitro* were used as bioinoculant combinations. Of all the screened isolates, 4 compatible bioinoculants (LGR1+LPGPR-1, LGR1+NE8, LGR2+LPGPR-1 and LGR2+NE8) were assessed *in vivo* for their antioxidant response and biocontrol potential against *Fusarium* sp.

The study site (wilt sick plot maintained for about 10 years for disease occurrence) was located at Pulses research farms, Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana, Punjab, India. Field experiment was conducted in Randomized block design (RBD) with three replications. Forty-five kg/ha seed was used for sowing the crop. The seeds of PBG-7 variety were treated with different microbial inoculant formulations (5ml liquid formulation, *Mesorhizobium* sp. and endophytic bacteria in the ratio of 1:1) containing 1×10^9 cells/ml of Luria Bertanni broth along with the recommended dose of the fungicide Captan (@3g/kg) as control treatment during *Rabi* season 2016-17.

Root samples were collected both before and after the appearance of disease in chickpea plants. Samples were uprooted from area of about 5 inches square at least 3 inches deep to include roots and soil. Soil and root layer was kept intact and gentle washing was given under tap water to remove adhered soil and debris. Various antioxidant enzymes were assayed in the root samples as per the standard protocols.

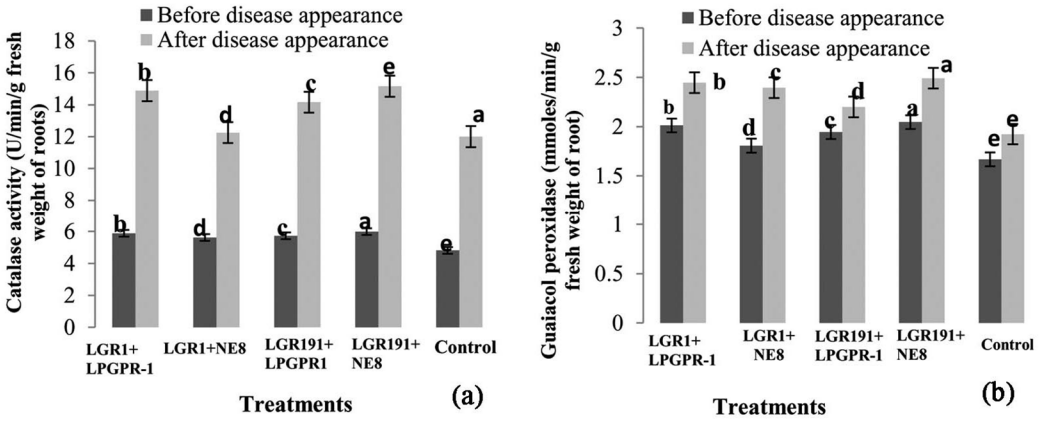
Catalase activity was assayed by the method of Aebi (1983). One gram of root tissue was homogenised in QB buffer (100 mM potassium phosphate buffer, pH 7.8, 15% glycerol, 1 mM EDTA, 1% Triton X-100) and homogenate was centrifuged at 15000 g for 15 min. The sample supernatant (10µl) was added to 2ml of the phosphate buffer, pH 7 and mixed. To start the reaction 1ml of 30mM H_2O_2 was added and decrease in absorbance was monitored after every 10sec for 1 min at 240nm using UV spectrophotometer and expressed as micromoles of H_2O_2 oxidized (U) per minute per g fresh weight of root. Peroxidase activity was determined using guaiacol as substrate as described by Chance and Maehly (1955). Root samples (0.5g) were crushed in 50 mM Na-phosphate buffer (pH 7) and centrifuged at 4°C at 15000g for 20 min and supernatant collected for assay. The reaction mixture consisted of 50 mM buffer, 10 mM guaiacol, 0.1 mM EDTA, and 10 mM H_2O_2. An increase in absorbance at 470 nm owing to guaiacol oxidation was expressed as mmoles tetraguaiacol formed/min/g fresh weight of root for 3 min.

The phenolics content in the frozen roots (1g) was estimated as described by Watermann and Mole (1994). The extraction was done thrice with 80% methanol at 4°C and centrifuged for 15 min (7000g). To 0.5ml of the supernatant, 0.5ml distilled water was added. Five ml Na_2CO_3 and 0.5ml of Folin-Ciocalteu reagent were added to the reaction mixture. Total phenolic content was estimated at 760 nm spectrophotometrically. Percent wilt control was assessed for all the treatments with respect to control treatment for assessing the effectiveness of inoculant combination treatments against the phytopathogen. Data was statistically analyzed using standard

ANOVA appropriate for RBD. Data was analyzed using CPCS-software. Differences were considered significant at 5% level of significance (p = 0.05).

Results and Discussion

The catalase activity of all inoculant combination treatments showed significant increase (p=0.05) after the disease appearance with respect to control treatment. Dual inoculant LGR191+NE8showed maximum catalase activity (5.99 U/min/g fresh weight of root) followed by LGR1+LPGPR-1 (5.88 U/min/g fresh weight of root) as compared to the control treatment (4.82 U/min/g fresh weight of root) (Fig. 1a). A 1.88% improvement in catalase activity was noticed with the appearance of disease symptoms in LGR191+NE8 treatment over recommended consortium inoculant treatmentLGR1+LPGPR-1. Bio-priming the plants with PGPR trigger induced systemic response (ISR) similar to that of systemic acquired response (SAR). ISR induced by the effect of biocontrol agents is a novel plant protection strategy (Nagpal *et al*. 2020). The results are in agreement with the findings of Jain *et al.* (2013) who demonstrated that the catalase activity in pea reached maximum after 72 hrs of pathogen challenge with maximum activity in dual inoculant treatment of *Bacillus subtilis* BHHU100 and *Pseudomonas aeruginosa* PJHU15.



Data are mean of three replications. Different alphabets indicate significantly different mean values.

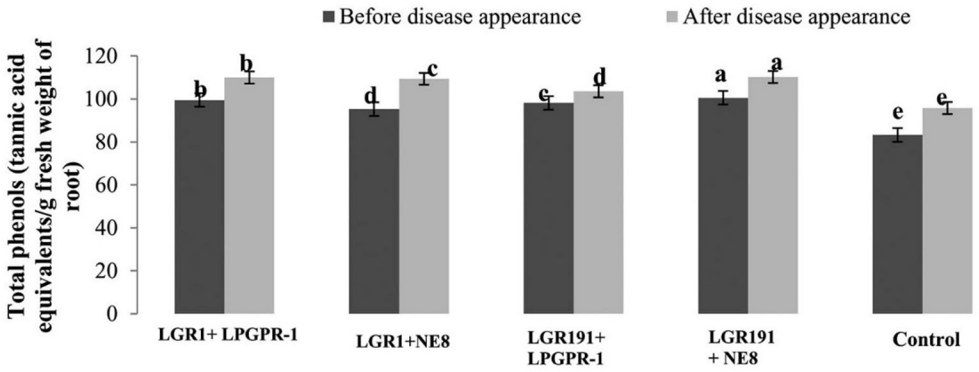
Fig. 1.Synergistic effect of *Mesorhizobium* sp. and PGPR (*Pseudomonas* sp.) on (a) catalase and (b) guaiacol peroxidase activity.

Peroxidase activity in all the dual treatments was recorded using guaiacol as hydrogen donor. Before the disease appearance, dual inoculant treatment LGR191+NE8 revealed higher peroxidase activity as compared to recommended consortium LGR1+LPGPR-1. A significant improvement in the peroxidase response was observed with the appearance of disease in all dual inoculant combinations. Maximum value for peroxidase activity was shown by LGR191+NE8 (2.492 mmoles/min/g fresh weight of root) followed by LGR1+LPGPR-1 (2.446 mmoles/min/g fresh weight of roots) as compared to the control treatment (Fig. 1b).

Peroxidase enzymes are involved in removal of reactive oxygen species (ROS) directly or indirectly through the regeneration of the two major redox molecules ascorbate and glutathione (Singh *et al.* 2011). The present findings are in close proximity with the results reported by Singh *et al.* (2013) who suggested significantly increased peroxidase activity with triple inoculation of the bioagents *Rhizobium* RL091+*Pseudomonas* PHU094+ *Trichoderma* THU0816 by 1.40-fold in

chickpea. A number of studies have confirmed that any of the discrepancy adept with SR defense via inoculation with PGPR can be overcome by using multiple strains (Pii *et al.* 2015).

The phenolics accumulation is one of the most crucial defense reactions in response to the fungal pathogens. The total phenol content (Fig. 2) was assayed for all the dual treatments using tannic acid as standard both before and after the appearance of disease symptoms. Maximum phenolics before the disease appearance was recorded with dual inoculant treatment LGR191+NE8 (100.56 tannic acid equivalents/g fresh weight of root) followed by LGR1+LPGPR-1 (99.52 tannic acid equivalents/g fresh weight of root). A significant upliftment ($p = 0.05$) in the activity with the onset of disease was recorded in all the dual inoculants treatments with maximum phenol content in dual inoculant treatment of LGR191+NE8. Recommended consortium inoculants LGR1+LPGPR-1 also produced superior phenolics as compared to chemical control treatment.



Data are mean of three replications. Different alphabets indicate significantly different means.

Fig. 2. Synergistic effect of *Mesorhizobium* sp. and PGPR (*Pseudomonas* sp.) on total phenolics.

Phenols act as antimicrobial, structural barriers, growth inhibitors, modulators of pathogenicity and activators of plant defense genes. Antagonistic potential of *P. fluorescens* strain 2-79 (NRRL B-15132) suppressing take-all disease of wheat was credited to the production of 2-acetamidophenol by Sarma *et al.* (2015). Combined inoculation of the biocontrol agents would work in an additive manner to accelerate the phenolics response against the evading pathogen. The present results are in agreement with Hemissi *et al.* (2013) who reported that the phenolics increased slowly reaching its peak at the 21st day of the infection with inoculation of bacterial isolate *PchAzm* over the control treatment. Co-inoculation of the roots with *Rhizoctonia solani* increased total phenolics level which was highest after three weeks of the fungal infection. Isoflavonoid like compounds such as phytoalexins interrupt metabolism or cellular structure of pathogens. Furanocoumarins common flavonoids like compounds are activated by ultraviolet light and can be extremely toxic and can lead to rapid cell death (Rao *et al.* 2015).

The improved activity of antioxidants is an excellent indicator of the ROS production and a build-up of a defensive machinery to diminish the oxidative damage triggered by the biotic stress experienced by the plants. The main facets of this changed host metabolism are the stimulation of a structural response at the sites of pathogen entry i.e. roots and atypical upsurge of electron-dense molecules in affected areas.

The phytopathogen retardation by potential antagonists applied in dual inoculation was assessed by recording the percent wilt control of all the treatments at 80, 100 and 120 days after

sowing (Table 1). A numeric increase in percent wilt control was observed for all the dual inoculants over recommended chemical fungicide as control. At 80DAS, 96.5% wilt control was recorded with LGR191+NE8 dual inoculation which was higher than chemical control treatment (91.9%). At 100 DAS, treatment LGR191+NE8 (95.9%) showed superior wilt control followed by recommended consortium of LGR1+LPGPR-1 (92.6%). The chemical control treatment of recommended fungicide showed 90.04% wilt control at 100DAS. After 120 days of sowing, significant rise (p=0.05) in the fungal suppression was documented with the dual inoculants. The present results are supported by the findings of Singh *et al. (*2016) who observed a reduction in wilt disease by 69% over the control treatment by the use of the biocontrol agents.

Table 1.Synergistic effects of *Mesorhizobium* sp. and PGPR (*Pseudomonas* sp.) on percent wilt control in chickpea.

Treatments	80DAS	100DAS	120DAS
LGR 1+LPGPR-1	94.93^{c}	92.57^{c}	94.17^{b}
LGR 1+NE8	95.50^{b}	94.40^{b}	93.60^{c}
LGR 191+LPGPR-1	94.42^{d}	92.57^{c}	92.88^{d}
LGR 191+NE8	96.53^{a}	95.89^{a}	95.77^{a}
Control (Recommended fungicide)	91.89^{e}	90.04^{d}	91.70^{e}

Data are mean of three replications. Different alphabets indicate significantly different mean values.

Foliar application of chemical fungicide to control the wilt disease caused by *Fusarium oxysporum* sp. *ciceris* is a widespread practice. But owing to an increased interest towards integrated disease management strategy which applies the concept of substituting the sole usage of chemical fungicides with the unified employment of biological and chemical tools for disease eradication is gaining wide acceptance. The study of diverse allelochemicals derived from the rhizospheric and endophytic microbes would provide greater understanding into the disease resistance mechanisms conferred by the host plant. Further comprehensive research into the mechanism of action of these allelochemicals would open multiple facets for disease suppression and pathogen elimination.

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