Int.J.Curr.Microbiol.App.Sci (2018) *7*(8): 4049-4057

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 08 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.708.420

Screening and Testing the Effect of Biocontrol Agents (*Bacillus* sp.) and *Fusarium oxysporum* f. sp. *ciceri* strains (Wilt) on Plant Defense Enzymes in Chickpea

Kiran K. Reddy* and K. Annapurna

Division of Microbiology, IARI, New Delhi-110012, India

**Corresponding author*

Keywords

Wilt, Days after germination (DAG), Chlorophyll

Article Info

Accepted:
22 July 2018
Available Online:
10 August 2018

A B S T R A C T

Wilt (*Fusarium oxysporum* f. sp. *ciceris*) is considered as one of the major factors for low productivity of chickpea (*Cicer arietinum* L.). In order to address the problem, germinated seeds of 'Desi' chickpea cv. JG-62 were inoculated with a conidial suspension (root dip) of highly virulent *Fusarium oxysporum* f. sp. *ciceris* (Foc) race 4, three days after germination (DAG). The extent of disease suppression was studied with two bacterial biocontrol agents B-36 and MSUC-2. Out of seventeen bacterial isolated screened for antifungal activity, strain B-36 reported maximum inhibition zone of 15 mm with fungal strain 101, which has shown maximum virulence (55.6% reduction in plant stand). Chlorophyll and nitrogen content has reduced by 25.2% and 66% respectively at 30 DAS in pathogen inoculated treatment. β 1,3 glucanse, chitinase, phenylalanine ammonia lyase activities had 1.9, 2.02 and1.72fold increase respectively in B-36 treated seeds compared to pathogen only treated seeds. 16S rDNA sequencing of both the bacterial cultures identified them as *Bacillus subtilis* (Acc No: KX 503819) and Bacillus sp (Acc No: KX 503820) respectively

Introduction

Chickpea (*Cicer arietinum*) is one of the most important food legumes grown worldwide, especially in dry areas of the Indian subcontinent (Saxena, 1990) and is one of the most important pulse crops cultivated in many countries of Asia and Africa. In addition to its importance as a food crop, it is valued for its beneficial effects in improving soil fertility and thus sustainability and profitability of production systems (Siva Ramakrishnan *et al.*, 2002). Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is a major factor restraining chickpea production worldwide. The disease is widespread in chickpea-growing areas of the world and is reported from at least 33 countries, causing 10–15% annual losses. The use of resistant cultivars is one of the most practical and cost-effective strategies for managing *Fusarium* wilt, but deployment of resistant varieties has not been extensive because of undesirable agronomic characteristics. Moreover, the high pathogenic variability in *F. oxysporum* f. sp. *ciceris* may limit the effectiveness of resistance (Haware and Nene 1982). These races are differentiated based upon their wilting symptoms caused by

them in the host plants. The most effective and practical method for management of the disease worldwide is the use of resistant cultivars (Jalali and Chand, 1992). However, the effectiveness of host resistance is curtailed by the occurrence of various pathogenic races. Seven Foc (*Fusarium oxysporum*f. sp. *ciceri*) races (0–6) have been identified (Jiménez-Díaz *et al.,* 1993). Races 1–4 were first described in India (Haware and Nene, 1982). Later, race 0 was reported in California (USA), Israel, Lebanon, and races 1 and 6 were identified in California, Israel, Morocco and Spain. Race 5, the most virulent of the races occurring in Spain, also occurs in California (Halila and Strange, 1996). The first objective of this study is to isolate different strains of *Fusarium oxysporum* f. sp. *ciceri* and study their virulence under pot trials. The second objective of this study is to select the best antagonistic bacterial agent against the fungus and to study their interaction effect on the plant defense enzyme perturbations.

Materials and Methods

Isolation of fungal strains

Total six strains of *Fusarium oxysporum* f. sp. *ciceri* belonging to the race 4 were collected from different regions of Andhra Pradesh and Sick plot of IARI. Wilted chickpea plants were surface sterilized with 0.1% $HgCl_2$ for 1 min. and the associated fungus was isolated on potato dextrose agar (PDA) (potato200 g, dextrose 20 g, agar 20 g and water 1 l) medium. Single spore culture of fungus was obtained by serial dilution method. The pure culture of the fungus was multiplied on autoclaved sorghum seeds in incubator at 28±1°C for 7 days (Fig. 2).

Validation of collected fungal strains

Fungal strains were validated for their identity of Fusarium genus by conforming under microscope (Carl Zeiss microscopy Inc. Axiocam 506 mono). Spore suspension in sterile milliQ water was prepared and observed for macro- and microconidia.

Screening for best virulent fungal strain

Desi chickpea variety JG-62 (Susceptible to fusarium wilt) along with cv. BG-212 (resistant to fusarium wilt) were used for screening. Seeds were surface sterilized in 0.1 % $Hgcl_2$ followed by two washes of 70% ethanol and seven washes of sterile water. Sterile pot culture mix (2:1:1 soil: sand: vermiculite) was used as base for filling in 4" plastic pots. Three seeds/pot were sown after germination. Different dose of inoculum was used ranging from 0 to 60 g inocula/kg potting mix. Each gram of inoculum (on sorghum base) consisted of 2.37 x 10^7 conidia, as measured by Hemocytometer.

Screening for best antagonistic bacterial agent

Total seventeen bacterial isolates were screened on potato dextrose agar for the zone of inhibition. A stab of most virulent fungal culture (Foc str. 101) was used. A streak of bacterial cultures maintained on the slant were used. The plates were incubated for one week at 28±2 °C

Pot experiment to unravel the tri-way communiqué vis-à-vis host plant, pathogen and antagonistic agent

A pot experiment was conducted with seven treatments (Table 2) in glasshouse at 28 °C. Three sterilized seeds/pot were used. 20g fungal inoculum (Foc str. 101) per kg of potting mix (2:1:1 soil: sand: vermiculite) was used, three days before sowing. Seeds were treated with *Bacillus sp.* cultures (B-36 and MSUC-2), which were grown in nutrient broth for 48 h at 37 °C in shaking incubator of 180rpm, till O.D of 0.8 was achieved.

Observations like chlorophyll content and nitrogen% in shoot and defense related enzymes were measured at 30 DAS.

Assay of enzyme activities

Peroxidase activity was assayed spectrophotometrically (Hartee 1955). The reaction mixture has 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature.

The change in absorbance at 420 nm was recorded at 30 sec intervals for 3 min and the boiled enzyme preparation served as blank. Phenylalanine ammonia lyase (PAL) assay was done as per the method described by Ross and Sederoff (1992).

The assay mixture containing 1 ml of enzyme, 5 ml of 50 mM Tris HCl (pH 8.8) and 6ml of 1mM L-phenylalanine was incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later 0.15 ml of toluene was added, vortexed for 30 sec, centrifuged (1000 rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. A standard curve was drawn with graded amounts of cinnamic acid in toluene

β-1, 3-glucanaseenzyme activity was colorimetrically assayed (Pan *et al.,* 1991). Crude enzyme extract of 6.25ml was added to 6.25 ml of 4% laminarin and incubated at 40°C for 10 min. The reaction was stopped by adding 3.75 ml of dinitrosalicylic acid (DNS) and heated for 5 min on boiling water bath (DNS prepared by adding 300 ml of 4.5% NaOH to 880 ml containing 8.8 g of DNS and 22.5 g potassium sodium tartarate). The resulting coloured solutions were diluted with distilled water, vortexed and the absorbance was read at 500 nm. The crude extract preparation mixed with laminar in at zero-time incubation served as blank. The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988). Reagents used consist of colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer. Chlorophyll content of the shoot was estimated by the technique of Arnon (1949) and nitrogen content of the shoot was estimated by the technique of Lindner (1944).

Results and Discussion

Anti-fungal activity of isolates

17 bacterial isolates were used from different sources to test their antifungal activity against *Fusarium oxysporum* f. sp. *ciceri* isolate 101 (Race 4). Out of 17, only 10 (59%) showed inhibition, rest 7 (41%) didn't exhibit inhibition zones (Table 1). Only 2 (11.7%) bacterial strains B-36 and MSUC-2 exhibited and inhibition zone above 1 cm and B-36 topped the list with maximum inhibition zone of 1.5 cm (Fig. 1). 7 isolates (41%)showed zone of inhibition $\geq$ 0.5 cm.

Several *Bacillus* spp. are known to suppress the soil-borne pathogens by various mechanisms viz., production of a wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with the pathogen for nutrient and for occupation of infection court, induced resistance, production of protease and fungal cell wall degrading enzymes (Perello *et al.*, 2003).

Selection of virulent fungal strain

Isolation of different Fusarium isolates (6 no.) from chickpea growing regions of India was done. The isolation was done from infected chickpea roots, following washing and surface sterilization on PDA media. The isolates were confirmed as *Fusarium* sps. based on observations of micro and macro-conidia

under 40 X microscopy (CarlZeiss) (Fig. 4). These isolates were tested for disease incidence and severity % in glass house. This is done to select the most virulent strain and also to standardize the inoculums dose. str. 101 has shown strong virulence, followed by str. 101> str. 105> str. 21> str. 52> str. 33= str. 38 in decreasing order of virulence.

Effect of different fungal strains on Germination % and plant stand % and optimization of inocula dosage

Out of the six fungal strains, strain 101 exhibited 10 % reduction in germination% at 4DAS and 54.6% reduction in plant stand at 30DAS (Table 2).

Inocula at high dosages (40 g/kg and 60 g/kg planting media) completely inhibited the chickpea growth. So 20 g/kg was selected was considered as optimum dosage for conduct of experiments with *Fusarium oxysporum* f. sp. *ciceri* strain 101 (Fig. 3).

Evaluating the effect of biocontrol agents on disease control parameters

Pot experiment was conducted with sterilized potting mixture (soil + vermiculite) and inoculated with fungal pathogen (one week before sowing). 4 seeds/pot are sown. Chlorophyll content was found to be highest when chemical seed treatment was done (T_6-8.99 mg/g FW) followed by *Mesorhizobium sp.* treated seed (T_2-7.58 mg/g FW). Lowest chlorophyll content was found with MSUC-2 treated seed (T_5-2.75 mg/g FW). B-36 treated seed reported 29.9% more chlorophyll content than pathogen only treated seeds (Fig. 5). Nitrogen content was found to be highest in T_1 (1.26 mg/g FW) and lowest in T_3 (0.42 m/g FW). B-36 treated seed has nitrogen content at par with T_1 (Fig. 6). Similar results were reported on fusarium wilt of lentil (Ahmed D *et al.*, 2017). The levels of different enzymes like β-1,3-glucanase, chitinase, peroxidase and phenylalanine ammonia lyase were analysed in root tissues at 30 DAS after biocontrol and pathogen inoculation.

Table.1 Zone of inhibition of different bacterial strains against *Fusarium oxysporum f. sp. ciceri* isolate 101

S. No	Culture No.	Zone of Inhibition (cm)
1	L-23	No inhibition
2	B-16	No inhibition
3	B-26	No inhibition
4	B-29	No inhibition
5	B-36	1.5
6	HKA-15	0.1
7	Wi-21	0.5
8	MSUC-2	1.1
9	Wi-2	0.3
10	HKA-121	0.8
11	B-33	No inhibition
12	F1-B	No inhibition
13	Wi-9	0.7
14	B-48	No inhibition
15	B-50	0.4
16	L-18	0.9
17	L-8	0.8

Table.2 Effect of *Fusarium oxysporum f. sp. ciceri* race 4 strains on germination and plant stand

Strain Name	Source	Germination (%) at 4 DAS	Plant stand (%) at 30 DAS
str. 101	Div. Of Plant Pathology	90	44.4
str. 105	Isolated from IARI soil (Sick plot)	100	66.6
str. 52	Isolated from IARI soil (Sick Plot)	100	77.7
str. 21	Chickpea grown region of A.P (Kurnool)	100	66.6
str.33	Chickpea grown region of A.P (Gadwal)	100	100
str. 38	Chickpea grown region of A.P (Maldakal)	100	100

Table.3 Effect of different treatments on enzyme activities in root at 30 DAS

Treatment	β-1,3 glucanase (µg of glucose/g fresh wt./min)	Peroxidase (change in absorbance/g fresh wt./min)	Chitinase (µg of glucose/g fresh wt./min)	Phenylalanine Ammonia lyase (n.mol of transcinnamic acid/g fresh wt./min)
T1-SV (control)	4.13	0.79	7.32	11.81
T2-SV+CPK-18	4.04	0.69	3.71	11.48
T3-SV+PG	6.91	2.97	8.98	17.68
T4-SV+PG+B-36	13.19	1.32	18.16	30.42
T5-SV+PG+MSUC-2	8.68	1.41	11.59	21.45
T6-SV+PG+Benlate @0.15 %	6.75	0.26	3.9	16.08
T7-RV+PG	18.45	0.18	13.50	26.14
C.D @ 5%	0.92	0.28	1.24	2.01

SV-Susceptible var. (JG-62); RV-Resistant variety (BG-212); Fungicide-Benlate at 0.15 %; PG-Pathogen; BCA-Biocontrol agent

Fig.1 Exhibits A, B, C, D showing Inhibition zones of various isolates

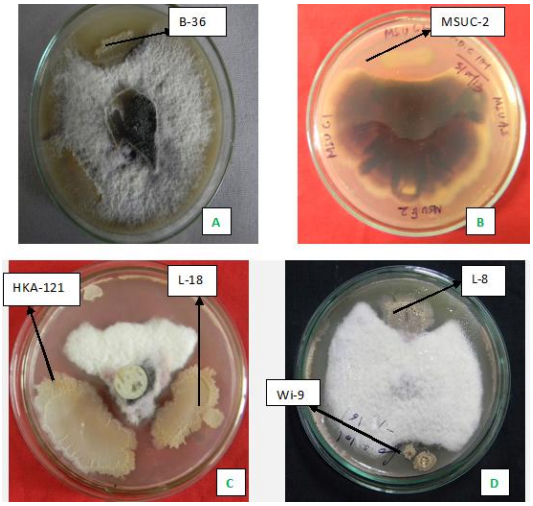


Fig.2 Fusarium fungus is grown on autoclave sorghum seeds. Spore count ranged between 2-6 x 10^7 spores/gm of sorghum seeds

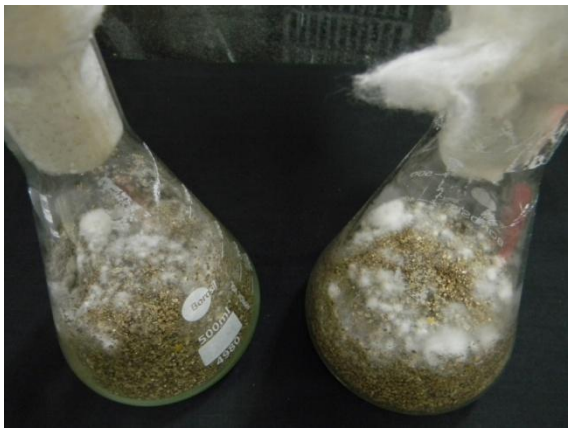


Fig.3 Effect of variable inocula dosage of *Fusarium oxysporum* f. sp. *ciceri* strain 101 on growth of chickpea. A) Only Susceptible variety JG-62 without inocula. B, C, D represent 20, 40, 60 g of inocula per kg of growth/planting media respectively

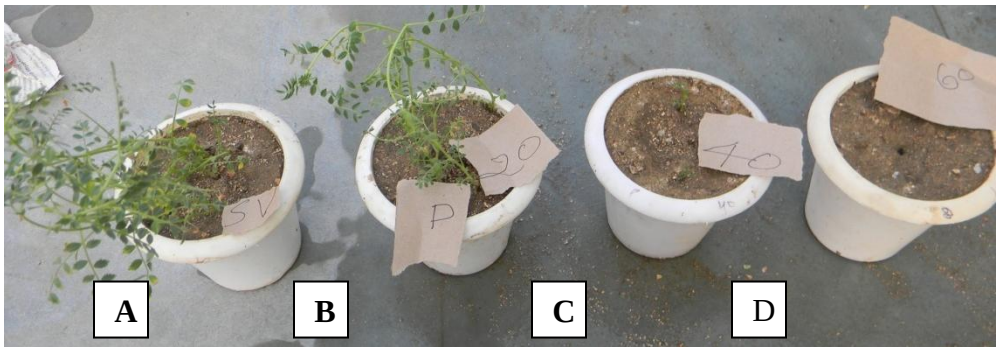


Fig.4 Micro- and macroconidia of different Fusarium strains observed under 40 X (Carl Zeiss microscopy Inc. Axiocam 506 mono) A) str. 101 B) str. 105 C) str. 52 D) str. 21 E) Str.33 F) str. 38

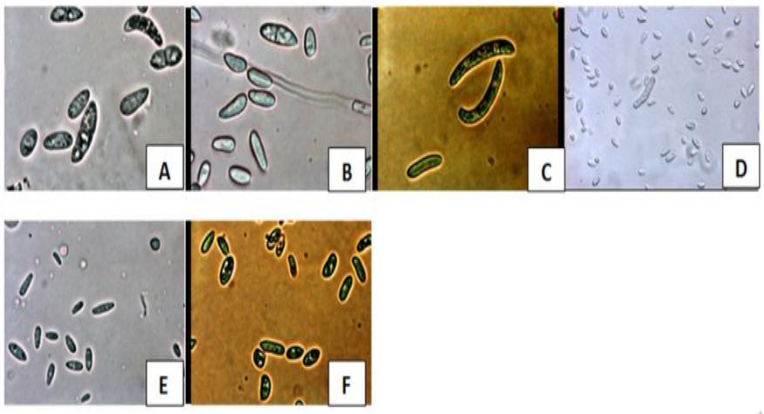


Fig.5 Chlorophyll content of shoot at 30 DAS

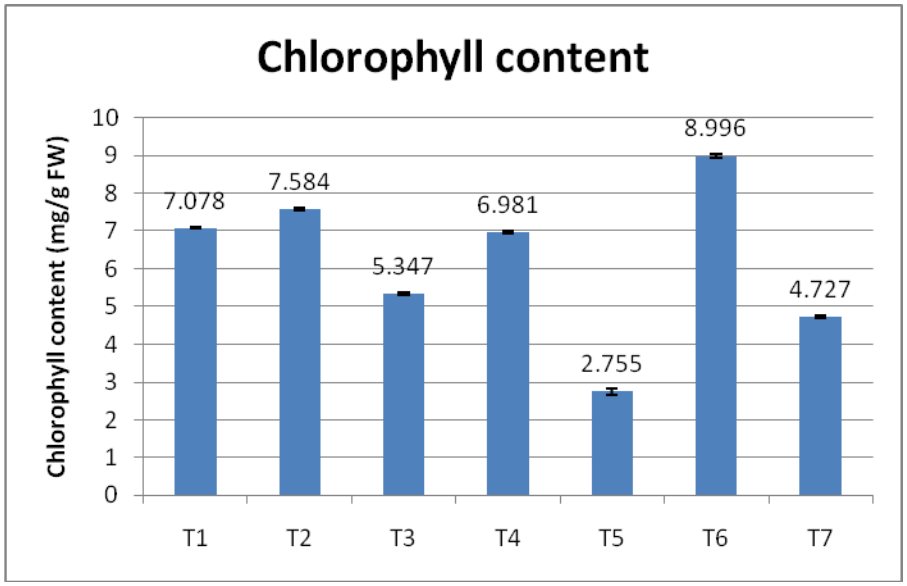
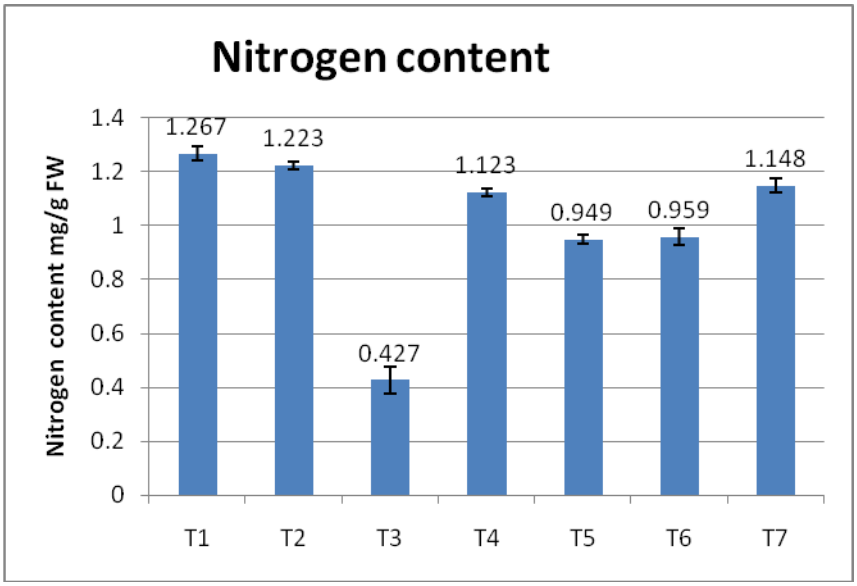


Fig.6 Nitrogen content of shoot at 30DAS



All the enzymes except peroxidase was found to be up surged by about 72-102% in B-36 treated seed compared to pathogen only treated seed. In T_7 treatment, there is a 1.9-3.1 fold increase in enzyme activities (Table 3).

This may to due to early triggering of the phenylpropanoid pathway. Similar results were reported in Rhizobium treated chickpea seed (Arfaoui *et al.*, 2005). Das *et al.*, (2003) has stressed that peroxidase enzyme is a key enzyme of the phenyl propanoid pathway, activated in response to pathogen infection. Changes in the activity of phenoloxidizing enzymes including peroxidise, plays a role in the regulation of metabolic pathways in diseased or injured tissues (Mehrotra and Aggarwal, 2003).

Fusarium oxysporum f. sp. *ciceri* race 4 strains vary widely with respect to their virulence abilities. Although chemical control and use of resistant varieties found to be effective in controlling the wilt in our experiment, they cause environmental damage and genetic resistance breakdown respectively. So, Biocontrol of wilt by seed treatment with B-36 strain in susceptible cultivars like JG-62 appears to be cheap and effective option.

Acknowledgment

The Author is thankful to DST for providing INSPIRE fellowship during the course of Ph.D work

References

Ahmed, D., and Shahab, S. 201. Effect of different inoculum levels of' *Fusarium solani'*(Mart.) sacc on plant growth, biochemical and nutrient parameters of lentil (*'Lens culinaris'* Medik.). *International Journal of Agriculture, Environment and Biotechnology*, *10*(2), 239.

Arfaoui, A., Sifi, B., El Hassni, M., El Hadrami, I., Boudabbous, A., and Chérif, M. 2005. Biochemical analysis of chickpea protection against Fusarium wilt afforded by two Rhizobium isolates. *Plant Pathology Journal*, 4(1), 35-42

Arnon D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology* 24: 1-15

Boller T, Mauch F. 1988. Colorimetric assay for chitinase. *Meth Enzymol* 161: 430435.

Das S., Aggarwal R., Singh D.V. 2003. Differential induction of defense related enzymes involved in lignin biosynthesis in wheat in response to spot bloth infection. *Indian Phytopathol.*56 (2): 129–133.

Halila MH, Strange RN, 1996. Identification of the causal agent of wilt of chickpea in Tunisia as *Fusarium oxysporum* f. sp. *ciceris* race. *Phytopathologia Mediterranea* 35, 67–74.

Hartee EF. 1955. Haematin compounds. In: Peach K, Tracy M, editors. Modern methods of plant analysis. New York: Springer-Verlag. pp 197-245.

Haware MP, Nene YL, 1982. Races of *Fusarium oxysporum* f. sp. *ciceris*. *Plant Disease* 66, 809–10.

Jalali BL, Chand H, 1992. Chickpea wilt. In: Singh US, Mukhopadhayay AN, Kumar J, Chambe HS, eds. *Plant Diseases of Cereals and Pulses*. Englewood Cliffs, NJ: Prentice Hall, 429–44.

Jiménez-Díaz RM, Alcalá-Jiménez AR, Hervás A, Trapero-Casas JL, 1993. Pathogenic variability and host resistance in the *Fusarium oxysporum* f. sp. *ciceris*/*Cicer arietinum* pathosystem. In: Arseniuk E, Goral T, eds. *Fusarium Mycotoxins, Taxonomy, Pathogenicity and Host Resistance. Proceedings of the 3rd European Seminar*. Radzikov, Poland: Plant Breeding and Acclimatization Institute, 87–94.

Lindner R.C., 1944. Rapid analytical method for some of the more common inorganic constituents of plant tissues. *Plant Disease* 66: 9-14.

Mehrotra R.S., Aggarwal R.S. 2003. Plant Pathology. Tata McGraw Hill Publishing Company Limited, New Delhi, 846 pp

Pan SQ, Ye XS, Kuc J. 1991. Association of β-1, 3-glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. PhysiolMolec Plant Pathol 39:25-39.

Perello A., Monaco C., Simon M.R., Sisterna M., Dalbello G. 2003. Biocontrol efficacy of *Trichoderma* isolates for tar spot of wheat in Argentina. Crop Prot. 22 (7): 1099–1106

Ross WW, Sederoff RR. 1992. Phenylalanine ammonia lyase from loblolly Pine: Purification of the enzyme and isolation of complementary DNA clone. Plant Physiol 98: 380-386

Saxena MC, 1990. Problems and potential of chickpea production in the nineties. In: *Chickpea in the Nineties. Proceedings of the Second International Workshop on Chickpea Improvement, 4–8 December 1989.* Patancheru, India: ICRISAT, 13–27

Singh, K. B. and Dahiya, B. S., Breeding for wilt resistance in chickpea. In Symposium on Problem and Breeding for Wilt Resistance in Bengal Gram, IARI, New Delhi, September 1973, pp. 13–14

Sivaramakrishnan, S., Kannan, S. and Singh, S. D., Genetic variability of *Fusarium* wilt pathogen isolates of chickpea (*Cicer arietinum* L.) assessed by molecular markers. *Mycopathologia*, 2002, 155, 171–178

How to cite this article:

Kiran K. Reddy and Annapurna, K. 2018. Screening and Testing the Effect of Biocontrol Agents (*Bacillus* sp.) and *Fusarium oxysporum* f. sp. ciceri strains (Wilt) on Plant Defense Enzymes in Chickpea. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 4049-4057.
doi: https://doi.org/10.20546/ijcmas.2018.708.420