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ORIGINAL ARTICLE

Destructive mycolytic suppression of *Fusarium oxysporum* causing wilt in chickpea by fungicide tolerant actinobacteria

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Abstract

The present study is aimed to evaluate the integrated effects of the chemical fungicide Bavistin (carbendazim 50% WP) on adaptive variants of rhizospheric actinobacteria in providing protection to the chickpea from wilt caused by *Fusarium oxsysporum.* Actinobacterial isolates recovered from the rhizospheric region were screened for their antibiosis against the fungal pathogen *F. oxysporum*. Among these actinobacteria two potent antagonistic isolates, *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01, showed a significant profile by producing extracellular lytic enzymes, hydrogen cyanide, siderophore, indole acetic acid (IAA) and solubilizing phosphate. Both of them caused hyphal deformation in *F. oxysporum* as observed through scanning electron microscopy (SEM). Before applying a blended form of biological agents and the chemical in the field, bavistin adaptive variants of both the actinobacterial isolates were obtained. Thereafter EC50 of bavistin to kill *F. oxysporum* was determined and applied with actinobacterial isolates during field trials. The effect of EC50 of Bavistin on the specific growth rate of isolates was also examined, which showed enhanced growth of isolates at concentrations close to EC50 of bavistin. During field trials, unsurpassed results were obtained using blends of the actinobacterial consortium with a low dose of chemical fungicide. This combination led to an increase in wilt protection by 2.66% and grain yield by 8.69% over full dose of chemical fungicide. These results advocate the efficiency of integrated formulation containing *Nocardiopsis* sp. KWC01, *Streptomyces* sp. KBR01 and low dose of bavistin in wilt management and productivity enhancement of chickpea plants.

Keywords Rhizospheric actinobacteria · *Nocardiopsis* sp. · *Streptomyces* sp. · *Fusarium oxysporum* · Chickpea

Introduction

Plant diseases and abiotic stresses have largely enervated the worldwide agricultural productivity (Atkinson and Urwin 2012). Chemical fungicide application and the use of resistant crop varieties have been viewed as the major solutions to control plant diseases. However, constant use of chemical pesticides or fertilizers has resulted in environmental pollution, harmful effects on non-targeted beneficial organisms or resistance development in insect pests or phytopathogenic fungi (Ntalli and Menkissoglu-Spiroudi 2011). These chemical compounds not only limit the soil health but are also unsafe to plants, animals and humans. Therefore, in the present scenario an urgent need is the sustainable use of natural resources and protecting soil fertility while getting better agriculture production. In recent years, the application of environment friendly alternatives to chemical pesticides in the form of biocontrol or the use of integrated pest management (IPM) that promotes a low quantity of chemical pesticide with biological agents has been encouraged to achieve high yields.

There is a large constituency of researchers who back biological approaches to disease management, as they ensure the safety of the environment as well. The activity of biocontrol agents, however, is affected by many factors, including sensitivity to environmental fluctuations, age of

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the inoculum or limited range of pathogen species they can control (Deacon and Berry 1993; Whipps 2001). A blended approach including both chemical and biological approaches can overcome challenges posed by individual applications of chemical fungicides or biocontrol agents (Salman and Abuamsha 2012). In combined systems (chemical and biological), the results can be improved because if one of the two approaches loses its effectiveness for whatever reason, the other will reinforce. IPM focuses on minimum use of chemical pesticides and maximum dependence on natural regulatory mechanisms to keep pest populations below the level at which they can cause economic loss (Gray et al. 2009).

Chickpea (*Cicer arietinum* L.) is an important cool-season food legume grown in over 55 countries on an area of about 14 million hectares (FAOSTAT 2017). Presently chickpea is the second most important food legume in the world after common bean. In India it is the top most important food legume accounting 71% of the global chickpea production (Gopalakrishnan et al. 2017). In 2019, India produced 9,937,990 tonnes of chickpeas, which is the second highest production after year 2018 since 1961 (FAOSTAT 2019). Several diseases like Ascochyta blight, Fusarium wilt, Stem rot and Powdery mildew etc. are known to limit worldwide production of chickpeas, in which wilt caused by *Fusarium oxysporum* is one of the most important fungal diseases. Fusarium wilt is prevalent in almost all chickpea-growing areas of the world and its incidence varied from 14 to 32% in the different states of India (Dubey et al. 2010).

Management of Fusarium wilt of chickpea has been challenging due to its prolonged saprophytic and fungicide resistant properties and this makes its suppression in natural farming fields difficult. Currently, biological control of this soil and seed-borne plant pathogenic fungi has been addressed using bacterial and fungal antagonists but the role of biological antagonists in the presence of chemical fungicide is yet to be explored to a larger extent, particularly the use of rhizospheric actinobacteria. As plant disease control agents, actinobacteria are excellent choices due to their ability to produce a broad range of secondary bioactive metabolites, which are reported to be involved in direct or indirect biocontrol mechanisms (Cheng et al. 2015). These are more suitable to adjust to various soil environments because of spore production as compared to other bacteria (Yandigeri et al. 2012). Besides this, earlier studies have proved high tolerance of actinobacteria towards fungicides (Alekhya and Goplalkrishnan 2017; Gopalakrishnan et al. 2013).

Taking into account the aforementioned facts, an attempt was made to get a way to maximize the chickpea production and decrease wilt. To accomplish this, fungicide adaptive actinobacteria were isolated and the possibility of synergy between these isolates was examined. Furthermore, a comparative study was conducted to assess antagonistic rhizospheric actinobacteria alone and their effect when combined with chemical fungicides in disease control in order to reduce the amount of chemical fungicides used while maintaining high yields and disease control.

Materials and methods

Isolation and culture conditions of fungal pathogen and actinobacteria

Wilt infected roots of chickpea were procured from standing crop at local farmers' fields of Haridwar (29.95° N, 78.16° E), Uttarakhand, India. The fungus was isolated and characterized by comparing it with a known culture of *F. oxysporum*. Further, the identity of the fungus was re-confirmed by Agharkar Research Institute, Pune, Maharashtra, India. For the isolation of actinobacteria, rhizospheric soil from the healthiest plants was collected and isolates were obtained on Actinomycetes isolation agar (AIA) using the serial dilution method. The fungal and actinobacterial cultures were stored at 4 ºC for their further applications during experimental work.

Screening for potent antagonistic actinobacteria

The biocontrol activities of actinobacterial isolates against *F. oxysporum* were determined by the modified dual culture method as described by Gopalakrishnan et al. (2011) and radial growth inhibition of the fungus was recorded.

Study of post-interaction events through SEM

Small pieces of agar (> 1 cm) containing mycelia were picked out from the zone of interaction and collected in a petriplate. Specimens were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) at 4 ºC followed by washing thrice in phosphate buffer. The specimens obtained were dehydrated by serially passing through 70, 80, 90 and 100% ethanol and air dried as described by Lopez-Llorca and Valiente (1993). The samples were mounted on stubs followed by coating with gold, the coated specimens were observed at 15 kV in a LEO 435 VP scanning electron microscope.

Characterization and identification of actinobacteria

The selected actinobacterial isolates were characterized on the basis of phenotypic, physiological and biochemical characteristics (Wink 2012). The identity of the isolates was confirmed by 16 S rRNA gene sequencing. Genomic DNA of the isolates was extracted following Green and Sambrook

(2012). 16 S rRNA amplification was carried out using universal eubacterial primers 27 F 5′ AGAGTTTGATCMTGG CTCAG 3′ and 1492R 5′ TACGGYTACCTTGTTACG ACTT 3′. All the sequences were compared with 16 S rRNA gene sequences available in the GenBank databases of NCBI by BLASTn search and phylogenetic analysis was performed using MEGA7 (Kumar et al. 2016). Accession numbers were obtained for isolates by submitting the 16 S rRNA sequences to GenBank Database.

PGP attributes and mycolytic enzyme production

The plant growth promoting attributes such as the production of indole acetic acid (IAA), siderophore and phosphate solubilization in actinobacterial isolates were assessed following Kumar et al. (2010). For the estimation of mycolytic enzyme production culture supernatants of the screened actinobacterial isolates were determined as a source of enzymes. Chitinase (Reisslig et al. 1955), protease (Meyers and Ahearn 1977) and β-1, 3 glucanase (Singh et al. 1999) activities were measured by determining the release of N-acetyl-D-glucosamine, tyrosine and glucose per hour, respectively. Hydrogen cyanide (HCN) production was determined by the method of Bakker and Schippers (1987).

Recovery of fungicide adaptive variants of actinobacteria

The selected antagonistic isolates KWC01 and KBR01 were grown in chemical fungicide amended media with different concentrations (10–5000 µg/ml) of the active ingredient of fungicide bavistin (BASF, India Ltd.) and the percentage mortality of antagonistic actinobacteria was recorded against different concentrations of bavistin. The sub-lethal (LC50) dose of the fungicide was calculated using probit analysis. The adaptive variants of KWC01 and KBR01 were obtained by raising them against the sub-lethal concentrations (LC50) of the fungicide by transferring the surviving colonies to culture medium and medium amended with sub-lethal (LC50) concentrations of bavistin (Saraf and Sood 2002). The mycolytic activities of chemical fungicide adaptive variants of KWC01 and KBR01were examined as described above.

Determination of 50% effective concentration (EC50) of chemical fungicide against *F. oxysporum*

The EC50 dose of chemical fungicide bavistin for inhibiting the spore germination in *F. oxysporum* was determined as described earlier by Lorito et al. (1994). *F. oxysporum* (10^5 conidia/ml) was incubated in potato dextrose agar medium amended with different concentrations of the fungicides (1–500 µg/ml) and incubated at 30 °C for 96 h. The samples from each concentration were analyzed for spore germination.

The EC50 dose of the fungicide for the reduction in fungal biomass was also determined. The reduction in fungal biomass was measured against different concentrations of the fungicide as described by Singh and Chhatpar (2011). EC50 dose for inhibition of fungal spore germination and fungal biomass reduction was determined by regression analysis of dose response plot of fungicide concentration vs. fungal reduction.

Effect of fungicide and antagonistic actinobacteria on seed germination in vitro

Seeds of chickpea var K850 of similar shape and size were surface sterilized with 70% ethanol (3–5 min) with intermittent washes using sterile distilled water. For seed treatments with actinobacteria, 50 ml suspension (1×10^8 spores ml^{-1}) of KWC01 and KBR01 prepared in 1% methyl cellulose was used for coating 100 seeds and for fungicide seed treatment two different concentrations of bavistin viz. EC50 (25 µg ml^{-1}) and recommended dose (100 µg ml^{-1}) prepared in 1% methyl cellulose were used. After curing for about 4 h, the seeds were placed on water agar tubes containing 10^5 spores/ml of *F. oxysporum*. The tubes were incubated in the dark for 96 h and observed for germination. Agar tubes without fungal spores served as control.

Assessment of yield parameters and disease reduction

To evaluate the effect of *Nocardiopsis* sp. KWC01and *Streptomyces* sp. KBR01 that were applied singly or in different combinations, in disease suppression and growth promotion of chickpea plants, a field experiment was conducted in a rain irrigated and Fusarium sick plot at Haridwar, India (78° 16´ E, 29° 94´ N) during two consecutive seasons, 2016–17 and 2017–18. In both seasons, chickpea seeds were sown in the second week of October and harvested in the second week of March. Seeds of Chickpea var K850 were direct-seeded by hand-planting in a completely randomised plot design in eight sets of treatments with three replicates of each treatment: (I) non bacterized seeds and without chemical fungicide (diseased control), (II) non bacterized seeds and without chemical fungicide sown in wilt free plot (healthy control), (III) seeds bacterized with KWC01, (IV) seeds bacterized with KBR01 (V) seeds bacterized with strains KWC01 and KBR01 (VI) non bacterized seeds treated with recommended dose of chemical fungicide (VII) non bacterized seeds treated with EC50 of chemical fungicide (VIII) seeds bacterized with strains KWC01 and KBR01 + EC50 of chemical fungicide.

The recommended dose 100 µg/ml and EC50 25 µg/ml of the active ingredient of chemical fungicide were applied for seed treatment. Seeds were sown at the rate of 60–80 Kg/ha with a row-to-row spacing of 30 cm. Average depth for sowing seeds was 5–8 cm. The data were recorded after 30 days intervals till harvesting. During both seasons, harvesting was done after 150 days of sowing.

At the final harvest, pod number, grain yield, stover yield and harvest index were recorded. The data on disease incidence were collected at 30 days intervals from sowing till harvesting. The following formulae were used to calculate these parameters:

$$\text{Grain yield(t/ha)} = \frac{\text{GrainYield(kg) per subplot/1000}}{\text{Area of subplot}} \times 1000$$

$$\text{Stover Yield(t/ha)} = \text{Total Biomass Yield(t/ha)} - \text{Grain Yield(t/ha)}$$

$$\text{Total Biomass yield(t/ha)} = \frac{\text{Total Biomass Yield(kg) per subplot/1000}}{\text{Area of subplot}} \times 1000$$

$$\text{Harvest Index} = \frac{\text{Grain Yield}}{\text{Total Biomass Yield}} \times 100$$

$$\text{Disease Reduction (\%)} = \frac{\text{No. of plants infected in disease control} - \text{No. of plants infected in treatent}}{\text{No. of plants infected in treatent}} \times 100$$

Statistical analysis

All the experiments were independently repeated thrice. For in vitro evaluation of PGP attributes and mycolytic enzyme production by actinobacteria, data were analyzed using the analysis of variance (ANOVA) and means were separated using Fisher's protected least significant difference (LSD) test at 5% level of significance. For field trials, obtained data were analyzed statistically by using ANOVA and Fisher's LSD test to determine the statistical significance at $p < 0.05$ and $p < 0.01$.

Results

Isolation, screening and identification of actinobacteria

In total 16 morpho-taxonomically diverse rhizospheric actinobacteria were screened for their antagonistic ability against *F. oxysporum*. Based on preliminary testing, four actinobacterial isolates exhibited antagonism against the fungal pathogen (Table 1). These isolates produced chitinase and β-1, 3 glucanase, however, protease activity was observed in only two isolates KWC01 and KBR01. All the four isolates showed the ability to produce IAA in a range of 7.05–23.7 µg ml^{-1} and secreted HCN except the isolate KWB01. Only two actinobacteria, KBR01 and KWC01, were found active in phosphate solubilisation. Siderophore production was confirmed by the formation of orange halo of varying size around KWA01, KBR01 and KWC01 in the specific medium indicating positive results.

Among all these, two potent antagonistic actinobateria, KWC01 and KBR01 were selected for further study. Based on the molecular analysis of isolates KWC01 and KBR01,

Table 1 *In-vitro* evaluation of isolated actinobacteria for their multiple functions against fungal pathogen (+ve and –ve indicates positive and negative results, respectively)

Isolates	Fungal growth inhibition in Dual culture (%)	Chitinase (Uml^{-1} h^{-1})	β-1,3 Glucanase (Uml^{-1} h^{-1})	Protease (Uml^{-1} h^{-1})	HCN	IAA (μg^{-1})	Phosphate (μg^{-1})	Siderophore
KWA04	32.32^{d}	2.65^{e}	10.45^{c}	0.0^{a}	+ve	16.32^{c}	0.0^{a}	-ve
KBR01	64.28^{b}	3.56^{d}	13.53^{b}	6.33^{c}	+ve	18.41^{b}	15.5^{b}	+ve
KWB01	28.14^{e}	5.21^{b}	6.32^{d}	0.0^{a}	-ve	7.05^{d}	0.0^{a}	+ve
KWC01	45.23^{c}	4.6^{c}	11.19^{c}	8.23^{b}	+ve	23.7^{a}	12.23^{c}	+ve
Control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	-ve	0.0^{a}	0.0^{a}	-ve
SEM	2.56	0.13	0.23	0.38	–	0.56	0.35	–
LSD 5%	2.73	0.24	0.58	0.43	–	1.02	0.03	–

Values are mean of three replicates; means with the same letter within a column are not significantly ($P > 0.05$) different according to Fisher's protected LSD test

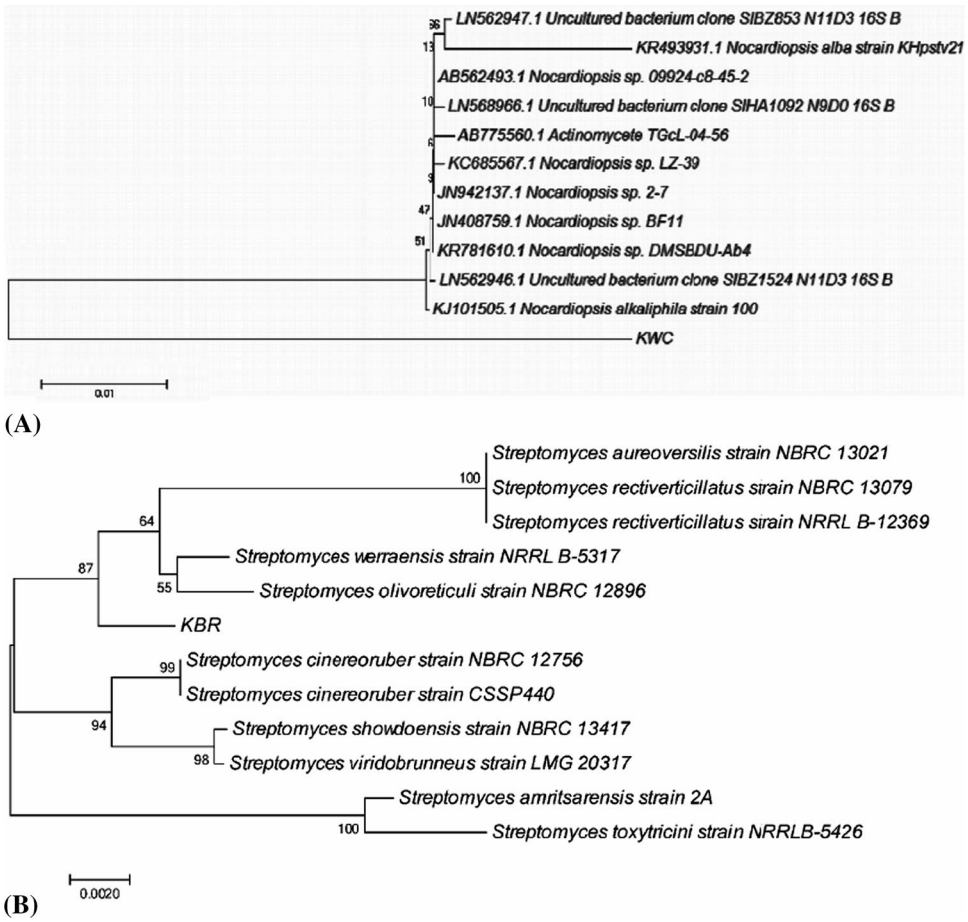

Fig. 1 Neighbour joining dendrograms using 16 S rRNA gene sequences of **a** KWC01 and **b** KBR 01

the closest homologues of these strains were found to be *Nocardiopsis* sp. 09924-c8-45-2 with 90% 16 S rRNA gene similarity and *Streptomyces werraensis* NRRL B-5317 with 99% 16 S rRNA gene similarity, respectively. According to the phylogenetic tree of both the isolates (Fig. 1a, b), KWC01 was identified as *Nocardiopsis* sp. and KBR01 as *Streptomyces* sp. The 16 S rRNA genes of isolates KWC01 and KBR01 were deposited in Gene Bank under accession numbers MF184926 and KY655215, respectively.

Study of post-interaction events by SEM

During interaction with *F. oxysporum*, both isolates viz. *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 caused deformities in *F. oxysporum*. Fungal hyphae showed perforation, lysis and distortion. No such abnormalities were observed in the control (Fig. 2). On the other hand when treated hyphae were transferred for their growth assessment on a fresh culture medium, no growth was observed indicating complete killing (fungicidal) of hyphae during the suppression process.

Recovery of fungicide adaptive variants of actinobacteria

Nocardiopsis sp. KWC01 and *Streptomyces* sp. KBR01 were checked for their susceptibility to different concentrations of bavistin. A graph was plotted based on these observations (Fig. 3) and the sub lethal (LC50) dose of bavistin was calculated using Probit analysis. LC50 of bavistin was found 34 µg/ml and 32 µg/ml for *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 respectively, as 50% mortality of the isolates was recorded at these concentrations. Fungicide adaptive variants of these isolates, that were grown by raising them at LC50 of the fungicide, showed that 20–50 µg/ml and 18–25 µg/ml of bavistin was observed optimal for the maximum specific growth rate of *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01, respectively (Fig. 4).

Determination of EC50 of chemical fungicide against *F. oxysporum*

The EC50 value of the fungicide was determined as the concentration that gives a response half-way between the baseline and maximal by fitting a dose-response curve. The EC 50 dose for fungal spore inhibition was 24.95 µg/ml and

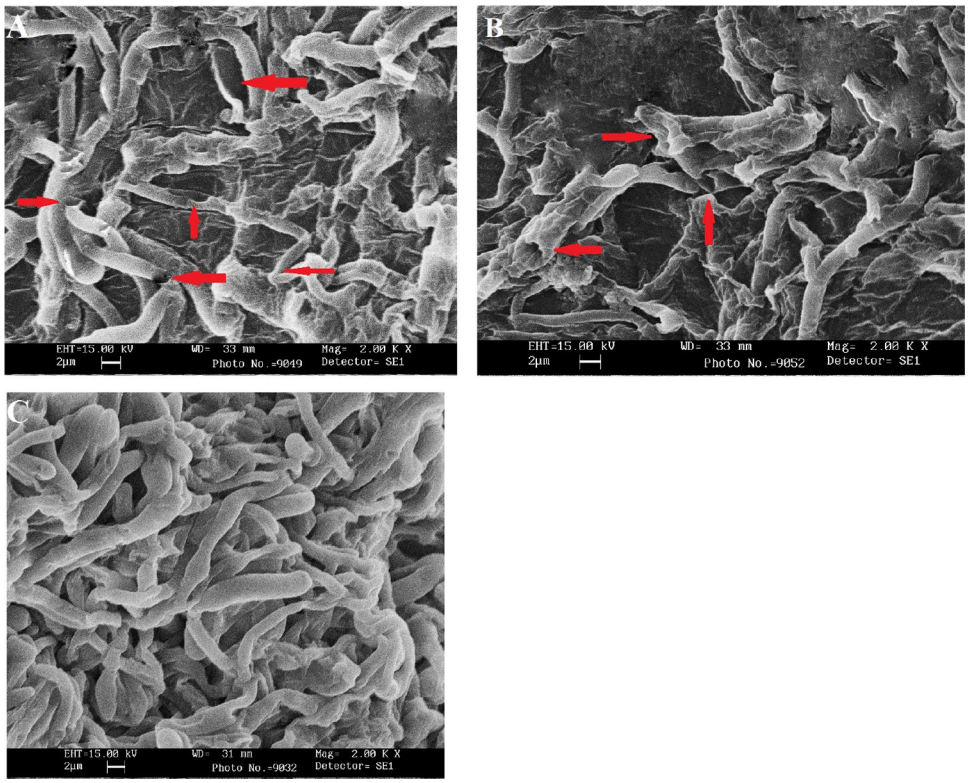

Fig. 2 Scanning electron microscopic photographs showing fungal mycelial destruction by antagonistic interaction effects of *Nocardiopsis* sp. KWC01 (**a**), *Streptomyces* sp. KBR01 (**b**) and Control (**c**). Arrows indicating the hyphal destruction, perforation and shrinkage

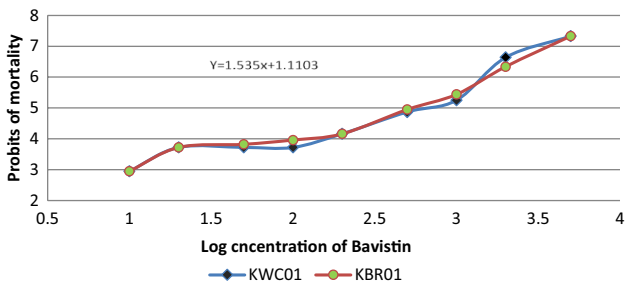

Fig. 3 Effect of graded concentration of bavistin on mortality of KWC01 and KBR01

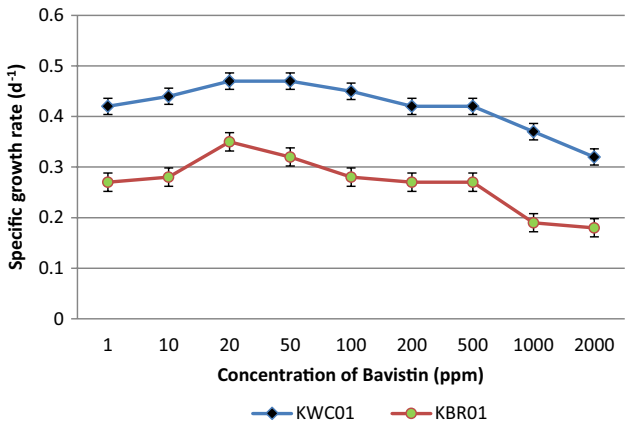

Fig. 4 Effect of graded concentrations of bavistin on specific growth rate of *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01. Bars represent ± standard error

25.05 µg/ml for fungal biomass reduction. The average concentration of 25 µg/ml was determined as the EC50 dose of the fungicides for fungus inhibition.

Effect of fungicide and antagonistic actinobacteria on seed germination in vitro

In fungal challenged conditions primed seeds of *C. arietinum* var K850 using KWC01 and KBR01 showed 76.6% and 80% seed germination respectively. On the other hand, the consortium of KWC01 + KBR01 led to seed germination up to 89.6%. However, maximum seeds germination was observed in treatments with the consortium (KWC01 + KBR01) + EC50 fungicide (95%) followed by the recommended dose of fungicide (93.3%). No seed germination was observed in untreated seeds, since the mortality of all seeds was observed.

Assessment of yield parameters and disease reduction

Both the actinobacterial isolates (KWC01 and KBR01) enhanced plant growth and disease reduction whether used individually, in consortium or blended with EC 50 of chemical fungicide, as compared to control. However, the effects of these actinobacteria were at their peak when blended with the EC50 of fungicide (Table 2). During season 2016-17 the highest grain yields were achieved in plants treated

Table 2 Effect of different treatments on crop yield and wilt reduction in chickpea plants 150 DAS

Treatments	No of Pods (plant^{-1})		Grain Yield (t ha^{-1})		Stover Yield (t ha^{-1})		Harvest index		Per-cent disease reduction over control	
	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18
KWC01	25.94**	25.02**	1.176**	1.104**	1.823**	1.8**	39.21	38.02	43.43	43
KBR01	28.6**	28.8**	1.35.4**	1.370**	1.981**	1.993**	40.6	40.74	57.12	57.32
Consortium	32.61**	33.32**	1.874**	1.881**	2.112**	2.198**	47.01**	46.11**	65.12	65.92
Full dose Fungicide	34.4**	32.4**	2.043**	1.995**	2.239**	2.205**	47.71**	47.49**	67.21	67.23
Ec50 Fungicide	21.04**	20.24*	1.104*	1.007	1.274	1.417**	46.43**	41.54	40.05	39.62
Ec50 fungicide + consortium	36**	38.12**	2.123**	2.266**	2.281**	2.418**	48.21**	48.37**	69	71
Healthy Control	25.23	26.13	1.169	1.158	1.798	1.712**	39.4	40.34	–	–
Diseased Control	15.01	13.91	0.574	0.487	0.986	0.936**	36.8	34.39	–	–
SEM	2.5	2.72	0.19	0.27	0.16	0.21	1.63	1.6		
LSD 1%	3.05	4.51	0.41	0.7	0.69	0.71	8.97	9.35		
LSD 5%	4.03	3.4	0.31	0.53	0.52	0.53	6.76	7.04		

Values are means of 10 randomly selected plants from each set

SEM standard error of mean, *LSD* least significance difference

*Significant at 5% LSD

*Significant at 1% level of LSD as compared to diseased control using analysis of variance (ANOVA) followed by Fisher's LSD. Consortium = KWC01 + KBR01

with the integrated application of actinobacterial consortium (KWC01 + KBR01) and EC50 fungicide. The observed disease reduction was also recorded highest (69%) in the plants treated with this combination.

During season 2017–18, again the treatments with KWC01 + KBR01 + EC50 fungicide resulted in better grain yield over other treatments. On the contrary, a decline in performance was recorded in treatments with the recommended dose of fungicide irrespective of their results in the previous year. A 71% disease reduction over control was observed in treatments with actinobacterial consortium + EC50 fungicide, while at that of recommended dose of fungicide was recorded at 67.23%.

Discussion

The main focus of the present research was to check the efficiency of the actinobacterial strains for suppressing the wilt in chickpeas. In search of an effective control measure, rhizospheric actinobactria were isolated and subjected to dual culture analysis against *F. oxyporum.* Based on in vitro evaluation, only two isolates out of sixteen (*Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01) expressed maximum potential to inhibit fungal growth. Such fungal suppressions may involve several mechanisms deployed by actinobacteria including the production of lytic enzymes like chitinase (Jog et al. 2014), β-1,3 glucanase (Sakdapetsiri et al. 2016) and protease (Palaniyandi et al. 2013), production of volatile HCN (Noori and Saud 2012) and siderophores (Macagnan et al. 2008). The release of siderophore not only suppresses the phytopathogen by sequestering iron in the rhizosphere but also helps the plant in uptaking metals such as zinc, iron, and copper (Kumar et al. 2019) and contributes to plant growth. In present study, the selected actinobacterial isolates were not only found to have the above mentioned antagonistic and indirect PGP activities, but also presented their potential in direct PGP activities such as IAA production and phosphate solubilisation (Table 1). Microorganisms possessing IAA production and phosphate solubilisation properties have been found to stimulate plant growth (Ali et al. 2020). In the present investigation, since the selected actinobacteria produced a variety of extra cellular mycolytic enzymes and growth-promoting hormones, it can be concluded that these isolates may serve as excellent biocontrol and PGP agents.

In the present study, isolates *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 caused mycelial deformities and hyphal degradation in *F. oxysporum*, which were observed through electron microscopic examination. Many strains of *Streptomyces* were reported to reduce the incidence of plant diseases caused due to fungi by inflicting abnormalities in them (Wang et al. 2013; Wu et al. 2015). In the present investigation, the fungal hyphae that were in contact with actinobacterial isolates showed perforation, lysis and distortion. Such hyphal abnormalities were also observed by Zhao et al. (2012) in *F. oxysporum* treated with *Streptomyces bikiniensis* HD-087. In addition to degrading enzymes secreted by actinobacterial isolates, secondary

metabolites could also be responsible for such hyphal abnormalities, as observed by Tamura et al. (2019). Moreover, in the present research when treated hyphae were transferred to a fresh culture medium, no growth was observed indicating the fungicidal nature of extracted metabolites. These findings are in accordance with Abdullah et al. (2021), who revealed the fungicidal effect of secondary metabolites from *Streptomyces plumbeus* isolate F31D against *F. oxysporum.*

Before using the biocontrol agent with chemical, mortality determination of actinobacteria in the presence of chemical fungicide is a prerequisite as described by Maheshwari et al. (2010). Although actinobacteria are prokaryotes and seem to be naturally compatible with bavistin, in our findings higher concentrations of this fungicide i.e. 3500 ppm proved toxic to both isolates, resulting in their complete growth loss. Fawole et al. (2010) also observed that the application of the Carbendazim-Mancozeb fungicidal mixture at a higher concentration reduced the actinobacterial population significantly. The effect of pesticides on microflora is affected by the chemical nature of the pesticide, frequency of use, dose levels, bioavailability and the mechanism of action (John and Shaike 2015).

To make an integration of actinobacteria with chemical fungicide, chemical adaptive variants of actinobacterial strains were developed by raising them repeatedly against the LC50 of the chemical fungicide. Repeated applications lead to adaptation of microorganisms towards carbendazim which is due to the growth of actinobacteria under selective pressure caused by bavistin as revealed by Yunlong et al. (2009). When no death was recorded at LC50 bavistin, isolates were further subjected to check the effect of chemical fungicide on their specific growth rate and it was observed that 20–50 µg/ml and 18–25 µg/ml of bavistin was found effective for the maximum specific growth rate of *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 respectively. Higher specific growth rates of these actinobacteria in the presence of bavistin can be explained by the fact that some bacteria can use pesticides as nutrients at particular concentration and therefore, grow nicely in presence of chemical pesticide. Fang et al. (2012) reported *Pseudomonas* sp. strain CBW utilizing carbendazim as the sole carbon and nitrogen source.

Considering the single application of actinobacterial isolates, *Streptomyces* sp. KBR01 showed better performance during *in vitro* and *in planta* experiments than that of *Nocardiopsis* sp. KWC01, which was probably contributed by production of high amount of either PGP metabolites or other hydrolytic enzymes by *Streptomyces* sp. KRR01 as also observed by Anusha et al. (2019) who found a direct correlation between experimental performance of the biocontrol agents and the amount of metabolite produced by them. Earlier many reports indicated PGP and disease suppressive potential of *Streptomyces* sp. Awla et al. (2017) recorded 80% reduction in rice blast caused by *Pyricularia oryzae* due to *Streptomyces* sp. UPMRS4. On the other hand Jacob et al. (2018) reported antifungal activity of *Streptomyces* sp. RP1A-12 against *Sclerotium rolfsii* and helped in the growth improvement of peanut plants.

In the current study, when both of these actinobacteria were applied jointly, results were improved rather than when they were applied individually. Co-inoculation of plant growth promoting bacteria or biocontrol agents has been reported to confer stimulatory effects of the microbes on each other leading to improved biological activities of the microbes which results into enhanced plant growth and disease suppression (Thakkar and Saraf 2015). However, it is not necessary that an additive or synergistic effect is achieved every time when a microbial consortium is used (Sarma et al. 2015). Cumulative synergistic effects of consortia have been observed in the present study, wherein mixed culture of *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 showed better results for crop yield and wilt reduction over individual microbial culture application. These results are in agreement with an earlier reported study by Jambhulkar et al. (2018), who observed that microbial consortia suppressed blast and bacterial leaf blight of rice in a synergistic mode of action. In the present study, significant plant growth promoting and biocontrol outputs from the actinobacterial consortium may be attributed to the cumulative effects of various bioactivities contributed by both the microorganisms participating in the consortium. In such interactions induction of silent pathways may result in the synthesis of novel secondary metabolites that are not produced in single-culture and may help to unmask hidden and poorly expressed metabolites.

The combined application of actinobacterial consortium (KWC01 + KBR01) and bavistin, during in vitro study resulted in maximum seed germination under fungal challenged conditions, suggesting the formation of a more effective protective layer around the seeds and preventing fungal invasion. Subsequently, in field trials, maximum wilt reduction was observed when applying the same combination. Moreover, enhanced grain yield revealed the significance of integrated use of co-inoculated actinobacterial isolates with a reduced dose of bavistin. As a result of this intervention, total grain yield was improved by 8.69% and stover yield by 5.73% compared with treatments with full doses of bavistin. Our results are in accordance with Dubey et al. (2015) who reported chickpea seeds treated with bio agent *Trichoderma harzianum* Pf 80 and *Mesorhizobium ciceri* along with vitavax in providing protection against Fusarium wilt and subsequently to attain higher yields. Abd-El-Khair et al. (2019) also reported successful controlling of *F. solani* and *F. oxysporum* in dry beans using the combined application of *Trichoderma* spp. and thiophanate-methyl. The blending of fungicide adaptive isolates KWC01 and KBR01, and

chemical fungicide bavistin worked synergistically. These effects may occur as a result of co-cultivation based secondary metabolite elicitation as observed by Singh and Chhatpar (2011).

The overall improved performance in the integrated application of actinobacteria and chemical pesticide also opens up the possibility of utilizing carbendazim as a source of carbon and nitrogen by actinobacteria. Earlier many reports depicted the involvement of actinobacteria in utilizing carbendazim as their energy source (Arya and Sharma 2015; Sun et al. 2014). *Nocardioides* sp. isolated from soil which has undergone repeated applications of carbendazim, exhibited its hydrolysis (Pandey et al. 2010). This enhanced degradation can be attributed to the stimulation of microbial activity and community structure in rhizosphere soil as stated by Xiao et al. (2013). These facts also provide a support to our results where we find an unexpected behaviour, in which the yield of chickpea got increased significantly in chemical fungicide applied treatments as compared to healthy control. This might be due to positive microbial-mediated ecosystem functions, in which other microflora present in the rhizosphere of chickpeas may be hydrolyzing chemical fungicide and providing nutritional elements to the plants for growth promotion. In our finding, the reduced dose of chemical fungicides with actinobacterial isolates proved more effective than full dose of chemical fungicide alone, in reducing Fusraium wilt and leading to increased yield in chickpea. Similar results were reported by Ruano-Rosa et al. (2018), where combining low concentrations of fluazinam and *Trichoderma* spp. led to control of avocado white root rot. Our results undoubtedly allow reduction in chemical use in the presence of *Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01 strains for field application without compromising the efficiency of integrated biocontrol.

Conclusion

Since farmers are dependent on synthetic fungicides to reduce food losses caused by fungi, they need to have an effective alternative to avoid negative impacts due to the extensive use of these fungicides. In recent years, the focus has been on developing biocontrol agents (BCA) as alternatives to conventional fungicides. But when a biological control is applied to the plant rhizosphere, competition with microflora other than the target species may occur and this fluctuation in the surrounding environment affects the activity of the biocontrol agent. Therefore, hybrid control measure like IPM is proposed by involving all the advantages of both approaches (BCA and chemical fungicides) while removing the cons of each. Utilizing an integrated management framework, in the present study, we have uncovered two potent actinobacterial strains (*Nocardiopsis* sp. KWC01 and *Streptomyces* sp. KBR01) that are compatible with the chemical fungicide bavistin and showed excellent PGP and disease suppressive properties against *F. oxysporum*. The consortium of these actinobacteria with a low dose of fungicide was not only found successful in combating Fusarium wilt in chickpea but also resulted in enhanced chickpea yield. Apart from this, further investigations, including long-term field trials are also needed in order to assess the applicability of biocontrol agents with a reduced dose of chemical fungicides in circumventing the inconsistent efficacy of biocontrol and resistance development in phytopathogens against the sole use of chemical pesticides.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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