The Pharma Innovation Journal 2022; SP-11(7): 3232-3236

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(7): 3232-3236
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www.thepharmajournal.com
Received: 25-04-2022
Accepted: 11-06-2022

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Biological management of *Fusarium* wilt of tomato by AM fungi in conjunction with other bio-control agents and their impact on growth and yield of tomato

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Abstract
In order to fulfill the needs of the human consumption, farmers are growing the tomato throught out the year in India. In India, it is grown as transplanted crop. Initially it can be grown on nursery beds and then transplanted to main field. At seedling stage, it is severely attacked by various pests, pathogens and insects which results in the death of seedlings. Biological management is one of the best biological method for the plant in which efficient and useful living organisms can be used against harmful pathogens which cause severe infection and death of plant. Now-a-days use of bio-control agents is the safest method for soil as well as plant to manage the diseases for protection of plant against attack of harmful pathogens. In recent years, biological management is accepted as major practice in agriculture which seeks to veer away from environmental and health contamination brought about by persistent pesticide and chemical fertilizer usage. In the present study the bio-control agents such as *Bacillus* sp., *Trichoderma* sp., were isolated from tomato rhizospheric soil, characterized and screened for antagonistic activity against *Fusarium oxysporum*. Out of 60 bacterial isolates, isolate KBI 36 showed highest per cent inhibition where as among 30 fungal isolates KTI 16 showed significantly highest per cent inhibition against *Fusarium oxysporum.* During pot experiment studies, these isolates were inoculated along with AM fungi. The plants inoculated with AM fungi + *B. s* ref. + *T. h* ref. showed least per cent disease incidence and highest growth parameters and yield followed by plants inoculated with AM fungi + KBI 36 + KTI 16. Thus from the present study, it is concluded that combined use of bio-control agents is an effort to shift microbiological equilibrium in favor of increased plant growth production as compared to single inoculants as well as uninoculated plants.

Keywords: AM fungi, KTI 18, KBI 36, *Fusarium oxysporum*, *Bacillus* sp., *Trichoderma* sp. Etc.

Introduction
Vegetables are rightly called “Vital nutritious, healthy foods” because of rich source of nutrients such as vitamins, minerals and dietary fiber but low in fat and carbohydrates. Now-a-days, most of the vegetables are grown all over the world as climate permits and can be cultivated in protected environment in suitable location. Among various vegetables grown, tomato is the edible berry of the plant *Lycopersicon esculentum*. Now rightly it is botanically called as *Solanum lycopersicum* L. It belongs to the family Solanaceae, kingdom Plantae and has rich source of nutrients. Tomato is considered as “Culinary Vegetable” because of low sugar content and it is typically served as part of salad or main course of a meal. In India, different cultivars of tomato can be grown. The genus *Solanum* consists of approximately 2000 species. Among them, most common edible species are *S. lycopersicum*, *S. nigram*, *S. melongena*, *S. macrocarpom, S. ferox*, *S. aethiopicum* are used for drying, culinery and curry purpose (Bajaj *et al*. 1979) [2].
Initially it can be grown on nursery beds and then transplanted to main field. At seedling stage, it is severely attacked by various pests, pathogens and insects. It is also severely attacked by various abiotic factors such as heavy temperature, rainfall, toxic metals and abnormal precipitation which results in the death of seedlings. Among various pathogens, *Fusarium oxysporum* is an ascomycetes fungus, well presented among all communities of soil borne fungi in every type of soil throughout the world. Once the pathogen causes infection, the plants become stunted; their leaves turn pale green to golden yellow and later started to wilt, wither, die, and drop off progressively upward from the stem base. Dark streaks occur in the xylem vascular tissue of the roots and lower stem, and the roots may decay. Infected seedlings wilt and die.
Use of Bio-control is one of the best biological method for the plant in which efficient and useful living organisms can be used against harmful pathogens which cause severe infection and death of plant.

In the present study the effective bio-control agents such as *Bacillus* sp., *Trichoderma* sp., were used against *Fusarium oxysporum.* The bacterial bio-agent called *Bacillus* sp. is rod shaped, endospore forming, aerobic Gram positive bacteria. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment. The *Trichoderma*, which is of fungal origin, present in most of the soils where they are the most prevalent culturable ascomycetes fungi and belongs to the family of Hypocreaceae. *Trichoderma* spp. are present in nearly all soils. As a bio-control agents, they can control most of the various soil borne diseases through mechanisms such as synthesis of enzymes (Chitinase, Xylanse, Phenylalanine etc.), secretion of antimicrobial metabolites, antibiotics which are toxic to the pathogens (James *et al*. 2019) [5]. Arbuscular Mycorrhizal Fungi is most commonly, popularly used endomycorrhizal fungi which forms unique structures *viz*., vesicles, arbuscules, hyphae and spores. AM fungi mainly take part in uptake and translocation of nutrients *viz.* phosphorus, sulphur, nitrogen, other micronutrients from the soil to plants. Apart from this, it colonizes the root and show positive influence on increasing the plant productivity, improvement in host nutrition and promote resistance in plants (Mariola *et al*., 2013) [7]. By looking into the importance of these bio-control agents, the present study was conducted to isolate, characterize and screening of bio-control agents along with AM fungi and their influence on crop growth and yield.

Material and methods

Isolation and characterization of *Bacillus* sp.

The *Bacillus* sp. was isolated from rhizospheric soil of tomato by using the method given by Al-Humam (2016) [1]. Four gram of soil sample was suspended in 96 ml sterile distilled water and shaken vigorously for 2 min. The samples were heated at 60^0 C for 60 min in water bath. Then the suspension was kept on bench surface at room temperature, for two hours for soil particles to settle. 100 µl of the suspension was spread on the nutrient agar medium plates enriched with 1% dextrose by using streak method. The plates were incubated at 28–30^0 C for 24–40 h for colony development.

The *Bacillus* isolates were morphologically characterized based on their colony morphology such as shape, colour, number of colonies and Gram reaction as per the standard procedures mentioned by Graham and Parker (1964) [4]. A loopful of pure culture was thinly spread on glass slide. The smear was air dried and heat fixed. Two drops of crystal violet dye were dropped over the smear and left for 1 min. The stain was washed under running water and left for drying. Then the smear was flooded with iodine solution for a minute. The iodine solution was drained off and the cells were decolourized with 95 per cent ethanol for 30 seconds. The smear was washed with water and blot dried carefully, then counter stained with safranin. Finally, the smear was rinsed with water, air dried and observed under microscope. The stained microscopic slides were examined through a bright field microscope under oil immersion for Gram reaction and cell morphology.

They were further characterized functionally by conducting bio-chemical tests such as citrate utilization, indole test, oxidase test.

Indole test

The freshly grown bacterial isolates were inoculated into test tubes containing sterilized tryptophan borth and add few drops of Kovac's reagent and incubated for 24 hr at 30 °C. The presence of red colour ring on top of test tube indicates the positive reaction (MacWilliams, 2009) [6].

Oxidase test

Small piece of filter paper was soaked in 1% Kovacs oxidase reagent (Tetra methyl para phenylenediamine dihydrochloride) and allowed to dry. With the help of loop, well isolated colony was picked from fresh (18-24 hr culture) bacterial plate and rub onto treated filter paper. Observe for colour changes. Microorgansims were oxidase positive when the color changes to dark purple within 5-10 seconds. Microorganisms were delayed oxidase positive when the color changes to purple within 60-90 seconds. Microorganisms were oxidase negative if the colour does not change or it takes longer than 2 minutes (Shields and Cathcart, 2016) [8].

Citrate utilization

The bacterial isolates were inoculated into test tubes containing Simmon's citrate agar medium and incubated for 72 hr at 35° C. Simmons citrate agar contains citrate as its only carbon and energy source. The presence of growth and change of colour from green to blue due to pH change indicates positive reaction (Seeley and Vandemark, 1981) [9].

Isolation and characterization of *Trichoderma* sp.

The serial dilution and plating technique was used to isolate the *Trichoderma* sp. from the samples collected. The collected samples were air dried in shade and finely grind before serial dilutions. *Trichoderma* Specific Medium (TSM) was used for isolation. In each test tube, 9 ml of water was poured. Test tube was plugged with non-absorbent cotton and was sterilized in an autoclave as mentioned earlier. After cooling, initial dilution was prepared in test tube labeled as 10^{-1} by addition of 1 g representative soil sample into the first test tube containing 9 ml of sterilized water. From the first dilution, 1 ml of suspension was transferred to the tube labeled as 10^{-2} having 9 ml sterilized water. Again same procedure was repeated, till the original sample will be diluted to 10^{-5}. The medium was poured over soil water suspension in Petri plate with rotary motion of the plate to mix it thoroughly. Medium was poured at the rate of 20 ml/plate. Plates was labeled and incubated in BOD incubator at 28±10 ^{0}C up to week's period. Incubated plates were watched every day for the growth of *Trichoderma* spp.

All the isolates were characterized initially based on their colony morphology such as shape, colour, number of fungal colonies and microscopic observations like presence or absence of conidia.

Screening of *Bacillus* sp. and *Trichoderma* sp. against *Fusarium oxysporum* for antimicrobial activity

The screening of bacterial and fungal isolates was carried out by dual culture assay against pathogenic fungi. The bacterial strains were streaked individually as straight and fungal bits were placed individually in dual culture assay on plates containing potato dextrose agar medium (Appendix-I) and incubated at 28 °C for 5 days. After observing a good ribbon-like growth of the bacteria on the Petri plates, the pathogen was spotted at right angles to the original streak bacteria and incubated at 28 °C ± 2 °C. The inhibition per cent was measured after 24 and 48 h. A control plate was also

maintained without inoculating the bacteria, to assess the normal growth of the fungal pathogen. Based on the results of antagonistic activity, the strains were selected for further studies (Gangwar *et al.,* 2011) [3]. Per cent inhibition over the control was calculated by using the formula given by Vincent (1972) [10].

Antagonistic effect = [(A-B) ÷ A] × 100

A – Diameter of mycelial growth of *Fusarium oxysporum* in control
B - Diameter of mycelial growth of *Fusarium oxysporum* in treated plates

Mass multiplication of best isolates of bacteria and fungi for pot experiment studies
Mass multiplication of *Bacillus* sp.
In order to produce the inoculum, *Bacillus* sp. was mass multiplied by using the procedure given by TNAU, Coimbatore. During the mass multiplication, mother culture need to be prepared. The medium used was nutrient broth. The medium was dispensed in conical flasks and sterilized at 15 lb pressure for 15 minutes. A loop of *Bacillus* culture was inoculated into the medium and incubated for 2 days. This serve as the mother culture.

Mass multiplication
The nutrient broth was prepared in fermentor and sterilized at 15 lb pressure for 15 minutes. Then the mother culture was added @ 1 l / 100 l of the medium and incubated at room temperature for 2 days. The medium containing the bacterial growth of *B. subtilis* was used for mixing with talc powder.

Mass multiplication of *Trichoderma* sp. – *Trichoderma* sp. was mass multiplied by using the procedure given by TNAU, Coimbatore.

Preparation of mother culture
The molasses yeast medium was prepared and dispensed into conical flasks and sterilized at 15 lb pressure for 15 minutes in an autoclave. After the medium gets cooled it was inoculated with 10 days old fungal disc of *Trichoderma* and then incubated for 10 days for fungal growth. This serves as mother culture.

Mass multiplication
Molasses yeast medium was prepared in fermentor and sterilized as described earlier. Then after the medium was cooled, the mother culture was added to the fermentor @ 1.5 l / 50 l of the medium and incubated at room temperature for 10 days. Then the incubated broth containing the fungal culture was used for commercial formulation preparation using talc powder.

Mass multiplication of AM fungi- i.e UASD AMF consortium (*Glomus macrocarpum, Gigaspora margarita* and *Acaulospora laevis*) was used in the present study and it was mass multiplied by using ragi as host crop. The fifty gram culture of UASD AMF consortium was taken from Department of Agricultural Microbiology, UAS, Dharwad and were mass multiplied by inoculating 50 g culture in pots with 950 g of vermiculite. Later ragi seeds were sown and allowed to grow for 90 days. Hoagland's nutrient solution was given at weekly intervals. After 90 days of growth, infective propagules were observed, spores were isolated and identified.

Influence of best isolates on growth of tomato under pot culture conditions
The pot experiment was conducted in a green house at Department of Agricultural Microbiology, College of Agriculture, University of Agricultural Sciences, Dharwad.

Nursery raising
The cultures of best bacterial, fungal isolates, AM fungi and *Fusarium oxysporum*. Were inoculated by following treatment details

a. T_1 – Absolute control
b. T_2 - Only UASD AMF Consortium (UAC)
c. T_3 – Only KBI 36
d. T_4 - Only KTI 16
e. T_5 - UAC + KBI 36
f. T_6 - UAC + KTI 16
g. T_7 – KBI 36 + KTI 16
h. T_8 - UAC + KBI 36 + KTI 16
i. T_9 - *Bacillus subtilis* reference strain (*B.s* ref.)
j. T_{10} - *Trichoderma harzianum* reference strain (*T.h* ref.)
k. T_{11} - UAC + *B.s* ref
l. T_{12} - UAC + *T.h* ref
m. T_{13} - *B.s* ref + *T.h* ref
n. T_{14} - UAC + *B.s* ref + *T.h* ref
o. T_{15} - Only *Fusarium oxysporum*

As per the above treatment details, the nursery pots were filled with sterilized soil, then cultures of bio-control agents were inoculated @ 5.2g/pot, AM fungi was inoculated @ 32g/pot and finally seeds were sown to pots at the rate of five per pot. The pots were watered regularly to maintain optimum moisture.

Transplantation of seedlings
During transplantation, forty five days old seedlings were transplanted from nursery pots to the experimental pots @ two seedlings per pot. The *Fusarium oxysporum* was inoculated @ 4% of the substrate per pot. After two weeks of transplantation, fertilizers were applied to all the pots at recommended dose uniformly. After 10 days of transplantation, thinning operation was carried out to retain one plant per pot. The experiment was continued until the crop harvest. The per cent disease was recorded at 45 DAT and growth parameters such as root length, plant height and dry weight and yield of tomato plants were recorded at 90 DAT.

Results and discussion
Biochemical characterization of bacterial isolates
The results indicated that all 60 isolates showed positive results for citrate utilization test. Some isolates showed negative results for indole test, oxidase test (Table 1).

Table 1: Biochemical characterization of bacterial isolates

Isolate code	Indole test	Oxidase test	Citrate utilization	Isolate code	Indole test	Oxidase test	Citrate utilization
ABI 1	+	+	+	IBI 31	+	-	+
ABI 2	+	+	+	IBI 32	+	+	+
ABI 3	+	+	+	IBI 33	+	+	+
BBI 4	+	+	+	KBI 34	+	+	+
BBI 5	+	+	+	KBI 35	+	-	+
BBI 6	+	+	+	KBI 36	+	+	+
BBI 7	-	+	+	KBI 37	+	+	+
DBI 8	+	+	+	KBI 38	+	+	+
DBI 9	+	+	+	MBI 39	-	+	+
DBI 10	+	-	+	MBI 40	-	-	+
DBI 11	+	+	+	MBI 41	-	+	+
GBI 12	+	+	+	NBI 42	-	+	+
GBI 13	+	+	+	NBI 43	-	+	+
GBI 14	-	+	+	NBI 44	-	+	+
GnBI 15	-	+	+	NBI 45	+	+	+
GnBI 16	-	+	+	NBI 46	+	+	+
GnBI 17	-	+	-	SBI 47	-	+	+
GnBI 18	-	+	+	SBI 48	-	+	+
GnBI 19	+	+	+	SBI 49	-	+	+
HBI 20	+	+	+	SBI 50	-	+	+
HBI 21	-	+	+	SBI 51	-	+	+
HBI 22	-	+	+	UBI 52	-	+	+
HBI 23	+	+	+	UBI 53	-	+	+
HrBI 24	+	-	+	UBI 54	-	-	+
HrBI 25	+	+	+	UBI 55	-	+	+
HrBI 26	+	+	+	UBI 56	-	+	+
IBI 27	-	+	+	VBI 57	-	+	+
IBI 28	+	+	+	VBI 58	+	+	+
IBI 29	-	+	+	VBI 59	-	+	+
IBI 30	+	+	+	VBI 60	+	+	+

Screening of *Bacillus* sp. and *Trichoderma* sp. against *Fusarium oxysporum* for antagonistic activity.

The *Bacillus subtilis* ref. strain was recorded highest per cent inhibition followed by isolate KBI 36. The least per cent inhibition was showed by SBI 48. The per cent inhibition showed by isolate BBI 8 was on par with the per cent inhibition showed by the isolate DBI 8. All the isolates were significantly differ with each other except the isolates (GBI 4and GnBI 15) which were not significantly differ with respect to per cent inhibition (Table 2).

The highest per cent inhibition was showed by *Trichoderma harzianum* ref. followed by KTI 16 which was again followed by HTI 11. The least per cent inhibition was showed by isolate GTI 7. The same per cent inhibition was observed in two isolates such as HTI 12 and ITI 14 which were significantly not differ. The remaining isolates were significantly differ with each other with respect to per cent inhibition (Table 2).

Table 2: Estimation of per cent inhibition by bacterial and fungal isolates

Bacterial Isolate code	Per cent inhibition (%)	Fungal Isolate code	Per cent inhibition (%)
ABI 1	53.26	BTI 1	29.27
GBI 04	50.04	BTI 2	26.21
GnBI 15	49.17	BTI 3	34.09
GnBI 16	44.17	DTI 4	10.97
GnBI 19	51.17	DTI 5	17.68
KBI 36	68.23	DTI 6	11.46
VBI 57	42.37	GTI 7	7.24
BBI 4	38.03	GTI 8	32.31
BBI 7	35.5	GTI 9	25.38
BBI 8	48.57	HTI 10	20.38
SBI 48	1.75	HTI 11	43.85
DBI 8	48.59	HTI 12	30.77
Bacillus subtilis ref.	61.04	HTI 13	34.61
S.Em.±	0.865	ITI 14	30.77
C.D @ 1%	0.296	ITI 15	27.70
		KTI 16	58.46
		KTI 17	46.16
		KTI 18	42.69
		MTI 19	21.46
		Trichoderma harzianum ref.	49.84
		S.Em.±	0.584
		C.D @ 1%	1.675

Influence of best isolates and AM fungi on per cent disease incidence @ 45 DAT

The plants inoculated with only *Fusarium oxysporum* had significantly highest per cent disease incidence where as plants inoculated with UAC + *B.s* ref. + *T.h* ref. had no disease incidence. The similar trend was followed in plants inoculated with UAC + KBI 36 + KTI 18 which did not showed any disease incidence. The uninoculated plants also showed some per cent disease incidence. The per cent disease incidence in plants inoculated with UAC+ KBI 36 was on par with the plants inoculated with UAC + KTI 18 There was no significant difference between the plants inoculated with UAC + *B.s* ref. and the plants inoculated with UAC + *T.h* ref. with respect to per cent disease incidence (Table 3).

Influence of best isolates on growth and yield of tomato under pot culture conditions

The plants inoculated with UAC + *B.s* ref. + *T.h* ref. recorded significantly highest plant height and yield followed by plants inoculated with UAC + KBI 36 + KTI 18 at 90 DAT. The plants inoculated with only *Fusarium oxysporum* recorded least plant height and yield at 90 DAT. The significantly highest plant height and yield were observed in all plants inoculated with other treatments as compared to uninoculated plants at 90 DAT (Table 3).

Mohammad *et al*. (2003) opined that the significant increase in growth parameters such as root length, plant height, shoot length and dry weight is mainly due to positive interactions showed by beneficial microbes by secreting essential compounds required by the plant at all stages of their life cycle.

The results of the present study concluded that combined use of bio-control agents is an effort to shift microbiological equilibrium in favor of increased plant growth production as compared to single inoculants as well as uninoculated plants.

Table 3: Effect of inoculation of AM fungi, efficient bio-control agents on per cent disease incidence at 45 DAT, plant height and yield at 90 DAT

Treatments	Details	Per cent disease incidence (%)	Plant height (cm/plant)	Yield (No. of fruits per plant)
T_1	Absolute control	10.5	80.5	4.00
T_2	Only UASD AMF Consortium (UAC)	8.5	75.5	6.50
T_3	Only KBI 36	12	83.0	8.00
T_4	Only KTI 18	11	91.5	6.20
T_5	UAC + KBI 36	9.5	90.0	7.50
T_6	UAC + KTI 18	9.0	85.6	8.30
T_7	KBI 36 + KTI 18	8.0	87.8	11.0
T_8	UAC + KBI 36+ KTI 18	0	84.5	12.7
T_9	*Bacillus subtilis* reference strain (*B.s* ref.)	10.5	95.5	10.50
T_{10}	*Trichoderma harzianum* reference strain (*T.h* ref.)	13.00	90.4	8.50
T_{11}	UAC + *B.s* ref.	12.00	75.5	9.30
T_{12}	UAC + *T.h* ref.	11.00	88.8	9.00
T_{13}	*B.s* ref + *T.h* ref.	15.20	72.5	11.5
T_{14}	UAC + *B.s* ref. + *T.h* ref.	0.00	105.2	15.0
T_{15}	Only *Fusarium oxysporum*	80.00	54.5	0.00
	S.Em.±	0.804	0.413	0.520
	C.D@1%	2.335	1.199	1.509

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