Bulgarian Journal of Agricultural Science, 28 (No 2) 2022, 238–241

Evaluation of mechanism of plant protective attributes of root colonizing bacteria against phytopathogenic fungi

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Abstract

Upadhyaya, Ch. & Patel, I. (2022). Evaluation of mechanism of plant protective attributes of root colonizing bacteria against phytopathogenic fungi. *Bulg. J. Agric. Sci., 28 (2)*, 238–241

A presented study was commenced to assess the plant protective mode of actions of root colonizing bacteria *Bacillus subtilis* and *Bacillus licheniformis* against phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani*. The dual inoculation assay showed the antagonistic effect of *B. subtilis* and *B. licheniformis* on both the fungal pathogens. Although, *B. licheniformis* revealed higher percent inhibition against *R. solani* (97.21±0.15%) and *F. oxysporum* (70.00±1.20%) than *B. subtilis*. Lipopeptides and volatile compounds produced by both *Bacillus* strains & responsible for biocontrol activity were determined by using HPTLC and GC-MS analysis respectively. The biocontrol treated *Vigna radiata* seeds were checked for the rate of seed germination in presence of a pathogen which revealed 4.26 fold greater disease resistance in seeds treated with both the bacterial strains in combination compared to untreated seeds and 12.73% higher resistance than chemical fungicide carbendazim (1mg/l) drenched seeds. Consequently, biocontrol formulation from selected bacterial strains can be applied to protect plants from fungal diseases.

Keywords: biocontrol; root colonizing bacteria; phytopathogenic fungi

Introduction

The plant pathogenic fungi can cause severe diseases and yield losses which impose a great risk to crop productivity and agronomic sustainability across the globe. The *Fusarium oxysporum* cause wilt disease in a range of plants as well as is very difficult to manage and consequently gathers high economic attention (Jagir et al., 2019). Amongst many, *Rhizoctonia solani* possesses commercial importance because of its vast host range and potency to impose devastating diseases viz. root rot, color rot, wire stem, etc. (Menzies J.D., 2020). There are countless strategies available to mitigate pathogenic invasion in plants out of which biocontrol agents were investigated immensely because of their efficacy and broad-spectrum fungicidal actions (El_Komy et al., 2015). Moreover, they are preferred due to the realization of hazardous effects of chemical fungicides and pesticides viz. environmental pollution and destruction of plant beneficial microflora (Alencar et al., 2020). The root colonizing rhizobacteria has been proven as a potential biocontrol agent as well as has plant growth-promoting activity (Mohammed & Toama**,** 2018; Eljounaidi et al., 2016). Hence, the presented analysis was governed by the thirst to deduce the mechanisms utilized by two such organisms *Bacillus subtilis* and *Bacillus licheniformis* for exerting the antagonistic effects on *F. oxysporum* and *R. sonani*. The *in-vivo* experiment on *Vigna radiata* seeds was governed by treating them with selected *Bacillus* strains as separate and combined formulations which were co-incubated with pathogenic fungus and further checked for disease resistance. Such an innovative dual inoculation assay will provide new insight into the development of a more efficacious biocontrol agent against phytopathogens.

Materials and Methods

The dual inoculation assay in solid and liquid media was done by incubating selected pathogenic fungal strains along with bacterial strains to check the antagonistic effect. The % growth inhibition of phytopathogen was calculated as following formula (Singh et al., 2010):

$$\% \text{ growth inhibition} = [\frac{C - T}{C}] \times 100, \quad (1)$$

where C = Radial growth of fungus in control,T = Radial growth of fungus in dual culture

The mechanisms by which selected organisms exert biocontrol activity were deduced by, hydrolytic enzyme production viz. chitinase and cellulase (Chaiharn et al., 2008). The selected biocontrol strains were also checked for the production of HCN (Hydrogen cyanide) (a potent plant defense inducer and act on pathogenic cells by blocking the respiratory chain protein – cytochrome c oxidase) (Miller and Higgins, 1970), quantifying biofilm formation capability (successful colonization for effective antagonism) by microtiter plate assay and exopolysaccharide (EPS) production in solid and liquid media (O'Toole, 2011). The EPS quantification was governed from Luria Bertani (LB) broth inoculated with 500µl bacterial inoculum and after 5 days of incubation centrifuged at 8000 rpm for 15 min and the supernatant was added with 20ml chilled acetone to obtain EPS precipitates after 2 h of incubation. The precipitates were filtered by filter paper, dried completely, and weighed. The content of EPS was deduced by measuring an increase in the weight of filter paper. For examining antifungal lipopeptide production and isolation, the procedure followed from Pathak et al. (2012) and the methanolic fractions from both the strains were analyzed by HPTLC analysis utilizing the HPTLC system (SICART, Anand) as well as their antifungal activity was checked by disc diffusion assay on PDA agar plates. Both bacterial strains were analyzed for the production of volatile compounds which have antifungal activity against phytopathogenic fungus. The volatile compounds identification was governed by GC-MS analysis (SICART, Anand). The half Petri dish with N-agar medium was inoculated with a bacterial extract containing disc at the center and another half part of the Petri dish was inoculated with pathogenic fungal biomass at the center. Both the halves of the Petri dish were put together and sealed by parafilm to avoid losses of volatiles and checked for antagonistic effect on fungal growth and % growth inhibition was calculated according to equation 1. The effect of biocontrol strains on the rate of seed germination of *Vigna radiata* was governed by treating them with both bacterial strains separately and in combination as T1: seeds coated with 1% CMC + *B. subtilis* + *R. solani*; T2: seeds coated with 1% CMC + *B. licheniformis*+ *R. solani;* T3: seeds coated with 1% CMC + *B. subtilis* + *F. oxysporum*; T4: seeds coated with 1% CMC + *B. licheniformis* + *F. oxysporum*; T5: seeds coated with 1% CMC + *B. subtilis* + *B. licheniformis*+ *R. solani*; T6: seeds coated with 1% CMC + *B. subtilis* + *B. licheniformis*+ *F. oxysporum*; T7: seeds coated with 1% CMC + *R. solani;* T8: seeds coated with 1% CMC + *F. oxysporum*; T9: seeds coated with 1% CMC (untreated negative control), T10: seeds coated with 1% CMC + Carbendazim (1mg/l antifungal chemical) + *F. oxysporum*. The *in-vivo* analysis was done via surface sterilization of seeds, coating with 1% CMC and air drying following treating them with bacterial and pathogenic strains as mentioned in T1 to T10 and kept 10 seeds in Petri dish contained with Whatman No. 1 to which after every 12 h interval distilled water was sprayed to give humidity for germination. Analysis governed as triplicate and results were denoted as mean ± SD and significance of difference was evaluated following Duncan's multiple range test by Graphpad prism and $p < 0.05$ was considered as significant.

Table 1. Antagonistic effects of *B. subtilis* and *B. licheniformis* on selected phytopathogenic fungal strains

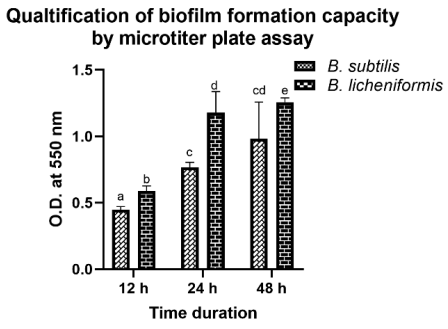
Antagonistic effect	Fungal strain	*B. subtilis*	*B. licheniformis*
Percent growth inhibition, %	*R. solani*	82.75±1.15^{a}	97.21±0.18^{b}
	F. oxysporum	58.92±2.00^{c}	70.00±1.20^{d}
Hydrolytic enzyme production (Zone size), mm	Chitinase	20.50±1.15^{a}	15.50±1.04^{c}
	Cellulase	21.70±1.25^{a}	25.70±1.80^{b}
EPS quantification, mg/ml	–	20.06±2.00^{a}	35.50±1.60^{b}
Antifungal activity of lipopeptides (Zone of inhibition), cm	*R. solani* (5 µl)	1.00±0.07^{a}	3.00±0.05^{b}
	R. solani (10 µl)	1.75±1.50^{a}	3.47±1.25^{c}
	F. oxysporum (5 µl)	0.05±0.10^{b}	1.05±0.06^{a}
	F. oxysporum (10 µl)	0.08±0.07^{c}	1.70±0.12^{a}
% growth inhibition of volatile compounds, %	*R. solani*	60.00±2.10^{a}	87.52±1.30^{c}
	F. oxysporum	65.23±1.24^{b}	77.80±0.55^{d}

Different letter(s) on superscript in a raw denote statistically different ($p < 0.05$) values

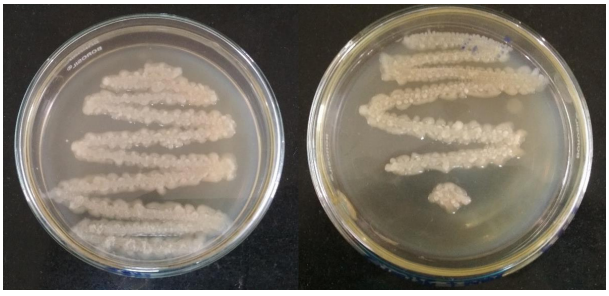
Result and Discussion

The antagonistic effect of both bacterial strains was confirmed by obtaining a zone of inhibition and mycelial growth inhibition using a liquid medium. The maximum mycelial growth inhibition of 70.00±1.20% of the fungus *F. oxysporum* and 97.21±0.15% of *R. solani* was observed by the *B. licheniformis* strain which was higher than the *B. subtilis* as illustrated in Table 1.

Hydrolytic enzyme production assay revealed the production of cellulase and chitinase by both the selected bacterial strains. The cellulase production was observed by zone formation on CMC agar plates and was maximum (25.70±1.80 mm) in the case of *B. licheniformis*. Whereas, chitinase production was deduced by utilizing colloidal chitin substrate containing plates via measuring zone of hydrolysis and was highest (20.50±1.15 mm) in the case of *B. subtilis* (Table 1). *B. subtilis* did not show HCN production whereas *B. licheniformis* showed HCN production upon 48 h and 72 h of growth. The microtiter plate assay showed significant biofilm formation by *B. licheniformis* and *B. subtilis* at 12, 24 and 48 h incubation which is illustrated in Figure 1a and both strains gave signif-

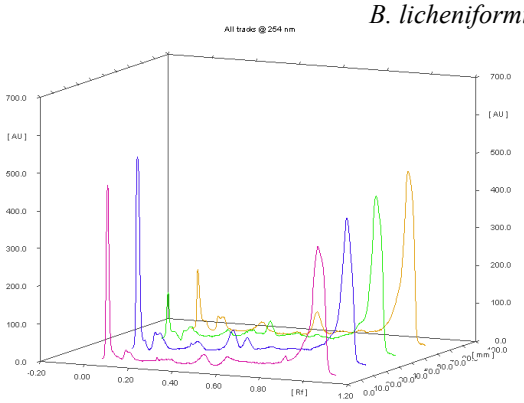


a)

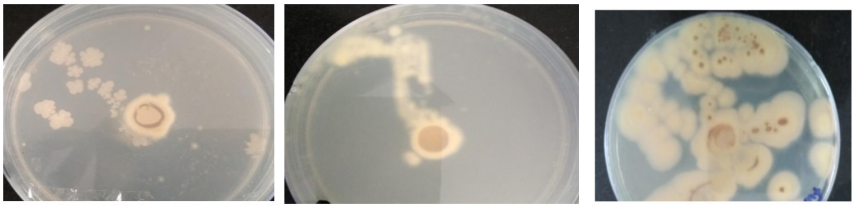


b)

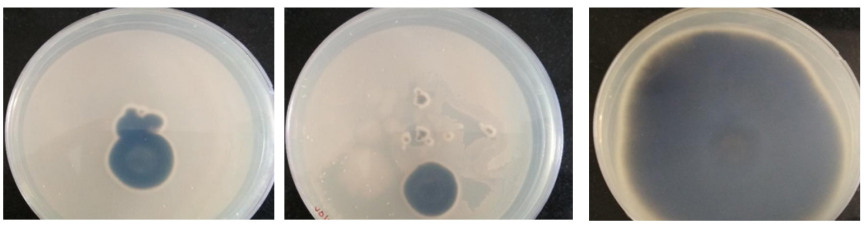
B. licheniformis *B. subtilis*



c)



B. subtilis + F. oxysporum *B. licheniformis + F. oxysporum* *F. oxysporum (Control)*



d)

B. subtilis + R. solani *B. licheniformis + R. solani* *R. solani (Control)*

Fig. 1. Assay of *B. subtilis* and *B. licheniformis* (a) biofilm assay (b) EPS production (c) Bands and 3D image of HPTLC assay (d) Effect of volatile compounds on the growth of phytopathogenic fungus

Table 2. Effect of selected bacterial strains on the rate of seed germination of *Vigna radiata* upon treatment of selected pathogenic fungi

	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Rate of seed germination, %	80.6±1.20^{bc}	77.5±2.42^{c}	68.9±1.25^{d}	74.5±0.95^{c}	96.2±2.00^{ab}	94.3±1.55^{b}	24.5±1.70^{e}	17.9± 2.52^{de}	99.1±1.00^{a}	82.3±0.89^{cd}

Different letter(s) on superscript in a raw denote statistically different ($p < 0.05$) values

icant EPS production (Figure 1b). Although, higher content of EPS was produced by *B. licheniformis* as compared to *B. subtilis* (Table 1).

The HPTLC analysis of extracts of both the bacterial strains showed lipopeptide production in form of prominent bands. The separated bands of lipopeptides were evaluated by a TLC visualizer and were also qualitatively assessed by a TLC scanner which revealed the production of iturin A, surfactin, and fengycin like lipopeptides by both bacterial strains (Figure 1c). The antifungal activity of crude lipopeptide extracts of both strains is shown in Table 1 which was higher in the case of *B. licheniformis* extract against both fungal isolates. The GC-MS analysis for volatile compounds in both bacterial extracts indicated the presence of 1-butanol, acetol, linalool, and ϒ-crotolactone volatiles having a respective retention time of 8.2, 14.5, 22.7, and 33.8 min. Their antagonistic effect on both phytopathogens was demonstrated in Figure 1d. The antifungal activity of volatiles was represented as % growth inhibition which was higher in the case of *B. licheniformis* than *B. subtilis* (Table 1). Thus, there are various means by which these two potent biocontrol agents exert the antifungal activity against phytopathogenic fungi whose outcome was following Borriss R. (2011). The *in vivo* antifungal activity of selected bacterial strains is exemplified in Table 2 which showed the highest potency in the case of T5 and T6 than the negative and positive controls. As a result, the present analysis revealed that both the bacterial strains in combination provided the highest protection against selected fungal pathogens and can be further utilized in the development of efficacious biocontrol agents.

Conclusion

The present study revealed the significant antagonistic effect of both bacillus strains against selected phytopathogenic fungi. Both the strains were reported to produce significant antifungal compounds viz. hydrolytic enzymes, exopolysaccharides, HCN, lipopeptides, and volatile compounds to exert the antagonistic effect. The antifungal potency of both the strains and produced compounds was deduced which gave substantial growth inhibition of phytopathogenic fungi. Out of two, *B. licheniformis* possess higher antagonistic potential than *B. subtilis* and higher antagonism was reported against *R. solani* than *F. oxysporum*. The *in vivo* analysis of bacterial inoculum treatment on *Vigna radiata* seeds showed the highest fungal resistance in seeds treated with both bacterial inoculum in combination and can be applied for formulating efficacious biocontrol agents for protecting plants from fungal pathogens.

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Received: October 20, 2021; *Accepted:* Februray 9, 2022; *Published:* April, 2022