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In-vitro compatibility of *Bacillus amyloliquefaciens* IIHR BA2 with commercial biocontrol agents and agrochemicals

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ABSTRACT: *Bacillus amyloliquefaciens* IIHR BA2 strain was tested *in vitro* for its compatibility with five different bio-control agents *viz*., *Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma viride, Paecilomyces lilacinus* and *Pochonia chlamydosporia.* Among them, *T. viride* and *B. subtilis* showed maximum compatibility of 62.80% and 60.00% respectively followed by *Pochonia chlamydosporia* (50.90%)*, Paecilomyces lilacinus* (48.60%) and *Pseudomonas fluorescens* (25.00%). Compatibility of *B. amyloliquefaciens* IIHR BA2 was also tested with seven commercial agrochemicals *viz.,* K-cyclin, captan, mancozeb, copper oxychloride, fenamidone + mancozeb, carbendazim and wettable sulphur at different concentrations. The results revealed that carbendazim and wettable sulphur were highly compatible at the concentration of 500 ppm but K-Cyclin, captan, mancozeb, copper oxychloride, fenamidone + mancozeb exhibited high toxicity and observed no growth at all the tested concentrations with *B. amyloliquefaciens* IIHR BA2. These results are very crucial for decision making in Integrated Nematode Management programmes and also in development of consortia formulations.

Keywords : *Bacillus amyloliquefaciens,* Bio-Control Agents, Agrochemicals.

INTRODUCTION

Among the IPM tactics, antagonistic microorganisms are one of the most important components, which include several potential strains of bacterial and fungal biocontrol agents (BCAs) which are commercially produced (Siddiqui and Mahmood, 1999; Radnedge *et al*., 2003; Rao *et al.,* 2009; Köhl *et al*., 2011). Since usage of synthetic chemicals might have deleterious effects on the non-targeted antagonists, an understanding on the effect of chemicals would give information to select the chemicals and chemical resistant antagonists. Further, addition of specific chemical compounds can influence the antagonistic properties of BCAs (Kay and Stewart, 1994; Naar and Kecskes, 1998).

Several BCAs used in disease management strategies along with chemical fungicides at lower rates had considerably increased disease control, compared to treatments with BCAs alone (Frances *et al.,* 2002; Buck, 2004). Integrated application of BCAs such as *Bacillus megaterium* with reduced dose of carbendazim was effective against *Fusarium* root rot of tomato (Omar *et al*., 2006) and *Pseudomonas aeruginosa* with lesser concentrations of chlorothalonil (Kavach®) controlled the late leaf spot of groundnut (Kishore *et al.,* 2005).

Correspondingly, it is also essential for the selected bioinoculant to compete with diverse populations of antagonistic strains that are already present in the crop niche. Hence, compatibility between different BCAs needs to be considered.

Amutha *et al.* (2009) demonstrated the positive effects of co-inoculating different species of *Azospirillum* on the growth of rice. Rao *et al*., (2004) reported that combination of *Pochonia chlamydosporia* and *Pseudomonas fluorescens* was effective in controlling *Meloidogyne incognita* in bell pepper.

However, the quantum of studies on the BCAs are inadequate for their compatibility with commercial microbial formulations and other agrochemicals to integrate the inputs as a package. Keeping all these in view, the present study aims to test the compatibility of *B. amyloliquefaciens* IIHR BA2 with commonly used fungal and bacterial bio-agents and chemical bactericides and fungicides at different concentrations under *in vitro* conditions.

MATERIALS AND METHODS

Test microbial cultures:

All the test microbial strains used in this study were native isolates and maintained at the laboratory

of Nematology, ICAR-IIHR, Bengaluru, Karnataka, India. The three fungal bio- agents used in the study *viz., Trichoderma viride* (IIHR-Tv5, ITCC-6889), *Paecilomyces lilacinus* (IIHR-Pl2, ITCC-6887) and *Pochonia chlamydosporia* (IIHR-Vc3, ITCC-6898) were maintained in potato dextrose agar (PDA) medium. The three bacterial bio-agents used in the study *viz., Bacillus subtilis* (IIHR-Bs2, NAIMCC-B-01211), *B. amyloliquefaciens* (IIHR-BA2, NAIMCC-TB2216) and *Pseudomonas fluorescens* (IIHR-Pf2, ITCC- B0034) were maintained in nutrient agar (NA) and as well in nutrient broth (NB) medium.

Test commercial agro chemicals

Six commercial fungicides *viz.,* captan (Captaf 50% WP, Rallis India Ltd., Mumbai), mancozeb (Dithane M-45 75% WP, Dow Agrosciene India Pvt Ltd., Mumbai), copper oxychloride (Blitox 50% WP, Rallis India Ltd., Mumbai), fenamidone + mancozeb (Sectin 60 WG - Fenamidone 10% + mancozeb 50% w/w WG, Bayer Crop Science Ltd., Gujarat), carbendazim (Bengard 50% W.P, Agricare., Panoli) and wettable sulphur (Nagsulp-P 80% W.P, Multiplex, Bengaluru) and one bactericide K-Cyclin (Streptomycin sulphate I.P. 90% w/w; Tetracyclin hydrochloride I.P. 10% w/w., Karnataka Antibiotics and Pharmaceuticals Ltd., Bengaluru) were used for this study. Each agro-chemical as prepared at different concentrations (Fungicide: 2000 ppm, 1500 ppm, 1000 ppm and 500 ppm; Bactericide: 200 ppm, 150 ppm, 100 ppm and 50 ppm) by dissolving in sterile culture media and tested in this study.

Compatibility of *B. amyloliquefaciens* IIHR BA2 with bacterial bio-agents

Agar well diffusion was done to test the compatibility of *B. amyloliquefaciens* IIHR BA2 with *B. subtilis* and *P. fluorescens* (Valgas *et al*., 2007). Sterile NA media plate was mixed with 200 µl of *B. amyloliquefaciens* IIHR BA2 broth culture before solidification. After solidification, 3 mm diam. disk was removed twice aseptically at a distance of 20 mm apart with a sterile cork borer. It was then loaded with 20 µl of *B. subtilis* and *P. fluorescens* maintained in separate plates with broth culture in one well and 20 µl of sterile water in another well and incubated for 48 h at 25±2 °C. Sterile water served as control. Observations were recorded on the growth or inhibition of test strains. The whole experiment was repeated twice and replicated three times. The per cent compatibility of *B. amyloliquefaciens* IIHR BA2 was calculated based on water control.

Per cent compatibility = [(X - Y)/Y] × 100

X is radial growth of bacteria in well (mm); Y is radius in water control (mm)

Compatibility of *B. amyloliquefaciens* IIHR BA2 with fungal bio-agents

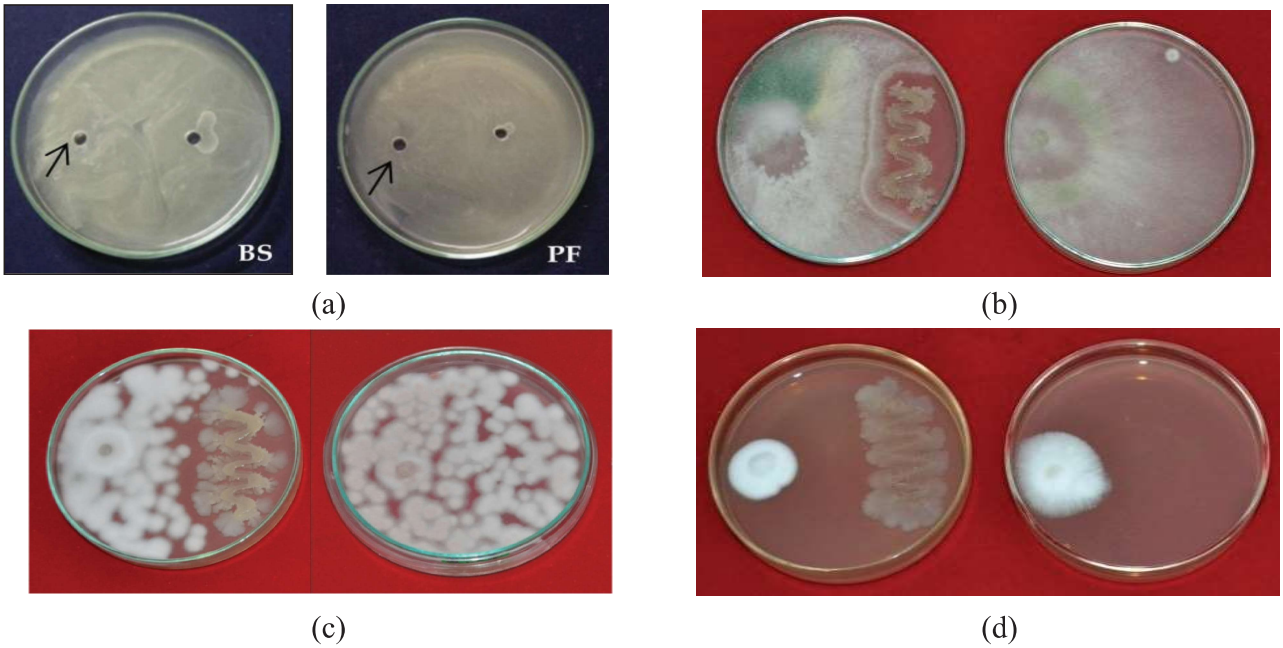

Figure 1. Compatibility of *B. amyloliquefaciens* IIHR BA2 with BCAs: (a). lef to right - *B. amyloliquefaciens* IIHR BA2 + *B. subtilis* (BS), *B. amyloliquefaciens* IIHR BA2 + *P. fluorescens* (PF) (Arrow mark indicate water control) (b). *B. amyloliquefaciens* IIHR BA2 + *T. viride*, (c). *B. amyloliquefaciens* IIHR BA2 + *P. lilacinus* (d). *B. amyloliquefaciens* IIHR BA2 + *P. chlamydosporia*

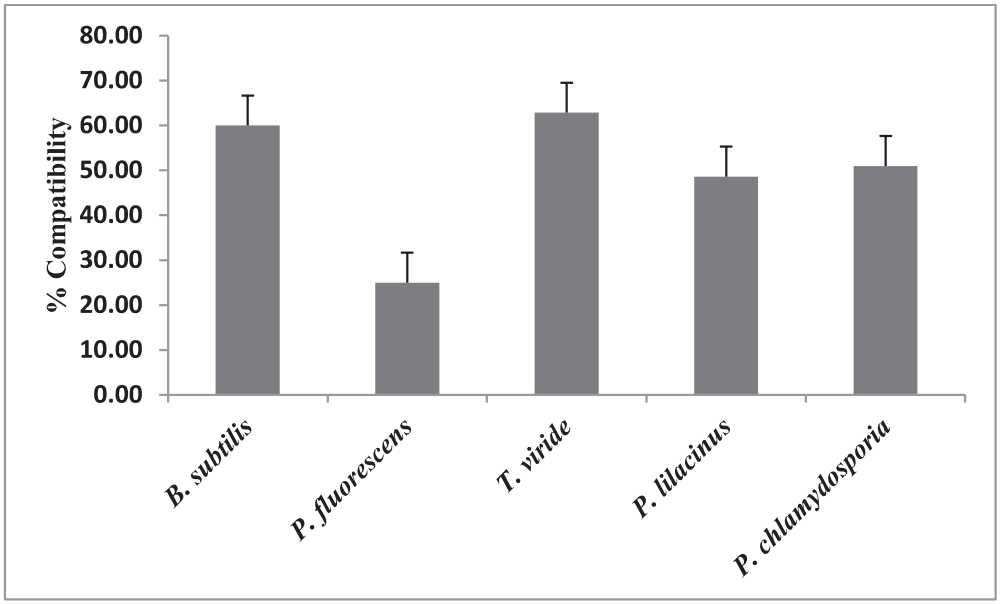

Figure 2. Percent compatibility of *B.* amyloliquefaciens IIHR BA2. Bars indicate standard error (SE).

Dual culture experiment was carried out to study the interaction of *B. amyloliquefaciens* IIHR BA2 with *T. viride, P. lilacinus* and *P. chlamydosporia* separately (Ji *et al*., 2013); Mycelial disks of 5 mm diam. were cut from the target fungi and placed on one edge of freshly prepared sterile PDA media containing Petri plate and *B. amyloliquefaciens* IIHR BA2 was streaked at 30 mm distance from the fungal disk. Petri plates without bacterial strains served as control. Three replications were maintained for each experiment and the whole experiment was repeated twice. All the plates were incubated at 25±2 °C for 4 days and examined for the fungal growth. The per cent compatibility of *B. amyloliquefaciens* IIHR BA2 was calculated based on the fungal mycelia growth diameter in control plate as per Sundar *et al*. (1995).

Per cent compatibility = $[(R_1 - R_2)/R_1 \times 100]$-100

R_1 - represents the mycelial growth of fungus in control plate; R_2 - the mycelial growth of the fungus in plates with bacteria

Compatibility of *B. amyloliquefaciens* IIHR BA2 with agrochemical

Poisoned food technique method was followed to test the *in-vitro* compatibility of *B. amyloliquefaciens* IIHR BA2 with agrochemicals (Grover and Moore, 1961). The chemical bactericide K- Cyclin at the concentration of 200 ppm, 150 ppm, 100 ppm and 50 ppm were prepared with autoclaved NA media. Similarly chemical fungicides captan, mancozeb, copper oxychloride, fenamidone + mancozeb, carbendazim and wettable sulphur were prepared at the concentration of 500 ppm, 1000 ppm and 2000 ppm. One day old *B. amyloliquefaciens* IIHR BA2 liquid culture was serially diluted and 1 ml was inoculated to NA media plate by pour plate technique. NA plates without agrochemical served as control and the treated plates were incubated at 28±2ºC. After 48 h, colony characters were observed in the plates each concentration and colony forming unit (CFU x 10^8/ml) was recorded. The experiment was repeated twice and three replicats were maintained for each experiment. The per cent inhibition was calculated based on the number of cfu compared to control as per the following formula.

Per cent Inhibition = $[(T1 - T2)/T1] \times 100$

T1- cfu in control plates; T2 - cfu in treated plates

Statistical analysis

All the data were statistically analyzed (Gomez and Gomez 1984). The percentage data were arc sine transformed before ANOVA and the means were compared by Duncans Multiple Range Tests.

RESULTS AND DISCUSSION

The present study revealed that *B. amyloliquefaciens* IIHR BA2 displayed varying compatible reactions with test bio-control agents and commercial agrochemicals.

Compatibility of *B. amyloliquefaciens* IIHR BA2 with other BCAs

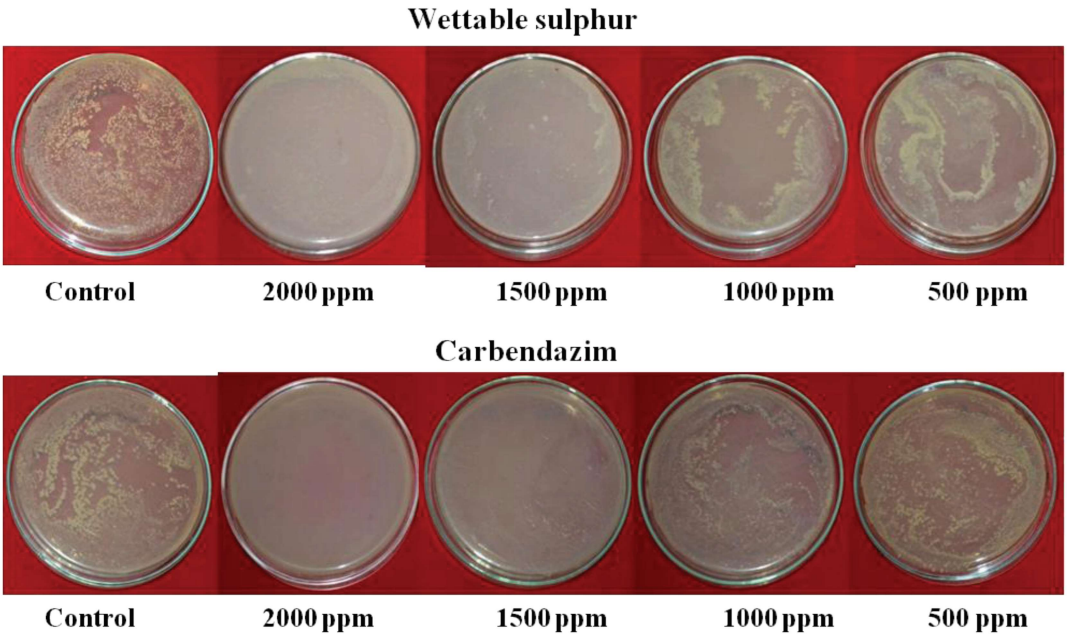

Figure 3. Response of *B. amyloliquefaciens* IIHR BA2 with commeercial agrochemicals

Table 1. Percent inhibition of *B. amyloliquefaciens* IIHR BA2 by commercial agrochemicals

Treatment	Inhibition in cfu				CD (0.01)	SEd (±)	CV (%)
	2000 ppm	1500 ppm	1000 ppm	500 ppm			
Captan 50% WP	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	98.53 (83.03)	NS	-	-
Mancozeb 75% WP	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	NS	-	-
Copper oxychloride 50% WP	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	NS	-	-
Fenamidone 10% + mancozeb 50% w/w WG	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	NS	-	-
Carbendazim 50% WP	100 (89.71)	98.56 (83.06)	10.82 (19.14)	7.22 (15.57)	0.67	0.22	0.58
Wettable sulphur 80% WP	91.76 (72.30)	74.12 (59.50)	12.35 (21.43)	11.50 (19.36)	4.29	1.40	4.20
K-cyclin (Streptomycin sulphate I.P. 90% w/w + Tetracyclin hydrochloride I.P. 10% w/w)	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	100.00 (89.71)	NS	-	-

Figures in parentheses are arcsine transformed values

Agar well diffusion plate test showed compatibility of *B. amyloliquefaciens* IIHR BA2 with *B. subtilis* and *P. fluorescens*. *B. amyloliquefaciens* IIHR BA2 showed significantly higher compatibility with *B subtilis* (60.00%) than *P. fluorescens* (25.00%) (Fig. 1 and 2). This might be due to its sequence similarity (>99%) with *B. subtilis* and both carry an evolutionary compact (Jeyaram *et al*., 2011; Zhang *et al*., 2016; Hanafy *et al*., 2016). In contrast *B. amyloliquefaciens* IIHR BA2 showed moderate compatibility with *P. fluorescens.* Earlier studies have reported that *B. amyloliquefaciens* harbours huge gene clusters involved in synthesis of antimicrobial lipopepetides and polyketides (Chen *et al*., 2009). Hence secretion of these chemotoxins might have reduced the growth of competitive strains.

In dual culture experiment *B. amyloliquefaciens* IIHR BA2 showed different degrees of compatibility with all the three test fungal BCAs *viz*., *T. viride, P. lilacinus* and *P. chlamydosporia*. Among them, *T. viride* showed the maximum mycelial growth and recorded the highest compatibility (62.80%) followed by *P. chlamydosporia* (50.90%) and *P. lilacinus* (48.60%) (Fig. 1 and 2). Similarly Fuga *et al*. (2016) studied the compatibility between the isolates of *Bacillus* spp. and *Trichoderma* spp. and resulted 12 out of 40 combinations were incompatible. However Sicuia *et al*. (2014) reported that *B. amyloliquefaciens* OS 17 inhibited the mycelial growth of entomopathogenic fungi *Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae*, *Verticillium lecanii* and *Isaria farinosa* (sin. *Paecilomyces farinosus*). Several studies reported that *B. amyloliquefaciens* produce a wide range of antifungal lipopeptides *viz.,* iturin, surfactin, fengycin, difficidin, macrolactin, *etc*., which might have inhibited the growth of the test fungal BCAs in the present study.

Compatibility of *B. amyloliquefaciens* IIHR BA2 with agrochemicals:

In the present study, *B. amyloliquefaciens* IIHR BA2 was found highly sensitive and completely incompatible with chemical fungicides captan, mancozeb, copper oxychloride and fenamidone + mancozeb at all the tested concentrations. Whereas carbendazim and wettable sulphur revealed comparatively less toxicity and minimum inhibition to *B. amyloliquefaciens* IIHR BA2 at lower concentrations of 500 and 1000 ppm. However higher concentrations of 1500 and 2000 ppm were relatively more toxic and highly inhibitive (Table 1 and Fig. 3). With bactericide K-cylin, no growth of *B. amyloliquefaciens* IIHR BA2 was observed at all the tested concentrations and proved highly toxic (Table 1). These results fall in line with Khan and Gandopadhyay (2008) who reported that *P. fluorescens* strain PFBC-25 was highly inhibited by captan whereas carbendazim was less inhibitory. Also Keshgond and Naik (2014) reported that carbendazim was less toxic to *P. putida* while mancozeb and captan were highly toxic. Compatible reactions occurred with carbendazim and wettable sulphur because a few bacteria can use pesticides as nutrients and they can survive under high chemical concentrations (Kishore and Jacob, 1987; Aislabie and Jones, 1995). The differential response of the *B. amyloliquefaciens* IIHR BA2 with fungicides and bactericides might be the reason of variation in their innate ability to digest these agrochemicals.

Thus the present study indicated that *B. amyloliquefaciens* IIHR BA2 expressed compatible reactions with other BCAs like *B. subtilis* and *T. viride*. It is necessary to understand further the interaction between these microbes at molecular level to have a fundamental clue on the observed compatibility in dual plate. This can help to develop better consortia formulation of microbes to target multiple pests and pathogens with differential modes of action. Also this study suggests to approach cautiously with highly incompatible agrochemicals like K-cyclin, captan, mancozeb, copper oxychloride and fenamidone + mancozeb for *B. amyloliquefaciens* IIHR BA2. Hence *B. amyloliquefaciens* IIHR BA2 can be applied in integration with other BCAs and selective agrochemicals for management of soil borne plant pathogens and nematodes.

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