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ORIGINAL ARTICLE

Development of *Mesorhizobium ciceri*-Based Biofilms and Analyses of Their Antifungal and Plant Growth Promoting Activity in Chickpea Challenged by Fusarium Wilt

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Abstract Biofilmed biofertilizers have emerged as a new improved inoculant technology to provide efficient nutrient and pest management and sustain soil fertility. In this investigation, development of a *Trichoderma viride–Mesorhizobium ciceri* biofilmed inoculant was undertaken, which we hypothesized, would possess more effective biological nitrogen fixing ability and plant growth promoting properties. As a novel attempt, we selected *Mesorhizobium ciceri* spp. with good antifungal attributes with the assumption that such inoculants could also serve as biocontrol agents. These biofilms exhibited significant enhancement in several plant growth promoting attributes, including 13–21 % increase in seed germination, production of ammonia, IAA and more than onefold to twofold enhancement in phosphate solubilisation, when compared to their individual partners. Enhancement of 10–11 % in antifungal activity against *Fusarium oxysporum* f. sp. *ciceri* was also recorded, over the respective *M. ciceri* counterparts. The effect of biofilms and the *M. ciceri* cultures individual on growth parameters of chickpea under pathogen challenged soil illustrated that the biofilms performed at par with the *M. ciceri* strains for most plant biometrical and disease related attributes. Elicitation of defense related enzymes like L-phenylalanine ammonia lyase, peroxidase and polyphenol oxidase was higher in *M. ciceri*/biofilm treated plants as compared to uninoculated plants under pathogen challenged soil. Further work on the signalling mechanisms among the partners and their tripartite interactions with host plant is envisaged in future studies.

Keywords Biofilms · Biocontrol · Biochemical characterization · Defense enzymes · Pathogen challenge

Introduction

In the last few decades, excessive application of chemical fertilizers and fungicides to control plant diseases and improve plant productivity has led to severe damage to soil microbial communities and fertility. The use of bioinoculants as a nutrient management strategy has emerged as a potential solution towards sustainable agriculture and crop productivity. Not only nutrients, these bio-inoculants can provide a vast array of plant growth promoting substances as well as antifungal compounds which protect plants from various phyto-pathogens and improve yield [1–3]. But this strategy needs new innovative technologies which can perform consistently under different agro-climatic zones. In the last few years the focus has shifted from single inoculation to coinoculation to use of consortium of microorganisms to obtain maximum benefits. Another strategy developed is the biofilmed biofertilizers wherein fungi, cyanobacteria or bacteria are used as matrix and agriculturally important bacteria like *Azotobacter*, *Rhizobium*, *Bacillus* and *Pseudomonas* are used as associate partners [4–8].

A biofilm is an assemblage of multiple microbial species associated with a surface or any interfaces, often enclosed

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in self-produced extracellular polymeric substances (EPS) matrix [9] which provides enhanced survival ability to the species under adverse environmental conditions [10]. Biofilm formation provides its partners with better nutrient availability by syntrophism, protection against environmental stresses (UV radiation, desiccation, acidity, alkalinity and osmotic shock), antimicrobial compounds and acquisition of new genetic traits [11, 12]. Microbial interaction within a soil microhabitat is a dynamic process which often determines the successful establishment of bio-inoculants [13]. Biofilm comprising bacteria and fungi are known to enhance growth and survival of bacterial inoculants in soil. Biofilm formation on fungal surface may be beneficial in different ways like bacteria can exploit fungi as nutrient source directly or it can degrade complex substrates through production of extracellular enzymes. Bacterial cells colonizing fungal hyphae may use it to reach and colonize new microhabitats in soil [14, 15]. Biofilm colonizing plant root surface provide better plant growth promotion as well as protection against several soil borne fungal pathogens [16].

Rhizobia are well known for their symbiotic nitrogen fixing ability in legumes, which in turn increases soil fertility and productivity. Although the basic tenet of *Rhizobium* inoculation is nitrogen fixation, rhizobia can also effectively control various soil-borne plant pathogenic fungi. Fungal pathogens of the genera *Fusarium*, *Rhizoctonia*, and *Macrophomina* are reported to be controlled by *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* [2, 3, 17]. Potential mechanisms include mycoparasitism, competition for nutrients, production of antifungal metabolites like hydrogen cyanide (HCN), antibiotics, siderophore and induction of plant defense mechanisms [18–20]. A number of studies showed higher elicitation of enzymes like L-phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) in plants pretreated with rhizobial strains, which play a significant role in induction of systemic resistance against several phytopathogens [21–23]. Rhizobia can effectively form biofilm with common soil fungi [6]. Studies showed that biofilmed biofertilizers may provide better results as compared to their conventional monoculture or mixed culture counterparts [24–26].

Trichoderma based formulations are most commonly used as biocontrol agents against plant diseases, however their combination with rhizobia are less investigated. In this study we focus towards in vitro development of *Rhizobium* based biofilms using *Trichoderma viride* as fungal matrix and evaluate its efficiency as bioinoculant having dual functions, biofertilizer as well as biocontrol agent in pathogen challenged chickpea crop. The hypothesis underlying this investigation is that the synergy between *Trichoderma*, a proven biocontrol agent and its combination with biocontrol properties possessing rhizobia can be a better option to the use of either separately.

Materials and Methods

Growth and Maintenance of Cultures

Two strains of *Mesorrhizobium ciceri* A13 and CR24 specific to chickpea were obtained from germplasm collection of the Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi. *Trichoderma viride* (ITCC 2211) was obtained from Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi, India. *Mesorhizobium ciceri* cultures were grown on Yeast Extract Manitol (YEM) broth and grown in shaking incubator (120 rpm) at 28 °C for 48 h, whereas *T. viride* was grown on Potato Dextrose Agar plates at 30 °C for seven days as stationary cultures.

Optimization of Medium for Biofilm Formation

Five different media—Nutrient broth (NB), Pikovskaya medium (Piko), Yeast extract manitol broth (YEM), Yeast extract peptone broth (YPG), and Jensen's medium (JEN) were used to optimize the growth of biofilm. Erlenmeyer flasks (1000 mL) containing 300 mL broth were autoclaved and after cooling inoculated with 2 mL of rhizobial culture (10^9 CFU/mL). Experiment was set up in triplicate and flasks were incubated at rotary shaker (120 rpm) at 28 °C for 48 h. After incubation, flasks were inoculated with 2 mL of *T. viride* spore suspension (2.2×10^7 spores/mL) and incubated under static condition at 30 °C for 2 weeks. Biofilms were harvested, washed three times with sterile distilled water to remove non adherent cells and centrifuged at 2000 rpm for 5 min. Biofilm fresh weight and dry weight (after oven drying at 70 °C for 24 h) were recorded. To get a uniform suspension, biofilms were homogenized by vortexing with glass beads for 10 min. Population count of each biofilm partner was recorded on plates containing appropriate antibiotics.

Development of Biofilmed Formulations

Mesorhizobium ciceri based biofilms were developed using *T. viride* as the fungal matrix following the method described by Triveni et al. [27]. Modified Jensen's broth supplemented with 1 % yeast extract was selected and used for biofilm development. Erlenmeyer flasks (1000 mL) containing 300 mL broth were autoclaved and after cooling inoculated with 2 mL of 48 h grown rhizobial culture (10^9 CFU/mL). Flasks were incubated at rotary shaker (120 rpm) at 28 °C for 48 h. After

incubation, flasks were inoculated with 2 mL of *T. viride* spore suspension (2.2×10^7 spore/mL) and incubated under static condition at 30 °C for 2 weeks. After 14 days, biofilm mats were collected, washed three times with sterile water to remove non adherent cells and centrifuged at 2000 rpm for 5 min. Biofilms were homogenized and the uniform suspension was used for population count and further analysis.

For biochemical tests, single cultures (10^9 CFU/mL for *Mesorhizobium ciceri* and 10^7 CFU/mL for *T. viride*) were compared with the samples of biofilm developed.

Seed Germination Assay

Chick pea seeds (variety JG-62) were surface sterilized by treating with 0.1 % mercuric chloride for 3 min followed by 70 % ethanol for 1 min and washed repeatedly with sterile distilled water. This seeds were soaked in different cultures/biofilm for 10 min and incubated on 1 % water agar plates (10 seeds per plate, 3 replications per treatment) for 3 days. Seeds soaked in sterile distilled water served as control.

Determination of Antifungal and Plant Growth Promoting Attributes

In Vitro Antifungal Activity

Cultures/biofilms were tested for their antifungal activity in vitro using the dual culture technique [28]. A fungal disc (4 mm) from 7 days old fungal plate was placed at the centre of a PDA plate and incubated at 30 °C for 24 h. The plates were inoculated with cultures/biofilms equidistantly on the margins and incubated at 28 °C for 5 days. Percent growth inhibition of fungal culture was calculated using the formula of Whipps [29].

$$\% \text{ Inhibition} = (R - r)/R \times 100$$

where r is the radius of the fungal colony opposite the bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony.

HCN Production

Production of hydrogen cyanide (HCN) was estimated qualitatively by the method of Bakker and Schippers [30]. Cultures/biofilms were streaked individually on plates containing King's B medium amended with 4.4 g/L glycine. A filter paper (Whatman No. 1) soaked in 0.5 % picric acid in 2 % (w/v) sodium carbonate was placed on lid of the petri plate. Plates were incubated at 28 °C for 72 h. Change in colour of the filter paper from yellow to light brown or reddish brown indicated HCN production.

Ammonia Production

Production of ammonia by the cultures was estimated by the method of Dye [31]. Cultures/biofilms were grown in peptone water for 96 h at 30 °C. After incubation 1 mL of Nessler's reagent was added to each tube. The development of faint yellow colour is indicative of small amount of ammonia, while deep yellow to brownish colour indicates greater production of ammonia.

IAA Production

IAA production by the biofilms was estimated using nutrient broth, supplemented with tryptophan solution (50 µg/mL broth). Sterile broth was inoculated with 100 µL of culture/biofilm suspension and incubated at 30 °C for 96 h. Experiment was designed in triplicate with appropriate uninoculated control.

The amount of IAA produced was estimated by the method of Hartmann et al. [32]. The intensity of pink colour at 530 nm was measured using a calibration curve of standard IAA stock solution (10–100 µg/mL) prepared in 50 % ethanol. IAA was quantified spectrophotometrically (Perkin Elmer spectrophotometer, model Lambda E2201) and expressed as µg IAA produced after 48 h.

Phosphate Solubilisation

Phosphate solubilisation activity was tested qualitatively on plates containing Pikovskaya's medium. Distinct clearing zone around the colonies indicated positive result. For quantitative estimation of phosphate solubilisation, Pikovskaya's broth was inoculated with cultures/biofilms and incubated on a rotary shaker (120 rpm) at 30 °C for 5 days. Experiment was set up with three replicates for each treatment and appropriate uninoculated control. After incubation, broth cultures were centrifuged at 5000 rpm for 10 min. Phosphate solubilisation was estimated by the method of King [33], improved by Jackson [34]. Intensity of the blue colour developed was measured at 660 nm using Perkin Elmer spectrophotometer. The quantity of phosphate solubilized was expressed as µg/mL P solubilized.

Evaluation of Biocontrol Potential of Cultures/Biofilms

A pot experiment was designed to evaluate the biocontrol potential of the cultures/biofilms against *Fusarium* sp. (wilt pathogen) challenged chick pea plants in the National

Phytotron Facility, with day/night temperature of 22–24/18 °C and humidity of 60 %. Chickpea seeds (variety JG-62) were surface sterilized and pre-germinated for 2 days in petri dishes containing 1 % water agar. The pots were autoclaved at 10^5 kg m^{-2} pressure and 121 °C temperature for 1 h on three consecutive days, and then inoculated with *Fusarium* sp, at the rate of 5 g/kg soil containing 5×10^3 spores kg^{-1} soil. The pre-germinated seeds were then transplanted into plastic pots (6 inch diameter, 3 seeds per pot) containing *Fusarium*-inoculated soil mixture (positive control) after 24 h. Seeds were inoculated with 1 mL of either *M. ciceri* culture (10^8 CFU/mL) or *M. ciceri* based biofilm (containing 10^7–10^8 CFU of *M. ciceri* and 10^6–10^7 CFU for *T. viride*) per seedling at sowing time. Seeds treated with Carbendazim (1.5 g/kg seeds) were used as chemical control and seeds treated with commercial formulation of *Trichoderma viride* were used to compare biocontrol potential of the biofilmed formulation. Pots with uninoculated seeds and pots without fungal inoculum served as negative controls. The experiment was set up with three replications for each treatment for a period of 8 weeks. Depending upon the visual observations, plants were watered and fertilized with 100 mL of nitrogen free seedling nutrient solution weekly. Disease intensity index (DII) was calculated as described by Cachinero et al. [35]. Plant growth parameters like shoot, root length and biomass as well as nodule dry weight were recorded. Acetylene reduction assay to determine nitrogenase activity of the nodules were performed by the method of Hardy et al. [36].

Elicitation of Defense-Related Enzymes in Host Plant

Elicitation and accumulation of defense related enzymes—L-phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) in host plant were studied as influenced by seed treatment with *Mesorhizobium ciceri* and its biofilms under pathogen challenge by Fusarium wilt. Experiment was done in CRD with seven treatments and three replications. Enzyme assay was done at 7 days interval up to 28 days after sowing (DAS).

L-Phenylalanine Ammonia Lyase (PAL) (EC 4.3.1.5)

Samples (3 g) of fresh leaves were ground using a pre-cooled (4 °C) mortar and pestle with 9 mL of sodium borate buffer mixed with 2-mercaptoethanol (0.8 mL/L of buffer). The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was used as enzyme extract for the assay. PAL activity was determined according to Sadasivam and Manickam [37].

Peroxidase (POX) (EC 1.11.1.7)

Enzyme extract was prepared by grinding 3 g of leaf samples in pre-cooled mortar and pestle, using 9 mL of 0.1 M phosphate buffer (pH 7.0). After centrifugation at 10,000 rpm for 10 min, the supernatant was used for the enzyme assay. POX activity was estimated by the method of Thimmaiah [38].

Polyphenol Oxidase (PPO) (EC 1.14.18.1)

The enzyme extract was prepared by grinding 3 g of leaf sample in pre-cooled mortar and pestle containing 6 mL of 0.1 M sodium phosphate buffer (pH 7.1). The content was centrifuged at 10,000 rpm for 10 min and the supernatant was used for the enzyme assay. PPO activity was estimated as described by Sadasivam and Manickam [37].

Statistical Analysis

The data was analysed using AGRIS statistical software. The tables and figures are provided with rankings, based on test of significance (0.01 probability), with 'a' being the highest.

Results

Optimization of Medium for Biofilm Formation

Biofilm development in nutrient broth (NB) and Pikovskaya medium (Piko) was slow and poor, as compared to other media (Table 1). Biofilm in Yeast extract mannitol (YEM) medium showed higher sporulation by the fungal partner, hence less attachment of bacterial cells to the fungal matrix was observed. Although biofilm development was low in Jensen's medium, addition of 1 % yeast extract stimulated biofilm formation significantly. Population count of each partner in biofilm was also found much higher in case of biofilm developed in Jensen's medium (Table 1).

Population Count of Partners in Biofilms

In this study, individual rhizobial cultures A13 (1.4×10^9 CFU/mL), CR24 (1.2×10^9 CFU/mL) and *T. viride* (2.2×10^7 spore/mL) were used for biofilm preparation as well as biochemical tests. In Biofilm1 (*T. viride–Mesorhizobium ciceri* A13) population counts of 1.62×10^8 CFU/mL for *Mesorhizobium ciceri* sp. A13 and 1.2×10^7 CFU/mL of *T. viride* was recorded, whereas Biofilm 2 (*T. viride–Mesorhizobium ciceri* CR24)

Table 1 Optimization of biofilm formation using different media

Medium	Biofilm fresh weight (g)	Biofilm dry weight (g)	Population count (CFU/mL)	
			Bacteria	Fungi
NB	10.21^{d}	7.67^{d}	1.10×10^{5}	8.20×10^{6}
Piko	10.34^{d}	8.20^{d}	1.62×10^{5}	5.60×10^{6}
YEM	19.11^{c}	10.50^{c}	1.78×10^{6}	1.20×10^{8}
YPG	23.94^{b}	12.22^{b}	2.31×10^{6}	1.11×10^{8}
JENY	28.29^{a}	13.10^{a}	1.20×10^{7}	1.62×10^{8}
LSD (0.01)	1.23	0.560		

Details of medium: *NB* nutrient broth, *Piko* Pikovaskaya medium, *YEM* yeast extract mannitol broth, *YPG* yeast extract peptone broth, JENY, Jensen's medium + 1 % yeast extract

exhibited population count of *Mesorhizobium ciceri* sp. CR24 (1.92×10^{8} CFU/mL) and *T. viride* (1.58×10^{7} CFU/mL).

Seed Germination Assay

Germination percentage in chickpea seeds was taken as an index of plant growth promoting activity of the cultures individually and their biofilmed counterparts (Fig. 1a). Treatment of seeds with individual cultures of rhizobia or *Trichoderma* could not influence the per cent germination significantly whereas biofilms of both the rhizobial cultures were significantly superior to medium treated seeds (control) in terms of % germination.

Biochemical Tests

All the cultures individually and their biofilms exhibited antagonistic activity against *Fusarium oxysporum* f. sp. *ciceri* (Table 2). Biofilmed formulations showed higher antifungal activity than their individual partners.

In this study, all the cultures and their biofilms, except *Mesorhizobium ciceri* isolate A13 exhibited HCN production (Table 2), with biofilms showing higher production than individual cultures. Similar results were observed for ammonia production (Table 2). All the cultures used in this study were found positive for IAA production, with values ranging from 2.03 to 19.50 µg/mL (Fig. 1b). Individual rhizobial cultures showed higher production of IAA as compared to their biofilmed formulation. The highest IAA production was found in rhizobial isolate A13 (19.50 µg/mL) followed by its biofilmed counterpart (17.34 µg/mL).

All the individual cultures and biofilms were found to be efficient solubilizers of inorganic phosphate (Fig. 1c). Distinct clearing zone was observed on Pikovskaya's medium plates (Fig. 2). Phosphate solubilizing activity ranged from 2.42 µg/mL (*T. viride*) to 15.50 µg/mL (Biofilm1). Biofilms exhibited significantly higher solubilisation potential than their individual partners.

Evaluation of Biocontrol Potential of Cultures/Biofilms

In the present study, all the treatments exhibited enhancement in growth parameters of chickpea plants as compared to the control plants challenged with *Fusarium* wilt (Table 3; Fig. 3). Rhizobial isolate A13 showed highest increase in shoot length (83.11 %) and shoot weight (119.66 %) as compared to control. It also showed highest increase in root length (101.8 %) and root weight (104.8 %). Both the rhizobial isolates showed better performance than their biofilmed counterpart in terms of shoot and root length and biomass; although observations revealed that Biofilm1 and rhizobial isolate CR24 were statistically at par. Seed treatment with *T. viride* and carbendazim performed fairly well as compared to control under fungal infection and were found statistically at par. Root nodules were formed only in plants treated with rhizobial isolates and biofilms and nitrogenase activity was found perfectly correlated with nodulation. Plants grown in pots without fungal inoculum were better than plants from *T. viride* and carbendazim treated plants in terms of growth parameters, but found less vigorous than rhizobia/biofilm inoculated plants.

Studies on Elicitation of Defense-Related Enzymes in Host Plant

L-Phenylalanine Ammonia Lyase (PAL)

L-Phenylalanine ammonia lyase activity was low initially (7 DAS), but increased sharply at 14 DAS and after that it gradually decreased (Fig. 4a). Both *Mesorhizobium ciceri* strains and their biofilms treated plants exhibited higher elicitation of enzyme as compared to control (uninoculated) plants. Enzyme activity was found significantly higher in biofilm treated plants than their rhizobial counterpart. Seeds treated with *T. viride* and Carbendazim showed lower amount of PAL activity. Lowest enzyme

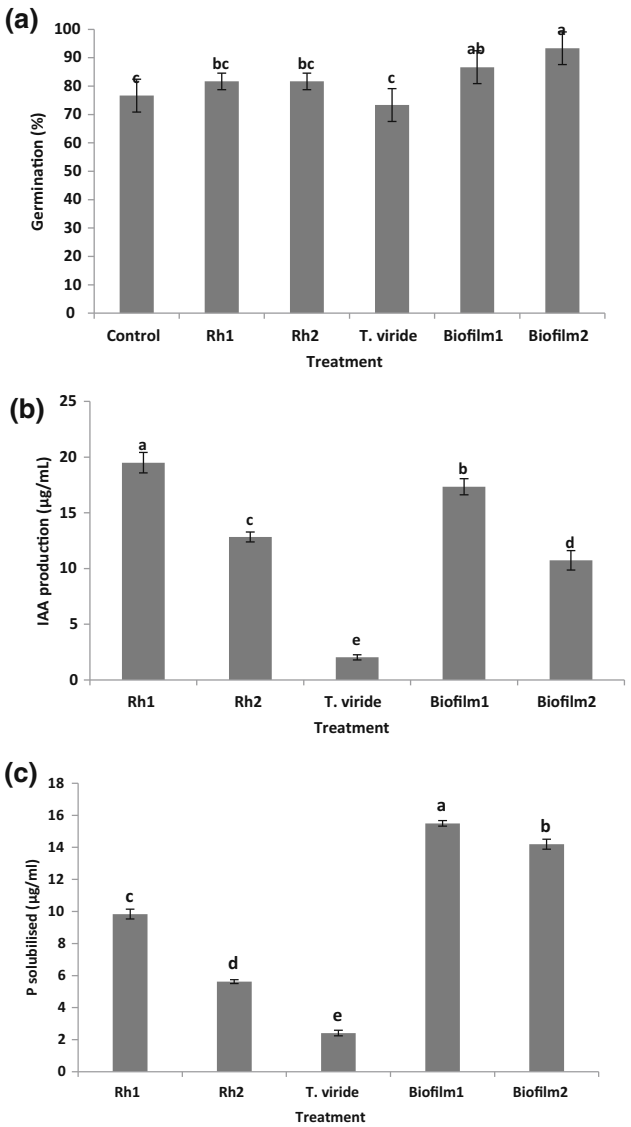

Fig. 1 Plant growth promoting activity exhibited by biofilms and their partners. **a** Percent germination of chickpea seeds, as influenced by biofilms and their partners; **b** IAA production exhibited by individual cultures and biofilms; **c** Phosphate solubilisation exhibited by individual cultures and biofilms. *Superscripts* in the histogram, denoted by *common letter* are not significantly different at 1 % level of probability by Duncan's Multiple Range Test (DMRT). Details of Treatments Control, Sterile water; Rh1, *M. ciceri* A13; Rh2, *M. ciceri* CR24, *T. viride*; Biofilm1, *T. viride-M. ciceri* A13; Biofilm2, *T. viride-M. ciceri* CR24

activity was observed in plants without any inoculation or only fungal challenge.

Peroxidase (POX)

Increase in peroxidase activity was observed up to 21 DAS and then decreased till 28 DAS in plants treated with biofilms and individual cultures (Fig. 4b). Although, in case of uninoculated plants grown in pathogen infested soil, enzyme activity remained statistically at par in all the days of observation.

Biofilm 2 (*T. viride–Mesorhizobium ciceri* CR24) exhibited highest enzyme activity followed by biofilm 1 (*T. viride–Mesorhizobium ciceri* A13). *T. viride* treated and carbendazim treated plants showed lower enzyme activity as compared to plants treated with biofilms and its partners.

Polyphenol Oxidase (PPO)

Polyphenol oxidase activity in all the treatments increased up to 14 DAS and after that activity decreased gradually till 28 DAS (Fig. 4c). Highest enzyme activity was observed in biofilm 1 (*T. viride–Mesorhizobium ciceri* A13) treated plants on 14 DAS. Observations revealed that enzyme activity in *Mesorhizobium ciceri* CR24 and biofilm 2 (*T. viride–Mesorhizobium ciceri* CR24) treated plants were statistically at par. Although the total enzyme activity was lower, *T. viride* treated and carbendazim treated plants also showed increase in activity till 14 DAS and decreased thereafter. Lowest enzyme activity was observed in plants without any inoculation or fungal challenge.

Discussion

Bioinoculants are being used for over a century to improve crop productivity and soil health [39]. A number of agriculturally important microbes have been identified for their plant growth promoting and antagonistic activity against phytopathogens and being commercialised as bioinoculants [40]. A micro-organism may function optimally under laboratory conditions, but successful establishment and growth of that microbe under different agro-ecological situations is still a challenging task. In that context, new and improved bioinoculant technologies are warranted which can reproduce the beneficial effects consistently in field level, wherein a large number of factors, besides environmental and native flora and the buffering capacity of soil play key roles. Root colonization is the most important step for effective interaction with the plant, and rhizobia with their inherent capacity to form symbiotic associations, particularly with legumes are most suitable. Microbes generally grow as biofilms around the roots or soil particles in the rhizosphere, helping to maintain contact with the plants.

There are several reports on rhizobia exhibiting antifungal activity against pathogenic fungi and reducing the disease incidence in plants [41–43]. Pathogenic control by rhizobia is attributed to mycoparasitism, production of antifungal metabolites like hydrogen cyanide (HCN), antibiotics, siderophores, competition for nutrients and

Table 2 Antifungal attributes exhibited by biofilms and their individual partners

Treatments	Antifungal activity against *Fusarium oxysporum* f. sp. *ciceri* (% inhibition)	HCN production[a]	Ammonia production[b]
T1			
Mesorhizobium ciceri A13	32.50	–	+
T2			
Mesorhizobium ciceri CR24	30.14	+	+
T3			
Trichoderma viride	26.57	+	+
T4			
Biofilm1	35.71	++	+++
T5			
Biofilm 2	33.33	+++	+++

[a] Degree of activity (+ + + + + > + + + + > + + + > + + > +)

[b] Colour, ranged from yellow (+) to dark brown (+ + + + +)

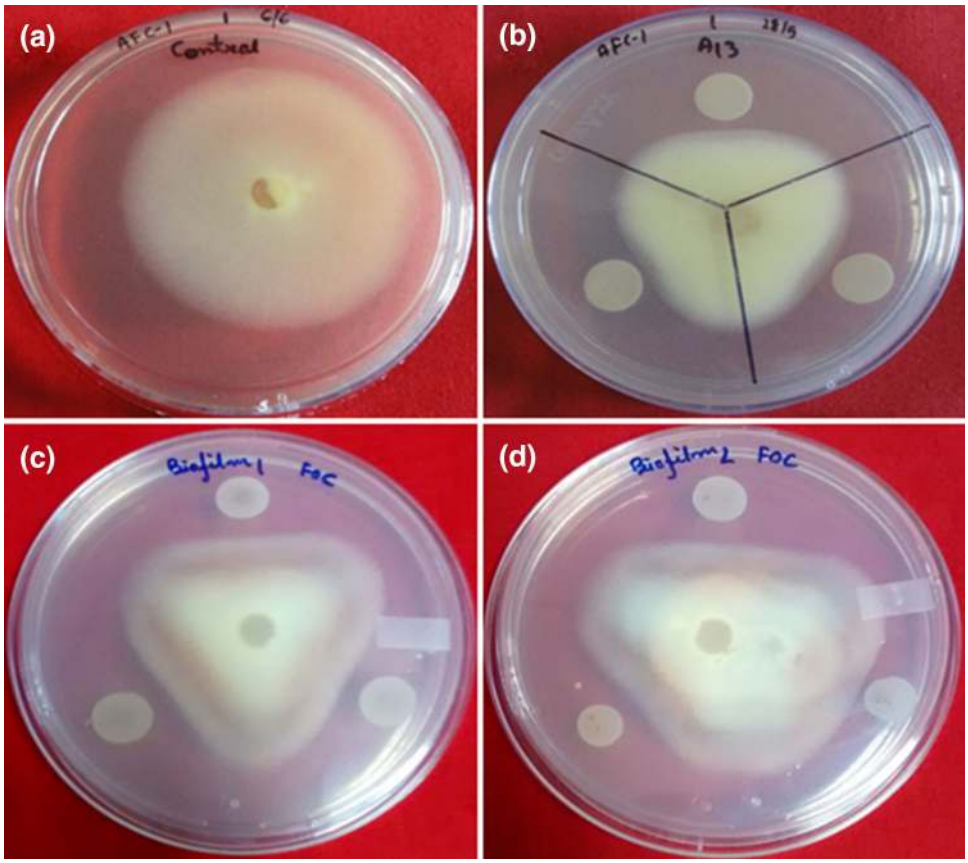

Fig. 2 *In vitro* anti-fungal activity shown by biofilms and its partners against *Fusarium oxysporum* f. sp. *ciceri*. **a** Control; **b** *M. ciceri* A13; **c** Biofilm 1, *T. viride-M. ciceri* A13; **d** Biofilm 2, *T. viride-M. ciceri* CR24

induction of plant defense mechanisms [20, 44, 45]. In our study, both the rhizobial isolates and *T. viride* alone exhibited good antifungal property in terms of growth inhibition of fungal mycelium (32.5, 30.14 and 26.57 % respectively). But observations revealed that there was a significant increase in inhibition, when biofilms were applied (35.71 and 33.33 % respectively). Biofilms also showed increase in HCN and ammonia production as compared to their individual partners, which are correlated to antifungal activity. Similar results were observed by Triveni et al. [7] in case of *B. subtilis–T. viride and P. fluorescens–T. viride* biofilms. Arfaoui et al. [17] has reported suppression of *Fusarium* wilt of chickpea under in vitro and in vivo conditions by six rhizobial strains

Table 3 Effect of biofilms and their partners on the growth of *Fusarium* wilt challenged chickpea plants

Treatment	DII (%)	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Nodule dry weight (g)	ARA (μmol C_2H_4/g dry wt. nodules/h)
Mesorhizobium ciceri 1 (A13)	5.5	$26.13^{a} \pm 0.67$	$9.16^{a} \pm 0.25$	$14.61^{a} \pm 0.20$	$5.08^{a} \pm 0.15$	$0.35^{a} \pm 0.01$	$3.17^{a} \pm 0.04$
Biofilm 1	10	$24.32^{b} \pm 0.46$	$8.55^{b} \pm 0.15$	$13.30^{b} \pm 0.26$	$4.43^{c} \pm 0.14$	$0.28^{b} \pm 0.01$	$2.80^{b} \pm 0.02$
Mesorhizobium ciceri 2 (CR24)	8.3	$24.30^{b} \pm 0.62$	$8.26^{b} \pm 0.10$	$13.34^{b} \pm 0.50$	$4.64^{b} \pm 0.12$	$0.24^{c} \pm 0.02$	$2.10^{c} \pm 0.01$
Biofilm 2	11.11	$22.77^{c} \pm 0.38$	$7.87^{c} \pm 0.08$	$11.70^{c} \pm 0.26$	$3.57^{d} \pm 0.07$	$0.21^{d} \pm 0.01$	$1.94^{d} \pm 0.02$
Control (without *M. ciceri)*	19.4	$14.27^{g} \pm 0.30$	$4.17^{e} \pm 0.35$	$7.24^{f} \pm 0.22$	$2.48^{e} \pm 0.15$	ND	ND
T. viride	13.8	$19.47^{e} \pm 0.30$	$7.43^{d} \pm 0.14$	$8.49^{e} \pm 0.17$	$3.40^{d} \pm 0.04$	ND	ND
Chemical control	10	$18.49^{f} \pm 0.29$	$7.37^{d} \pm 0.10$	$8.66^{e} \pm 0.15$	$3.43^{d} \pm 0.10$	ND	ND
Absolute Control (without (*M. ciceri* and fungal challenge)	0.0	$21.48^{d} \pm 0.70$	$8.38^{b} \pm 0.13$	$10.50^{d} \pm 0.10$	$3.48^{d} \pm 0.03$	ND	ND

Means in the columns followed by same superscript letters indicate no significant difference ($p = 0.05$) by Duncan's multiple range test

ND not detected

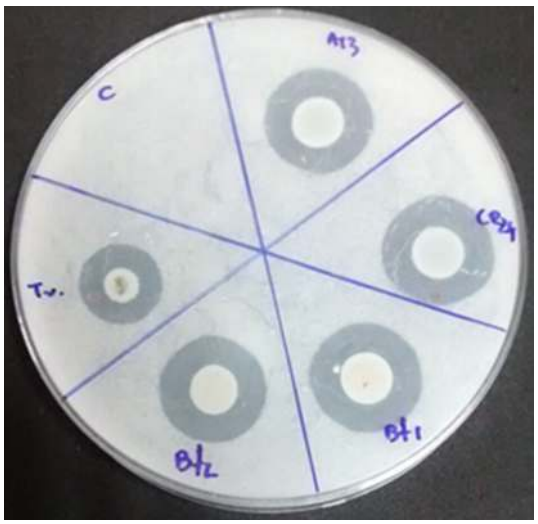

Fig. 3 Phosphate solubilisation activity shown by individual cultures and biofilms in Pikovskaya medium

positive for HCN production. Chandra et al. [45] also observed growth inhibition of *Sclerotinia sclerotiorum* causing white rot in *Brassica campestris* by HCN producing *Rhizobium loti* MP6.

Biofilm formation is quite common in natural environments which provide its component microbial cells a certain degrees of protection and helps in their growth, survival and successful colonization [46]. Often biofilms exhibit higher amount of plant growth promoting activity than its partners individually. The increased cell density within a biofilm provides the opportunity to exhibit attributes that single cells cannot achieve efficiently [7].

In this study, biofilm application led to higher germination percentage of chickpea seeds compared to individual partners. Enhancement in germination percentage reveals that the synergistic interaction between the partners provides better growth promoting environment. Similar type of enhanced seed germination percentage was observed by Qurashi and Sabri [47] in chick pea and Buddhika et al. [48] in maize. Biofilmed biofertilizers have already shown its potential in several crops like rice, cotton, wheat, tea, soybean and mung bean [5, 6, 8, 49].

Biofertilizers mediated increase of plant growth and yield is often attributed to production of growth regulators like IAA, gibberellins and cytokinins [50, 51]. Cassán et al. [52] reported increase in growth of maize and soybean by strain of *Azospirillum brasilense* and *Bradyrhizobium japonicum* producing IAA. Besides its role as phytostimulator, IAA acts as signalling molecule in plant–microbial interactions and biofilm development [53, 54]. In this study, IAA production increased up to 19.44 % in biofilms as compared to its partners. This reveals better signalling and compatibility between the bacterial and fungal partners. Similar observations were recorded by Jayasinghearachichi and Seneviratne [25].

Phosphate solubilisation is one of the important plant growth promoting activities performed by microbes in soil. Although phosphate fertilizers provide most of the available P required by plants, some of it gets fixed and excessive application of chemical fertilizer is not only expensive, but also hazardous to environment. A number of studies reveal that rhizobia can efficiently solubilize inorganic phosphates through production of organic acids [55, 56]. Seneviratne [16] have reported that biofilm formation may enhance phosphate solubilisation ability. In our study, all the cultures were found positive for phosphate production. Phosphate solubilisation was enhanced significantly in biofilmed formulations as compared to individual cultures. Similar type of enhancement was observed by Jayasinghearachichi and Seneviratne [57] in

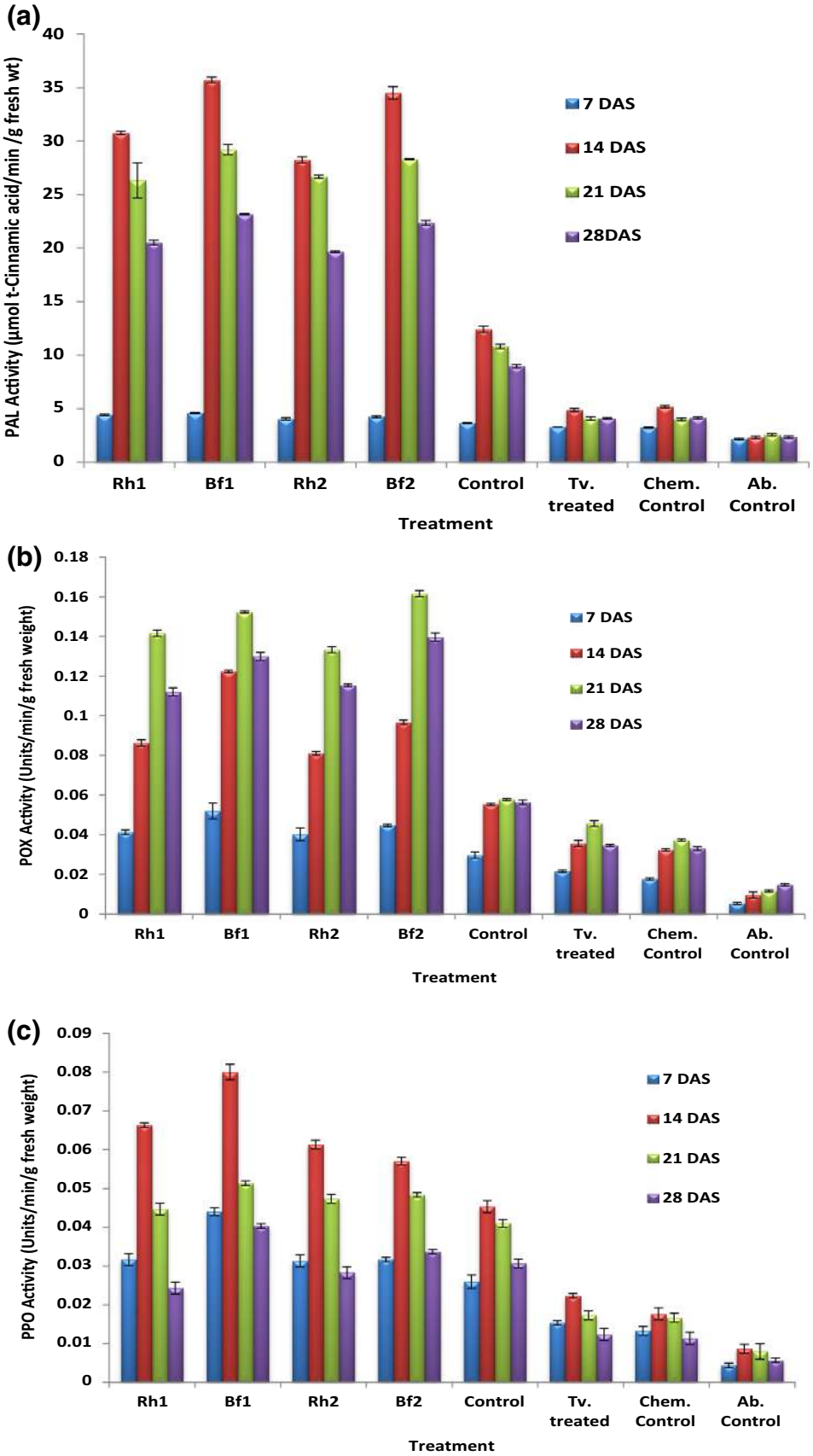

Fig. 4 Influence of biofilms and their partners in elicitation of different defense enzymes in chickpea plants. **a** PAL activity, **b** POX activity, **c** PPO activity. Treatments denote Rh1, *M. ciceri* A13; Bf1, *T. viride-M. ciceri* A13 biofilm; Rh2, *M. ciceri* CR24, Bf2, *T. viride-M. ciceri* CR24 biofilm; Control, seeds without *Rhizobium* inoculation; Tv. treated, seeds treated with commercial formulation of *T. viride*; Chem. control, seeds treated with Carbendazim (1.5 g/kg seed); Ab. Control, without *M. ciceri* or fungal challenge

case of *Pleurotus ostreatus–Bradyrhizobium elkanii* SEMIA 5019 biofilm.

Several examples illustrate the potential of rhizobia in biocontrol of disease causing plant pathogens and improve plant growth. Application of rhizobial isolates as seed coating or as soil drench reduced disease incidence of *M. phaseolina*, *R. solani* and *Fusarium* spp. in okra plants [58]. Deshwal et al. [42] reported that several rhizobial and bradyrhizobial isolates can effectively suppress the growth of *Macrophomina phaseolina* causing charcoal rot of ground nut. In our study, both the biofilmed and their counterpart rhizobial isolates were found to reduce disease incidence and increase plant growth parameters like shoot and root length and biomass in *Fusarium* inoculated pots. Similar type of observations were recorded by Ganesan et al. [59], where dual inoculation of *Rhizobium* with *Trichoderma harzianum* not only reduced incidence of collar rot in groundnut caused by *Sclerotium rolfsii*, but also increased several growth parameters like root, shoot length and plant biomass. Dual inoculation of *Rhizobium* and *Trichoderma* have also been reported to reduced damping off and root rot diseases and increase yield components in broad bean, chickpea and lupine plants [60].

A number of studies have revealed the significant role of defense related enzymes like L-phenylalanine ammonia lyase, peroxidase and polyphenol oxidase in disease resistance [21, 61]. In our study, greater increase in the activity of PAL, POX and PPO enzymes was observed in culture/biofilm inoculated plants as compared to non-inoculated control as well as plants without any inoculation or fungal challenge. Enzyme activity was observed much higher in case of biofilms treated plants compared to single inoculation throughout the study period. This suggests that biofilms are much efficient in elicitation of induced systemic resistance in plants thereby, reducing disease incidence. Similar type of increase in defense enzyme activity has been reported by Mabrouk et al. [22] and Dutta et al. [62] in pea and pigeon pea against fusarial wilt. Osdaghi et al. [23] also reported induction of resistance in common bean when bacterized with *Rhizobium leguminosarum* bv. *phaseoli* against common bacterial blight caused by *Xanthomonas axonopodis* pv. *phaseoli*.

Studies suggest that combining PGPR strains with diverse mode of plant growth promotion and antagonistic activity against phyto-pathogens are more effective than monoculture inoculum [60, 63]. Our study illustrates the promise of *Mesorhizobium ciceri* based biofilms with *T. viride* having both the potential of plant growth promotion and biocontrol of plant pathogens. Biofilmed inoculants having dual attributes can therefore be a novel means to contribute to increased plant growth and productivity in an environment-friendly manner.

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Compliance with Ethical Standards

Conflict of interest The authors state no conflicts of interest.

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