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RESEARCH ARTICLE

ANTAGONISTIC ACTIVITY BETWEEN *BACILLUS SUBTILIS*, *PSEUDOMONAS* SP. RC, *AZOSPIRILLUM BRASILENSE*, *RHIZOBIUM MELILOTI* AND CERTAIN FUNGAL PATHOGENS UNDER LABORATORY CONDITIONS

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ABSTRACT

The principa This study conducted the tests in vitro with *Bacillus subtilis*, *Pseudomonas* sp. RC, *Azospirillum brasilense* and *Rhizobium meliloti* against *Bipolaris spicifera*, *Curvularialunata*, *Fusarium* spp., *Nigrospora oryzae, Exserohilum rostratum, Alternari a*spp. And *Thanatephoruscucumeris* via the dual culture technique, and found that each bacterium was varied in its inhibitory effect on each pathogen. The interaction between pathogens conidia, sclerotia germinated and *B.subtilis*, *Pseudomonas sp*. RC, *A.brasilense* and *R.meliloti* showed abnormal hyphal swelling, lyses and completed degradation of the hyphal tip. The results have been seen that the controls where no cultures were incubated in the wells, the fungal culture continued to grow and covered the 9 cm petri dishes in 7 days. Clear inhibition zones were observed in the interaction area between *N.oryzae* R9+*B.subtilis*, *B.spicifera* R15+*B.subtilis, A.alternata* R18+*B.subtilis, N.oryzae* R9+*Pseudomonas* sp. RC, *T.cucumeris* R12+*Pseudomonas* sp. RC*, A.alternata* R18+*Pseudomonas* sp. RCand *A.alternata* R20+ *Pseudomonas* sp. RC. And found that there were a great inhibition zones that were observed between pathogens by approximately 83.33% respectively around the fungal culture. *R.meliloti* revealed moderate inhibition activity towards *T.cucumeris* R2, and gave value roughly 77.77%. However, *A.brasilense* was determined as the bacteria with the weakest inhibitory activity against *N.oryzae* R9; it recorded the lowest level of inhibition which was scored 66.66%.

INTRODUCTION

The development of new strategies to control *B.spicifera*, *C.lunata*, *Fusarium* spp., *N.oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris*such as the application of the biological control agents BCAs like *B.subtilis*, *P.fluorescens*, *A.brasilense* and *R.meliloti*, however require more work towards understanding their mode of action and eco-physiology with suitable formulation that may be applied as beneficial rhizosphere biofertilizer to help increase growth and health to control pathogens, as well as provide a natural safe alternative towards the use of synthetic chemicals (Antounand Pre Vost, 2005; Yasuda *et al.,* 2009; Latha *et al.,* 2011; Yu-xiang *et al.,* 2011; Chowdappa *et al.,* 2012; Hamdia *et al.,* 2016c). As an alternative to using *B.subtilis*, *P.fluorescens*, *A.brasilense* and *R.meliloti* microorganisms directly in soil to achieve the above purpose, excellent microbial sources with good enzyme activity have been identified as a source for application in large scale enzyme production via microbial cells (Mohammed *et al.,* 2014; Pérez-Montaño *et al.,* 2014). *B.subtilis*, *P.fluorescens*, *A.brasilense* and *R.meliloti* have many advantages such as good suppression of rice pathogens, adaptability to wide soil pH, availability of these organisms in all soils types with abilities to secrete hydrolytic enzymes and cause mycoparasitism of plant fungal pathogens and enhanced plant growth and productivity (Abeysingne, 2007; Walters *et al.,* 2013). The enzyme activity from these bacteria has multiple functions and one potential application is as a soil conditioner that may be added together with fertilizers to keep unwanted pathogens population under check as well as to promote growth (Schirmbock *et al.,* 1994; Lorito *et al.,* 1996).Many mechanisms have been reported on the mode by which these biological control agents may control the spread of phytopathogens, and exploited *B.subtilis*, *P.fluorescens*, *A.brasilense* and *R.meliloti* as biocontrolagents (Titiya *et al.,* 2007; Wiwattanapatapee *et al.,* 2007; Cummings *et al.,* 2009; Kumar *et al.,* 2009). Moreover, several investigations found these agents with the ability to release different kinds of antimicrobial compounds, including antibiotic peptides and hydrolytic enzymese.g. glucanase enzyme activity produced

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by *B.subtilis*, *P.fluorescens* (Katz and Demain, 1977; Tabernero, 1994; Mawadza, 2000;Ganeshan and Kumar, 2005). *P.fluorescens, A.brasilense* and *R.meliloti* are responsible for releasing indole-3-acetic acid (IAA) by consist of various isoenzymeswith different molecular weights (Gopalakrishnan *et al.,* 2015). Bioactive compounds exuded from *B.circulans* IAM 1165 and *B.subtilis* NSRS 89-24 in vivo have been shown to have the potential to be applied as fungicides to control blast and sheath blight diseases of rice plants (Tabernero, 1994; Leelasuphakul *et al.,* 2006; Idris *et al.,* 2007; Yadi *et al.,* 2013).*B.subtilis*, *P.fluorescens*, *A.brasilense* and *R.meliloti* strains have been played crucial role to protect fruits and vegetable crops from post-harvest diseases (Sinclair, 1989; Mari *et al.,* 1996). More recently, *Bacillus* strains with high potential to excrete heat-proteins have been used successfully to reduce rice blast disease (De Vleesschauwer *et al.,* 2008; Karthikeyan and Gnanamanickam, 2008). In addition *B.subtilis* was used as products of antagonistic strains and is commercially available (Vasudevan *et al.,* 2002).The aim of this study is to determine the best antagonistic ability of *B.subtilis*, *Pseudomonas* sp. RC, *A.brasilense* and *R.melilot*ias biocontrol agents against *B.spicifera*, *C.lunata*, *Fusarium spp*., *N.oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris* via dual culture technique on the growth rate of pathogens under laboratory conditions.

MATERIALS AND METHODS

Preparation of Causal Organisms

The laboratory experiments were conducted using *B.subtilis*, *A.brasilense*, *R.meliloti* and *Pseudomonas* sp. RC (isolated for the first time in this study) against *B.spicifera*, *C.lunata* isolates, *Fusarium spp*., *N.oryzae, E.rostratum, Alternaria* spp. and *T.cucumeris* isolates that were isolated from rice plant (Hamdia *et al.,* 2016a and b). Pure cultures from fungal mycelium and spores of above rice pathogens were sub-cultured on potato dextrose agar (PDA) plates and incubated at 28±2 °C for one week to obtain fresh mycelium.

Preparation of Biological Control Agents

Pure culture of *B.subtilis*, *Pseudomonas* sp. RC, *A.brasilense* and *R.meliloti* were cultured on Nutrient Broth Agar (NBA) media, and incubated at 28±2 °C to obtain fresh fungal cell. A single colony of gram positive bacteria *B.subtilis* which was isolated from Alfalfa plant (*Medicago sativa* L.), transferred from nutrient agar (NA) plate to the sterilized flasks containing 100 mL nutrient broth (NB) in an aseptic manner (Titiya *et al.,* 2007). *A.brasilense* is a genus of gram positive bacteria was isolated from Wheat plant (*Triticum aestivum*), a single colony transferred to the following media: 5gm of Malic acid, 0.5gm of KH_2PO_4, 0.2gm of $MgSO_4.7H_2O$, 0.1gm of NaCl, 0.02gm of $CaCl_2$, 0.002gm of $Na_2MoO_4.2H_2O$, 0.01gm of $MnSO_4.H_2O$, 0.01gm of $FeCl_3.6H_2O$, 4.5gm of KOH, 2ml of bromo themol blue, 17gm of Agar, 0.02gm of yeast extract and 4gm of NH_4Cl(Bashan and Bashan, 2011). As for *R.meliloti* (gram negative bacteria) which was isolated from Alfalfa plant (*Medicago sativa*) also, a single colony cultured inside sterilized flasks including 100 mL from media containing the following compounds: 10gm of manitol, 0.5gm of KH_2PO_4, 0.2gm of $MgSO_4.7H_2O$, 0.2gm of NaCl, 0.05gm of $FeCl_3.6H_2O$, 1gm of yeast extract and 20gm of Agar (Bissonnette *et al.,* 1986). *Pseudomonas* sp. RC which is considered gram negative bacteria was isolated from local rice variety cv. Mashkhab/Najaf and used for the first time in this study also transferedto theflasks containing 100 mL media 20gm of Glucose,1gm of $(NH_4)_2SO_4$, 0.5gm of $MgSO_4.7H_2O$, 0.2gm of yeast extract, 0.1gm of $FeCl_3$,0.1gm of $MgSO_4.7H_2O$and 5gm of $Ca_3(Po_4)_5$ (Hoberg *et al.,* 2005).These cultures were grown in a 37°Cincubator shaker with agitation at 150 rpm for 36 hours (Titiya*et al.,*2007).

Antagonistic activity between *B.subtilis*, *Pseudomonas* sp. RC, *A.brasilense*, *R.meliloti* and Rice Pathogens

T.cucumeris R1, *T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7, *C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 were individually cultured where 5 mm disc of their mycelium were placed in the middle of a 9 cm petri dish and allowed to grow for about 3 cm from the center. There were holes made aseptically in these plates using a cork borer (Titiya *et al.,* 2007). Ten (10) µL of each bacteria suspension was added into each hole, and 10 µL of deionized distilled water was used as control treatment (Figure 1). These additions were made once fungal growth achieved its desired growth radius. Three (3) days post-incubation, antagonistic activity against the pathogens was estimated on solid medium by scoring as denoted in (Figure2 to5).Three replicates were used for each pathogen; inhibition zone percentage was periodically checked and calculated after 7 days by using the following formula according to the Mojica Marin *et al.,* (2008).

$$\% \text{ Inhibition} = \frac{\text{Control growth} - \text{Fungal growth (cm)}}{\text{Control growth}} \times 100$$

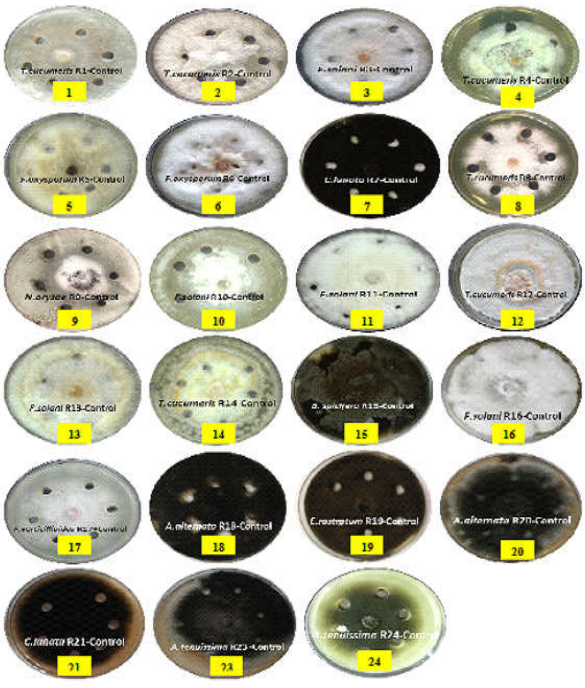

Figure 1. Rice pathogens control.(1) *T.cucumeris* R1.(2) *T.cucumeris* R2.(3) *F.solani* R3.(4) *T.cucumeris* R4.(5) *F.oxysporum* R5.(6) *F.oxysporum* R6.(7) *C.lunata* R7.(8) *F.solani* R8.(9) *N.oryzae* R9.(10) *T.cucumeris* R10.(11) *F.solani* R11.(12) *T.cucumeris* R12.(13) *F.solani* R13.(14) *T.cucumeris* R14.(15) *B.spicifera* R15.(16) *F.solani* R16.(17) *F.verticillioides* R17.(18) *A.alternata* R18. (19)*E.rostratum* R19.(20) *A.alternata* R20. (21) *C.lunata* R21.(23) *A.tenuissima* R23.(24) *A.tenuissima* R24.

RESULTS

Antagonistic activity between *Bacillus subtilis* and Rice Pathogens

Figure 2 shows the results obtained when we used *T.cucumeris* R1, *T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7, *C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 together with *B.subtilis*. However as we can see from (Figure 2), the mycelium growth of pathogens as presented above that can cover the entire 9 cm petri dish in 7 days (Figure 1) were inhibited and affected completely by *B.subtilis*as growth inhibition zones were evident surrounding the *F.oxysporum* R5, *F.solani* R8,*T.cucumeris* R14, *A.tenuissima* R23, *C.lunata* R21, *N.oryzae* R9, *B.spicifera* R15 and *A.alternata* R18 plug in the middle of the plate (Figure 2). The inhibition zone was roughly 66.66, 66.66, 66.66, 66.66, 77.77, 83.33, 83.33 and 83.33% respectively in comparison with control plates (Table1), however as we can see from Figure 2,the mycelium growth of *F.oxysporum*R6 which provided the lowest inhibition value as approximately 38.88% as in comparison with above pathogens.

Table 1. Antagonistic activity between *B.subtilis* and *B.spicifera*, *C.lunata*, *Fusarium* spp., *N. oryzae, E.rostratum, Alternaria spp*. And *T.cucumeris* under laboratory conditions

Treatments	*Inhibition Zone after 7 days
T.cucumeris R1+ *B.subtilis*	50
T.cucumeris R2+ *B.subtilis*	55.55
F.solani R3+ *B.subtilis*	44.44
*T.cucumeris*R4+ *B.subtilis*	55.55
*F.oxysporum*R5+ *B.subtilis*	66.66
*F.oxysporum*R6+ *B.subtilis*	38.88
C. lunata R7+ *B.subtilis*	44.44
F.solani R8+ *B.subtilis*	66.66
N. oryzae R9+ *B.subtilis*	83.33
*T.cucumeris*R10+ *B.subtilis*	55.55
*F.solani*R11+ *B.subtilis*	44.44
T.cucumeris R12+ *B.subtilis*	44.44
*F.solani*R13+ *B.subtilis*	50
T.cucumeris R14+ *B.subtilis*	66.66
*Bipolaris spicifera*R15+ *B.subtilis*	83.33
*F.solani*R16+ *B.subtilis*	55.55
F.verticillioides R17+ *B.subtilis*	50
*A.alternata*R18+ *B.subtilis*	83.33
*E.rostratum*R19+ *B.subtilis*	44.44
*A.alternata*R20+ *B.subtilis*	61.11
C.lunata R21+ *B.subtilis*	77.77
*A.tenuissima*R23+ *B.subtilis*	66.66
*A.tenuissima*R24+ *B.subtilis*	61.11

*Inhibition zone after 7 days according to Mojica-Marin *et al.,* (2008).

Antagonistic activity between *Pseudomonas* sp. RC and Rice Pathogens

Figure 3 shows the results of *T.cucumeris* R1,*T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7,*C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 reacted to *Pseudomonas* sp. RC. The laboratory experiment exhibited *Pseudomonas* sp. RC greater efficiency of reducing radial growth of *T.cucumeris* R2, *F.solani* R11, *F.solani* R16, *A.tenuissima* R23, *A.tenuissima* R24, *N.oryzae* R9, *T.cucumeris* R12, *A.alternata* R18 and *A.alternata* R20 by roughly 77.77, 77.77, 77.77, 77.77, 77.77, 83.33, 83.33, 83.33 and 83.33% respectively (Table 2) in comparison with control treatment which was calculated after 7 days according to Mojica-Marin *et al.,* (2008) as stated in section materials and methods. However, the *Pseudomonas* sp. RC showed the lowest level of inhibition on the causal agents as compared to the rest pathogens was with *T.cucumeris* R4, *F.solani* R8 which gave roughly 44.44% for each one (Figure 3).

Table 2. Antagonistic activity between *Pseudomonas* sp. RC and *B.spicifera*, *C.lunata*, *Fusarium* spp., *N. oryzae, E.rostratum, Alternaria spp*. And *T.cucumeris* under laboratory conditions

Treatments	*Inhibition Zone after 7 days
T.cucumeris R1+ *Pseudomonas* sp. RC	66.66
T.cucumeris R2+ *Pseudomonas* sp. RC	77.77
F.solani R3+ *Pseudomonas* sp. RC	61.11
*T.cucumeris*R4+ *Pseudomonas* sp. RC	44.44
*F.oxysporum*R5+ *Pseudomonas* sp. RC	61.11
*F.oxysporum*R6+ *Pseudomonas* sp. RC	55.55
C. lunata R7+ *Pseudomonas* sp. RC	61.11
F.solani R8+ *Pseudomonas* sp. RC	44.44
N. oryzae R9+ *Pseudomonas* sp. RC	83.33
*T.cucumeris*R10+ *Pseudomonas* sp. RC	55.55
*F.solani*R11+ *Pseudomonas* sp. RC	77.77
T.cucumeris R12+ *Pseudomonas* sp. RC	83.33
*F.solani*R13+ *Pseudomonas* sp. RC	61.11
T.cucumeris R14+ *Pseudomonas* sp. RC	66.66
*Bipolaris spicifera*R15+ *Pseudomonas* sp. RC	66.66
*F.solani*R16+ *Pseudomonas* sp. RC	77.77
F.verticillioides R17+ *Pseudomonas* sp. RC	55.55
*A.alternata*R18+ *Pseudomonas* sp. RC	83.33
*E.rostratum*R19+ *Pseudomonas* sp. RC	44.44
*A.alternata*R20+ *Pseudomonas* sp. RC	83.33
C.lunata R21+ *Pseudomonas* sp. RC	50
*A.tenuissima*R23+ *Pseudomonas* sp. RC	77.77
*A.tenuissima*R24+ *Pseudomonas* sp. RC	77.77

*Inhibition zone after 7 days according to Mojica-Marin *et al.,* (2008).

Antagonistic activity between *Azospirillum brasilense* and Rice Pathogens

Figure 4 refers to the results obtained when we used *T.cucumeris* R1, *T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16, *F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7,*C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19, *A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 together with *A.brasilense*.

However as we can see from (Figure 4) the mycelium growth of these pathogens were inhibited and affected by *A.brasilense* as growth inhibition zones were evident surrounding the *F.solani* R3, *B.spicifera* R15, *A.tenuissima* R24 and *N.oryzae* R9, and the inhibition zone was gave roughly 61.11, 61.11, 61.11 and 66.66% respectively in comparison with control (Table 3).

However, as seen in Figure 4, the mycelium growth of *F.oxysporum*R6, *C.lunata* R7 and *C.lunata* R21were gave less inhibition score which was roughly 22.22% compared to high value was shown by *N.oryzae* R9.

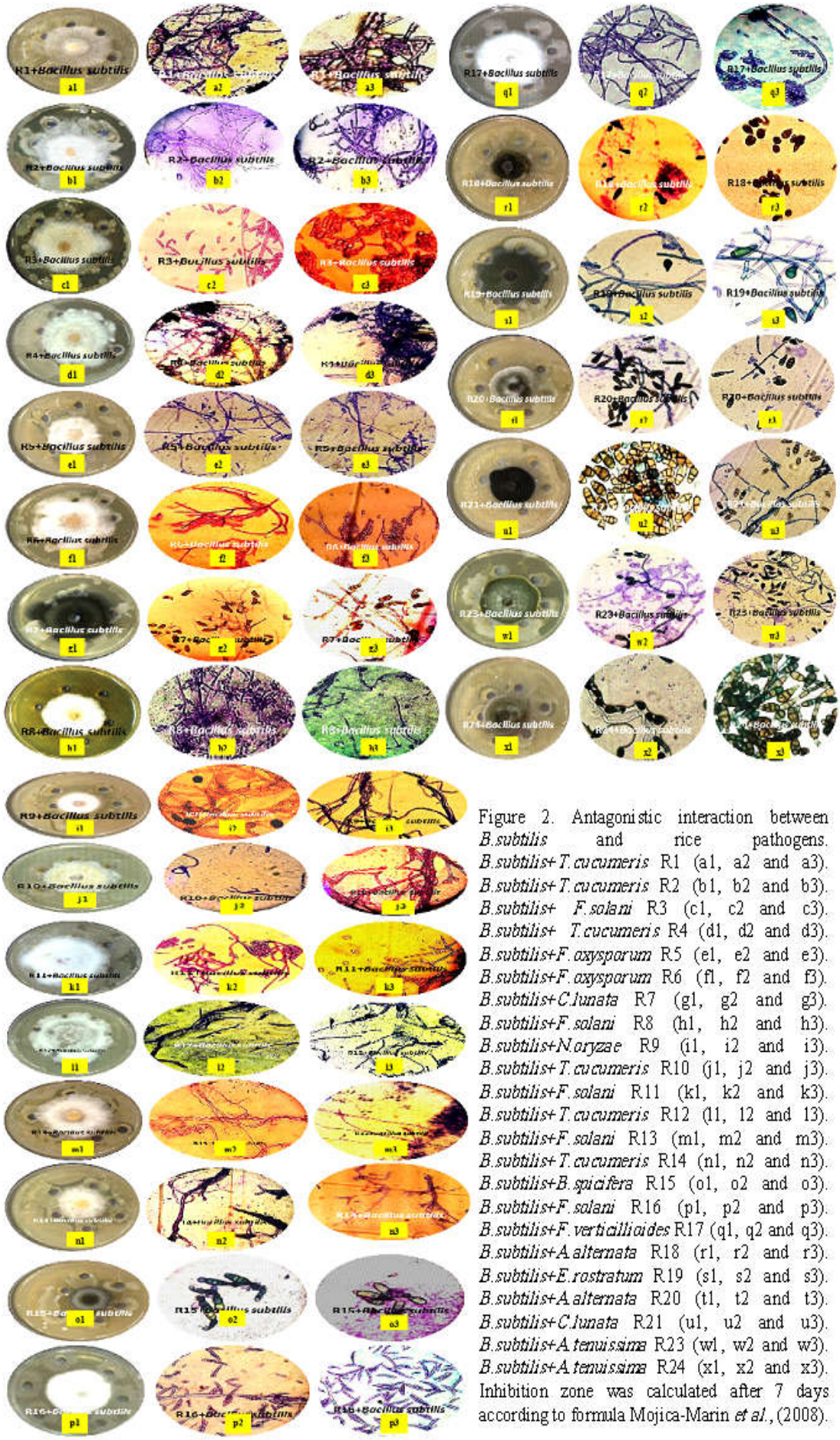

Figure 2. Antagonistic interaction between *B.subtilis* and rice pathogens. *B.subtilis*+*T.cucumeris* R1 (a1, a2 and a3). *B.subtilis*+*T.cucumeris* R2 (b1, b2 and b3). *B.subtilis*+ *F.solani* R3 (c1, c2 and c3). *B.subtilis*+ *T.cucumeris* R4 (d1, d2 and d3). *B.subtilis*+*F.oxysporum* R5 (e1, e2 and e3). *B.subtilis*+*F.oxysporum* R6 (f1, f2 and f3). *B.subtilis*+*C.lunata* R7 (g1, g2 and g3). *B.subtilis*+*F.solani* R8 (h1, h2 and h3). *B.subtilis*+*N.oryzae* R9 (i1, i2 and i3). *B.subtilis*+*T.cucumeris* R10 (j1, j2 and j3). *B.subtilis*+*F.solani* R11 (k1, k2 and k3). *B.subtilis*+*T.cucumeris* R12 (l1, l2 and l3). *B.subtilis*+*F.solani* R13 (m1, m2 and m3). *B.subtilis*+*T.cucumeris* R14 (n1, n2 and n3). *B.subtilis*+*B.spicifera* R15 (o1, o2 and o3). *B.subtilis*+*F.solani* R16 (p1, p2 and p3). *B.subtilis*+*F.verticillioides* R17 (q1, q2 and q3). *B.subtilis*+*A.alternata* R18 (r1, r2 and r3). *B.subtilis*+*E.rostratum* R19 (s1, s2 and s3). *B.subtilis*+*A.alternata* R20 (t1, t2 and t3). *B.subtilis*+*C.lunata* R21 (u1, u2 and u3). *B.subtilis*+*A.tenuissima* R23 (w1, w2 and w3). *B.subtilis*+*A.tenuissima* R24 (x1, x2 and x3). Inhibition zone was calculated after 7 days according to formula Mojica-Marin *et al.*, (2008).

Figure 2.

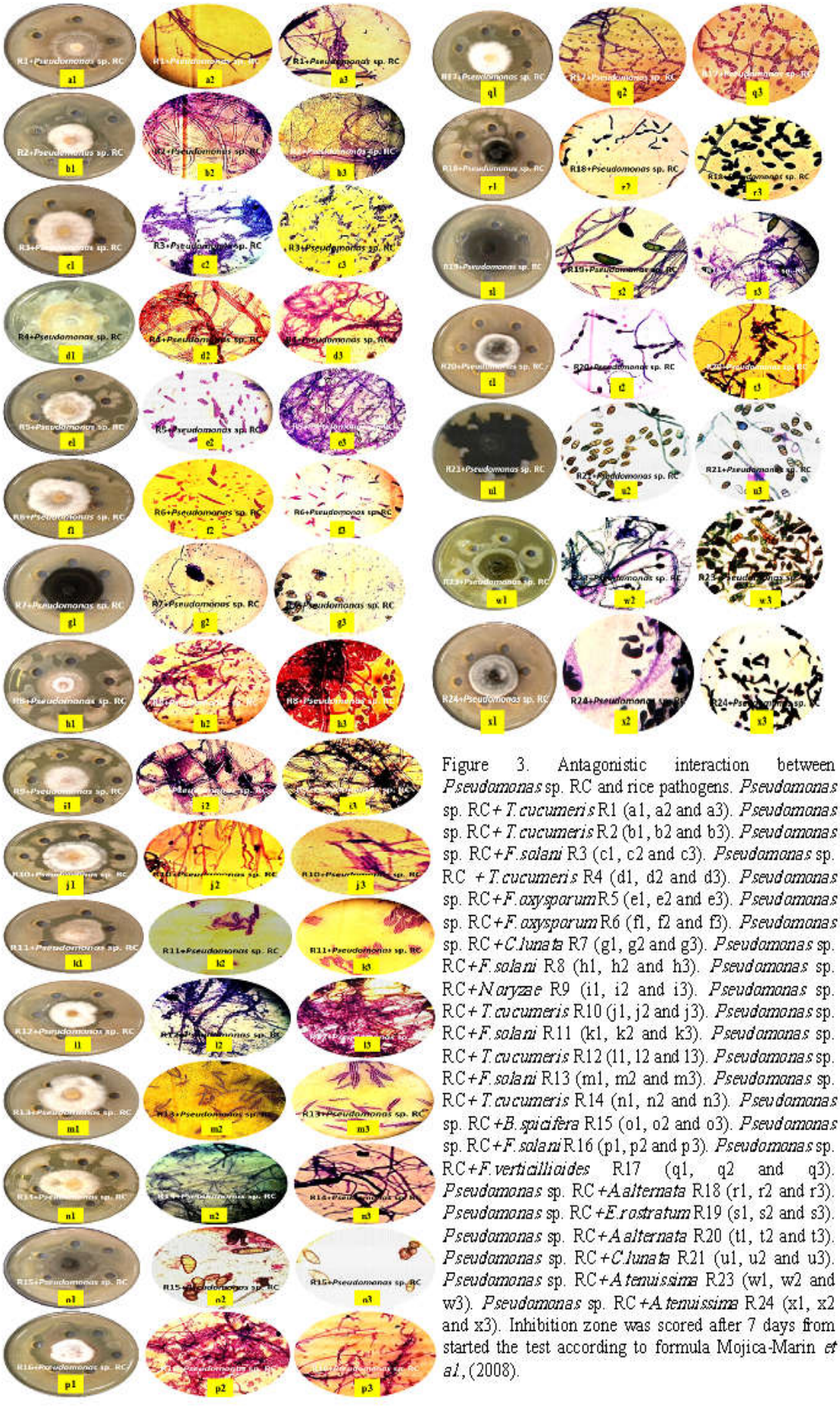

Figure 3. Antagonistic interaction between *Pseudomonas* sp. RC and rice pathogens. *Pseudomonas* sp. RC+*T.cucumeris* R1 (a1, a2 and a3). *Pseudomonas* sp. RC+*T.cucumeris* R2 (b1, b2 and b3). *Pseudomonas* sp. RC+*F.solani* R3 (c1, c2 and c3). *Pseudomonas* sp. RC +*T.cucumeris* R4 (d1, d2 and d3). *Pseudomonas* sp. RC+*F.oxysporum* R5 (e1, e2 and e3). *Pseudomonas* sp. RC+*F.oxysporum* R6 (f1, f2 and f3). *Pseudomonas* sp. RC+*C.lunata* R7 (g1, g2 and g3). *Pseudomonas* sp. RC+*F.solani* R8 (h1, h2 and h3). *Pseudomonas* sp. RC+*N.oryzae* R9 (i1, i2 and i3). *Pseudomonas* sp. RC+*T.cucumeris* R10 (j1, j2 and j3). *Pseudomonas* sp. RC+*F.solani* R11 (k1, k2 and k3). *Pseudomonas* sp. RC+*T.cucumeris* R12 (l1, l2 and l3). *Pseudomonas* sp. RC+*F.solani* R13 (m1, m2 and m3). *Pseudomonas* sp. RC+*T.cucumeris* R14 (n1, n2 and n3). *Pseudomonas* sp. RC+*B.spicifera* R15 (o1, o2 and o3). *Pseudomonas* sp. RC+*F.solani* R16 (p1, p2 and p3). *Pseudomonas* sp. RC+*F.verticillioides* R17 (q1, q2 and q3). *Pseudomonas* sp. RC+*A.alternata* R18 (r1, r2 and r3). *Pseudomonas* sp. RC+*E.rostratum* R19 (s1, s2 and s3). *Pseudomonas* sp. RC+*A.alternata* R20 (t1, t2 and t3). *Pseudomonas* sp. RC+*C.lunata* R21 (u1, u2 and u3). *Pseudomonas* sp. RC+*A.tenuissima* R23 (w1, w2 and w3). *Pseudomonas* sp. RC+*A.tenuissima* R24 (x1, x2 and x3). Inhibition zone was scored after 7 days from started the test according to formula Mojica-Marin *et al.*, (2008).

Figure 3.

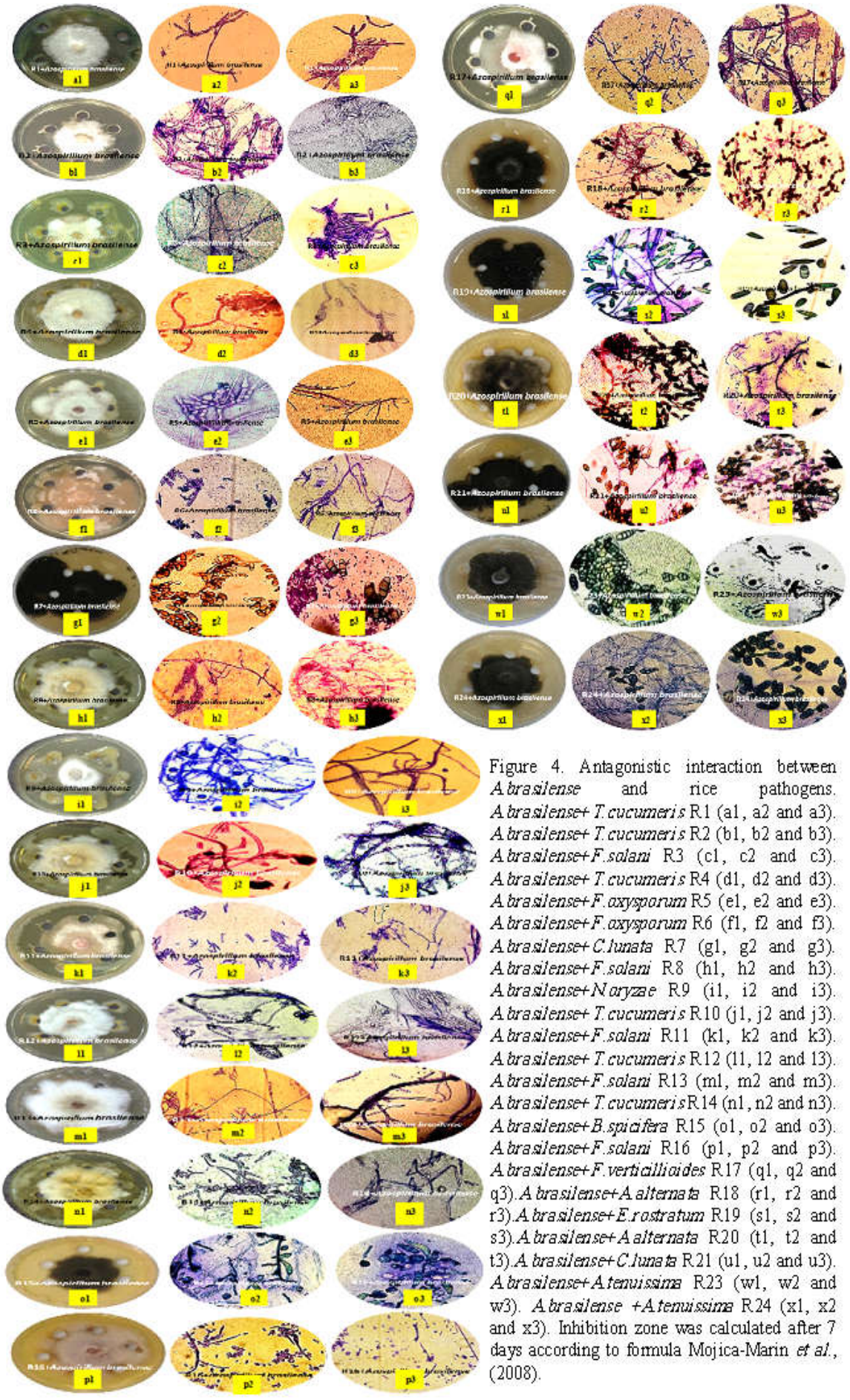

Figure 4. Antagonistic interaction between *A brasilense* and rice pathogens. *A brasilense*+ *T.cucumeris* R1 (a1, a2 and a3). *A brasilense*+ *T.cucumeris* R2 (b1, b2 and b3). *A brasilense*+*F.solani* R3 (c1, c2 and c3). *A brasilense*+ *T.cucumeris* R4 (d1, d2 and d3). *A brasilense*+*F.oxysporum* R5 (e1, e2 and e3). *A brasilense*+*F.oxysporum* R6 (f1, f2 and f3). *A brasilense*+ *C.lunata* R7 (g1, g2 and g3). *A brasilense*+*F.solani* R8 (h1, h2 and h3). *A brasilense*+*N oryzae* R9 (i1, i2 and i3). *A brasilense*+ *T.cucumeris* R10 (j1, j2 and j3). *A brasilense*+*F.solani* R11 (k1, k2 and k3). *A brasilense*+ *T.cucumeris* R12 (l1, l2 and l3). *A brasilense*+*F.solani* R13 (m1, m2 and m3). *A brasilense*+ *T.cucumeris* R14 (n1, n2 and n3). *A brasilense*+*B.spicifera* R15 (o1, o2 and o3). *A brasilense*+*F.solani* R16 (p1, p2 and p3). *A brasilense*+*F.verticillioides* R17 (q1, q2 and q3). *A brasilense*+*A alternata* R18 (r1, r2 and r3). *A brasilense*+*E.rostratum* R19 (s1, s2 and s3). *A brasilense*+*A alternata* R20 (t1, t2 and t3). *A brasilense*+*C.lunata* R21 (u1, u2 and u3). *A brasilense*+*A tenuissima* R23 (w1, w2 and w3). *A brasilense* +*A tenuissima* R24 (x1, x2 and x3). Inhibition zone was calculated after 7 days according to formula Mojica-Marin *et al.*, (2008).

Figure 4.

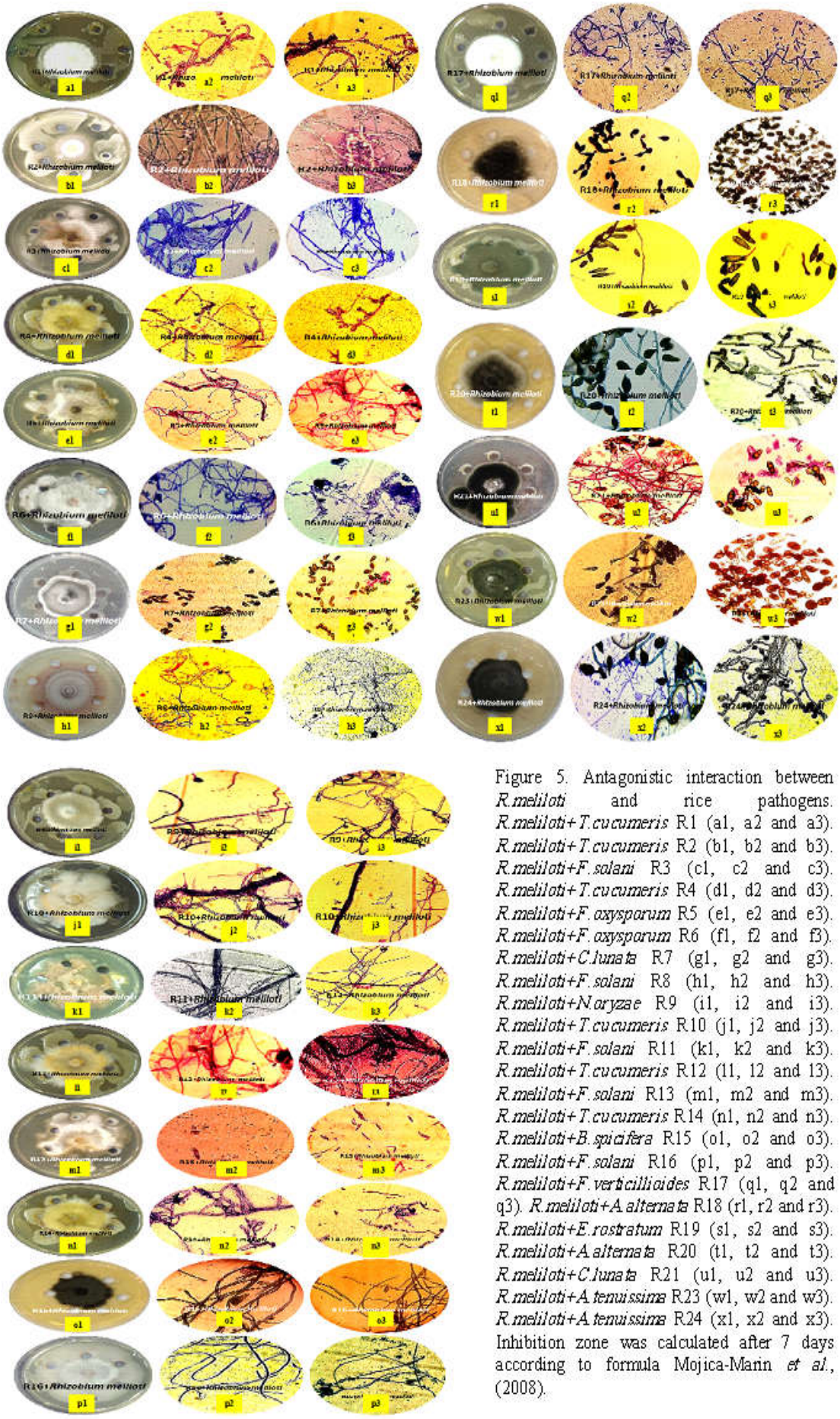

Figure 5. Antagonistic interaction between *R.meliloti* and rice pathogens. *R.meliloti+T.cucumeris* R1 (a1, a2 and a3). *R.meliloti+T.cucumeris* R2 (b1, b2 and b3). *R.meliloti+F.solani* R3 (c1, c2 and c3). *R.meliloti+T.cucumeris* R4 (d1, d2 and d3). *R.meliloti+F.oxysporum* R5 (e1, e2 and e3). *R.meliloti+F.oxysporum* R6 (f1, f2 and f3). *R.meliloti+C.lunata* R7 (g1, g2 and g3). *R.meliloti+F.solani* R8 (h1, h2 and h3). *R.meliloti+N.oryzae* R9 (i1, i2 and i3). *R.meliloti+T.cucumeris* R10 (j1, j2 and j3). *R.meliloti+F.solani* R11 (k1, k2 and k3). *R.meliloti+T.cucumeris* R12 (l1, l2 and l3). *R.meliloti+F.solani* R13 (m1, m2 and m3). *R.meliloti+T.cucumeris* R14 (n1, n2 and n3). *R.meliloti+B.spicifera* R15 (o1, o2 and o3). *R.meliloti+F.solani* R16 (p1, p2 and p3). *R.meliloti+F.verticillioides* R17 (q1, q2 and q3). *R.meliloti+A.alternata* R18 (r1, r2 and r3). *R.meliloti+E.rostratum* R19 (s1, s2 and s3). *R.meliloti+A.alternata* R20 (t1, t2 and t3). *R.meliloti+C.lunata* R21 (u1, u2 and u3). *R.meliloti+A.tenuissima* R23 (w1, w2 and w3). *R.meliloti+A.tenuissima* R24 (x1, x2 and x3). Inhibition zone was calculated after 7 days according to formula Mojica-Marin *et al.*, (2008).

Figure 5.

Table 3. Antagonistic activity between *A.brasilense* and *B.spicifera*, *C.lunata*, *Fusarium* spp., *N.oryzae, E.rostratum, Alternaria* spp. and *T.cucumeris* under laboratory conditions

Treatments	*Inhibition Zone after 7 days
T.cucumeris R1+ *A.brasilense*	50
T.cucumeris R2+ *A.brasilense*	55.55
F.solani R3+ *A.brasilense*	61.11
*T.cucumeris*R4+ *A.brasilense*	44.44
*F.oxysporum*R5+ *A.brasilense*	33.33
*F.oxysporum*R6+ *A.brasilense*	22.22
C. lunata R7+ *A.brasilense*	22.22
*F.solani*R8+ *A.brasilense*	44.44
N. oryzae R9+ *A.brasilense*	66.66
*T.cucumeris*R10+ *A.brasilense*	50
*F.solani*R11+ *A. brasilense*	38.88
T.cucumeris R12+ *A.brasilense*	55.55
*F.solani*R13+ *A.brasilense*	27.77
T.cucumeris R14+ *A.brasilense*	38.88
*Bipolarisspicifera*R15+ *A.brasilense*	61.11
*F.solani*R16+ *A.brasilense*	55.55
F.verticillioides R17+ *A.brasilense*	38.88
*A.alternata*R18+ *A.brasilense*	50
*E.rostratum*R19+ *A.brasilense*	50
*A.alternata*R20+ *A.brasilense*	27.77
C.lunata R21+ *A.brasilense*	22.22
*A.tenuissima*R23+ *A.brasilense*	55.55
*A.tenuissima*R24+ *A.brasilense*	61.11

*Inhibition zone after 7 days according to Mojica-Marin*et al.,* (2008).

Antagonistic activity between *Rhizobium meliloti* and Rice Pathogens

Table 4 shows the antagonistic activity between *R.meliloti* and *T.cucumeris* R1, *T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16*, F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7*, C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19*, A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24 individually.

Table 4. Antagonistic activity between *R.meliloti* and *B.spicifera*, *C.lunata*, *Fusarium* spp., *N. oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris* under laboratory conditions

Treatments	*Inhibition Zone after 7 days
T.cucumeris R1+ *R.meliloti*	55.55
T.cucumeris R2+ *R.meliloti*	77.77
F.solani R3+ *R.meliloti*	33.33
*T.cucumeris*R4+ *R.meliloti*	50
*F.oxysporum*R5+ *R.meliloti*	38.88
*F.oxysporum*R6+ *R.meliloti*	27.77
C. lunata R7+ *R.meliloti*	61.11
F.solani R8+ *R.meliloti*	50
N. oryzae R9+ *R.meliloti*	66.66
*T.cucumeris*R10+ *R.meliloti*	27.77
*F.solani*R11+ *R.meliloti*	22.22
T.cucumeris R12+ *R.meliloti*	55.55
*F.solani*R13+ *R.meliloti*	33.33
T.cucumeris R14+ *R.meliloti*	50
*Bipolaris spicifera*R15+ *R.meliloti*	50
*F.solani*R16+ *R.meliloti*	61.11
F.verticillioides R17+ *R.meliloti*	61.11
*A.alternata*R18+ *R.meliloti*	55.55
*E.rostratum*R19+ *R.meliloti*	27.77
*A.alternata*R20+ *R.meliloti*	44.44
C.lunata R21+ *R.meliloti*	33.33
*A.tenuissima*R23+ *R.meliloti*	61.11
*A.tenuissima*R24+ *R.meliloti*	61.11

*Inhibition zone after 7 days according to Mojica-Marin *et al.,* (2008).

However as we can see from (Figure 5) the mycelium growth of certain pathogens e.g. *N.oryzae* R9 and *T.cucumeris* R2 were inhibited and affected by *R.meliloti* as growth inhibition zones were evident surrounding the mycelium and spores of these pathogens, and the inhibition zone was roughly 66.66 and 77.77% respectively in comparison with control (Table4), inhibition zone was calculated after 7 days according to formula Mojica-Marin *et al.,* (2008). *F.solani* R11 exhibited the lowest percentage of inhibition zone rate 22.22% compared to the other pathogens used in this study e.g. *T.cucumeris* R2 (Figure 5).

DISCUSSION

In this study we observed from Figure 2,3,4 and 5 the interaction between *B.subtilis*, *Pseudomonas* sp. RC, *A.brasilense*, *R.meliloti* and twenty-three rice pathogens (*T.cucumeris* R1,*T.cucumeris* R2, *T.cucumeris* R4, *T.cucumeris* R10, *T.cucumeris* R12, *T.cucumeris* R14, *F.solani* R3, *F.oxysporum* R5, *F.oxysporum* R6, *F.solani* R8, *F.solani* R11, *F.solani* R13, *F.solani* R16*, F.verticillioides* R17, *N.oryzae* R9, *C.lunata* R7*,C.lunata* R21, *B.spicifera* R15, *E.rostratum* R19*, A.alternata* R18, *A.alternata* R20, *A.tenuissima* R23 and *A.tenuissima* R24. Based on the laboratory findings of the antagonistic ability of the four bacteria as donated in Figure 2, 3, 4 and 5, however revealed more effective and excellent potential in inhibition activity which was observed in petri dishes treated with *B.subtilis*, *Pseudomonas* sp. RC to reduced radial growth for each pathogen as compared with *A.brasilense* and *R.meliloti*.

Figure 2,3,4 and 5 however showed that when the wells were inoculated with 10 µl of *B.subtilis*, *Pseudomonas* sp. RC, *A.brasilense* and *R.meliloti* cultures, inhibition pursued of *B.spicifera*, *C.lunata*, *Fusarium spp*., *N.oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris* mycelium resulted in an inhibition zones that roughly 83.33, 83.33, 77.77 and 66.66% respectively. These results were in accordance to previous research findings that showed these bacteria can inhibit the radial growth of *F.solani* and *F.oxysporum* by 61% (Montealegre *et al.,* 2003; Yang *et al.,* 2009). Besides, these results are in agreement with previous studies that showed these bacteria when used as biological control agent (BCA) against *Pyricularia grisea* and *Rhizoctonia solani* in dual culture test showed inhibition of approximately 60 % to the growth of both pathogens due to the secretion of antifungal compounds by the BCAs (Papavizas, 1985; Leelasuphakul *et al.,* 2006). Lippi and Monaco (1994) also reported similar findings with *B.subtilis*, where they reported the release of several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin with inhibition effect on causal agents. In addition, investigators have reported that *B.subtilis*, *P.fluorescens*, *A.brasilense*, *R.meliloti* have high potential to attack the pathogens, and completely surrounding the mycelium and spores and prior to gradually destroyed them through producing extracellular lytic enzymes and antibiotics that act as a strong biocontrol agents against fungias we can see in Figure 2,3,4 and 5, also their effects are enhanced when used with other antagonistics organisms as we can see with these pathogens (Hossain, 2007; Behdani *et al.,* 2012; Bao*et al.,*2013; Saraf *et al.,* 2014). Furthermore *B.subtilis* which also has the potential of producing cell wall degrading enzymes (CDWEs),also has been shown to be an efficient biocontrol agent to inhibit pathogens and linking the fungal pathogens by sugar linkage through releasing of extracellular enzymes e.g. protease and lipase (Hamdia *et al.,* 2014). Moreover, *P.fluorescens*, *B.subtilis* and *R.meliloti* have good ability to

produce secrete siderophores and hydrogen cyanide which are very toxic to pathogenic organisms (Deshwal *et al.,* 2003; Nagarajkumar *et al.,* 2004; Gopalakrishnan *et al.,* 2015; *El-Hendawy and Abo-Elyousr, 2016*). Researchers have also documented a large array of secondary products that are produced by these organisms such as phenazine-1-carboxilic acid (PCA), 2,4-pyrrolnitrin,2,4-Diacetylphloroglucinol(2,4-DAPG) and oomycin (Soleimani *et al.,*2005; Zaghloul *et al.,*2007; Moubarak and Abdel-Monaim, 2011). Figure 2 and 3 which show the effect of *B.subtilis* and *Pseudomonas* sp. RC on rice pathogens show very clearly the inhibition zone 83.33% of these bacteria on pathogenic fungi by extensive degradation of *N.oryzae* R9*, B.spicifera* R15*, A.alternata* R18*, T.cucumeris* R12*, A.alternata* R18 and *A.alternata* R20. *B.subtilis*, *P.fluorescens* have been reported by several researchers as the mode of action have a great impact on above pathogens by releasing several enzymes such as chitinase, glucanase and protease that have high ability to degrade cell wall structure of *B.spicifera*, *C.lunata*, *Fusarium spp*., *N.oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris* which are consisting of chitin and glucan (Chet, 1981; Handelsman and Parke, 1989; Berg *et al.,* 2002).

Conclusion

As a consequence of dual culture assays of *Pseudomonas* sp. RC*, B.subtilis, R.meliloti* and *A.brasilense* were determined the *Pseudomonas* sp. RC and *B.subtilis* were most effective biocontrol agents (BCAs) were used in this experiment to inhibit and reduce redial growth of *B.spicifera*, *C.lunata*, *Fusarium spp*., *N.oryzae, E.rostratum, Alternaria spp*. and *T.cucumeris*. Ultimately, we believe that the efficacy shown by *B.subtilis* and *Pseudomonas* sp. RC will need to be tested later on above pathogens under greenhouse conditions on rice plant as a means of controlling the spread and disease severity of these fungi in economically important crops under greenhouse and normal field conditions.

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