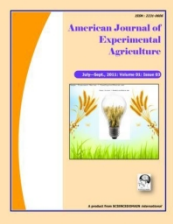

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Interaction of Fluorescent Pseudomonads with *Pythium ultimum* and *Rhizoctonia solani* in Cucumber Roots

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Authors' contributions

This work was carried out in collaboration between all authors. Author MS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RA and SB. managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

The effects of the fluorescent pseudomonad isolate CW2 on hyphae of *Pythium ultimum* (Trow) and *Rhizoctonia solani* (Kühn) and root colonization were studied in gnotobiotic systems under scanning electron microscopy (SEM). Results of SEM revealed that the frequency of bacterial colonization was higher in cucumber roots infested with the fungi than in healthy roots. Isolate CW2 caused irregular and abnormal fungal growth. Swellings and shrinkages of *P. ultimum* and *R. Solani* hyphae were obvious when cucumber roots were drenched with CW2. SEM studies were also conducted to evaluate the effect of CW2 on hyphae of both fungi on PDA medium. The hyphae of *P. ultimum* and *R. solani* showed distinct morphological alterations and degradation compared to untreated healthy control hyphae of *Pythium* or *Rhizoctonia* which were slender and uniform in shape. The results provide direct evidence of bacterial attachment and colonization to fungal hyphae of both tested spp. and show that fungal growth to be significantly reduced in the presence of isolate CW2.

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Keywords: SEM; colonization; fluorescent pseudomonas; attachment; antifungal, biocontrol.

1. INTRODUCTION

The application of microorganisms as biocontrol agents has been known for several decades [1]. Plant growth-promoting rhizobacteria (PGPR) promote plant growth following inoculation onto seeds or subterranean plant parts [2]. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line of defense against pathogenic microbes [3,4,5]. There are several species of micro-organisms that have been tested as potential biocontrol agents of damping-off, including bacteria such as *Pseudomonas* spp. [6,7], *Bacillus* spp. and *Streptomyces griseoviridis* as well as fungi, for instance *Trichoderma* spp. (*T. virens*, *T. harzinum*), *Gliocladium* spp. and *Pythium oligandrum* [8].

Fluorescent pseudomonads are one group of rhizospheric bacteria that have been described as biological control agents and showed great promise with respect to protecting plant roots by reducing the incidence of fungal-induced diseases [9,10]. Fluorescent Pseudomonads produce versatile catabolic and secondary metabolites which include antifungal compounds. They have excellent root-colonizing abilities allowing them to be effective in the vicinity of plant roots. The soil-borne fluorescent pseudomonads have received special attention and emerged as the largest and potentially most promising group involved in biocontrol of plant diseases [1,11,12].

Currently, *Pseudomonas* spp. are particularly suitable for applications as agricultural biocontrol agents since they: (1) can use many exudate compounds released by roots as a source of nutrients [13]; (2) are abundantly present in natural soils, in particular on plant root systems, which is indicative of their adaptive potential [14]; (3) have a high growth rate relative to many other rhizosphere bacteria; (4) possess diverse mechanisms by which they can exert inhibitory activity towards phytopathogens and thereby mediate crop protection [13,14]; (5) are easy to grow *in vitro*; (6) can subsequently be reintroduced into the rhizosphere by seed bacterization [14]; (7) are susceptible to mutation and molecular manipulation techniques[15]; and (8) their growth temperatures are well suited to soil temperatures ranging from 10 to 35ºC [16].

In recent years, several studies were conducted to evaluate the efficacy of fluorescent pseudomonad species against plant pathogens. The fluorescent pseudomonad isolate CW2 proved to be a promising biocontrol agent against many plant pathogens including *P. ultimum*, *R. solani* [17], *Fusarium oxysporum* f. sp. *Lycopersici* [18]. The mechanism of action of the bacterium was studied intensively. The bacterium produces antibiotics such as 2,4-diacetylphloroglucinol (DAPG) and other phenazine derivative [17]. In addition, it was possible to apply the bacterium in combination with chemical fungicides to control *P. ultimum* damping-off in tomato [19]. However little information is available about colonization of plant roots by this bacterium. The aim of this work was to provide direct evidence for the attachment and colonization of cucumber roots by the *Pseudomonas fluorescens* isolate CW2, in presence or absence of *P. ultimum* or *R. solani*. In addition, the study aimed at assessing the ability of the antagonistic bacterial isolates to control the hyphal growth of both fungal spp. by the isolate CW2.

2. MATERIALS AND METHODS

2.1 Cultivation and Maintenance of Antagonistic Fluorescent pseudomonads

The antagonistic *P. fluorescens* isolate CW2 was obtained from the Institute of Phytomedicine, University of Hohenheim, Germany. Stock culture of the bacterium was prepared by growing the bacterium in 125-ml Erlenmeyer flasks containing 25 ml King's B [20] liquid medium on a rotary shaker (150 rpm) for 20 h at 28ºC. Isolates were stored as 1ml-aliquots in 20% glycerol at - 80ºC.

2.2 Fungal Isolates

The oomycete *P. ultimum* (Trow) and *R. solani* (Kühn) were obtained from the Institute of Phytomedicine, University of Hohenheim. The fungi were grown on potato dextrose agar medium (PDA) at 22ºC for 4 days. Stock cultures were stored at 4ºC and sub cultured routinely every 2 weeks.

2.3 Effect of Bacteria on Mycelial growth of *P. ultimum* and *R. solani*

Single 0.5 cm diameter agar disks grown with 4 days old *P. ultimum* or *R. solani* were transferred into 125 ml Erlenmeyer flasks containing 25 ml PDB and incubated on a rotary shaker at 110 rpm and 22ºC. After 3 days of incubation, 0.1 ml of CW2 (1×10^9cfu ml^{-1}) was added to the fungal cultures and incubated for an additional 3 days. Mycelial dry weights were determined after filtration through preweighted Whatman # 1 filter paper and drying overnight at 80ºC (modification of Nelson et al., 1986) [21].

2.4 Colonization of Cucumber roots by *P. fluorescens*

To study the population size of the *P. fluorescens* isolate CW2, cucumber seeds were pre-germinated between towel papers for two days. The germinated seedlings were then transplanted in 9-cm diameter pots filled with humosoil®: sand mixtures (2:1 v/v) and incubated under greenhouse conditions. For inoculation of the seedlings, the bacterium was grown for 24 h in KB medium at 28ºC with shaking (150 rpm). Two methods of application were used. For soil drenching, bacterial suspensions (1×10^9cfu ml^{-1}; 30 ml pot^{-1}) were poured over the soil of the pots of cucumber seedlings. For root dipping, the roots of germinated seedlings were soaked in 24 h old (1×10^9cfu ml^{-1}) bacterial suspensions for 30 min before transplanting them in the pots. After different periods of time (1, 3, 7, 14 and 21 days) roots of cucumber were washed gently under running tap water, cut into 1 cm long pieces and dipped in 100 ml KB liquid medium [18]. After incubation for two hours at 28ºC on a rotary shaker with 200 rpm, serial dilutions were made from 1 ml of the KB suspension.

100 µl of suspension were spread on solid NPC medium (KB medium plus Novobiocin 45.0 mg L^{-1} Penicillin G 35.2 mg L^{-1} and Cycloheximide 75.0 mg L^{-1}) prepared according to Sands and Rovira (1970)[22] and incubated for 3 days at 28ºC. Bacterial colonies were visualized under UV light at 360 nm. Colonies showing green fluorescence were counted.

2.5 Effect of Bacteria on *P. ultimum* and *R. solani* hyphae (scanning electron microscopy)

Dual cultures of *P. fluorescens* isolate CW2 and *P. ultimum* or *R. solani* were grown in 9 cm Petri dishes containing PDA medium for 3 days at 22ºC [17]. For scanning electron microscopy (SEM), 0.5 cm agar pieces with *P. ultimum* or *R. solani* were fixed with glutaraldehyde (4% w/v) in phosphate buffer (50 mM; pH 6.8) for 12 h at 4°C and rinsed 5 times with the same buffer for 20 min. Samples were post fixed in osmium tetraoxide (1%) in phosphate buffer (50 mM; 6.8 pH) for 12 h at 4ºC and rinsed with phosphate buffer (4X, 15 min each). After dehydration in graded acetone series, samples were critical-point dried, mounted on stubs, sputter-coated with gold-palladium, and observed using a Zeiss DSM100 scanning electron microscope operating at 15 kV [23].

2.6 Interaction of the *P. fluorescens* isolate CW2 with *P. ultimum* and *R. solani* in the Rhizosphere of Cucumber seedlings

A gnotobiotic system described by Simons et al. (1996) [24] was used in studying the interaction of CW2 with *P. ultimum* and *R. solani*. Briefly, cucumber seeds were surface sterilized by soaking in 1% (v/v) sodium hypochlorite for 5 min followed by five thorough rinses with sterile water. Seeds were then germinated between autoclaved Whatman #1 filter paper in 9 cm diameter Petri dishes in the dark at room temperature. Germinated seedlings were planted in tubes filled with sand [24] before application of bacteria or fungi. One ml of bacterial suspension (cells of isolate CW2 from a 24 h culture in KB medium) was washed in plant nutrient solution (PNS) and adjusted to a concentration of 1×10^9 cfu ml^{-1}. After two days of incubation, the tubes were infested with PDA disks grown with 4 day old *P. ultimum* or *R. solani*. Control experiments included untreated seedlings, seedlings treated with CW2 alone, and seedlings treated either with *P. ultimum* or *R. solani*. Tubes were then incubated at room temperature for three days.

For SEM, plantlets were removed gently from the tubes and the roots were washed with water and sectioned in about 1 cm pieces. Preparation of SEM samples was carried out as mentioned above.

2.7 Data Analysis

Statistical significances were computed with Xlstat (Adinosoft) using Tukey test after significant F-test (P = 0.05) of the analysis of variance (ANOVA). Means indicated with different letters are significantly different.

3. RESULTS

3.1 Root Colonization

The population sizes of CW2 reisolated from the rhizosphere of cucumber seedlings were slight higher but not significantly different ($F= 9.74$; df 9, 19; $P< 0.001$) between the root dipping and soil drenching methods (Fig. 1). The determined bacterial concentrations of CW2 were 6.6 and 5.9 log cfu g^{-1} root fresh weight, following root dipping and soil drenching, respectively. After 3 days of application as well as after 7 and 14 days, the bacterial number determined on roots did not differ between soil drenching and root dipping methods. After the third week of application, bacterial populations were significantly reduced in in cucumber

roots after soil drenching and root dipping (Fig. 1). Significant reduction of bacterial concentration was recorded after 21 days of the experiment.

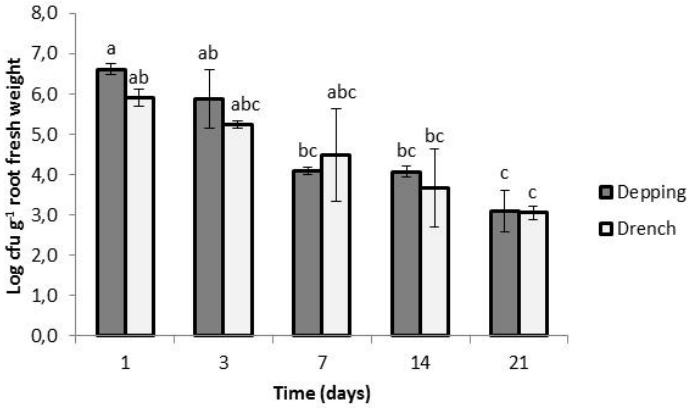

Fig. 1. Comparison of root colonization of roots of cucumber plants by fluorescent pseudomonads isolates CW2. Bacterial suspensions (1×10^9cfu ml^{-1}) were applied either as root dipping or as soil drench. Data are represented as the log cfu/g root fresh weight. Bars represent standard errors (SE)

3.2 Effect of Bacteria on Mycelial Growth

Growth rate of *P. ultimum* and *R. solani* was not significantly different (F= 71.99; df 3, 19; P < 0.0001) after 3days of incubation in PDB medium at 110 rpm and 24ºC (Fig. 2). However, in the presence of *P. fluorescens* isolate CW2; the growth rate was significantly reduced. The percent of reduction of mycelial weight was 60.5 and 63.6 % for *P. ultimum* and *R. solani* respectively.

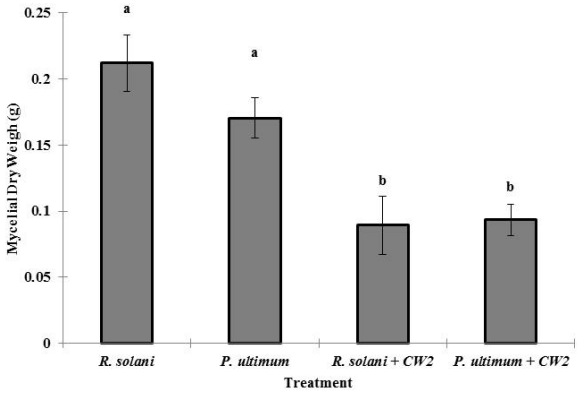

Fig. 2. Effect of the fluorescent pseudomonads isolate CW2 (1×10^9cfu ml^{-1}) on mycelial growth of *P. ultimum* and *R. solani* in liquid PDB medium. The experiment was done in triplicates and repeated three times. Data with different letters are significantly different after Tukeys HSD test using ANOVA at $P < 0.05$. Bars represent standard errors (SE)

3.3 Scanning Electron Microscope Studies (SEM)

These studies were carried out using the *P. fluorescens* isolate CW2. The effect of the bacterial isolate has been studied on mycelial growth of *P. ultimum* and, *R. solani* in dual cultures on PDA medium. The interaction of this bacterial isolate with *P. ultimum* and, *R. solani* in cucumber rhizosphere was conducted in a gnotobiotic system as described in Simons et al. [24]. Results are detailed below:

3.3.1 Effect of CW2 on *P. ultimum*

SEM examinations of *P. ultimum* hyphae, three days after co-culture with CW2 on PDA medium showed irregular mycelial morphology of *P. ultimum* (Fig. 3). Untreated *Pythium* hyphae (Fig. 3a) were slender, smooth, parallel, little or no tip branching, and uniform in shape. However, in the presence of isolate CW2 hyphae exhibited irregular swellings, rough surface, curling of hyphal tips and loss of apical growth, increased tip branching (Fig. 3b) with shorter and swollen hyphal cells (Fig. 3c).

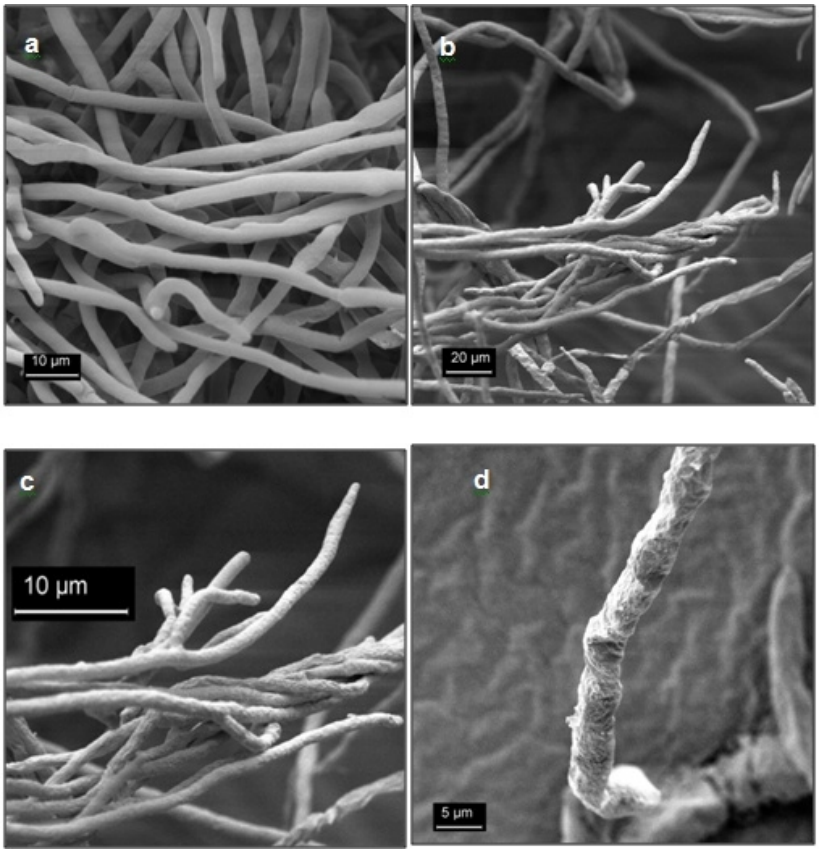

Fig. 3. Scanning electron micrographs showing hyphae of *P. ultimum* after three days of co-cultivation with fluorescent pseudomonadsisolate CW2. (a) Normal growth in the absence of CW2 (control). (b) Irregular mycelial shape and increased branching at the hyphal tips in the presence of cells of CW2. (c) Magnification of b. (d) Shrinkage of hyphae. Samples were taken from inhibition zones

3.3.2 Effect of CW2 on *R. solani*

Untreated control *R. solani* hyphae were slender, well septated, uniform in shape and the hyphal tip was round shaped (Fig. 4a). Isolate CW2 also affected shape and growth of *R. solani* hyphae (Fig. 4). CW2 caused irregular shrinkage of hyphae and increased lateral branching (Fig. 4b). In addition increased septation was observed near the hyphal tips which have a pin-like shape (Fig. 4c).

3.4 Interaction of CW2 with *P. ultimum* and *R. solani* in Cucumber rhizosphere

SEM micrographs showed that CW2 was able to colonize cucumber roots (Fig. 5a-i). As shown in Fig. 4, CW2 was distributed as single cells on the root surface and many cells were found in the grooves between epidermal cells of cucumber roots (Fig. 5d). Three days after infestation with *Pythium* or *Rhizoctonia*, cucumber roots were highly colonized with CW2. The bacteria were found in high numbers and it was shown that mycelia of both fungi were affected by the bacterial cells. Shrinkage of hyphae and irregular mycelial shapes were prevailing in the presence of CW2-cells (Fig. 5f-i). On the other hand, normal hyphae of *P. ultimum* and *R. solani* were observed in the absence of the bacteria (Fig. 5b-c). The hyphae of both fungi were severely affected by the CW2 isolate; the bacterial cells were attached to the hyphae and formed a dense colonization pattern on the hyphoplane (surface of hyphae) of *P. ultimum* and *R. solani* (Fig. 5 e- i).

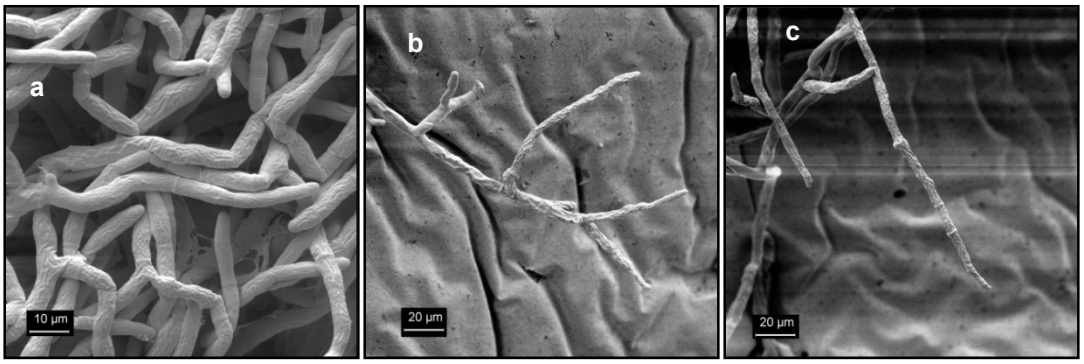

Fig. 4. Scanning electron micrographs showing hyphae of *R. solani* after three days of co-cultivation with the fluorescent pseudomonads isolate CW2. (a) Normal growth in the absence of CW2 (control). (b) Irregular hyphal shape and increased lateral branching in the presence of isolate CW2. (c) Pin-like shaped hyphal tip. Samples were taken from inhibition zones

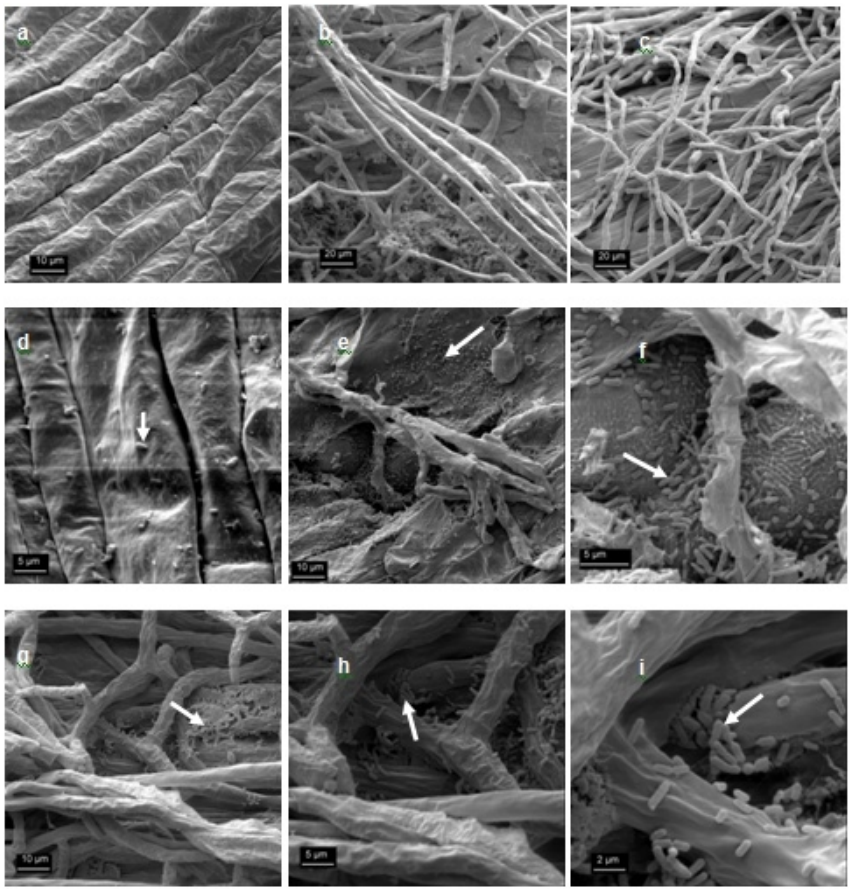

Fig. 5. Scanning electron micrographs showing an effect of the interaction of the fluorescent pseudomonad (arrows) isolate CW2 with *P. ultimum* and *R. solani* in the rhizosphere of cucumber plants. (a) control un-treated cucumber roots; (b) cucumber roots inoculated with *P. ultimum*; (c) cucumber roots inoculated with *R. solani*; (d) cucumber treated with isolate CW2; (e) cucumber roots inoculated with *P. ultimum* and treated with isolate CW2; (f) magnification of e; (g) cucumber roots inoculated with *R. solani* and treated with cells of CW2: (h) magnification of g; (i) magnification of h

4. DISCUSSION

For a strain which acts via antibiosis one may assume that proper colonization is needed to deliver antifungal compounds along the entire root system [14]. Root colonization by biocontrol agents and more specifically fluorescent pseudomonads is critical for the expression of their beneficial effects. The survival kinetics of introduced bacteria is a dynamic function of root growth and differentiation on one hand, and bacterial growth and death on the other. Therefore, bacterial distribution along a root system is expected to vary according to space and time. Variation in bacterial densities along host roots has been assumed to follow the patterns of exudates and their concentrations. According to Gamalero et al. (2004) [25], the proportion of culturable bacteria is expected to be affected by the concentration and composition of root exudates.

It was found that CW2 isolate was able to colonize cucumber roots as revealed by enumeration of the total cfu. Total cfu g^{-1} root fresh weights of cucumber were determined 1, 3, 7, 14 and 21 days after treatment with bacteria either by soil drenching or seed treatment. However, the method is relatively elaborate, time consuming and does not provide a representative visual depiction of spatial colonization patterns relative to the host plant root [26]. These studies typically have been limited to a description of a given bacterial strain as populations per entire root system or as populations on root segments from specific regions of the root system [27].

Colonization may involve simply root surface development but, endophytic colonization of the root is also known, and the degree of endophytic colonization depends on bacterial strain and plant type [4]. When colonizing a root environment, an organism is confronted with a complex array of parameters such as water content, temperature, pH, soil types, and composition of root exudates, mineral content, microorganisms, and other factors [12]. Evidence of colonization are provided by EM as seen in Fig. 5 where bacterial cells appear as colonies rather than being evenly and randomly distributed along the hyphae which offers a reasonable explanation for the increased bacterial numbers associated with cucumber roots in the presence of the fungal pathogen. It is also consistent with the views of Gamalero et al. (2004) [25]; the proportion of culturable bacteria is expected to be affected by the concentration and composition of root exudates.

For the detection of the pseudomonads on the surface of the cucumber roots, scanning electron microscopy (SEM) was used. SEM offers the advantage to detect local interactions and /or the adhesion places of the bacteria directly on the roots [28,29]. SEM investigations showed that the CW2 isolate formed micro-colonies at furrow-like site on the root surface. Similar observations were made also by Brown and Beringer (1983) [30] and Chin-A-Woeng et al. (1997) [29] using other root-colonizing bacteria.

It is obvious from SEM studies that the bacteria may have more than one control mechanism against *P. ultimum* and *R. solani*. It was shown that the bacterium was able to colonize fungal hyphae. Lysis, swelling and increased branching of the fungal hyphae were noticed in dual cultures of bacteria and fungi grown on PDB medium (light microscopy data are not shown).

Multiple mechanisms of biocontrol have been described in some fluorescent pseudomonads strains [9]. Modes of action include: inhibition of the pathogen by antimicrobial compounds (antibiosis), competition for iron through production of siderophores, competition for colonization sites and nutrients supplied by seeds and roots; induction of plant resistance mechanisms, inactivation of pathogen germination factors present in seed or root exudates, degradation of pathogenicity factors of the pathogen such as toxins, parasitism that may involve production of extracellular cell wall-degrading enzymes, for example, chitinase and ß-1,3-glucanase that can lyse pathogen cell walls [4].

The most commonly reported mechanisms of biocontrol by fluorescent pseudomonads species include production of antibiotics, hydrogen cyanide (HCN), lytic exoenzymes, competition for iron mediated by siderophores, competition for carbon and induced systemic resistance [9].

5. CONCLUSION

In conclusion the capacity of *P. fluorescens* isolate CW2 to colonize the mycelia of phytopathogens used in this study and possibly other similar phytopathogenic fungi, allowing them to release their anti-fungal agents directly in the vicinity of the fungus maximizing its effect. Therefore, plants benefit from the protective advantage imparted by pseudomonads similar to CW2. The efficacy of field applications of these biocontrol agents is being determined in field applications on cucumber.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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