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Efficacy of Culture Filtrate from *Fusarium oxysporum* F221-B against Plant Pathogenic Fungi *In Vitro* and Fusarium Root Rot and Wilt Disease in Hydroponics

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Abstract The purpose of this research was to evaluate the potential of culture filtrate (CF) of non-pathogenic *Fusarium oxysporum* (F221-B) against 10 plant pathogenic fungi namely, *Curvularia* sp. (C11, C12), *F. semitectum* (F113), *F. oxysporum* f.sp. *lactucae* (F221-R, F422-G), *Rhizoctonia* spp. (R111, R112, R113) and *R. solani* (R11, R12) *in vitro* and *Fusarium* root rot disease in hydroponics. *In vitro* antifungal activity analysis indicated that all test concentrations of F221-B culture filtrate could slightly inhibit mycelial growth of all tested fungal pathogens less than 39 % over control. Interestingly, the CF at all test concentrations revealed the greatest spore germination inhibition 100% over control against F113, F221-R and F422-G whereas spore germinations of C11 and C12 were completely inhibited by the CF at the concentrations of 60, 80 and 100%. In addition, spore abnormalities such as swelling and lysis were noted. Regard to antifungal analysis in hydroponics, the most striking and remarkable result was obtained. The non-pathogenic *F. oxysporum* F221-B was proved to be efficient in reducing disease severity of *Fusarium* root rot in lettuce more than 90% and producing significantly better growth than that in non-treated lettuces. Moreover, no significant degree of difference in terms of lettuce growth was noted between using culture filtrate and spore suspension of F221-B.

Keywords: culture filtrate, non-pathogenic *Fusarium oxysporum* F221-B, *F. oxysporum* f.sp. *lactucae*

Introduction

Biological control of plant diseases including fungal pathogens has been considered as environmentally safe and viable alternative measure to chemical control (Baker, 1987; Cook, 1993; Tjamos *et al*., 1992). Non-pathogenic species of the fungal genus *Fusarium* were numerously reported to successfully control Fusarium wilt of various crops worldwide (Elmer, 2004; Larkin and Fravel, 1998 and 2002; Nel *et al*., 2006; Silva and Bettiol, 2005). In Thailand, non-pathogenic *F. oxysporum* (F221-B), originally isolated from root of lettuce grown in hydroponics, has shown its ability as plant growth promoting fungus (PGPF) on various crops suchas

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Butterhead, Cos, Green oak, Red oak lettuces, kale and mung bean (Thongkamngam *et al*., 2013; Thongkamngam and Jaenaksorn, 2015a) and as biocontrol agent (BCA) against plant pathogenic fungi, namely *Curvularia* spp., *Colletotrichum* spp. and *F. oxysporum* f. sp. *lactucae* (Thongkamngam and Jaenaksorn, 2015a). Non-pathogenic F221-B showed remarkable consistency in retaining its morphology characteristics and its efficacy as BCA and PGPF during 24 months storage (Thongkamngam and Jaenaksorn, 2015a). In addition, F221-B was proved to be able to colonize on root surface (epiphyte) as well as inside the root of lettuce (endophyte) without producing any disease symptoms (Thongkamngam and Jaenaksorn, 2015b).

Fusarium root rot and wilt disease was regarded as one of the most seriously disease of lettuce grown in soil and hydroponic cultivation (Cabral *et al*., 2014; Chinta *et al*., 2014; Malbran and Mourelos, 2014; Scott *et al*., 2014; Thongkamngam *et al*., 2012). The disease was caused by *Fusarium oxysporum* f.sp. *lactucae*. Effective control methods against this disease are limited whereas chemical control has adverse effect to the consumer and environment. On this regard, non-pathogenic F221-B would be one of the strong candidates as biological control agent to solve this problem. Based on the above-mentioned good characteristics of F221-B as PGPR and BCA from which the feature of antibiosis was also observed in confrontation experiment between F221-B and test fungal pathogens at the early stage of incubation period (Thongkamngam and Jaenaksorn, 2015a), it is interesting to further research on the antifungal ability of secondary metabolites in culture filtrate of non-pathogenic F221-B.

Therefore, the research was conducted to determine the potential of culture filtrate (CF) of non-pathogenic *Fusarium oxysporum* (F221-B) against 10 plant pathogenic fungi namely, *Curvularia* sp. (C11, C12), F. semitectum (F113), *F. oxysporum* f.sp. *lactucae* (F221-R, F422-G), *Rhizoctonia* sp. (R11, R12, R13) and *R. solani* (R11, R12) *in vitro* and Fusarium root rot disease in hydroponics.

Materials and methods

Fungal isolation

The test fungal pathogens were isolated from naturally infected disease plants i.e. *Curvularia* spp. (C11, C12) and *F. semitectum* (F113) from rice seeds showing dirty panicle disease, *F. oxysporum* f.sp. *lactucae* (F221-R, F422-G) from root of lettuce grown in hydroponics showing root rot disease, *Rhizoctonia* spp. (R111, R112, R113) from rice leaf shealth showing blight disease and *R. solani* (R11, R12) from rice seedling, by tissue transplanting technique on water agar. The fungi were purified and maintained on potato dextrose agar (PDA) and stored at 4°C.

Non-pathogenic *Fusarium* F221-B was obtained from the previous research of Thongkamngam *et al*. (2013).

Preparation of culture filtrate (CF) of F. oxysporum (F221-B)

Regard to our research aiming at assessment for antifungal activity of secondary metabolites in culture filtrate of non-pathogenic *F. oxysporum* (F221-B), only the complete cell-free culture filtrate has to be used in the experiment. Therefore, two types of preparation for obtaining the cell-free culture filtrate were carried out for this regard: i) using micro-filter (Minisart®; Sartorius stedim biotech, 0.2 µm) and ii) using moist-heat from autoclave.

To obtain filtrates of the fungal culture, 5 mycelial agar plugs (0.5 cm in diameter) of F221-B were inoculated into 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB) and the culture was incubated at room temperature. After 7 days, the biomass was separated from the broth, which contained the fungal metabolites, by filtering through Whatman filter paper no.1 before being centrifuged at 10,000 rpm for 15 minutes. Then, the filtrates were undergone through the process of getting the complete cell-free CF by 2 types of preparation procedure mentioned-above. After that, the obtained cell-free CF was diluted with sterile distilled water to give concentrations of 20, 40, 60 and 80 % ready to be used.

Effect of cell-free culture filtrate (CF) of F. oxysporum (F 221-B) against plant pathogenic fungi by agar well diffusion assay

To determine the antifungal activity of F221-B culture filtrate at different concentrations (0, 20, 40, 60, 80 and 100%) on mycelial growth of 10 plant pathogenic fungi, the experiment was conducted in duplicate; i) employing cell-free CF prepared by using micro-filter and ii) employing cell-free CF prepared by using moist heat of autoclave. Randomized Completely Block Design (RCBD) with 3 replications was employed.

Ten plant pathogenic fungi (C11, C12, F113, F221-R, F422-G, R11, R12, R111, R112, and R113) were cultured on PDA plate and incubated for 3 days. Then, 6 holes of 5 mm in diameter were punched with sterile cork borer aseptically into the agar outside the periphery of pathogen colony. 30 µl of each concentration of F221-B culture filtrate was introduced into each bored agar well. All plates were incubated at room temperature. The radial growths of tested pathogens were daily measured and the percentage of growth inhibition of tested pathogens was calculated.

Effect of cell-free culture filtrate (CF) of F. oxysporum (F 221-B) on spore germination of plant pathogenic fungi

To investigate the inhibitory effect of F221-B culture filtrates at different concentrations (0, 20, 40, 60, 80 and 100%) on spore germination of 5 fungal plant pathogens, namely C11, C12, F113, F221-R and F422-G, the experiment was performed in duplicate; i) employing cell-free CF prepared by using micro-filter and ii) employing cell-free CF prepared by using moist heat of autoclave. Completely randomized design with 2 replications was employed. 30 µl of the fungal spores (1×10^6 spore/ml) were placed onto concave glass slides in the presence of 30 µl of culture filtrates (concentration of 0, 20, 40, 60, 80 and 100 percent) and incubated in the moist chamber in the dark at room temperature (25±2°C). After 12, 24 and 48 h, the percentage of spore germination of fungal spores was determined by light microscopy. In addition, the occurrence of spore abnormalities was monitored.

Effect of culture filtrate (CF) of F. oxysporum (F 221-B) for controlling root rot of lettuce in hydroponics

Antifungal activity of F221-B culture filtrate was further determined for controlling *Fusarium* root rot disease of lettuce grown in hydroponics. The concentration of F221-B culture filtrate to be used in hydroponic experiment was selected from the agar well diffusion assay and spore germination test from which showing high potential of antifungal activity. The experiment was conducted using completely randomized design of 6 treatments with 3 replications and 3 plants per replication. Treatments comprised of:

Treatment 1: Healthy Control (Non-inoculated control)
Treatment 2: Inoculated control (with pathogen F422-G)
Treatment 3: Culture filtrate of F221-B
Treatment 4: Culture filtrate of F221-B + F422-G
Treatment 5: Spore suspension of F221-B
Treatment 6: Spore suspension of F221-B + F422-G

Seeds of Cos lettuce were sown in moistened sponge in seed tray and watered with tap water for a week and then moved into mini DFT system and fertilized with nutrient solution (EC=1 ms/cm2, pH=5.8-6.2). After 2 weeks, 3 seedlings were transplanted into a rectangular plastic container (25×34×15 cm) containing 5 liters of nutrient solution (EC=1.6 ms/cm^2, pH=5.8-6.2).

At 14 days, lettuces were treated with non-pathogenic F221-B by immersing their root into either culture filtrate or spore suspension of F221-B (5 ml/plant) for 5 minutes and the rest of F221-B was poured back into the growing container. Sterile water was used for control. Three days later,

pathogen inoculation was made by pouring 15 ml of spore suspension (10^6 spores/ml) of F422-G into a growing container. Sterile water was used for control.

Data collection and analysis

Disease index was rated up to 6 levels (0: healthy root, 1: reddish brown root, 2: reddish brown root and became rotten, 3: rotten root, with slight wilting, 4: rotten root with severe wilting, 5: plant became dead).

Disease incidence (DI) and disease severity (DS) of tested plant were calculated by equation 1 and 2, respectively.

$$\% \text{ DI} = \frac{\text{Number of plants showing infected root}}{\text{Number of total plants}} \times 100 \quad (1)$$

$$\% \text{ DS} = \frac{\sum (\text{Number of plants showing infected root} \times \text{disease index})}{\text{Number of total plants} \times \text{the highest disease index}} \times 100 \quad (2)$$

DI, DS, as well as plant growth parameters such as leaf number, leaf size, chlorophyll content (SPAD value) on the leaf were weekly monitored after inoculation whereas fresh and dry weight of shoot and root were recorded at the end of trial.

All collected data were statistically analysed by analysis of variance. Treatment means were separated using Duncan's multiple range tests.

Results and Discussion

Effect of cell-free culture filtrate (CF) of F. oxysporum (F 221-B) against 10 plant pathogenic fungi by agar well diffusion assay

Culture filtrate of F221-B was checked for its effect against mycelial growth of 10 fungal plant pathogens by using agar well diffusion assay. Among 2 types of CF of F221-B, the results revealed that cell-free culture filtrate prepared by using either micro-filter or moist-heat from autoclave still showed the similar level of antifungal activity against all tested fungi except for R11 and R112 (Fig. 1). This indicated the thermo-stable nature of these antifungal metabolites in culture filtrate produced by F221-B. Surprisingly, autoclaved CF gave higher inhibition against R11 and R112 than those from non-autoclaved CF.

Results shown in Fig. 1 revealed that the cell-free culture filtrate of F221-B contained secondary metabolites such as some bioactive compounds that inhibited the *in vitro* fungal pathogen growth whereas this inhibitory effect varied depending on the concentrations of CF tested. F221-B culture filtrate at all tested concentrations exhibited slight inhibitory effect against mycelial growth of all tested fungal pathogens in the range of 6-39 percent over the untreated control. Overall, among the tested concentrations, culture filtrates at 80 and 100 % gave the significant highest inhibition.

Curvularia spp. (C11 and C12) were shown to be most sensitive to F221-B culture filtrate.

The inhibitory effect of F221-B culture filtrate was in accordance with those of Benhamou *et al.* (2002); Halmann and Sikora, (1994); and Khurshid *et al*. (2014) who reported that various non-pathogenic *Fusarium* isolates have shown their ability to suppress plant pathogens. Tayung *et al.* (2010) reported that the metabolite produced by endophytic *Fusarium* (MTCC-9622) showed significant antifungal activity against 3 postharvest pathogens including *F. oxysporum*.

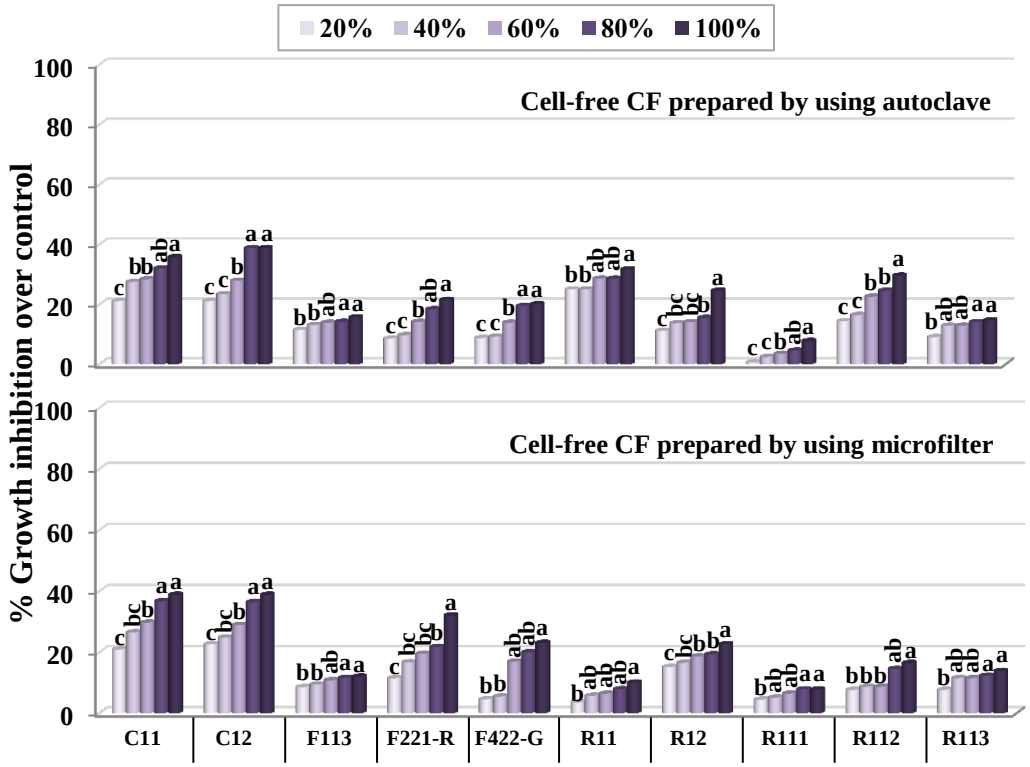

Figure 1. Effect of cell-free culture filtrate produced by non-pathogenic *Fusarium oxysporum* F221-B against mycelial growth of 10 plant pathogens by agar well diffusion assay. (C11, C12: *Curvularia* spp.; F113: *F. semitectum*; F221-R and F422-G: *F. oxysporum* f.sp. *lactucae*; R11 and R12: *R. solani*, R111, R112 and R113: *Rhizoctonia* spp.)

Effect of cell-free culture filtrate (CF) of F. oxysporum (F221-B) on spore germination of 5 plant pathogenic fungi

Culture filtrate of F221-B was tested for its antifungal activity on spore germination of 5 fungal plant pathogens. The result was in line with the afore-mentioned experiment using agar well diffusion method that CF of

F221-B prepared using either micro-filter or moist-heat from autoclave resulted in the same level of antifungal activity against the germination of test fungi (Fig. 2). This reconfirmed once again that the toxic metabolites in culture filtrate produced by F221-B are having thermo-stable nature.

A significant inhibitory effect (100 % inhibition over control) of all test concentrations (20, 40, 60, 80 and 100%) of cell-free culture filtrate of F221-B on germination of spores of *F. semitectum* (F113) and *F. oxysporum* f.sp. *lactucae* (F221-R, F422-G) was evident throughout the experiment (12-48 h) (Fig. 2). However, for the case of *Curvularia* spp. (C11 and C12), significant antifungal activity (100 % inhibition over control) of the CF of F221-B was observed only at the concentration of 60, 80 and 100 %. In addition, the level of inhibition of germination of their spores added to F221-B cell-free CF at the low concentration of 20 and 40 % declined with increasing time (Fig. 2). This decline could be held responsible for the decreasing levels of volatile sporostatic factors produced by F221-B. As soon as the appearance of volatile compound was ended, a persistent level of inhibition of spore germination caused by a stable inhibitor from non-volatile compound was shown. Furthermore, abnormalities of spores were detected such as swelling, lysis (Fig. 3).

In the current study, we observed the significantly higher degree of inhibitory activity of F221-B culture filtrates against spore germination of all tested fungi than those of their mycelial growth. This may be attributed to the direct exposure of spore to the culture filtrate.

Findings from *in vitro* experiments concerning the inhibitory effects of culture filtrates of F221-B are in agreement with several previous works by Aydi-Ben Abdallah *et al.* (2014); Lemanceau *et al*. (1993); Sonawane *et al*. (2015). In addition, the filtrate of non-pathogenic *F. oxysporum* was reported to counteract the growth of *Staphylococcus aureus* causing dermatological disease (Ahmed Sheikh, 2010).

On the basis of our findings from *in vitro* studies, it is assumed that the culture filtrate of F221-B tested may also contain antibiotics, toxins and/ or enzymes against the test fungal plant pathogens. Although the antifungal metabolites in culture filtrate produced by F221-B have not been so far identified chemically, it is still noteworthy that thermo-stability of such metabolites has been observed from our *in vitro* experiments. Additional studies are required to identify the active antifungal compound in F221-B culture filtrate.

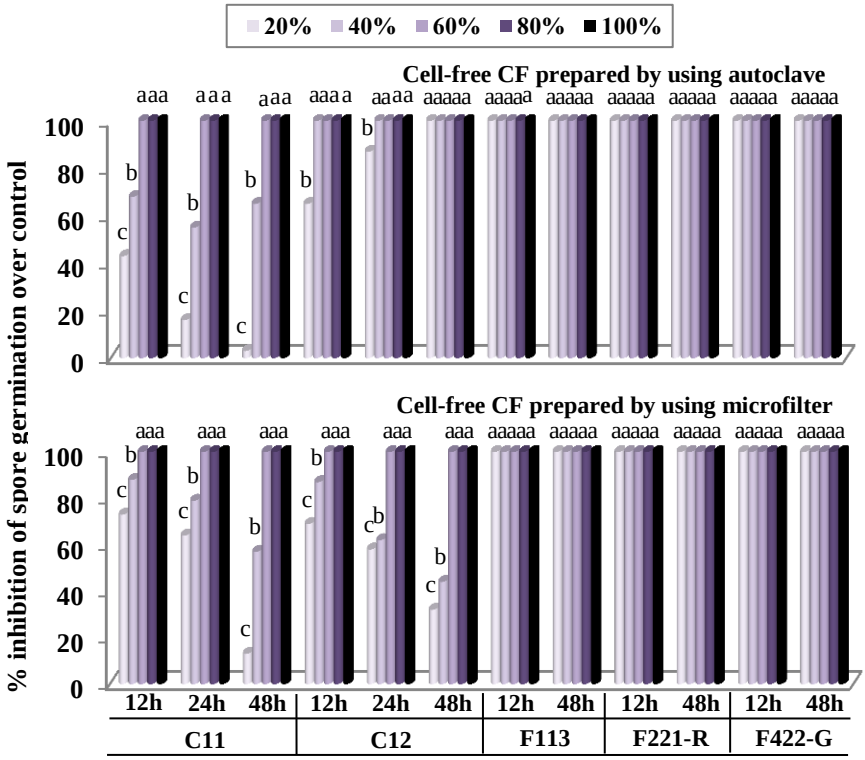

Figure 2. Effect cell-free culture filtrate produced by non-pathogenic *F. oxysporum* F221-B at different concentrations (20, 40, 60, 80 and 100 percent) against spore germination of 5 plant pathogenic fungi (3 days after inoculation)**.**

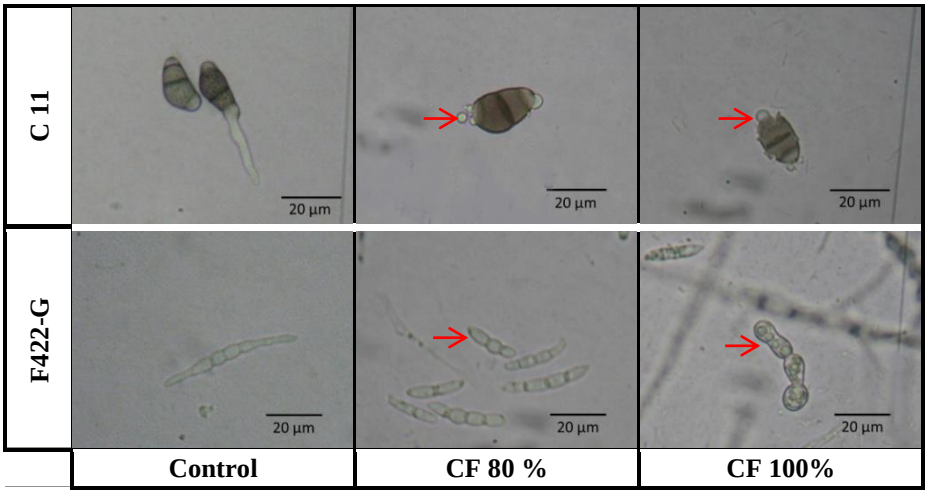

Figure 3. Abnormal spores of *Curvularia* sp. (C11) and *Fusarium oxysporum* f.sp. *lactucae* (F422-G) occurred in culture filtrate of non-pathogenic F221-B at the concentration of 80 and 100 percent, at 48 h.

Effect of culture filtrate (CF) of F. oxysporum (F 221-B) for controlling Fusarium root rot disease of lettuce in hydroponics

The experiment was conducted to investigate the effect of culture filtrate of non-pathogenic F221-B in controlling *Fusarium* root rot disease of lettuce in hydroponics. The result showed that lettuce from non-inoculated (healthy) control grew and developed normally while lettuce treated only with F221-B (culture filtrate or spore suspension) did not show any disease symptoms throughout the trial (Table 1) and grew significantly better than that of non-inoculated control (Fig. 4).

At 7 days after inoculation (DAI), the inoculated lettuce with F422-G began to show symptoms of root rot with 64 % disease incidence (DI) and 56.1 % disease severity (DS) whereas the lettuce treated with culture filtrate and spore suspension of non-pathogenic *F. oxysporum* (F 221-B) prior to pathogen inoculation showed significant low percentage in DI and DS about 32, 16 and 6.6, 3.3 percent, respectively. Regard to inoculated control, percentage of DI and DS increased with increasing incubation time; lettuces showed severe symptoms of root rot and wilt with 100% DI at 14 DAI and DS reached highest of 93.6% at 28 DAI. At 28 DAI, pre-treatment with either culture filtrate or spore suspension of F221-B exhibited a significant inhibition over control of more than 90 % in DS (Table 1).

In terms of plant growth, the most pronounced and statistically significant reduction in all the growth parameters such as leaf number, leaf size and SPAD value was recorded only in lettuces in inoculated control throughout the trial (Fig. 4) while the treatments with F221-B (either CF or spore) prior to pathogen (F422-G) inoculation resulted in the same normal growth as that in non-inoculated control (healthy control) and in F221-B treated lettuce. For overall, non-pathogenic F221-B-treated lettuces showed a more vigorous root system without apparent of root rot symptom and finally resulted in significantly much better growth (fresh weight of stem and root) than that in non-treated lettuces. In addition, spore of F221-B tended to be more efficient than the culture filtrate since the fresh weight and dry weight of stem and root of lettuce treated with spore of F221-B was shown to be significantly greater than that in non-inoculated control (Fig. 4).

Based on our results, non-pathogenic *F. oxysporum* (F 221-B) was proved to be efficient in reducing disease severity of *Fusarium* root rot in lettuce and producing greater growth with no significant degree of difference between using culture filtrate or spore suspension of F221-B. These results are agreed with earlier reports of their success in using non-pathogenic *F. oxysporum* to control Fusarium wilt of various vegetables (Reddy, 2013) such as asparagus (Elmer, 2004), basil (Minuto *et al*., 1997 and Larkin and Fravel, 2002), cucumber (Mandeel and Baker, 1991; Paulitz *et al*., 1987), pea (Benhamou and Garand, 2001), radish (Toyota *et al*., 1995), spinach (Katsube and Alaska, 1997), sweet potato (Ogawa and

Komada, 1984) and tomato (Larkin and Fravel, 1996 and Silva and Bettiol, 2005). In addition, our result is similar to that of Horinouchi *et al*. (2010), who reported that Fusarium wilt of spinach was effectively controlled by PGPF *F. equiseti* GF183.

These results provide evidence of the antagonistic activity of F221-B in controlling root rot disease caused by *F. oxysporum* f.sp. *lactucae* (F422-G) and promoting the overall growth in lettuce.

Table 1. Effect of non-pathogenic *F. oxysporum* (F221-B) in controlling root rot of lettuce grown in hydroponics.

Treatment	% Disease incidence (DI)				% Disease severity (DS)				% Inhibition in DS over control
	7 DAI[1/]	14 DAI	21 DAI	28 DAI	7 DAI	14 DAI	21 DAI	28 DAI	28 DAI
Healthy control	0[2/]	0	0	0	0	0	0	0	-
Inoculated control (F422-G)	64a	100a	100a	100a	56.1a	68.3a	74.6a	93.6a	0
F221-B (CF)	0	0	0	0	0	0	0	0	-
F221-B (CF)+ F422-G	32b	32b	32b	32b	6.6b	6.6b	6.6b	6.6b	93.4
F221-B (Spore)	0	0	0	0	0	0	0	0	-
F221-B (Spore)+F422-G	16b	16b	16b	16b	3.3b	3.3b	3.3b	3.3b	96.7

[1/]DAI: Day after inoculation, Inoculation was made at the age of plant of 17 days.
[2/]Means in a column followed by the same letters are not significantly different according to Duncan's Multiple Range Test at P=0.05.

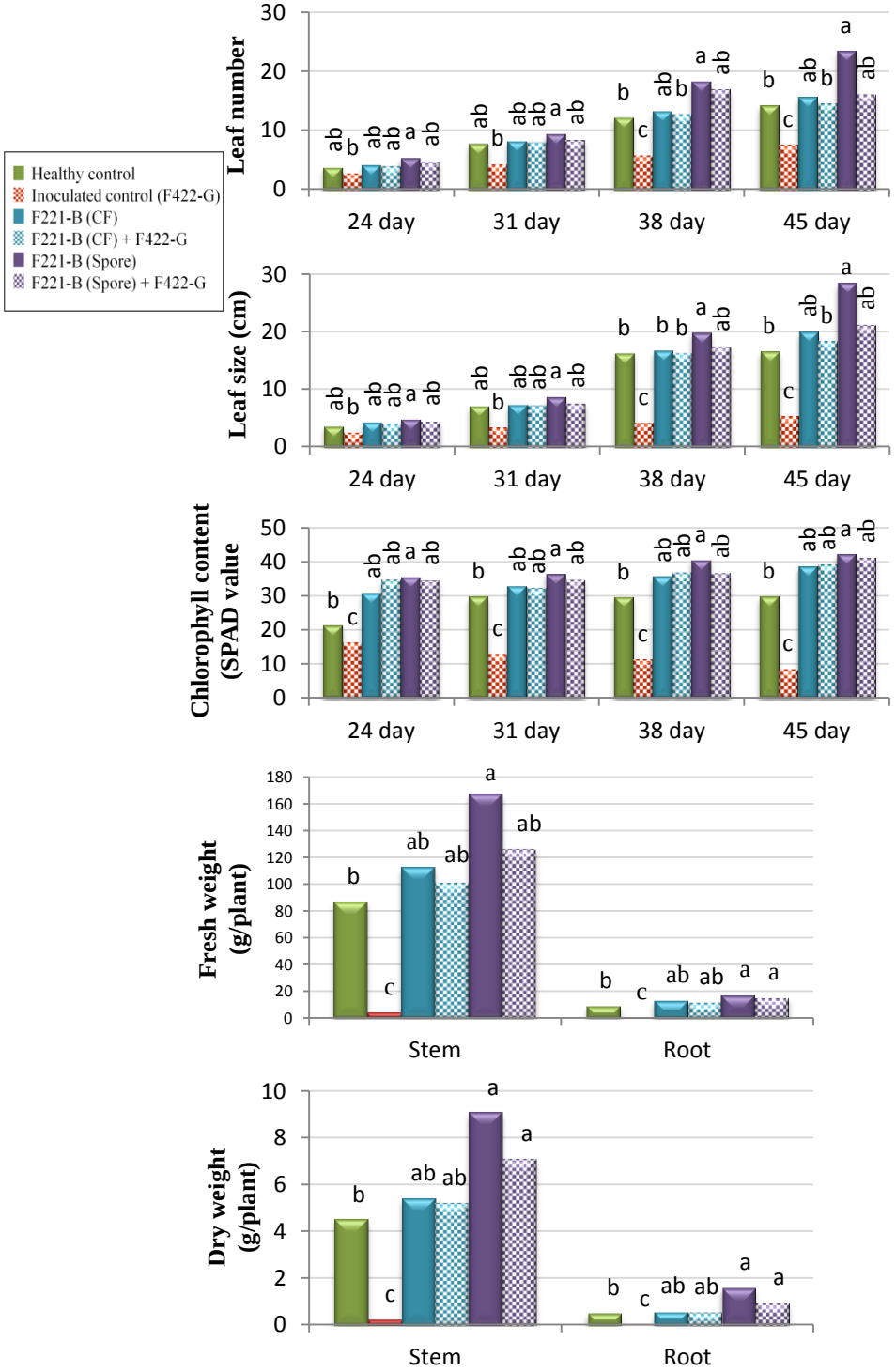

Figure 4. Effect of non-pathogenic *F. oxysporum* (F221-B) on growth of lettuce in hydroponics.

Figure 5. Effect of non-pathogenic *F. oxysporum* F221-B (culture filtrate and spore suspension) for controlling root rot disease of lettuce grown in hydroponics (45 days after inoculation).

Conclusion

In this research, the potential of cell-free culture filtrate of non-pathogenic *F. oxysporum* (F221-B) was evaluated against 10 plant pathogenic fungi. Among 2 types of cell-free culture filtrate used, autoclaved CF for overall still exhibited the same level of antifungal activity against test fungi as those from non-autoclaved CF (using micro-filter). This should be noted that thermo-stability of antifungal metabolites in culture filtrate of F221-B has been observed from our *in vitro* experiments. For agar well diffusion assay, all test concentrations of culture filtrate could slightly inhibit mycelial growth (about 6-39 percent over control) of all tested fungal pathogens while the promising fungitoxic effect was recorded only at the concentrations of 80 and 100 percent of CF. Similar but more promising results were obtained from the spore germination test. F221-B culture filtrate at all test concentrations showed excellent spore germination inhibition 100 % over control against F113, F221-R and F422-G whereas only at 60, 80 and 100 % of F221-B culture filtrate were shown to completely inhibit the spore germination of C11 and C12.

Taken together, non-pathogenic *F. oxysporum* F221-B in the form of either culture filtrate or spore suspension was significantly efficient in controlling root rot disease of lettuce. In addition, it significantly exhibited beneficial effect on overall growth of lettuce.

Acknowledgements

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