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Development of *Beauveria bassiana*-Based Bio-Fungicide Against Fusarium Wilt Pathogens for *Capsicum Annuum*, a Promising Approach Toward Vital Biocontrol Industry in Gaza Strip

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Abstract

Wilting or damping off disease is caused by a pathogenic fungi (*Fusarium oxysporum*), which considered as as one of the most serious diseases, which lead to a serious threat in the production of one of the most dependent crops in Gaza strip, *Capsicum annuum*. Two fungi were isolated, *Beauveria bassiana* which used as biological control agent and *F. oxysporum* which causes wilt disease. They were cultured and tested in vitro and applied under field conditions. This study approved that the entomopathogenic fungi especially, *b. bassiana* is safe and significant approach for the biological control of wilt disease caused by *F. oxysporum.* Evaluation of *B. bassiana* spores activity, as a biological control agent was carried out using liquid-semi solid fermentation techniques and the entomopathogenic of *B. bassiana* against growing *F. oxysporum* was proven. Our results showed a highly significantly effects of *B. bassiana* against *F. oxysporum*.

Keywords:
B. bassiana,
F. oxysporum,
C. annuum,
Biocontrol,
Gaza strip.

1. Introduction:

Fusarium wilt, also called Yellows, widespread plant disease caused by a number of highly specialized forms of the soil-borne fungus *Fusarium oxysporium*. Infected plants are usually stunted; their leaves turn pale green to golden yellow and later wilt, wither, die, and drop off progressively upward from the stem base (Monica, 2010; Marshal, 2012). *F. oxysporum* is a major wilt pathogen of many economically important crop plants such as the *Solanaeae* family, including "potato, tomato, and pepper", other commercially important plants including basil, beans, carnation, chrysanthemum, peas, and watermelon, strawberry, Palms and woody ornamentals are also affected (Belgrove, 2011). The *Fusarium* wilt fungi are all soil borne and difficult to control, eradication of the pathogen are limited by the ability of their spores to survive in soil for long periods, with or without a host plant, so, crop rotation is generally not effective. Soil fumigation with a broad-spectrum fungicide provides good initial control, but re-colonization of the soil occurs very quickly (Yousaf & Khalid, 2007; Đorđević, 2011). Thus, bio-control is alternative strategy as it has a potential for the management of *Fusarium* wilt diseases. Currently, in terms of agriculture, USDA defines biological control as “The involvement of the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause.”

Beneficial microorganisms that fit this definition are also known as biological control agents (BCAs). BCAs play an important role in forestry as many chemical fungicides are being faced out, and organic production is encouraged aiming at improving sustainable plant production. These are ideal, safe, cheap, long lasting and eco-friendly when compared with chemicals In this respect a variety of microorganisms have been isolated from rhizo-sphere of cultivated plants, and have demonstrated antagonistic activity against the *Fusarium* wilt (Kidane, 2009).

Capsicum annuum is a very widespread species under the family of *Solanaceae* (Shaha *et al.,* 2013). It's sensitive to high temperatures so crops are cultivated in a temperature of ≥34 °C in day and ≥21 °C at night (Rylski & Spigelman, 1982; Erickson *et al.,* 2002).

Capsicum annuum is one of the most important cultivated crops in Gaza strip, in which it enters into many fields of food industries. Many ways were used all over the world in controlling diseases rewrite. Some are chemical methods including using chemicals such as mercuric chloride, sodium azide, or using gaseous compounds such as ethylene oxide, propylene oxide, or methyl bromide. (Wolf *et al.,* 1994). Others are biological methods such as biological soil disinfestations (Momma & Noriaki, 2008). Biological control is the most distinctive method used in controlling diseases and was initiated in 1762 (Shahid *et al.,* 2012).

Chemicals has been used for decades, but their dangerous influence on non-target organisms, groundwater and food crops encouraged ministry of agriculture in Gaza strip to develop alternative control measures, which are promising because they don't need to be ingested but only act by contact (Shahid *et al.,* 2012). *B. bassiana* is considered as a biological control agent, a fungus which is widely used in biological control fields. It showed its effectiveness against wilt disease caused by *F oxysporum*. It has been reported as a strong biological agent against fungal plant pathogens such as *F. oxysporum* (Ownley *et al.,* 2010).

This fungus infects plants through roots and invades vesicular tissue causing wilting, yellowing and eventually collapsing of leaves and other parts of the plant (Harman, 2000) *B. bassiana* grows as a white mould. It produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells (El kichaoi *et al.,* 2016). It's able to produce secondary metabolites beside antibacterial, antifungal, cytotoxic and insecticidal activities (Ownley *et al.,* 2010).

2. Methodology:

2.1 Protocols:

2.1.1 Isolation of *B. bassiana* and *F. oxysporum*:

F. oxysporum was isolated in the ministry of agriculture in Gaza strip and was used in this experiment while *B. bassiana* was isolated in biology and biotechnology department of the Islamic university in Gaza (El kichaoi *et al.,* 2016)

2.1.2 Culture of *B. bassiana* and *F. oxysporum*:

Using potato dextrose agar (PDA) medium, *F. oxysporum* was re-cultivated for further use. Dodine acetate 2 DOC2 used as a selective media for *B. bassiana* isolation (Shin *et al.,* 2010). For more *B. bassiana* growth, PDA plates were incubated in total darkness at 25 °C.

2.1.3 Preparation of spore suspensions:

PDB liquid medium was prepared and used for the growing of fungi and production of spores which was used as spore suspension for further experiments. Liquid medium was inoculated in flasks with *B. bassiana* and *F. oxysporum* propagated on PDA after scraping 2-3 weeks old surface cultures. Flasks were held on shaker at 110 rpm for 5-6 days at 25°C. to remove mycelia and culture debris after culturing in PDB. The two suspensions were stirred and filtered by a single layer of linen. *B. bassiana* spores concentration was adjusted to 2.5×10^{7} spores/ml using hemocytometer (Gindin *et al.,* 2006).

2.1.4 Morphological identification of fungal isolates:

Cultures were examined from time to time and separated into groups based on their morphological characteristics on PDA including colony color and texture, growth rates, growth pattern and pigmentation (Guo *et al.,*1998; El kichaoi *et al.,* 2016) after culture sporulation observed, small plaques from the center and the edge were taken onto glass slide to study their hypha structure and color, shape and size of conidia and conidiophores (Yu, 2010). These vegetative and reproductive structures were examined using compound light microscope.

2.1.5 Evaluation of *B. bassiana* against *F. oxysporum*:

2.1.5.1 Testing of *B. bassiana* effect under lab conditions:

B. bassiana isolate was tested for their ability to inhibition of *F. oxysporum,* on agar plate as illustrate by Weller and Cook (Weller & Cook, 1986) and Wong and Baker (Wong & Baker, 1984). The pathogenic was transferred to regular Petri dishes containing fresh PDA to produce fungal mycelium plugs. *b. bassiana* isolate was streaking on PDA with streaking of *F. oxysprium* at the same time, and incubated at 27±1 C^0 for 72hr. Other application by wells for spore suspension of *B. bassiana* and 1 piece of *F. oxysporium* in the center of Petri dish and incubated at 27±1 C^0 for 72hr. Each previous test was demonstrated on control. The size of inhibition zone for fungal was used as a measure of the ability of those fungal to inhibit (Weller & Cook, 1986).

2.1.5.2 Dividing groups under field conditions:

Capsicum annuum seedlings were planted in 20 plastic bags in each group, and were divided into four groups as the experiment proceeded. These groups include: (20 plot of *Capsicum annuum* as negative control, 20 plots of treated *Capsicum annuum* with *B. bassiana* at the same time , 50 plots of *Capsicum annuum* as positive control, 50 plots of treated *Capsicum annuum* after three days of infection and 50 plots of *Capsicum annuum* treated with chemical fungicide (Dimethomorph Mancozeb). Nothing was added to the negative control group, it was grown normally, while the positive control group was only infected with *F. oxysporum*. Chemical fungicide samples were treated.

2.1.6 Data collection and statistical analysis:

Data were subjected and computed using version 22 of statistical package for social science, (SPSS). One way Anova was the main statistical test used in our study.

3. Results:

3.1 Isolation of *B. bassiana* and *F. oxysporum*:

B.bassiana was isolated in biology and biotechnology department in the Islamic University of Gaza and it was used in this study. The two isolates are shown in Figures 1 and 2.

Figure 1 *Culture of b. bassiana on DOC2 selective media*

Figure 2 *Culture of F. oxysporum on PDA media*

3.2 Culture of *B. bassiana* and *F. oxysporum* and their cultural characterization:

Isolates of *B. bassiana* and *F. oxysporum* were cultured to increase their quantity using PDA media and their cultural characteristics were studied. *B. bassiana* grows as white mold producing powdery conidia with white spore balls composing of conidiogenous cells as in Figure 3. *F. oxysporum* grows as white aerial mycelia producing dark violet pigment on PDA.

Figure 3 *Culture of B. bassiana on PDA*

3.3 Microscopic examination:

The examined microscopic characters of *B. bassiana* were shape, size, color and thickness of hyphae, conidiophore and conidium.Results showed that hyphae size was about 1-2µm and conidiogene cells was about 3-6µm in size. Conidiogene cells, which have bottle like form, small neck then were branched from hyphae up to more than 20 µm long and 1µm wide. Circular fertile hypae of *B. bassiana* was found on branch with normal thickness and mycelium was white and insulated. Microscopic characters are shown in Figure 4.

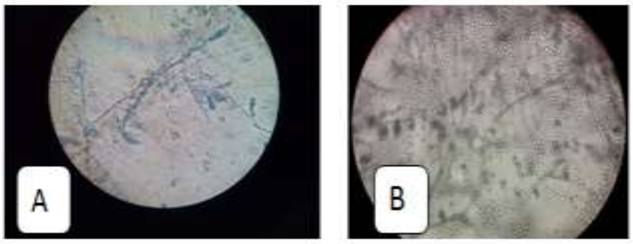

Figure 4 **A:** *F. oxysporum* & **B:** *B. bassiana* spores under microscope

F. oxysporum isolate was studied under microscope. Microconidia size was about 5.0~12×2.2~3.5 µm, which forms on microconidiophores. They have elliptical shape and no septate, while macroconidia size was 27~46×3.0~4.5 µm, they have 3 septates and appears between straight to curved. The optimum PH for mycelia growth was 7.0.

3.4 Spore suspensions and enrichment

Cultivated of *B. bassiana* was done using selective media that contains dodine while *F. oxysporum* was cultured on PDA medium. Concentration of *B. bassiana* was adjusted to 2.5×10^7 spore/ml which was the lethal dose for *F. oxysporum*.

3.5 Evaluation of *B. bassiana* potential against *F. oxysporum*

After adjusting the concentration of *B. bassiana* as 2.5×10^7 spore/ml, it was tested in vitro against *F. oxysporum*. Several examinations and studies on *B. bassiana* effect against *F. oxysporum* were done using two types of methods, streaking and well diffusion methods. Each test was compared to a control. Results are shown in Figure 5.

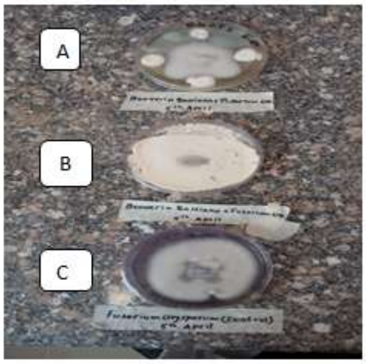

Figure 5 **A**: *Illustrate the effect of B.bassiana on F. oxysporum by wells*; **B**: *Illustrate the effect of B. bassiana on F. oxysporum by streaking;* **C**: *Illustrate positive control*

3.6 Evaluation of *B. bassiana* potential against *F. oxysporum* under field conditions comparing with the efficiency of chemical fungicide:

3.6.1 Comparison between the effect of *B. bassiana* and chemical fungicide on branches number of *Capsicum annuum* infected by *Fusarium* wilt:

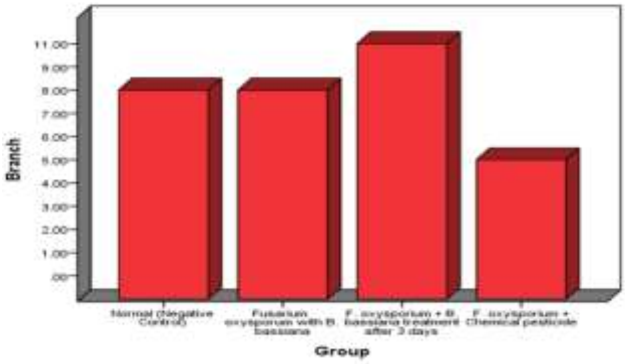

Figure 6 *The influence of B. bassiana on Fusarium wilts disease comparing to chemical fungicide according to branches number*

Depending on the branches number the result in Figure 6 show that *B. bassiana* positively affect the growth of plant infected by *Fusarium* wilt disease comparing to the influence of chemical fungicide. The best result was when *B. bassiana* added after 3 days of infection.

3.6.2 Comparison between the effect of *B. bassiana* and chemical fungicide on mean of stem and root length of *Capsicum annuum* infected by *Fusarium* wilt disease:

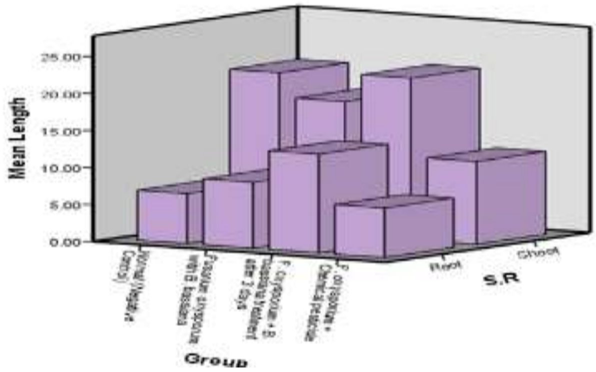

Figure 7 *The influence of B. bassiana on Fusarium wilt comparing to chemical fungicide according to mean of stem and root length*

The result in Figure 7 concerning the mean of stem and root length confirm the result in Figure 6 on the branches number where *B. bassiana* affected positively the growth (especially the shoots measurements) in both cases.

3.6.3 Comparison between the effect of *B. bassiana* and chemical fungicide on the mean of fresh weight of *Capsicum annuum* infected by *Fusarium* wilt:

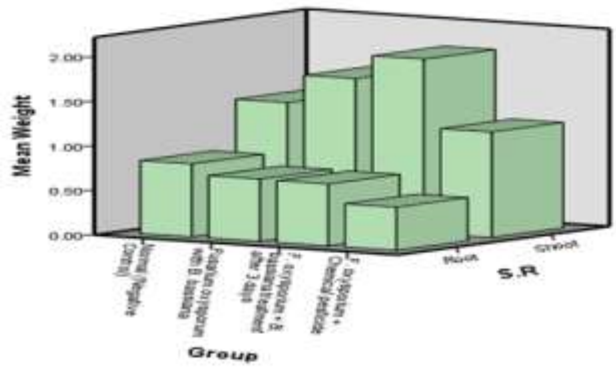

Figure 8 *The influence of B. bassiana on Fusarium wilt disease comparing to chemical fungicide according to mean of fresh weight*

As shown in Figure 8, concerning the fresh weight of plant, we can observe that the influence of *B. bassiana* is always better than the effect of the chemical pesticide and the best result of *B. bassiana* was when added after 3 days of infection by *Fusarium* wilt disease.

3.6.4 Comparison between the effect of *B. bassiana* and chemical fungicide on the mean of dry weight of *Capsicum annuum* infected by *Fusarium* wilt disease:

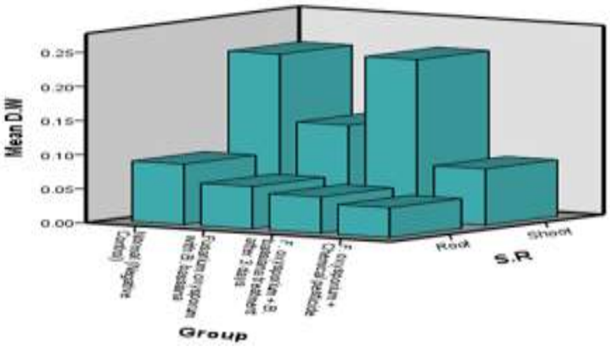

Figure 9 *The influence of B. bassiana on Fusarium wilt disease comparing to chemical fungicide according to mean of dry weight*

Depending on the most important measurements for plant growth, which is dry weight, (especially the shoots measurements) the results in Figure 9 confirm the above results where it appears that the impact of *B bassiana* much better than chemical pesticide in terms of their ability to prevent the negative impact of *Fusarium* wilt disease on *Capsicum annuum* growth.

4. Discussion:

Different approaches are used for controlling pests worldwide. A promising environmentally friendly mean is biological control, which is non-chemical measure that has been reported to be effective against many pests (El Kichaoui *et al.,* 2016). It is the use of special microorganisms to control pathogens, it is safer than chemical usage which harmful to human health and environment. Moreover, limited agricultural areas with intensive agriculture are particularly in need of such entomopathogenic fungi in order to limit the use of chemical fungicide and reduce the ground water pollution. Gaza strip is good example for such areas with agriculture representing a backbone for population life. *B. bassiana* is one of the most potential biological control agent that used in controlling economically important pests (Booth *et al.,* 2000; Shapiro *et al,* 2002;), it is show an effective influence as entomopathogenic fungi (Stafford & Allan, 2010) as its prolific producers of proteins, enzymes, and bioactive secondary metabolites, which are responsible for their virulence activities against other organisms (Molnar *et al.,* 2010; García *et al.,* 2016). In this regard, this study investigated the influence of *B. bassiana* isolate on pathogenic fungi *F. oxysporum*, which caused the wilt disease in some plants. *B. bassiana* have shown to have an effective antifungal activity against *F. oxysporum* which cause wilt disease (Parine et al, 2010). The present research was agrees with many studies that beneficial fungi such as *B. bassiana* have a high effectiveness with no effects on human public health (Costa *et al.,* 2011; El Kichaoui *et al.,* 2016). The use of chemical fungicides has become risky, it can cause serious disease and symptoms to human, environmental pollution and long-term residue on food crops issues (Shahid *et al.,* 2012; Zhao *et al.,* 2013). Comparing to chemical fungicides, B. bassiana is less toxic and environmentally safe (Rebek *et al.,* 2012; El Kichaoui *et al.,* 2016). According the present study will be of great benefit in establishing a role of entomopathogenic fungi in encouraging plant growth with similar efficiency as chemical fungicide. The results in our research will be encouraging decision makers to adopt strategies for isolating, growing and using bio-control agent, so this fits with the main objective for the biological control unit, which aspires to get rid of chemical pesticides and replace with biological fungicide.

5. Conclusion:

This research as a part of general project that aims to solve some of environmental and health problems by reducing the use of chemical fertilizers, pesticides and drugs and replace them by natural enemy or organisms.. The properties of biological control should be taken into account when designing fungicide for control plant diseases with no adverse effects on human health and environment. Therefore, the use of the bio-fungicide is one of the best solutions and safe approach for plant disease management and this lead to reduce the costs of pest control, care human health and environment from pollution which caused by chemical fungicides usage. Uses of *B. bassiana* pre-injury work to prevent and controlling *Fusarium* wilt disease in Gaza strip.

Acknowledgements:

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تطوير مبيد فطري من البفاريا باسيانا لمكافحة مسببات الذبول الوعائي ، طريق واحد باتجاه قطاع صناعي حيوي للمكافحة الحيوية في قطاع غزة.

نظراً للأهمية العالمية لمرض الذبول الوعائي الفيوزاري فقد تم توثيقه بشكل كبير ، و كونه يؤثر على مستوى معيشة ملايين الأشخاص حول العالم بسبب تدميره لمحاصيل مهمة على رأسها نباتات العائلة الباذنجانية كالطماطم والفلفل وغيرها من نباتات العائلة مما يجعله ذو أهمية خاصة. و مع ذلك فإن الخيارات الفعالة و المتوفرة لمكافحته قليلة و لا يوجد حتى الآن طريقة واحدة فاعلة للتحكم أو الحد من انتشاره . في الوقت الراهن و نظراً لأن هناك اهتمام كبير باتجاه سلامة المستهلك و البيئة ، الانتاج العضوي ، متبقيات المبيدات الكيميائية ، تعتبر " وسائل المكافحة الحيوية " من أعظم الحلول و جهود المكافحة تتجه نحو الاستفادة من هذه الوسائل الحيوية. تم عزل اثنين من الفطريات وهي فطر البفاريا باسيانا والذي يستخدم كعوامل مكافحة حيوية وفطر فيوزاريوم أوكسيسبوريم وهو المسبب لمرض الذبول الوعائي. تم تقييم كفاءة فطر البفاريا في المختبر من خلال زراعته مع فطر الفيوزاريوم مع وجود عينة ضابطة لكل فطر ومن ثم الانتقال إلى مرحلة الحقل والتطبيق الميداني من خلال تقسيمها إلى مجموعات. وقد أثبتت هذه الدراسة كفاءة فطر البفاريا كعوامل مكافحة حيوية ضد مرض الذبول الوعائي في نبات الفلفل الحار وأنها تمتلك كفاءة عالية ضد مسببات الذبول الوعائي مقارنة بالمبيدات الكيميائية.

كلمات مفتاحية:
بفاريا باسيانا
فيوزاريوم أوكسيسبوريم
الفلفل الحار
المكافحة الحيوية
قطاع غزة.