The Use of Endophyte *Beauveria Bassiana* for Bio-protection of Date Palm Seedlings against Red Palm Weevil and *Rhizoctonia* Root-Rot Disease

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Abstract:

Ten *Beauveria bassiana* local strains were isolated from cadavers of different date palm insects, then screened enzymatically to select the highest virulent isolate. Two-week old axenically date palm seedlings were grown in petri-dishes and subjected to direct injection with two rates of *Beauveria bassiana* spore suspension (0.5×10^7 or 1.5×10^7 spores/ml). The endophytic fungus was recovered from sites distant from the point of inoculation 6 months after application. This indicate that the fungus has the potential to move throughout the plant tissues. One week after endophytic colonization, date palm seedlings were infected with the pathogen *Rhizoctonia solani*. The treated seedlings exhibited 70.6% reduction of root rot disease incidence.

On the other hand, date palm pulp of the endophytically colonized seedlings was used in a laboratory diet for the larvae of the red palm weevil (RPW). The larval mortality during 14 days achieved 80.3 %, under laboratory conditions.

Key Words: *Beauveria bassiana,* Date-palm root-rot, Endophytes, Induced disease resistance, Red palm weevil (*Rhynchophorus ferrugineus*), *Rhizoctonia solani,*.

Introduction:

Date-palm is targeted by a large number of pests, including fungi, insects, and nematodes (Carpenter and Elmer, 1978). Some of these pests are serious and difficult to control such as red palm weevil (*Rhynchophorus ferrugineus* Oliv., Coleoptera: *Curculionidae*) (El-Sufty *et al.,* 2007). Chemical control approaches of red palm weevil have proven inefficient. The use of biological control with microorganisms can play a significant role in the management of many insect pests and mycopathogens. The entomopathogenic fungi are considered an important component in the integrated control of different chewing and sucking insect pests (Gallego and Gallego, 1988). In this respect, the fungus *Beauveria bassiana* (*Deuteromycotina: Hyphomycetes*) has been reported as a suppressive bio-agent for several insect species worldwide (Ferron, 1981). It is the anamorph stage of *Cordyceps bassiana,* a teleomorph in the *Ascomycetous: Clavicipitaceae*) (Sung *et al*., 2007). Strains have been collected from

different insect cadavers and cultured to obtained the commercial product use, Boverin (Lysenko and Kucvran, 1971). Entomopathogenic fungi are efficacious only with prerequisites of high ambient moisture and optimal temperature (Lord, 2005). However, not all the developing hyphae of *Beauveria bassiana* on the leaf surface penetrate the insect cuticle (Wanger and Lewis, 2000). Leaf surface of some plants such as date-palm leaves were inconvenient for *Beauveria bassiana* conidial colonization (Asensio *et al*., 2005).

On the other hand, Rhizoctonia root-rot of date-palm seedlings wherever the plant is grown is caused by the soil borne pathogen *Rhizoctonia solani* (El-Deeb *et al.*, 2006).The pathogen can survive for long periods in all soil types. As the disease becomes apparent, it is often too late to apply control measures (Frank and Murphy, 1977).

Recent researches reported that *Beauveria bassiana* could be used as a fungal endophyte to induce plant resistance against some insect pests and some mycopathogens. Some crop plants such as corn, cotton, coffee, and banana, which has been examined in several studies (Bing and Lewis, 1992, Griffin, 2007, Posada *et al*., 2007 and Akello *et al*., 2008). The present investigation was carried out to find out successful approach to control red palm weevil and Rhizoctonia-root-rot of date-palm seedlings using the endophytic fungus *Beauveria bassiana.*

Materials and Methods:

Isolation and identification of *Beauveria bassiana* strains:

Beauveria bassiana was isolated from dead larvae of red palm weevil procured from an infested date-palm orchard in Sharkia and Giza governorates, Egypt. The small larval segment were externally sterilized in 100 % ethanol for about one minute and allowed to air dry for another minute. Sterilized surface segments were put into PDA medium in Petri-dishes. The isolates of entomopathogenic fungus, *Beauveria* spp. were identified according to Glare and Inwood (1998). The isolates were then propagated on yeast extract peptone supplemented liquid medium according to Haraprasad *et al.* (2001). The mycelial mat was harvested from 12-day-old cultures and lyophilized. Random amplified polymorphic DNA (RAPD) analysis was applied to detect *Beauveria bassiana* strains at the Botany Dept., Fac. Agric., Al-Azhar Univ., according to the method described by Hegedus and Khachatourians (1996). It was used later for rapid detection of the endophyte fungus inside date-palm tissues. The isolates were enzymatically screened to select the highest virulent isolate.

Histological rapid staining was applied to date-palm tissues for detection of the endophytic fungus establishment according to the method of Saha *et al*. (1988) using rose Bengal stain light microscopy examination.

Enzymatically screening for the highest virulent *B bassiana*:

Five isolates of the entomopathogenic fungus, *B bassiana* were tested for chitinase enzyme activity. Each isolate was cultured in a liquid synthetic medium containing chitin- azure as a sole carbon source and incubated at 25± 2 ºC for 10 days according to Kang *et al*. (1999).

The amount of dye release from chitin-azure by chitinase producing *B bassiana* isolates was determined densitometrically and used as a measure of chitinolytic activity according to the method described by Evrall, *et al*. (1990). The highest chitinase activity was associated with the highest entomopathogenic strain.

Fungal inocula preparations:

Suspension of the most virulent isolate of *B. bassiana* was prepared from 12-day-old liquid culture in sterile water and adjusted at the rates of 0.5×10^7 or 1.5×10^7 spores/ml to be used to inject 2-week-old date-palm seedlings.

The inocula of *R.solani* isolated from rotted roots of date-palm seedlings was prepared by growing on autoclaved rye grain for 15 days at 20 ºC according to Acharya *et al*. (1984).

Potted sandy-loam soil was infested by adding the inoculum at the rate of 5 % of soil weight and mixed thoroughly with the upper surface (5 – 8 cm depth), then covered with 1 cm thick layer of sterilized soil. The pots were regularly watered for one week to ensure even distribution of the inoculated fungus.

After 3 months from inoculation, date-palm seedlings were cautiously unearthed to estimate root-rot disease incidence. Above ground, Rhizoctonia root-rot symptoms include yellowing of leaves. Below ground, a dark brown-gray starts under the crown and sprayed over the root surface (Zimmer, 1988).

Endophytically colonization of date-palm seedlings:

One hundred 2-week-old axenically reared date-palm seedlings cv. Medjhool grown in Petri-dishes were inoculated with the two levels of *B. bassiana* conidial suspensions, *i.e* 0.5×10^7 or 1.5×10^7 spores/ml using injection method according to Usuki *et al*. (2002). One week after endophytic colonization, date-palm seedlings were transplanted into potted

sandy-loam soil (one seedling/pot) infested with *R.solani* inoculum (5g/Kg soil).

The treated date-palm seedlings (endophytically colonized and *Rhizoctonia* - infected) were compared with another group of date-palm seedlings that was not endophytically colonized, grown in similar potted infested soil, and with endophytically colonized seedlings grown in non- infested pottedsoil.

All treatment was replicated four times. All the seedlings were left up to 6 months under greenhouse conditions of Fac. Agric., Al-Azhar Univ., Cairo. Disease assessment was carried out 3 months after inoculation. Re-isolation was carried out from infested tissues and isolated fungi were compared with the original culture used.

Incorporation of endophytically date-palm tissues into synthetic diet of red palm weevil larvae:

100 healthy larvae of red palm weevils at the fourth instar were collected by hand or by cutting log section of infested date-palm trees. The larvae were kept in a climatic chamber (70±5 RH and 27±2 ºC) for 14 days without light. The larvae were fed onto broken wheat kernels mixed with endophytically date-palm tissues (200mg tissues/Kg wheat) after one week from infestation with the two level of *B bassiana* conidial suspensions, i.e. ($0.5x\ 10^7$ or c). Each diet treatment was replicated four times.

Observations were performed daily and the recording of larvae mycosis rate began when the percentage of the mortality exceeded 50% according to the procedure described by Leckie *et al.* (2008). Untreated larvae were fed on broken wheat kernels only.

Resultes and Discussion:

Isolation and identification of *Beauveria bassiana* strains :

Fifteen isolates were identified as *Beauveria* but five only of them were detected as *B. bassiana* strains according to RAPD analysis.

Enzymatically screening for the highest virulent *B. bassiana*:

Data presented in Table (1) show that the highest chitinase activity was associated with *B. bassiana* isolate No.2 of dead larvae procured from Giza governorate, Egypt.

Table (1):
Chitinase activity and protein content in culture filtrates of of *B. bassiana*.

No of *B. bassiana* Isolate	Chitinase activity (Units /ml)
1	4.3
2	6.2
3	5.5
4	4.6
5	4.7

Endophytically colonization of date-palm seedlings:

Rapid staining of date-palm tissues injected with *B. bassiana* conidial suspension indicate that the entomopathogenic fungus was established inside date-palm tissues until the end of the 6 months trial (table 2). The direct injection with the spore suspension yielded high post colonization, recovered from sites distant from the point of inoculation. This indicates that the fungus has the potential to move throughout the plant tissues.

Table (2):
Date-palm Rhizoctonia root-rot disease incidence on 3-month-old seedlings cv. Medjhool under greenhouse conditions.

Treatment	Root-rot disease %
B. bassiana colonization + pathogen	05
Free *B. bassiana* colonization + pathogen	17
B. bassiana colonization without pathogen	00
Free *B. bassiana* colonization without pathogen	00
L.S.D. at 50 %	3.0

The obtained result agree with the findings of Gomez-Vidal *et al.* (2006) who observed that, some endophytic fungi survived inside leaf tissues of date-palm and sparsely detected within the vascular tissues. Date-palm seedling growth did not decrease when the elevated *B. bassiana* concentration (1.5×10^7 spores/ml) was used.

This is in harmony with Carroll (1998) who stated that endophyte is any microorganisms inhabit plants without causing visible disease symptoms.

One hundred treated seedlings exhibited 5 seedlings with rotted roots were compared with 17 diseased seedlings in control treatment (70.6 % reduction of root-rot disease incidence). The root-colonized by *B. bassiana* can achieve biocontrol effect based on induced disease resistance in whole plant tissues. These results are in agreement with Griffin (2007) findings on cotton tissues, and with Ownley *et al*. (2008) on tomato tissues. In this

respect, Klingeman *et al*. (2008) observed that hyphae of *B. bassiana* were coiled around hyphae of the pathogen of tomato root-rot, *Pythium myriotylum.*

Incorporation of endophytically date-palm tissues into synthetic diet of red palm weevil larvae:

Laboratory bioassay of red palm weevil larvae feeding on synthetic diet of powdered wheat grains mixed with date-palm tissues containing *B. bassiana* conidial spores (Table 3) indicated that diet colonized with the entomopathogenic fungus reduce insect survival. L T_{50} values of the diet test was achieved between 3-5 days. Mortality was high compared to control (77-80.8 % during 14 days) for larvae fed the diet containing 1.5 x 10^7 *B. bassiana* spores/ml. Larval mortality rate was lower (50-70 %) for larvae fed the diet containing 0.5 x 10^7 *B. bassiana* spores/ml.

Table (3):
Effect of red palm weevil larvae feeding on synthetic diet mixed with date palm tissues colonized with two levels of *B. bassiana* inoculum on larval mortality percentage during laboratorial incubation for 14 days at 27 ± 2 ºC.

Diet mixed with date palm tissues	Larval mortality %	
	After 7 days	After 14 days
Coloniztion with high fungal inoculum *	77.0	80.8
Colonization with low fungal inoculums	50.0	70.0
Non colonized tissues	2.0	5.0
L.S.D. at 50 %	4.5	4.8

*Low inoculum = 0.5 x 10^7 *B. bassiana* spores/ml. while the high inoculum = 1.5 x 10^7 spores/ml

Application of *B. bassiana* as an artificial endophyte inside date-palm plants can be important component in the integrated control of red palm weevils. The potential insecticidal action of *B. bassiana* application includes toxic principles production especially Beauvercin (Gupta *et al*., 1995 and Charnley, 2003), chitin enzymatically degradation (St -Leger *et al*., 1986), metabolic acids production (Bidochka and Khachtourians, 1991), lictin pinding characteristics (Pendland and Boucias, 1986), and volatile organic compounds release such as diisopropyl naphthalene and sesquiterpenes (Crespo *et al*., 2008).

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الحماية الحيوية لبادرات نخيل البلح ضد الآفات المرضية والحشرية بواسطة الفطر الداخلي بوفيريا باسيانا

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الملخص:

تم عزل 10 عزلات محلية للفطر بوفيريا باسيانا الممرض للحشرات من الأجساد الحشرية الميتة التي تهاجم نخيل البلح في مصر، وتم انتخاب أكثرها شراسة من خلال الاختبار الإنزيمي لتحليل الشيتين.

تمت تربية بادرات نخيل البلح صنف مجهل تحت ظروف معملية معقمة في أطباق بتري، وتم حقنها عند عمر أسبوعين بتركيزين مختلفين من معلق جراثيم الفطر بوفيريا باسيانا (0.5× 107 جرثومة/مل، 1.5× 107 جرثومة/مل). تم الكشف عن وجود فطر بوفيريا في مواضع بعيدة عن موضع الحقن مما دل على قدرة الفطر المحقون على التحرك داخل أنسجة النخيل وحثه على إحداث مقاومة جهازية ضد الإصابة المرضية أو الهجوم الحشري. وبعد أسبوع من المعاملة تم تقسيم البادرات إلى قسمين:

القسم الأول من البادرات تمت زراعته في أصص ملقحة بلقاح الفطر رايزوكتونيا سولاني المسبب لمرض عفن جذور البادرات بمعدل (5٪ لقاح/كجم تربة)، وتمت رعاية وحضانة البادرات تحت ظروف الصوبة لمدة 3 أشهر، تم بعدها حساب نسبة البادرات المصابة، ولوحظ أن البادرات المحقونة بالبوفيريا أظهرت نقصاً في نسبة الإصابة بعفن الجذور بلغ 70.6٪.

القسم الثاني من البادرات المحقونة تم خلط مهروس أنسجته بمطحون حبوب القمح وتم تقديمه لتغذية يرقات سليمة من سوسة النخيل الحمراء وتم تحضينها تحت ظروف المعمل بينما تم إمداد معاملة المقارنة بمطحون حبوب القمح فقط، وعند وصول اليرقات إلى نسبة 50 ٪ موت وكان ذلك خلال 3- 5 أيام من التحضين، تم حساب نسبة الموت اليرقي خلال 14 يوما، وقد لوحظ أن النسبة الأعلى للموت (77–80.8٪) كانت تخص المعاملة المغذاة على نسيج النخيل المحقون بالمعلق الجرثومي الأعلى

تركيزاً بينما كانت نسبة الموت الأقل تخص المعاملة المغذاة على نسيج النخيل المحقون بالمعلق الجرثومي الأقل تركيزاً (50– 70٪).

الكلمات المفتاحية: أعفان جذور نخيل البلح، سوسة النخيل الحمراء، فطر بوفيريا باسيانا، فطر ريزوكتونيا سولاني، الفطريات الداخلية، مقاومة مستحثة للأمراض.