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FIRST REPORT OF PEANUT CROWN ROT DISEASE CAUSED BY *Aspergillus niger* IN IRAQ AND ITS BIO- CONTROL

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Effective Microorganisms EM-1

Bioagents

ABSTRACT

The objective of this study was to identify and isolate the pathogen of peanut crown rot disease from infested fields of karbalaa governorate and evaluate the efficiency of water extracts of Sage *(Salvia officials* L.), Common hornwort (*Ceratophylum demersum* L.) EM-1 and Seabloom29 in disease control. As results various isolates of *Aspergillus niger* were isolated from the infected plants. Also, establishment of Koch postulate revealed that the tested isolates caused significant reduction in percentage seed germination and increasing disease incidence and severity. Water extracts of Sage and Common hornwort significantly inhibited *A. niger* isolates, this inhibition was 34.8 – 100.0% at 1-15% of extracts respectively. The results of the study revealed that Effective Microorganisms (EM-1) had highly antagonistic activity against *A.niger* at 15% concentration compared with the control. Also the bio-fertilizer Sea bloom29 caused highest reduction in mean fungal growth at the same concentration. Under lath house condition all the bio-agents used for study induced significant reduction in both incidence and severity of peanut crown rot disease, furthermore plant extracts increased rate of seed germination to 93.33-100% and reduced root rot incidence and severity to 13.3-33.3%, 12.5-33.3% respectively as compared to pathogen alone. EM-1 and Sea bloom29 were very effective in reducing disease in plants. All Bio-control treatments increased plant fresh and dry weight. These results showed possibility of using the plant extracts and bioagents as alternative methods to control plant pathogens and decrease using of chemicals and their problems.

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1 Introduction

Crown rot disease of peanut (*Arachis hypogaea* L.) caused by *Aspergillus niger* van Tiegham is an important disease in several temperate countries (Carina et al., 2006). Annual world yield loss caused by disease is more than 10% (Pande & Rao, 2000) and is more prevalent in soils with low moisture content and high temperature, approximately 30ºC (Kishore et al., 2007), but in Iraq the disease has not previously reported. Diseases attacking in very devastating on stem tissues near ground surface and causing rot, witling and plant death (Pande & Rao, 2000). Biological control is an alternative approach because of the increasing concerns about pesticide use in general. Many reports indicate that certain rhizobacteria can promote plant growth and reduce detrimental effects of various stresses under controlled conditions.

Effective microorganisms (EM) is a water based mixed culture of many microorganisms, including yeasts, lactic acid bacteria, photosynthetic bacteria, actinomycetes, and fermenting fungi (Higa, 1994; Merfield et al., 1999; Ojha et al., 2008). On the other hand, there are many researches which consider the use of plant extracts because of containing effective secondary metabolic substances to control pathogenic fungi (Chowdhury, 2005; Monaim et al., 2011, Islam & Faruq, 2012). These substances have good characteristics such as rapid degradation, low toxicity to human and animals and high specificity (Lokendra & Sharma, 1978).

Therefore, present study has been undertaken to isolates the local pathogenic agents of crown rot disease of peanut from karbalaa governorate, Iraq and to test the biocidal efficacy of locally available plant extracts and bio-agents.

2 Materials and Methods

2.1 Isolation and Identification of Pathogen

Several diseased peanut plants which showed evident symptoms of crown rot (Fig.1) were collected from different fields in Karbala city (100km south of Baghdad, Iraq) during April 2012. Affected peanut tissues were cut into approximately 0.5-1cm and washed under running tap water. After washing the infected tissue were taken for surface sterilized in 0.5% sodium hypochlorite for 1 min. and placed on the surface of Potato sucrose agar (PSA) supplemented with 50 mg\L streptomycin sulfate. After 48-72 h of incubation at 25°C, single spore isolation from each developing colony was done to have pure isolates of *Aspergillus*. *Aspergillus* isolates were transferred and cultured on potato carrot agar (20: 20: 20/ L of water) slants and stored at 4°C. Isolates of *Aspergillus* were identified to species level according to their cultural and morphological features (Klich, 2002).

2.2 Pathogenicity test of *A. niger*

Pots (12.5 cm diameter) containing 1kg\pot autoclaved soil were inoculated with 1% *A. niger* inoculum grown on millet seeds and sown with surface sterilized peanut seeds (5 seeds/pot). Seeds sown in uncontaminated sterilized soil were serving as control. The pots were irrigated with water as needed and maintained under lath house condition in a complete randomized design (CRD) and replicated three times. The percentage of seed germination was estimated four days after seedlings emergence. Data on plant dry weight, disease incidence and disease severity were calculated. The disease severity were calculated on 0-4 point scale (according to the scale: 1= non infected plants, 2= rot on one side of the crown, 3= infection around the crown and 4= dead plant). The disease severity was calculated according to Mckinney formula (Mckinney, 1923) as shown below:

Severity (%) = ((Plants in 1 degree *1+… Plants in 4 degree *4)/ all plants *4) * 100%.

Figure 1 Crown rot disease on peanut caused by *A. niger* (Infected plants were collected from Karbalaa city).

2.3 Preparation of Plant extracts

Leaves of Sage *(S. officials* L.) were obtained from local market and common hornwort (*C. demersum* L) plants were collected from Al-Musaib, Babylon city (80 km south of Baghdad). Plants and leaves extracts were prepared at room temperature using sterile distilled water. The fresh leaves of *C. demersum* were rinsed, blotted dry in a shaded, well-aerated place for 15 days, and then thoroughly grounded by mixer and grinder (type Retsch Gumb H, sk1). The resulting powder was soaked in sterile distilled water (1/2 w/w) for 24 hrs with shacking. Leaf debris was removed by filtration through cheesecloth and then by filter paper. The extract was heated at 50°C to remove excessive water into quarter. All extracts were used immediately or stored at 4°C.

2.4 Preparation of EM-1 and Sea Bloom 29 Product

The microorganisms in the stock solution are normally dormant and therefore it needs to be activated before use. The activation of EM has done in the ratio of 5:5:95 EM-1 standard solution, molasses and warm water respectively. Container fill up half with activated EM then filled with water. After fitting a cap, container was shacked thoroughly for mixing all ingredients. No air gap was left inside the container. The container was sealed firmly to ensure no air contact with the liquid. This could be done by placing a small polythene piece on the opening, squeezing it in with the help of the inner cap and then screwing the main cap. The container was stored in an incubator at 37°C. The cap was open daily for a few seconds to release gases, which was formed during the fermentation period. After the interval of 7-10 days a layer of yeast started to appear on the surface of the suspension with a pleasant smell, and the product was ready to use. Whereas Sea Bloom 29 was added directly with required concentrations.

2.5 Effect of plant extracts, EM-1 and Sea Bloom 29 on *A. niger* growth

Plant extracts, EM-1 and Sea Bloom 29 activity was tested by the method described Dixit et al (1976) at concentrates 1, 5, 10 and 15% of extracts in sterilized PSA medium, and then poured in 9 cm diam petri dishes. Three dishes were used for every treatment as replicates. After medium become solid the center of each dish was inoculated with 0.5 cm disk *A. niger* 7 days old culture (AN1, AN2 isolates). Dishes of PSA medium was inoculated with disks were used of fungal culture as control. All dishes were incubated at 25±1°C. Results were recorded after the fungal growth in control treatment was covering the whole plate, inhibition ratio was according to:

$$\% \text{ Inhibition} = [(R - r)/ R \times 100]$$

Where, r is the radius of the fungal colony against the bioagents and R is the radius of the fungal colony without the bioagents.

2.6 Lath house experiment

Pots (12.5 cm diameter) containing sterile soil (1kg /pot) were sown with peanut seeds (5 seed/pot). Pots were distributed under lath house conditions in compete randomized design with three replicates. Treatments were as following 1- *Aspergillus niger* (AN1) inoculum alone; 2-AN1+ *S. officnalis* extract (Soe); 3- AN1+ *C. demersum* extract (Cde); 4- AN1+ Effective Microorganism (EM-1), 5- AN1+ Sea Bloom 29; 6- AN1+ EM-1+Soe ; 7- AN1+ EM-1+Cde ; 8-AN1+Beltanol. The treatments were similarly repeated with other highly pathogenic isolate *A. niger* (AN2) to get insisted results. Absolute control treatments were non-treated and non-pathogen contaminated soil, the plant extracts, bioagents (EM-1 and Sea Bloom29) alone. Pathogen inoculum grown on millet seeds was added to the pots at 1% (w/w) before sowing. The plant extracts were added at a rate of 25ml\pot. 3 days before sowing, EM-1 and Sea Bloom29 were added at 10ml\pot 3 days before sowing whereas, beltanol was used at 1ml\ L. 1day after pathogen addition. The percentage of germination was calculated when seedlings emergence of control was complete. The plants were carefully removed, two months after sowing and assessed for crown rot severity according to previously indicated scale. Disease severity was calculated according to Mckinney formula (Mckinney, 1923). Fresh and dry weights of plants were also determined.

3 Results and Discussion

3.1 Isolation and identification of pathogen

Aniger has been detected in all collected samples of peanut plants at karbalaa area. The finding was in agreement with seed test which showed that *A. niger* was associated with almost all the tested seeds. This may indicate that this pathogen come from seeds. *A. niger* was not previously reported as causing of crown rot disease of peanut in Iraq before. pathogenicity test showed that *A. niger* was highly pathogenic to peanut plants (Table 1 and Fig.2). Results showed that local isolates of *A. niger* did not significantly (p=0.05) affected the 1mouth old plants. The pathogen caused 100% disease incidence and plant death. Two selective tested *A. niger* isolates decreased plant growth compared with absolute control (without pathogen).

3.2 Effect of plant extracts EM-1 and Sea Bloom29 on pathogen growth

Extract of *S. officnalis* and *C. demersum* inhibited the growth of *A. niger* isolates on PSA medium (table 2). Growth rate of AN1 isolate in Soe and Cde was significantly lower (3.33 and 5.23cm respectively) and inhibition was 34.8-100.0% at 1-15% of extracts concentration respectively. Growth rate of AN2 isolate was also inhibited on PSA medium contained different concentrates of plant extracts. EM-1 at 15% concentration had good antagonism ability against the test pathogenic fungi which inhibited growth of AN1 by 67.8% and that of *AN2* 73.3% compared to the control.

Table 1 Effect of *A. niger* isolates on peanut seed germination, crown rot incidence and severity and peanut growth under lath house condition.

isolates*	Germination (%)	Disease incidence (%)	Severity (%)	Plant Height (cm)	Plant fresh weight (g)	Plant Dry weight (g)
AN1	75	100	100	21.0	2.70	**0.43**
AN2	85	90	70	24.75	3.65	**0.70**
Control	90	0	0	30.5	6.33	**1.13**
L.S.D.(*P*= 0.05)	16.86	10.66	3.55	1.28	0.35	**0.18**

*AN1, AN2=*A. niger* isolate 1 and 2 respectively, each treatment replicate trice.

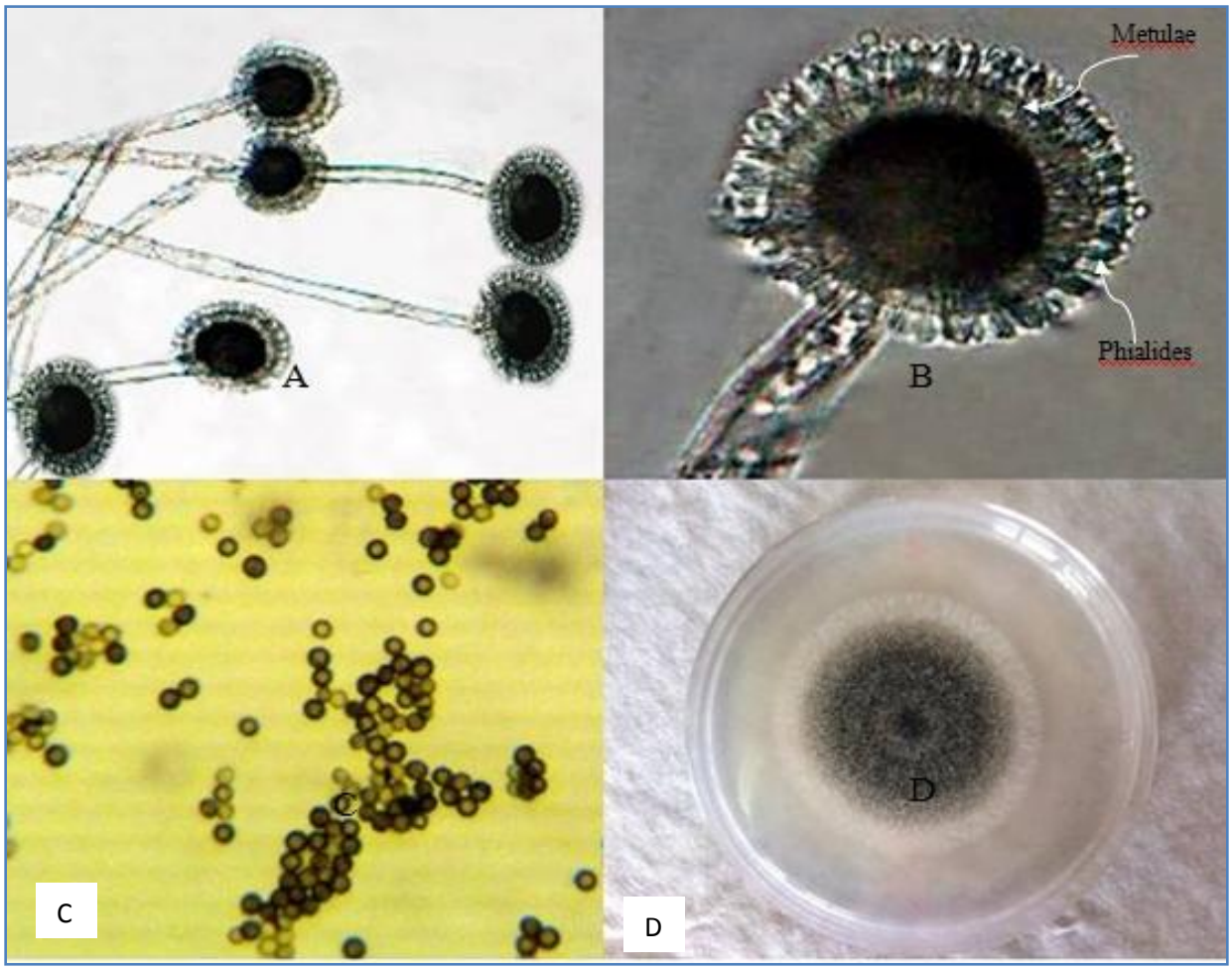

Figure 2 *A. niger* characteristics (A), conidiophores and conidial heads. (B), Metulae and phialides.(C), Conidiospores. D), Colony on PSA.

There was significant difference reported growth in different test concentration except *S. officnalis* extract on AN1 at 10 and 15% which inhibited fungal growth completely. The bio-fertilizer Sea Bloom 29 reduced fungal growth at 15% concentrates and the reduction was 65.9 and 78.9% for AN1 and AN2 sp. respectively. On the other hand Beltanol prevented fungal growth completely.

3.3 Lath house experiment: Effect of some plant extracts and Bioagents on peanut crown rot disease.

All the bio-agents used in this study induced significant (p=0.05) reduction in both incidence and severity of peanut grown rot disease, caused by *A. nige* isolates under lath house conditions (Table 3).

S. officnalis and *C. demersum* extracts increased the rate of seed germination (93.33-100%) and reduced root rot incidence and severity from 13.3-33.3 % , 12.5-33.3% respectively compared to the check containing individual pathogen. The treatment containing EM-1 and Sea Bloom 29 were very effective in reducing disease incidence in plant and all treatments showed increased plant fresh wet (5.10-6.57g) and dry weight (1.23-1.86g) respectively. However Beltanol was superior to another treatment by reducing root rot incidence and severity which was reflected on increased growth of plants. Plant extracts and bio-agents increased wet and dry weight when added to pots alone without the pathogens, The integrated control treatment (EM-1+ *S. officnalis* and EM-1+ *C. demersum* extracts) was superior to another treatments as showed the increased rate of seed germination 100% and fresh wet and dry weight 7.6-7.7g and2.8-2.8g respectively. These results were significantly different compared with control treatment (without pathogen).

Discussion

A. niger caused crown rot of peanut plants, the fungus started to attack the crown tissues of plant and severely destroyed. *A. niger* induced crown rot of peanuts due to seed infection under specific hot, humid growth conditions (Anderegg et al., 1976; Gautam et al., 2011). Results of this study were similar to the previously reported results that indicating the isolation of pure culture of *A. niger* from crown rot infected field grown groundnut plants (Sailaja et al.,1997; Youssef et al., 2008).

Table 2 Influence of various concentrations of plant extracts, EM-1 and Sea Bloom29 on the growth and inhibition of on *A. niger* cultured on PSA medium.

Treatments	Concentration (%)	Growth rate (cm)	Inhibition (%)
AN1+ Soe*	1	3.33	**63.0**
	5	1.67	**81.4**
	10	0.00	**100.0**
	15	0.00	**100.0**
AN1+ Cde	1	5.23	**34.8**
	5	2.57	**71.5**
	10	0.00	**100.0**
	15	0.00	**100.0**
AN1+ EM-1	1	6.87	**23.7**
	5	5.84	**35.2**
	10	4.83	**46.3**
	15	2.90	**67.8**
AN1+ Sea Bloom	1	7.10	**21.1**
	5	6.10	**32.2**
	10	4.57	**49.3**
	15	3.10	**65.9**
AN2+ Soe	1	4.00	**55.6**
	5	1.33	**85.2**
	10	0.00	**100.0**
	15	0.00	**100.0**
AN2+Cde	1	5.03	**44.0**
	5	3.57	**59.3**
	10	0.67	**92.8**
	15	0.00	**100.0**
AN2+ EM-1	1	6.33	**29.6**
	5	5.33	**40.7**
	10	4.17	**53.7**
	15	2.40	**73.3**
AN2+Sea Bloom	1	6.57	**27.0**
	5	5.73	**37.0**
	10	2.37	**72.6**
	15	1.90	**78.9**
AN1+Beltanol	1	0.00	**100.0**
AN2+Beltanol	1	0.00	**100.0**
Control (AN1alone)	0	9.00	**0.0**
Control (AN2alone)	0	9.00	**0.0**
L.S.D(p=0.05)	-	**0.41**	**4.84**

AN1, AN2 =*A. niger* pathogenic isolates. Soe= *S. officnalis* extract, Cde= *C. demersum* Control = plants without pathogen.

The biocontrol agents used in study were inhibited the growth of pathogen and decreased disease incidence and severity. These results were in accordance with Castro et al. (1995) those indicated that EM-1 had high antagonism ability and inhibited growth of some plant pathogenic species of bacteria and fungi.

EM-1 consists of mixed cultures of beneficial naturally occurring microorganisms that can be applied as inoculants to increase the microbial diversity of soils and plants. The basic groups of microorganisms in EM are lactic acid bacteria, purple bacteria (photosynthetic bacteria) and yeasts (Higa, 1991). Research has shown that the inoculation of EM cultures to the soil or plant ecosystem can improve soil quality, soil health, and the growth, yield, and quality of crops (Higa & Wididana, 1991). The results of the current study showed biocidal efficacy of Sea Bloom 29 first time and the formulation have given good inhibition of fungal growth of both *A. niger* isolates. The effects of Sea Bloom 29 was because of containing of many minerals, organic maters, growth regulators which extracted from Sea plants and these natural compounds induced systemic resistance against pathogen and increase growth plant. Extract of *S. officnalis* and *C. demersum* also inhibited the growth of *A. niger* isolates on PSA medium and decreased disease and increased plant growth under lath house conditions, these results were in agreement with Fareed et al. (2008) who has reported higher influences of plant extracts such as hornwort (*C. demersum*) on growth of some plant pathogenic bacteria and fungi. Water and alcohol extracts of sage (*S. officnalis*) also inhibited the growth of some plant pathogenic fungi (San Aye & Matsumoto, 2011) . These results are in conformity with the finding of Badiee et al. (2012) in which they reported very high antifungal activities of *S. officinalis*, and could serve as a natural alternative to synthetic fungicides for the control of some important fungal diseases. Accordingly *S. officinalis* extract could be considered as potential sources of antifungal compounds for plant disease control. Such extracts showed maximum activity, even at very low concentrations, these extract were showing similar fungicide effects as chemical fungicides (Abo-Shanab et al., 2004). Results of present study are in conformity with Kishore et al. (2007) who found that seed treatment with the test compounds had no effect on the incidence of crown rot in peanut in *A. niger*-infested soil. Also the fungicide Beltanol was very affected on pathogen and inhibited it completely. The effects of Beltanol was reported to combined with copper mineral which is transfer in plant tissues and kill the pathogens (Meister, 2000).

Results of this work demonstrated that the plant extract and bio-formula used in this study are promising in the management of crown rot diseases of peanut in Iraq.

Table 3 Evaluation of plant extracts and bioagents on incidence and severity of peanut crown rot disease under lath house conditions.

Treatment*	Germination (%)	Disease (%)		Weight (g)	
		Disease Incidence	Disease Severity	Fresh	Dry
AN1	73.33	100.0	81.2	2.50	**0.47**
AN1 +Soe	93.33	20.0	20.8	6.27	**1.67**
AN1+Cde	93.33	33.3	33.3	5.40	**1.46**
AN1+EM-1	86.67	43.3	33.3	5.10	**1.43**
AN1+EM-1+ Soe	100.00	13.3	8.3	6.37	**1.76**
AN1+EM-1+ Cde	100.00	13.3	8.3	6.30	**1.80**
AN1+Sea Bloom	86.67	53.3	50.0	4.83	**1.23**
AN1+Beltanol	100.00	6.7	4.2	6.63	**1.93**
AN2	80.00	80.0	72.9	3.67	**0.79**
AN2+ Soe	93.33	13.3	12.5	5.93	**1.77**
AN2+Cde	100.00	26.7	22.9	5.43	**1.36**
AN2+EM-1	93.33	40.0	31.2	5.37	**1.37**
AN2+EM-1+ Soe	100.00	13.3	8.3	6.57	**1.83**
AN2+EM-1+ Cde	100.00	6.7	0.0	6.03	**1.86**
AN2+ Sea Bloom	80.00	60.0	58.3	5.27	**1.53**
AN2+ Beltanol	100.00	6.7	0.0	6.80	**1.96**
Control	100.00	0.0	0.0	6.93	**2.27**
Soe	100.00	0.0	0.0	7.30	**2.63**
Cde	100.00	0.0	0.0	7.13	**2.50**
EM-1	100.00	0.0	0.0	7.56	**2.76**
EM-1+ Soe	100.00	0.0	0.0	7.70	**2.90**
EM-1+ Cde	100.00	0.0	0.0	7.60	**2.80**
Sea Bloom 29	100.00	0.0	0.0	7.50	**2.73**
L.S.D.(*P*= 0.05)	**10.47**	**12.67**	**10.20**	**0.33**	**0.11**

AN1, AN2 =*A. niger* pathogenic isolates. Soe= *S. officnalis* extract, Cde= *C. demersum* Control= plants without pathogen.

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