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Induced Resistance in Khella Plants (*Ammi visnaga* L.) Against Damping-off Disease using some Antioxidants, Salts and Plant Extracts

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Eight isolates of *Fusarium* spp. were isolated from naturally infected khella roots collected from different localities in Minia governorate. The isolates were purified and identified as *Fusarium solani*. All the isolates were pathogenic to khella plants cv. Balady however, pathogenic isolates varied in their virulence. In general, isolates FK7 and FK8 obtained from Minia were highly pathogenic causing pre- and post-emergence damping-off followed by isolate FK3 which was isolated from Matay, while the lowest virulence was expressed by isolates FK5 and FK6 that was isolated from Samalout. Some chemicals, salts and plant extracts were evaluated for inducing resistance against damping-off in khella under greenhouse conditions. Results indicated that all the tested materials decreased the percentage of pre- and post- emergence damping-off compared to untreated plants. Salicylic acid (SA) (200 ppm), marjorana and neem extract (4%) and dipotassium phosphate (3%) reduced damping-off in khella plants. Also, all tested chemicals significant increased plant survival.

Keywords: Antioxidants, damping-off, induced resistance, khella, plant extracts and salts.

Khella (*Ammi visnaga* L) is a relative of Queen Anne's Lace and it is commonly known to Arabs as "toothpick" plant. Khella was originally cultivated by the ancient Egyptians who used it to treat many ailments, including urinary tract diseases (Assawah, 2002). Khella is a bitter, aromatic plant that is native to the Mediterranean area of North Africa and the Middle East, and it is cultivated in the United States, Mexico, Chile and Argentina (Gunaydin and Erim, 2002). The seeds contain a fatty oil, which includes khellin, and research that was conducted in the 1950s, led to its formulation in many commercial drugs for dilating blood vessels. Some of the constituents in Khella include coumarins, visnagin, visnadin, khellin, khellos glycoside, flavonoids, sterols and volatile oil (Jouad *et al.*, 2002).

Unfortunately, in Egypt and several countries many plants are infected by several soil borne fungi causing damping-off disease which affected both quantity and quality of the yield.

Soil-borne diseases are still major threat to plants in Egypt and all over the world because of the wide host range of the pathogens and their strong survival ability in the soil (Bokor, 2007).

Induction of resistance in plants became widespread nowadays to manage broad range of plant pathogens including bacteria, viruses, fungi and some insects (Sticher *et al.*, 1997). Application of many chemical compounds (Nafie and Mazen, 2008) and extracts of various plants have also been explored as natural resistance inducers such as, *Azadirachta indica* Juss against Alternaria leaf spot on sesame (Guleria and Kumar, 2006), *Datura metel* L. against *Rhizoctonia solani*, *Xanthomonas oryzae* pv. *oryzae*, and *Alternaria solani* (Satran *et al.*, 2004).

The present study, therefore, was conducted to: 1) isolate pathogen (s) of khella causing damping-off; 2) study the effect of chemical compounds and plant extracts on linear growth of pathogenic isolates and 3) the effect of some chemical inducers and plant extracts on controlling damping-off of khella plants.

Materials and Methods

Survey of khella damping-off disease:

Three weeks after khella planting, survey of root rot was initiated and continued till 6 weeks. Damping-off diseases of khella (*Ammi visnaga* L.) cv. Balady that grown at different localities in Minia governorate, namely El-Minia, Shousha, Matay and Beni-Mazar were surveyed during the growing season of 2009-2010. Three fields per each district were concerned and the diseased plants showing typical symptoms of root rot were surveyed in the exact location. Sampling sites were determined with a field map, 5 sampling sites were designated per field tested, one of each of the four corners plus one in the centre of the field. Sampling sites were located at least 5 m from the edge of the field. The average percentage of disease incidence was calculated as the number of rotted khella plants in relative to the total number of examined plants.

Isolation, purification and identification of the pathogen (s):

Naturally infected plant tissue showing root rot symptoms in seedling stage were collected from different fields in El-Minia governorate and the tissue was cut into 5 mm long and 2-3 mm thick pieces. These pieces were surface sterilized with 1% NaOCl solution for 2 minutes followed by thorough washing with sterilized water. These surface sterilized pieces were transferred to potato dextrose agar (PDA) medium in 9 cm diameter Petri dishes and incubated at 28±2°C for 5 days. Purification of the isolated fungus was carried out using hyphal tip techniques after Dhings and Sinclair (1985). The pure culture then transferred to PDA slants and identified according to their morphological characters as described by Nelson *et al.* (1983). The pure cultures were maintained in refrigerator at 4°C and confirmed by Agric. Res. Centre (ARC) Plant Pathol., Res. Inst., Mycology and Plant Diseases Survey Department, Gamma Street, Giza, Egypt.

Preparation of fungal inocula:

Inocula of the most frequent fungus were prepared from one week old culture of recovered fungi in flask (500 ml) containing 150 g autoclaved sorghum medium, (sorghum, sand and water in 1:1:4 ratio) according to Abdel-Rehim (1984) and incubated at 28±2°C for 15 days.

The resulted cultures were used for soil infestation in a greenhouse experiment for studying the pathogenicity test.

Pathogenicity tests:

The pathogenicity test was done in the greenhouse of Department of Plant. Pathol., Fac. of Agric., El-Minia Univ. Pots (30 cm in diameter) were always sterilized by immersing in 5% formalin for 15 minutes and then dried for 15 days, the pots were filled with 8 kg containing autoclaved sandy loam soil. Soil infestation was carried out by adding the inoculum of the fungus tested at the rate of 3% of soil weight. Fungal inocula were thoroughly mixed with the soil and regularly watered every day for a week before planting.

Soil mixed alone with the same amount of autoclaved sorghum medium served as a checked control. Khella seeds were surface sterilized by immersing them in 1% sodium hypochlorite solution (NaOCl) for 2 min then washed several times with sterilized water. Ten seeds were sown in each pot and maintained in a greenhouse. Five pots were used for each treatment.

All plants were observed daily and examined for pre and post emergence damping-off. Percentages of pre- and post-emergence damping-off as well as healthy survival percentages in each treatment were determined 3 and 6 weeks after sowing using the next formula according to Mikhail *et al.* (2005).

$$\text{Pre-emergence (\%)} = \frac{\text{Number of non germinated seeds}}{\text{Total number of sown seeds}} \times 100$$

$$\text{Post-emergence (\%)} = \frac{\text{Number of dead seedlings}}{\text{Total number of sown seeds}} \times 100$$

$$\text{Survived seedlings (\%)} = \frac{\text{Number of survivals}}{\text{Total number of sown seeds}} \times 100$$

Re-isolation of the pathogen from the infected plants was also done to confirm the causal agent of wilting.

Effect of antioxidants, salts and plant extracts on controlling root rot under greenhouse conditions:

Three antioxidant compounds, i.e. ascorbic acid (AA), citric acid (CA) and salicylic acid (SA), four plant extracts, *i.e.* basil leaves (*Ocium basillicum*), marigold leaves (*Tagetes erecta*), marjorana herb (*M. hortensis*) neem seeds (*Azadirachta indica*) and three salts, *i.e.* calcium chloride, monopotassium phosphate and dipotassium phosphate, were examined.

Three different concentrations of each tested compounds were used; 50 ppm, 100 ppm, 200 ppm of antioxidants, 1, 2, 4% of plant extract and 1, 2, 3% of the salts. Only deionised distilled water (DDW) was served as control. Seeds of khella cv. Balady were disinfested by dipping in 0.1 ml mercuric chloride solution for 2 min then washed thoroughly by DDW and blotted dried immediately before soaking in the test solution. Sterilized seeds were soaked in the test solutions separately for 2.5 hrs before planting. Treated seeds were sown in the infested soil with the isolate FK7 at the rate 10 seeds/pot. Seeds soaked in tap water were sown in pots to serve as check. Five pots for each treatment were used as replicates. Pre-emergence and post – emergence damping-off were recorded after 3 and 6 weeks from sowing, respectively.

Preparation of plant extracts:

Ten grams of basil leaves (*Ocium basillicum*), marigold leaves (*Tagetes erecta*), marjorana leaves (*Marjorana hortensis*) neem seeds (*Azadirachta indica*) were prepared in the Department of Plant Pathology, Faculty of Agriculture, El-Minia University. These sample separately mixed in 100 ml distilled water in an electric blender for three minutes, then left for 72 hr before filtration through muslin cloth and centrifuged for 10 min at 5000 rpm. Sterilization was made by 0.2 µm Millipore filter. Each filtrate was considered as standard solution(S) of 100% concentration and then kept in a freezer until using.

Statistical Analysis:

All experiments were performed twice. Analyses of variances were carried out using MSTAT-C program (version 2.10, 1991). Least significant difference (LSD) was employed to test for significant difference between treatments at $P \leq 0.05$ (Gomez and Gomez, 1984).

Results

Survey of khella damping-off in El-Minia governorate:

Data in Table (1) showed that various distributions of khella damping-off disease occurred among El-Minia districts. The highest damping-off was recorded in Minia (22%) followed by Matai (18%) while the least damping-off (10%) was recorded in Samalout.

Table 1. Mean percentage of naturally infection by damping-off disease in khella during 2009-2010 growing season at different localities of Minia Governorate

Collection origin	Damping-off (%)*
Beni-Mazar	16
Matai	18
Samalout	10
El-Minia	22

* Damping-off was recorded, 6 weeks after sowing.

Pathogenicity test and identification of the causal organism (s):

Eight fungal isolates were obtained from naturally infected khella plants collected from different locations in El-Minia governorate and showing root rot symptoms in seedling stage.

Pathogenicity test (Table 2) revealed that, all isolates were pathogenic to khella plants showing pre-and post-emergence damping-off, but their virulence was varied. Isolate FK7 was the highest virulent causing pre-and post-emergence damping -off (42% and 24% respectively), followed by isolate FK8 (32 and 20%, respectively), while the lowest virulence was expressed by isolates FK5 and FK6 (12% and 6 % respectively). All the obtained isolates were identify as *Fusarium solani* according to Nelson *et al.* (1983) and verified in Mycol. and Plant Dis. Survey Dept., Plant Pathol. Res. Inst., Agric. Res. Centre (ARC), Giza, Egypt.

Table 2. Pathogenicity test of *Fusarium solani* isolated from khella roots collected from El- Minia governorates under greenhouse conditions

Isolate	Location	Damping-off (%)		Survival plants (%)
		Pre-emergence*	Post- emergence**	
FK1	Beni-Mazar	22	10	68
FK2	Beni-Mazar	16	12	72
FK3	Matai	24	14	62
FK4	Matai	22	12	66
FK5	Samalout	12	6	82
FK6	Samalout	10	4	86
FK7	El-Minia	42	24	34
FK8	El-Minia	32	20	48
Control		0.0	0.0	100
LSD at 0.05		6.253	4.57	5.21

* Pre-emergence damping-off was determined, 3 weeks of planting.
** Post- emergence damping-off was determined, 6 weeks of planting.

Effect of soaking khella seeds in some chemical inducers for controlling root rot under greenhouse conditions:

a) Effect of some antioxidants:

Data presented in Table (3) showed that all tested antioxidants significantly decreased the percentage of pre- and post-emergence damping-off caused by *F. solani* and increased of survival plants compared with control. However, SA was the most effective inducer for decreasing percentage of pre- and post-emergence damping-off, being 10 and 6.67% followed by AA being 14 and 9.33% on the average, respectively. On the other hand, CA showed less ones. The highest redaction of pre- and post emergence damping –off were recorded in case of SA at 200 ppm followed by AA at 200 ppm.

A) Effect of some salts:

Data in Table (4) revealed that soaking khella seeds in any of the solutions of the three salts decreased significant pre-and post emergence damping-off. However, phosphate dipotassium (K_2HPO_4) was the most effective inducer at 3% for decreasing percentage of pre- and post-emergence damping-off, being 6 and 4% compared with 38% and 22% in control, respectively. Meanwhile, calcium chloride ($CaCl_2$) gave the least effect ones especially at lowest concentration (1%) while recoded 20% pre-emergence and 18% post-emergence damping-off. On the other hand, the effect of these salts increased by increasing of concentrations.

B) Effect of some plant extracts:

Data in Table (5) indicate that soaking khella seeds in solutions of any tested plant extracts decreased significant pre- and post-emergence damping-off and increased plant survival. Marjorana herb at 3% gave the highest redaction of percentage of pre- and post-emergence damping-off (4 and 6%) followed by neem seeds at 3% (8 and 6%, respectively), while the weakest effect was recorded by marigold at 1% (24 and 18%, respectively).

Table 3. Effect of seed soaking of khella in some antioxidants solutions on controlling damping-off grown in infested soil with *Fusarium solani* under greenhouse conditions

Antioxidant	Con. (PPM)	Damping-off (%)		Survival plants (%)
		Pre-emergence	Post-emergence	
Ascorbic acid	50	22	14	64
	100	12	8	80
	200	8	6	86
	Mean	14	9.33	76.67
Salicylic acid	50	14	10	76
	100	10	6	84
	200	6	4	90
	Mean	10	6.67	83.33
Citric acid	50	32	20	48
	100	20	16	64
	200	16	12	72
	Mean	22.67	16	61.33
Control		38	22	40
LSD at 5% Antioxidants (A)=		2.71	1.69	2.89
Concentrations (C)=		2.95	186	1.79
Interaction (IXC)=		5.19	2.37	3.58

Table 4. Effect of seed soaking of khella in some salts solutions on controlling damping -off grown in infested soil with *Fusarium solani* under greenhouse conditions

Salts	Con. (%)	Damping-off (%)		Survival plants (%)
		Pre- emergence	Post- emergence	
K_2HPO_4	1	10	10	80
	2	10	8	82
	3	6	4	90
	Mean	8.67	7.33	84
KH_2PO_4	1	16	14	70
	2	12	10	78
	3	10	10	80
	Mean	12.67	11.33	76
$CaCl_2$	1	20	18	62
	2	16	14	70
	3	12	10	78
	Mean	16	14	70
Control		38	22	40
LSD at 5% Salts (S)=		2.71	1.69	2.89
Concentrations (C)=		2.95	186	1.79
Interaction (SXC)=		5.19	2.37	3.58

Table 5. Effect of seed soaking of khella in some plant extracts solutions on controlling damping-off incidence grown in infested soil with *Fusarium solani* under greenhouse conditions

Plant extract	Con. (%)	Damping-off (%)		Survival plants (%)
		Pre-emergence	Post-emergence	
Neem	1	12	14	74
	2	10	10	80
	4	8	6	86
	Mean	10	10	80
Basil	1	18	12	70
	2	12	12	76
	4	10	10	80
	Mean	13.33	11.33	75.33
Marigold	1	24	18	58
	2	16	12	72
	4	10	8	82
	Mean	16.67	12.67	70.67
Marjorana	1	10	8	82
	2	8	8	84
	4	4	5	91
	Mean	7.33	7	85.67
Control		38	22	40
LSD at 5% Plant extracts (P)=		2.42	1.46	2.79
Concentrations (C)=		2.15	0.81	2.14
Interaction (PXC)=		Ns	1.81	ns

Discussion

Khella (*Ammi visnaga* L.) is one of the most important medicinal and ornamental plants as the fruits contain hormone bitter principles such as khellin and visnagin, khellin is a potent selective coronary vasodilator and bronchodilator. It used in the treatment of coronary insufficiency, angina pectoris and in bronchial asthma (Al-Badawy, 1990). Soil borne diseases including damping-off, wilt and root rot cause important considerable losses in yield.

In this study extensive survey was conducted thought four locations in El-Minia Governorate (Beni-Mazar, Matai, Samalout and El-Minia) to determine the occurrence and frequency of fungi associated with damping-off of khella plants. The survey showed differences in the frequency of isolates isolated from diseased plants.

In general, eight isolates of *Fusarium solani* was isolated from naturally rotted roots of khella plants in seedling stage. This fungus had been recognized as the main causative agents of damping-off and wilt diseases on different plants in the majority of cultivated areas all over the world (Sagitove *et al.*, 2010).

In Egypt the pathogen was also isolated from different plants suffering from seedling and root rot disease (Abdel-Monaim *et al.*, 2011 and El-Far *et al.*, 2010). These results are in harmony with those reported by other researchers (Hilal *et al.*, 1998 and Abdel-Monaim *et al.*, 2011).

Pathogenicity test of eight isolates isolated from diseased plants proved that they were pathogenic and virulent for khella plants. Pre and post emergence damping-off were varied according to the fungus isolate. Isolates FK7 and FK8 isolated from El-Minia caused the highest percentage of pre- and post- emergence damping-off while the weakest was caused by isolates FK5 and FK6 isolated from Samalout. Induced resistance has attracted the attention of researchers worldwide as a possible strategy for integrated plant disease management (Achuo *et al.*, 2004). Control of damping-off and root rot/wilt diseases was tried using antioxidants, *i.e.* coumaric, citric, salicylic acids and propylgalate, as alternatives to fungicides application for avoiding environmental pollution (Galal *et al.*, 2001). Results indicated that all tested inducers reduced damping-off, root rot /wilt by artificial infection with the tested isolate fungus, when applied as seed soaking.

Generally, the pest control in this study was the tested solutions applied as seed soaking with high concentration (200 ppm) for the tested antioxidants, 4% for plant extracts and 3% for salts. Salicylic acid was better than the other antioxidants followed by ascorbic acid. Similar results were reported by Galal and Abdou (1996) who found that application of salicylic acid or ascorbic acid was the best to control Fusarial diseases of cowpea.

Also, Mostafa (2006) reported that soaking cumin seeds or soil drenching with antioxidant solutions (salicylic, ascorbic, coumaric, benzoic acids, and propylgalate) before planting resulted resistant cumin seedlings against infection with the *Fusarium oxysporum cumini* and *Acremonium egyptiacum*. Abdel- Monaim (2008) showed that soaking lupine seeds in antioxidant solutions reduced of damping -off and root rot diseases caused by *Fusarium solani* and *Macrophomina phaseolina*.

The mode of action of antioxidants was reported in many host-pathogen interactions, i.e. many oxidative enzymes such as peroxidase, catalase, ascorbate oxidase and polyphenol oxidase were detected as a result of infection with many pathogens (Clark *et al.*, 2002 and Abdel-Monaim, 2008) or as a result of treatments with different antioxidants (Ragab *et al.*, 2009 and Abdel-Monaim *et al.*, 2011).

The efficacy of various plant extracts to induce resistance was studied in these experiments; overall results showed that neem and marjorana leaf extracts at the highest dose (3%) rates can provide protection against khella damping-off.

The role of plant extracts to induce resistance against plant pathogens has been reported by different researchers. Ghazanfar *et al.* (2011) found that the maximum disease reduction (43.5%) against disease in the plant extracts was observed by applying *Azadirachta indica* leaf extract, but *Datura. metel* and *Allium sativum* extracts were not effective. Guleria and Kumar (2006) found that aqueous leaf extract of neem controlled Alternaria leaf spot of sesame. The efficacy of neem leaf extract has also been reported by Paul and Sharma (2000) and it had the same effect

as bavistin in managing leaf stripe pathogen of barley. Antimicrobial activity and induction of systemic resistance by *Datura metal* leaf extract against *Rhizoctonia solani and Xanthomonas oryzae* pv. *oryzae* in rice has been investigated (Kagale *et al.*, 2004) and it was found that foliar application of leaf extract effectively reduced the incidence of sheath blight and bacterial blight of rice. Satya *et al.* (2007) applied aqueous leaf extract of zimmu (*Allium sativum* L. *x Allium cepa* L.) to first and second leaves of cotton plants that induced systemic resistance in third and fourth leaves and reduced the number of lesions up to 73% after challenged infection with *Xanthomonas campestris pv. malvacearum* compared with water-treated control plants. It has been effectively used against different plant pathogens, such as *Alternaria solani, Pseudomonas syringae pv. tomato, Xanthomonas vesicatoria,* and *Clavibacter michiganensis* subsp. *michiganensis* of tomato and induce disease resistance (Balestra *et al.*, 2009). Results in this study are in harmony with those reported by Roth *et al.* (2000) studied the effects of an extract of *Lychnis viscaria* L. seeds that contains brassinosteroids, an aqueous application enhanced by 36% resistance to tobacco, cucumber, and tomato against viral and fungal pathogens by increasing PR- proteins, peroxidase, chitinase, and β-1,3-glucanase. Salts were used as SAR induction against many pathogenic agents.

Different types of phosphate salt via dibasic and tribasic sodium or potassium phosphates were highly effective in SAR induction with a pH value greater than 7 (Gottstein and Kuc, 1989). Foliar application of these phosphate salts induced systemic resistance (ISR) in broad bean (Walters and Murray, 1992), pepper (Reuveni and Reuveni, 1998), rice (Manandhar *et al.*, 1998) and lupine (Abdel-Monaim, 2008) against different pathogens belonging to various taxonomic groups.

The present results showed that the dipotassium hydrogen phosphate (K_2HPO_4) was the most effective inducer at 4% for decreasing percentage of pre- and post-emergence damping-off of khella plants under greenhouse conditions. These results are in agreement with those reported by Abdel-Monaim (2008) and Ragab *et al.* (2009).

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المقاومة المستحثة في نباتات الخلة ضد مرض موت البادرات باستخدام بعض الكيماويات والمستخلصات النباتية

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يعتبر نبات الخلة البلدي من النباتات الطبية الهامة والتي لها فوائد كثيرة ومتنوعة وهذا النبات يهاجم بالعديد من الفطريات الممرضة التى تسكن التربة فى طور البادرة وتسبب له العديد من الخسائر الاقتصادية الكبيرة لذلك تم اجراء هذا البحث بهدف حصر مرض سقوط البادرات في ٤ مراكز داخل محافظة المنيا هي بني مزار- مطاي- سمالوط والمنيا واظهرت النتائج ان العزلات المتحصل عليها من المنيا كانت الاعلي في القدرة المرضية بينما العزلات المعزولة من مركز سمالوط كانت الاقل في القدرة المرضية. كما أظهر العزل والتعريف ان الفطر فيوزاريوم سولانى هو المسبب الرئيسي لهذا المرض.

تم دراسة تأثير نقع بذور الخلة قبل الزراعه في تركيزات مختلفة من مضادات الاكسدة مثل حمض الساليسيلك وحمض الاسكوربيك وحمض الستريك وذلك بثلاثة تركيزات هي ٥٠ ، ١٠٠ ، ٢٠٠ جزء في المليون وايضا املاح البوتاسيوم الاحادية والثنائية وكلوريد الكالسيوم بتركيزات ١ و ٢ و ٣% وكذلك اربع مستخلصات نباتية هي أوراق الريحان والاقحوان وبذور النيم واوراق البردقوش بتركيزات ١ و ٢ و ٤% تحت ظروف الصوية

أظهرت النتائج المتحصل عليها في هذه الدراسة ان جميع المواد المستخدمة لها فعالية كبيرة في خفض نسبة الاصابة بمرض موت البادرات تحت وفوق سطح التربة وزيادة نسبة النباتات السليمة مقارنة بالنباتات الغير معاملة حيث كانت معاملات حمض الساليسيلك بتركيز ٢٠٠ جزء في المليون وكذلك املاح البوتاسيوم الثنائية بتركيز ٣% ومستخلص البردقوش بنسبة ٤% هي افضل المعاملات المختبرة في هذه الدراسة لخفض نسبة الاصابة بمرض سقوط بادرات الخلة وزيادة النباتات السليمة وتشير النتائج الي امكانية استخدام بعض المستحثات الكيماوية والمستخلصات النباتية في مكافحة مرض سقوط بادرات الخلة البلدي فى مصر.