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Impact of some Resistance Inducers on the *in vitro* Growth and Sporulation of *Fusarium oxysporum* f.sp. *lycopersici* the Causal Agent of Tomato Wilt in Kazakhstan

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The efficacy of the aqueous extracts of garlic (*Allium sativum* L) bulbs and seeds of black pepper (*Piper nigrum*) at 0.5, 1.0, 2.0, 3.0 and 4.0% concentrations and salicylic acid and riboflavin at 0.1, 0.5, 1.5, 5.0 and 10mM concentrations were investigated in order to determine their inhibitory effects on the *in vitro* growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici* (*FOL*), the causal agent of tomato wilt disease in Kazakhstan. The garlic extracts inhibited completely the *in vitro* growth and sporulation at conc. 3%, while, riboflavin and salicylic acid did that at 3 and 10 mM conc., respectively. The black pepper extract was the least effective one in this respect where the fungus could grow and produced appreciable number of spores even at its highest conc. (4%).

Keywords: Black pepper, garlic extract, *Fusarium oxysporum*, resistance inducers, riboflavin, salicylic acid and tomato.

Tomato (*Lycopersicon esculentum* Mill.) is one of the most cultivated, popular and important vegetable crops in the world. Tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* became one of the limiting factors in the production of tomato and accounts for yield losses annually. As for the pathogen persists indefinitely in infested soils, it became one of the most prevalent and damaging diseases wherever tomatoes are grown (Holguin-Pena, 2005). No fungicides are labelled for control the tomato Fusarium wilt (Edmunds and Pottorff, 2009). The tomato Fusarium wilt can be controlled by using the resistant cultivars (Reis *et al.*, 2005; Takken and Rep, 2010), biological control (Larkin and Fravel, 2002 and Aba Alkhail, 2005). Some resistance inducers salts can be used also for controlling the root rot and wilt pathogens (Attitalla *et al.*, 1998; Özgönen *et al.*, 2001; Griffiths *et al.*, 2002; Benkeblia, 2004; Aba Alkhail, 2005; Abo-Elnaga and Ahmed, 2006 and Saikia *et al.*, 2006).

This study aimed to determine the influence of some resistance inducers, *i.e.* garlic, black pepper, salicylic acid and riboflavin, at different concentrations, on the *in vitro* growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici*, the causal of tomato wilt disease in Kazakhstan.

Material and Methods

Source of the Fusarium wilt pathogen:

An aggressive isolate of *Fusarium oxysporum* f.sp. *lycopersici* (*FOL*) isolated from wilted tomato plants grown under glasshouse conditions in Almaty province of Kazakhstan was used in this study. The used *FOL* isolate proved pathogenic ability on several tomato cultivars (Under publication).

Preparation of tested plant resistance inducers:

Extracts of garlic (*Allium sativum* L.) and black pepper (*Piper nigrum*) (Benkeblia, 2004 and Lylian *et al.*, 2006) as well as, salicylic acid (SA) [2-Hydroxybenzoic acid], Molecular formula [$C_7H_6O_3$], Molar mass (138.12 g mol−1] and riboflavin [7,8-dimethyl- 10-((2R,3R,4S)- 2,3,4,5- tetrahydroxypentyl) benzo [g] pteridine- 2,4 (3H,10H)- dione], Molecular formula [C17H20N4O6], Molar mass [376.36 g/mol] (Attitalla *et al.*, 1998; Dong and Beer, 2000; Saikia, 2006 and Zhang *et al.*, 2009) were selected as resistance inducers based on references of their antifungal activities and the previous results with soil borne pathogens (Quarles, 2000) particularly *F. oxysporum* f.sp. *lycopersici* (Attitalla *et al.*, 1998 and Özgönen *et al.*, 2001). Bulbs of garlic and seeds of black pepper were obtained from the local market of Almaty province, Kazakhstan. Garlic bulbs were peeled while black pepper seeds were grounded to fine powder. The peeled bulbs and powdered seeds were extracted by blending 400 g of each in 1000 ml of sterile distilled water for 10 min., filtered firstly through two layers of muslin cloth and filtered again through Whattman No. 1 filters paper. These crude extracts were set as original concentrations (40%). The extracts were stored at -40°C until used.

The effect of the prepared plant extracts (garlic and black pepper) as well as, salicylic acid and riboflavin on the *in vitro* growth and sporulation of the tested isolate of *FOL* were investigated. All tested materials were sterilized by 45 Millipore filter immediately before adding their desired concentrations, *i.e.* 0.5, 1, 2, 3 and 4% (v/v) from each plant extract and 0.1, 0.5, 1.5, 5.0 and 10mM from each of salicylic acid and riboflavin, to the autoclaved PDA warmed-medium immediately before solidification and pouring in the sterilized Petri-dishes, 9 cm diameter (15 ml/dish).

Antifungal activity assay:

Agar culture circle disks (5 mm diameter) of *F. oxysporum* f.sp. *lycopersici* were placed in the centre of each Petri dish and incubated at 28°C. Petri dishes containing untreated PDA medium served as control. Each treatment was replicated three times. The experiment was terminated when fungal growth reached the edges of any Petri-dish in any treatment. The fungal growth was measured by averaging the two diameters taken at right angles for each colony. Also, the spore account per millilitre for each treatment was calculated by microscopic enumeration with a cell-counting haemocytometer slide. Percentage of inhibition or stimulation in the fungal growth or its sporulation compared to the untreated control was calculated according to the following formula:

$$\text{Inhibition or stimulation (\%)} = [T - C] / C * 100$$

Whereas: T = treatment and C = Control.

Statistical analysis:

Most of data were analyzed statistically according to Gomez and Gomez (1984).

Results

The inhibitory effect of the tested inducers on the linear growth:

The linear growth of the tomato wilt fungus, *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*), was significantly affected by different tested inducer's treatments [garlic (G), black pepper (BP) extracts, salicylic acid (SA) and riboflavin (R)], their concentrations as well as by the interaction between them (Tables 1 & 2). Regardless concentrations, the garlic extracts recorded the highest inhibitory effect on the *FOL* growth (55.43%), followed by black pepper extracts (35.03%), riboflavin (33.03%), and salicylic acid (26.41%), respectively. Concerning concentrations, the *FOL* growth was reduced by 14.23, 7.53, 34.44, 77.02 and 91.63% at the 1st, 2nd , 3rd, 4th, and 5th concentrations, respectively (Table, 2). Comparing to the untreated control, the linear growth was completely inhibited (100% inhibition) by using garlic extracts at 2.0%, salicylic acid at 10.0mM, and riboflavin at 5.0mM. The black pepper extracts at 4.0% conc. decreased the linear growth by 66.51%. However, garlic extracts at 0.5 and 1.0%, black pepper at 3.0%, salicylic acid and riboflavin at 0.1 and 0.5mM significantly increased the linear growth comparing to the untreated control.

Table 1. *In vitro* effect of different concentrations of resistance inducers on growth of tested *FOL* isolate

Treatment	Linear growth (mm) at different concentrations*					Control	Mean
	1st Conc.	2nd Conc.	3rd Conc.	4th Conc.	5th Conc.		
Garlic (G)	59.0	51.0	0.0	0.0	0.0	65.7	29.28
Black pepper (BP)	51.7	57.7	36.3	22.7	22.0	65.7	42.67
Salicylic acid (SA)	55.7	65.3	65.7	37.7	0.0	65.7	48.33
Riboflavin (R)	59.0	69.0	70.3	0.0	0.0	65.7	44.00
Mean	56.33	60.75	43.08	15.08	5.50	65.67	

L.S.D. at 5% for: Treatments (T)= 0.23, Concentrations (C)= 0.15, Interaction (TxC)= 1.36

* 1st , 2nd , 3rd , 4th and 5th concentrations were 0.5, 1.0, 2.0, 3.0 and 4.0%, for garlic (G) and black pepper (BP) extracts and 0.1, 0.5, 1.5, 5.0, and 10.0mM for salicylic acid (SA) and riboflavin (R), respectively.

Table 2. *In vitro* growth inhibition or stimulation (%) of tested *FOL* isolate as affected by different concentrations of tested induces

Tested inducer	Inhibition or stimulation (%) of linear growth at tested five concentrations*					Control	Mean
	1st Conc.	2nd Conc.	3rd Conc.	4th Conc.	5th Conc.		
Garlic (G)	-10.20	-22.37	-100.00	-100.00	-100.00	0.00	-55.43
Black pepper (BP)	-21.31	-12.18	-44.75	-65.45	-66.51	0.00	-35.03
Salicylic acid (SA)	-15.22	-0.61	0.00	-42.62	-100.00	0.00	-26.41
Riboflavin (R)	-10.20	5.02	7.00	-100.00	-100.00	0.00	-33.03
Mean	-14.23	-7.53	-34.44	-77.02	-91.63	0.00	

* As described in footnote of Table (1).

The *FOL* linear growth at known concentrations, *i.e.* 1.0% black pepper extracts, 0.5 and 1.5mM salicylic acid and riboflavin was significantly higher than lower concentration of these inducers.

The inhibitory effect of the tested inducers on the sporulation:

As described before, the spore count (10^6/ml) produced by the tomato wilt fungus, *F. oxysporum* f.sp. *lycopersici* (*FOL*), was responded similarly against different tested inducer's treatments [garlic (G), black pepper (BP) extracts, salicylic acid (SA) and riboflavin (R)] (Tables 3 & 4). Regardless concentrations, the riboflavin treatment recorded the highest inhibitory effect against the *FOL* sporulation (39.11%), followed by the garlic extracts (38.34%), salicylic acid (37.73%) and black pepper (6.55%), respectively. Concerning concentrations, average of sporulation was decreased by 8.98% at the 1st. concentration, increased by 9.45% at the 2nd concentration then decreased progressively by 38.23, 60.39 and 84.45% at the 3rd, 4th and 5th concentrations, respectively (Table 4).

Table 3. *In vitro* effect of some inducers at different concentrations on sporulation of *F. oxysporum* f.sp. *lycopersici* (10^6/ml)

Tested inducer	Tested concentration*					Control	Mean
	1st Conc.	2nd Conc.	3rd Conc.	4th Conc.	5th Conc.		
Garlic (G)	1.111	0.933	0.000	0.000	0.000	0.757	0.467
Black pepper (BP)	0.467	0.791	0.827	0.933	0.471	0.757	0.708
Salicylic acid (SA)	0.708	0.782	0.316	0.267	0.000	0.757	0.472
Riboflavin (R)	0.472	0.809	0.729	0.000	0.000	0.757	0.461
Mean	0.689	0.829	0.468	0.300	0.118	0.757	
L.S.D. at 5% for:	Treatments (T) = 0.015 Concentrations (C)= 0.010 Interaction (T x C)= 0.088						

* As described in footnote of Table (1).

Table 4. *In vitro* effect of different inducers [garlic (G), black pepper (BP) extracts, salicylic acid (SA) and riboflavin (R)] at different concentrations on sporulation of *F. oxysporum* f.sp. *lycopersici*

Tested inducer	Tested concentration*					Control	Mean
	1st Conc.	2nd Conc.	3rd Conc.	4th Conc.	5th Conc.		
Garlic (G)	46.70	23.24	-100.00	-100.00	-100.00	0.00	-38.34
Black pepper (BP)	-38.34	4.46	9.15	23.24	-37.79	0.00	-6.55
Salicylic acid (SA)	-6.55	3.29	-58.34	-64.79	-100.00	0.00	-37.73
Riboflavin (R)	-37.73	6.81	-3.76	-100.00	-100.00	0.00	-39.11
Mean	-8.98	9.45	-38.23	-60.39	-84.45	0.00	

* As described in footnote of Table (1).

Comparing to the untreated control, the spore production by the *FOL* was completely inhibited by using garlic extracts at 2.0%, salicylic acid at 10.0mM, and riboflavin at 5.0mM. However, the highest concentration of the black pepper extracts (4.0%) decreased the *FOL* sporulation only by 37.79%. The *FOL* sporulation was significantly higher at 0.5 and 1.0% garlic extracts, 3.0% black pepper, 0.1 and 0.5mM salicylic acid than sporulation in the untreated control. Whereas, using the riboflavin at the first three concentrations, 0.1, 0.5 and 1.5mM had no significant effect on the *FOL* sporulation compared to the untreated control.

Discussion

In this study, aqueous extracts of bulbs of garlic (*Allium sativum* L) , dried seeds of black pepper (*Piper nigrum*) , salicylic acid and riboflavin were investigated for their inhibitory effects on the *in vitro* growth and sporulation of *Fusarium oxysporum* f.sp. *lycopersici* (*FOL*) the causal of tomato wilt disease in Kazakhstan. The garlic extract, riboflavin and salicylic acid completely inhibited the *in vitro* growth and sporulation at concentrations of 2%, 5mM and 10 mM, respectively. However, the black pepper extract was the least effective in this respect as the fungus could grow and produced appreciable number of spores even at its highest concentration, 4%. Generally, the *in vitro* growth and sporulation of *FOL* was significantly lower at concentration of 0.5% for the black pepper extract and 0.1mM for riboflavin and salicylic acid more than at the concentrations of 2 & 3% for plant extracts and 0.5 & 1.5mM for riboflavin and salicylic acid. Thus, the *in vitro* growth and sporulation of *FOL* were relatively enhanced at the latter concentrations. This might be due to the synergistic effects between fungus metabolites and a known substance at the mentioned concentrations. These findings agree with those obtained by Özgönen *et al.*, (2001) who stated that salicylic acid (SA) completely inhibited the mycelial development of *FOL in vitro* at concentrations from 0.6 mM to 1.0 mM. Aba Alkhail (2005) reported that the garlic (*Allium sativum*) extract was the most effective against *FOL*. Also, Abo-Elnaga and Ahmed (2006) found that the antifungal properties of cloves extract was more effective than black pepper in inhibiting mycelial growth and disease incidence of onion neck rot disease caused by *Botrytis allii*. Saikia *et al.* (2006) found that riboflavin caused induction of systemic resistance in chickpea against Fusarium wilt and charcoal rot diseases. The dose effect of 0.01 to 20 mM riboflavin showed that 1.0 mM concentration was sufficient for maximum induction of resistance; higher concentration did not increase the effect. At this concentration, riboflavin neither caused cell death of the host plant nor directly affected the pathogen's growth. Abbasi *et al.* (2010) recorded that *Piper nigrum,* commonly known as ''Black-pepper'', has gained a global consideration because of its volume in the spice industry. This plant has shown great potential for the discovery of novel biologically active compounds and need for techniques to enhance the production of high quality consistent plant material for feasible accumulation of metabolites. Based on the present results, further studies are needed for controlling the tomato Fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*) under gashouse conditions, at Kazakhstan, using some of the tested inducer treatments.

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تأثير بعض محثات المقاومة على نمو وتجرثم الفطر فيوزاريوم اوكسيسبورم ليكوبيرسيكى المسبب لمرض ذبول الطماطم في كازاخستان

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في هذه الدراسة ، تم اختبار فعالية المستخلصات المائية لأبصالِ الثوم وبذور الفلفل الأسود بتركيزات ٠,٥، ١,٠، ٢,٠، ٣,٠ و٤,٠% ومحاليل حمض السالسيلك والريبوفلافين بتركيزات ٠,١، ٠,٥، ١,٥، ٥,٠ و١٠ ملليمول على نمو وتجرثم الفطر المسبب لمرض ذبول الفيوزاريوم في الطماطم (فيوزاريوم اوكسيسبورم ليكوبرسيكى) في كازاخستان. ولقد أظهرت النتائج أن مستخلصات الثوم كانتْ الأكثر فاعليةً حيث تم تثبيط نمو وتجرثم الفطر تماما عند تركيز ٣% يليه في ذلك مركب الريبوفلافين عند تركيز ٣ ملليمول وحمض السالسيلك عند تركيز ١٠ ملليمول، على التوالي بينما كان مستخلص الفلفل السود الأقل فعالية في هذا الخصوص حيث تمكن الفطر من النمو وتكوين أعدادا محسوسة من الجراثيم عند أعلى تركيز لها (٤%).