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Virulence of *Alternaria* strains toward potato and tomato cultivars

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SUMMARY

Alternaria alternata is one of the causal agents of the early blight, a dangerous disease of potato and tomato, which is common for almost all regions, where these crops are grown. In this study the virulence and aggressiveness of *A. alternata* isolates, obtained from the leaves and tubers of potato and leaves and fruits of tomato, has been studied on 13 potato cultivars of different maturity groups and 5 large-fruited tomato cultivars. In the case of two isolates, obtained from tomato leaves and significantly differing in their virulence, the activity of subtilisin- and tripsin-like serine proteases has been also analyzed.

The performed study has revealed intraspecific differences in the virulence and aggressiveness of *A. alternata* toward the leaves of different potato and tomato cultivars. Some isolates successfully infected cultivars, highly resistant toward other isolates that probably evidences some potato and tomato cultivars have genes of specific resistance to *A. alternata.*

In addition, the revealed difference in the activity of serine proteases is observed on both interspecific and intraspecific levels and correlated with the virulence and aggressiveness of isolates. A high level of tripsin-like activity of secreted serine proteases is observed in highly pathogenic isolate. Thus, this parameter can be used as a marker for the study of the virulence and aggressiveness of *A. alternata* isolates.

KEYWORDS

Alternaria alternata, virulence, aggressiveness, serine protease activity

INTRODUCTION

Alternaria alternata is a common saprotrophic or parasitic fungus belonging to Ascomycetes. Like *A. solani*, *A. alternata* is a causal agent of the early blight of potato and tomato, a dangerous disease presenting in almost all cultivation areas of these crops.

Till recently it was considered that plants are infected with only large-spored *A. solani*, whereas small-spored *A. alternata* represents a secondary plant pathogen able to either infect plants together with *A. solani*, or to leave as a saprotroph on the necrotic lesions caused by other plant pathogens. However, today we have some data that *A. alternata* is able to infect plants independently, though the relation between the variability of the virulence and aggressiveness of different strains and their species-specific and organotropic specialization still remain unclear.

Hydrolytic enzymes secreting by necrotrophic plant pathogens provide the availability of macromolecular compounds as nutrients and, therefore, play an important role in the nutrition of such pathogens, including the early blight causal agents. This group of enzymes includes different proteases. Extracellular proteases, secreted by fungi, are able to macerate plant tissues and destroy cell wall components that allow a pathogen to overrun the natural resistance of a host plant. Thus, proteases not only act as digestive enzymes of fungi, but also participate in pathogenetic processes.

Some recent data allow us to suppose that serine exoproteases secreting by some plant pathogenic fungi can be considered as pathogenicity factors. For example, Dunaevskii *et al.* (2006) showed that plant pathogenic fungi, including *A. alternata*, secrete tripsin- and subtilisin-like proteases. Though the synthesis of proteases is a constitutive process, their repertoire in plant pathogens, including necrotrophic ones, should differ from that in saprotrophs. In this study we supposed that differences in the level of secretion of tripsin-like and subtilisin-like proteases occur at both interspecific and intraspecific levels and are able to serve as the markers of virulence and aggressiveness of isolates.

MATERIALS AND METHODS

Host plants

Thirteen Russian and Belorussian potato cultivars of different maturity groups were used in the study: first early (Zorachka and Lileya Belorusskaya), second early (Briz, Manifest, Romano, and Nevskiy), early maincrop (Volat, Lad, Skarb, and Yanka), and maincrop (Vektar Belorusskiy, Zhuravinka, and Ragneda). In addition, five commercial large-fruited tomato cultivars were used: Dubrava, Tomsk, La-la-fam, Verlioka, and Bych'e Serdtse.

Healthy meristemal plants were planted in enriched peat soil and kept in a greenhouse at 20-24°C and natural photoperiod. Watering was performed as required. No fertilizers were used during vegetation. To perform inoculation, the leaves of the 4-6 layers from the top were used. Leaves were placed in sterile Petri dishes onto object plates covered with wet filter paper.

Isolate sources

A. alternata and *A.solani* isolates were isolated from infected fresh leaves of potato and fresh leaves and fruits of tomato (Table 1). In addition, some isolates were collected from infected potato tubers within 10 days after the harvesting; isolation was performed only from conidiophores appeared on the live tissues of tubers. Infected samples were placed in wet chambers; isolation was carried out from a border of live and healthy tissue. The strain PPL 31 was kindly provided by the colleagues from the Laboratory of mycology and plant pathology of the All-Russian Research Institute of Plant Protection.

Table 1. *Description of Alternaria isolates used in the study*

Name	Species	Region of collection	Host plant, cultivar, organ	Year of collection
RPL 16	*A. alternata*	Ryazan region	Potato, cv. Ragneda, leaves	2012
RPL 21	*A. alternata*	Ryazan region	Potato, cv. Ragneda, leaves	2012
KPT 1	*A. alternata*	Kostroma region	Potato, cv. Udacha, tuber	2013
KPT 4	*A. alternata*	Bryansk region	Potato, cv. Bryanskaya Roza, tuber	2013
METL 5	*A. alternata*	Mariy-El Republic	Large-fruited tomato, leaves	2007
METL 12	*A. alternata*	Mariy-El Republic	Large-fruited tomato, leaves	2007
MTF 7	*A. alternata*	Moscow region	Large-fruited tomato, fruit	2013
PPL 31	*A. solani*	Primorye (Russian Far East)	Potato, leaves	2006
VTL 16	*A. solani*	Voronezh region	Large-fruited tomato, leaves	2014

Inoculum preparation and inoculation

To obtain inoculum (conidia), isolates were grown on potato-carrot agar up to the conidia formation (7-10 days). In the case of *A. alternata*, conidia were washed off from colonies by 10 ml of sterile water. In the case of *A. solani*, conidia were obtained by another way: aerial mycelium was removed after 7-10 days of incubation, and then Petri dishes were placed into refrigerator (+5°C) overnight and treated with UV radiation. After 3 days of incubation conidia were washed off from colonies by 10 ml of sterile water. The concentration of suspension used for inoculation was 100 conidia/μL. A drop (10 μL) of conidia suspension or sterile water (control) was placed on leaf surface, inoculated leaves were incubated for 7 days at room temperature, and then the level of infection was assessed. All experiments were performed in three replications.

Enzyme activity assay

The maintenance and cultivation of fungi was performed by submerged cultivation in liquid potato broth medium as described earlier (Valueva *et al.*, 2013). After 5, 10, and 18 days of cultivation mycelium was harvested on a weighed Whatman No. 41 filter paper, washed with a small volume of warm distilled water, heated overnight in an oven at +90°C, then cooled in a desiccator and weighed again. The longer drying did not result in a further weight loss. A crude culture filtrate obtained after the harvesting of mycelium was used for the enzyme activity assay.

The enzymatic activity of serine proteases was determined by the Erlanger's method (Erlanger *et al.*, 1961), using synthetic substrates BAPNA (Nα-benzoyl-DL-arginine-nitroanilide) and Z-AALpNA (N-carbobenzyloxy-L-Ala-L-Ala-L-Leu-pNa) in the assay of the tripsin-like and subtilisin-like activity, respectively. The initial substrate concentration was 0.5 mM. One unit of enzyme activity (U) was equal to the amount of enzyme hydrolyzing 1 nmol of the substrate per 1 min. Both BAPNA and Z-AALpNA substrates were purchased from Sigma-Aldrich (USA); all other commercially available reagents were of the highest grade. All experiments were performed in three replications.

RESULTS AND DISCUSSION

Virulence and aggressiveness of Alternaria isolates toward different potato and tomato cultivars

All isolates collected and isolated from tomato and potato leaves, tomato fruits, and potato tubers were able to infect potato and tomato leaves. Analysis of variance (ANOVA) show that

factors of cultivar, strain, and their combination influence the diameter of necrosis ($P<0.05$ for all variants). At the same time, we observe significant differences in the virulence of isolates toward the leaves of different cultivars of host plants. For example, all tested *A. alternata* isolates and *A. solani* strain used as a control strain were able to infect the following potato cultivars: Nevskiy, Lad, Volat, Briz, and Zorachka (Table 2). The maximum resistance was observed in the cvs. Lileya Belorusskaya and Yanka, which were infected with only one isolate, and also in the cv. Ragneda (infected with two isolates). Note that these three cultivars and also cvs. Romano and Zhuravinka were not infected by the control *A. solani* strain.

The study on tomato leaves showed that only cv. Dubrava was infected with all isolates studied; cvs. Bych'e Serdtse and Verlioka were infected with only one of three tested *A. alternata* strains and with the control *A. solani* strain (Table 3).

None of the studied isolates was virulent toward all studied potato cultivars. The highest virulence was observed in two isolates from potato tubers (KPT 1 and KPT 4) and one isolate from tomato leaves (METL 5), which were able to infect leaves of 10-11 cultivars. The lowest virulence was observed in the isolate obtained from tomato fruit (MTF 7), which infected only seven cultivars. The control *A. solani* isolate (PPL 31) did not show high virulence and infected the leaves of 8 out of 13 potato cultivars.

The same situation was observed concerning the virulence of the studied isolates toward different tomato cultivars. Only *A. solani* isolate was able to infect leaves of all tested tomato cultivars. Isolates of *A. alternata* collected from tomato fruits (MTF 7) and leaves (METL 5) infected leaves of four cultivars, whereas the METL 12 isolate infected only one tomato cultivar.

Table 2. *Pathogenicity of the tested isolates toward different potato cultivars*

Potato cultivar	Average diameter of necrotic lesions, mm								Number of virulent isolates
	Alternaria alternata							A. solani	
	Potato leaves		Potato tubers		Tomato fruits	Tomato leaves		Potato leaves	
	RPL 16	RPL 21	KPT 1	KPT 4	MTF 7	METL 5	METL 12	PPL 31	
Nevsky	18	20	15	10	15	10	3	10	8
Lad	4	4	25	24.5	3	5	15	8	8
Volat	3	3	6,5	10	3	10	3	10	8
Briz	10	10	30	5	25	15	20	20	8
Zorachka	3	3	8	15	3	4	3	7	8
Manifest	2	2	3	5	3	8	0	15	7
Vektar belor.	8	0	15	0	8	20	15	5	6
Zhuravinka	5	5	0	20	0	10	10	0	5
Romano	0	2	5	20	0	10	2	0	5
Skarb	0	5	20	6	0	4	0	27	5
Ragneda	0	0	0	20	0	20	0	0	2
Yanka	0	0	7	0	0	0	0	0	1
Lileya belor.	0	0	0	5	0	0	0	0	1
Total number of infected cultivars	8	9	1	11	7	11	8	8	
Average necrosis diameter, mm	4.1±1.7*	4.2±1.7	10.4±3.1	10.8±2.6	4.6±2.4	9±2.1	5.5±2.2	7.8±2.7	

* - a confidence interval for the significance level 0.05.

Table 3. *Pathogenicity of the tested isolates toward different tomato cultivars*

Tomato cultivar	Average diameter of necrotic lesions, mm				Number of virulent isolates
	Alternaria alternata			A.solani	
	Tomato fruits	Tomato leaves		Tomato leaves	
	MTF 7	METL 5	METL 12	VTL 16	
Dubrava	10	18	10	35	4
Tomsk	10	5	0	15	3
La-la-fam	5	5	0	5	3
Verlioka	5	0	0	5	2
Bych'e Serdtse	0	20	0	5	2
Total number of infected cultivars	4	4	1	5	
Average necrosis diameter, mm	6±2.1*	9.6±4.5	2±2	13±6.7	

* - a confidence interval for the significance level 0.05.

In this study only one component of aggressiveness was assessed: the diameter of necrotic lesions on infected leaves. According to the obtained results, isolates KPT 1 and KPT 4, obtained from potato tubers, and isolate METL 5, obtained from tomato leaf, were the most aggressive toward potato leaves of different cultivars: the average necrosis diameter was 8.9-10.8 mm. The same strains were also virulent toward the maximum number of potato cultivars (10-11 out of 13 cultivars; Table 2). METL 5 and KPT 4 isolates caused significant necrosis (20 mm) of leaves of cv. Ragneda, which was not infected with other isolates, including *A. solani*. The KPT 4 isolate was the only isolate successfully infected cv. Lileya Belorusskaya, whereas the KPT 1 isolate was the only isolate infected cv. Yanka.

Significant differences in the diameter of necrotic lesions were also observed for infected tomato cultivars (Table 3). The METL 12 strain was able to infect leaves of the cv. Dubrava, but did not infect other tomato cultivars. The METL 5 strain caused a strong infection of leaves of cvs. Dubrava and Bych'e Serdtse (18-20 mm), whereas the *A. solani* strain caused significant necroses on leaves of the cv. Dubrava (35 mm).

Serine protease secretion

In this study, we assessed the protease secretion by isolates, differing in their virulence toward potato and tomato; therefore, the studied isolates were cultivated on liquid medium based on thermostable proteins of potato. The study was carried out using two isolates, METL 12 and METL 5, collected from tomato leaves and significantly differing in their virulence and aggressiveness toward both potato and tomato leaves.

According to the obtained results, the dynamics of secretion of serine proteases was similar in both isolates: sharp increase in the activity from 5th to 10th days of growth and sharp decrease to the 18th day. In both isolates, the maximum activity of secreted proteases was observed at the 10th day of growth (Table 4).

The separate assessment of the activity of tripsin-like and subtilisin-like proteases resulted in an interesting observation. On the 10th day, the more aggressive METL 5 isolate demonstrated a higher activity of tripsin-like proteases than that of the less aggressive METL 12 isolate. At the same time, the activity of subtilisin-like protease was higher in less pathogenic METL 12 strain. These results correspond to the results of other studies. For example, Dunaevskii *et al.* (2006) showed that saprotrophic species *Trichoderma harcianum*, *Penicillium terlikowskii*, and

Penicillium chrysogenum are characterized by high activity of subtilisin-like proteases and do not produce tripsin-like proteases, whereas phytopathogenic species *Alternaria alternata*, *Botrytis cinerea*, and *Ulocladium botrytis* produce proteases of both types.

Table 4. *Dynamics of secretion of serine proteases by METL 5 and METL 12 isolates of Alternaria alternata*

Isolate	Average activity of serine proteases, U/g of dried mycelium*					
	Tripsin-like (BAPNA substrate)			Subtilisin-like (Z-AALpNa substrate)		
Day of growth	5	10	18	5	10	18
METL 12	8	162	4,2	22	14092	433
METL 5	0	298	2.9	46	1635	36

* U - the amount of enzyme that hydrolyzes 1 nmol of a substrate per 1 min.

Thus, the performed study demonstrated some intraspecific difference in the virulence and aggressiveness of *A. alternata* toward leaves of different potato and tomato cultivars. Some isolates were able to infect cultivars resistant to other isolates that makes it possible to suppose the presence of the corresponding specific resistance genes in potato and tomato cultivars.

Our study also showed that differences in the activity of serine proteases are observed not only at interspecific (Dunaevskii *et al.*, 2006) but at intraspecific levels and are connected with the virulence and aggressiveness of isolates. Though both isolates studied were isolated from alive tomato leaves, they probably differ in their trophic substrate: the METL 5 isolate, which is more pathogenic and has a high level of tripsin-like activity, is able to infect live tissues, whereas the less aggressive and virulent METL 12 isolate, characterized by a high level of subtilisin-like activity, grows on dead tissues and actively utilizes the substrate via the saprotrophic way. Therefore, a high level of the tripsin-like activity of extracellular serine proteases is observed in highly pathogenic isolates, and this parameter can be used as a marker for the study of the virulence and aggressiveness of *A. alternata* isolates.

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