DIAGNOSTICS OF POTATO BACTERIAL PATHOGEN *DICKEYA DIANTHICOLA*

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Abstract: the host range and diagnostic methods where tested for bacterium Dickeya dianthicola, the causative agent of blackleg ofpotato, that has been recently been found in Russia. Artificial inoculation has identified additional host plants of D. dianthicola: tomato, tobacco, and iris. This should be considered at planning of protective measures. We obtained specific to D. dianthicola polyclonal antibodies, which provided in specific ELISA sensitivity about 10^5 cells /ml of the tuber extract. Specific probe ADE3 designed for the primers ADE1ADE2 could be used to identify bacteria of the genus Dickeya by real-time PCR.

Key words: black leg of potato, Dickeya dianthicola, enzyme-linked immunoenzyme assay real-time PCR.

Black leg is the most harmful bacterial disease of potatoes, and appears as a necrotization of plant stem in field and soft rot of tubers stored after harvest [2, 4].

This disease is caused by three separate but closely related species of pectolytic bacteria of family Enterobacteriaceae:

- *Pectobacterium carotovorum* subsp. *carotovomm* (*Pcc*, syn. *Erwinict carotovora* subsp. *carotovora)* [15];

-*P. atrosepticum (Pa,* syn. *E. carotovora* subsp. *atroseptica)* [16];

- *Dickeya* spp. (D, syn. *E. chrvsanthemi* or *P. chrysanthemi)* [22].

The first two species are common pathogens of potato at the territory of the former USSR [2, 5, 7, 9, 10, 11]. Bacteria of the genus *Dickeya* differ significantly from other pathogens causing soft rots. For the first time these bacteria were described in the early 1950-s and called *Erwinia chrysanthemi* so as their first host was Chrysanthemum plant. In the 1980-s the bacteria were found to cause diseases of other crops, including potatoes [18].

Some later, *E. chrysanthemi* was transferred to a new genus *Dickeya* and divided into six species [22]. Bacteria of the genus *Dickeya* can damage a wide range of plants in diverse climatic conditions [23].

Since 2004, strains Dickeya spp. were causing significant economic losses in potato production in Western Europe. *D. dcidcmthii* and *D. zeae* (biovar 3) the most harmful pathogens of potatoes in hot-climate areas. *D. dianthicola* (biovars 1 and 7) is more adapted to the moderate climate and widely distributed in Europe [22]. Recently, this pathogen was found in Spain [20] and Finland [17]. European Organization of Plant Quarantine and Protection (EPPO) included the phytopathogenic bacteria of genus *Dickeya* into the list A2 of Plant Quarantine organisms [24].

In the last few years researchers have found an emergence of new genetic group of highle aggressive strains that are proposed to call *D. solcmi* [23].

In 2009, analyzing infected potato tubers from the Lipetsk region, we found for the first time in Russia bacteria of the genus *Dickeya.* A number of microbiological and molecular tests has proved similarity of the isolates to species *D. dianthicola* [6]. Potato seeds were the main source of infection. In many countries, the infection of potato by *Dickeya* was associated with planting material imported from the Netherlands [23]. In Russia, the risk may increase after entry to Word Trade Organization (WTO), and increasing import of planting material produced in countries with different phytosanitary situation. We need a reliable methods of detection with high sensitivity and specificity for this pathogen to reduce the possible damage from introduction of this emerging pathogen.

The aim of the work was to clarify the range of potential host plants of Russian strains of *Dickeya* sp. and develop methods of molecular and serological diagnostics of the pathogen.

Materials and methods

Bacteria were isolated from infected potato samples, obtained from several regions of Russian Federation in 2009-2010 on Potato agar (PDA) or nutrient broth with yeast extract (NBY) in Petri dishes. The plates were incubated for 2-3 days at 27°C. Flat, slightly translucent colonies were sub-cultured on Logan's medium, and checked for pectate layer liquefaction. The selected pectolytic isolates were stored in 15% glycerol at -70°C and in sterile tap water at room temperature. All the isolates were analyzed with biochemical and physiological tests according to Laboratory guide for identification of plant pathogenic bacteria [13]. Reduced carbohydrates formed from sucrose and indole synthesis were tested as well. Pathogenicity of strains was evaluated by the ability to rot potato slices at 28 ° C in 24 and 48 h after infection.

To determine the virulence of the bacteria, we carried out inoculation of plants from different families, including carnation (*Dianthus caryophylhis* L., cv. "Margarita"). dahlia (*Dahlia variabilis* Desfi, cv. “Figaro FT’), barley (*Hordeum vulgaris* L., cv. “Mikhailovsky”), wheat (*Triticum aestivum* L., cv. “Moskovskaya 39"). clover (*Trifolium repens* L., cv. “Red”), flax (*Linum usitatissimum* L., cv. “Smolensk”), white lupine *(Lupinus albus* L., cv. “Gamma”), oats (*Avena sativa* L., cv. “Horse”), vetch (*Vicia sativa* L., cv. “L'govskaya 22”), rape (*Brassica napus* L., cv. “Griffin” grade), triticale (*Triticale* Wittm. & A. Camus, cv. “Valentin”), iris (*Iris germanica* L., cv. “Rimefire”), potato *(Sola mtm tuberosum* L., cv. “Luck”), tomato *(Lycopersicon esculentum* L., cv. “Belyi naliv”), tobacco (*Nicotiana tabacum* L., cv. “Samsun”).

For inoculation, the bacterial suspension with concentration 10^8 cells / ml was infiltrated by sterile syringe into leaf axils. Four plants of each species were inoculated by each strain, including *D. dianthicola* D9, D33, and *P. atrosepticum* Eca 393, sterile tap water was used as a negative control. If symptoms appeared on the inoculated plant, re-isolation and pathogen identification was done to fulfill the Koch's triad.

Specific polyclonal antiserum was obtained by immunization of rabbits with bacterial suspension prepared for *D. dianthicola* strains D9 and D17 grown on YDC medium for

48 h at 27°C according to Allan and Kelman [12]. Chinchilla rabbits (age 4-5 months) were immunized according to the method developed at Potato Research Institute (Moscow) for bacterial antigens. Immunoglobulins were isolated on chromatographic column filled with Protein A of *Staphylococus aureus* immobilized on sepharose as affine sorbent [3]. Antibodies were adjusted to a concentration of 1 mg/ml and stored as 1 ml aliquots at -20°C.

The antibodies titer was measured by indirect immuno-anzyme assay (ELISA) using anti-rabbit donkey antibodies labeled by peroxidase (Research Institute by Gamaleya, Moscow).

The obtained antibodies were conjugated with horseradish peroxidase by method of Nakane [19]. For *P. citrosepticum* we used a commercial ELISA kit produced by the Potato Research Institute.

The sensitivity of antibodies was determined by ELISA against the strains of *Dickeya* sp., *Pa, Pcc,* and strain of *Clavibacter michiganensis* subsp. *sepedonicus* Cms204 as a negative control. All bacteria were applied at concentration of 10^8 cells / ml.

Sensitivity of the ELISA was evaluated against homologous strain of *D. dianthicola* D9.

The suspension of D9 was prepared from one-day age culture in the buffer and in potato tuber extract at range of concentrations up to 10^9 cells / ml and applied in reaction against the antibody adsorbed on standard 96-wells plate in series of ten-fold dilutions down to concentration of 10 cells / ml.

ELISA was performed by "double sandwich" method [1] and repeated 3 times. Optical density of ELISA enzymatic reaction product was measured at 450 nm using a microplate photometer (Bio-Rad, model 680).

DNA from bacteria was isolated by commercial kit "Proba-GS ("DNA-Technology", Moscow) according to manufacturer's recommendations. Collection of DNA for strains of all species of genus *Dickeya* was kindly provided by Dr. van der Wolf (Plant Research International, Wageningen, the Netherlands) [6].

Real-time PCR analysis was made with published primers ADE1/ADE2, specific to the *pectatlyase (pelB)* gene of *Dickeya* spp. [14], and original probe ADE3 (FAM-gcg ccg teg tgc tgc aca tat ttt teg ccg-BHQl). Strains of *D. dianthicola* (D9, D17) were used as positive control, and strains *Pcc* and *Pea (Ecc 3, Ecc 36,* and *Eca 393*) - as negative one. DNA concentration was adjusted to 10 ug / ml.

MasterMix ("Dialat Ltd", Moscow) and «iCycler iQ5» (Bio-Rad Laboratories, USA) were used for PCR with following temperature-time profile: initial denaturation - 95°C, 9 min, 40 cycles of: denaturation at 94°C - 1 min, annealing - 57°C - 1 min and elongation - 72°C - 2 min.

The data were analyzed by MANOVA and compared by Duncan's tests using SPSS 15.

Results and Discussion

Potato plants with typical symptoms of black leg collected at different regions of Russia in 2009-2010 were used for isolation of bacterial isolates. Colonies obtained on potato agar gentian violet had pale white center with a transparent mucous border. The same colonies onNBY medium were flat and almost transparent. Typical of *Dickeya* spp. colonies were sub-cultured on Logan's medium with sodium polypectate. Strains of *Pectobacterium* and *Dickeya* softened the pectate layer. Among majority of *Pectobacterium* colonies we have found a number of bacteria, identified as *Dickeya* spp. by biochemical profile.

All the isolates were tested for hypersensitivity reaction (HR) in tobacco leaves, and HR-positive ones were used for pathogenicity test on potato slices. Classic PCR with specific primers for *pelB* gene ADE1/2 was applied to confirm identification of the *Dickeya*

spp. isolates (table 1), isolates of *Pcc* gave no PCRproduct with ADE1/2, although, they did not differ in reaction of indole reduced compounds synthesis, and in pectolytic activity.

Table 1

Strains of genera *Pectobacterium* and *Dickeya* used in this work

Genus, Species	Strain	Origin, time of isolation	Host plant
Collection of Laboratory of plant protection of RGAU-MSKHA by K.A. Timiryazev			
Dickeya sp.	D9Tr	Moscow, 2010	Tomato
Dickeya sp.	D23,	Moscow region, 2010	Potato
Dickeya sp.	$D23_2$	Moscow region, 2010	Potato
Dickeya sp.	$D23_3$	Moscow region, 2010	Potato
Dickeya sp.	$D23_4$	Moscow region, 2010	Potato
Dickeya solani	D.Fil	Voronezh region, 2010	Potato
Collection of Russian Research Institute of Phytopathology			
Dickeya dianthicola	D33	Nizhni Novgorod region, 2009	Potato
Dickeya dianthicola	D9	Nizhni Novgorod region, 2009	Potato
Dickeya dianthicola	D8	Nizhni Novgorod region, 2009	Potato
Dickeya dianthicola	D17	Nizhni Novgorod region, 2009	Potato
Dickeya sp.	D3B.,	Voronezh region, 2010	Potato
Dickeya sp.	$D3B_2$	Voronezh region, 2010 г	Potato
Dickeya sp.	$D3B_3$	Voronezh region, 2010	Potato
Dickeya sp.	D1	Voronezh region, 2010	Potato
Dickeya sp.	D9B	Voronezh region, 2010	Potato

Artificial inoculation of carnations, dahlias, barley, wheat, clover, flax, lupines, oats, vetch, canola, and triticale did not produce any visible symptoms (table 2).

Table 2

Evaluation of pathogenicity and range of host plants infected by *D. dianthicola* and *P. atrosepticum* at artificial inoculation

Вид растения	*D. dianthicola,* D9, D33	*D. solani* *DFil*	*P. atrosepticum,* Eca 393, Eca21, Eca31a	Контроль (H_20)
Potato	+	+	+	—
Carnation	-	-	-	-
Tobacco	+	+	-	-
Tomato	+	+	-	-
Dahlia	-	-	-	-
Iris	+	+	+	-
Barley	-	-	-	-
Wheat	-	-	-	-
Clover	-	-	-	-
Flax	-	-	-	-
White lupine	-	-	-	-
Oats	-	-	-	-

Continued

Вид растения	*D. dianthicola,* D9, D33	*D. solani DFil*	*P. atrosepticum,* Eca 393, Eca21, Eca31a	Контроль (H_20)
Vetch	-	-	-	-
Rape	-	-	-	-
Triticale	-	-	-	-
Mustard	-	-	-	-

Note:. "+" Typical symptoms of wilting and necrosis, no reaction.

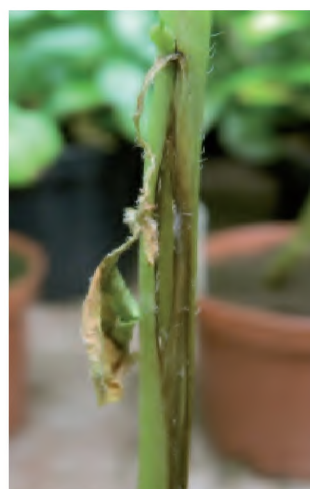
Iris plants and potatoes were affected by strain D9, D33, and Eca 393. In 4-5 days after inoculation, potato plants had a dark blurring spreading stem spot. Bacteria spread through the vascular system, caused wilting of the leaves, which eventually dried up completely.

Iris plants in 2-3 days turned black around the point of inoculation with bacterial exudates, leaves softened, and further spreading of the pathogen was accompanied by darkening of the entire leaf surface (fig. 1).

Plants of tomato and tobacco were not damaged by the strain Eca 393, but affected by D9 and D33. Plant stems of tomato were broken in the site of inoculation in 5-6 days, and leaves were wilted. Plant stem of tobacco darkened and lost turgor, the bacteria spread up from the site of inoculation in 3-4 days (see fig. 1).

Experiments conducted recently in Spain showed that *Dickeya* spp. can cause disease of potatoes, com, onions, chicory, and African violet [21]. The obtained results allow us to include in this list tobacco, tomato, and iris. This improves our understanding of the pathogen

a)



b)

c)

d)

Fig. 1. Symptoms caused by *D. dianthicola* after artificial plant inoculation: a - iris, b-potato, c-tobacco, d-tomato

circulation in nature shows the importance of spatial isolation of potato from other host plant species.

ELISA results showed that *D. dianthicola*, *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* were serologically distinct. The strains of *D. dianthicola* and *Pa* reacted only with homologous antiserum and did not cross-reacted. *Pcc* strains gave negative results agains both two antibodies (table 3). Thus, it was established that those three pathogen causing blackleg of potato are serologically different and can be easily distinguishable by ELISA.

Sensitivity of ELISA in detection of *D. dianthicola* showed no difference for buffer- and tuber-extract (table 4). In both variants, detection thresholds were around

Table 3

ELISA reaction of the black leg pathogens against different antibodies
(optical density at 450 nm)

Species	Strain	Antibodies against:				
		D. dianthicola		*P. atrosepticum*		
		D9	D17	Eca 393	Eca 31 a	Eca 21
D. dianthicola	D9	0,832 c	0,657 b	0,026 a	0,016 a	0,018 abc
	D8	0,871 d	0,638 b	0,021 a	0,021 a	0,015 ab
	D17	0,759 b	0,662 b	0,020 a	0,015 a	0,010 ab
	D33	0,827 c	0,650 b	0,025 a	0,028 a	0,026 abc
P. atrosepticum	Eca 21	0,020 a	0,023 a	0,300 b	0,118 c	0,229 d
	Eca 393	0,030 a	0,020 a	0,841 c	0,300 d	0,546 e
	Eca 31[a]	0,047 a	0,017 a	0,320 b	0,128 c	0,256 d
P. carotovorum subsp. Carotovorum	P39	0,013 a	0,013 a	0,008 a	0,009 ab	0,009 a
	P3-2	0,038 a	0,023 a	0,030 a	0,053 a	0,010 ab
	P3-3	0,030 a	0,020 a	0,026 a	0,014 ab	0,015 ab
Negative *contro\-C.michiganensis* ssp. *sepedonicus*	Cms204	0,037 a	0,019 a	0,024 a	0,034 ab	0,021 abc

Note. Values with the same letter are not significantly different by Duncan's test (95% probability).

Table 4

ELISA sensitivity against *D. dianthicola* strain D9 at buffer and tuber extract

Concentration of D. dianthicola, CFU/ml (Factor A)	Optical density at 450 nm, A_{450} (Factor B)		Average for Factor A LSD_{05}= 0,080
	buffer	tuber extract	
10^6	2.934c	2.117c	2.525c
10^5	0.745b	0.710b	0.727b
10^4	0.266a	0.288a	0.277a
10^3	0.257a	0.227a	0.242a
10^2	0.234a	0.223a	0.228a

Continued

Concentration of D. dianthicola, CFU/ml (Factor A)	Optical density at 450 nm, A_{450} (Factor B)		Average for Factor A LSD_{05} = 0,080
	buffer	tuber extract	
10^1	0.220a	0.228a	0.224a
10^0	0.217a	0.229a	0.223a
10^{-1}	0.230a	0.239a	0.234a
Negative control - phosphate Buffer	0.235a	0.237a	0.236a
Average for Factor B $F_f < F_{05}$	0.593	0.500	

LSD_{05} for pairs wise difference – 0,253.

10^5 cells / ml. This is indicated a high specificity of the obtained antibodies and the absence of reaction inhibitors in potato tuber extracts. Thus, *Dickeya sp.* could be detected by ELISA directly in tuber extract without labor-intensive isolation of bacterial pure culture.

The real-time PCR assay was developed based on the sequence of DNA gene *pelB* of *Dickeya* sp. and other enterobacteria available in the genebank (www.ncbi.nlm .nih.gov). Fluorescent bye-labeled probe ADE3 was selected and the assay sensitivity and specificity has been evaluated in analysis of DNA of different species within genera *Dickeya* and *Pectobacterinm.*

The threshold crossing values for 20 strains of *Dickeya* including D9 were in average at 25th cycle (with range 21-37 cycles) (table 5), ie we observed the genus-specific reaction.

Such result shows that the probe ADE3 in combination with the primers ADE1/ADE2 can be used for reliable detection of bacteria of genus *Dickeya,* and can be included in diagnostic kit. Reliable identification of species within the genus *Dickeya* still available only by sequencing fragments of *dnaX* gene [6].

The threshold cycle value (Ct) in real time PCR at different bacterial cell

Table 5

Threshold crossing values C, for real-time PCR of *Dickeya* strains with primers ADE1/ADE2 and probe ADE3

Strains *Dickeya* spp.	Threshold crossing cycles, Ct
2132	23,67 ± 1,47
2124	22,26 ± 0,03
2126	21,26 ± 0,16
2117	23,16 ± 0,25
2115	24,83 ± 0,15
2118	23,64 ± 0,02
2127	33,07 ± 0,19
2121	28,04 ± 0,50
2122	24,46 ± 0,16
2120	22,01 ± 0,54
2133	23,68 ± 0,01
2094	22,51 ± 0,01
2128	37,57 ± 0,41
2222	22,38 ± 0,11
2131	23,14 ± 0,11
2119	25,82 ± 0,03
2125	23,01 ± 0,04
2116	24,51 ± 0,19
2129	22,88 ± 0,11
D9	26,84 ± 0,32
Negative control – *Pcc*	n.r.*
Negative control – *Pa*	n.r.*

* No reaction.

concentration revealed a reliable decrease of Ct for increasing bacteria concentrations. The obtained curve (fig. 2) was used for calculation of PCR efficiency [8] with obtained

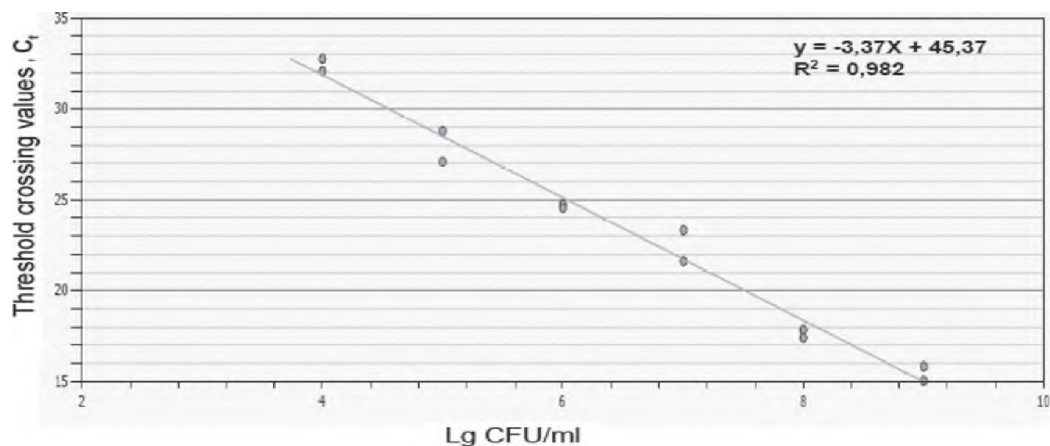


Fig. 2. Correlation between C_t and concentration of *D. dianthicola* cells

value 1.98 (incensement of copy number per cycle). Thus, the developed primer/probe combination has efficiency close to maximum for PCR and can be used for practical application.

Conclusions

1. The host plants for *D. dianthicola* include tomato, tobacco and iris. The isolation of potato fields from these species should be considered as a protective measure.
2. ELISA kit was developed for detection of *D. dianthicola*, and provided a high specificity and sensitivity around 10^5 cells / ml in the tuber extract.
3. Matched fluorescent probe ADE3, which in combination with primers ADE1/ADE2 can be used for rapid detection of bacteria of the genus *Dickeya* PCR in real time.

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This work was supported by the Federal Program "Scientific and scientific-pedagogical stafflimovative Russia", a government contract and grant P2380 ISTC № 3431 and 3978.

ДИАГНОСТИКА БАКТЕРИАЛЬНОГО ПАТОГЕНА КАРТОФЕЛЯ
DICKEYA DIANTICOLA

Аннотация: исследовали круграстений-хозяев и возможность диагностики недавно обнаруженного в России возбудителя черной ножки картофеля — бактерии Dickeya dianthicola. При искусственном заражении было показано, что помимо картофелярастениями-хозяевами D. dianthicola являются томат, табак и ирис. Это необходимо учитывать при планировании защитных мероприятий. Были получены специфичные к D. dianthicola антитела, которые обеспечивали при диагностике методом ПФА высокую специфичность и порог чувствительности 10^5 клеток/мл в клубневом экстракте. Подобрана проба ADE3, которая в сочетании с праймерами ADE1ADE2 может быть использована для выявления бактерий рода Dickeya методом ПЦР в реальном времени.

Ключевые слова: черная ножка картофеля, Dickeya dianthicola, иммуноферментный анализ, ПЦР в реальном времени.

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