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Identification and Prevalence of Ralstonia Solanacearum from Potato Fields of Kyrgyzstan

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Abstract For the first time in Kyrgyzstan Raltsonia *solanacearum* bacterium as a pathogen of bacterial wilt (quarantine for the country object**)** was obtained and identified by enzyme-linked immunosorbent assay (ELISA) and biochemical methods. Three potato (*Solanum tuberosum*) cultivars: Picasso, Sante and Nevskiy were used for isolation of pathogen, which were collected from different regions of Kyrgyzstan. Detection and identification of the pathogen by ELISA performed directly from diseased potato shoots and leaves, and from pure culture of *Ralstonia solanacearum* isolated from tubers of potato seed during storage. For ELISA was used *Ralstonia solanacerium* PathoScreen R Kit DAS ELISA (Agdia product, USA).Isolated races of *Ralstonia solanacearum* by biochemical characteristics were classified as a 3-biotype.

Keywords *the* potato (*Solanum tuberosum*) cultivars, identification of *Ralstonia solanacearum* , ELISA assay, biochemical tests.

1. INTRODUCTION

Ralstonia solanacearum is a soil-borne pathogen that naturally infects roots. It exhibits a strong and tissue-specific tropism within the host, specifically invading, and highly multiplying in the xylem vessels [1, 2]. It causes a wilt disease in more than 450 plant species of 54 botanical families across the globe [3,4,5]. *Ralstonia solanacearum* has been studied intensively both biochemically and genetically, and has long been recognized as a model system for the analysis of pathogenicity [6]. It is well adapted to life in soil in the absence of host plants [7], thereby providing a good system to investigate functions governing adaptation to such an ecological niche. Considering the genetic diversity among the strains responsible for wilting disease in different plants, the pathogen is now termed as *Ralstonia solanacearum species* complex [8].In a traditional way this pahogen has been classified into five races with respect to their host specificity and six biovars according to their biochemical properties [9].

The first signs of the disease are shown in the beginning of the flowering and tuber formation. Plants suddenly wilt; the leaves turn yellow, shrivel and droop. The lower basal part of the stem softens and rots. A typical feature of brown rot is the splitting of the stems, the cross-cut of them follow a drop of bacterial exudates. Subsequently, the bacteria penetrate into the stolon, then into young tubers, causing browning of the vascular ring. From sections of the affected vessels and tubers follows brown mucus [10]. Bacterial wilt occurs mainly in tropic, sub-tropic and warm temperature zones [11]. However, this disease has extended to more temperate areas [12].

Ralstonia solanacearum is a b-proteobacterium and whose complete genome sequence was presented by analysis of strain GMI1000. The 5.8-megabase (Mb) genome is organized into two replicons: a 3.7-Mb chromosome and a 2.1-Mb mega plasmid. The genome encodes many proteins potentially associated with a role in pathogenicity. [13].

Brown slimy bacterial bacteriosis of potatoes (bacterial wilt, or wilt) caused by *Ralstonia solanacearum potatoes (RS)* is a relatively a new disease in the fields of Kyrgyzstan. There are still no data and records of the scientists and experts on the biology and distribution of this disease in the potato crops regions of Kyrgyzstan. There are suggestions that this bacterial disease was brought with imported planting material to Kyrgyzstan from neighboring countries. So, the disease has been found in Russia in 1999 by quarantine inspection only in the area of 0.06 hectares, planted with imported varieties Santa, then the infestation of potato has been found in many regions of Russia : in the Urals, Far East, Western and Eastern Siberia [14].

In Kyrgyzstan, the potato (*Solanum tuberosum*) is a staple product for the population**.** Recently, in different regions the farms start to grow the varieties such as Picasso, Sante, Nevskiy, which were imported from Russia and other countries of the world, besides to local potato varieties. Approximately 32% of potatoes yields are lost per year due to viral, bacterial, fungal, and pest attack to potato tuber and potato plant [15]. There is a particular threat to potato production (especially the seed production) because of asymptomatic cases of these bacterial diseases; as apparently healthy tubers have a margin hidden (latent) infection and pose a threat to crops next year, so it is important to be able to identify it in the contaminated material. Still, the prevalence and host range of races and biovars of *Ralstonia solanacearum* is unknown in potatoes cultivated regions of Kyrgyzstan, but it is becoming increasingly clear that this species causes disease in vegetation period and in storage after harvesting.

The objective of this study was to develop simple and reliable tools to distinguish the biovars of *Ralstonia solanacearum* by using biochemical and ELISA tests and to determine the prevalence of pathogen races in commercial potatoes fields of Kyrgyzstan.

2. MATERIALS and METHODS.

2.1. Origin of isolates. For direct isolation of *Ralstonia solanacearum* were used potatoes tubers of Picasso, Sante and Nevskiy varieties, which were collected in the fall 2010 and 2011 from Issuk-Kul and Chy regions of country. All isolates from potatoes fields came from individual tubers of different plants. Each tuber was placed in an individual plastic bag after harvest.

2.2. Cultural characterization. The infected part of tubers was cut using a sterile sharp knife. A suspension from plant ooze and exudates was prepared in sterile distilled water and then streaked onto Kelman's tetrazolium chloride (TZC) agar and 2% sucrose peptone agar (SPA).After incubation at 28°C for 24 to 36 h, chartered colonies of *Ralstonia solanacearum* were selected on mediums. Isolates of *R. solanacearum* were maintained in sterile distilled water for following identification steps and stored at room temperature. Pure cultures were tested by biochemical and enzyme-linked immunosorbent assay (ELISA) methods. A mobility, gram negative reaction, catalase, amylolytic and lecithinase activity, liquefaction of gelatin, saccharolytic enzymes, the formation of indole and other biochemical properties were determined. For pigment formation tests the liquid mediums: meat-peptone broth and tryptophan broth were used. More consistent results were obtained when L-tyrosine was added to the medium. The ability to denitrification was tested using **the s**emi-solid medium: 10% peptone, 5% NaCL, 2,0% KNO_3, 3,0 % Bacto agar and Hiss reagent.

2.3. Test to determine the mobility. Cells of virulent races of *Ralstonia solanacearum* are motile when viewed microscopically, while avirulent races cells are immobile. The mobility was observed using the medium: 0, 1 % tryptone, 0, 1 % glycerol, 10% phosphate buffer, 3, 5 % Bacto agar.

2.4. **The biovars test.** The pathogen species is subdivided into races based on host range. Currently, polymerase chain reaction (PCR) is used for definitive identification of pathogen race. To identify the biovar of pathogen species we have used biochemical method based on the utilization of the disaccharides: cellobiose, lactose and maltose and oxidation of the hexose alcohols: dulcitol, mannitol, and sorbitol [9, 16].

2.5. Accumulation of Ralstonia solanacearum isolates in the host-plant tissue.

Healthy potato tubers of different varieties were used for accumulation the pathogen culture in the host cell. The tubers were washed profusely and thoroughly with water, and then were sterilized in 96% ethyl alcohol, after that thoroughly rinsed in sterile water and cut into pieces and placed in Petri dishes, on wet sterile filter paper. Bacterial suspensions at a concentration of 10^8 CFU/ml were infiltrated into potato slices. Inoculated slices were incubated at optimal temperature (28^0 C) for the bacteria. The optimum moisture ensured the rapid growth of bacteria.

2.6. Pathogenesis assays on potato seedlings and plants. Three potato (*Solanum tuberosum)* cultivars were used for pathogenicity tests: Picasso (highly sensitivity), Sante (medium resistant) and Nevskiy (highly resistant) .Three-week old plants grown in soil were inoculated by soil drench without root severing. The concentration of bacterial inoculums was 10^8 CFU/ml. The experiment was repeated at least two times, giving a total of six test plants. Inoculated plants were kept in a room condition with natural light and mean temperature at 28°C. Percentage of plants showing the wilting symptom was recorded during 28 days.

2.7. Tolerance to NaCL, 2, 0 % tests. To determine the sensitivity of *Ralstonia solanacearum* isolates to sodium chloride different media were included in this study : TTC medium (1% peptone, 0.1% casein hydrolysate, 0.5% glucose, 1,5% Bacto agar, 0.005% TTC); potato medium without gentian – violet (2.0% potato extract, 2,0% Bacto agar); peptone –yeast (0, 5% yeast extract , 1,0% peptone , 2,0% Bacto agar); extract sucrose- peptone (2,0 % saccharose, 0,5% peptone, 0,05% potassium phosphate dibasic, 0,025% magnesium sulfate, 2,0% Bacto agar) with the addition of 2,0 % sodium chloride.

2.8. Immunoblot ELISA test (Agdia). The *Ralstonia solanacearum (*RS) ELISA test was used with plant samples exhibiting symptoms of Rs and with bacterial culture samples. According to protocol of DAS ELISA of Agdia the samples were added to microplate coated with monoclonal antibodies to EPS of Rs. If EPS is present in the sample, it is bound by antibodies and captured on the microplate during the incubation period. After incubation, the plate was washed to remove unbound sample. An enzyme conjugate solution, containing a monoclonal antibody conjugated to peroxidase, is added and binded to any captured EPS. After incubation the plate is washed to remove any unbound conjugate. This final binding creates a sandwich of the target analyte between the two specific antibodies. Weels in which a blue color developed was indicated positive results. Wells in which there was no significant color development indicated negative result. Test results were valid only
if positive control wells give a positive result and buffer wells remain colorless.

3. RESULTS and DISCUSSION.

3.1. Origin of isolates and Organism Characteristics. We have analyzed potato tubers of Picasso, Sante, Nevskiy varieties. *Ralstonia solanacearum* - as a pathogen of bacterial wilt was obtained from Picasso variety. 12 isolates from potato fields of Issuk-Kul and 7 isolates from Chy regions were identified as Ralstonia *solanacearum species.*

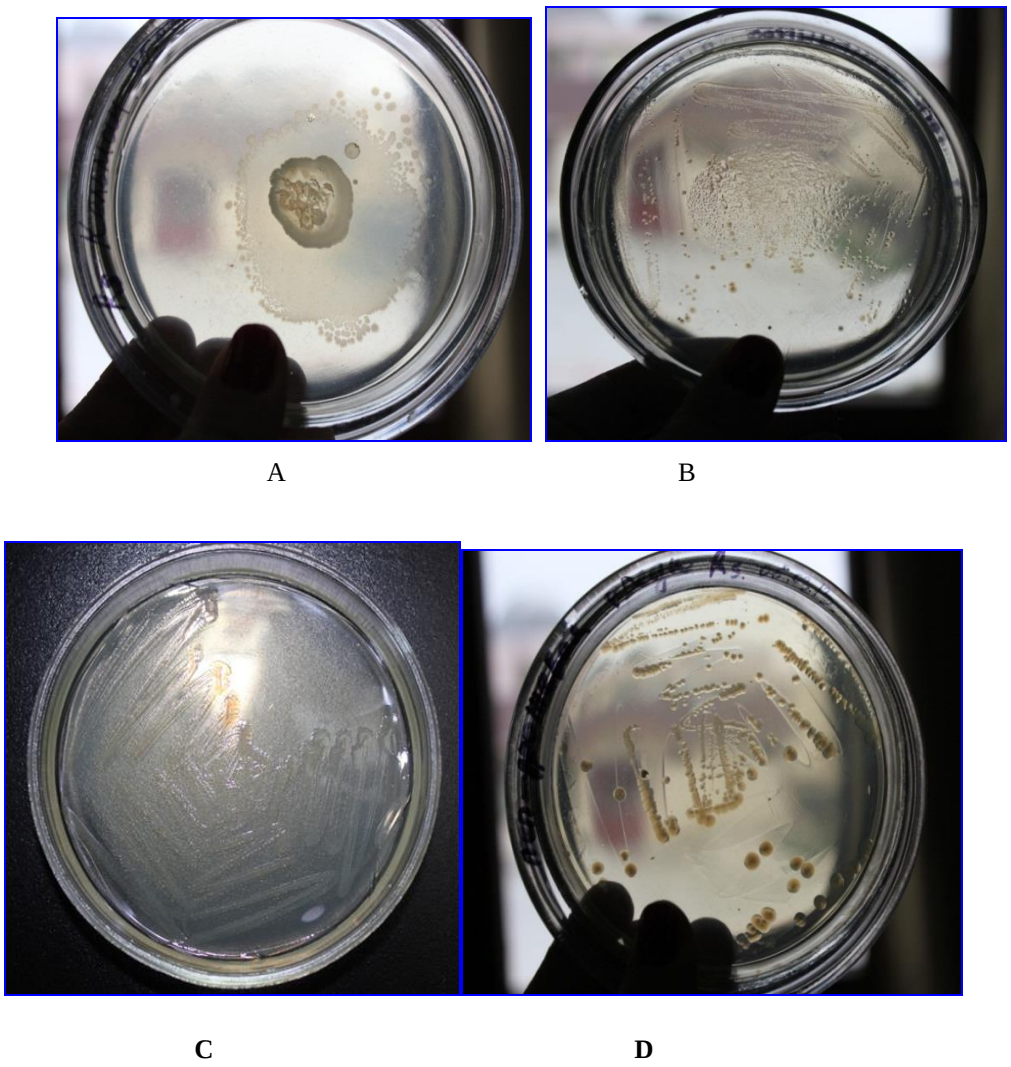
Large, elevated, fluidal and white colonies of isolated bacteria were grown after two days on TZC medium, and white, fluidal with whorls characteristic colonies were appeared on SPA. The organism was capable to grow at 28 - 36 ° C temperatures aerobically and does not form endospores. The bacterium is slightly thick sticks with dimensions of 0.7-0.9 microns, gram – negative, motile and is non-encapsulated. Cells of obtained isolates *Ralstonia solanacearum* were motile when viewed microscopically, that is indicated to it's the ability of virulent. The isolates were catalase and oxidase positive.

New isolates of *Ralstonia solanacearum* were able to reduce nitrate to nitrite. Changing the medium color to red and formation a layer of foam from an intensive gas release indicate to a complete reduction of nitrate and denitrification (fig.1).

Fig.1. Formation a layer of foam from an intensive gas release indicate a complete reduction of nitrate and denitrification by *Ralstonia solanacearum*

3.2. Sensitivity to NaCL, 2, 0 % tests. The causative agent of potato brown rot is more sensitive to the presence of salt in the environment than other no spore forming plant pathogen bacteria. Typically, bacteria of *Pseudomonas* genus can develop tolerance to 3 % or more of sodium chloride [17]. Whereas *Ralstonia solanacearum* isolates have a sensitivity to 2% NaCL, even some species can prevent their growth in the presence in the medium only 1.0% salt.

The sensitivity of *Ralstonia solanacearum* isolates to sodium chloride was different in used media. Isolates have formed colonies differ in shape, size and color; also differ in the intensity of growth. The growth of bacteria was inhibited on the TTC medium with 2, 0% NaCL, so the colonies were slightly noticeable (fig.2, A). Whereas, the growth of bacteria colonies on the potato medium without gentian – violet and peptone –yeast medium was normal with a high visibility (fig.2, D, C). On the extract sucrose- peptone the growth of bacteria was slight, but the growth has not stopped and further continued (fig.2, B). Different compositions of used media with the same content of sodium chloride have a different effect on the sensitivity of *Ralstonia solanacearum* isolates to salt. The pathogen isolates have showed a considerable tolerance in potato medium without gentian – violet and peptone –yeast medium. The isolates have showed a very low tolerance on the TTC medium, a resistant tolerance was visible on the extract sucrose- peptone medium.



A B

C D

Fig.2. A- a growth inhibition on the TTC medium with 2, 0% NaCL, B- *inhibited* but continued growth on saccharose peptone medium with 2, 0% NaCL, C- a normal growth on the on Genthian violet medium with 2, 0% NaCL ; D- a normal growth on the peptone-yeast medium with 2,0 % NaCL, for 48 hours

3.4. The biovars test. Specific host range and distribution of *Ralstonia solanacearum* depends on the race and the biovars of the pathogen. In table 1. the data related to the relationship of race, biovars, host range, and geographic distribution of *Ralstonia solanacearum are* summarized. It is known the five races of potato brown rot. The most dangerous is a race 3 that is affecting the potatoes in low temperature. The infection persists for a long time in plant debris and potato tubers (in a latent form), and it is common in temperate regions. It's main sources are infected soil, crop residues, weeds of the genus Solanaceae [16].

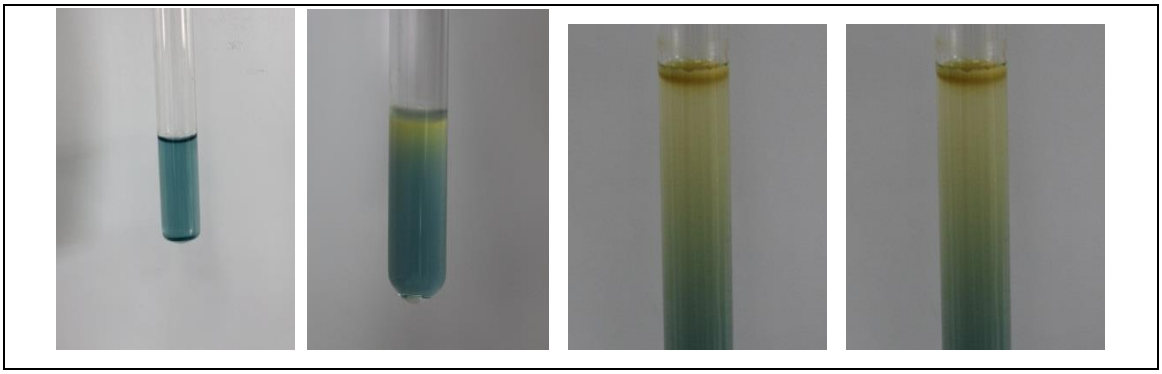
Table 1. Races and biovars of *Ralstonia solanacearum.*(Adapted from Daughtrey 2003) [10]

Race	Host Range	Geographic distribution	Biovar
1	Wide	Asia, Australia	3, 4
		Americas	1
2	Banana, other *Musa* spp.	Caribbean, Brazil, Worldwide	1
3	Potato, some other *Solanaceae,* Geranium; few other species	Worldwide except US and Canada	2
4	Ginger	Asia	3,4
5	Mulberry	China	5

Isolated races of *Ralstonia solanacearum* by biochemical characteristics were classified as a 3-biotype, so they were able to oxidize the disaccharide: cellobiose, lactose and maltose and the hexose alcohols: dulcitol, mannitol, and sorbitol. Table 2 illustrates the classification into biovars based on this method. When bromomethyl Blau was used as an indicator the medium becomes yellow as a result of oxidation , and when Andred indicator was used , the medium has changed to red. Transformation of these substrate by isolates has occurred slowly, for example as shown in Fig.3 in the presence of bromomethyl Blau indicator an oxidation of dulcitol was occurred only after 12 days (fig.3 A).

Table 2. Classification of *Ralstonia solanacearum* into biovars. (Adapted from French et al, 1995)[9]

	Biovars				
Physiological Tests	**1**	**2**	**3**	**4**	**5**
Utilization of disaccharides					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Oxidation of alcohols					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	



2 days 4-5 days 7-9 days 10-12 days

Fig.3 An oxidation of dulcitol by *Ralstonia solanacearum isolates* in the presence of bromomethyl Blau indicator

3.5. Accumulation of Ralstonia solanacearum isolates in the host-plant tissue.

In many cases *Ralstonia solanacearum* bacteria are closely interrelated with secondary pathogens such as the causative agent of soft rot *Erwinia carotovora var. atroseptica* [18].This creates some difficulties for the isolation of a pure culture of *Ralstonia solanacearum* from the affected tissue. For the accumulation of the culture of the pathogen in the host cell and to determinate it's virulence, bacterial suspensions of *Ralstonia solanacearum* at a concentration of 10^8 CFU/ml were infiltrated into sterile healthy potato slices. They were incubated at lower temperatures (22^0 C), in moisture chamber. The optimum moisture ensured the rapid growth of bacteria. The organism quickly began to multiply in infected host cells. On the third day a dark ringed circles were appeared on potato slices. Gradually, a rotting of the entire surface of potato slices has started. In 5 days there was a complete decay, with the release of odors and turning into mucous (fig. 4).

Of all the varieties tested only Picasso showed high sensitivity to rotting at low temperatures. These results allowed us to identify which varieties are more resistant or more susceptible to this disease. It is important to provide advice to farmers which varieties are the best to grow in different climatic zones of the republic. This test additionally has confirmed that obtained *Ralstonia solanacearum* new isolates are belong to biovar 3, which can survive at low temperatures. Some researchers have noted in their results that, high

temperatures and high soil moisture generally favors *Ralstonia solanacearum*, the exception being certain Race 3 strains that are pathogenic on potato and are able to grow well at lower temperatures [9].

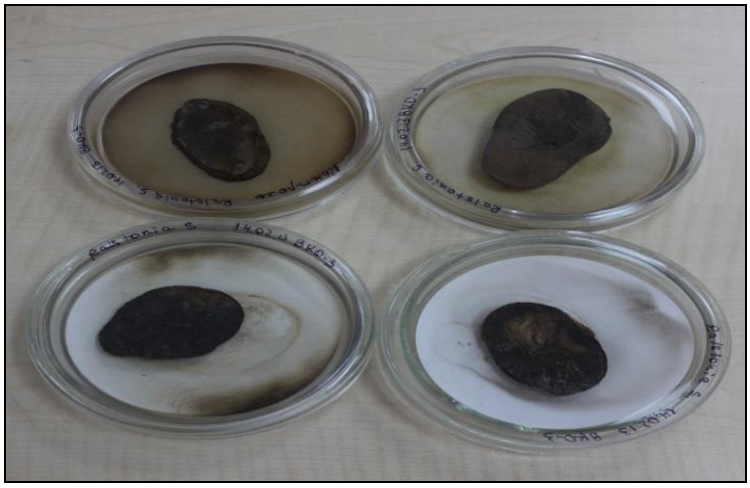


Fig.4. Rotted potato tubers of Picasso variety in 5 days after infiltration of a pathogen suspension.

3.6. Pathogenesis assays on potato seedlings and plants. Three potato (*Solanum tuberosum)* cultivars were used for pathogenicity tests: Picasso (highly sensitivity), Sante (medium resistant) and Nevskiy (highly resistant). In between 3-6 days began to appear the symptoms of disease in the Picasso variety plants. The first symptoms of the disease were wilting leaves on the ends of branches. During disease development, the leaves turn chlorosis and eventually necrotic. Close to the ground part of the stem of infected plants turn gray-brown. This is a characteristic symptom of potatoes brown rot (fig.5 A and B). In the variety of Sante the symptoms of disease began to appear in 2 weeks, and the lower leaves are browned and dry, turn yellow and chlorosis. Stems have stood relatively for long time, and then 4 weeks later started to bend. Nevskiy variety was resistant to the pathogen infected dose. Within 6 weeks there were no signs of disease. The specific symptoms: wilting of the leaves at the end of the day with recovery at night, the edges of the leaves turned black and curled were observed within 5 to 10 days, but no symptoms were observed on control plants treated with sterile water.

A B

Fig.5. A: close to the ground part of the stem of infected became grey brown; B: infected plants show yellowing, wilting, and browning of lower leaves followed by necrosis;

3.7. Immunoblot ELISA test (Agdia). Using the ELISA technology has allowed to identify *Ralstonia solanacearum* bacteria from diseased leaves of potato at a concentration of 10^3-10^4 cells\ml. Detection and identification of the pathogen by ELISA (Agdia product, USA) performed directly from diseased potato stems and leaves at a concentration of 10^3-10^4 cells\ml. Weels in which a blue color developed was indicated positive results. The bacterium was reisolated from the infected leaves and stems and identified as described above (fig.6).

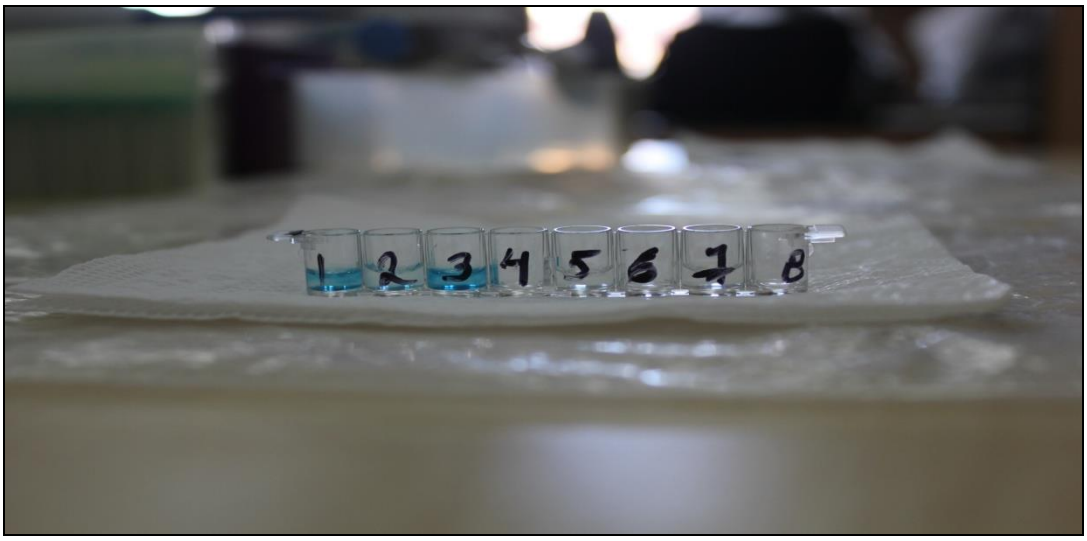


Fıg.6. Weels with a blue color developed is indicated positive results from diseased potato stems and leaves at a concentration of 10^3-10^4 cells\ml.

In this study, we have used well known, efficient methods and bioassay for systematic screening of *R. solanacearum* for identification phenotypic and biochemical profile, also for pathogencity and virulence. As a result, an aggressive race, biovar 3 was most isolated from the potato fields of Tup district of Issyk-Kul region, especially in fields where Picasso variety was grown. This area is characterized by wet and temperate climate than other areas of the Issyk-Kul region. The low percentage of affection with this agent was noted in Sante variety. The pathogen was no almost obtained from Nevskiy variety plants and tubers. In this region, the pathogens were isolated from growing plants with character symptoms and tubers after harvest in storage, they were available for sale.

In Chuy oblast, where the climate is hot and the humidity is relatively low [15], pathogen races of *R. solanacearum* were obtained from Picasso and Santa potato varieties. In this region, essentially isolates were relieved from the tubers for sale, or in storage.

We have not found R. *solanacearum species as* causative agents of wilt in local potatoes varieties (red and white crumbly) grown in mountainous areas of Kochkor district, This indicates that the disease has penetrated into Kyrgyzstan from neighboring countries together with planting material.

Our results for the first time in Kyrgyzstan have revealed the presence *Ralstonia solanacearum* bacterium as a pathogen of bacterial wilt **(**quarantine for the country object**)** in the potato fields of Issyk-Kul and Chy regions. As well as our results have allowed to determine which varieties are most susceptible to the disease and in which district a threat constitutes to most of its wide dissemination. This is important to prevent farmers, which varieties they should buy as planting material. The areas in which have not yet been introduced commercial varieties should be remaining clean zones from this disease.

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