Original article

Detecting pathogenic bacterial wilt disease of potato using biochemical markers and evaluate resistant in some cultivars

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A B S T R A C T

Bacterial wilt caused by *Ralstonia solanacearum* (Smith), is one of the chief severe diseases of potato in warm temperate regions, tropics and subtropics of the world. The study was conducted to isolate and identify bacterial pathogens and select the most resistant cultivars and avoid the decrease in the total value of Egyptian potato exports to the European Union (EU) due to the quarantine restrictions imposed by the EU on potato tubers exported from Egypt affected by bacterial wilt. The results of traditional identification through morphological and serological studies showed that the five isolates were isolated and identified as *Ralstonia solanacearum*. Furthermore, the results illustrated that RS5 isolate showed the lowest percentage of disease incidence reduction on the three tested potatoes cultivar Bellini, Spunta and Mondial recorded 9.64%, 15.41% and 34.12%, respectively. While, RS8 isolate exhibited the highest effective one the percentage of disease reduction on all tested potato cultivars. This isolate reduced disease incidence 60.60%, 63.21% and 71.66%, compering to the healthy control treatment. The result of molecular identification represent that the probe used in Taq-man (PCR) was of the type (B2) capable to detect only biovar 2 of *R. solanacearum* bacterial wilt. Furthermore, primer and probe are specific for detection of the race 3 biovar 2 strain. Positive results were obtained in all assays used including IFAS, protein content and SDS-PAGE with all five isolates. So the isolate (RS5) was the most virulence one, followed by RS1, RS3, RS2 and RS8, registered that the tested isolates were *R. solanacearum* race 3, biovar 2. Also, studies focused on the form of genetic distances and similarities based on pathogenic and plant growth parameters. The results illustrate that the highest genetic similarity (0.998) was found between Bellini and Spunta cultivars as the closest but the lowest value (0.946) was found between Mondial and Bellini as most distant. These results were similarity with genetic distances and SDS-PAGE profile of the three tested potato cultivars**.**

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1. Introduction:

Potato (*Solanum tuberosum* L.) is take up a high class among essential vegetable crops in the world and consistently ranks fourth among the most important staple food crops after wheat, corn and rice. (Birch et al., 2012). Potato is a steady nutrition safety crop with great achievable for difficulty alleviation and combating malnutrition in the developing world (Devaux et al., 2020). Currently, the total value of Egyptian potato exports lurch due to quarantine con-straint on the potato bacterial wilt by the EU (Makled and Elkodosy, 2018). The bacterial wilt disease was first recorded in Egypt from potato tubers that had symptoms of rot (Sabet, 1961).

Bacterial wilt of potato plants and brown rot on potato tubers induced by *Ralstonia solanacearum* Smith, this bacterium is one of the furthermost threatening diseases of potato production in Asia, Africa, and Central and South America (Charkowski et al., 2020). These bacteria are considered soil borne, so it's usually attack plants through the roots and colonizes xylem vessels (García et al., 2019). As the vascular bundles in the diseased plants are filled with bacteria impede that transport of water and nutrients, and thus the symptoms appear, which are yellowing of the leaves, redness of the vascular bundles, necrosis, and ultimately the total wilt of the infected plants, then followed by physiological modifications in the diseased plants, such as increasing the respiratory rate and reducing transpiration and photosynthesis (Karim and Hossain, 2018).

The bacterium that causes wilt in the potato plant is classified into five races according to the host range, and into five biovars according to the capability of races to oxidize three hexose sugar alcohols and three disaccharides (Hayward, 1964). The race (3) and biovar (2) of *R. solanacearum* is responsible for attacking the potato crop, as it was discovered in the Andean region of South America (Prior and Fegan, 2005). R3bv2 was introduced to northern Europe around 1972, which increased the incidence and severity of disease and caused significant damage (Chávez et al., 2012). Consequently, governments passed quarantine legislation requiring the destruction of the affected crops and the rest of the affected fields (Janse, 1996). Serological methods can deficiency particularity because of cross-reactions of polyclonal antibodies with other bacteria and may have defined susceptibility (Puaprasert et al., 2018). Immunofluorescent antibody stain and *Tag-Man* (PCR) were used by Janse et al. (2004). A fluorogenic (*TaqMan*) PCR analysis was developed to detect *R. solanacearum* strains. Two fluorogenic probes were utilized in a multiplex response; one broad-range probe (RS) revealed all biovars of *R. solanacearum*, and a second further specific probe (B2) was applied to only detect biovar 2A (Chen et al., 2010).

Bacterial wilt attacks more than two hundred plant species, including more than fifty plant families (Aslam et al., 2017; Uwamahoro et al., 2020). The pathogen of bacterial wilt is the most destructive bacterial pathogen due to its infestation, uncommonly wide host range, perseverance, and wide topographical distribution (Wei et al., 2018). The disease is common to occur in the wet equatorial areas, sub-equatorial and some temperate areas of the world which has been estimated to affect about 3.75 million acres in relatively 80 countries throughout the world with international damage estimates actually over $950 million per year (Charkowski et al., 2020).

Bacterial wilt is the most significant one of bacterial disease on potato, and announced many years ago in Egypt (Farag et al., 2017). As the disease incidence and severity varies between potato cultivars, due to the presence of genetic differences between ones. Studies have shown that Spunta and Bellini cultivars are among the most susceptible to the disease, unlike Mondial cultivar (Karim and Hossain, 2018). So the studies illustrate that the highest genetic similarity was found between Bellini and Spunta as the closest but the lowest value was found between Mondial and Bellini as most distant (ELHetawy, 2018). Genetic distance and Similarity coefficient were estimated between the three cultivars of potato by d determining the protein contents (Amelio and Tagarelli, 2018). So, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is the most one techniques to differ the protein. This technique is most one used to evaluate the proteins in complex extracts, and resulting from the discontinuous SDS-PAGE system first mention by Laemmli (1970). This system relies on two types of gels - a solvent gel (aka running) in which the proteins are crucial based on their molecular weights (MWs) and a stacking gel in which proteins are centered prior to entering the resolving gel (Pavlova et al., 2018). Genetic multiplicity is the source for the existence of plants in nature and for improved yields. By studying the genetic differences between potato varieties, it helps the breeder to obtain information on diversity in plant genetic resources, thus increases the chance to improve and produce new varieties with desirable characteristics (Priya et al., 2018). Therefore, the research aimed to isolate the causal pathogen of bacterial wilt and differentiate between three varieties of potatoes that are widely cultivated in Egypt in terms of their susceptibility infection and resistance to benefit from these results in the breeding programs.

2. Materials and Methods:

2.1. Isolation of R. Solanacearum from different potato fields in Egypt:

Brown rot samples of potato tuber cultivars (Spunta, Bellini, Lady rosetta and Mondial) collected from different potato districts at different governorates *i.e.* Beni-Suef (Al Maymun village), which located between the latitude: 29.0667° N, longitude: 31.0833° E, Menoufia (Telia1, 2 and El-Basha village), which located between the latitude: 30.5972° N, longitude: 30.9876° E, Beheira (El-Bostan and Om Sabir village), which located between the latitude: 30.8481° N, longitude: 30.3436° E, and El- Sharqia (El-salhia city), which located between the latitude: 30.7327° N, longitude:31.7195° E, were washed in running spout water, surface disinfected by submersion in ethyl alcohol 95% and flamed. For each sample cores of 5–10 mm in diameter and 5 mm in length, containing main vascular and cortical tissues were macerated in 10 ml sterilized water. Then serial-diluted and streaked on triphenyl tetrazolium chloride medium (Kelman'TZC, 1954). Petri-dishes were incubated at 28 ± 2 °C for 24–48 h. This medium was proposed by Kelman (1954), and modified by Sujeet et al., 2017.

2.2. Morphological and physiological properties of isolated Ralstonia solanacearum isolates:

Previously isolated bacteria from potato brown rot tubers were identified based on the morphological, physiological and cultural studies, as described by Kelman (1954). Cultural and physiological properties, as well as morphological characterizations were studied through grown on three types of media SMSA, King's B and TZC petri-dishes at 28 ± 2 °C for 48 h (Mutimawurugo et al., 2019). The colony's colour, shape, size, surface, margin, elevation, sporulation, gram reaction and motility were esteemed. As stated by Timila and Manandhar (2016) the isolates of *R. solanacearum* were characterizes into biovars on the basis of oxidation of disaccharides and hexose alcohols including (Lactose, Maltose, Cellobiose, Mannitol, Sorbitol and Dulcitol).

The biochemical properties of the isolated bacterial isolates were submited to various biochemical tests including Gram's stain, oxidase test (Pradhanang et al., 2000), gelatin and starch hydrolysis (Fahy and Persley, 1983), and arginine dihydrolase activity (Zhou et al., 2012).

2.3. Molecular characteristics:

Serological characteristics such as Immunofluorescence antibody stains (IFAS) and Quantitative, Real-time, Fluorogenic PCR (*Taq*-Man) were carried out as stated by Puaprasert et al., 2018; García et al., 2019 .This procedure was conducted at Potato Brown Rot Project (PBRC), Agric. Res. Center, Giza- Egypt. *Taq*-Man probe and primers (thermo scientific cat No. 0721), Bangalore, India, were used as shown in Table 1. Primers and probe were prepared by using Primer Express version 1.0 software by

Table 1
list of primers and *TaqMan* probe used to detect pathogenic *Ralstonia solanacearum* (biovar 2/race 3).

Primers	
B2-1-FS'	TGG CGC ACT GCA CTC AAC − 3'
B2- 11- R5'	AAT CAC ATG CAA TTC GCC TAC G − 3'
Probe	
B2-P5' - [VIC]	TTC AAG CCG AAC ACC TGC TGC AAG - [TAMRA]-3 '.

adapting previous *R. solanacearum* PCR protocols. *Taq*-Man probe and primers for biovar 2A-specified assess were illustrate from the biovar 2A-specified DNA sequence. The system use an oligonucleotide identified probe, prepared to link to amplified target DNA between a pair of target-specified PCR primers. The probe is marked at each end with two dyes: a reporter and a quencher. While, the probe is flawless, any fluorescences released by the reporter dye was absorbed by the quencher. When the two dyes be spatially separated (e.g. if the probe was degraded), then fluorescence was released. This generic precept is named Fluorescence Resonance Energy Transfer (FRET). Throughout a *Taq*-Man assess, if target DNA is ready, the primers bind to it and DNA amplification occurs, as in PCR. This releases the reporter dye from the quencher, and rises in fluorescence is identified, detection the sample is positive for the target organism.

2.4. Susceptibility of Spunta, Bellini and Mondial three potato cultivars against bacterial wilt, caused by Ralstonia solanacearum.

Inoculum preparation: The inoculums of previously identified five pathogenic isolates of *R. solanacearum* (RS1, RS2, RS3, RS5, and RS8) were used. The inoculum was prepared by incubating the bacteria for a day in nutrient broth on a shaker running at 120 rpm. Then the bacteria concentration was adjusted to 10^8 cells ml $^{-1}$ by spectrophotometer measures. All suspensions were directed to the cell count technique which gives a reliable count for only life cells (Andreas et al., 2009).

Potato tubers of Spunta, Bellini and Mondial cultivars were obtained from Potato Brown Rot Project (PBRC), Agric. Res. Center, Giza- Egypt, to the assess their susceptibility. The inoculum of these isolates was adjusted to 10^8 and inoculated four weeks old healthy seedlings of potato plant cultivars grown in 25 cm diameter plastic pots containing 10 kg pest-free sandy-clay soil (1/1, v/v). Soil used in the experiment was previously bacteriologically checked to ensure *R. solanacearum* free. Inoculation was done using the previously prepared bacterial suspension 10^8cfu/ml by the stem puncture technique described by Kumari and Ranjan (2019), Artificial infection of soil was done by soaking 250 ml of bacterial suspension per potting soil (25 ml / kg soil). Similarly, control treatments were carried out using sterile water instead of bacterial inoculum. The inoculated plants in pots were coated with polyethylene bags for three days, at 28 ± 2 °C under green-house conditions, then bags were detached and the pots were irrigated daily. Four pots as replicates were used for each source of tested isolate and arranged in a randomized complete block design. Inoculated potato seedlings were observed for disease assessment plant growth parameters such as number of tubers and weight of tubers / plant, as well as percent reduction of disease incidence and disease severity were calculated as follows:

Disease assessment: The percentage of disease incidence (DI) was recorded 40 days after inoculation with the pathogen.

The percentage of disease severity (DS) was calculated from disease rating for each plant periodically according to the scale proposed by Messiha et al. (2007) describing the wilt symptoms in the plant foliage as follows:

0 = no symptoms 1 = up to 25% 2 = 26–50%
3 = 51–75% 4 = 76–100% 5 = dead plants
DS was calculated by the following formula:

$$DS = \frac{\sum R.T}{5xN} x100$$

T = Total number of wilted plants with each category
R = Disease ratting scale R (R = 0, 1, 2, 3, 4 and 5)
N = Total number of tested plants
Percent reduction of disease incidence and severity were calculated by the following formula:

$$Percent\ Reduction = \frac{C - T}{C} X100$$

C = control T = treatment

2.5. Assessment of potato cultivars by evaluate protein contents using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE):

This procedure was conducted at the Biotechnology Lab at Cairo University Research Park, Faculty of Agriculture, (CURP). SDS-PAGE is an electrophoresis method that allows protein separation by weight according to (Laemmli, 1970). To evaluate the three cultivars of potato which used in this experiment, the test was carried out in several steps.

Newly grown leaves were taken from the control treatment in this experiment for the three potato cultivars and extracted by using 1.0 ml of 0.05 M Tris –Hcl buffer. Twenty microliters of β mercapto ethanol added to partitionate the proteins to small sub-units. The mixture was centrifuged at 12000 rpm for five minutes.

Fifteen microliters of each protein extracts of each potato cultivars genotype were resolved in 12% poly acrylamide –SDS resolving gel, to separate the protein sub-units according to their molecular weights.

Sample loading, electrophoresis and gel staining: For each one of slot gels, 15 μl of each leaves extract sample was loaded, electrophoresis and gel staining according to (Kim and Cho, 2019). The gels were photographed and the protein bands were scored. The electrophoretic products compared with protein bands of a ladder, from the Bio- Rad manual for the Gel Slab Dryers model 483.

2.6. Genetic distances and similarities based on pathogenic and plant growth parameters:

The genetic similarity coefficient among potato cultivars based on differences in pathogenic and plant growth parameters was calculated according to the Pearson coefficient using the IBM SPSS statistics program (George and Mallery, 2019). The genetic distance was estimated, and the phylogenetic dendrogram was generated by the clustering analysis method (Amelio and Tagarelli, 2018) using STATISTICA 8 program (Weiβ, 2007).

2.7. Genetic distances and similarities based on SDS-PAGE profile:

Patterns of the SDS-PAGE bands were recorded as present (1) or absent (0), and each was treated as independent. Genetic diversity was determined by comparing the banding patterns of all genotypes at the standard level of protein banding patterns. Genetic similarity among genotypes was calculated according to Dice coefficient measurement (Dice, 1945) using the same program as previously mentioned in Genetic distances and similarities based on pathogenic and plant growth parameters.

2.8. Statistical analysis:

The results of the previous experiments were statistically analyzed according to the procedures reported by Sneedecor and Cochran (1980) and Statistix 9 computer program was then used to analyze statistically the obtained data and mean separation as stated by Duncan's multiple range test (p < 0.5).

3. Results

3.1. Isolation of R. Solanacearum from different potato fields in Egypt:

Eighty samples were obtained from different naturally diseased potato plants screening symptoms of bacterial wilt collected from Spunta, Bellini, Lady-rosetta and Mondial cultivars at different localities in four governorates (Beni-suef, Menoufia, Beheira and El-sharqia). Data in table 2 show the number of isolates obtained from different localities. Only nine isolates among the collected samples were positive against Ralstonia solanacearum. Four virulence isolates from Spunta cultivars, including three isolates achieved of Al-Maymun village, Beni-suef governorate and one isolate acquired from Telia (1), Menoufia governorate. Four isolates from Bellini cultivars, two from Al Maymun village, Beni-suef governorate and two from Om Sabir village, Beheira governorate and one isolate from Lady-rosetta obtained from Telia (2), Menoufia governorate. On the other hand, 71 isolates were negative. Fig. 1 show the different between healthy and artificially typical infected wilted potato plants (Spunta cv.) as well as the bacterial ooze and the character of virulence colonies isolated from infected naturally plants. As the vascular bundles in the diseased plants are filled with bacteria that impede the transport of water and nutrients, and thus the symptoms appear, which are yellowing of the leaves, redness of the vascular bundles, necrosis, and ultimately the total wilt of the infected plants.

3.2. Detection of pathogenic isolates:

3.2.1. Morphological and physiological properties of the isolated Ralstonia solanacearum.

Nine isolates from different habitats were subjected to complete identification up to species level. All identified isolates were Gram negative, short rods, motility and oxidase positive reaction. These isolates were non-sporulation, negative reaction for Arginine dihydrolase, starch hydrolysis and gelatin liquefaction. Fluorescent pigments could be detected with UV on King's B medium, but production of brown pigments on glucose nutrient agar medium was detected. No variation could be detected among the isolates in these characteristics except for the motility test in which the phenotype conversion mutants (PC- type) variant isolates were more motile, and for the oxidation/fermentation (O/F) test in which the virulent isolates were aerobic while the PC-type variant ones were microaerophilic. As for the utilization of carbon sources is concerned all the pathogenic isolates mentioned were utilized glucose, lactose, maltose and cellbiose. On the other hand, none of the examined isolates was able to utilize sorbitol, mannitol and dulcitol.

Cultivation on SMSA, King's B and TZC media: To differentiate between virulence (RS5) and avirulence (RS7) isolate of *R. solanacearum*, the isolates were cultivated on SMSA, King's B and TZC media. Data in table 3 and Fig. 2 explained the characters of RS5. Virulent colony isolate developed on the SMSA medium were slimy, white, irregular and fluidal with pink-red coloration in the center, whereas the PC-type variant ones was less fluidal or a fluidal, uniformly round, butyrous, dry and ultimately deep pink to red as shown in Fig. 3. Triphenyl tetrazolium chloride medium (TTC or TZC), containing 2, 3, 5 triphenyl tetrazolium chloride was used to differentiate between the virulent (wild-type) and avirulent (PC-type variant) isolates of the bacterium. Differentiation between the two forms were easily noticed on media containing 2,3,5 triphenyltetrazolium chloride. Colonies of the avirulent isolate (RS7) was uniformly round, butyrous and deep red in color due to the formation of formozan on tetrazolium-containing medium, contrary to the virulent ones. But the virulent one was highly fluidal, slimy, irregular, mucous creamy-white with pink centers. The virulence colonies on King's B medium had the same characteristics on modified SMSA and TZC media. But the coloration of virulence and non–virulence colonies on King's B medium were brownish and white, respectively.

3.3. Molecular characteristics:

Identification methods for the pathogenic isolates were carried out through using Immunofluorescence antibody stains (IFAS) and Quantitative, Real-time, Fluorogenic PCR (*Taq-Man*). The most five *R. solanacearum* pathogenic isolates showed fluorescent short rod shape cells and evenly stained as bright green fluorescent under immunofluorescence microscope (Fig. 4). Also, the result represent that the probe used in *Taq-man* was of the type (B2) capable to detect only biovar 2 of *R. solanacearum* bacterial wilt. Furthermore, primer and probe are specific for detection of the race 3 biovar 2 strain. Positive results were obtained in both assays with all five isolates. The curve approached from number (1) on horizontal axis which indicates to cycle number was the most virulence isolate compared to negative control. . So the isolate (RS5) was the most virulence one, followed by RS1, RS3, RS2 and RS8, representative that the tested isolates were *R. solanacearum* race 3, biovar 2.

Table 2
Number of positive *Ralstonia solanacearum* samples collected from different cultivars at different localities in four Egyptation governorates.

Governorate	No. of sample	Cultivar							
		Spunta		Bellini		Lady rosetta		Mondial	
		(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Beni-suef	15								
Al Maymun		3	1	2	3	–	–	*	6
Menoufia	30								
Telia (1)		1	*	*	4	*	6	*	*
Telia (2)		*	*	*	6	1	*	–	–
El-Basha		*	8	*	*	*	*	*	4
Beheira	25								
El-Bostan		*	7	*	9	*	5	–	–
Om Sabir		*	*	2	*	*	2	–	–
El-Sharkia	10								
El-salhia city		*	4	–	–	–	–	*	6
Total	80	4	20	4	22	1	13	0	16

(*) Samples don't collected from this site.
(−) Negative reaction during isolation.
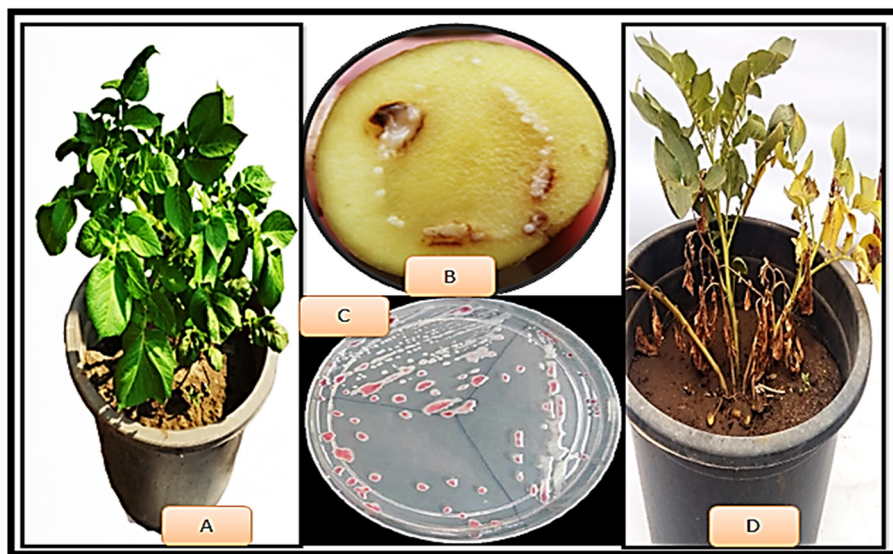(**+**) Number of positive reaction during isolation

Fig. 1. Typical symptoms of bacterial wilt disease on potato plants (Spunta cv.), caused by *R. solanacearum*. (A) Healthy plant, (B) bacterial ooze and colony growth (C) virulence colonies of bacteria and (D) wilted plant.

Table 3
Cultural characterization of virulent (v) and avirulent (av) *Ralstonia solanacearum* isolates on the used three different media.

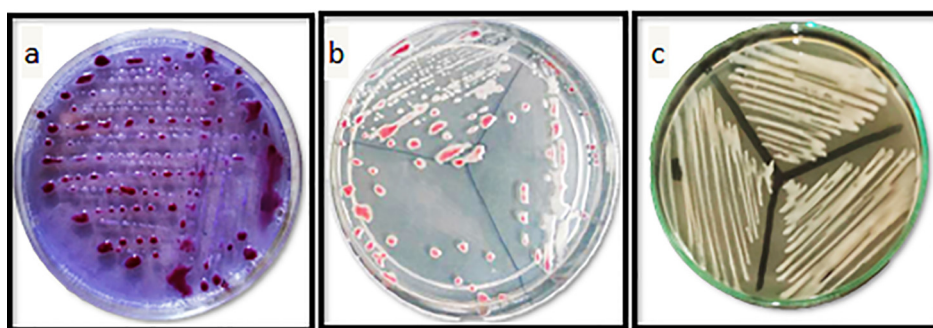| Colony character	Medium					
	SMSA		TZC		K. B	
	v	av	v	av	V	av
Margin	Entire	Entire	Entire	Entire	Entire	Entire
surface	smooth surface	smooth surface	smooth surface	smooth surface	smooth surface	smooth surface
Form	Irregular	Circular	Irregular	Circular	Irregular	Circular
Elevation	Convex	Convex	Convex	Convex	Convex	Convex
colour	White with pink centers	Red	White with pink centers	Red	Brownish	white

Fig. 2. Colony characters of virulent *Ralstonia solanacearum* isolate (RS5) on different media (a) modified SMSA, (b) TZC and (c) King's B medium.

3.4. Susceptibility of Spunta, Bellini and Mundial cultivars to bacterial wilt, caused by Ralstonia solanacearum.

Results in Table 4 demonstrate that significant differences between the treatments. The isolate RS5 showed the lowest percentage of disease incidence reduction on the three potatoes cultivar Bellini, Spunta and Mondial recorded 9.64%, 15.41% and 34.12%, respectively. Followed by the isolate RS1that recorded 18.14%, 23.78% and 45.32%, respectively, while RS3 isolate registered 29.71%, 40.43% and 52.79, respectively. RS2 isolate calculated 45.25%, 56.36% and 60.77%. Finally, RS8 isolate exhibited the highest effective one the percentage of disease reduction on all tested potato cultivars. This isolate recorded 60.60%, 63.21% and 71.66%,

compering to the healthy control treatment. Results presented that potato cultivars reacted differently and varied significantly in their degree of susceptibility. Bellini cv. was the highest in susceptibility, so decreased the average of percentage disease incidence and severity reduction which showed 32.66% and 38.88%, respectively. Followed by Spunta cv. calculated 39.81% and 45.62%. While Mondial cv. was the moderately susceptible this registered 52.94% and 60.16%. Furthermore, the quality of potatoes cultivars reduced with increasing the disease incidence and severity. So the production of potatoes cultivars decreased and thus associated with the number and weight of tubers decreased. The data revealed that the number of tubers in the three cultivars of potato was close, while differences were observed in the weights of the tubers due
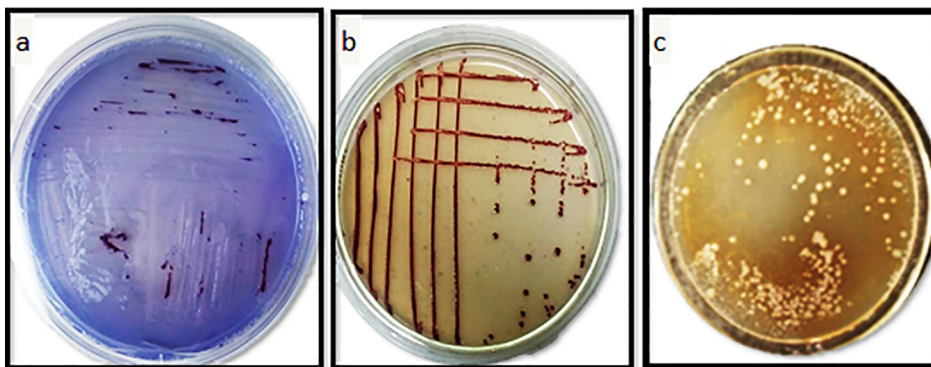
Fig. 3. Colony characters of avirulent *Ralstonia solanacearum* isolate (RS7) on different media (a) modified SMSA, (b) TZC and (c) King's B medium.
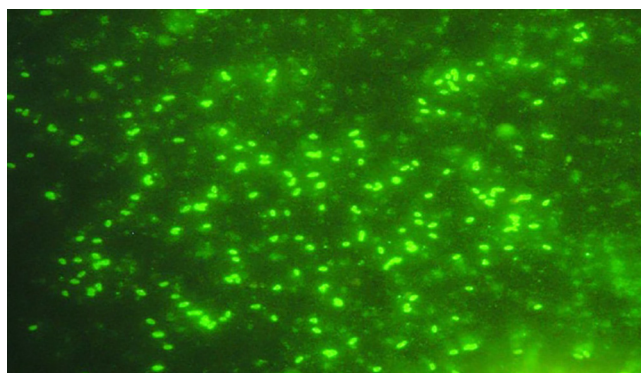

Fig. 4. Positive reaction of race (3), biovar (2) of *R. solanacearum* isolate (RS5) in the serological immunofluorescent antibody staining (IFAS) test.

to their size. Mondial cultivar was the largest average of weight and recorded that 86.50 g, followed by Spunta cultivar was calculated 71.84 g, and finally Bellini cultivar was the lowest one and recorded 64.37 g.

3.5. Genetic distances and similarities based on pathogenic and plant growth parameters:

The genetic distance and genetic similarity among potato cultivars based on pathogenic and plant growth parameters were indicated in Table 5. The results illustrate that the highest genetic similarity (0.998) was found between Bellini and Spunta as the closest but the lowest value (0.946) was found between Mondial and Bellini as most distant. The same results show that the highest genetic distances value (19.0) was found between Bellini and

Table 4
Pathogenic and plant growth parameter of the three potato cultivars inoculated with the five virulent *Ralstonia solanacearum* measured as percentage reduction of disease incidence and disease severity, number of tubers and weight of tubers.

Treatment	Pathogenic parameter		Plant growth parameter	
	DI redution (%)	DS reduction (%)	No. of tubers	Weight of tubers (g/plant)
Spunta + RS1	23.68 ± 1.1	31.80 ± 1.5	3.25 ± 0.03	62.43 ± 1.2
Spunta + RS2	56.36 ± 2.3	60.46 ± 1.6	4.25 ± 0.02	89.85 ± 1.3
Spunta + RS3	40.43 ± 1.2	45.11 ± 1.4	3.00 ± 0.04	63.12 ± 1.9
Spunta + RS5	15.41 ± 0.9	20.39 ± 0.7	3.00 ± 0.03	55.12 ± 1.5
Spunta + RS8	63.21 ± 2.5	70.68 ± 2.2	4.25 ± 0.05	88.70 ± 2.1
Average	39.81 ± 1.6	45.62 ± 0.8	3.55 ± 0.04	71.84 ± 2.6
Spunta	100 ± 2.5	100 ± 3.5	4.50 ± 0.03	128.18 ± 4.2
Bellini + RS1	18.14 ± 0.8	23.58 ± 1.1	3.25 ± 0.04	59.23 ± 2.1
Bellini + RS2	45.25 ± 1.4	51.10 ± 2.1	4.00 ± 0.02	73.03 ± 2.6
Bellini + RS3	29.71 ± 1.5	35.02 ± 2.3	3.75 ± 0.03	64.50 ± 2.9
Bellini + RS5	9.64 ± 0.7	17.25 ± 0.9	2.75 ± 0.01	43.97 ± 2.4
Bellini + RS8	60.60 ± 2.2	67.86 ± 3.2	4.50 ± 0.04	81.16 ± 2.8
Average	32.66 ± 1.2	38.88 ± 2.8	3.65 ± 0.03	64.37 ± 2.8
Bellini	100 ± 1.9	100 ± 2.5	4.75 ± 0.03	127.53 ± 3.9
Mondial + RS1	45.32 ± 1.8	50.90 ± 1.6	3.00 ± 0.02	71.08 ± 2.8
Mondial + RS2	60.77 ± 1.6	70.24 ± 2.1	4.25 ± 0.03	112.31 ± 4.6
Mondial + RS3	52.79 ± 1.7	61.02 ± 2.3	3.50 ± 0.01	81.65 ± 3.1
Mondial + RS5	34.17 ± 1.2	40.49 ± 2.2	2.25 ± 0.02	47.39 ± 1.5
Mondial + RS8	71.66 ± 2.8	78.14 ± 3.1	4.25 ± 0.03	120.08 ± 4.2
Average	52.94 ± 2.4	60.16 ± 2.9	3.45 ± 0.04	86.50 ± 3.5
Mondial	100 ± 3.6	100 ± 2.92.8	5.00 ± 0.03	145.26 ± 4.9
LSD 0.05 for		Interaction		
Cultivar 2.319		cultivar*isolates 5.036		
Isolates 2.082		DI for cultivar*isolates 1.037		
DI for cultivar 1.059		No. of tuber for cultivar*isolates 1.380		
DI for isolates 1.293		weight for cultivar*isolates 15.729		
No. of tuber for cultivar 1.924				
No. of tuber for isolates 2.035				
weight for cultivar 12.684				
weight for isolates 9.572				

Table 5
Genetic distance and similarity coefficient (Pearson similarity) calculated based on differences in pathogenic and plant growth parameters between the three potato cultivars.

Cultivar	Spunta	Bellini	Mondial
Genetic distance			
Spunta	0.0		
Bellini	2.9	0.0	
Mondial	16.7	19.0	0.0
Similarity coefficient			
Spunta	1.000		
Bellini	0.998	1.000	
Mondial	0.958	0.946	1.000

Mondial as the most distant genetically, while the lowest value (2.9) found between Bellini and Spunta cultivars.

The phylogenetic tree based on the genetic distance (Fig. 5) showed two clusters according to the data scored from pathogenic and plant growth parameters, clustering analysis subdivided the investigated cultivars into two groups. Each of Spunta and Bellini cultivars formed cluster I, and Mondial formed an independent cluster (cluster II).

3.6. Genetic distances and similarities based on SDS-PAGE profile:

SDS-PAGE profiles of total dissolved proteins contents obtained from the resistant and susceptible plants of three potato cultivars, as well as the control is presented in Fig. 6. SDS-PAGE analysis illustrated that nine protein bands with various molecular weights (MWs) reached from 275 to 6.5 kDa. The results in Table 6 explain that the genetic distance and similarity coefficient (Dice similarity) calculated based on differences in protein banding patterns between the three potato cultivars. The highest genetic distances value (2.00) was found between Mondial and Bellini as the most distant genetically, while the lowest value (1.73) found between Bellini and Spunta cultivars, but the highest similarity value (0.842) was found between Bellini and Spunta as the closest but the lowest value (0.778) was found between Mondial and Bellini as most distant.

The phylogenetic tree based on the genetic distance between three cultivars of potato by banding patterns of protein SDS-
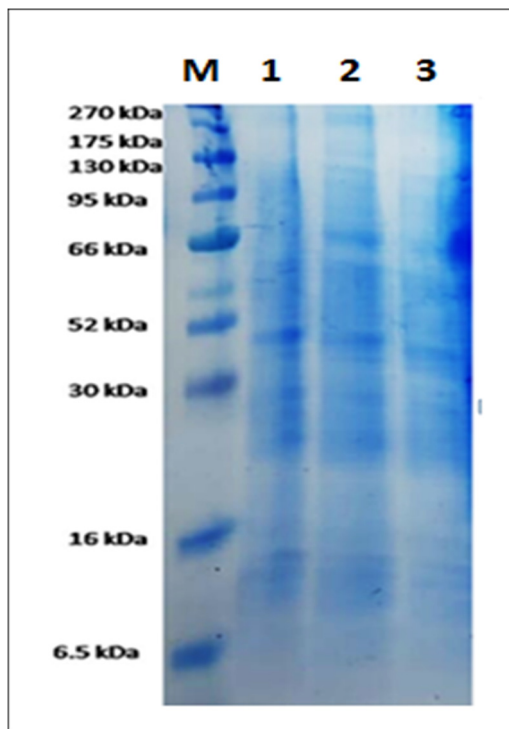

Fig. 6. Differentiate between potato cultivars by protein SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (M) Marker, (1) Spunta cultivar, (2) Bellini cultivar and (3) Mondial cultivar.

PAGE is shown in Fig. 7. the dendrogram separated all cultivars into two clusters. First cluster divided into two sub-clusters formed a separate sub-cluster with Spunta, and the second sub-cluster included Belini. While the second cluster included one cultivar Mondial. These results were consistent with the pathological studies, as they showed that the potato cultivars Spunta and Bellini were the closest to each other genetically. Therefore, their sensitivity to disease was the closest while it was high, as was shown by the pathological studies.
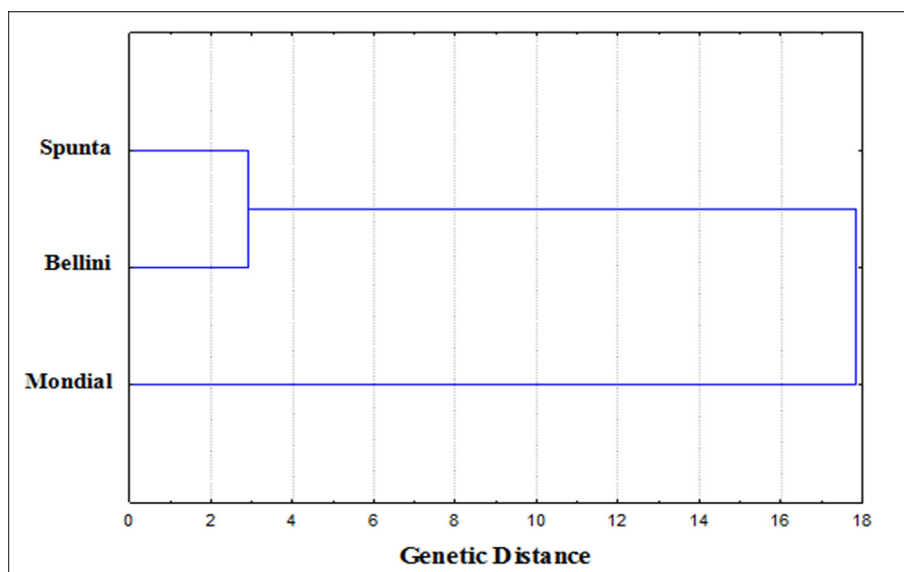

Fig. 5. Phylogenetic tree (Linkage dendrogram) of studied potato cultivars based on their pathogenic and plant growth parameters.

Table 6
Genetic distance and similarity coefficient (Dice similarity) calculated based on differences in protein banding patterns between the three potato cultivars.

Cultivar	Spunta	Bellini	Mondial
Genetic distance			
Spunta	0.00		
Bellini	1.73	0.00	
Mondial	1.73	2.00	0.00
Similarity coefficient			
Spunta	1.000		
Bellini	0.842	1.000	
Mondial	0.800	0.778	1.000

4. Discussion:

Ralstonia solanacearum the causal pathogen of potato bacterial wilt, has been recorded earlier in Egypt many years ago (Farag et al., 2017) based on symptomology only. *R. solanacearum* is measured one of the greatest serious problems of potato production (Ramesh et al., 2009). In Egypt disease caused economic losses that affect potato yield and threatens potato exportation to European Union (Farag et al., 1999).

Under those circumstances, *R. solanacearum* is listed as a regulatory pathogen in A2 group (the group of quarantine pest which can be present in the location but cannot be widely distributed there and has to be officially controlled) quarantine organism (Database, 2017) and is listed by Asia and Pacific Plant Protection Commission (APPPC) and International Association of Professional Security Consultants (IAPSC). The occurrence of different races and strains of the pathogen with varying virulence under different environmental conditions presents a serious danger to European and Mediterranean potato and tomato production. Therefore, the absence of the bacterium is an important consideration for countries exporting seed potatoes (CABI, 2017). Moreover, these bacteria attacks more than two hundred plant species, including more than fifty plant families (Aslam et al., 2017; Uwamahoro et al., 2020). The pathogen of bacterial wilt is the most destructive bacterial pathogen due to its infestation, uncommonly wide host range, perseverance, and wide topographical distribution (Wei et al., 2018). Therefore, we are confronted to study this disease. The disease was limited to some governorates of Egypt where potato cultivation is abundant.

In this study eighty samples were accessed from different naturally infected potato plants exhibition symptoms of bacterial wilt obtained from different cultivars (Spunta, Bellini, Lady-rosetta and Mondial) at different localities in four governorates (Beni-suef, Menoufia, Beheira and El-Sharqia). The results showed that the highest number isolates were obtained from Spunta and Bellini cultivars, due to their highly susceptible to bacterial wilt pathogen. The highest percentage of positive samples were obtained from in Beni-suef governorate. Isolates obtained during isolation were detected as *Ralstonia solanacearum* by using morphological and physiological properties (Kubota et al., 2008).

Using the traditional identification methods based on morphological, physiological and biochemical characteristics of bacteria. The results indicate that all tested isolates were non-spore, short rods, motile bacteria and Gram negative. The isolates exhibited positive reaction for oxidase activity and negative reaction for Arginine dihydrolase, starch hydrolysis and gelatin liquefaction. As for the utilization of carbon sources is concerned all pathogenic isolates were capale to utilize glucose, lactose, maltose and cellbiose. On the other hand, none of the observed isolates were capable to utilize sorbitol, mannitol and dulcitol. Such result was identical with (Pradhanang et al., 2000). *R. solanacearum* divided into five biovars based on ability of races to oxidize three hexose sugar alcohols (dulcitol, mannitol, and sorbitol), and three other of disaccharides (cellobiose, lactose, and maltose), according to Mutimawurugo et al., 2019. Accordingly, biovar (1) can't analyze these sugars, while biovar (2) can analyze only disaccharides, biovar (3) can analyze all of them and biovar (4) analyze only hexose alcohols, while biovar (5) can analyze all sugars except dulcitol and sorbitol as stated by Sagar et al. (2014).

Growth of pathogenic *R. solanacearum* on three differentiated media as used to between virulence and avirulence. The characters of virulent isolate (RS5) colony developed on SMSA medium were slimy, white, irregular and fluid with pink-red coloration in the center. Whilst using TZC medium, to differentiate between virulent and avirulent isolates. Colonies of the avirulent isolate (RS7) were
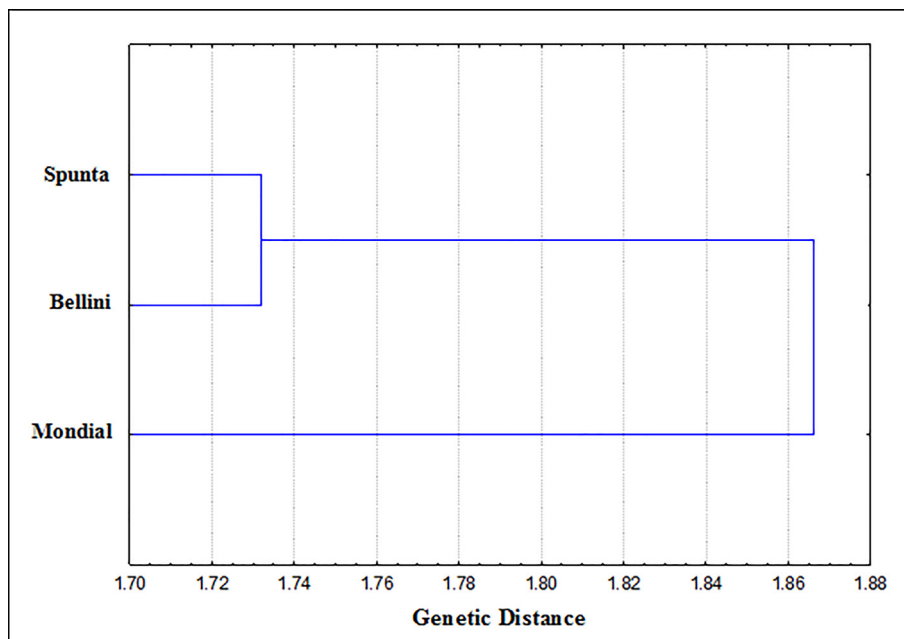

Fig. 7. Phylogenetic tree (Linkage dendrogram) of studied potato cultivars based on their protein banding patterns.

consistently round, butyrous and deep red in color due to for-mozan formation on tetrazolium-containing medium, contrary to the virulent ones. But the virulent ones were highly fluidal, slimy, irregular, mucoid creamy-white with pink centers. The virulence colonies on King's B medium had the same characteristics on mod-ified SMSA and TZC media. But the coloration of virulence and non– virulence colonies on King's B medium were brownish and white, respectively. The methods newly advanced for the revealing of the pathogen in different habitats have qualitatively improved the pathological studies. The obtained results were consistent with Sujeet et al. (2017) results showed that single colonies of these bacteria appear after their growth for 36 to 48 h at a temperature of 28 °C and the virulence colonies were characterized by white or cream colored, irregularly shaped, highly fluidal and opaque. Infre-quently, these types of non-virulence colonies appear and were characterized by round, smaller and butyrous or dry. A Kelman's selective nutrient tetrazolium chloride (TZC) medium (Kelman, 1954) capable to discriminate between the two colony categories on this medium. Strains of *R. solanacearum* have been classified as mentioned before into five biovars (Santiago et al., 2017), and five races (Bin et al., 2010).

The causal pathogen of potato brown rot *R. solanacearum* is clas-sified nowadays into five races affecting hosts in different plant families (Ravelomanantsoa et al., 2018). Race 3 biovar 2, the so-called potato race, is the dominant strain in Egypt (Serag et al., 2020) which is characterized by low virulence to tobacco and lower optimum temperature (Buddenhagen and Kelman, 1964).

Furthermore, the results revealed that fluorescent short rod shape cells of pathogenic bacteria stained evenly as bright green fluorescent under the immunofluorescence antibody stain (IFAS) to confirm the characterization of *R. solanacearum* in potato tissue and is considered as another achievement technique (Janse, 1988). According to Janse et al. (2004), using an immunofluorescence antibody stain to *R. solanacearum* biovar 2 was shown to be detect-able as few as 10^4 CFU per mL in potato tissue. The sensitivity of serological methods for the detection of *R. solanacearum* in potato or in soil can be increased by applying an enrichment procedure, for example by inoculating extracts in a selective broth (Shahbaz et al., 2015; Sujeet et al., 2017).

The hierarchical classification is sub-divided into four phylo-types (genetic groups) according to analysis of the 16S-to-23S internal transcribed spacer region, and each of them is further sub-divided into smaller groups called sequevars. Every phylotype reverse the topographical origin of varieties. The fraction of phylo-type II embraces race 3 biovar 2 (Williamson et al., 2002). The strain obtained results indicated that *R. solanacearum* in Egypt belong to phylotype II sequevar I, which is also included the Amer-ican strains (Pardo et al., 2019).

The result represent that the probe used in *Taq-man* was of the type (B2) capable to detect only biovar 2 of *R. solanacearum* bacte-rial wilt. The isolate (RS5) was the most virulente one, followed by RS1, RS3, RS2 and RS8, representative that the tested isolates were *R. solanacearum* race 3, biovar 2. *Tag-Man* is a molecular revelation method to join polymerase chain reaction (PCR) with fluorescent detection of the amplicon (Puaprasert et al., 2018; Birhane et al., 2020). The RS primers and probe detected all biovars and races of virulent *R.solanacearum* whereas the B2 primer and probe are specific for identification of the phylotype II sequevar I, equivalent to Race 3 biovar 2 strain. These results agree with the finding of (Malko et al., 2019).

This study planned to clear up the sensibility of potato varieties to *R. solanacearum* was applied to screen variety Egyptian potato varieties for their sensibility to the pathogen as foundation to recover the administration of potato bacterial wilt. No resistance against *R. solanacearum* is obtainable in the three tested potato cul-tivars in the present research, however the level of susceptibility

diverse significantly amongst them. As all known potato cultivars in Egypt are susceptible; breeding programs should to research of resistance in the wild solanum types that display tolerance or resistance the disease to accomplish positive results (Poudel and Neupane, 2018).

The results of the study aimed that there are losses in potato production in infected plants compared to healthy ones, due to the severity of the disease and spreading the bacterial pathogen into plant tissues. *R. solanacearum* is a soil-dwelling bacterium that invades plants through roots and colonizes xylem vessels (Karim and Hossain, 2018). This study was compatible with some other studies, the potato production and yield losses due to bacterial wilt as high as 100 per cent have been reported in parts of tropical Africa (Shivani, 2016). In Kenya, the potato industry is threatened by bacterial wilt (BW) because soils in most production areas are infested with the wilt causing bacterium and over 50% yield losses have been reported (IPDN, 2014). The farmers reported experienc-ing yield losses ranging from 5% to 80% due to bacterial wilt. According to some recent studies, the disease is found in all the potato growing areas of Kenya and the country is affecting 77% of potato farms which had been introduced with tuber seeds imported from Europe (Kaguongo et al., 2010). Potato yield losses in Uganda estimated about 30% (IPDN, 2014)), with more severe losses being 100%. Kabeil et al. (2008) reported that potatoes were one of the largest exported crops in Egypt. Yet, the total value of Egyptian potato exports fell from a peak value of US$ 102.12 mil-lion in 1995 to $US 7.7 million in 2000 mainly due to this organism related quarantine restrictions imposed by the European Union (EU) which used to account for about 70–90% of Egyptian potato exports and it represented a drop from approximately 419,000 metric tons to 48,500 tons.

The results illustrated that through the phylogenetic tree based on the genetic distance of both the recorded data from the patho-gen and plant growth parameters, as well as from the results of the protein analysis (SDS-PAGE) of the three potato cultivars. Data showed two groups from clustering analysis divided the studied items into two groups. The Spunta and Bellini varieties each formed the first group, and Mondial formed an independent group (Group II). Therefore, their sensitivity to disease was the closest while it was high, as was shown by the pathological studies. This is due to the similarity in terms of upbringing and also some genetic characteristic, so slight significant differences in disease severity between three cultivars were detected and calculated, these due to the common between the varieties in some character-istics. Bellini cultivar was crossed by (Felsina × Mondial), medium early for maturity and Netherlands for origin according to Spanoghe et al. (2015).While, spunta cultivar was crossed by (Bea × USDA 96–56), intermediate early for maturity and Nether-lands for origin, as mentioned by Khidr et al. (2017). Mondial cul-tivar was crossed by (Spunta × SVP VE 66–295), intermediate late for maturity and Netherlands for origin, as stated by Eriksson et al. (2016).

Meanwhile, the potato varieties with the least susceptibility to the disease would be studied when implanting potatoes in fields with a history of bacterial wilt. Therefore, we must go to build up programs to discover resistant and / or tolerant potato varieties (Shimira et al., 2020). SDS-PAGE is an electrophoresis method that allows protein by mass to be separated (Mouzo et al., 2018).This recent method of differentiation between plants. Constructed on the results of this research, we can conclude that the intra-cultivar variances in protein polymorphism may be detected through the electrophoresis patterns applied to content protein through SDS-PAGE in potato varieties. For efficient breeding pro-gram information, genetic diversity within crop varieties is funda-mental. It is especially appropriate for describing individual approached and cultivars and as a general guide in the chosen of

the parents for hybridization. Genetic fingerprinting has been adopted traditionally through the use of isozymes, total seed proteins, tuber storage proteins and further newly through various types of molecular markers (Zhang et al., 2017; Sogbohossou et al., 2018). The diversity within and between identified species is the basis of all crop amelioration programs. Whether all the individuals in the species would have been analogous, thereafter possibly there could not have been any scope for amelioration in plant for different traits. Since the inception of plant breeding programs, the natural variability and divergence between crops has been widely defined and used in the advancement of crop cultivars. However, as time progressed, the natural variability was reduced due to unbalanced breeding practices centering on the progression of only a few traits, the continued use of minor selected genotypes as heterogeneous parents of the amelioration program and the addition of a few salient lines to Many countries through this broaden the genetic similarities between current crop cultivars (Bhandari et al., 2017).

5. Conclusion

In this study, bacterial pathogens was isolated and defined, as well as select the most resistant cultivars, thus avoiding a decrease in the total value of Egyptian potato exports. Molecular genetics methods such as SDS-PAGE profile was used to differentiate between varieties by protein content and determine the genetic diversity between them, which is useful in breeding programs to produce disease-resistant varieties.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

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Data curation, AM Khairy, MRA Tohamy, MAS Ali; Investigation, MRA Tohamy, MA Zayed, MAS Ali ; Methodology, AM Khairy, MRA Tohamy; Resources, AM Khairy, MRA Tohamy, MAS Ali; Software, MA Zayed; Supervision, MRA Tohamy, MA Zayed; Validation, AM Khairy, MRA Tohamy, MAS Ali; Writing – original draft, AM Khairy, MRA Tohamy, MAS Ali.

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