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Characterization of *Ralstonia solanacearum* causing bacterial wilt from major chili growing areas of Pakistan

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ABSTRACT: For proper disease management, accurate diagnosis of the pathogen is essential. Therefore, in the present study *Ralstonia solanacearum* causing bacterial wilt of chili was characterized to determine the distribution of biovars of the bacterium in the eight agroecological zones with varying climatic conditions and edaphic factors. Among all the 114 isolates of *R. solanacearum*, 77% showed mucoid growth while 23% isolates gave non-mucoid growth. Similarly, the isolates with mucoid growth were found positive for hypersensitivity response (HR), while those with non-mucoid growth showed negative HR. All the isolates grew well at 37 °C, while none of the isolates produced its colony at 41 °C. All the 114 isolates of *R. solanacearum* showed positive responses for all the biochemical tests used for confirmation of the bacterium. Out of 114 *R. solanacearum* isolates, 81% were identified as Biovar III while the remaining 19% were recognized as Biovar IV. Biovar III constituted 37 and 70% in the provinces of Punjab and Sindh, respectively, while Biovar IV formed 19 and 30%. On the other hand, in Khyber Pakhtunkhwa and Balochistan, only Biovar III was recorded. Similarly, Biovar III was observed from all the eight agroecological zones of the four provinces of the country and found to be predominant. On the other hand, Biovar IV was recorded from four agroecological zones located in the provinces of Punjab and Sindh. All the isolates yielded a 750-bp band that corresponded to *R. solanacearum*. It is concluded that Biovar III is widely prevalent in the country warranting stringent control measures.

Key words: *Capsicum annuum*, hypersensitive response, distributional variability, Biovar III, agroecological zones.

INTRODUCTION

Bacterial wilt, among bacterial diseases of plants, incited by the bacterium *Ralstonia solanacearum*, is regarded as economically the most important biotic factor in the world posing serious threat to the lucrative production of solanaceous crops. The bacterium, formerly named as *Pseudomonas solanacearum* (Smith) (Smith 1914) and then *Burkholderia solanacearum* (Smith) (Yabuuchi et al. 1992), belongs to the class Betaproteobacteria of the phylum Pseudomonadota comprising gram negative bacteria (Garrity et al. 2005). It is rod-shaped, gram negative, anaerobic, and usually non-motile (Martin and French 1985). The species is widespread in geographical distribution, showed remarkable genetic diversity and infected a large number of host plants. There are upwards of 450 plant species from 54 different botanical families which have reportedly been attacked by the bacterium including ornamentals and cause significant reductions in yield and production of economically important crops like chili, banana, potato, and tomato (Kelman et al. 1994, Wicker et al. 2007).

The losses caused by *R. solanacearum* vary depending on the severity of the infection, the plant species affected, and the local environmental conditions. However, in severe cases, the losses can be as high as 100%, resulting in a total crop failure (Nisa et al. 2022). Because of its diversified and complex nature, it has been characterized and divided into five races (Buddenhagen et al. 1962, Pegg and Moffett 1971, He et al. 1983) and six biovars (Hayward 1964, 1991,

1994, He et al. 1983, Kelman et al. 1994). *R. solanacearum* is regarded as a "species complex" as it has exhibited great genetic variability among the species, and a new hierarchical classification system consisting of species, phylotypes, sequevars, and clones was proposed for the bacterium (Fegan and Prior 2005). The number of sequevars of the bacterium identified until now is 55 (Li et al. 2016, Liu et al. 2017). Moreover, on the basis of polyphasic taxonomic system, the *R. solanacearum* species complex consists of three genospecies, namely *R. pseudosolanacearum*, *R. solanacearum* and *R. syzygii* (Safni et al. 2014).

Biovars I and II are particularly common throughout the world, while Biovars III, IV, and V are prevalent mainly in Asian countries, but in the near past Biovar III was also reported from Florida, state of the United States of America (Ji et al. 2007). Among these biovars and races, race 3 Biovar II is highly destructive strain of potato brown rot and geranium wilt. It is a quarantine pathogen in Canada and Europe as this strain is more adapted to cooler temperatures, but this strain has never been reported from the United States of America after 1999, when it was first introduced in the country from the imported cutting of geranium from Guatemala (Williamson et al. 2002). The pathogen was listed as select agent in the United States of America due to its potential threat to agricultural crops (Lambert 2002).

In Pakistan, crop production is seriously threatened by bacterial wilt caused by *R. solanacearum*, as the pathogen is widening its host range and becoming more aggressive (Shahbaz et al. 2015, Aslam et al. 2017a, 2017b, 2019). Since its first report from Pakistan, subsequent surveys revealed its presence from all the provinces causing severe losses to vegetables and fruits (Begum et al. 2012). The bacterium is soil borne, and its possible dissemination through seed is becoming the worst menace to the cultivation of solanaceous vegetables especially chili in the Punjab and Sindh provinces of the country (Shahbaz et al. 2015). During monsoon seasons, bacterial wilt of chili is aggravated, but this problem is often concealed and mystified with other disease problems. *R. solanacearum* has also been found associated with other soil borne pathogens resulting into disease complexes. As a result, the additive associations among *R. solanacearum* and *Meloidogyne* species resulted in much higher yield losses than the individual associations and made the host plants more prone to the disease (Asghar et al. 2020, Mukhtar and Kayani, 2019, 2020, Shahid et al. 2022, 2023).

Among the major chili growing countries, Pakistan is ranked fifth in cultivation and tenth in terms of production in the world (FAO 2012). The bacterium has been found as a major hindrance for the lucrative cultivation of solanaceous crops including chili in Pakistan. The average per hectare yield of chili (2.54 tons/h) is fairly low in Pakistan than developed countries, which are getting many times higher yields. Among various biotic factors responsible for this low yield, *R. solanacearum* is considered as the major restriction.

For the proper management of the disease, accurate identification, diagnosis, and virulence of the pathogen are essential. As very little information is available in Pakistan about the prevalence and distribution of biovars of *R. solanacearum*, the present study was carried out to characterize the bacterium and to determine the distribution of biovars present in the eight agroecological zones with varying climatic conditions and edaphic factors. The information will help the farming community to devise management strategies accordingly to abate yield losses.

MATERIALS AND METHODS

Collection of *Ralstonia solanacearum* isolates

In toto, 114 isolates of *R. solanacearum* infecting chili were obtained from 14 main chili producing districts of the eight agroecological zones located in the four provinces of the country (Fateh et al. 2022). Chili plants with typical symptoms of the disease were dug up cautiously with rhizospheric soil and brought to the laboratory for further analyses. The common symptoms of bacterial wilt included wilting, yellowing, or browning of leaves, stunted growth, and death of the plant. In some cases, bacterial ooze was visible on the stems or at the base of the plant. Other symptoms included leaf drop, necrosis, and stem rot. The bacterial infection with the diseased plants was confirmed serologically (Opina and Miller 2005). All the isolates were coded accordingly.

Isolation of *Ralstonia solanacearum*

The isolation of *R. solanacearum* was made primarily from the infected stems collected from surveyed fields of each district of eight agroecological zones. The infected stems from the collar region were cut into 10-cm lengthwise sections followed by surface sterilization using 70% ethanol and further chopped into small fragments. The chopped pieces were then placed in sterilized distilled water in a shaker at room temperature for 5 min with continuous shaking. An aliquot of 100 µL of bacterial suspension from each isolate was individually processed on triphenyle tetrazolium chloride (TTC) medium, distributed homogeneously and placed in an incubator set at 28 °C for two days for the growth of bacteria (Englebrecht 1994).

Purification and confirmation of *Ralstonia solanacearum*

Pure cultures of the bacterium were procured from a single colony obtained from each bacterial culture by inoculating aseptically onto nutrient agar and TTC media. The individual colonies were again inoculated on selective medium South Africa media amended with bacitracin, cyclohexamide, penicillin, and TZC to keep from any contamination. Further confirmation of the pure cultures of 114 isolates of *R. solanacearum* was done serologically (Opina and Miller 2005) and by their hypersensitivity response.

Hypersensitive response

Serologically confirmed isolates were assessed for their hypersensitive response on *Nicotiana tabacum*. Bacterial culture (10^8 cfu/mL suspension) from each isolate in sterilized distilled water was made and injected into leaf mesophyll of *N. tabacum* plants with the help of sterilized syringe. For positive control, only distilled water was infiltrated. Each leaf of *N. tabacum* was inoculated twice, and for each isolate, bacterial suspensions were infiltrated in the leaves of three plants by following the same method. Inoculations of tobacco plants were made at 28 °C and assessed after 24 and 48 h for their hypersensitive response, *i.e.*, development of necrosis on the leaves of inoculated plants. After confirmation, the isolates were assigned codes accordingly.

Characterization of *Ralstonia solanacearum*

The isolates of the bacterium were further characterized on the basis of morphology, *i.e.*, by their growth patterns (mucoid and non-mucoid growth) and biochemical tests (Atiq et al. 2022, Khurshid et al. 2022) viz. gram reaction, catalase activity, Levan production (Schaad 1988, Rahoo et al. 2022), KOH loop test (Suslow et al. 1982), oxidase activity (Kovacs 1956), lipase activity, pigment production (King et al. 1954), arginine dihydrolase reaction (Thornley 1960), gas production (Van den Mooter et al. 1987), oxidation, and fermentation activity (Hayward 1964).

Molecular confirmation

For molecular confirmation, the DNAs from the 114 purified isolates of *R. solanacearum* were isolated, quantified and amplified by using the primer pair JHFegl: 5'GACGATGCATGCCGCTGGTCGC 3' and JHRegl: 5' CACGAACACCACGTTGCTCGCATTGG 3'. The polymerase chain reaction (PCR) products electrophoresed through a 1% agarose gel were visualized with ultraviolet light after ethidium bromide staining (Anwar et al. 2022, Ashraf et al. 2022).

Identification of biovars

The bacterial isolates were identified into biovars on the basis of utilization of different sugars. One gram of each disaccharide (maltose, cellobiose, lactose) and hexose alcohol (dulcitol, mannitol, sorbitol) was mixed with 9 mL of sterilized distilled water to make 10% of the solutions. The sugars were sterilized by filtering through 0.2-µm pore size filters (orange

scientific, GyroDisc CA-PC sterile, endotoxin-free, Hydrophilic with catalogue No. 1520012 having cellulose Acetate membrane 30 mm), and from each sugar and carbohydrate, 10 mL was added in 190 mL of Ayer's medium, distilled water serving as control. The medium containing agar was plated, a suspension of bacterial culture (10^8 cfu/mL) was prepared, and 25 µL was taken and inoculated onto the surface of Ayer's mineral base medium amended with carbohydrates. The plates were incubated at 28 °C and observed for the absence or presence of bacterial growth (Hayward 1964, He et al. 1983).

RESULTS

Growth pattern and hypersensitive response of *Ralstonia solanacearum* isolates

Of all the 114 isolates of *R. solanacearum*, 88 (77%) showed mucoid growth, while 26 (23%) isolates gave non-mucoid growth. Similarly, the isolates with mucoid growth were found positive for hypersensitive response, while those with non-mucoid growth showed negative hypersensitive response (Table 1). All the isolates grew well at 37 °C, while none of the isolates produced its colony at 41 °C.

Biochemical characterization

All the 114 isolates of *R. solanacearum* showed positive responses for all the biochemical tests viz. gram reaction, catalase activity, Levan production, KOH loop test, oxidase activity, lipase activity, pigment production, arginine dihydrolase reaction, gas production, oxidation and fermentation activity used for confirmation of the bacterium, as shown in Table 2. Likewise, all the isolates yielded a 750-bp band that corresponded to *R. solanacearum*.

Biovar distribution

Out of 114 *R. solanacearum* isolates, 92 (81%) were identified as Biovar III, while the remaining 22 (19%) were recognized as Biovar IV throughout the country. In the provinces of Punjab and Sindh, Biovar III constituted 37 and 70%, respectively, of all the isolates, while Biovar IV formed 19 and 30%. On the other hand, in Khyber Pakhtunkhwa and Balochistan, only Biovar III was recorded (Fig. 1). Similarly, Biovar III was observed from all the eight agroecological zones of the four provinces of the country and found to be predominant. On the other hand, Biovar IV was recorded from four agroecological zones located in the provinces of Punjab and Sindh (Fig. 2). In the same way, Biovar III was found in all the 14 districts, while Biovar IV was present in nine of the districts except Attock, Nowshera, Karak, Loralai, and Barkhan (Fig. 3). The details of all the isolates are given in Table 1.

Table 1. Details of *Ralstonia solanacearum* isolates with hypersensitive reaction and growth pattern in eight agroecological zones of Pakistan.

Zone	District	Isolates with +ve HR and mucoid growth		Isolates with -ve HR and non-mucoid growth	
		Biovar 3	Biovar 4	Biovar 3	Biovar 4
Indus delta	Thatta	RsTt2, RsTt4, RsTt6, RsTt8, RsTt9	RsTt1, RsTt3, RsTt5, RsTt10	RsTt7	-
	Badin	RsBd3, RsBd4, RsBd5, RsBd6, RsBd7, RsBd9, RsBd10	RsBd1, RsBd2	-	RsBd8
Southern irrigated plain	Mir Pur Khas	RsMp1, RsMp2, RsMp3, RsMp4, RsMp5, RsMp6, RsMp8, RsMp10	RsMp7, RsMp9	-	-
	Umer Kot	RsUm3, RsUm4, RsUm5, RsUm6, RsUm8	RsUm1, RsUm7	-	RsUm2
Sandy deserts	Bahawalpur	RsBw1, RsBw2, RsBw3, RsBw7, RsBw8	RsBw5	RsBW4, RsBw6	-
	Sanghar	RsSg1, RsSg2, RsSg5, RsSg7, RsSg8, RsSg9	RsSg4	RsSg3	RsSg6

continue...

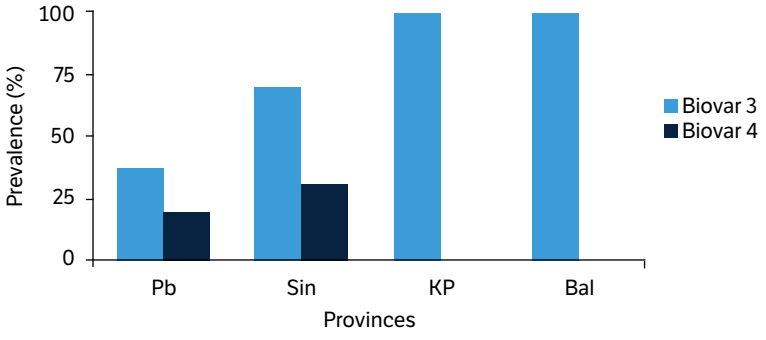
Table 1. Continuation...

Zone	District	Isolates with +ve HR and mucoid growth		Isolates with -ve HR and non-mucoid growth	
		Biovar 3	Biovar 4	Biovar 3	Biovar 4
Northern irrigated plain	Multan	RsMn3, RsMn6, RsMn7, RsMn8	RsMn5	RsMn1, RsMn4	RsMn2
	Pakpattan	RsPP3, RsPP4, RsPP8, RsPP9	RsPP2, RsPP6	RsPP1, RsPP7	RsPP5
	Kasur	RsKr1, RsKr2, RsKr5, RsKr7, RsKr8, RsKr9	RsKr4	RsKr3	RsKr6
Barani areas	Attock	RsAk1, RsAK2, RsAk3, RsAk5, RsAk7, RsAk8	-	RsAK4, RsAK6	-
Wet mountains	Nowshera	RsNw1, RsNw3, RsNw7, RsNw6, RsNw5	-	RsNw2, RsNw4	-
Western dry mountains	Karak	RsKK1, RsKK4, RsKK2, RsKK5	-	RsKK3, RsKK6	-
	Loralai	RsLi1, RsLi2, RsLi4, RsLi6	-	RsLi3, RsLi5	-
Sulaiman piedmont	Barkhan	RsBn1, RsBn4, RsBn5	-	RsBn2, RsBn3, RsBn6	-
No. and % of isolates		72 (63%)	16 (14%)	20 (18%)	6 (5%)

HR: hypersensitivity response.

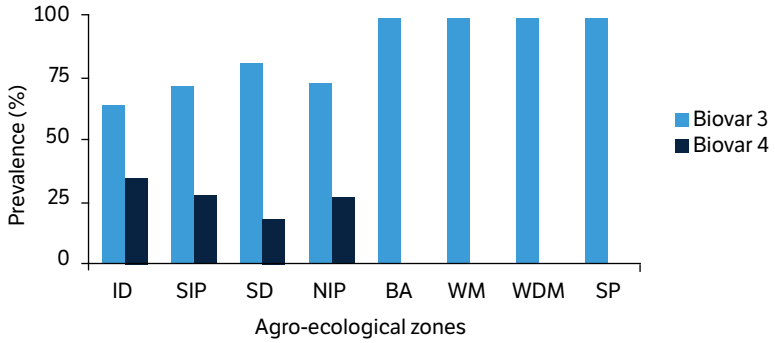
Table 2. Summary of biochemical characterization of *Ralstonia solanacearum* strains.

Sr. No.	Biochemical test	Response
1	Gram staining	All isolates were found gram negative
2	Catalase activity	All isolates were positive for catalase activity
3	KOH Loop Test	All isolates produced loop when mixed with 3% KOH
4	Oxidase activity	All isolates produced purple color and were considered as positive
5	Levan production	No Levan production by any strains and found to be positive
6	Pigment production	All isolates were positive for pigment production
7	Lipase activity	All isolates were positive for lipase activity
8	Gas production	All isolates were positive for gas production
9	Growth at 37 ˚C	All strains grew well at 37 ˚C
10	Growth at 41 ˚C	None of the strains produced its colony at 41 ˚C
11	Aerobic growth	None of the strain tested conferred anaerobic growth. It showed the strict aerobic nature of the bacterium
12	Catalase oxidase test	The fresh pure colonies of R. solanacearum produced gas bubbles when mixed with a drop of hydrogen peroxide and confirmed the bacterium as gram negative



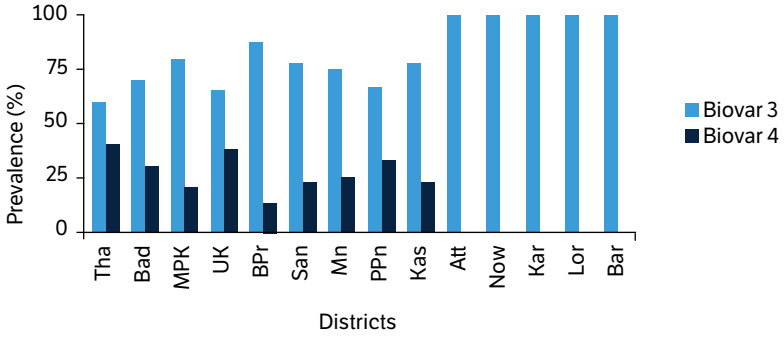
Pb: Punjab; Sin: Sindh; KP: Khyber Pukhtunkhwa; Bal: Balochistan.

Figure 1. Prevalence of biovars of *Ralstonia solanacearum* in the four provinces of Pakistan.



ID: Indus delta; SIP: Southern irrigated plain; SD: sandy deserts; NIP: Northern irrigated plain; BA: Barani areas; WM: wet mountains; WDM: Western dry mountains; SP: Sulaiman Piedmont.

Figure 2. Prevalence of biovars of *Ralstonia solanacearum* in the eight agroecological zones of Pakistan.



Tha: Thatta; Bad: Badin; MPK: Mir Pur Khas; UK: Umer Kot; BPr: Bahawalpur; San: Sanghar; Mn: Multan; PPn: Pakpattan; Kas: Kasur; Att: Attock; Now: Nowshera; Kar: Karak; Lor: Loralai; Bar: Barkhan.

Figure 3. Prevalence of biovars of *Ralstonia solanacearum* in the 14 districts of Pakistan.

DISCUSSION

Ralstonia solanacearum, causing bacterial wilt of chili, is widespread in warm temperate, tropical, and subtropical regions of the world. In Asia, it has been reported from almost all the countries. *R. solanacearum* does not have uniform biology, host range and acts as complex variants. As it does not behave as single bacterium, that is why it is described into biovars, races, groups, sub-races, and strains.

In the present study, variations in hypersensitivity response and growth were observed among 114 isolates of *R. solanacearum* collected from eight agroecological zones of Pakistan. Of all the 114 isolates of *R. solanacearum*, 88 showed positive hypersensitive response and mucoid growth, while 26 isolates gave negative hypersensitive response with non-mucoid growth.

The variations in these parameters might be due to differences in temperature, moisture, soil types, and other edaphic factors of various districts of eight agroecological zones of the country. Morphological variability in terms of growth has also been reported by many workers among different isolates of *R. solanacearum* (Smith 1920, Kelman 1953, Denny and Hayward 2001, EPPO 2004) which corroborated our findings.

Two types of morphological colony of *R. solanacearum* can be typically observed on agar plates: fluidal or mucoid, and afluidal or non-mucoid (Smith 1920, Kelman 1953, Denny and Hayward 2001, EPPO 2004). The mucoid substance is produced by the accumulation of an exopolysaccharide (EPS), which causes these mucoid colonies to exhibit a typical irregularity of their surfaces (Smith 1920), often with characteristic whorls in the center. Under certain conditions, *R. solanacearum* colonies spontaneously undergo a change in morphology from fluidal to afluidal and are linked to a great reduction in disease-inducing capacity of these cells (Kelman 1954, Buddenhagen and Kelman 1964, Brumbley and Denny

1990). This phenomenon is known as phenotypic conversion (PC) (Denny et al. 1994, Poussier et al. 2003) and occurs in most *R. solanacearum* strains (Kelman 1954). PC-type variants can be easily observed by prolonged culture on agar plates (Kelman 1954, Buddenhagen and Kelman 1964) and when the organism is grown in a non-aerated liquid medium with glucose and an organic source of nitrogen (Kelman and Hruschka 1973).

It has been reported that all strains of *R. solanacearum* with mucoid colonies are virulent and produce EPS (Kelman 1954, Buddenhagen and Kelman 1964, Boucher et al. 1992, Poussier et al. 2003), while EPS-deficient mutants (non-mucoid colonies) are avirulent. *R. solanacearum* EPS appears to be highly heterogeneous, since it has a varying composition among strains (Drigues et al. 1985). *In planta*, EPS would probably act by occluding xylem vessels, interfering directly with normal fluid movement of the plant, or by breaking the vessels due to hydrostatic overpressure (Schell 2000). On the other hand, EPS I might also favor stem colonization by the pathogen, since EPS I-deficient mutants have been shown to multiply more slowly, and colonize poorly the stem of infected plants (Saile et al. 1997, Araud-Razou et al. 1998). In that sense, EPS I would be contributing to minimizing or avoiding the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defense mechanisms (Young and Sequeira 1986, Araud-Razou et al. 1998). As EPS-deficient mutants can infect and multiply to some extent in planta without inducing wilting symptoms, EPS might take part mainly in late stages of the process, modulating disease severity rather than the infective ability of the bacterium. In *R. solanacearum*, EPS is thought to be the main factor accounting for virulence of the pathogen (Schell 2000, Hikichi et al. 2007).

In the present study, 114 isolates of *R. solanacearum* collected from different agroecological zones of the country were classified into biovars. The study revealed that there were no Biovars I, II and V in the country, and only Biovars III and IV were found prevalent with varying proportions in different agroecological zones. In the country, 81% isolates were identified as Biovar III, and the remaining 19% were detected as Biovar IV. Biovar III was found in all the agroecological zones as against Biovar IV, which was observed from four agroecological zones. The predominance of Biovar III in the country is attributable to favorable climatic conditions, edaphic factors, cropping pattern as against Biovar IV, which seems to be impervious to most of these factors.

Biovar II, which is a bio-agent in the United States of America and Europe, is favored by cold environment. Contrarily, the conditions for this biovar in Pakistan are not propitious. On the other hand, Biovars III and IV are mainly Asian strains, and grow well in the country as conditions for their development, infection, pathogenesis, and dissemination are favorable. Biovar III was found dominant in all the provinces and agroecological zones of the country mainly in Sindh and Punjab provinces. The main reasons for its dominance are the cultivation of multiple crops.

Almost all the solanaceous vegetables grown throughout the year are the hosts of *R. solanacearum*. In the present study, Biovar III was found to be predominant, and 81% isolates were identified as Biovar III and corroborated the findings of Shahbaz et al. (2015), who reported that 84% of the isolates of *R. solanacearum* were Biovar III. In the present study, 19% of the isolates were recognized as Biovar IV, which has been observed for the first-time infecting chili. The dominance of Biovar III as compared to Biovar IV is due to the fact that Biovar III is more adaptable to fluctuating environmental components and it is not much affected by changing edaphic factors, as Biovar III is more aggressive than Biovar IV and can induce wilting rapidly in different hosts (Kumar et al. 2004, 2014). The incidence and prevalence of bacterial wilt (Biovar III) has been found the highest in soils where dissolved organic carbon content was relatively high and was related to substrate availability (Messiha et al. 2007, 2009).

CONCLUSION

It was concluded that Biovar III is widely prevalent in the country as the environmental conditions, and other factors mentioned are favorable for its spread. Therefore, control strategies should be adopted accordingly to minimize losses caused by this biovar and its spread.

AUTHORS' CONTRIBUTION

Conceptualization: Aslam, M. N. and Mukhtar T.; **Methodology:** Aslam, M. N. and Mukhtar T.; **Investigation:** Aslam, M. N.; **Writing – Original Draft:** Aslam, M. N.; **Writing – Review and Editing:** Mukhtar T.; **Funding Acquisition:** Aslam, M. N.; **Supervision:** Mukhtar T.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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