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Virulence of *Phytophthora infestans* isolates from potato in Spain

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Abstract: The oomycete *Phytophthora infestans* is responsible for the disease known as late blight in potato and tomato. It is the plant pathogen that has caused the greatest impact on humankind so far and, despite all the studies that have been made, it remains the most important in this crop. In Spain during the last years a greater severity of the disease has been observed in both, potato and tomato, probably due to genetic changes in pathogen populations described recently. The aim of this study was the characterization of the physiological strains of 52 isolates of *P. infestans* obtained in different potato-growing areas in Spain. For this purpose, inoculations on detached leaves were performed in order to determine compatibility or incompatibility reactions. A total of 17 physiological races were found. The less frequent virulence factors were *Avr5* and *Avr8*. By studying the epidemiology of the pathogen, a specific breeding program for late blight resistance can be implemented.

Keywords: physiological strains; late blight; *Solanum tuberosum*; virulence

The oomycete *Phytophthora infestans* (Mont.) de Bary is responsible for the disease known as late blight in potatoes and is the plant pathogen that has caused the greatest impact on humankind so far. The disease has been known for a long time and was introduced in Europe at 1840 when it destroyed all potato crops in Ireland causing the famous Irish Potato Famine in which more than one million people died (Goss et al. 2014).

Despite all studies that have been performed on this pathogen, it is still the most important one in potato. According to estimates from the International Potato Center (CIP), even if resistant cultivars and high doses of fungicides are used, this disease implies a global yield loss of 16% of the potato crop, representing an annual economic loss of 5.2 billion euros worldwide (Haverkort et al. 2009).

Virulence is the genetic ability of an oomycete to overcome host resistance and cause disease. The *P. infestans*/plant host patosystem can be explained by a gene-to-gene model, i.e. for each gene that determines the resistance of the host, there is a specific gene that determines its pathogenicity or virulence. The plant resistance gene is only effective if there is a corresponding avirulence gene in the pathogen (Birch & Whisson 2001). The virulence spectrum is the range of *Avr* genes expressed by the isolate when inoculated in a differential series of genotypes with *R* resistance genes. Isolates showing the same spectrum are called physiological races, and are considered as very complex if they show a broad spectrum of virulence. The characterization of *P. infestans* isolates in pathotypes or physiological races is performed on the basis of their virulence on the *R* genes

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of a group of differential genotypes. Thus the race (1.3) is virulent in cultivars with *R1* and *R3* genes. These genotypes carry 11 vertical resistance *R* genes derived from the wild species *S. demissum* identified by Black et al. (1953) and Malcosom and Black (1966). All resistance loci in these lines have been mapped except for locus *R4* (van Poppel et al. 2009).

Four of these genes, *R1, R2, R3a*, and *R3b* have been cloned (Hein et al. 2009). *R1* was the first characterized gene, which confers resistance in leaves and tubers (Ballvora et al. 2002). It has a domain over R proteins with CC, NBS and LRR type structures that regulate the expression of resistance genes. Genetic mapping also determined that *R3* is formed by two closely linked genes, *R3a* and *R3b* with 82% similarity and located on chromosome XI (Huang et al. 2004). *R3a* encodes the CC-NBS-LRR proteins that confer leaf resistance. The *R3b* gene is related to specific resistance proteins in both, leaves and tubers, but does not recognize the avirulence factor *Avr3a.* This indicates that despite their high similarity, both genes do not share the same specificity for an avirulence factor (Li et al. 2011). *R10* and *R11* genes were also mapped by Bradshaw et al. (2006).

The severity of the disease produced by this oomycete is mainly due to the complexity and aggressiveness of the existing strains, which makes it one of the most difficult plant pathogens to control. The continuous changes in populations worldwide have made the control of late blight increasingly complicated, due to the gradual reduction of the effectiveness of fungicides, a consequence of the continuous applications (Jaramillo 2003). Cooke et al. (2011) conduct a review of pathogen epidemiology and control in Europe. They show a great diversity of populations that include both types of mating and sexual reproduction, although they describe great differences between regions.

In Spain, during the last years, a greater severity of the disease has been observed in both, potato and tomato, probably because of recently described genetic changes related to the presence of the two mating types A1 and A2 (Alor et al. 2014). This leads to an increase in the variability of the pathogen and consequently the emergence of new races with higher virulence. It should also be noted that the introduction and subsequent migration of new pathotypes increase the aggressiveness (Fry 2008; Hannukkala et al. 2008). The aim of this study was to characterize the *P. infestans* isolates collected in potato fields in Spain in order to determine the physiological strains and virulences using Black's differentials.

MATERIALS AND METHODS

***P. infestans* isolates.** The characterization of the physiological races was performed using 52 isolates of *P. infestans* sampled between 2003 and 2014 from potato leaves in different fields of Spain (Table 1). A total of 33 isolates came from previous surveys carried out in southern Spain and 19 were collected in the northern regions and in Tenerife Island (Alor et al. 2019). We also used as control the reference strain MP324 (A1) which was kindly provided by the Plant Breeding and Acclimatization Institute, Mlochów Center (Poland). Infected potato leaves were transferred to tuber slices from cv. Bintje, and then incubated at 90% relative humidity and room temperature for five days. Afterwards, they were grown in the rye B agar medium (Caten & Jinks 1968) and maintained in a growth chamber at 18 ± 2 °C and 16 h photoperiod. Every 2–3 months, depending on the growth rate of the isolate, subcultures of rye agar were made with mycelium, transferring it to new Petri dishes.

Identification of physiological races. The virulence of each isolate was verified using potato leaflets collected from the *P. infestans* susceptible cv. Bintje (Tooley et al. 1989) and Black's 11 differentials series with single *R* genes ranging from *R1* to *R11* from *Solanum demissum* (Black et al. 1953; Malcolmson & Black 1966; Malcolmson 1969).

The plant material consisted of micropropagated seedlings of the differential clones (R1 to R11) for the determination of the physiological races. The culture medium used was MS (Merck, Germany) (Murashige & Skoog 1962) with a pH between 5.65–5.75. The propagated material was placed in tubes with MS and incubated in a culture chamber for 21 days, at 22 ± 2 °C, and photoperiod of 16 hours. When the seedlings reached a size of approximately 10 cm length, 8–10 knots per seedling were cut for multiplication. The replication was done in vessels with MS medium during 21 days until seedlings with well-developed roots and leaves were obtained. These were then acclimatized in trays with perlite and nutritive solution for 12 days. Finally, they were transplanted into pots with sterile substrate in a greenhouse at a temperature between 20–24 °C and 16 h of light.

For the identification of the physiological races, five leaflets from the middle part of each differential were inoculated with each isolate. The evaluation of the virulence spectrum consisted in determining the reaction of compatibility or mycelial

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Table1. Origin and mating types of the 52 Spanish *Phytophthora infestans* isolates used in this study

Code	Location	Province	Mating type
AL-01	Iturrieta	Alava	A2
AL-02	Arkaute	Alava	A2
AL-03	Iturrieta	Alava	A2
AL-04	Heredia	Alava	A1
AL-05	Zuazo de San Millan	Alava	A1
AL-06	Gauna	Alava	A2
AL-07	Iturrieta	Alava	A1
CA-01	Sanlucar de Barrameda	Cadiz	A1
CA-02	La Barca	Cadiz	A1
CA-03	La Barca	Cadiz	A1
CA-04	Oca-Sanlucar	Cadiz	A2
CA-05	Oca-Sanlucar	Cadiz	A2
CA-06	Oca-Sanlucar	Cadiz	A1
CA-07	Oca-Sanlucar	Cadiz	A2
CA-08	Oca-Sanlucar	Cadiz	A2
CA-09	Oca-Sanlucar	Cadiz	A1
CA-10	Oca-Sanlucar	Cadiz	A2
CA-11	Oca-Sanlucar	Cadiz	A2
CA-12	Oca-Sanlucar	Cadiz	A1
CA-13	Oca-Sanlucar	Cadiz	A2
CA-14	Oca-Sanlúcar	Cadiz	A2
CA-15	Oca-Sanlucar	Cadiz	A1
CA-16	Oca-Sanlucar	Cadiz	A2
CA-17	Oca-Sanlucar	Cadiz	A1
CA-18	Chipiona	Cadiz	A2
CA-19	Jerez	Cadiz	A1
CA-20	Jerez	Cadiz	A1
CA-21	Sanlucar de Barrameda	Cadiz	A2
CA-22	Sanlucar de Barrameda	Cadiz	A2
CO-01	Villarrubia	Cordoba	A1
CR-01	Villamanrique	Ciudad Real	A1
CR-02	Villamanrique	Ciudad Real	A1
CR-03	Villamanrique	Ciudad Real	A1
CR-04	Villamanrique	Ciudad Real	A1
JA-01	Alcala La Real	Jaen	A1
OR-01	Xinzo de Limia	Orense	A2
OR-02	Xinzo de Limia	Orense	A2
OR-03	Xinzo de Limia	Orense	A2
SE-01	Lora del Rio	Sevilla	A2
SE-02	Maribañez	Sevilla	A1
SE-03	Brenes	Sevilla	A2
SE-04	Sevilla	Sevilla	A2
SE-05	Sevilla	Sevilla	A1
SE-06	La Rinconada	Sevilla	A1
SE-07	Guillena	Sevilla	A1

Table1. to be continued

Code	Location	Province	Mating type
TE-01	La Matanza	Tenerife	A2
TE-02	La Matanza	Tenerife	A2
TE-03	La Matanza	Tenerife	A2
TE-04	La Victoria	Tenerife	A2
TE-05	La Victoria	Tenerife	A2
TE-06	La Victoria	Tenerife	A2
TE-07	Cerro Gordo	Tenerife	A2

growth and sporulation, against the incompatibility or presence of necrosis or hypersensitivity. A visual evaluation was made, and the pathogen was subsequently confirmed by microscopic observation. The isolate was considered virulent if at least 3 of the 5 leaflets showed compatibility reaction, otherwise it was considered negative as described by Barquero et al. (2005).

Preparation of leaflets and inoculum. The inoculum for each isolate was prepared from a suspension obtained from isolates grown for 4 weeks by washing the rye agar plates in double-distilled water. The liquid phase was collected in a sterile test tube to estimate the spore concentration, using a Neubauer chamber. The concentrations of all isolates were then adjusted to 4×10^4 sporangia/mL. Spore germination was induced by thermal shock at 4 °C for 2 h to stimulate the spread of zoospores and then for 30 min at room temperature to reactivate the released zoospores. Leaflets from the upper part of the plant were collected early in the morning and placed in a hunched position on filter paper wetted with sterile water using a grid as support. The plastic containers in which they were placed had dimensions of 40 × 30 × 5 cm and contained 80 mL of sterile water to create a wet chamber. The infection of the differential clones was performed on the abaxial face of the leaflets, placing 40 μL of the inoculum. The trays were placed in the growth chamber at 18 ± 2 °C, and a photoperiod of 18 h, until the susceptible control Bintje (R_0) showed 100% of the surface affected.

Standarized diversity indices. Seven days after inoculation, the virulence of the isolates was assessed by presence or absence of sporangia. The virulent isolates were able to sporulate and cause lesions on more than 3% of the leaf area. Race diversity was calculated by two normalizaed indices: the Shannon and Gleason indices (Goodwin et al. 1995).

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The Shannon index (*HS*) was calculated using the following Equation (1):

$$HS = -\Sigma j\left(p_j \ln p_j\right);\ j = 1 \ldots Np \quad (1)$$

where: p_j – the frequency of the *j-th* race in the population; *Np* – the number of races identified.

The range of values is between 0 and ln*Np*, where 0 indicates no diversity, all isolates belonging to the same race, and ln*Np* shows the highest level of diversity, indicating that each isolate is a different race.

The Gleason index (*HG*) was calculated using the following formula:

$$HG = \frac{(Np - 1)}{\ln(ni)} \quad (2)$$

where: *Np* – the number of identified races; *ni* – the number of evaluated isolates.

This index reflects the richness of diversity as the number of different phenotypes present in the population. A correction factor was used to compare the locations and reduce the effect due to the difference in sample size. This was applied when the size was less than 100 individuals, thus calculating the relative Shannon index (*HSR*) and relative Gleason index (*HGR*) as proposed by Andrivon (1994).

$$HSR = \frac{HS}{HS\max} \quad (3)$$

where: $HS\max = \frac{HS}{\ln(ni)}$

and $HGR = \frac{HG}{HG\max} \quad (4)$

where: $HG\max = \frac{(Np-1)}{(ni-1)}$

*HG*max – the highest possible value of *HG*; *HS*max – the highest possible value of *HS* in a sample of *ni* individuals, where *ni* – the number of evaluated isolates; *Np* – the number of identified races.

The complexity of the race of an isolate was estimated from the number of differential clones in which the isolate induced the disease.

RESULTS

Table 2 shows the observed compatibility or incompatibility of each studied *P. infestans* isolate on the set of Black's differential clones. The susceptible control Bintje (R_0) was infected by all isolates after inoculation and confirmed therefore the pathogenicity of the 52 isolates. Likewise, the known virulence spectrum of control strain MP-324 (A1) validated the use of the differential clones, since only the expected incompatibilities with clones R_5, R_8 and R_9 were detected. Table 2 shows beside the individual reactions also the number of virulence factors of the different isolates. Pathotype OR-02 showed the lowest virulence spectrum with only 3 factors compared to the isolates from Cadiz with 11 factors, followed by isolates from Alava, Orense, Tenerife and Seville which presented a total of 10.

The derived absolute frequencies of virulence factors in the collection of isolates are also indicated in Table 2. The smallest absolute frequencies were observed for *Avr9* which occurred in only 33 isolates (63.5%) and for *Avr5* and *Avr8*, both present in 38 isolates (73.1%). On the other hand the highest frequencies revealed factor *Avr7* which was present in 50 isolates representing 96.2%, followed by factors *Avr1* and *Avr11* observed in a total of 48 and 47 isolates, representing 92.3 and 90.4%, respectively.

The complexity of an isolate is given by the spectrum of virulence, which can be determined by the number of *Avr* factors it expresses (Table 3). It can be seen the number of isolates of each race and the absolute frequencies for each race by province for the total set of 52 isolates. The identification of simple and complex races should be highlighted. A total of 17 physiological races were found among the 52 inoculated isolates. Two simple strains came from Alava (1.3.4.7.8) and Orense (2.3.7) and the most complex strain with all 11 virulence factors (1.2.3.4.5.6.7.8.9.10.11), which occurred also with the highest frequency (25%), was an isolate from Cadiz. The locations of Alava, Cadiz and Seville showed three physiological strains each with different virulence spectra between them.

Table 4 shows the relative frequencies of each virulence factor per origin, with the *Avr1* factor being on average the most frequent (89%) and factors *Avr9* and *Avr5* were the less frequent with 68% for the sampled provinces. With respect to the diversity in term of virulence factors, Cadiz presented the highest average frequency with all virulence factors (*Avr1*–*Avr11*) of 90%, while Orense had the lowest relative frequency of 70%.

Table 5 shows the diversity indices for each origin. Cadiz presents a higher richness and uniformity in its isolates with a value of 3.09 for the *HSR*,

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Table 2. Compatibility and incompatibility reactions of the *P. infestans* isolates with Black's differential set of *R* genes

Isolate	Differential clon of *Solanum demissum*												
	r[1]	*R1*	*R2*	*R3*	*R4*	*R5*	*R6*	*R7*	*R8*	*R9*	*R10*	*R11*	NoVirF
MP-324[2]	+	+	+	+	+	–	+	+	–	–	+	+	8
AL-01	+	+	+	+	+	+	+	+	+	–	+	+	10
AL-02	+	+	+	+	+	+	+	+	+	–	+	+	10
AL-03	+	+	+	+	+	+	+	+	+	–	+	+	10
AL-04	+	+	–	+	+	–	–	+	+	–	+	+	7
AL-05	+	+	–	+	+	–	–	+	+	–	+	+	7
AL-06	+	+	–	+	+	–	–	+	+	–	–	–	5
AL-07	+	+	+	+	+	+	+	+	+	–	+	+	10
CA-01	+	+	+	–	+	–	+	+	+	–	+	+	8
CA-02	+	+	+	–	+	–	+	+	+	–	+	+	8
CA-03	+	+	+	–	+	–	+	+	+	–	+	+	8
CA-04	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-05	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-06	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-07	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-08	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-09	+	+	+	+	+	+	+	+	–	+	–	+	9
CA-10	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-11	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-12	+	+	+	+	+	+	+	+	–	+	-	+	9
CA-13	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-14	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-15	+	+	+	+	+	+	+	+	–	+	–	+	9
CA-16	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-17	+	+	+	+	+	+	+	+	–	+	–	+	9
CA-18	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-19	+	+	+	–	+	–	+	+	+	–	+	+	8
CA-20	+	+	+	–	+	–	+	+	+	–	+	+	8
CA-21	+	+	+	+	+	+	+	+	+	+	+	+	11
CA-22	+	+	+	+	+	+	+	+	+	+	+	+	11
CO-01	+	+	+	+	+	–	+	+	+	–	+	+	9
CR-01	+	+	+	+	–	+	–	+	+	+	+	–	8
CR-02	+	+	+	+	–	+	–	+	+	+	+	–	8
CR-03	+	+	+	+	–	+	–	+	+	+	+	–	8
CR-04	+	+	–	+	+	–	+	–	–	+	+	+	7
JA-01	+	+	+	–	+	+	+	–	+	+	+	+	9
OR-01	+	+	+	+	–	+	+	+	+	+	+	+	10
OR-02	+	–	+	+	–	–	–	+	–	–	–	–	3
OR-03	+	+	+	+	–	+	+	+	+	+	+	+	10
SE-01	+	+	+	+	+	+	+	+	+	+	–	+	10
SE-02	+	–	+	+	+	–	–	+	+	+	+	+	8
SE-03	+	+	+	+	+	+	+	+	+	+	–	+	10
SE-04	+	+	+	+	+	+	+	+	+	+	–	+	10
SE-05	+	+	-	+	+	+	+	+	–	+	+	+	9
SE-06	+	–	+	+	+	–	–	+	+	+	+	+	8
SE-07	+	–	+	+	+	–	–	+	+	+	+	+	8
TE-01	+	+	+	+	+	+	+	+	–	+	+	+	10

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Table 2. to be continued

Isolate	Differential clon of *Solanum demissum*												
	r[1]	*R1*	*R2*	*R3*	*R4*	*R5*	*R6*	*R7*	*R8*	*R9*	*R10*	*R11*	NoVirF
TE-02	+	+	–	+	+	+	+	+	–	–	+	+	8
TE-03	+	+	–	+	+	+	+	+	–	–	+	+	8
TE-04	+	+	–	+	+	+	+	+	–	–	+	+	8
TE-05	+	+	–	+	+	+	+	+	–	–	+	+	8
TE-06	+	+	–	+	+	+	+	+	–	–	+	+	8
TE-07	+	+	+	+	+	+	+	+	–	+	+	+	10
FVirF	52	48	42	46	46	38	42	50	38	33	43	47	

[1]susceptible cv. Bintje; [2]control strain with known virulence factors; NoVirF – number of virulence factors per isolate; FVirF – absolute frequency of virulence factors in the 52 isolates

Table 3. Physiological races of *Phytophthora infestans* isolates and absolute frequencies of virulence factors collected from potato samples in Spain

Area	Isolates	Races	Number of isolates per race[1]	Frequency (%)[2]
Alava	7	1.2.3.4.5.6.7.8.10.11	4 (57.1)	7.7
		1.3.4.7.8	1 (14.3)	1.9
		1.3.4.7.8.10.11	2 (28.6)	3.8
Cadiz	22	1.2.3.4.5.6.7.8.9.10.11	13 (59.1)	25.0
		1.2.4.6.7.8.10.11	5 (22.7)	9.7
		1.2.3.4.5.6.7.9.11	4 (18.2)	7.7
Ciudad Real	4	1.2.3.5.7.8.9.10	3 (75.0)	5.8
		1.3.4.6.9.10.11	1 (25.0)	1.9
Cordoba	1	1.2.3.4.6.7.8.10.11	1 (100.0)	1.9
Jaen	1	1.2.4.5.6.8.9.10.11	1 (100.0)	1.9
Orense	3	1.2.3.5.6.7.8.9.10.11	2 (66.7)	3.8
		2.3.7	1 (33.3)	1.9
Tenerife	7	1.2.3.4.5.6.7.9.10.11	2 (28.6)	3.8
		1.3.4.5.6.7.10.11	5 (71.4)	9.7
Seville	7	2.3.4.7.8.9.10.11	3 (42.9)	5.8
		1.2.3.4.5.6.7.8.9.11	3 (42.9)	5.8
		1.3.4.5.6.7.9.10.11	1 (14.2)	1.9

[1]Absolut frequency of each race per location (values in brackets mean percentages); [2]absolut frequency of the race for the set of 52 isolates

indicating a higher similarity between the frequencies of the different phenotypes of the region. The *HGR* reflects the number of different phenotypes in the region. The isolates from Cadiz reached a value of 6.79, but in Cordoba and Jaen these values are zero, since they are represented by only one isolate per province.

DISCUSSION

In the present study involving 52 *P. infestans* isolates from 8 prospected provinces a total of 17 races were identified, based on their virulence patterns. The most complex race was detected in a group of isolates from Cadiz containing all 11 virulence factors (*Avr1* to *Avr11*). In Poland, Lebecka et al. (2007) described races with an average of 8 factors. In Japan Fukue et al. (2018) describe 13 races with a range of 5 to 8 virulence factors. The Estonian races showed an average of 7.2 virulence factors per isolate (Runno-Paurson et al. 2016). However, in other countries such as Algeria, Beninal et al. (2009) have also described pathotypes with all factors, as in our study. Casa-Coila et al. (2020) found

Table 4. Relative frequencies of virulence factors in the prospected geographical areas

Area	Relative frequency											
	Avr1	*Avr2*	*Avr3*	*Avr4*	*Avr5*	*Avr6*	*Avr7*	*Avr8*	*Avr9*	*Avr10*	*Avr11*	Mean
Alava	1.00	0.57	1.00	1.00	0.57	0.57	1.00	1.00	0.00	0.86	0.86	0.77
Cadiz	1.00	1.00	0.77	1.00	0.77	1.00	1.00	0.82	0.77	0.82	1.00	0.90
Ciudad Real	1.00	0.75	1.00	0.25	0.75	0.25	0.75	0.75	1.00	1.00	0.25	0.70
Cordoba	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00	1.00	0.82
Jaen	1.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	0.82
Orense	0.67	1.00	1.00	0.00	0.67	0.67	1.00	0.67	0.67	0.67	0.67	0.70
Tenerife	1.00	0.29	1.00	1.00	1.00	1.00	1.00	0.00	0.29	1.00	1.00	0.78
Seville	0.57	0.86	1.00	1.00	0.57	0.57	1.00	0.86	1.00	0.57	1.00	0.82
Mean	0.89	0.84	0.82	0.75	0.68	0.78	0.82	0.73	0.68	0.87	0.85	0.79

Table 5. Racial diversity of the isolates from Spain based on normalized Shannon and Gleason indices

Origin	Isolates	Races	*HS*	*HRS*	*HG*	*HRG*
Alava	7	3	0.40	1.95	1.03	3.08
Cadiz	22	3	0.77	3.09	0.65	6.79
Ciudad Real	4	2	0.24	1.39	0.72	2.16
Cordoba	1	1	0.08	0.00	0.00	0.00
Jaen	1	1	0.08	0.00	0.00	0.00
Orense	3	2	0.20	1.10	0.91	1.82
Tenerife	7	2	0.35	1.95	0.51	3.08
Seville	7	3	0.41	1.95	1.03	3.08
Total	52	17				

HS – Shannon's index; *HRS* – Shannon's relative index; *HG* – Gleason's index; *HRG* – Gleason's relative index

in Brazil that the most complex race contained also all virulence factors, but represented only one isolate (1.3%). In contrast, the most frequent pathotypes were 1.3.4.7.8.10.11 and 1.3.4.6.7.8.10.11.

Three of the 11 virulence genes identified in the isolates showed lower frequencies (*Avr5, Avr8,* and *Avr9*), which is consistent with Hannukkala et al. (2008). These authors identified complex pathotypes (1.3.4.5.7.10.11) and observed an increase in these virulence genes from 5.6 to 7.5% in Finland and Russia. In addition, Andrivon et al. (2004) and González et al. (2006) mention that virulences *Avr2, Avr5, Avr8* and *Avr9* are rare in certain parts of Europe. The frequency of *Avr9* was also found to be low in Poland (Michalska et al. 2016).

Besides sexual recombination, other reasons for the greater complexity of the new races have been proposed by Goodwin (1997). The author suggests that there is a moderate mutation rate, producing billions of sporangia, with strong selection pressure imposed by resistance genes and fungicides. This would be sufficient for generating the observed variability in virulence and sensitivity to existing active materials. The same author mentions that US-1 was probably the only pathotype of *P. infestans* in Europe before 1970, before the arrival of mating type A2. The great variation of virulence towards the 11 *R* genes of the differential series was possibly the result of mutations within the clonal lineage of that pathotype. Hybridization of the pathogen with other species could be another potential source of genetic variability, since interspecific hybrids between *P. infestans* and *P. mirabilis* have been obtained in the laboratory (Goodwin & Fry 1994).

Another possible reason could be the effect of the ploidy change in the pathogen. Tooley et al. (1989) found A2 isolates in Poland with a high DNA content, which would correspond to pentaploid or hexaploid genotypes. Also Grünwald and Flier (2005) described the existence of British tetraploid isolates. These isolates could be carriers of both, the A1 and A2 locus in the same isolate, due to the duplication of their genome, which gives them the ability to produce oospores with both mating types.

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Another hypothesis is related to migration as the main source of increasing virulence and race diversity. In Canada, the United States and the United Kingdom, dramatic changes have been detected in the genetic and physiological structure of the population of this pathogen, describing the emergence of new lineages and increased complexity (Peters et al. 1998). Swiezynski et al. (2000) analysed isolates collected before and after the migrations to North America and Europe described by Fry et al. (1991), Goodwin (1997) and Forbes et al. (1997). These results confirmed the presence of 11 virulence factors in both populations, suggesting that their increase does not result from the introduction of new pathotypes, but could be caused by the presence of sexual recombination due to the existence of mating types A1 and A2 of the pathogen (Grünwald & Flier 2005). All these studies would indicate that the high complexity of the new races of *P. infestans* is not only due to sexual recombination *per se* (Oliva et al. 2002), but also to non-sexual recombinations such as interspecific somatic hybrids, parasexuality or migrations, which give the pathogen adaptive conditions to extreme situations.

The *HGR* was higher than the *HSR* for the locations Alava, Cadiz, Tenerife and Seville, indicating that the richness of the diversity of the isolates is relatively high. These data are consistent with those found by Dowley et al. (2000), which describe also a higher Gleason index than the Shannon index with values of 3.02 and 1.67, respectively in Nepalese *P. infestans* populations.

Lebreton and Adrivon (1998) mentioned that variations in diversity and complexity were evident between populations of different years, as well as between those of the same year, but from different origins. In Brittany, the complexity found was greater in 1994 than in the previous year. The diversity of races in French populations was, however, moderate compared to other European populations, such as in the Netherlands, where 25 races were found among the 77 evaluated isolates (Schöber & Turkensteen 1992).

However, the genetic diversity observed in the present study is not as high as described for the central region of Mexico, where sexual reproduction of the pathogen predominated. The populations showed her even higher heterogeneity, detecting more than 20 genotypes in one experimental field at Chapingo. Also Matuszak et al. (1994) identified 15 genotypes in 33 isolates in a single field. In Poland also a high diversity in the population was found, indicating sexual reproduction, since they detected at the same place and in the same year isolates of both mating types (Brylinska et al. 2016).

Alor et al. (2019) carried out the characterisation of Spanish isolates. However, this is the first study that analyses the complexity of *P. infestans* isolates in Spain. It represents a first step towards understanding the epidemiology of the pathogen here in Spain and useful, to initiate a specific potato breeding program for late blight resistance.

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