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Grass pea and pea phylogenetic relatedness reflected at *Fusarium oxysporum* host range

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Abstract

Vascular wilt, caused by the infection of the soil-borne pathogen *Fusarium oxysporum* (*Fo*), is one of the most destructive diseases of many crops, including legumes such as grass pea (*Lathyrus sativus*), with several *formae speciales* (ff. spp.) defined according to their hosts. Commonly described as host-specific, *Fo* could, in some cases, show a broader host range comprising related plant species, making its host range characterization an important aspect of epidemiology and crop protection. No information on identification and host range status of strains able to infect grass pea is available, nor whether grass pea could act as host to different *Fo* ff. spp. In this study, the host range of two *Fo* strains isolated from grass pea (*Fo* ex. *L. sativus* 1 and 2) was evaluated using related legume species: pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), and barrel medic (*Medicago truncatula*). In addition, the responses of grass pea to the causal agents of fusarium wilt in these legume species, *Fo* f. sp. *pisi*, *lentis*, *ciceris*, *phaseoli*, and *medicaginis*, were also investigated. Disease symptom evaluation by disease rating over time, its related area under disease progress curve (AUDPC) and disease progress rate (DIr), revealed that *Fo* ex. *L. sativus* 1 and 2 are host-specific, infecting only grass pea although with low aggressiveness. Grass pea could also be infected by *Fo* f. sp. *pisi* races, with race 2 the most virulent strain in grass pea, even more virulent than the *Fo* strains retrieved originally from grass pea. The phylogenetic relatedness between grass pea and pea may in part explain this observation, indicating that *Fo* f. sp. *pisi* can also infect legume-related species such as grass pea. Additionally, specialization might be occurring, with particular *Fo* isolates only virulent to grass pea, although with lower virulence than *Fo* f. sp. *pisi* race 2 itself. These cross-inoculation results reinforce the importance of performing host-range studies, even on specialist pathogens, to identify potential closely related alternative hosts and consequently improve or adapt disease control management.

Keywords: *Fusarium oxysporum*, grass pea, host range, disease management

1. Introduction

Fusarium oxysporum (*Fo*) is a ubiquitous soil-borne fungi (Agrios, 2005) that includes morphologically indistinguishable plant pathogenic and non-pathogenic strains (Lievens et al., 2008). The pathogenic strains promote vascular wilt in over 100 different host species (Di Pietro et al., 2003; Michielse and Rep, 2009). Despite the broad host range of the fungus species, individual strains are often characterized as highly host- specific, restricted to one or a few plant species, and grouped into more than 120 *formae speciales* (ff. spp.) (Armstrong and Armstrong, 1981; Michielse and Rep, 2009). However, a recent review stated that the *Fo* host range can also be broader for many ff. spp., and in some cases a single plant species can be infected by different *Fo* ff. spp. (Edel-Hermann and Lecomte, 2019). The authors concluded that only 50% of the 106 ff. spp. reviewed have a unique plant species as a host. Furthermore, this number could be even smaller if more potential hosts were tested (Edel-Hermann and Lecomte, 2019).

The absence of sexual reproduction, little aptitude for gene flow, and low mutation rate make *Fo* a pathogen with low evolutionary potential (McDonald and Linde, 2002). Nevertheless, the ability of *Fo* spores to remain in the soil for long periods, even in the absence of a host (Di Pietro et al., 2003), makes its management a difficult task. Chemical fungicides or biological control are among the most common measures applied, but they are generally ineffective (Yadeta and Thomma, 2013). Although successful control of soil-borne diseases requires the integration of different management procedures, the use of resistant cultivars is widely considered the safest, most economical, and most effective crop-protection method (Panth et al., 2020; Rubiales et al., 2015). The development of crop resistant *Fo* varieties is thus essential. The first step in the development of these varieties is the identification of resistance sources, a massive task in which all the possible sources should be considered, including related plant species. Consequently, the characterization of the pathogen host range is fundamental.

Fusarium wilt disease has a dramatic impact on a wide range of plant species and is considered fifth in the top 10 plant pathogens of scientific/economic relevance (Dean et al., 2012). In legumes, *Fusarium oxysporum* species complex causes devastating wilt worldwide (Sampaio et al., 2020). As an example, *Fo* ff. spp. *pisi*, *ciceris*, *lentis*, *phaseoli*, and *medicaginis* are, respectively, destructive pathogens worldwide of pea (*Pisum sativum*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), and alfalfa (*Medicago sativa*) (Alves-Santos

et al., 2002; Haglund and Kraft, 2001; Navas-Cortés et al., 2000; Ramírez-Suero et al., 2010; Taylor et al., 2007). Fusarium wilt can be important also in minor legume crops such as cowpea (*Vigna unguiculata*) (Summerell et al., 2011) and grass pea (*Lathyrus sativus*) (Campbell, 1997). Although considered underused, these crops are very important regionally, used as a staple food in many developing countries (Cullis and Kunert, 2016). In particular, grass pea is considered one of the most promising sources of calories and protein in drier areas of Asia and Africa (Vaz Patto et al., 2006b), and is produced to a lesser extent in some European countries, such as Portugal (Lambein et al., 2019). Yield losses by fusarium wilt reaching 25% were reported in Indian and Ethiopian grass pea growing areas (Campbell, 1997; Talukdar, 2013). We recently detected the presence of fusarium wilt at two different fields in Alvaiázere, a Portuguese region where grass pea has a long history of cultivation as part of its local heritage (Vaz Patto, 2009).

Grass pea has a close phylogenetic relationship with pea (Schaefer et al., 2012; Wojciechowski et al., 2004), so close that there are suggestions that the genus *Pisum* should be included in the genus *Lathyrus* (Schaefer et al., 2012). Grass pea and pea share ascochyta blight, powdery mildew, and rust pathogens (Barilli et al., 2016; Vaz Patto et al., 2006a; Vaz Patto and Rubiales, 2009), corroborating that related plant species are more prone to share pathogens (Gilbert et al., 2015) and, eventually, also resistance sources.

To design defense strategies in grass pea against *Fo*, for which soil eradication is a difficult task, understanding the impact of alternative hosts on the pathogen survival is crucial. However, no information is available about the host range of the *Fo* strains affecting grass pea and it is also not known if *Fo* strains affecting other legume crops can have grass pea as an alternative host.

The purpose of the present cross-inoculation study was (i) to determine the host range of the recently identified *Fo* strains infecting grass pea in Portugal (*Fo* ex. *L. sativus* 1 and 2) using related legume species and (ii) to determine the disease response of grass pea against the causal agents of fusarium wilt in these related legume species. This information could provide new insights on the origin of *Fo* ex. *L. sativus* 1 and 2. It could also contribute to fusarium wilt management in legumes by identifying alternative host species in which *Fo* can multiply but which could also be promising sources of resistance.

2. Material and Methods

2.1 Fungal strains and culture conditions

Fusarium oxysporum ex. *L. sativus* used strains (*Fo* ex. *L. sativus* 1 and 2) were isolated from naturally infected grass pea plants showing fusarium wilt symptoms, such as yellowing of the leaves starting from the bottom to the top of the plant, browning of roots and stems and a complete wilt, in two different grass pea field locations in Alvaiázere, Portugal, in 2016. The cultures were individually isolated from different plant parts, either roots and basal, middle and apical stems, following a protocol adapted from Lichtenzveig et al. (2006). The plant fragments were plated on Potato Dextrose Agar (PDA, *Merck*) containing 0.1 mg/mL^{-1} chloramphenicol (*Sigma-Aldrich*) and incubated at 28 ºC for three days. *Fo* colonies that emerged from the plant fragments, colonizing the PDA plate, were subcultured until fungal purification, confirmed by macro and microconidial morphological analysis. Afterwards the species was confirmed by sequencing of the Internal Transcribed Spacer region using the ITS4 primer or the D1/D2 region of the large subunit ribosomal DNA using NL1 and NL4 primers, at Biopremier, Lisboa, Portugal.

Different *Fo* strains, causal agents of fusarium wilt in other legume species, were also used in this study: *F. oxysporum* f. sp. *pisi* race 1 strain CBS 127.73 NRRL36628, provided by CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands); *Fo* f. sp. *pisi* race 2 strain R2F42, provided by Dr W. Chen (USDA-ARS Pullman, USA); *Fo* f. sp. *lentis* strain 10 and *Fo* f. sp. *ciceris* race 5 strain 8012, both provided by IAS-CSIC Cordoba, Spain; *Fo* f. sp. *medicaginis* strain 605, provided by Microorganismes d'Intérêt AgroEnvironmental (MIAE) (INRA Dijon, France); and *Fo* f. sp. *phaseoli* race 6 strain SP1, provided by Dr J. M. Díaz-Mínguez (Universidad de Salamanca, Spain).

The different fungal strains were stored as microconidial suspension at -80 ºC in 30% glycerol. For microconidia multiplication, cultures were grown in potato dextrose broth (PDB, *Sigma-Aldrich*) at 28 ºC, in a shake culture set at 170 rpm (Di Pietro and Roncero, 1998).

2.2 Plant material and growth conditions

The host range of *Fo* ex. *L. sativus* strains was studied using five different grain legume species besides grass pea, namely pea, lentil, chickpea, common bean, and the model legume, barrel medic. For each legume species, four different accessions were used, selected based on their reported susceptibility to their specific *Fo* f. sp. (Table 1). To determine the response of grass pea against the different *Fo* ff. spp. described in the previous section, the same four grass pea accessions described in Table 1 were used.

In each inoculation experiment, appropriate susceptible checks were included. Grass pea accession PI196001, pea accession P21, lentil accession 81S15, chickpea accession JG62, barrel medic accession PI249878, and common bean accession g654 were the susceptible checks used.

Seeds were germinated for two days on wet filter paper in a Petri dish at 4 ºC in the dark. The Petri dishes were then shifted to 26 ºC until seed germination. Germinated seeds were planted into plastic pots (6 × 6 × 8 cm), containing sterile vermiculite (1–3 mm diameter) and grown in a controlled environment chamber under 16/8 h light-dark period at 26 ± 2 ºC, 60% of relative humidity, and 200 µmol m^{-2} s^{-1} illumination. Plants were watered every two days with tap water.

Table 1: Plant species accessions used in this study and their classification against their own *Fo* ff. spp..

Legume species	Germplasm accession	Susceptible to their own *Fo* f. sp.	Reference
Grass pea	PI195605 PI196001 PI257589 PI358601	Na	
Pea	P21 JI1210 JI1213 Kebby	✓ ✓ ✓ ✓	(Bani et al., 2012; Bani, unpublished)
Lentil	BGE001402 ILL4774 ILL5490 81S15 (ILL5883)	✓ ✓ ✓ ✓	(Pouralibaba et al., 2016; Pouralibaba et al., 2015)
Chickpea	JG-62 P-2245 C-104 ICCV-2	✓ ✓ ✓ ✓	(Jiménez-Díaz et al., 2015)
Common bean	g654 g1636 g1955 g4164	✓ ✓ ✓ ✓	(Leitão et al., 2020)
Barrel medic	PI239878 PI516927 PI577607 A17	✓ ✓ ✓ Intermediate	(Rispail and Rubiales, 2014)

na: non-available

2.3 Plant inoculation and disease assessment

Three consecutive inoculation experiments were performed per fungal strain, with five to 10 plants per accession, in a complete randomized design. Five plants per susceptible check were included. All the legume

seedlings, with the exception of barrel medic, were inoculated when seven days old. Barrel medic seedlings were inoculated when ten days old due to their smaller size.

The inoculation was performed following a modified version of a dip technique from Haglund (1989). Briefly, roots were removed from the vermiculite, cleaned, trimmed by a third, and immersed for 5 min in an inoculum suspension of 5×10^6 conidia mL^{-1} of water. Five control plants per inoculation experiment were also included; they were treated in the same way but immersed in sterile water. Inoculated seedlings and controls were replanted in individual autoclaved vermiculite pots and maintained in the growth chamber under the same conditions mentioned above.

Disease assessment was performed every three days from the 7th to the 30th day after infection (dai). The symptom evaluation was performed by counting the number of yellow leaves per number of total leaves, allowing the calculation of the percentage of disease intensity (% DI) per plant (Bani et al., 2012). These data were used to calculate the area under the disease progress curve (AUDPC) with the formula:

$$\text{AUDPC} = \sum[(x_i + x_{i+1})/2] \times (t_{i+1} - t)$$

where x_i is the estimated portion of disease intensity at date i, x_{i+1} is the disease intensity at date $i + 1$, and $t_{i+1} - t_i$ is the interval of days between recording dates i and $i + 1$. Disease intensity percentage along the evaluation time was also used to calculate a linear regression, allowing the estimation of the disease progress rate (DIr) given by the slope of the regression line. The maximum % DI score obtained at 30 dai (DI30), AUDPC accounting for the disease intensity progression along time, and DIr, as the progression speed parameter, were the three traits used for susceptibility assessment.

At the end of the plant disease evaluation, *Fo* strains causing disease symptoms were reisolated as previously described, following an adapted protocol from Lichtenzveig et al. (2006) to confirm that the observed symptoms were due to pathogen colonization.

2.4 Statistical analysis

For statistical analysis, AUDPC, DI30, and DIr values of the three inoculation experiments were combined. Graphical inspection of residuals to assess normality and identification of outliers was conducted using Genstat 19th edition software.

Pathogen strains, plant species, or accessions within plant species were compared using non-parametric Kruskal-Wallis test due to absence of normally distributed residuals, even after data transformation. Dunn's multiple comparison test was used for means comparison at $P = 0.01$. These statistical analyses were performed using R software (3.5.2 version).

3. Results

3.1 *Fusarium oxysporum* ex. *L. sativus* 1 and 2 host range

Fusarium oxysporum ex. *L. sativus* 1 and 2 were very specific to grass pea (Table 2). Infection was negligible on pea, lentil, chickpea, common bean, and barrel medic, with AUDPC, DI30, and DIr values close to zero, in a clear incompatible interaction with the strains recently isolated from grass pea.

Table 2: AUDPC, DI30 and DIr average values and respective standard deviation per legume species and accessions within species for *Fo* ex. *L. sativus* 1 and 2. Data followed with different small letters, per column, represents significant differences ($P = 0.01$) among accessions within species according to Dunn's test. Data followed by different capital letters, per column, represents significant differences ($P = 0.01$) among species according to Dunn's test.

		***Fo* ex *L. sativus* 1**			***Fo* ex *L. sativus* 2**		
Species	**Accessions**	**AUDPC**	**DI30**	**Dir**	**AUDPC**	**DI30**	**DIr**
Grass pea	PI195605	346.6±65.0a	32.9±3.9b	1.3±0.1a	323.4±61.9b	32.8±2.0b	1.2±0.1b
	PI257589	378.9±71.6a	33.3±4.9ab	1.3±0.1a	310.1±51.2b	35.6±5.1ab	1.3±0.1ab
	PI196001	377.9±88.6a	36.1±5.7ab	1.4±0.2a	362.1±91.9ab	35.0±5.2ab	1.3±0.2ab
	PI358601	403.5±86.6a	39.3±5.9a	1.5±0.2a	443.7±32.8a	37.2±2.9a	1.4±0.1a
AVERAGE		376.7±78.0A	35.4±5.1A	1.4±0.1A	359.8±59.4A	35.1±3.8A	1.3±0.1A
Pea	JI1210	1.2±4.7b	0.0±0.0b	0.0±0.0a	1.8±5.4a	0.0±0.0a	0.0±0.0a
	JI1213	0.0±0.0b	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	Kebby	3.8±10.3ab	1.5±4.4ab	0.0±0.0a	3.1±6.0a	3.1±6.0a	0.0±0.0a
	P21	8.1±12.9a	4.5±5.6a	0.1±0.1a	1.9±4.2a	1.9±4.2a	0.0±0.0a
AVERAGE		3.3±7.0B	1.5±2.5B	0.0±0.0B	1.7±3.9B	1.2 ±2.5B	0.0±0.0B
Lentil	BGE001402	2.4±5.0a	2.4±5.0a	0.0±0.0a	0.7±2.9a	0.7±2.9a	0.0±0.0a
	ILL4774	1.8±4.3a	1.8±4.3a	0.0±0.0a	1.2±3.5a	1.2±3.5a	0.0±0.0a
	ILL5490	2.0±4.6a	2.0±4.6a	0.0±0.0a	2.0±4.6a	2.0±4.6a	0.0±0.0a
	81S15	1.9±4.5a	1.9±4.5a	0.0±0.0a	2.0±4.3a	2.0±4.3a	0.0±0.0a
AVERAGE		2.0±4.6B	2.0±4.6B	0.0±0.0B	1.5±3.8B	1.5±3.8B	0.0±0.0B
Chickpea	JG-62	1.6±3.7a	1.6±3.7a	0.0±0.0a	1.7±3.6a	1.7±3.6a	0.0±0.0a
	P-2245	1.4±3.5a	1.4±3.5a	0.0±0.0a	1.4±3.4a	1.4±3.4a	0.0±0.0a
	C-104	3.2±7.3a	2.4±4.3a	0.0±0.0a	3.6±5.7a	2.9±4.5a	0.0±0.0a
	ICCV-2	1.9±3.9a	1.9±3.9a	0.0±0.0a	1.2±3.2a	1.2±3.2a	0.0±0.0a

AVERAGE		2.0±4.6B	1.8±3.8B	0.0±0.0B	2.0±4.0B	1.8±3.7B	0.0±0.0B
Common bean	g654	0.8±4.1a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	g1636	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.9±4.3a	0.0±0.0a	0.0±0.0a
	g1955	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.8±3.9a	0.0±0.0a	0.0±0.0a
	g4164	0.7±3.7a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
AVERAGE		0.4±1.9B	0.0±0.0B	0.0±0.0B	0.4±2.0B	0.0±0.0B	0.0±0.0B
Barrel medic	PI239878	1.3±5.2a	0.0±0.0a	0.0±0.0a	1.1±4.6a	0.0±0.0a	0.0±0.0a
	PI516927	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	PI577607	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	A17	0.0±0.oa	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
AVERAGE		0.3±1.3B	0.0±0.0B	0.0±0.0B	0.3±1.1B	0.0±0.0B	0.0±0.0B

Although the observed grass pea infection levels were not so high, a significant interaction between grass pea accessions and the *Fo* ex. *L. sativus* strains was detected. All grass pea accessions developed disease symptoms when inoculated with these two *Fo* strains, but significant differences between accessions and the three assessed traits, AUDPC, DI30, and Dir, were only observed after *Fo* ex. *L. sativus* 2 infection. Grass pea accessions PI195605 and PI257589 with the lower AUDPC values were considered the less susceptible, while PI358601 with the higher AUDPC value was considered the most susceptible for *Fo* ex. *L. sativus* 2 (Table 2). The other traits assessed, DI30 and DIr, corroborate that PI195605 and PI358601 were the most contrasting accessions (Table 2). No significant differences were observed among grass pea accessions inoculated with *Fo* ex. *L. sativus* 1 considering AUDPC. This is in accordance with DIr but not with DI30, where PI195605 and PI358601 were also considered the most contrasting accessions as observed for *Fo* ex. *L. sativus* 2 (Table 2).

3.2 Grass pea susceptibility to different *Fusarium oxysporum* ff. spp.

Grass pea accessions could be infected by *Fo* ex *L. sativus* and by *Fo* f. sp. *pisi* but not by ff. spp. *phaseoli*, *medicaginis*, *lentis*, or *ciceris*. Reisolation of *Fo* strains causing disease symptoms, *Fo* ex *L. sativus* 1 and 2 and *Fo* f. sp. *pisi* races 1 and 2, confirmed *Fo* presence and that the observed symptoms were due to pathogen colonization.

By comparing the AUDPC average values obtained in grass pea it was possible to group the *Fo* strains according to the grass pea response (Table 3). The highest AUDPC value was obtained when grass pea accessions were inoculated with *Fo* f. sp. *pisi* race 2, this being the *Fo* strain considered the most virulent of the six tested in grass pea. This *Fo* f. sp. *pisi* race 2 strain was followed by the *Fo* ex *L. sativus* 1 and 2 and *pisi* race 1 strains group that demonstrated a compatible interaction with grass pea accessions but did not achieve high levels of AUDPC. Lastly,

AUDPC values obtained after infection with *Fo* f. sp. *phaseoli*, *medicaginis*, *lentis*, and *ciceris* were close to zero, revealing an incompatible interaction with grass pea.

Although grass pea accessions revealed the highest AUDPC after infection with *Fo* f. sp. *pisi* race 2 when compared with the other isolates, the obtained average value (944.4) is considered low when compared with the AUDPC average value of the highly susceptible pea accession to *Fo* f. sp. *pisi* race 2 (P21), 2218, used as susceptible control in this study.

Table 3: AUDPC average values and respective standard deviation per grass pea accession for each *Fo* strain. Data followed with different small letters, per column, represents significant differences (P = 0.01) among accessions for each *Fo* strain according to Dunn's test. Data followed by different capital letters, per row, represents significant differences (P = 0.01) among *Fo* strains according to Dunn's test.

	Fusarium oxysporum							
Accession	***pisi* race 2**	**ex. *L. sativus* 2**	**ex. *L. sativus* 1**	***pisi* race 1**	***phaseoli***	***Medicaginis***	***lentis***	***ciceris***
PI195605	701.8±86.6 c	318.4±64.3 b	337.4±74.3 a	283.3±107.0 a	5.3±9.1 a	4.7±9.6 a	5.2±8.2 a	2.5±5.9 a
PI257589	829.8±188.5 bc	311.6±80.1 b	400.1±119.8 a	338.9±71.4 a	5.7±10.6 a	6.2±11.1 a	5.4±10.2 a	4.6±9.6 a
PI196001	1053.5±210.0ab	340.0±114.3 b	400.4±109.3 a	360.6±98.9 a	15.7±12.5 a	7.8±10.6 a	12.9±12.6 a	8.8±10.4 a
PI358601	1192.6±248.3a	495.6±114.2 a	389.0±114.2 a	295.2±110.1 a	9.2±10.8 a	13.2±12.3 a	5.5±9.3 a	13.1±11.1 a
AVERAGE	944.4±183.4 A	366.4±93.2 B	381.7±104.4 B	319.5±96.9 B	9.0±10.8 C	8.0±10.9 C	7.2±10.1 C	7.2±9.2 C

A significant interaction between grass pea accessions and *Fo* strains was detected for AUDPC (Table 3). The tested grass pea accessions were mostly differentiated after infection with *Fo* f. sp. *pisi* race 2 and ex. *L. sativus* 2. The AUDPC values revealed that PI358601 was the most susceptible accession for both strains; PI195605 was the less susceptible for *Fo* f. sp. *pisi* race 2 and one of the less susceptible accessions for *Fo* ex. *L. sativus* 2. Significant interaction between grass pea accessions and the *Fo* strains able to infect grass pea was also detected for the other susceptibility assessed traits, DI30 and DIr (Table 4). The same trend on contrasting accessions was detected when analyzing these traits. In *Fo* ex. *L. sativus* 2, the other two grass pea accessions (PI257589 and PI196001) behaved similarly for DI30 and DIr, and were indistinguishable from the most contrasting accessions (Table 4). However, their AUDPC values revealed that they were more similar to the less susceptible accession PI195605 (Table 3). In *Fo* f. sp.

pisi race 2, these two grass pea accessions revealed values of DI30 near to 100%, similar to the most susceptible accession (Table 4), which was not the case when considering the AUDPC values, where PI257589 behaved similarly to the less susceptible accession (Table 3).

Differences among grass pea accessions inoculated with *Fo* ex. *L. sativus* 1 were only detected on DI30, the maximum disease infection attained at 30 dai (Table 4). However, these contrasting values were not enough to cause differences in progression disease traits such as AUDPC and DIr.

No significant differences were observed among grass pea accessions after infection with *Fo* f. sp. *pisi* race 1 for any of the traits assessed.

Table 4: Maximum disease intensity, obtained at 30 dai (DI30) and disease progress rate (DIr) average values and respective standard deviation per grass pea accession for each *Fo* strain able to infect grass pea. Data followed with different small letters, per column, represents significant differences ($P = 0.01$) between accessions for each *Fo* strain according to Dunn's test. Data followed by different capital letters, per row, represents significant differences ($P = 0.01$) among *Fo* strains according to Dunn's test.

	Fusarium oxysporum							
	***pisi* race 2**		**ex. *L. sativus* 2**		**ex. *L. sativus* 1**		***pisi* race 1**	
Accession	**DI30**	**DIr**	**DI30**	**DIr**	**DI30**	**Dir**	**DI30**	**DIr**
PI195605	74.0±6.0b	3.0±0.5b	32.8±2.0b	1.2±0.1b	32.9±3.9b	1.3±0.1a	31.4±3.2a	1.1±0.2a
PI257589	93.2±7.3a	3.4±0.5ab	35.6±5.1ab	1.3±0.2ab	33.3±4.9ab	1.3±0.2a	32.5±4.1a	1.3±0.2a
PI196001	94.1±9.7a	3.7±0.4a	35.0±5.2ab	1.3±0.2ab	36.1±5.7ab	1.4±0.2a	30.8±5.5a	1.3±0.3a
PI358601	95.1±7.9a	3.8±0.5a	37.2±2.9a	1.4±0.2a	39.3±5.9a	1.5±0.2a	28.4±5.7a	1.1±0.3a
AVERAGE	89.1±7.7A	3.5±0.5A	35.1±3.8B	1.3±0.2B	35.4±5.1B	1.4±0.2B	30.8±4.6B	1.20B

The interaction between the different grass pea accessions and the different *Fo* strains able to infect grass pea is graphically represented in Figure 1. Grass pea accessions did not behave in the same way when infected with different *Fo* strains, indicating different responses depending on the *Fo* strain. Although able to cause infection in all grass pea accessions, neither *Fo* ex. *L. sativus* 1 nor *Fo* f. sp. *pisi* race 1 were able to distinguish among grass pea accessions. Nevertheless, significant differences were observed among grass pea accessions when infected with *Fo* ex. *L. sativus* 2 and *Fo* f. sp. *pisi* race 2, with the range of grass pea accessions responses much wider with the last strain with the identification of highly susceptible accessions (Figure 1).

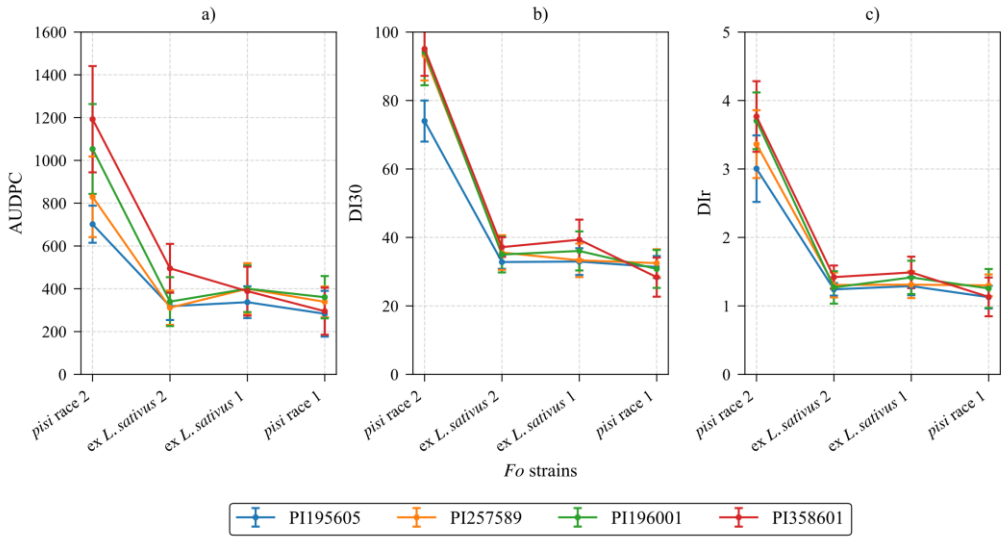

Figure 1: Average values of grass pea accessions in response to different *Fo* strains. a) AUDPC, b) maximum disease intensity at 30 dai (DI30), c) disease progress rate (DIr). Bars represent standard deviation.

4. Discussion

The drastic impact caused by fusarium wilt diseases on several legume crops (Infantino et al., 2006) and the difficulty in eradicating the pathogen (*Fo*) causing it from the soil makes the host range characterization of newly detected *Fo* isolates an important step in the development of appropriate crop protection strategies.

The current study aimed to determine the host range of *Fo* ex. *L. sativus* 1 and 2 recently isolated from naturally infected grass pea plants using differentially related legume species that are infected by different *Fo* ff. spp., such as pea, lentil, chickpea, common bean, and barrel medic. Likewise, this study also aimed to analyze the disease responses of grass pea plants to the different *Fo* ff. spp. affecting these related legumes, under controlled conditions. If host range overlap occurs, this information will have repercussions not only on legume fusarium wilt management, due to the presence of alternative hosts, but also on the search for resistance sources needed for the development of resistance varieties, due to the possibility of having to search a broader species base.

The results demonstrated that *Fo* ex. *L. sativus* 1 and 2, although causing moderate levels of infection, are specific to grass pea. They caused negligible symptoms in all other legumes tested that were considered non-hosts for these strains. AUDPC differences among pea accessions in response to *Fo* ex. *L. sativus* 1 have been detected, although

the values were overall negligible and the slight yellowing on older leaves was probably caused by the plant's natural aging.

Nevertheless, the two *Fo* f. sp. *pisi* races tested were able to infect all the grass pea accessions analyzed, causing similar (race 1) or greater (race 2) disease symptoms than the strains retrieved from grass pea plants. *Fusarium oxysporum* f. sp. *pisi* race 2 and *Fo* ex. *L. sativus* 2 were the strains that better distinguish grass pea accessions reactions, showing, in general terms, the same contrasting accessions.

Known as a destructive disease in numerous legumes, fusarium wilt is mainly characterized as being caused by host-specific strains (Kankanala et al., 2019). This host-specific characteristic is in accordance with the infection pattern obtained by the newly *Fo* strains retrieved from grass pea plants, *Fo* ex. *L. sativus* 1 and 2, where disease symptoms were only detected in grass pea. However, *Fo* f. sp. *pisi* does not behave equally, infecting pea but also grass pea accessions, confirming that the *Fo* host range can also be broader and the same plant species be infected by different strains (Edel-Hermann and Lecomte, 2019).

Probably due to the "deep-rooted" idea of a high specificity of this pathogen, literature in the search for potential alternative species hosts among *Fo* ff. spp. is very scarce. The available information on host range characterization of the different *Fo* ff. spp. used in this study is no exception. The continuous search for resistant leguminous genotypes against their own *Fo* is well documented, with several studies done on the characterization of species-specific germplasm collections (Bani et al., 2012; Leitão et al., 2020; Pouralibaba et al., 2015; Rispail and Rubiales, 2014; Sharma et al., 2005). However, this might not be enough to see if the *Fo* strain in question is able to infect others beyond their known host species and use related species as an alternative host. The presence of these alternative hosts can hinder disease management by allowing the pathogen's survival from one growing season to the next. In this way, characterizing the "broad" *Fo* ff. spp. host range, even if considered narrow a priori, is an important task for fusarium wilt management. Furthermore, alternative hosts can also contribute to the continuous search for resistance sources against these pathogens.

In *Fo* f. sp. *pisi*, four races (*Fop* race 1, 2, 5, and 6) are established, the first two distributed worldwide and the second two centered in western Washington State in the US (Infantino et al., 2006). Due to their worldwide spread, only race 1 and 2 were tested in the present study. Here, we found for the first time that *Fo* f. sp. *pisi* strains can also infect grass pea, with race 2 the most virulent strain in grass pea plants. Although this is the first report on *Fo* host sharing between pea and grass pea, these results are in line with previous studies, where it was demonstrated that other

pea fungal pathogens have the capability to infect grass pea plants. *Ascochyta pinodes*, the causal agent of pea ascochyta blight, is also able to infect grass pea, producing moderate or high levels of infection, while on the other hand, *A. fabae*, *A. rabiei* and *A. lentis*, the causal agents of ascochyta blight on faba bean, chickpea, and lentil, respectively, are not able to infect grass pea (Barilli et al., 2016). Similarly, grass pea can be severely infected by *Uromyces pisi*, the causal agent of rust in pea, but not by *U. ciceris-arietini* or *U. viciae-fabae* (Almeida et al., 2014; Vaz Patto and Rubiales, 2009). Compatible interaction between grass pea accessions and *Erysiphe pisi*, the causal agent of powdery mildew infection in pea, are also reported (Vaz Patto et al., 2006a). Nevertheless, most of these reported cases refer to weak specialized pathogens, with *A. pinodes* known as the less specialized *Ascochyta* spp. (Barilli et al., 2016; Le May et al., 2014) and *U. pisi* considered the less-specialized species of the genus with the broadest host range (Barilli et al., 2012; Rubiales et al., 2013).

Pathogen sharing is more likely to happen among related plant species (Gilbert et al., 2015), and the same also seems to be true for *Fo* strains. Interestingly, *Fo* f. sp. *pisi* has been previously described as also able to infect chickpea (De Curtis et al., 2014), suggesting a broader host range for this pathogen. Other *Fo* legume infecting f. sp., like *Fo* f. sp. *tracheiphilum*, the causal agent of fusarium wilt in cowpea (Armstrong, 1980), also promote disease in soybean, a species with high genome co-linearity with cowpea (Pottorff et al., 2012). However, the opposite was not described and reports of *Fo* f. sp. *glycines*, the strain responsible for the disease in soybean, are not existent in cowpea, hampering the comparison with our results. In other plant families, like *Cucurbitaceae*, the *Fo* host range overlapping is frequently described but seems more complex than in the case of *Fabaceae* species. *Fo* f. sp. *cucumerinum*, causing wilt in cucumber (*Cucumis sativus*) can also be pathogenic to both melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*). Melon and muskmelon (also *C. melo*) are hosts of *Fo* f. sp. *melonis* and watermelon is host of *Fo* f. sp. *niveum*, this last one also capable of infecting cucumber and melon (Koike et al., 2007).

The ability of *Fo* f. sp. *pisi* race 2 to infect with high virulence the grass pea accessions might in part be explained by the phylogenetic proximity of pea and grass pea (Schaefer et al., 2012; Wojciechowski et al., 2004). However, when compared with pea susceptibility, grass pea infection levels are not as severe. The susceptible pea accession used in this study (P21) revealed an AUDPC average of 2218, a value similar to the one obtained by Bani et al. (2012), 2274, under similar inoculation and incubation conditions. This is almost twice the AUDPC value of the most susceptible grass pea accession characterized in the present study. Acting as a host for *Fo* f. sp. *pisi* race 2 but with lower disease symptoms, grass pea can be a promising related species to search for resistance against this

pathogen. In fact, the four grass-pea-tested accessions demonstrated different levels of susceptibility when inoculated with *Fo* f. sp. *pisi* race 2. Grass pea accession PI195605 was consistent for all the susceptibility parameters analyzed, always showing lower disease levels than the other grass pea accessions. Furthermore, although the other three grass pea accessions tested showed similar final scores of disease intensity, with values near to 100%, differences in the disease intensity and speed of progression, with some accessions reaching almost 100% of disease intensity faster than others, suggests that different disease response mechanisms could exist among grass pea accessions.

Susceptibility of grass pea plants to *Fo* f. sp. *lentis* was reported by Talukdar (2013) in India, suggesting that grass pea could also be a host for *Fo* f. sp. *lentis*. However, this was not observed in the present study using a high virulent lentil strain from Iran (Pouralibaba et al., 2016). Differences in the virulence among *Fo* f. sp. *lentis* isolates were already reported, leading to the recent identification of eight races among *Fo* f. sp. *lentis* Indian isolates (Hiremani and Dubey, 2018). However, no race information about the *Fo* f. sp. *lentis* strain used in this study is available, nor is information available about the Indian virulent strain infecting grass pea identified by Talukdar (2013), limiting the discussion about race-specific responses.

The absence of disease symptoms in grass pea after inoculation with the *Fo* f. sp. *phaseoli*, *medicaginis*, *lentis* and *ciceris* tested make them not virulent in grass pea. These *Fo* ff. spp. are the causal agents of fusarium wilt in legume species phylogenetic distant from grass pea, and probably too distant to share *Fo* strains.

Despite the several *Fo* strains that have been already identified, the ubiquitous distribution of this pathogen makes the occurrence of novel *Fo*-host plant interaction a likely future event (Edel-Hermann and Lecomte, 2019). The occurrence of new *Fo* outbreaks in crops is highly influenced by human activities (Gordon and Martyn, 1997), such as the introduction of new crop species into production systems and/or the use of certain recurrent agricultural practices (Stukenbrock and McDonald, 2008). With the predicted increase in global temperature, more severe and frequent damaging epidemics are expected. By affecting pathogen development, increasing growth and survival rates, a rapid spread into new locations leading to a fast emergence of virulent strains can endanger plant resistance responses and allow contact with new potential hosts (Elad and Pertot, 2014; Garrett et al., 2006). In fact, an example of emergence of new variants by adaptation has recently reported in grass pea for *Ascochyta* in Italy, with new isolates very specific on grass pea reported that were considered derived from *A. lentis* on grass pea (Infantino et al., 2016). Therefore, both these new *A. lentis* var. *lathyri* (Infantino et al., 2016) and *A. pinodes* (Barilli et al., 2012) can infect grass pea, and they should be compared.

Although grass pea is considered an underused crop, some European countries are showing a renewed interest in it (Lambein et al., 2019). In Portugal, grass pea is an important source of revenue for some local economies (Vaz Patto et al., 2011). In the past, the Alvaiázere region of Portugal was an important producer of grass pea, however, over the years cultivation was reduced (Vaz Patto, 2009). More recently, grass pea production in this area has expanded again due to a renewed interest in the region's traditional gastronomic heritage. Grass pea has become more attractive to consumers and, consequently, to farmers, which has led to an increase in the number of growers and cultivated areas. The intensification of production without a proper crop rotation might be contributing to fusarium wilt grass pea outbreaks. Another possibility is that the fungus was already in the soil from ancient periods of cultivation and is now increasing in abundance due to the increased presence of a host, allowing symptoms to be detected.

For now, *Fo* ex. *L. sativus* 1 and 2 are not considered highly virulent or aggressive strains, but we cannot rule out that due to successive and long-term cultivation of grass pea in the same areas, the virulence of these strains might increase in the future. The fact that *Fo* f. sp. *pisi* has a broader host range suggests the possibility that *Fo* ex. *L. sativus* 1 and 2 might have evolved from this less specialized strain. Indeed, in the Alvaiázere region some farmers tend to cultivate grass pea and pea together in the same field and the presence of fusarium wilt has been detected on those pea plants (Bani et al., 2014). Unfortunately, the ITS sequence analysis did not allow us to confirm this suggestion, for although it confirmed the *Fo* identity it could not differentiate among the *Fo* legume strains under study. Despite the absence of phylogenetic relationship confirmation between *Fo* ex. *L. sativus* strains and *Fo* f. sp. *pisi* by ITS analysis, the results from the grass pea disease symptoms evaluation indicate that *Fo* ex. *L. sativus* 1 and 2 origin could have been due to a co-evolution of *Fo* with a new host. An example on acquired pathogenicity of a local *Fo* population was already reported on a *Fo* f. sp. *cubense* Brazilian population. Through horizontal gene transfer of pathogenicity genes from pathogenic strains, probably introduced, to non-pathogenic, new local pathogenic strains can evolve (Deltour et al., 2018).

Although this study was initiated due to a regional detection of fusarium wilt symptoms in Portuguese grass pea fields, grass pea cultivation is growing in marginal lands over three different continents (Lambein et al., 2019). Furthermore, grass pea plays an important role in several developing countries where, beyond its use as a source of dietary protein, it also provides an income to resource-poor farmers (Dixit et al., 2016; Lambein et al., 2019), making the knowledge gained here relevant for disease management worldwide.

Despite the limited number of accessions per species and the restricted geographical origin of the strains isolated from grass pea plants, results from cross-inoculation assays between *Fusarium oxysporum* vs. *Lathyrus sativus* and related legume species were reported here for the first time. Although not considered highly virulent, the presence of *Fo* in Portuguese grass pea was confirmed. For now, *Fo* f. sp. *pisi* race 2 is the most virulent strain characterized in grass pea. The variability detected in disease intensity among grass pea accessions suggests that variable response mechanisms to *Fo* f. sp. *pisi* race 2 may be present in grass pea, creating opportunities to develop resistant varieties against this pathogen.

5. Conclusions

Broader host range studies are important even if the pathogen is considered specialized. For a newly identified pathogen, the host range characterization can provide new insights on its origin and evolution, but overall, it can contribute to the improvement of disease management control, revealing which plant species could act as alternative hosts and as alternative resistant sources. The two *Fo* strains recently isolated from grass pea Portuguese fields (*Fo* ex. *L. sativus* 1 and 2) are host-specific, infecting only grass pea plants, with low aggressiveness. The causal agent of fusarium wilt in pea, *Fo* f. sp. *pisi* race 2, is the most virulent strain characterized at the moment in grass pea plants, more than the *Fo* strains retrieved from grass pea. Grass pea is now considered an alternative host for *Fo* f. sp. *pisi* and can be further explored to identify new sources of resistance against this pathogen.

CRediT authorship contribution statement

A.M.S.: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft. **D. R.**: Resources, Supervision, Writing - review & editing. **M.C.V.P.**: Conceptualization, Supervision, Writing - review & editing.

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