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RESEARCH ARTICLE

Age-related resistance to *Fusarium oxysporum* f. sp. *cepae* and associated enzymatic changes in seedlings of *Allium cepa* and *A. fistulosum*

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ABSTRACT

This research analysed the response of onion (*Allium cepa*) and *A. fistulosum* against *Fusarium oxysporum* f. sp. *cepae* (*Foc*) isolates and the associated changes in peroxidase, β-1,3-glucanase and chitinase activities. The response of *A. cepa* and *A. fistulosum* at different stages of seedling development were also evaluated. Several seedling tests were performed, and disease symptoms were evaluated 12-14 days after inoculation. *Allium fistulosum* behaved as more resistant than *A. cepa* cultivars by exposition to the most aggressive *Foc* isolates at sowing date. Increased levels of peroxidase and glucanase activities were found in the *A. cepa* and *A. fistulosum* seedlings exposed to the pathogen, and were positively correlated with disease symptoms. For chitinase activity, this correlation was found only for *A. cepa*. Two peroxidase isoforms were found to be specific for *A. fistulosum* roots after inoculation and could be involved in resistance. The inoculation at 7, 14 and 42 days after sowing showed that both host species were resistant to *Foc,* proving that onion susceptibility decreased promptly after germination. However, an increase in peroxidase and glucanase activities in 7-and 14-day-old inoculated seedling was detected only for *A. cepa,* suggesting an earlier acquisition of resistance in *A. fistulosum.*
Key words: bunching onion, chitinases, glucanases, onion, peroxidases.

INTRODUCTION

Fusarium basal rot affects onion (*Allium cepa* L.) and other *Allium* species worldwide (Cramer, 2000). The pathogen infects the roots or the basal plate of the bulb. The disease symptoms progresses from darkening of the stem-plate (necrosis), yellowish of older leaves, wilting during the season, up to bulb rotting during post-harvest storage causing important marketable yield losses (Cramer, 2000; Schwartz & Mohan, 2008). *Fusarium oxysporum* Schlecht. f. sp. *cepae* (*Foc*) is the most widespread and commonly found causal organism (Schwartz & Mohan, 2008), although *F. proliferatum* (Matsush.) Nirenberg and other *Fusarium* species have been also reported (Galván et al., 2008). In phylogenetic studies, *F. oxysporum* isolates from onion have been grouped into two clades, among several genetic clades known for other *formae specialis* of this species complex (Galván et al., 2008; Taylor et al., 2013; Southwood et al., 2012).

Fusarium may also cause damping-off after seedling emergency, or no seedling emergency, although onions become more resistant during vegetative growth (Cramer, 2000; Stadnik & Dhingra, 1995). The adult plants become susceptible again after the beginning of bulbing and consequently the disease may appear late in the season and persist until postharvest storage (Cramer, 2000; Schwartz & Mohan, 2008). The relationship between development and induction of resistance in plants has been known since long ago (Kahn & Libby, 1958), and has been found in diverse plant-pathogen interactions (Panter & Jones, 2002). Although the influence of onion developmental stage on *Foc* susceptibility is well known, there is scarce experimental data supporting and explaining this knowledge.

No complete resistance to *Fusarium* basal rot has been found within *Allium cepa*, although quantitative differences in susceptibility have been exploited in onion breeding (Cramer, 2000; Taylor et al., 2013). Higher levels of resistance to onion-pathogenic *Fusarium* isolates has been found in adult plants of *Allium fistulosum* (bunching onion, Welsh onion) in greenhouse tests (Holz & Knox-Davies, 1974; Galván et al., 2008). Hence, *Allium fistulosum* is a good candidate as a source of resistance

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in onion breeding, through bridge crossing with *A. roylei* (Khrustaleva & Kik, 1998).

It is known that plants react against pathogen attack by activating an array of defence mechanisms, including the synthesis of proteins with antimicrobial activity (Niks et al., 2011; Ferreira et al., 2007). Several reports reviewed by Develey Rivière & Galiana (2007) have related the developmental changes in resistance with the expression of genes that codify for pathogenesis-related (PR) proteins. Diverse enzymes including peroxidases, β-1,3-glucanases and chitinases are described as PR proteins (Passardi et al., 2005; Van Loon et al., 2006).

The high versatility of isoperoxidases allows them to be involved in a range of physiological and developmental processes (Passardi et al., 2005; Bakalovic et al., 2006). Among these, class III peroxidases (EC 1.11.1.7) can play a role in defence against pathogens by involvement in physiological responses, specific resistance responses, or induction during pathogenesis as a response to damage (Neale et al., 1990; Dowd & Johnson, 2005; Silvar et al., 2008). Chitinases or poly(1,4-N-acetyl-glucosaminyl-) glycanohydrolases (EC 3.2.1.14) catalyse the hydrolysis of the β-1,4 link between units of N-acetyl-glucosamine in the polysaccharide chitin. Their genetic expression and enzymatic activity is highly dependent on the organ and developmental stage. β-1,3-glucanases (EC 3.2.1.39) catalyse the hydrolysis of β-1,3-glycosidic links in β-1,3 D-glucans such as callose and laminarine present in the cell walls of plants and fungi. Glucanases are generally induced in response to pathogen attack or environmental stress (Simmons, 1994; Buchner et al., 2002). A proposed defence role for glucanases is the release of elicitors from the pathogen leading to the induction of defence responses (Lawrence et al., 2000). In some reports the constitutive levels of β-1,3-glucanases contributed to host resistance (Silvar et al., 2008) whereas other reports found that their induction was not associated with resistance (Pritsch et al., 2001).

Zappacosta et al. (2003) evaluated the changes in the enzymatic activities in calli of *Allium cepa* (susceptible) and *A. fistulosum* (resistant) against *Phoma terrestres*, the causal agent of pink root. The exposure to the pathogen increased peroxidase and β-1,3-glucanase activities in onion but not in *A. fistulosum*, which presented higher constitutive levels than onion. In onion plants exposed to *Botrytis allii* (McLusky et al., 1999) peroxidase activities increased next to the inoculated zone, and were associated to papillae development. Age related resistance in *A. cepa* and *A. fistulosum* against *Fusarium* pathogenic isolates may be related with the expression and changes in the enzymatic activities.

The aims of this research were: (i) to analyse the response of *A. cepa* and *A. fistulosum* against *Fusarium oxysporum* f. sp. *cepae* and the associated changes in enzymatic (peroxidase, β-1,3-glucanase and chitinase) activities; and (ii) to evaluate the response of *A. cepa* and *A. fistulosum* at different stages of seedling development.

MATERIALS AND METHODS

Plant material and *Fusarium* isolates

Seeds of *Allium cepa* cv. 'Pantanoso del Sauce CRS' (referred onwards as 'Pantanoso') and *Allium fistulosum* (accession UR05010, cultivated, collected in Uruguay) were produced at the Centro Regional Sur (CRS, Facultad de Agronomía, Progreso, Uruguay) and used in this study. The onion cv. 'Brava' was obtained from Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. 'Pantanoso' is an intermediate-day cultivar, whereas 'Brava' is a long-day onion cultivar. Seed lots did not receive fungicide treatments. Before each experiment, seeds were surface-disinfected during 1 min in a sodium hypochlorite solution (15 g/L), and washed two times with sterile destilled water.

The *Fusarium oxysporum* isolates UR06, UR17-8, EZA, NL109-2 and NL93186 were obtained from the strain collection of the Laboratório de Fitopatologia (Facultad de Agronomía, Universidad de la República, Uruguay). Several strains with different aggressiveness were used in order to obtain a range of disease and enzymatic responses. All strains were isolated from onion and had their pathogenicity confirmed. Their origins were described in Galván et al. (2008). Each *Fusarium* inoculum was produced as a suspension of conidia obtained from 10-15-day-old colonies grown on potato dextrose agar (PDA), filtered through sterilized cheesecloth and adjusted to 3×10^5 conidia/mL of sterile distilled water.

Response of *Allium* species against *Foc* isolates

In order to evaluate the response of *Allium* species against *Foc* isolates, two experiments were performed. In the first experiment, *A. fistulosum* and cv. 'Pantanoso' were tested against isolates UR17-8, NL109-2, and EZA. In the second experiment, *A. fistulosum* and cvs. 'Brava' and 'Pantanoso' were tested against UR06, EZA, and NL93186.

These responses were evaluated using seedlings tests as described by Krueger et al. (1989) and Calegiore-Gei et al. (2004) and modified as follows. Sterilized aluminium paper pots (0.18 L) filled with heat-sterilized sand were poured on with 20 mL of the corresponding suspension of conidia, and homogenized using a spoon (3×10^4 conidia/g of dry sand). Seeds of the corresponding *Allium* accession were sown and placed within an individual polyethylene bag to prevent dehydration and avoid cross contamination. For each combination of isolate and host accession, six pots with 30 sown seeds each were used. Non-inoculated pots poured on with sterile distilled water were included as control treatment. The pots were randomly distributed in a solarium maintained at 28-30°C with a 12 h of fluorescent light regime (75 mmol/m^2/s) during 14 days. At this time, the total number of emerging seedlings and the number of normal seedlings (defined as fully developed seedlings, with similar aspect and size than seedlings in the non-inoculated control) were recorded. Each complete experiment was run

two times. Normal seedlings were further collected and processed as described below for enzymatic analyses.

Tests at different seedling ages

Two experiments were carried out to evaluate the response of *A. fistulosum* and cv. 'Pantanoso' at different seedling ages against *Fusarium*. The first experiment evaluated the responses of 0-, 14- and 42-day-old seedlings, whereas the second experiment evaluated the responses of 0 and 7 days old seedlings. Inoculation at sowing (day 0) was performed as described before. For inoculation of 14 and 42 days old seedlings, these were produced in trays containing a sterilized mixture of horticultural substrate and sand (1:1) irrigated with sterile destilled water. Fourteen-day-old seedlings were transferred to pots filled with sand infected with a suspension of *Foc* UR17-8 (3×10^4 conidia/g dry sand). The same procedure was followed for the inoculation at day 42. Ten pots (replicates) per age treatment containing three seedlings each were included, and ten pots per age treatment were included as non-inoculated control. At the moment of transplantation the medium was watered with a solution of 2 g/L of Phostrogen (Bayer Garden). After 14 days the seedlings were counted as total and normal, as explained before. Normal seedlings were further collected and processed as described below for enzymatic analyses.

For inoculation of seven-day-old seedlings, *A. cepa* cv. 'Pantanoso' or *A. fistulosum* were sown in pots (30 seeds per pot, six replicates per treatment), irrigated with 10 mL of sterilized water, and placed into an individual plastic bag. After seven days, the inoculum (isolate NL93186, 20 mL) was distributed into the sand at several positions next to the seedlings, using a syringe. After 14 days, the seedlings were counted as total and normal ones. Non-inoculated control pots were poured on with sterile water. Normal seedlings were further collected and processed as described below for enzymatic analyses.

Disease evaluation and data analysis

A Damage Index (DI) was defined as 1 – (Inoculated/Control), where 'Inoculated' is the value of the variable (e.g., number of emerged normal seedlings) in the inoculated treatment, and 'Control' is the value of the variable in the control treatment. DI values close to zero indicate either high plant resistance or low virulence of the pathogen, whereas values close to one indicate host susceptibility or pathogen aggressiveness. Repeated experiments were considered as replications over time. Analysis of the variables involved mixed models and estimation of components of variance by restricted maximum likelihood (REML) using INFOSTAT (Universidad de Córdoba, Argentina).

Enzymatic analyses

Normal seedlings collected at the end of seedling tests were washed out with distilled water, kept frozen at -20°C or lyophilized and stored at -20°C until use. Two extracts were prepared pooling the seedlings from each treatment: one with the roots plus the basal plate (identified as 'root extracts'), and the other with the leaves and false stems (identified as 'leaf extracts'). Seedling tissues were extracted with 50 mM acetate buffer pH 5.6 (6:1 v/w) and the suspensions were centrifuged at 7000 g for 15 min at 4ºC. The cleared supernatants were used to determine soluble protein concentration and enzymatic activities.

Protein concentration (mg/ml) was determined using the Bradford method (1976) and bovine serum albumin as standard. The enzyme substrates laminarine, *o*-dianisidine, p-nitrophenyl-b-D-glucosamine, and Chitin Azure were from Sigma. All assays were run in triplicates.

Peroxidase (POX) activity was determined spectrophotometrically by recording the increase in absorbance at 460 nm due to the oxidation of *o*-dianisidine (Shannon et al., 1966). A molar extinction coefficient of 1.13×10^4 M/cm was used for the oxidized *o*-dianisidine. One enzyme unit (EU) was defined as the amount of enzyme causing decomposition of 1 µmol of *o*-dianisidine/min at 23ºC and pH 5.6. Enzymatic activity was expressed as EU/mL and the specific activity (SA) was defined as the ratio between enzymatic activity and enzyme concentration.

β-1,3-glucanase (EGA) activity was determined according to Abeles and Forrence (1970) using laminarine (1% in 50 mM acetate buffer pH 4.8) as substrate. The released reducing sugars were determined as glucose using the 3,5-dinitrosalicylic acid reagent at 540 nm (Bernfeld, 1955). One EU was defined as the amount of enzyme that produces 1 mg of glucose/min under the assay conditions.

Total chitinase (CHI) activity was determined using Chitin Azure as substrate according to Sung Kim et al. (2000) and modified as follows. A volume of 150 µL of chitin azure suspension (5 mg/mL in 50 mM acetate buffer pH 5.6) was incubated with 300 µL of sample for 3 h at 40ºC. The suspensions were centrifuged at 7000 g for 5 min at 4ºC. The released soluble dye was measured at 560 nm. Samples were run in triplicates. One EU was defined as the amount of enzyme that produces an increase of 0.01 units in the absorbance at 560 nm (Gómez Ramirez et al., 2004).

N-acetyl-β-glucosaminidase (NAG) activity, which catalyses the production of monomers of N-acetyl-β-glucosamine from dimers or oligomers derived from chitin degradation by endo- and exo-chitinases, was determined according to Frändberg and Schnürer (1994) and modified as follows. A sample volume of 10 µL was incubated with 90 µL of the substrate *p*-nitrophenyl-β-D-glucosamine (0.2 mM in 50 mM acetate buffer pH 5.6) for 30 min at 40ºC. The reaction was stopped with 10 µL of 1M NaOH and the release of *p*-nitrophenol was followed at 405 nm. A calibration curve was built up with *p*-nitrophenol (0-100 µM). One EU was defined as the amount of enzyme that catalyses the production of one nmol of *p*-nitrophenol/min under the assay conditions.

Electrophoretic analyses

Isoelectric focusing (IEF) was done using the Phast-System equipment (Pharmacia). Samples were

run in PhastGel IEF 3-9 according to the manufacturer's instruction and specifically stained for POX activity using *o*-dianisidine and H_2O_2 in the same concentration as in the soluble enzymatic assay. A pI 3-9 marker was included.

RESULTS

Response of *Allium* species against *Foc* isolates

Allium fistulosum and *A. cepa* cv. 'Pantanoso' were evaluated against three *Fusarium* strains in the first experiment (Figure 1). *Allium fistulosum* behaved as resistant against isolates UR17-8, NL109-2 and EZA, as the number of total and normal seedlings did not significantly differ from the non-inoculated treatment. The onion cv. 'Pantanoso' was moderately affected by UR17-8 and NL109-2 and severely affected by the EZA isolate (Figure 1A, B). Damping-off occurred either at pre- or post-emergency of seedlings.

The responses of *Allium fistulosum* and *A. cepa* cvs. 'Pantanoso' and 'Brava' against UR06, EZA and NL93186 isolates were also studied (Figure 1C, D). Both onion cultivars showed similar levels of susceptibility, with a slight decrease in the number of total and normal seedlings caused by UR06, without significant difference with the non-inoculated control. A significant reduction in the number of seedlings was caused by the EZA isolate, and no seedling emergence was found after inoculation with NL93186. *Allium fistulosum* behaved as resistant to the UR06 and EZA isolates, without significant difference to the control treatment, but were significantly affected by NL93186, although to a lesser extent than the onion cultivars.

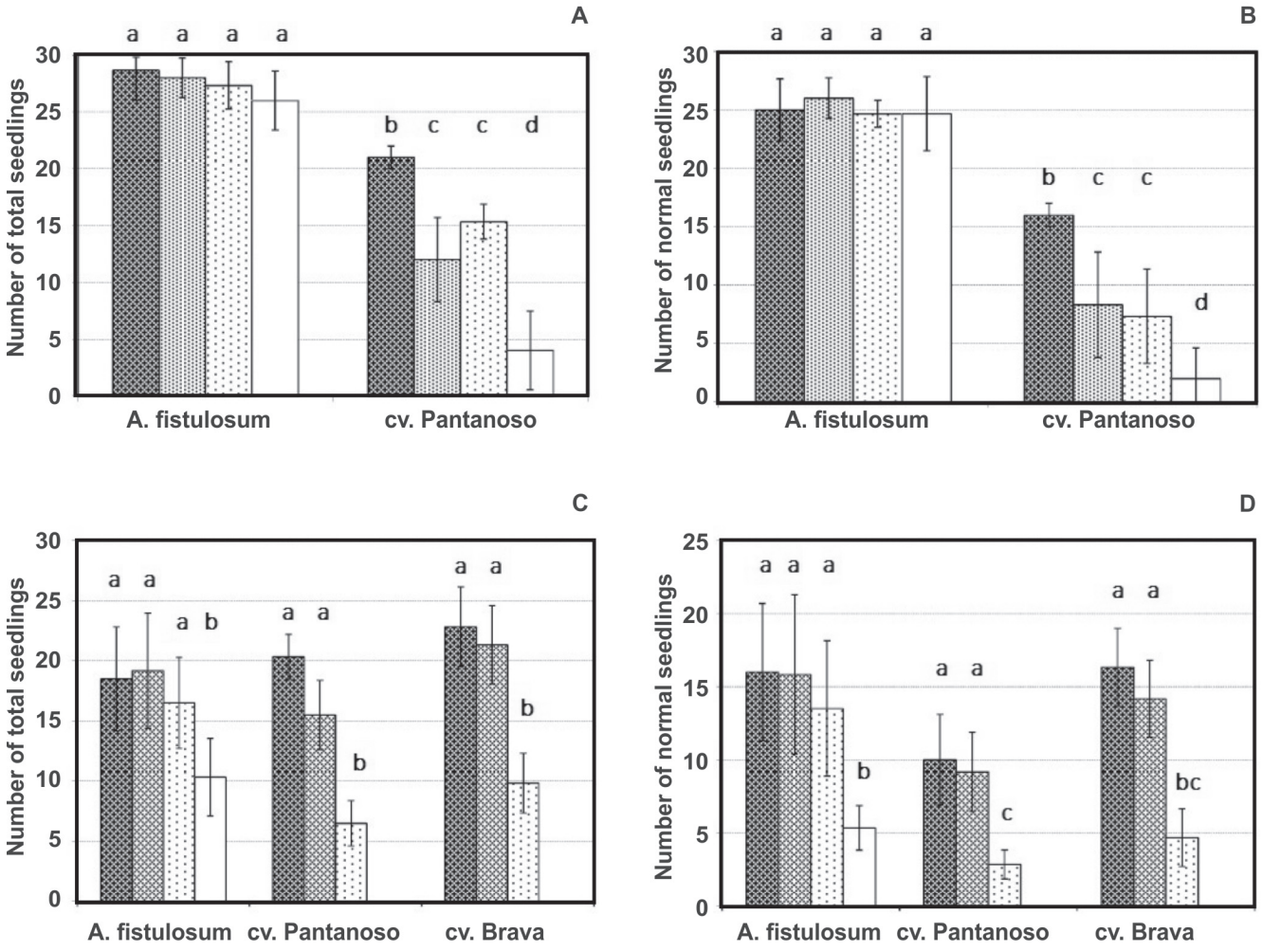

FIGURE 1 - Response of *Allium* species against *Foc* isolates. **A**, **C**: Total number of emerged seedlings. **B**, **D**: Number of fully developed normal seedlings. **A**, **B**: Non-inoculated control ( ); seedlings exposed to *Fusarium oxysporum* f. sp. *cepae* isolates UR17-8 ( ), NL109-2 ( ) and EZA ( ). **C**, **D**: Non-inoculated control ( ); seedlings exposed to UR06 ( ), EZA ( ) and NL93186 ( ) isolates. Lines with slashed ends represent the standard deviation for each mean. Columns with the same letter at the top do not differ significantly (REML analysis, $p<0.05$).

Enzymatic analyses were performed for root and leaf extracts prepared from the seedlings of *A. fistulosum* and *A. cepa* cvs. 'Pantanoso' and 'Brava' inoculated with isolates UR06, EZA and NL93186, as well as the control treatment. Peroxidase (POX), β-1-3-glucanase (EGA), chitinase (CHI) and N-acetyl-β-glucosaminidase (NAG) activities were determined in these extracts. The largest variations in enzymatic activities were detected in the roots, thus the results presented in Table 1 corresponds to root extracts only. The inoculation of onion cultivars 'Pantanoso' and 'Brava' with UR06, the mildest isolate, lead to increased POX and CHI activities compared with the controls (REML analysis, $p<0.05$). A significant increase in EGA activity was observed in cv. 'Brava' but not in 'Pantanoso', whereas no significant changes were observed for NAG activity. When the cultivars were exposed to the EZA isolate, the four enzymatic activities markedly increased in both *A. cepa* cultivars in comparison to the non-inoculated controls (REML analysis, $p<0.05$) (Table 1). Finally, the inoculation of onion cultivars 'Pantanoso' and 'Brava' with NL93186 caused total devastation of the seedlings, preventing the preparation of extracts.

Inoculation of *Allium fistulosum* with the UR06 and EZA isolates did not affect POX, EGA and NAG activities. CHI activity was increased following inoculation with EZA, while no change was observed after inoclation with UR06. Inoculation of *A. fistulosum* with NL93186, the most aggressive isolate, caused significant increases in POX, EGA and NAG activities and a decrease in CHI activity (Table 1).

In general, enzymatic activities increased as the damage index (DI) increased, and therefore larger changes were observed for the most aggressive *Foc* strains. An exception was the case of CHI activity in *A. fistulosum* seedlings exposed to NL93186, where the activity was lower than that of the control, as mentioned above. The increase in POX activity was positively correlated with the increase in DI, with Pearson correlation index R = 0.995 for cv. 'Pantanoso'; R = 0.987 for cv. 'Brava' and R = 0.981 for *A. fistulosum*. For EGA activity the correlations were, respectively, 0.990, 0.970 and 0.962, whilst for NAG activity R values were, respectively, 0.999, 0.985 and 0.959. For CHI activity R values were 0.976 for 'Pantanoso' and 0.978 for 'Brava', whereas this correlation was not significant for *A. fistulosum*.

The expression of isoperoxidases evaluated by isoelectric focussing (IEF) was assessed for *A. cepa* cvs. 'Pantanoso' and 'Brava' and for *A. fistulosum*. The patterns for both onion cultivars were similar (roots and leaves) except for the isoform corresponding to pI 8.0, which was expressed only in roots from cv. 'Pantanoso' (Figure 2A). *Allium fistulosum* profiles are different from *A. cepa* ones in root and leaf extracts. The expression profiles after inoculation with the *Foc* isolates UR06 and EZA are presented only for *A. cepa* 'Pantanoso' and *A. fistulosum* (Figure 2B). An isoform of pI 8.0 was repressed in the roots of cv. 'Pantanoso' after inoculation with the EZA isolate, while an isoform of pI 7.25 is slightly expressed in response to both *Foc* isolates. This isoform was also expressed in *A. cepa* 'Brava' (profile not shown). In the leaf extracts of *A. cepa* cv. 'Pantanoso', the strong expression of an acidic isoform of pI 3.65 was observed (also present in cv. 'Brava', data not shown). In *A. fistulosum* roots, inoculation with isolate EZA induced the expression of two isoforms of pI 8.3 and 8.55, whereas in the leaf extract it induced repression of the isoform of pI 8.15 (Figure 2B).

Tests at different seedling ages

The effect of seedling age in the resistance to the pathogen was investigated by exposing *A. cepa* cv. 'Pantanoso' and *A. fistulosum* to the UR17-8 isolate at days 0 (sowing), 14 and 42 (Table 2). Both accessions

TABLE 1 - Enzymatic activities in roots extracts from seedlings of *Allium fistulosum* and *A. cepa* cvs. 'Pantanoso' and 'Brava' inoculated with three *Fusarium oxysporum* f. sp. *cepae* isolates at sowing day.

Accessions	Treatments	DI [1]	POX [2]	EGA [2]	CHI [2]	NAG [2]
A. fistulosum	Control		27.5	474	72.5	643
	UR06	0.01	29.6	420	64.4	519
	EZA	0.16	30.1	461	157.8*	620
	NL93186	0.67*	70.2*	1255*	33.6*	1017*
Pantanoso [3]	Control		69.2	952	78.3	753
	UR06	0.08	131.9*	1083	125.7*	792
	EZA	0.72*	359.0*	1699*	222.7*	1290*
Brava [3]	Control		81.5	517	105.9	799
	UR06	0.13	173.2*	831*	208.1*	830
	EZA	0.71*	356.3*	1284*	375.1*	1004*

[1]Damage index calculated by counting normal seedlings 14 days after inoculation, as DI = 1 – (Inoculated/Control). DI values followed by an asterisk indicate significant difference between inoculated and control treatments (REML analysis, $p<0.05$).
[2]EU/g: enzyme units per gram of lyophilized weight material. Values followed by an asterisk indicate significant difference between inoculated and control treatments (REML analysis, $p<0.05$). POX, peroxidases; EGA, β-1-3-glucanases; CHI, chitinases; NAG, N-acetyl-β-glucosaminidase.
[3]Seedlings inoculated with NL93186 were completely lost and extracts could not be prepared.

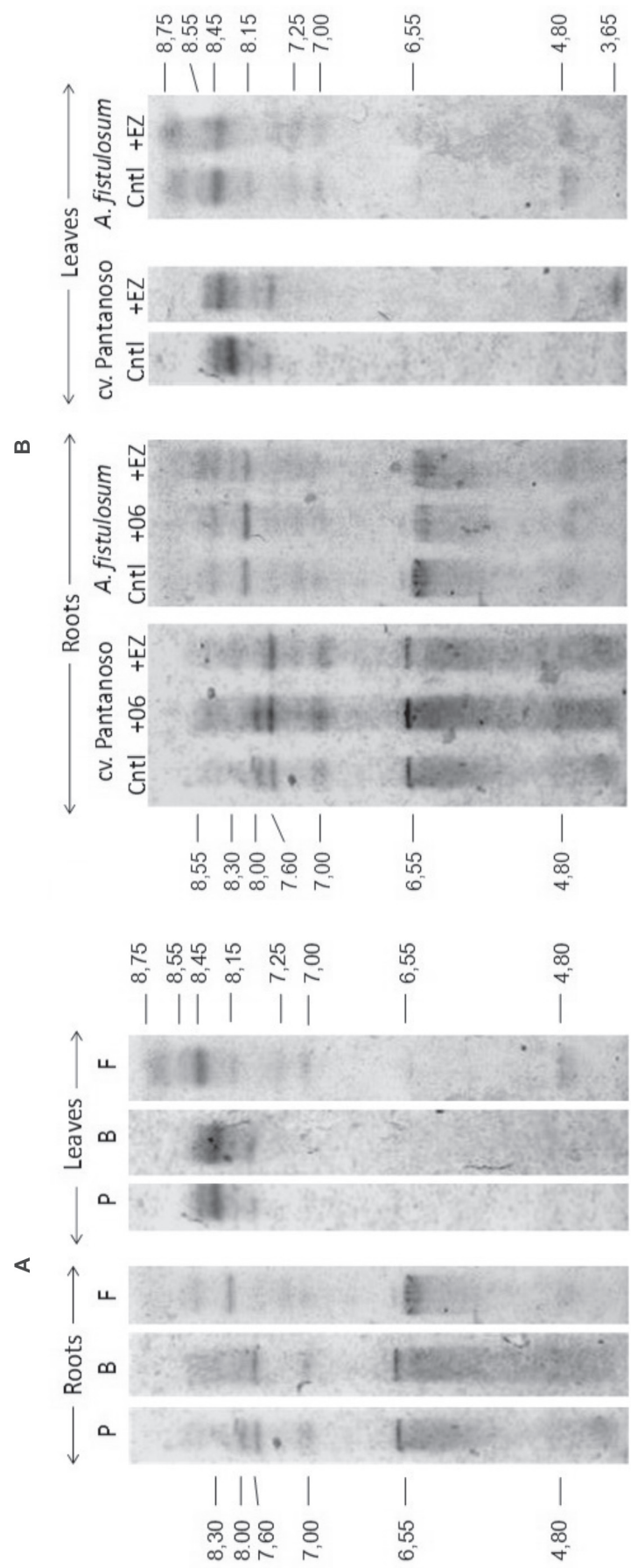

FIGURE 2 - POX isoforms of *Allium cepa* and *A. fistulosum* seedling extracts determined by isoelectric focusing (IEF). **A.** IEF profiles for the controls. **P**, cv. 'Pantanoso'; **B**, cv. 'Brava'; **F**, *A. fistulosum*. Lanes corresponding to root and leaf extracts are indicated at the top. **B.** IEF profiles for seedlings of *A. cepa* cv. 'Pantanoso' and *A. fistulosum* inoculated with *Foc* UR06 (+06) and *Foc* EZA (+EZ). **Cntl**, non-inoculated controls. Lanes corresponding to root and leaf extracts are indicated at the top.

TABLE 2 - Peroxidase (POX) and β-1-3-glucanase (EGA) activities in *A. cepa* cv. 'Pantanoso del Sauce CRS' and *A. fistulosum* seedlings inoculated with *Fusarium oxysporum* f. sp. *cepae* isolate UR17-8 at different seedling ages.

Age at inoculation	DI [1]	POX			EGA		
		C [2]	F [2]	R [3]	C	F	R
***Allium cepa* cv. 'Pantanoso'**							
0-day-old	0.64*	41.0 b	132.0 a	3.2*	827 b	2541 a	3.1*
14-day-old	0	21.8 c	42.2 b	1.9*	417 c	544 c	1.3
42-day-old	0	14.6 c	18.1 c	1.2	407 c	480 c	1.2
Allium fistulosum							
0-day-old	0.41*	33.5 b	134.0 a	4.0*	525 c	863 b	1.6*
14-day-old	0	36.9 b	40.0 b	1.1	386 cd	455 c	1.2
42-day-old	0	27.1 c	27.2 c	1.0	344 d	314 d	0.9

[1]Damage index calculated by counting the number of normal seedlings as DI = 1 – (Inoculated/Control). DI values followed by an asterisk indicate significant difference between inoculated and control treatments (REML analysis, $p<0.05$). In all cases DI were evaluated at 14 days after inoculation.
[2]Enzymatic units (EU/g) in (**C**) the non-inoculated controls and (**F**) the *Fusarium* inoculated treatment. For each enzymatic activity, means followed by the same letter are not statistically different (REML analysis, $p<0.05$).
[3]R: Enzymatic rates calculated as the ratio between EU/g in the *Fusarium* inoculated and EU/g in the control treatments. R values followed by an asterisk indicate significant differences between C and F treatments.

were susceptible at sowing. *A. fistulosum* had 15.1 normal seedlings in the inoculated treatment compared to 25.5 in the control (DI = 0.41), whereas cv. 'Pantanoso' had 7.8 and 22 seedlings, respectively (DI = 0.64). When the seedlings were exposed to the pathogen at 14 and 42 days after sowing, no disease symptoms were observed in any experiment and the number of seedlings did not significantly differ from the controls for both accessions (Table 2). Therefore, the response after inoculation at sowing markedly differ from the response when 14- and 42-day-old seedlings were inoculated, indicating that both accessions acquired resistance with age.

The basal levels of POX and EGA activities in the non-inoculated controls of *A. fistulosum* and onion cv. 'Pantanoso' significantly decreased with age (REML analysis, $p<0.05$). For both accessions, enzymatic activities in the treatment inoculated at sowing day significantly differed from the corresponding non-inoculated control (REML analysis, $p<0.05$). POX and EGA activities for cv. 'Pantanoso' increased 3.2 and 3.1 fold, respectively, whereas for *A. fistulosum* these activities increased 4.0 and 1.6 fold, respectively. For seedlings of cv. 'Pantanoso' inoculated 14 days after sowing, POX activity increased 1.9 fold while the increase in EGA activity was not significant (Table 2). The inoculation of onion seedlings at 42 days after sowing did not significantly affect POX and EGA activities. *Allium fistulosum* seedlings inoculated at 14 and 42 days after sowing did not display significantly changed enzymatic activities in comparison with the controls.

To investigate whether onion seedlings may acquire resistance even earlier than 14 days, seedlings were exposed to the pathogen at day seven after sowing using the most virulent *Foc* strain (NL93186) (Table 3). Whilst *A. cepa* cv. 'Pantanoso' was devastated by inoculation at sowing with null seedling emergency (DI = 1), it behaved as resistant when the inoculation was performed at seven days after sowing (DI = 0.07). *Allium fistulosum* was moderately affected when inoculated at sowing day (DI = 0.67), as found in previous experiments with the same isolate (Table 1), but was not affected when inoculated seven days later (Table 3).

Onion cv. 'Pantanoso' inoculated at sowing day was devastated and extracts could not be prepared. The inoculation at seven days after sowing showed no significant changes in CHI and NAG activities in comparison with the non-inoculated seedlings, whereas significant increases of POX and EGA activities were detected. *Allium fistulosum* seedlings showed moderate dumping off after inoculation at sowing day (DI=0.67) and significant increases of the POX, EGA, CHI and NAG activities. For the inoculation seven days after sowing, *A. fistulosum* showed no changes in enzymatic activities (Table 3).

DISCUSSION

This research confirmed that damping-off disease caused by *Fusarium* in onion is dependent on the age of the seedlings at the time of infection. This was demonstrated by the presence of symptoms only for seeds inoculated at the sowing day but not for germinating seedlings. Nevertheless, it should be taken into account that all evaluations were performed 14 days after inoculation, a time chosen as a compromise between visible symptoms development and the collection of enough plant material to produce extracts for enzymatic assays.

Age-related effects on onion susceptibility to *Foc* were reviewed by Cramer (2000), who summarized that vegetatively grown onion plants were more resistant than seedlings and dormant bulbs, and even that pre-germination of seeds is not a convenient technique when testing resistance, because susceptible lines may appear as resistant.

TABLE 3 - Enzymatic activities in root extracts from seedlings of *A. cepa* cv. 'Pantanoso del Sauce CRS' and *A. fistulosum* inoculated with *Fusarium oxysporum* f. sp. *cepae* isolate NL 93186 at two seedling ages.

Age at inoculation	DI [1]	POX[2]			EGA[2]			CHI[2]			NAG[2]		
		C[3]	F[3]	R[4]	C	F	R	C	F	R	C	F	R
***Allium cepa* cv. 'Pantanoso'**													
0-day-old	1.00 *	76.7	–[5]	–	895	–	–	50	–	–	844	–	–
7-day-old	0.07	39.8	84.8	2.1*	652	868	1.3*	121	143	1.2	902	957	1.1
Allium fistulosum													
0-day-old	0.67*	27.5	70.2	2.6*	474	1255	2.6*	72.5	33.6	0.5*	643	1017	1.6*
7-day-old	0.03	30.2	29.5	1.0	543	575	1.1	276	254	0.9	912	987	1.1

[1]Damage index calculated by counting the number of normal seedlings as DI = 1 – (Inoculated/Control). DI values followed by an asterisk indicate significant difference between inoculated and control treatments (REML analysis, $p<0.05$). In all cases DI were evaluated at 14 days after inoculation.
[2]POX: peroxidase; EGA: β -1,3-glucanase; CHI: chitinase; NAG: N-acetyl-glucosaminidase activities.
[3]Enzymatic units (EU/g) in (**C**) the non-inoculated controls and (**F**) the *Fusarium* inoculated treatment.
[4]R: Enzymatic rates calculated as the ratio between EU/g in the *Fusarium* inoculated and EU/g in the control seedlings. R values followeb by an asterisk indicate significant difference between C and F treatments.
[5] - , No data available. Seedling emergency was null, and extracts could not be prepared.

In addition, Stadnik & Dhingra (1995) inoculated seeds of several accessions and found that germination decreased by 28 to 100% in comparison with non-inoculated controls. Contrastingly, inoculated 30-day- old onion plantlets of the same accessions showed only a decrease in plant vigour in comparison with the controls, although they displayed local infection and rotting was often visible after harvest.

A decrease in onion susceptibility to *Foc* during the vegetative growing phase has been partially attributed to increased plant vigour and the consequent ability of the plant to overcome local infections (Cramer, 2000). Our results suggest that a decrease in susceptibility is acquired early during germination. Diverse plant-pathogen interactions, including *Fusarium* diseases, are influenced by the host developmental stage due to changes in gene expression associated with physiological changes and organ development (Neale et al., 1990; Develey Rivière & Galiana, 2007). Effects of plant age were observed for *F. oxysporum* f. sp. *apii* (Hart & Endo, 1981), as two-week-old celery seedlings wilted faster than six to eight weeks old ones. *F. oxysporum* f. sp. *pisi* caused severe wilting on peas inoculated between three to 14 days, but no symptoms were observed when plants were inoculated 21 days after sowing (Nyvall & Haglund, 1976).

Onion susceptibility to *Foc* during early seed germination and plant senescence may suggest that resistance is generated by actively growing leaves. However, the fact that very young seedlings were less susceptible to *Foc* supports the hypothesis that factors responsible for such decrease appear as soon as plant metabolism is triggered during germination. Even though distinct resistance mechanisms could co-exist, age-related resistance in pathosystems involving *Arabidopsis thaliana* and *Nicotiana tabacum* were found to be associated with the upstream and downstream activation of genes of the salicylic acid pathway, among others (Develey Rivière & Galiana, 2007; Carviel et al., 2009).

Wyatt et al. (1991) found that age-related resistance in *N. tabacum* against *Peronospora tabacina* was associated with the developmental expression of peroxidases, β-1-3-glucanases and chitinases. In our research, enzymatic activities evaluated in a pooled quantitative approach showed enhanced expression in response to the infection. However, these expressions did not represent an efficient defence response against the pathogen, because the levels of activities were positively correlated with DI. In addition, we found that *Foc* infected bulbs sampled at harvesting from a commercial field presented higher expression for CHI, POX and EGA than healthy bulbs, with the highest increase for EGA (REML analysis, $p<0.05$; data not shown). These observations contribute to support that enzymatic activities rose as a response to damage, but not as an efficient defence.

The activities of the same families of enzymes were evaluated by Zappacosta et al. (2003), as the response of calli and roots of the pink root susceptible onion cvs. 'Valcatorce' and 'T-412' and the resistant *A. fistulosum* cv. 'Nogiwa Negi' to sterile culture filtrates of *Phoma terrestris*. A high constitutive activity of glucanases and chitinases was proposed to explain the resistance of *A. fistulosum*. Contrastingly, in our experiments, basal levels of glucanases and chitinases in *A. fistulosum* seedlings were similar to those found in *A. cepa* cv. 'Pantanoso'. In agreement with the herein reported results, however, Zappacosta et al. (2003) speculated that the increase in enzymatic activities in 'Valcatorce' but not in the resistant *A. fistulosum*, apparently does not contribute to any defence reaction, at least in the later stage of pathogenesis.

According to our results, the acquisition of resistance with age is not explained by basal enzymatic activities, since older seedlings behaving as resistant to *Foc* showed lower enzymatic activities compared with the earlier, susceptible stages, with the exception of CHI. Nevertheless, a comprehensive study of basal expression of enzymes along

the onion life cycle will help to elucidate the relationship between these families of enzymes and resistance to *Foc* during the vegetative stage.

The expression of specific isoperoxidases was induced or repressed after the exposition to *Foc* isolates. When exposed to the pathogen, *A. fistulosum* roots expressed two isoforms that are not present in *A. cepa*, and which could be involved in an efficient resistance response. The most aggressive isolate (EZA) repressed the expression of an isoform in the roots of 'Pantanoso', and induced one isoform in the roots and one in the leaves of 'Pantanoso' and 'Brava'. The less aggressive isolate (UR06) did not repress any isoform, but induced the same two isoforms in both onion cultivars. The ability of the EZA isolate to repress the POX isoform of pI 8.0 in roots of cv. 'Pantanoso' may contribute to its higher aggressiveness in comparison with the UR06 isolate, but further research is needed to confirm these hypotheses.

The *Fusarium oxysporum* f. sp. *cepae* isolates used in this study differed in their virulence. The Uruguayan isolates UR17-8 and UR06 were the mildest ones, the Australian isolate EZA was consistently virulent, and the Dutch isolate NL93186 was the most virulent for all *Allium* accessions. Quantitative differences in virulence were reported by several authors, and suggest the presence of diverse mechanisms of pathogenicity (Saxena & Cramer, 2009; Southwood et al., 2012; Taylor et al., 2013). In our research, NL93186 was the only isolate able to inhibit CHI activity in *A. fistulosum* roots, suggesting a possible correlation between CHI inhibition and virulence.

The single *Allium fistulosum* accession tested in this research was less affected by *Foc* isolates than the onion cultivars, particularly when inoculated at sowing day with aggressive isolates. These results for *A. fistulosum* seedlings confirm the resistance reported for adult plants in greenhouse tests (Holz & Knox-Davies, 1974; Galván et al., 2008) and the potential of this species as a source of resistance in onion breeding. In comparison with most onion cultivars, *A. fistulosum* accessions could present higher level of resistance promptly expressed after germination. As the overall induction of peroxidases and glucanases was correlated with the level of symptoms and *A. fistulosum* reached lower levels than *A. cepa* during early germination, *A. fistulosum* seems to develop efficient defence mechanisms earlier than *A. cepa.* Future research involving molecular determinations is needed to confirm and identify resistance factors in *A. fistulosum*, and specific peroxidase, glucanase or chitinase isoforms as pathogenesis related proteins.

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