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Cellular changes and *PR-1* gene expression accompanying infection-related morphogenesis and **cell progression** during invasive growth *Pyricularia oryzae* pathotype *Triticum* in resistant and susceptible wheat cultivars

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ABSTRACT

Pyricularia oryzae pathotype *Triticum* (PoT) causes Wheat Blast, a devastating crop disease present in many wheat-producing countries. With the aim to contribute with the understanding of the mechanisms underlying pathogenesis of the fungus on wheat (*Triticum aestivum*), cytological and molecular studies were performed here.

Susceptible and resistant interactions of wheat-PoT in cultivars of different levels of resistance were investigated by cellular changes and the molecular responses of wheat leaves according *PR-1* expression. There was significant difference between susceptible (Apogee-PY15-PY34, Buck, MSINTA-PY15) and resistant (Buck-PY34, MSINTA-PY34) interactions in the responses tested. The two isolates of PoT analysed showed different ability to infect wheat plants at the cellular level during early tissue penetration analysed and documented by epi-fluorescence microscopy, patterns of *PR-1* genes, and wheat plant defense reaction.

Microscopic time-course analysis (24, 48, 72 and 96 h after inoculation) of individual interaction sites per leaf revealed different leaf invasion strategies of the more and less aggressive PoT isolates during the first stages of the course of pathogenesis.

The results and conclusions of the present study provides the first evidence that different *PR-1* spatial and temporal expression patterns occur in the early infective process of wheat-PoT, underlying two different defence mechanisms in plants according susceptible or resistant interactions. Thus, the results here laid a theoretical foundation for the future control of wheat blast using the different pattern of *PR-1* gene as markers for disease resistance during the first stages of infection providing a significant contribution to a more efficient selection of wheat genotypes in breeding studies.

1. Introduction

Among the cereal, wheat (*Triticum aestivum* L.) is the second largest grain worldwide based on total production volume (Kohli et al., 2011; Talbot, 2003). It is affected by several fungal diseases, like Wheat Blast (*Pyricularia oryzae)*, a new emerging threat the cereal production (Kohli et al., 2011; Talbot, 2003; Nizzoli et al., 2023). Wheat blast is one of the main fungal biotic limitations that affect wheat plants due to restrictions to production up to 100 % and reductions in grain yield and quality, positioning first among the top 10 fungal pathogens according to economic impact its causes (Nizolli et al., 2023).

On wheat, the *Triticum* pathotype cause blast symptoms on spikes that appear complete or partial bleaching and with no grain or shriveled grain production (Goulart et al., 2007). On the leaves, small green-gray lesions with dark edges firstly, that rapidly expand becoming necrotic and tan in color are the common symptoms of the disease (Debona et al., 2012).

The disease, was first observed in Brasil (1985) and then, wide-spreaded in other South American countries (Argentina, Bolivia, Paraguay) since the 1980 s (Perelló et al., 2017). Moreover, new reports of the disease indicate its spreading to Africa and Asia (Islam et al., 2019) (Tembo et al., 2020). Therefore, blast is now considered a major threat of global spread to wheat production worldwide, which could occur via infected seed or grain. In Argentina from 2017 until now

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different studies about the behavior of Argentinian cultivars in this pathosystem wheat-PoT demonstrated different expression of disease symptoms according cultivars and isolates challenged (Perelló et al., 2017; Martínez et al., 2019). However, in agreement with Cruz et al. (2016) and Ceresini et al. (2018), not enough studies were focused on wheat-*P. oryzae* pathosystem until now. New studies are necessary in Argentina involving new varieties and isolates taking in to account the risk of Wheat Blast increasing in the neighbors countries, like Brazil, Bolivia and Paraguay and the lack of resistance. Few studies were focused on wheat −*P. oryzae* pathosystem until now (Ceresini et al., 2018, Chaves et al., 2022) in contrasting with previous reports of blast infectivity involved rice plants (Meng et al., 2019, Marcel et al., 2010) or barley plants (Delventhal et al., 2014; Ulferts et al., 2015; Zellerhoff et al., 2006). As a hemibiotroph pathogen, *P. oryzae* firstly establishes a biotrophic interaction with the host at the early infection stages. The switch into the necrotrophic stage occurs between 24–36 h post-infection (hpi) with the invasive growth into the neighboring cells of the host (Cao et al., 2016; Eseola et al., 2021).

On the other hand, the plant pathogens have to suppress plant defense to be successful in the infection process (Hückelhoven, 2007) overcoming the host defense responses (McDonald and Linde, 2002; Almagro et al., 2009) that involves among others, the bio-synthesis of pathogenesis related proteins. In this sense, during the first stage of the pathogenesis process, the recognition of the pathogen by the plant is mediated by the production Pathogenesis-related (*PR*) proteins (Tao et al., 2003). Pathogenesis-related (*PR*) proteins, categorized into 17 families, tend to build up following pathogen attacks or similar conditions in various plant species (van Loon et al., 2006). Nevertheless, the biological and biochemical roles of these *PR* proteins in defense responses and growth processes remain uncertain (Hong et al., 2005). Within the *PR* gene family, *PR-1* genes are commonly employed as indicators of systemic acquired resistance across numerous plant species. All the same, only a fraction of *PR-1* genes have been thoroughly investigated among the numerous members within this family, leaving limited information available about the other members. In this study van Loon et al. (2006) out of the 22 genes identified, only one *PR-1* gene encoding a basic protein, has been confirmed to be responsive to pathogens. The remaining *PR-1* genes, according to reports, did not show any response to either bacterial or fungal pathogens (van Loon et al., 2006).

In plants, the *PR-1* gene shows significant induction in response to salicylic acid (SA) and serves as an indicator of SA-mediated defense reactions (Wang et al., 2005). *PR-1* proteins are known to localize in the apoplast or vacuoles, where they play a regulatory role in both abiotic and biotic stress responses (Breen et al., 2017). When plants are attacked by pathogens, *PR-1* proteins accumulate in the apoplast. Their role is to sequester sterols from oomycetes, which rely on plant-produced sterols for survival. This sequestration process ultimately inhibits the growth of the pathogens (Gamir et al., 2017). *PR-1* overexpression in plants results in increased resistance against pathogens (Han et al., 2023).

The studies related to the expression of *PR-1* gene were carried out focused on the interaction *Rice-P. oryzae*. In a study by Manandhar et al. (1999), both compatible and incompatible interactions were examined in the Rice-*P. oryzae* pathosystem, focusing on the accumulation of protein transcripts associated with pathogenesis. In the case of *PR-1*, transcript accumulation decreased only after 72 to 96 h. Notably, all these transcripts showed higher accumulation levels in compatible interactions compared to incompatible ones between rice and *P. oryzae*. The activation of *PR-1* genes when rice plants encounter *P. oryzae* infection is a crucial aspect of the plant's defense mechanism against this fungus. When the presence of the fungus is detected, rice plants initiated defense responses, including the upregulation of PR genes like *PR-1*. These genes encode proteins that have a role in either inhibiting the growth of the pathogen or signaling pathways involved in plant defense. Moreover, Tang et al. (2017) evaluated the expression of *PR-1* and *PR-10* when inoculating wild and mutant rice plants with *P. oryzae*. They determined an increase in the expression of both proteins 72 hpi in the plants. By using mutant plants which had an immunity protein called APIP12 deactivated, they determined a greater susceptibility of rice plants to virulent *P. oryzae* isolates in conjunction with a reduction in the expression of *PRs*, which indicates that these proteins are strongly related to the pathogenesis process. *PR-1* type genes are used as indicators for systemic acquired resistance (SAR) (van Loon and van Strien, 1999; Jayaraj and Punja, 2007) and could be potentially important markers to explore responses in wheat against blast disease.

Instead, the information available on mechanisms and strategies of the infection in the Wheat-PoT pathosystem, is also still limited. Even when their previous antifungal activity suggests a function in plant defense (Breen et al., 2017) the reports concerning the biological activity and function of the group *PR-1* in the pathosystem wheat-*P. oryzae* are scarcely documented (Cabot et al., 2018). So, this investigation helps to advance in the knowledge of *P. oryzae* infection on new wheat cultivars. Thus, the objectives were: to investigate the cytological features associated with compatible and incompatible interactions of new isolate/cultivar combinations that have not been tested before and providing insights of the role of *PR-1* in wheat defenses during the first steps of infection with two isolates of the fungus with contrasting behaviour.

2. Material and methods

2.1. Plant material and growth conditions

Apogee is an experimental variety of wheat that possess the shortest life cycle in wheat in the world − generating the spike about one month after planting when grown under controlled conditions (23 °C and continuous light) (Li et al., 2017). Apogee, a susceptible wheat cultivar against isolates of *P. oryzae* (Strugala et al., 2015, Portz et al., 2020) was used as positive control during the experiments. Buck Aparcero and MS INTA 215, were also selected according previous results of contrasting behavior against *P. oryzae* under greenhouse conditions in Argentina (data no shown). After a pre-germination of the seeds, the plants were grown in pots under 16–8 h light–dark cycles, 21 °C and 65 % HR. Six seedlings were growth per pot with soil type ED73 (Balster Einheitserdewerk GmbH). Three replications were carried out for each cultivar/isolate combination in the following assays.

2.2. Fungal growth, inoculation procedure and disease evaluation

Pathogenicity test were conducted with *Pyricularia oryzae* isolates PY15 and PY34, molecularly identified previously as *P. oryzae* pathotype *Triticum (*PoT) (WHEAT BLAST, 2020). The isolates were collected from wheat at field conditions in Argentina maintained at the Plant Pathology Research Center (CIDEFI), Buenos Aires, Argentina. The isolates were cultivated on oat meal agar "OMA" (20 g agar, 2 g yeast extract, 10 g 1 starch, 30 g oat fakes in 1 L of destilled water + 5 ‰ chloramphenicol) then incubated in a chamber with fluorescent tubes under 16/8h light/dark cycle and UV at 25 °C, as described by Martinez et al. (2019). One week old colonies were scraped with sterile bidistilled water (ddH_2O) and filtered. The suspension was mixed in a 1:2 ratio with Tween-20 as surfactant before inoculation. Inoculation of each isolate was performed by spaying approx. 1 ml of *P. oryzae* conidial suspension (concentration $2.5x10^5$ conidia ml^{-1}) onto the surface of the third expanded leave using a TLC-glass spray nozzle (0.8 bar of pressure and 40 cm of distance). Controls received sterile distilled water and 200 µl Tween 20 only. After the inoculation, plants were maintained in chamber with darkness (24 h) at 25 °C, then grown in the growth chamber under the same conditions described above and covered with polythene tents to maintain high humidity until evaluation. After 7 days post inoculation (dpi), the disease leaf area (%) and the number of lesions.cm^{-2} were evaluated. Ten leaves were cut for each cultivar-isolate interaction and placed on plates containing water agar medium (1 % agar in distilled water). Photographs of plates were taken using a Nikon D3100 digital camera and images used to quantify the diseased leaf and number of lesions using

the APS ASSES2.0 program.

2.3. Epi-fluorescence microscopy

In previous studies, induced autofluorescence has been utilized to facilitate the imaging of proteins and organelles within cell protoplasts, as well as to enable fluorescence imaging of fungal mycelium of *P. oryzae* during interactions with rice, barley, and wheat (Delventhal et al., 2014; Portz et al., 2020; Chaves et al., 2021). With the purpose to determine fluorescence at the pathogen penetration areas (Jarosch et al., 2005), three samples (each with 0.5 cm^2) per isolate and evaluation time (24, 48, 72 and 96 h post inoculation "hpi") were collected from the third leaf of each wheat cultivar and kept in a clearing solution (Zellerhof et al., 2008) for 48 h and saved in 50 % (v/v) glycerol until use. Epi-fluorescence microscopy (excitation 485 nm, dichroic mirror 510 nm, barrier 520 nm, Leica DMR, Germany) was used to analyze the samples. The evaluation consisted on the observation of 200 infection sites per leaf per time point, a digital camera JVC KYF 750 was used to take the pictures. The cellular interaction types were grouped into four categories of diseases progress adapting a previous classification of Ulferts et al. (2015): AP (appressorium), FW (fluorescent cell wall), FM (fluorescent mesophyll), CM (collapsed mesophyll cells). Experiments were carried out three times and frequency of each type of reaction per sites examined per replication and treatment was calculated.

2.4. Quantification of PR-1 gene expression

Leaves selected for quantify *PR-1* gene expression were subjected to the following steps. For quantification of *PR-1* gene expression were used three samples consisting of two leaves each. For RNA isolation, the protocol of Mogga et al. (2016) was followed. The concentrations of RNA were measured by Nanodrop (NanoPhotometer® P-Class (P330), Implen GmbH, München, Deutschland) and adjusted to 1 μg/μl; cDNA was synthesized using reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Fisher Scientific Inc., Germany) with Hint-AnchorT-Primer. Quantitative real time PCR was done using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc. USA), and the following gene specific primers:

Primer		Sequence	Source
TaPR1*	F (5-3)	GAATGCAGACGCCAAGCTA	Zellerhoff, (2009)
	R (5-3)	GCACGGGCAGCGTTGT	
TaEF1a**	F (5-3)	ATGATTCCCACCAAGCCCAT	McGrann et al. (2015)
	R (5-3)	ACACCAACAGCCACAGTTTGC	

* Target gene ** Housekeeping gene.

8 μl of a PCR Mastermix (5 μl iTaq Universal SYBR Green Supermix, forward primer 500 mM, reverse primer 500 mM and water) was pipetted into a 384-well RT-PCR plate afterwards 2 μl of the diluted cDNA (1:10) were placed in each well. An adhesive foil was placed to seal the plate and centrifuged at 1000 rpm. The plate was placed in the PCR-Real Time CFX96 (Bio-Rad Laboratories Inc. USA). Curves obtained were analyzed using the CFX Real-Time PCR Detection Systems Program and relative gene expression was calculated (Livak and Schmittgen, 2001).

2.5. Statistical analysis

All data were analyzed for homogeneity (Levene's test) and for conformation to a Normal distribution (Shapiro-Wilks's test). The statistical software InfoStat version 2020 (Grupo InfoStat, FCA, Universidad Nacional de Cordoba, Argentina) was used for the analysis (Di Rienzo et al., 2011). Data were presented as means ± standard deviation and one-way analysis of variance (ANOVA) was performed followed by the Tuckey's test with a significance of $p < 0.05$ to separate means that presented significant differences.

3. Results

3.1. Dynamics of cellular responses of wheat leaves infected by P. Oryzae

To establish the colonization process of the pathogen, microscopic histological observations were performed by observe the samples under bright field microscopy on located germinated conidia. The epi-fluorescent light allowed the observation of autofluorescence of the wheat leaves cells and the quantification of the sites of fungal invasion.

Different stages of *P. oryzae* leaf invasion were discriminated, involving the processes of its establishment at the leaf surface, leaf infection and colonization, development of disease and increasing tissue necrosis. Leaf infection by *P. oryzae* start when a conidium stick to the wheat leaf surface (Fig. 1 **A**), produces a germ tube and a melanized appressorium (AP) that penetrate the epidermal cell by mechanical and enzymatical piercing of the wheat cell surface (Fig. 1 **B,C**). The early infection stage was macroscopically symptomless. Microscopically, inside the first infected cell, dividing invasive hyphae become to developed (Fig. 1 **D,E**). Fungal spread cell to cell and continued growth in the mesophyll by advanced hyphal networks until a successful pathogen establishment after 96 hpi.

Fluorescence microscopy analysis of the *P. oryzae* penetration sites on epidermal wheat cells reveal that the appearance of fluorescence correlated with advancing fungal invasion and represents an indicator of plant defense responses as described for PoT infection on barley (Ulfers et al., 2015). The penetration of cells started with the appressorium differentiation (Fig. 2 **A**). Then, the autofluorescence of a part or the whole epidermal cell wall (Fig. 2 **B**) was observed. After that, the infection hyphae reach the mesophyll that still kept their regular shape (Fig. 2 **C**) and finally resulting in collapse and autofluorescence of the adjacent mesophyll cells (Fig. 2 **D-E**). This stage of the fungal development was still symptomless since necrosis was not visible at the macroscopic stage until the appearance of lesions, generally, by 6–7 days after inoculation.

The results showed from the three wheat cultivars statistical analyzed, the first epidermal fluorescent cells associated with pathogen appressoria (AP) were observed at 24 hpi, and until the 96 hpi (Fig. 3 **A-C**). At 48 hpi FW was observed in the foliar tissue of the three wheat cultivars, with statistic highest values in cultivar Buck Aparcero-PY15 and PY34 interaction (19 % and 22 % respectively). Moreover, also FM at low levels was also observed in the three cultivars with isolate PY15. And also, low values of FM were registered in the MS INTA 215-PY34 combination. At 72 hpi values of FM were increased in the three cultivars, and in cultivars Buck Aparcero and MS INTA 215 with isolate PY15 in particular, dead epidermal cells allowed the mesophyll invasion, followed by its cell death (CM) (9 % and 2 % respectively). At 96 hpi, the greatest significant differences among the cultivars and isolates were registered. At this time point, the highest values of CM (35 % and 39 %) were registered in cultivar Apogee with both isolates PY15 and PY34 respectively compared with the lowest value (2 %) of Buck Aparcero in combination with PY34. Moreover, at 96 hpi cultivars Buck Aparcero and MS INTA 215 a contrasting response according to the isolate challenged was observed.

3.2. Pathogenicity evaluation in response to the effect of P. Oryzae

The first blast symptoms appeared 72 hpi as small water-soaked lesions in the interaction MS INTA 215-PY15 (Fig. 4**A**). Then, these lesions increase with severe chlorosis around them. In the three cultivars, at 7 days after inoculation, the lesions became necrotic and the total leaf area expressed tissue death. On leaves, disease symptoms aspect were elliptical or rounded lesions with dark margins and gray center. However, variability in the disease expression level among the wheat cultivar analysed were observed. Moreover, differences were found according the *P. oryzae* isolate challenged (Fig. 4**B**). A significant highest susceptibility (between 51 to 55 % of diseased leaf area) was found in cultivar

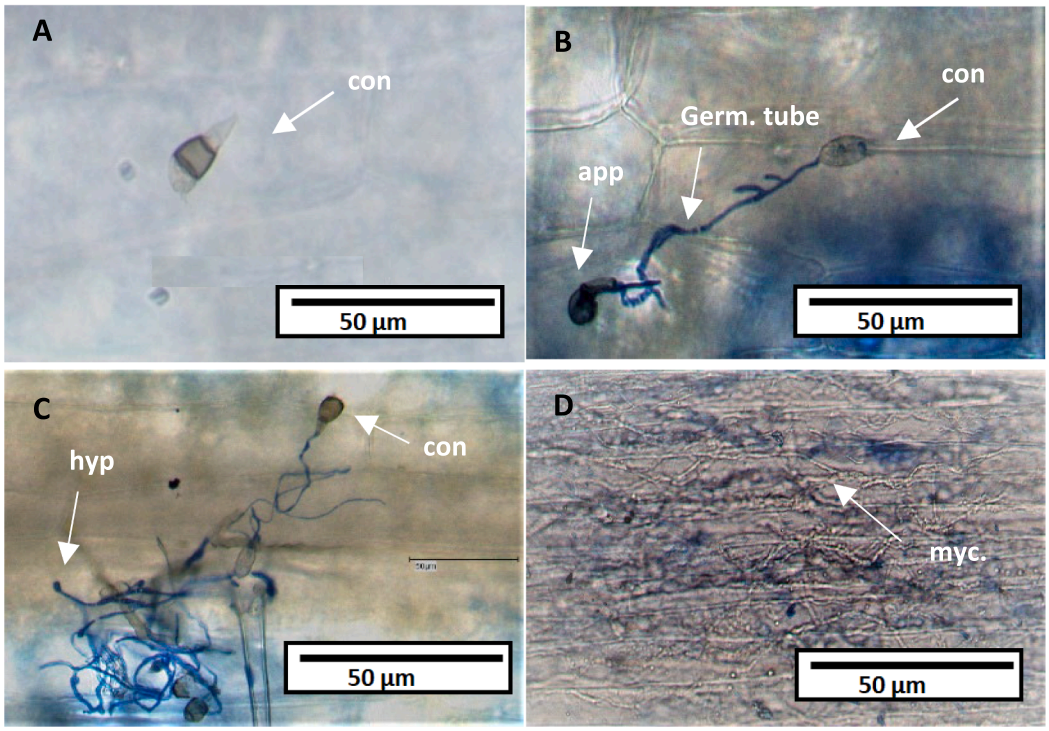

Fig. 1. Fungal infection process on wheat *cv* Apogee inoculated with *Pyricularia oryzae* pathotype *Triticum* PY15 isolate (PoT). At different time points after inoculation third leaf Apogee cultivar with conidia of *PoT*, was examined using a bright field microscopy for observations of the infection progress of *PoT*. The infected leaves were stained with methylene blue to observe the different structures developed by the fungus. Reference: **A,** conidia (24 hpi). **B,** appressorium (48 hpi) and germ tube (24–48 hpi). **C,** hyphae extension on and inside the leaf tissue (72–96 hpi). **D,** mycelial development on and inside the foliar tissue (96 hpi). app, appressorium; con, conidium; hyp, hyphae; myc, mycelia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Apogee challenged with both isolates, PY34 and PY15 respectively. Significant great differences were observed in wheat Buck Aparcero challenged with isolate PY15, that induced a disease severity of 41 %, that reached only 5 % challenged with isolate PY34. The inoculation of the cultivar MS INTA 215 with the isolates PY15 and PY34 revealed a significant difference in disease severity (46 and 14 % respectively). The untreated control did not present symptoms, therefore a diseased leaf area value of 0 % was established. For this reason, it was not included in the graphs. Individual plant-fungus interactions inspected microscopically under stereoscopic microscope on leaves seven dpi, indicated significant differences in lesions cm^{-2} (NL), according to the cultivar and isolate analysed (Fig. 5). In this sense, cultivar Apogee showed the highest medium values (14 lesions cm^{-2}) induced by isolate PY15 respect of those induced by isolate PY34 (9 lesions cm^{-2}). For cultivar Buck Aparcero the difference in the behaviour between the both isolates were even greater (6,6 and 1,4 lesions cm^{-2} respectively). In the case of cultivar MS INTA 215 challenged with *P. oryzae* isolates PY15 and PY34, the values were of 7,8 and 2,2 lesions cm^{-2} respectively. In summary, these results are indicative of the greater NL induced by *P. oryzae* isolate PY15 compared with isolate PY34.

3.3. Pathogenesis related protein PR-1 after the fungal infection

Spatially and temporally differential expression patterns of the wheat *PR-1* gene were found at appropriate times (0, 24, 48, 72 and 96 hpi) for each cultivar/isolate interaction (Fig. 6 **A-C**). Two different expression profiles, with an increase or decrease in transcript abundance of *PR-1* at 72 hpi were observed in the three inoculated cultivars.

The first pattern, observed for the interaction Apogee with both isolates and Buck and MS INTA in combination with PY15, was characterized by an increase of the *PR-1* expression with a maximum at 72 hpi. In contrast the expression of wheat *PR-1* gene differed for the interaction BUCK-PY34 and MS INTA-PY34. Here an increase of the expression was observed up to 96 hpi.

For the interaction between Apogee with PY15 and PY34, the temporal pattern of increase during the evaluation time was similar; The maximum wheat *PR-1* transcript abundance was reached at 72 hpi. Further, the values decreased abruptly, which was much more marked in the interaction Apogee-PY34 comparing with Apogee-PY15. At 96 hpi, the *PR-1* expression decline (Fig. 6 **A**).

In the case of Buck Aparcero-PY15 and PY34, the relative expression of *PR-1* was the one that showed the highest relative values during the entire observation time, compared to the values of the two tested wheat cultivars. Even when no significant statistical differences were observer, the expression pattern followed the same trend descripted for cultivar Apogee, that is, a gradual increase until 72 hpi when the maximum value was achieved with PY15, which then declined at 96 hpi. In contrast, the relative expression of *PR-1* was increasing in the different time points of the combination Buck Aparcero-PY34, from 0 hpi to 96 hpi (Fig. 6 **B**).

Analysing the interaction MS INTA 215 infected with isolates PY15 and PY34, the results showed that *PR-1* were expressed with null or very low values at 0 hpi and 24 hpi, even this low relative expression detection follow until 48 hpi in the interaction with isolate PY34. The expression pattern of *PR-1* with PY15 followed the same model as showed for cultivar Apogee infected with both isolates, and Buck Aparcero infected with PY15, this means the maximum level at 72 hpi and then, decreasing. However, the pattern of progress of MS INTA 215 with isolate PY34 was increasing during the whole time of observation, with a maximum value at 96 hpi (Fig. 6 **C**).

Results here indicate quantitative differences in the expression of *PR-1* comparing the three cultivars analyzed. The highest values were seen in cultivar Buck Aparcero throughout the whole evaluation time regarding the results showed by cultivars Apogee and MS INTA 215. Particularly, the highest value reached of *PR-1* transcript abundance was

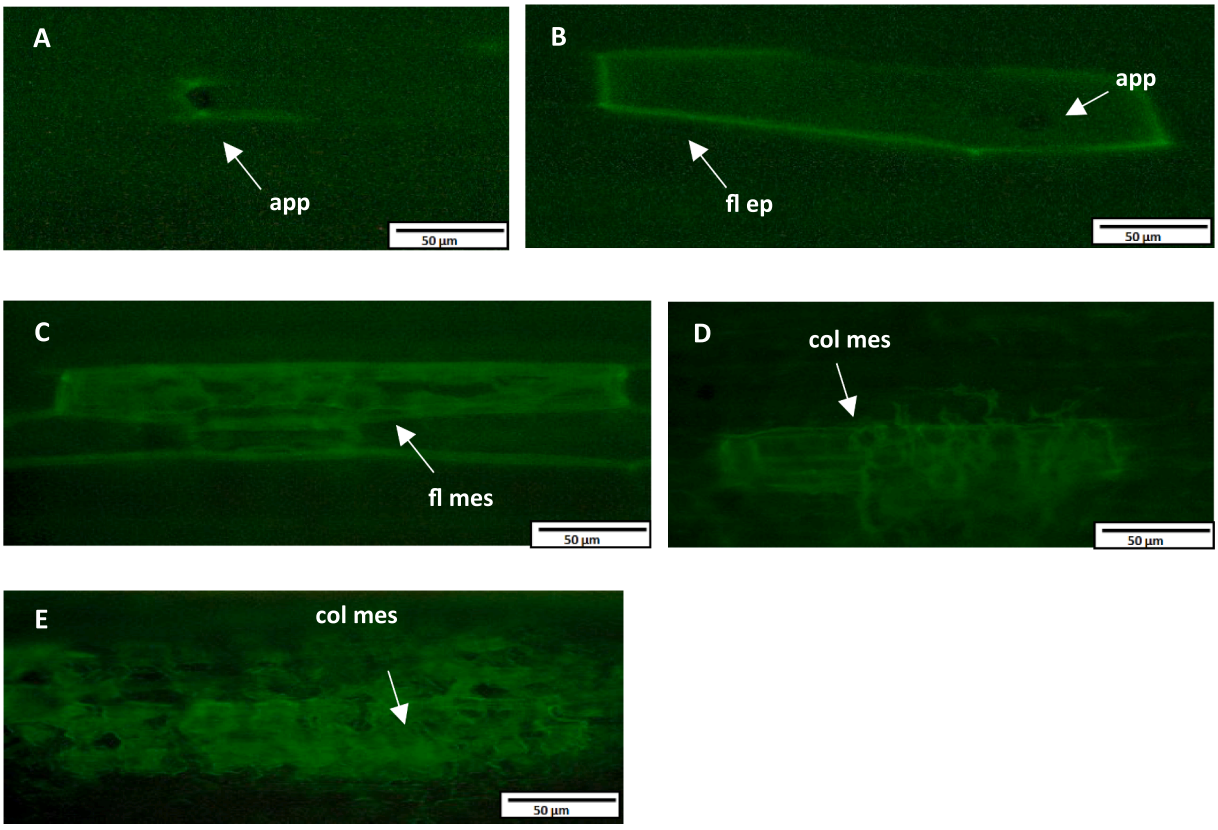

Fig. 2. Microscopic phenotype reaction on *cv* Apogee inoculated with *PoT* under fluorescent microscopy. After the inoculation with the isolates PY15 and PY34 three samples (each with 0.5 cm^2) per isolate for each evaluation time (24, 48, 72 and 96 hpi) were collected from the third leaf of each wheat cultivar and kept in a clearing solution for bleaching for 48 h and subsequently stored in 50 % (v/v) glycerol until use. Samples were then placed and fixed with the adaxial side up on slides containing drops of distilled water and observed by epi-fluorescence microscopy for visualization of the presence of fluorescent compounds. These figures show fluorescence microscopy images of infected epidermal cells on wheat leaves at different time-points after inoculation with *PoT*. **A**. apressorium; **B**. Fluorescent epidermal cell wall; **C** Fluorescent mesophyll; **D-E**. Collapsed mesophyllum with well-developed invaded hyphae in the epidermal cell beneath the appressorium producing several branches and colonizing neighboring epidermal cells. App, appressorium; col mes, collapsed mesophyllum; fl ep, fluorescent epidermal cell wall; fl mes, fluorescent mesophyllum.

shown in the interaction Buck Aparcero-PY34 at 96 hpi.

4. Discussion

In this work, new insights in the knowledge of the early *P. oryzae* infection, causal agent of wheat blast, were revelated by results arrived from the combination of histopathological examination on leaf tissue during infection, a molecular approach of the *PR-1* activity and the phenotypical expression of the disease in the specific interactions isolate per cultivar tested. Of particular interest were our results obtained here of both *PR-1* expression together with the infection sites and pathogenicity. It is noteworthy that the expression of wheat defense proteins in response to *P. oryzae* infection observed by genetic tools showed accordance with the phenotypic response of wheat seedling. This indicate the potential usefulness of these genes as suitable markers for leaf pathogenesis, concluding that wheat *PR-1* was a potential efficient antifungal protein against *P. oryzae* on wheat.

In agreement of that found on rice by Wilson and Talbot (2009) the fungus began its infection process adhering the conidium to the wheat leaf surface and after its germination, forms an appressorium enabling it to penetrate host cells and establish colonization. The fungus colonizes the wheat cells in a biotrophic manner by branched of the infection hyphae. *P. oryzae* followed a sequential pattern of invading viable wheat cells and eventually switchs into a necrotrophic growth. This phase is marked by the development of lesions and the onset of sporulation stage.

As was reported by Lattanzio et al., (2006), several compounds exist constitutively in healthy plants acting as chemical barriers to fungal pathogens and that protect plants against its attack. According our results, the presence of fluorescent compounds accumulating at the site where infection could suggest the presence of innate wheat defense responses. Ulferts et al., (2015) conducted a quantitative microscopic analysis of the infection of *P. oryzae* on barley. In agreement with those results and our results here, it appears that as barley, also wheat has the capability to impede or slow down the progression of *P. oryzae* infection at both the penetration and post-penetration stages. These stages were monitored here by observing the presence or absence of invasive hyphae within the attacked epidermal cells and its correlation with the appearance of autofluorescent plant material. It is highlight that here we could observe the same sites of cellular interaction that those described by Ulferts, (2015) but without the papilla formation in any of the cultivars analyzed in the wheat-PoT interaction. We also observed the formation of appressoria and the first mycelia penetration without macroscopic symptoms, considering this as a biotrophic stage. The appearance of fluorescent tissues due to the colonization of hyphae, could be considered as necrotrophic stage where the first symptom formation begins.

Our results show that differences among the progress of the infection in the three tested wheat cultivars exist. On top of that they imply that the specific interaction with each tested isolate of *P. oryzae* influenced in the timing and degree of disease severity variation. Moreover, the microscopic and macroscopic observation here suggests that the infection seems to be associated to different infection strategies of the fungus *P. oryzae* on wheat. This accords to the tested wheat cultivars and *Pyricularia* isolates demonstrating varying degrees of resistance and

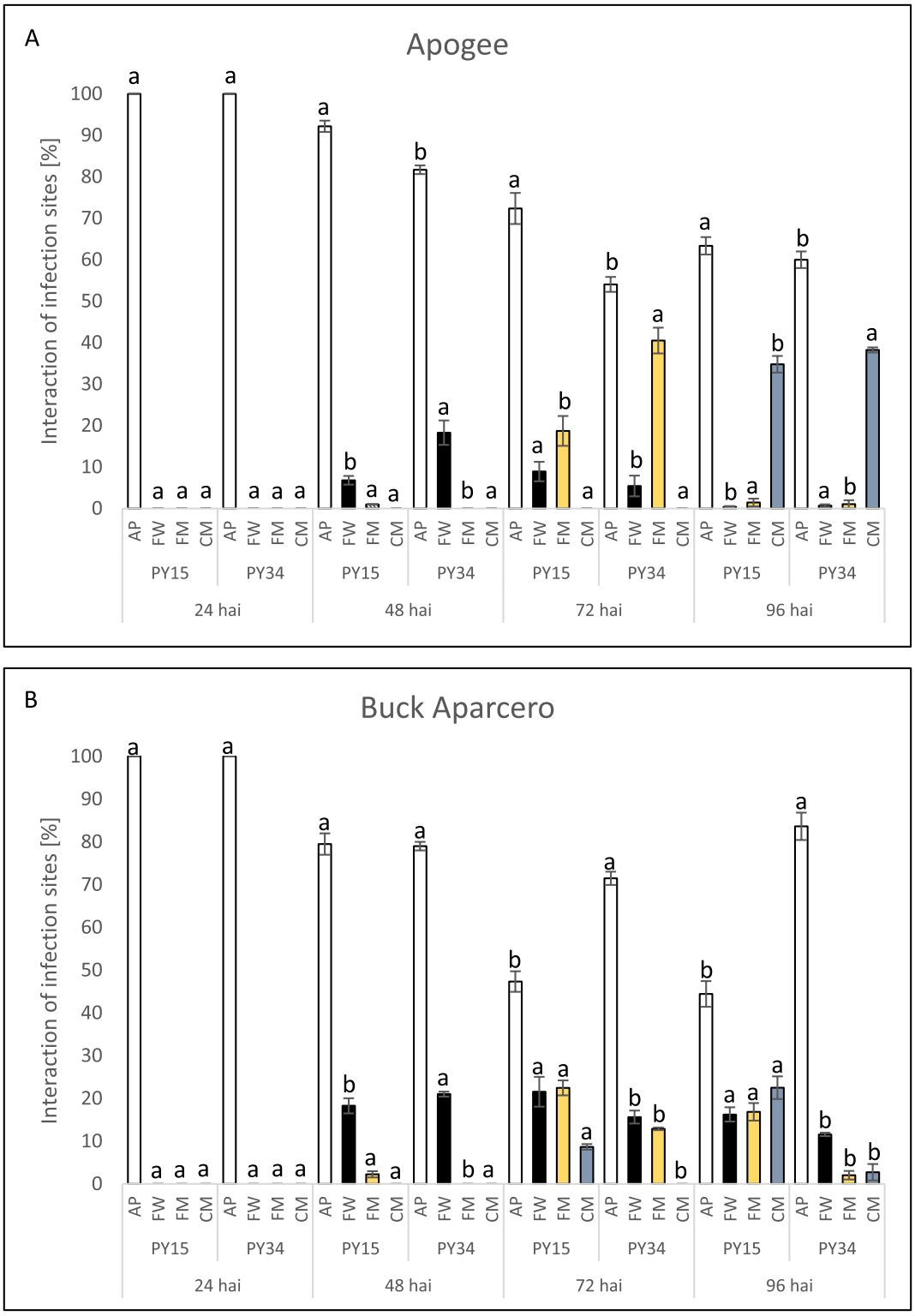

Fig. 3. Microscopic quantification of the interaction between *PoT* with wheat cultivars. After the inoculation of the third leaves of wheat plants with the virulent *PoT* isolates PY15 and PY34 were harvested at different time points (24, 48, 72 and 96 hpi) and analysed by brightfield and epi-fluorescent microscopy after bleaching. A total of 200 infection sites were inspected per leaf and time point. The cellular interaction types were grouped into the following categories according to the development of fungal infection structures: AP (appressorium), FW. (Fluorescent epidermal cell wall), FM (fluorescent mesophyll), CM (collapsed mesophyll cells). Experiments were repeated in triple with similar results. The frequency of each type of cellular reaction for the appressorial sites examined per each replication and treatment was calculated. The bars represent the mean values and the standard deviations from frequency of infection sites. In fig, (**A**) shows the interaction between Apogee and PY15 and PY34; in figure (**B**) the interaction between Buck Aparcero and PY15 and PY34; (**C**) shows the interaction between MS INTA 215 with PY15 and PY34. *Significant differences ($p < 0.05$) are marked with different letters.*

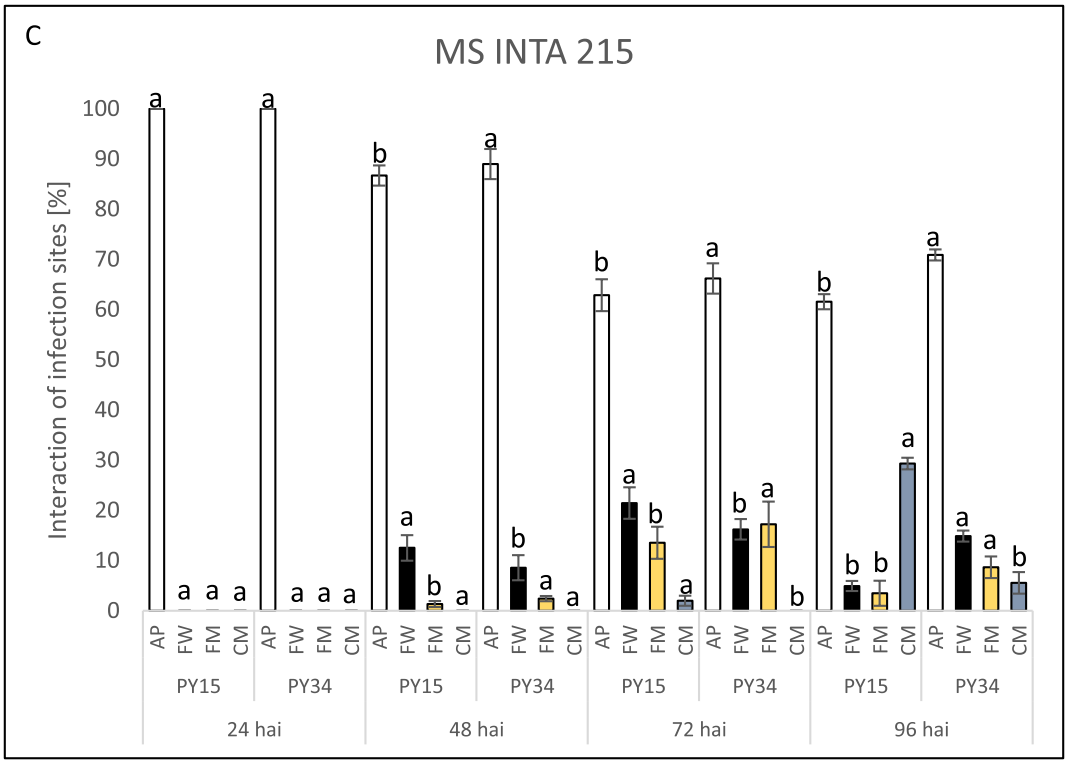

Fig. 3. (*continued*).

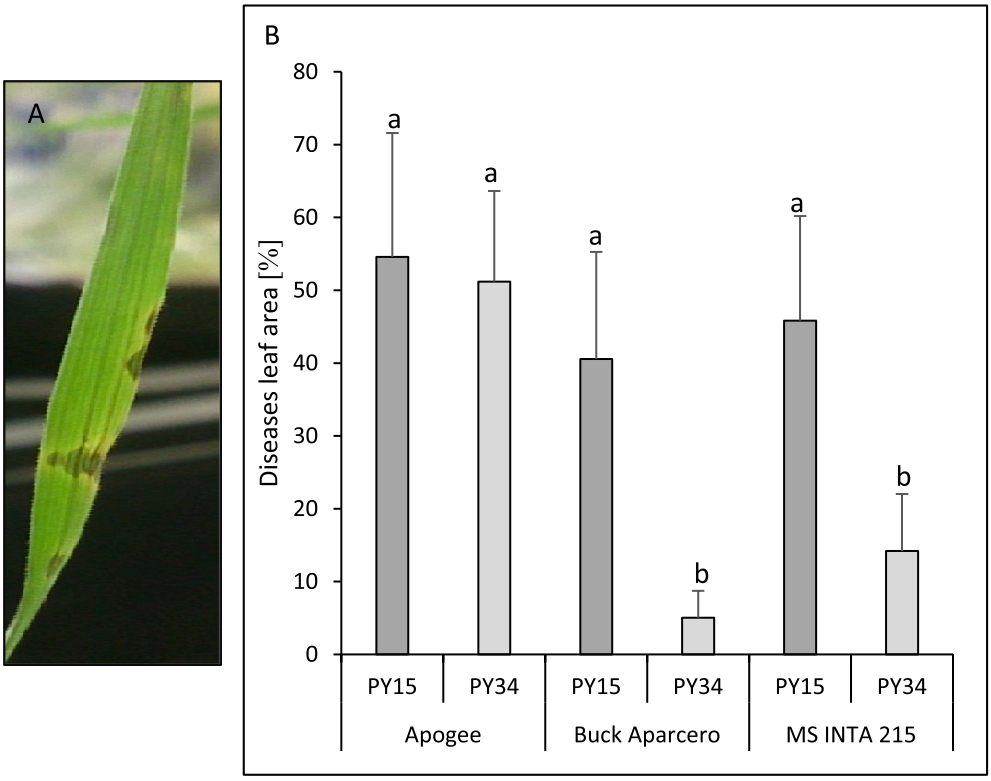

Fig. 4. Leaf infection with isolates PY15 and PY34 on wheat plants. Inoculation of each isolate was performed by spaying approx. one ml of *PoT* conidial suspension at concentration of 250,000 conidia ml^{-1} onto the surface of the third expanded leave using a TLC-glass spray nozzle (0.8 bar of pressure and 40 cm of distance). The controls were sprayed with sterile distilled water under the same conditions. Seven days after inoculation, diseased leaf areas were quantitatively scored on third leaves by taking photographs from ten plants and evaluated with the software APS Assess 2.0. (A) Water-soaked lesions in the interaction MS INTA 215-PY15 at 72 hpi; (B) The bars show the mean of diseased leaf area value (%) with their standard deviation from 10 leaves for each combination. *Significant differences ($p < 0.05$) are marked with different letters.*

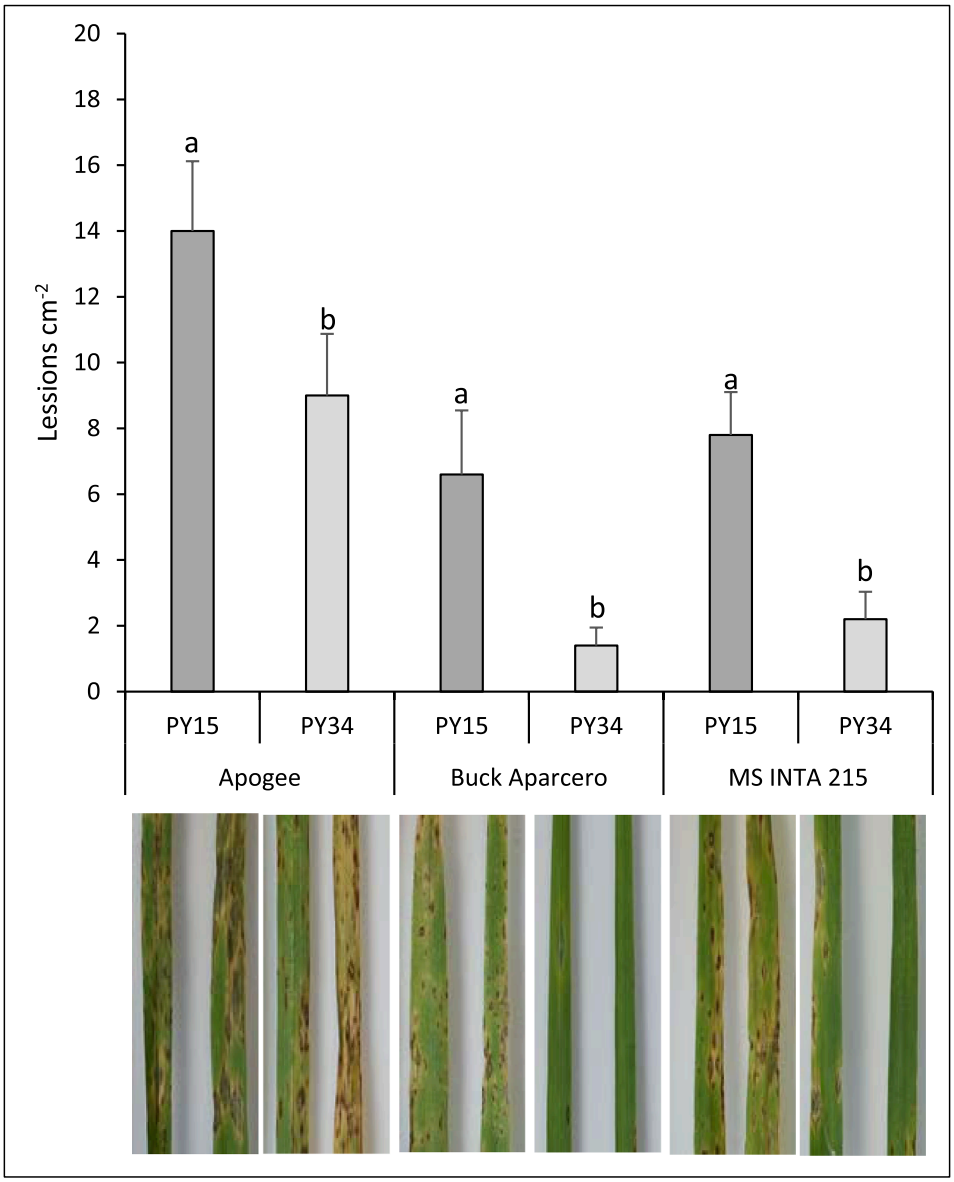

Fig. 5. Individual plant-fungus interaction inspected macro and microscopically under stereoscope microscope. Seven days post inoculation ten leaves were cut of cultivars Apogee, Buck Aparcero and MS INTA 215 in response to *PoT* isolates PY15 and PY34 challenge and the number of lesions cm^{-2} was evaluated using the image analysis software APS ASSES2.0. The bars show the mean value of lesions cm^{-2} with their standard deviation. Two representative photos are shown for each interaction. Results shown are from a single experiment. *Significant differences ($p < 0.05$) are marked with different letters.*

virulence, respectively.

The findings of this paper, also put light on the rol of pathogenesis-related (*PR*) protein 1 when the pathogen *P. oryzae* infects wheat leaves. *PR-1* gene expression constitutes the main family of the *PR* proteins induced in response to a variety of fungal pathogens. However, even having important antifungal activities, their function remains elusive in Wheat-blast disease. Also, there is not previous information on the role of *PR-1* proteins specifically in the Wheat-PoT pathosystem. *PR* genes are thought to be induced on the recognition of the presence of pathogens, and are induced in both susceptible and resistant combinations, although *PR-1* gene expression is higher in resistant combinations. As we tried to elucidate what happens with two varieties of Argentine wheat when faced with the attack of this pathogen, it seemed novel to have indications of resistance and susceptibility based on the accumulation of *PR-1* genes. For those reasons, we decided to study and analyze the expression of this defense gene. Recent previous results of our group also showed that *PR-1* was correlated with SAR and that its expression was significantly affected by the inoculation with *P. oryzae* (Cabot et al., 2018). Our findings illustrate that *PR-1* is highly expressed in both hypersensitive and resistant responses of the wheat plants tested. Members of the *PR* family are among the most prominently produced proteins in plants under attack by pathogens, *PR-1* gene has traditionally served as an indicator of salicylic acid-mediated disease resistance (Breen et al., 2017). Our gene expression analysis indicates that *PR-1* protein plays a role in the defense mechanisms of wheat plants and exhibits antifungal activity against *P. oryzae*. Our hypothesis includes that high constitutive levels of *PR-1 in vivo*, allows resistant wheat cultivars the activation of additional defense responses, which in turn restrict the spread of the disease. The findings here are of significant importance, particularly in the context of understanding wheat defenses against early Wheat Blast disease development. This also indicates the steps of how the disease is spreading into leaves. On the other hand, a quantitative restriction of the pathogen growth was linked to delayed and/or diminished visible symptom development on infected wheat plants.

In this study, the RTq-PCR analysis revealed that the activation of *PR-1* was differentially regulated according two distinct wheat plants

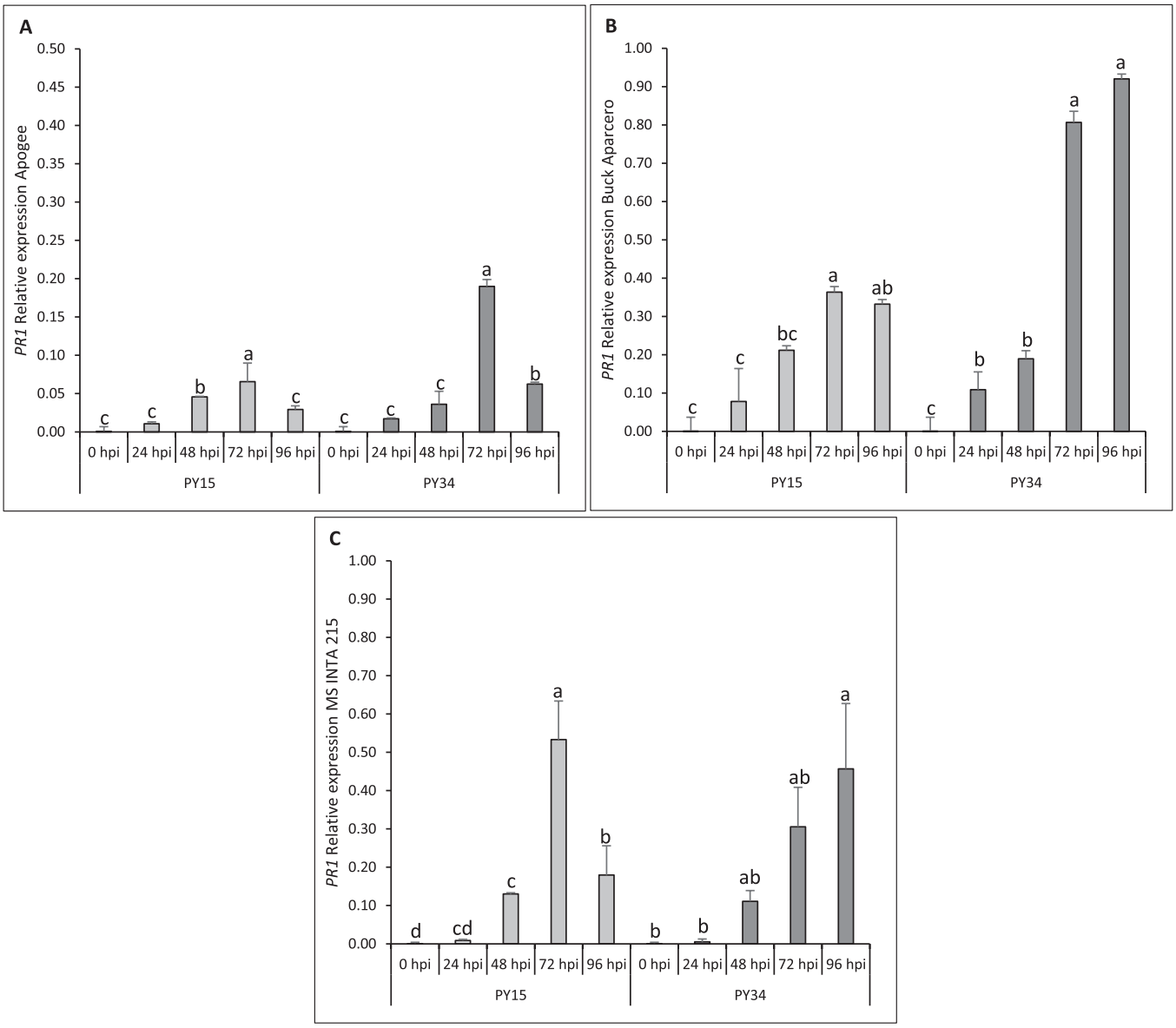

Fig. 6. RT-PCR–based temporal expression analysis of PR1 expression in wheat infected with PoT. Plants of wheat of three cultivars, Apogee (A), Buck Aparcero (B) and MS INTA 215 (C) were inoculated with the two isolates of MoT PY15 and PY34 using a concentration of $2.5x10^5$ conidia ml^{-1}. The third leaf of each individual plant was harvested at 0, 24, 48, 72 and 96 hpi and used for RNA isolation. RNA was transcribed to cDNA and used for quantification of PR1 by RT-qPCR. The relative expression of PR1 was calculated to EF1α using the mathematical models of Livak and Schmittgen (2001). Values shown represent the mean and the standard deviation of three samples consisting of two leaves each. Results shown are from a single experiment. Significant differences ($p < 0.05$) are marked with different letters.

responses categorized as partial resistance and high susceptibility, and therefore, two different pattens of progress, (i) an increased transcript accumulation in infected leaves tissue with a maximum value performed at 72 hpi and then showing decreased values and (ii), an increased transcript accumulation in infected tissue maintained during all the hole period of observation showing the highest value reached at 96 hpi. In agreement, the first model showed here, was coincident with cultivar x isolate interaction that expressed high disease severity after 7 dai. Contrast, the second pattern of expression profile was observed in the two combinations showing the lowest disease severity at 7 dai. Thus, these cultivars exhibited an outstanding resistant or susceptible phenotype suggesting that the differences are a matter of the timing and magnitude of the defense response. In this context, the terms "partial resistance" and "susceptibility" refer to genotypes that consistently exhibit low or high levels of wheat blast severity, respectively. In the temporal observation of *PR-1* expression, Wang et al., (2005) found that the infection's success in a particular interaction plant- pathogen could be linked to the delayed and/or insufficient activation of these defense genes. Here, in our results it was evidenced by the response of the Apogee cultivar to the two isolates. This cultivar presented the lowest *PR-1* expression values compared to the other cultivars and the highest severity values 7 dai. Interestingly, the *PR-1* transcript pattern, correlated with results observed in the sites of infection analysis for this cultivar; In this sense, at 24 hpi only appressorium were observed in both interactions. At 48 hpi there was FW in both interactions and the *PR-1* increased with respect to 24 hpi. The increase was somewhat higher in the interaction with PY15 which also already presented some FM. At 72 hpi Apogee-PY34 in particular presented high values of FM which would explain the higher value of the transcript abundance. In agree with Lin et al. (2023) one of the reasons of these differences may be attributed to the fact that structurally conserved plant pathogenesis-related *PR-1* and *PR-1-like* (*PR-1L*) proteins are both involved in plant

defense and fungal virulence, respectively. Moreover, we speculated that the differences of gene expression could be attributed not only to the different resistant genetic backgrounds of the varieties used (Apogee, Buck and MS INTA) but also due to the difference in virulence of the strains challenged (PY15 and PY34) activating differents downstream signaling pathways.

In the pathosystem wheat-*P. oryzae*, a variety of mechanisms come into play that determine the outcome of the interaction. This can result in compatibility, leading to susceptibility, or incompatibility, leading to resistance, as observed in the study conducted by Wei et al., (2013). Thus, we can speculate that resistant plants expressing latter the maximal induction level of *PR-1* on wheat leaves when compared to susceptible varieties. Moreover, resistant wheat plants demonstrate a constitutive activation of basal defense responses, which likely includes the accumulation of hydrogen peroxide and an up-regulated expression that activates downstream immune signaling processes, as described by Gupta et al. (2020).

In this context, a clear correlation is evident between *P. oryzae* leaf colonization and the development of leaf symptoms in the susceptible cultivar Apogee. This cultivar exhibited the highest fungal accumulation (as collapsed mesophyll, severity values and number of lesions cm^{-2}) and the lowest values in *PR-1* expression. Also, a susceptible behaviour was shown in the combinations MS INTA 215-PY15 and Buck Aparcero-PY15 that expressed the highest relative values of *PR-1* at 72 hpi, comparing with the results obtained in Apogee. In the case of resistant interactions (Buck Aparcero and MS INTA 215 with PY34) lowest severity values and lesions cm^2 were observed. Interestingly, the *PR-1* expression patterns in both cases were similar, growing up 96 hpi where the major values were reached. In coincidence, both interactions had the lowest values of CM compared to the rest of the analyzed interactions indicating a resistant behavior of the hosts infected with this isolate (**Table 1**).

In conclusion, the results presented here demonstrate that differential defense signaling crosstalk and the expression of the *PR-1* gene are key components in the cultivar-specific resistance of wheat plants to Blast disease. Moreover, resistance is closely linked to the activation of defense genes. The *PR-1* analysis could contribute as a tool in a breeding program obtaining wheat cultivars with a high level of tolerance to the infection caused by the fungus *P. oryzae*. Moreover, the analysis presented here identified for a first time a novel pattern of *PR-1* gene in wheat-*P. oryzae* that may play a vital role in the response to this threat. Further studies of how *PR-1* genes are implicated in wheat plant defense will provide more comprehensive view of the uncover the underlying mechanisms of resistance or tolerance in connection with wheat blast disease.

The authors did not perform any study with animals or humans' participants to produce this manuscript.

The authors read and approved the final version of the manuscript.

The authors A. Perelló and I. Martínez contributed equally form to the paper as follows: study conception and design; data collection; analysis and interpretation of results; draft manuscript preparation.

Both authors reviewed the results and approved the final version of the manuscript.

The authors declare that this research was carried out in a manner that respected the rights, dignity and welfare of its participants and other interested parties.

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Uncited references

Dean et al. (2012), Kavanashree et al. (2020), Sung et al. (2021), Wang et al. (2004), Zellerhoff et al. (2008).

CRediT authorship contribution statement

Sergio I. Martinez: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Analía Perelló:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

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