**Free polyamine and polyamine regulation during pre-penetration and penetration
resistance events in oat against crown rust (*Puccinia coronata* f.sp. *avenae*)**

Gracia Montilla-Bascón¹, Diego Rubiales¹, Teresa Altabella^{2,3} and Elena Prats¹.

¹CSIC, Institute for Sustainable Agriculture, 14004 Córdoba, Spain.

²Plant Physiology Unit, Faculty of Pharmacy, University of Barcelona, E-08028
Barcelona, Spain

³Center for Research in Agricultural Genomics (CRAG) (CSIC-IRTA-UAB-UB),
Campus UAB. E-08193 Cerdanyola. Barcelona. Spain

*To whom correspondence should be addressed:

Elena Prats

Institute for Sustainable Agriculture, CSIC, Avda. Menéndez Pidal s/n. 14004. Córdoba,
Spain.

E-mail: elena.prats@ias.csic.es

Tel: +34 957499291

Fax: +34 957499252

ABSTRACT

During recent years a wealth of studies has focussed on the role of polyamines in plant-environmental stress interaction. However, less is known about the role of these compounds in plant disease resistance, and most of this knowledge is related to the hypersensitive response. Here we examined the involvement of polyamines in the oat-crown rust interaction focussing on the mesophyll cell pre-penetration and penetration resistance mechanisms. We used two different oat cultivars: one resistant (Saia) and other susceptible (Araceli) to crown rust (*Puccinia coronata* f.sp. *avenae*). Differential increases in polyamines between oat cultivars upon rust inoculation were observed and associated with increased pre-penetration and penetration resistance. Elevated levels of spermidine and spermine, together with increases of the cell wall bound diamine- and polyamine oxidase activities, and concomitant increases of the polyamine oxidation product, DAP, were observed in the resistant cultivar at early stages of the infection process. This suggests an involvement of polyamines and derived H₂O₂ during penetration resistance. The putative role of polyamines in oat resistance to crown rust is supported by the increased resistance observed in the susceptible cultivar Araceli, as a result of reduced appressorium formation, after treatment with exogenous polyamines. Since ADC has been reported as a key polyamine biosynthesis enzyme in plants exposed to stress, ADC transcript levels and enzyme activity were also evaluated, revealing an increase of ADC activity in cv. Saia at the key time points for the pre-penetration and penetration resistance responses, which suggest an involvement of this enzyme in the resistance response.

INTRODUCTION

Puccinia coronata f.sp. *avenae* is an obligated fungal pathogen that causes crown rust on oat (*Avena spp*). It is the most important disease of the oat crop provoking high yield and grain quality losses worldwide. Because its spread mechanisms are very efficient, usual crop management practices are inefficient for their control. Thus, breeding for resistance is one of the most effective, economical, and environmentally friendly means for its control. However, the obtained resistance, based often on major genes leading to hypersensitive response, is rapidly defeated by new virulent populations due to selection pressure on the pathogen as a result of large-scale and long term cultivation practices (Niks & Rubiales 2002, Sánchez-Martín *et al.*, 2012). Resistance mechanisms engaged prior to hypersensitive response in oat cultivars are important as they are mostly non-race dependent and are usually under polygenic control (Qi *et al.*, 2000). Such resistance is expected to be more durable than single gene controlled race-specific resistance; however particular targets, such as genes, proteins or metabolites, related with those pre-penetration and penetration resistance responses need to be defined for ease selection of resistance genotypes.

Polyamines are low-molecular-weight organic polycations positively charged at physiological pH that are present in a wide range of living organisms. In plants, polyamines are involved in different physiological processes, such as growth, development, and response to abiotic and biotic stresses (Tiburcio *et al.*, 2014). The diamine putrescine, the triamine spermidine and the tetramine spermine are the main

polyamines found in higher organisms. In plants, putrescine is synthesized from ornithine by ornithine decarboxylase (ODC, EC 4.1.1.17) or, via agmatine, from arginine by arginine decarboxylase (ADC, EC 4.1.1.19). Spermidine and spermine are synthesized by addition of aminopropyl groups, arisen from the decarboxylation of S-adenosylmethionine, to putrescine and spermidine respectively, by spermidine and spermine synthases. Free polyamine levels in plant cells depend not only on their biosynthesis but also on their transport, conjugation and degradation. In nature, polyamines often occur as free molecules (soluble polyamines, S), but they can also be conjugated to low-molecular mass compounds (conjugated soluble polyamines, SH) or bound to different macromolecules (conjugated insoluble polyamines, PH) (Tiburcio *et al.*, 1997). Polyamines are also catabolized through the activity of amine oxidases, including the copper-containing amine oxidases (DAO; EC 1.4.3.6), with high affinity for diamines, and the FAD-dependent polyamine oxidases (PAO; EC 1.5.3.3), that oxidize spermidine and spermine at their secondary amino groups. In monocotyledonous plants, PAOs catalyse the terminal catabolism of polyamines oxidizing the carbon at the end-side of the N⁴-nitrogen of Spd and Spm to produce an aminoaldehyde, in addition to 1,3-diaminopropane (DAP) and H₂O₂ (Cona *et al.*, 2006).

In recent years, there has been a growing interest in the study of polyamines involvement in the plant responses against different environmental stresses. Thus, several works reported the induction and accumulation of polyamines associated with the improvement of plant tolerance to a wide range of abiotic stresses (i.e. Liu 2007). However, relatively little work has been carried out in the dissection of the role of free

polyamines and their metabolism during plant-pathogen interactions (Walters 2000), and much of what has been conducted relates to the hypersensitive response (Walters 2003).

The present work aims to unravel the role of polyamines in oat defense against the rust fungus *Puccinia coronata* f.sp. *avenae*, focussing on the different resistance responses engaged before and during mesophyll cell attempt penetration, and hence engaged prior to hypersensitive response..

MATERIAL AND METHODS

Plant material

For this study 2 commercial cultivars supplied by the Andalusian Network of Agriculture Experimentation (RAEA) were used. Oat cultivars studied were Araceli, and Saia that had previously shown to be susceptible and resistant to the used rust isolate (Sánchez-Martín *et al.*, 2012). Plants were grown individually in 30 x 110 mm plastic centrifuge tubes (with two 5 mm drainage holes) filled with peat:sand (3:1). Tubes stood in trays filled to *ca* 50 mm depth with the same compost which was watered freely throughout. Plants were grown in a room at 20°C, 65% relative humidity and under 12 h dark/12 h light with 250 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ photon flux density supplied by high-output white fluorescent tubes. Fully expanded first-formed leaves of approximately 12 day-old plants (their second-formed leaf was unrolled) were used in all experiments.

Inoculation

P. coronata f.sp. *avenae* (*Pca*) isolate Co-04, was used for inoculation (Sanchez-Martin *et al.*, 2012). Urediospores were multiplied on plants of oat cv. Araceli, which were highly susceptible to this isolate. One day before experimental inoculation, spores were collected and kept overnight in a desiccator.

Plants with the first leaf completely expanded were inoculated with urediospores mixed with pure talcum (1:1, w/w) by dusting them over the plants to give approximately 30 spores mm⁻² (checked by counts made from glass slides laid adjacent to leaves). Homogeneous inoculation was ensured by arranging the tubes with the plants around the base of a settling tower where leaves were laid flat, adaxial surface up. After inoculation, plants were incubated for 9.5 hours in darkness at 100 % RH and 18°C, and thereafter returned to the growth room.

Microscopic observations

For microscopic assessment of fungal development, middle segments of 1-3 cm² from each inoculated leaf still attached to the plant (four leaves per treatment) were excised at 84 hours after inoculation (h.a.i.) and stained with Uvitex (Ciba, Barcelona, Spain), according to Niks & Rubiales (1994). Plants were observed by ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm emission) using a Leica DM LS phase contrast microscope (Leica Microsystems, Wetzlar, Germany; 100x objective).

Percentages of germinated urediospores were determined from 100 random urediospores per leaf segment. In addition, on each leaf segment, 100 infection units (growth arising from individual urediospore) that successfully germinated were scored and classified according to their developmental stage i.e. whether they reached a stomata and formed an appressorium/substomatal vesicle but did not form infection hyphae (pre-penetration resistance), formed the substomatal vesicle and one or more primary infection hyphae but colony growth had ceased forming less than six haustorial mother cells due to mesophyll penetration resistance, and infection units with six or more haustorial mother cells which were considered established. In addition, the presence of host cell death associated with the impaired (early cell death) or established (late cell death) colonies was recorded. Death of plant cells was recognized by yellow whole-cell fluorescence under violet incident light (420 nm excitation/490 nm emission).

Polyamine quantification

The standard polyamines, putrescine, spermine, spermidine, and 1,3-diaminopropane (DAP) were obtained as their hydrochlorides (Sigma) whereas agmatine was obtained as its sulfate (Sigma). HTD (1,7-diaminoheptane) was used as internal control (Sigma).

For total polyamine quantification, non-inoculated controls and inoculated plant tissue fixed at 24, 48 and 72 h.a.i. was pulverized in liquid N₂ and homogenized in cold 5% HClO₄ in (0.1 w/v) according to Flores & Galston (1982). Extracts were incubated at

4°C during 1 h and centrifuged at 13000 g at 4°C during 20 min. Resulting supernatant collected in a plastic tube was stored at -20°C until use.

In order to quantify excreted polyamines to the leaf surface at 24 h.a.i., leaves were sequentially washed by dripping 4 ml of 5% perchloric acid twice on the leaf surface with a pipette (Prats *et al.*, 2002). The washing solution, containing the polyamines excreted onto the leaf surface was concentrated under vacuum, resuspended in 1mL perchloric acid, and stored at -20°C until use.

Standards and plant extracts were benzoylated according to Redmond & Tseng, (1979); one mL 2 N NaOH was mixed with 500 µL of HClO₄ extract. After addition of 10 µL benzoyl chloride, vortexing for 10 s, and incubation for 20 min at room temperature, 2 mL saturated NaCl were added. Benzoyl polyamines were extracted in 2 mL diethyl ether, vortexing for 15 s and incubating 5 min at 4°C. Thereafter 1 mL of the ether phase was collected, evaporated to dryness under a stream of warm air, and redissolved in 100 µL methanol (MERCK; HPLC grade). Standards were treated in a similar way, with up to 50 nmol of each polyamine in the reaction mixture. The benzoylated samples were stored at -20°C. High performance liquid chromatography analysis of benzoyl-polyamines was performed according to Slocum *et al.*, (1989), using an Agilent 2100 Series HPLC. The benzoylated extracts were eluted at room temperature through a 4.6 x 250 mm, 5 µm particle size reverse-phase (C18) column (Altex-octadecylsilane) and detected at 254 nm. 20 µL of benzoyl-PAs were manually injected at a flow rate of 1 mL/min. The solvent system consisted of methanol:water, run

1 isocratically at 50 to 100% methanol following the Programme I described by Slocum *et*
2 *al.*, (1989).

4 **Polyamine bioassay**

5 To assess the effect of polyamines on the different resistance responses, according
6 to previous literature (i.e. Tanou *et al.*, 2014) 1mM solution of each polyamine with 0.1%
7 Tween 20 was sprayed over the entire plant until surface runoff was observed.
8 Approximately, three mL of solution were applied to each plant. Thus, the concentration
9 of polyamines applied was within the range usually detected in plants after rust
10 inoculation. Control plants were similarly treated with 0.1% Tween 20 in water.
11 Treatment was applied twice a day during two days and the third day plants were
12 inoculated and fixed for microscopic inspection at 84 h.a.i. as explained above.

14 **Gene expression analysis by real-time qRT-PCR**

15 Total RNA was extracted from 100 mg of ground leaf tissue according to
16 Chomczynski & Sacchi (1987). RNA was cleaned with RNeasy® Minelute Cleanup Kit
17 (Qiagen) and contamination of residual genomic DNA verified. Prior to retrotranscription
18 experiments, the concentration and integrity of RNA were verified by an optical density
19 at 260 nm (OD260)/OD280 absorption ratio in a NanoDrop ND-1000 spectrophotometer
20 (Thermo scientific). First and second-strand of complementary DNA (cDNA) were
21 synthesized using SuperScript® III First-Strand (Invitrogen) and DNA Polymerase I
22 (BioLabs), respectively, according to manufacture's instructions. cDNA was cleaned by
23 Quick PCR Purification Kit (Qiagen).

1

2 Relative expression of oat ADC was quantified by using the following primers:

3 5'-TACGGCGATGTGTACCATGT-3' and 5'-GTCCTTGTTACGGCAAAGT-3';

4 designed with the Universal Probe Library Assay Design Center (Roche applied Science)

5 from the sequence published in NCBI database (X56802.1; Bell & Malmberg 1990).

6 Previous to test the expression of ADC, four additional genes were tested for using as

7 reference genes; glyceraldehyde-3-phosphate dehydrogenase (GADPH), beta-tubulin

8 (TUBB), alpha-tubulin (TUBA) and 18S ribosomal RNA (10S rRNA) according to

9 Jarosova & Kundu (2010). Following preliminary assays, GADPH was selected as the

10 endogenous normalization measure due to the high stable expression showed in our oat

11 samples. Real-time qRT-PCR was performed at least with 3 independent biological plus 3

12 technical replicated cDNA templates in StepOne Real-Time PCR System (Applied

13 Biosystems) using FastStart Universal SYBR Green Master (Rox) (Roche) according to

14 the manufacturer's recommendations. The reaction mixture contained: 10 µl of SYBR

15 Green master mix, 6 µL of each primer, and 4 µL of cDNA as template. The

16 amplification conditions were 95°C for 10 min, followed by 40 cycles of amplification at

17 95°C for 15 s, 60°C for 1 min. Following amplification, a melting curve program 95°C

18 for 15 s, 60°C for 1 min and 60 to 95°C with a heating rate of 0.3°C/min was run. The

19 melting point analysis was performed at the end of the real-time RT-PCR to confirm the

20 amplification of a unique product for each gene. The fold changes of ADC gene

21 transcripts in different treatments versus control (i.e., non-inoculated plants) were

22 normalized using the CT and efficiency obtained for the GADPH amplification run on the

23 same cDNA templates according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

1

2 **Analysis of ADC activity**

3 ADC activity was determined in leaf extracts according to Tiburcio *et al.*, (1986),
4 using L-[U-¹⁴C]arginine (Amersham, UK) as substrate, and measuring the ¹⁴CO₂
5 resulting from the specific decarboxylation of arginine catalysed by ADC. Enzyme
6 activity was expressed as nmol ¹⁴CO₂ released/h mg protein. Protein was determined
7 according to the method of Bradford (1976). Bovine γ-globulin (Sigma) was used as
8 standard.

9

10 **Analysis of DAO/PAO activity**

11 Diamine oxidase (DAO) (EC1.4.3.6.) and polyamine oxidase (PAO) (EC1.4.3.4.)
12 activities were measured spectrophotometrically based on the colorimetric assay of Δ-
13 pyrroline using putrescine and spermidine as substrates for DAO and PAO activities,
14 respectively (Asthir *et al.*, 2004, Holmstedt *et al.*, 1961). Briefly, as a cleaning step each
15 leaf was homogenized 1:2 (w:v) in 100 mM K-phosphate buffer (pH 7) containing 5mM
16 dithiothreitol at 4°C, and centrifuged at 16,000g for 20 min at 4°C. In order to measure
17 soluble DAO/PAO activities the pellet was extracted with 100 mM K-phosphate buffer
18 (pH 7) containing 20 mM EDTA (1:1, p:v), incubated 10 min at 4°C and, after
19 centrifugation 20 min at 16,000g at 4°C, the supernatant was collected. This step was
20 repeated twice. The remaining pellet was used directly to quantify wall-bound DAO/PAO
21 activities. For the enzyme activity assay, extract (0.75 μL of supernatant for soluble or
22 0.30 mg of the pellet for wall-bound activity) was combined with 50 units of catalase, 75
23 μL of 2-aminobenzaldehyde (0.1%) and 75 μL of substrate: 10 mM putrescine in 50mM

K-phosphate buffer (pH 7.5) for DAO or 10 mM spermidine in 50mM K-phosphate buffer (pH 6) for PAO. The reaction was carried out at 30°C for 3h, then 10% perchloric acid (v:v) was added and the mix was incubated 15 min at RT in order to stop the reaction. After centrifugation at 5,000 rpm for 15 min at RT, the supernatant was used to determine the formation of the reaction product Δ -pyrroline by reading the absorbance at 430 nm. Inactivated enzymes by heating at 95°C for 5 min were used as control reactions. Enzyme activity was expressed in ng of Δ -pyrroline min⁻¹g⁻¹FW.

Statistical analysis

Four leaves (from four different plants) per genotype were studied in a complete randomized block design. For statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \arcsin [\sqrt{(\%/100)}]$) to normalize data and stabilize variances throughout the data range. Data were then subjected to analysis of variance using SPSS software after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett's test were performed to test normality and homogeneity of variances respectively. Multiple comparison among all genotypes were carried out according to Tukey test ($p < 0.05$).

RESULTS

Microscopic characterization of resistance responses of oat cultivars to *Puccinia coronata* f.sp. *avenae* attack

No differences in *P. coronata* spore germination were observed between cultivars. However histological characterization confirmed the susceptibility of cv. Araceli with 68.5% of established colonies (Table 1). Cultivar Saia showed as highly resistant with the resistance responses engaged during the early stages of the infection process. Thus, 34% of rust attacks were hampered during appressorium and substomatal vesicle formation, and 37% were hampered during the mesophyll penetration attempt, impairing colony establishment. Thus, cv. Saia showed near 75% fewer established colonies than the susceptible control Araceli (Table 1). Only a very low proportion (ca. 10%) of cells triggered a programmed cell death in Saia following pathogen attack and this was mainly a late cell death (Table 1).

Polyamine content

At most crucial times of the infection process significant differences were observed in levels of particular polyamines between the oat cultivars. Overall, the highest significant differences among cultivars were found for spermine and spermidine, agmatine and DAP ($p<0.001$; Fig. 1). Thus, Saia displayed significantly, 4 fold higher, constitutive levels of spermidine and spermine, than the susceptible genotype Araceli. Agmatine content was also overall higher in Saia respect to Araceli ($p=0.013$). In addition statistical analysis showed interaction between genotype and time post inoculation for most polyamines indicating a different trend between the susceptible and resistant cultivars. Decreased levels of spermine and spermidine 24 and 48 h.a.i. in cv. Saia correlated with an increase of the catabolism product DAP at these time points (Fig. 1).

1 The level of polyamines excreted to the leaf surface was less than 10% of the total
2 content for all assessed polyamines (Fig. 2). Interestingly, excreted polyamines to the leaf
3 surface increased 24 h.a.i. in both susceptible and resistant cultivars compared with non
4 inoculated controls ($p<0.001$). However, there was a significant interaction for most
5 polyamines between cultivars and treatments indicating that the two cultivars did not
6 respond similarly to the rust attack. Thus, cv. Saia showed a significantly higher increase
7 in excreted spermine and spermidine after inoculation compared with the susceptible cv.
8 Araceli. Particularly, whereas no significant increase of excreted spermidine was
9 observed in Araceli, more than two fold increase was observed in Saia. In addition Saia
10 showed a 2-fold increase in excreted agmatine respect to Araceli. No significant
11 differences in excreted DAP or putrescine were found between cultivars following rust
12 inoculation (Fig. 2).

14 **Exogenous polyamine bioassay**

15 In order to test whether the naturally excreted polyamines to the leaf surface upon
16 inoculation might interfere with the rust infection process, polyamines were applied
17 exogenously to the susceptible cv. Araceli and their effect in arresting the early fungal
18 developmental stages was recorded.

20 Exogenous application of polyamines resulted in an increase of pre-penetration
21 resistance in the susceptible cv. Araceli for all polyamines applied, particularly for
22 spermine, spermidine and putrescine ($p<0.001$; Fig. 3). In addition, the percentage of
23 early aborted colonies increased 4- and 3-fold related to the control following application

of putrescine and spermine, respectively. Thus, a significant decrease of established colonies was observed after polyamine application to the susceptible cv. Araceli, showing putrescine, spermine, and spermidine the highest reduction in colony establishment (Fig. 3). On the contrary, cell death response was not modified in cv. Araceli following polyamine application (data not shown).

Relative expression of *ADC* gene

Since ADC plays a key regulatory role in the polyamine biosynthesis pathway, its regulation in oat was assessed at both gene expression and enzyme activity level at key time points following rust inoculation.

Quantitative RT-PCR experiments were carried out to assess ADC regulation at transcript level during the response to rust attack in the different cultivars. *ADC* expression was overall down-regulated in both cultivars following rust inoculation, at the very early stages of the infection process (Fig. 4). However, significant differences were observed between cultivars. Thus, *ADC* expression was strongly down-regulated during the first 72 h.a.i in cv. Araceli, whereas in Saia, albeit *ADC* expression was down-regulated at 24 h.a.i., it was significantly higher compared with Araceli at that time point. Moreover, in cv. Saia the *ADC* expression was significantly up-regulated at 48 h.a.i. ($p=0.002$) whereas at 72 h.a.i. it was similar to control, non-inoculated levels (Fig. 4). Thus, during all the assessed time points, *ADC* expression in Saia was significantly higher than in Araceli.

1 **ADC activity**

2 ADC activity showed steady state levels in susceptible Araceli during 24, 48 and
3 72 h.a.i. whereas Saia showed differences over the time following inoculation.
4 Particularly, constitutive levels of the ADC activity and also levels at the earliest time
5 points after inoculation (i.e. 24 and 48 h.a.i.) were significantly higher in Saia than in
6 Araceli (about 2-fold, $p<0.001$). At 72 h.a.i. the ADC activity in Saia decreased to reach
7 similar levels than those observed in Araceli (Fig. 5).

8

9 **Soluble and cell-wall bound DAO and PAO activities**

10 Similar levels of soluble and cell-wall bound DAO activities were found in the
11 healthy, non-inoculated cultivars (Fig. 6). However, the soluble diamino oxidase activity
12 decreased significantly in cv. Saia at 24 h.a.i., whereas no changes were observed in the
13 susceptible cv. Araceli. Differences were also observed in the cell-wall bound DAO
14 activity between inoculated susceptible and resistant cultivars. Thus, a 4 fold activity
15 increase was observed in the resistant cv. Saia at 48 h.a.i. respect to the control non-
16 inoculated plants ($p=0.01$). A significant increase of bound-wall DAO activity was also
17 detected in cv. Araceli, but in a lower proportion and later than in Saia (72 h.a.i., $p=0.014$)
18 (Fig. 6). The cell-wall bound DAO activity decreased in Saia at 72 h.a.i. No significant
19 differences in soluble PAO activity were observed between non-inoculated Saia and
20 Araceli plants (data not shown). However, slight increases of cell-wall bound PAO
21 activity were observed in Saia at 24 and 72 h.a.i. compared with Araceli.

22

23 **DISCUSSION**

1

2 There are numerous reports about the role of polyamines in plant-abiotic stress
3 interaction. However, little information exists on how polyamine metabolism may
4 influence plant resistance responses to pathogens (Walters 2003), and this is mainly
5 focussed on the barley-powdery mildew (Walters 2000, Walters 2003) and in the
6 tobacco-tobacco mosaic virus (Yamakawa *et al.*, 1998) interactions. Few works can be
7 found about plant-rust interaction and those are related to polyamine changes during
8 infection in compatible interactions (Aldesuquy *et al.*, 2000,) or during incompatible
9 interaction but without focussing on particular mechanisms. Thus, detailed studies
10 dissecting the role of polyamines during the engagement of specific resistance responses,
11 and particularly those that may confer durable resistance are needed. In this sense,
12 resistance mechanisms acting before or during mesophyll cell attempt penetration may
13 prove more durable and difficult to be overcome than resistance based on HR and hence
14 are desirable from a breeding point of view.

15

16 Our data show that in the oat resistant cv. Saia, characterized by pre-penetration
17 and penetration resistance responses to crown rust, the content of free intracellular
18 polyamines, such as agmatine, spermidine and spermine, as well as the polyamines
19 excreted to the leaf surface, increased at crucial times of the crown rust infection process,
20 whereas no change or lower increases were observed in the susceptible cv. Araceli.
21 Constitutive excreted polyamines were similar in both cultivars and, interestingly,
22 increased levels of excreted agmatine, putrescine and spermine were detected in both
23 cultivars following inoculation. Assuming an involvement of the polyamines in the

1 resistance process, this might explain the basal levels of pre-penetration resistance
2 observed also in the susceptible cultivar Araceli. Moreover, the levels of different
3 polyamines were significantly higher in the resistant cv. Saia when compared with cv.
4 Araceli, suggesting a role for these polyamines in the pre-penetration responses. Pre-
5 penetration resistance mechanisms may be particularly relevant during the plant-rust
6 interaction due to the relatively extended growth of the rust germ tube on the leaf surface
7 until reaching a stomata for appressorium formation (Prats *et al.*, 2007). It is known that
8 topographical signals mediate the processes leading to appressorium formation (Hoch &
9 Staples 1987). However, physical features are not the only important determinant of rust
10 germ tube growth and appressorium formation. Specific plant chemical signals may also
11 influence appressorium differentiation. Thus, in resistant onion varieties it has been found
12 that phenolic compounds excreted from dead cells to the surface prevent urediospore
13 germination of *Colletotrichum circinans* (Walker & Stahmann 1955) and coumarins
14 excreted to sunflower leaf surface prevent germination and appressorium formation of
15 *Puccinia helianthii* (Prats *et al.*, 2002, 2007). The early excretion of the polyamines upon
16 rust attack coinciding with the time of germ-tube growth and appressorium formation
17 suggest that the excreted polyamines might act as a chemical signal able to interfere with
18 the germ-tube growth and appressorium formation.

19
20 The exogenous application of the different polyamines to the leaf surface
21 confirmed their ability to hamper the early stages of the infection process. Thus, all
22 polyamines tested contributed to a poor growth of the germ tube that often did not reach
23 the stoma, because of a disoriented germ tube growth or continued germ tube growth

1 across stomata and the inability of the germ tube to form an appressorium when finding a
2 stoma. No correlation was found between a particular polyamine and the prevalence of
3 one of these three responses. These results agree with previous *in vitro* observations on
4 *Magnaporthe grisea* showing that polyamines did not affect conidial germination but
5 specifically impaired appressorium formation (Choi *et al.*, 1998). These authors observed
6 high levels of polyamines, particularly of spermidine, in freshly collected spores,
7 decreasing during conidial germination and growth. Thus, changes in the levels of
8 polyamines within the fungus, provoked by the excreted polyamines from the host might
9 lead to the inhibition of the appressorium formation observed in cv. Saia.

10
11 Total polyamine content, particularly of agmatine, spermine and spermidine
12 remained higher in Saia during the earliest stages of the rust infection process compared
13 to the susceptible control. This together with the increase of aborted following exogenous
14 polyamine application, suggest also a role for polyamines during mesophyll penetration
15 resistance. This is supported by the increase of DAP in Saia at 24 and 48 h.a.i. together
16 with increases in the DAO and PAO cell-wall bound activities. There is evidence that
17 DAO and PAO are predominantly located in the cell wall (Angelini *et al.*, 1993). As a
18 result of the diamine and polyamine oxidase activities, polyamines are oxidised yielding
19 the catabolism products DAP and H₂O₂ (Bagni & Tassoni 2001). It has been recently
20 demonstrated that polyamines serve as the source of hydrogen peroxide during the HR in
21 tobacco plants infected with *Pseudomonas cichorii* (Pellegrini *et al.*, 1994). H₂O₂ is also
22 required as a signal molecule for the orchestration of the papilla constituents and for the
23 oxidative cross-linking of components such as phenolics and proteins into the papilla and

1 associated cell wall region (Pellegrini *et al.*, 1994, Vanacker *et al.*, 2000) to enhance
2 penetration resistance to pathogen infection (Angelini *et al.*, 1993). Indeed, it has been
3 suggested that the H₂O₂ generated by the cell-wall bound DAO and PAO could be
4 utilised by the cell wall peroxidases for cell wall strengthening and lignification (Angelini
5 *et al.*, 1993, Yoda *et al.*, 2009). The increased cell-wall DAO and PAO activities together
6 with the increases in DAP in Saia suggest the generation of H₂O₂ that might contribute to
7 the higher penetration resistance observed in this cultivar. In addition, the higher levels of
8 polyamines found in cv. Saia might also contribute to its penetration resistance, since
9 polyamines can be found directly associated with pectin polysaccharides and are also
10 believed to control lignin deposition and cell wall pH (Angelini *et al.*, 1993, Dorazi &
11 Bagni, 1987), having an essential role in strengthening the links between the cell wall
12 components (Berta *et al.*, 1997).

13
14 Regulation of ADC activity is one of the key steps in controlling polyamine
15 biosynthesis in plants, mainly when the plant is under stress, whereas ODC appears to be
16 associated with actively dividing cells in meristematic and reproductive tissues (Ruiz *et al.*, 2000). In agreement with this, our results shown a high increase of ADC activity in
17 Saia compared with Araceli at the key time points for the pre-penetration and penetration
18 resistance responses. In addition, ADC activity seems to be regulated at multiple levels
19 by different stimuli. Thus, some reports showed that changes in mRNA levels correlated
20 with enzyme activity during plant development, whereas others showed that the changes
21 in mRNA and protein levels could not account for those observed in enzyme activity.
22 Thus, when possible, it is advisable to study both, transcript and enzyme activity levels,

1 in order to understand the regulatory mechanism in the particular combination of
2 species/stress-resistance response to tackle possible candidate targets for further
3 selection. In our work, the values of *ADC* relative expression do not completely match
4 those of ADC activity. This lack of correlation between ADC transcript levels and
5 enzyme activity is mainly observed in infected cv. Saia at 24 and 48 h.a.i. It has been
6 reported that oat ADC is synthesized as an inactive proenzyme that is post-translational
7 processed to give the active enzyme (Malmberg & Cellino, 1994). Thus, the observed
8 differences between *ADC* transcript levels and enzyme activity could be explained by this
9 post-translational regulation.

10
11 Overall our data show a role for free polyamines during resistance events prior to
12 hypersensitive response, influencing both, growth of the germ tube and appressorium
13 formation over stomata and the penetration of mesophyll cells. Further involvement of
14 conjugated polyamines or the polyamine catabolic product, hydrogen peroxide, in
15 penetration resistance still need to be clarified. However, the already proved role of free
16 polyamines in pre-penetration and penetration resistance responses offers opportunities
17 for breeding for a more durable resistance.

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20
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Tables

Table 1: Microscopic characterization of resistance responses of oat cultivars to *Puccinia coronata* attack.

Cultivar	%Pre-penetration Resistance*	%Penetration resistance	%Early cell death	%Late cell death	%Established colonies
Araceli	24.02±1.26	7.44±1.45	0.00±0.00	0.0±0.00	68.54±2.08
Saia	33.87±4.06	37.07±5.04	3.03±1.84	7.74±5.02	18.29±1.87
	<i>l.s.d</i> 4.4	4.7	3.8	5.8	3.8

*Infection units (growth arising from individual urediospore) successfully germinated were scored and classified according to their developmental stage i.e. whether they reached a stomate and formed an appressorium or substomatal vesicle but did not form infection hyphae (pre-penetration resistance); formed the substomatal vesicle and one or more primary infection hyphae but colony growth had ceased forming less than six haustorial mother cells due to mesophyll penetration resistance; and infection units with six or more haustorial mother cells which were considered established. In addition, the presence of host cell death associated with the impaired (early cell death) or established (late cell death) colonies was recorded.

1 **Figure Legends**

2 **Figure 1. Total polyamine content in rust susceptible and resistant oat cultivars.**

3 Agmatine (AGM), putrescine (PUT), spermidine (SPD), spermine (SPM) and 1,3-
4 diaminopropane (DAP) were quantified in susceptible Araceli (white bars) and resistant
5 Saia (black bars) plants during a time course following inoculation with the host fungus
6 *Puccinia coronata* f.sp. *avenae*. Data are mean of 5 replicates $\pm$ standard error.

7

8 **Figure 2. Excreted polyamines to leaf surface in rust susceptible and resistant oat**

9 **cultivars.** Agmatine (AGM), putrescine (PUT), spermidine (SPD), spermine (SPM) and
10 1,3-diaminopropane (DAP) were quantified in healthy (white bars) and inoculated (black
11 bars) susceptible Araceli and resistant Saia plants 24 hours following inoculation with the
12 host fungus *Puccinia coronata* f.sp. *avenae*. Data are mean of 5 replicates $\pm$ standard
13 error.

14

15 **Figure 3. Effect of exogenous application of polyamines on *P. coronata* f.sp. *avenae***
16 **development and plant resistance responses.** All parameters were microscopically
17 evaluated on the second leaf 84 hours after inoculation. Values are the means of five
18 independent replicates $\pm$ standard error.

19

20 **Figure 4. Relative expression of arginine decarboxylase (ADC) gene in rust**
21 **susceptible and resistant oat cultivars.** Real time RT-PCR was carried out in oat leaves
22 of susceptible (Araceli) and resistant (Saia) plants during a time course following
23 inoculation with the host fungus *Puccinia coronata* f.sp. *avenae* at 24 (white), 48 (grey)

and 72 (black) hours after inoculation. Data are mean of 3 biological replicates and 3 technical replicates $\pm$ standard error.

Figure 5. Arginine decarboxylase (ADC) activity in oat leaves of rust susceptible and resistant cultivars. ADC activity was measured in oat leaves of susceptible Araceli (white bars) and resistant Saia (black bars) plants during a time course following inoculation with the host fungus *Puccinia coronata* f.sp. *avenae*. Data are mean of 5 biological replicates $\pm$ standard error.

Figure 6. Diamine oxidase (DAO) activity in oat leaves of rust susceptible and resistant cultivars. Soluble (A,B) and cell-wall bound (C,D) DAO was measured in healthy (white bars) and inoculated (black bars) leaves of susceptible Araceli and resistant Saia oat cultivars during a time course following inoculation with the host fungus *Puccinia coronata* f.sp. *avenae*. Data are mean of 5 biological replicates $\pm$ standard error.