Changes in polyamine profile in host and non-host oat-powdery mildew interactions

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Short title: Polyamines in host and non host interactions

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ABSTRACT

Oat (*Avena sativa* L.) crop constitutes a rich source of biologically active secondary metabolites. Most of these compounds act as chemical signals and defense metabolites and constitute a potential source for the development of control methods for specific diseases. Polyamines are low molecular organic cations involved in various physiological events, particularly those related to abiotic stress responses, albeit recently their potential in disease resistance has being investigated. In this work we monitored the polyamine content in leaves of both resistant and susceptible oat cultivars in response *Blumeria graminis* f.sp. *avenae* (*Bga*, host interaction) and with *Blumeria graminis* f.sp. *hordei* (non-host interaction). Our results show significant differences between the resistant and susceptible cultivars for specific free polyamine levels, and also with respect to the non-host interaction at crucial stages of the infection process. In addition, polyamine degradation products, such as 1,3-diamino propane increased following pathogen challenge, suggesting a role for reactive oxygen species derived from this pathway in resistance. Exogenous application of polyamines to leaf surface increased penetration resistance of oat against *Bga.* Overall, data support both, a direct and indirect role for polyamines in resistance in host and nonhost interactions, in responses of oat against appropriate and inappropriate powdery mildew *formae speciales*.

Keywords: disease resistance, host and non-host interactions, oat, polyamines, powdery mildew,

1. Introduction

Plants produce a wide number of phytochemicals useful in its interaction with the environment including biotic and abiotic stress factors. Polyamines can be considered as one of the earliest known secondary metabolites in biochemistry (Galston and Sawhney, 1990) and are considered to be ubiquitous in all living cells. These low molecular weight compounds are positively charged at physiological pH and hence initially their biological function was associated with the capability of binding to negatively charged molecules (Cohen, 1998). However, in addition to stabilizing macromolecular structures, polyamines also act as regulatory molecules in many fundamental cellular processes including cell division, embryogenesis, as well as in senescence and in response to stress (Martin-Tanguy, 1997). Recent studies indicate that polyamines may act as cellular signals in intricate cross talk with hormonal pathways, such as abscisic acid and ethylene, integrated with processes of hydrogen peroxide and nitric oxide signaling (An et al., 2008; Toumi et al., 2010; Yamasaki and Cohen, 2006).

Polyamine biosynthesis is initiated from the basic amino acids ornithine and arginine, which are decarboxylated by ornithine decarboxylase (ODC; EC 4·1.1·17) and arginine decarboxylase (ADC; EC 4·1.1·19), respectively, to yield the diamine putrescine. Putrescine then serves as the substrate for the formation of the tri- and tetra-amines spermidine, spermine and other derived polyamines. The earliest reported changes in polyamines were associated with the response to abiotic stresses as reviewed by Alcazar et al. (2010). However, recent studies also show polyamine accumulation in response to pathogens (Walters, 2003). However the physiological significance of these responses, the dynamics of polyamines at the very early stages of the infection, or whether same polyamines have a similar role during resistance responses in different plant species remains unclear. Furthermore, very little is known about the role of polyamines in a non-host interaction with the sole evidence of their involvement during the attempted infection of bacteria to non-host tobacco plants (Yoda et al., 2009).

Oat powdery mildew (*Blumeria graminis* f. sp. *avenae, Bga*) is a biotrophic fungus that develops reasonably synchronously through a highly ordered morphogenetic sequence slightly delayed with respect barley powdery mildew (*Blumeria graminis* f. sp. *hordei, Bgh*) reviewed by Green et al. (2002). Emergence of a short primary germ tube is followed by that of the second, appressorial germ tube that elongates and differentiates a hooked, apical appressorium. A penetration peg emerging beneath the appressorium (14-16 hours after inoculation) attempts to breach the plant epidermal cell

wall. If successful, it enters the cell lumen where its tip swells and differentiates (20-24 hours after inoculation) a mature haustorium. This absorbs nutrient from the epidermal cell to support further fungal growth. Alternatively plants may hamper the infection process to limit fungal multiplication. The best known mechanisms by which cereal defend against powdery mildew are by forming papillae - cell wall appositions deposited on the inner surface of epidermal cell walls directly beneath appressoria that impede fungal penetration within the epidermal cell, and by the death of penetrated cells.

The objective of this work is to reveal changes on specific free polyamines during the resistance response of oat against *Blumeria graminis* f.sp. *avenae* focusing in the very early changes occurred following inoculation, and the role of each specific polyamine in the different resistant mechanisms (i.e. penetration resistance, cell death). Furthermore, we investigated the role of polyamines during the non-host interaction between oat and the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*).

2. Materials and methods

2.1. Plant material and inoculation

Seedlings of the oat (*A. sativa*) cultivars Charming (resistant to powdery mildew) and Selma (susceptible to powdery mildew) were used. Seedlings were grown in plastic pots with peat:sand (3:1) in a growth chamber with 20 ºC, 65% relative humidity and under 12 h dark/12 h light with 200 µmol m^{-2} sec^{-1} photon flux density supplied by high-output white fluorescent tubes.

For host interaction studies, both oat cultivars were inoculated with *Blumeria graminis* f.sp. *avenae* race 5. For non-host interactions cultivar Selma was inoculated with *Blumeria graminis* f.sp. *hordei* isolate CC1 according to Prats et al. (2005).

2.2. Microscopic observations

For histological studies, leaves were fixed at 48 hours after inoculation and cleared as described by Carver et al. (1994). Fungal structures were stained with aniline blue in lactoglycerol (0.1%) according to Lyngkjaer and Carver (1999). Observations were made with a Leica DM LS phase contrast microscope (Leica Microsystems) fitted with differential interference contrast and incident fluorescence attachments (blue

exciter filter, max transmittance 480 nm; dichroic mirror and barrier filter transmittance >530 nm).

2.3. Polyamine quantification

The standard polyamines, putrescine, spermidine,spermine and 1-3,diamino propane (DAP) were obtained as their hydrochlorides (Sigma) whereas agmatine was obtained as its sulfate (Sigma) and norspermidine was used as free base (Aldrich). At 12, 24 and 48 hours after inoculation oat leaves were fixed in liquid nitrogen and stored frozen until use. Plant extracts were obtained by homogenizing the plant tissue in perchloric acid (0.1 w/v) according to Flores and Galston (1982). Standards and plant extracts were benzoylated according to Redmond and Tseng, (1979). High performance liquid chromatography analysis of benzoyl-PAs was performed according to Slocum et al. (1989), using an Agilent 2100 Series HPLC.

2.4. Analysis of arginine decarboxylase activity

Arginine decarboxylase activity in the leaf extract was determined as previously described by (Tiburcio et al., 1986), using L-[U-^{14}C]arginine (Amersham, UK) as substrate, and measuring the $^{14}CO_2$ resulting from the specific decarboxylation of arginine catalysed by this enzyme. Enzyme was expressed as nmol $^{14}CO_2$ released/h mg protein. Protein was determined according to the method of Bradford (Bradford, 1976). Bovine γ-globulin (Sigma) was used as a standard.

2.5. Polyamine bioassay

To assess the effects of polyamines on the different resistance mechanisms, a 1mM solution of each polyamine with 0.1% Tween 20 was sprayed over the entire plant until surface runoff was observed. Control plants were similarly treated with 0.1% Tween 20 in water. Treatment was applied twice a day during two days and the third day plants were inoculated and fixed for microscopic inspection.

In addition a second polyamine application was performed by removing the abaxial leaf epidermis and floating the leaf segments on solution, to bathe the mesophyll and facilitate a wide access of polyamine solution to the adaxial (inoculated) epidermis (Lyngkjær et al., 1997; Zeyen et al., 2002b). Therefore, the abaxial epidermis was removed and the central 30 mm leaf segment was excised and floated adaxial (intact) surface up, in a randomised design, on individual 10 ml aliquots of the appropriate

solution 1mM of each polyamine contained in wells of multi-compartment boxes. Segments were held for 1 h for uptake before inoculation using a settling tower placed directly over the floating segments. Transparent lids were fitted to the boxes which were placed in the growth cabinet for 36 hours incubation before segments were fixed as stated above.

2.6. Statistic

Five replications were used for experimentation. For statistical analysis, percentages from microscopy studies were transformed to arcsine square roots to normalize data and stabilize variances throughout the data range. Data were subjected to analysis of variance using SPSS sofware, after which residual plots were inspected to confirm data conformed to normality. Significances of mean differences were assessed following contrast analysis (Scheffe's).

3. Results

3.1. Microscopic characterization of resistance responses to Bga attack

Histological characterization shown in Table 1 confirmed the susceptibility of cultivar Selma with approximately 70% of established colonies. No cell death was evident, and failed attempted penetration due to papilla formation was observed in the 30% of the cases. In contrast, cultivar Charming was resistant with only 9% established colonies, with a combination of penetration resistance (44%) and hypersensitive response. Approximately 47% of cells triggered a hypersensitive response leading to the death of the cell. From this, 26.7% of the cells showed death symptoms before any haustorium could be observed and the response was considered an early and rapid hypersensitive response whereas in 20% of the cells a small haustorium was observed in the dead cell and the response was considered a late hypersensitive response.

3.2. Polyamine content and arginine decarboxylase activity in oat leaves following Bga attack (host interaction)

Detailed quantification of polyamines showed significant differences between the resistant and the susceptible cultivar, regarding specific polyamines and time-frame during the infection process (Fig. 1). Putrescine levels were slightly but significantly increased in both cultivars at 24 hours after inoculation In addition, an early increase at 12 hours after inoculation in DAP was observed in Charming following *Bga* inoculation

(Fig.1). Selma overall showed higher levels than Charming (P<0.001) albeit no changes with inoculation were observed. By contrary Charming showed significantly higher levels of the polyamine spermidine than Selma (P<0.01) and in addition showed a significant increase at 24 hours after inoculation of more than 40% respect to constitutive levels (P<0.05).

Arginine decarboxylase activity was significantly higher in Charming compared with Selma (P<0.05) with a mean of 55.6±2.74 and 46.3±3.93 nmol $^{14}CO_2$ released h^{-1} mg $prot^{-1}$, respectively. Following inoculation arginine decarboxylase activity was significantly higher in inoculated Charming leaves compared to Selma at 12 and 24 hours after inoculation although a significant decrease was observed at 48 hours after inoculation (Fig. 2A).

3.3. Polyamine content and arginine decarboxylase activity in oat leaves following Bgh attack (non-host interaction)

Following non-host inoculation of oat (Selma) leaves with *Bgh* an increase in putrescine was observed during the early stages of the interaction (12 and 24 h.a.i). These changes were correlated with an increase in agmatine also at very early stages of the oat-*Bgh* interaction (Fig. 3). Interestingly also an increase in norspermidine was observed at late stages of the interaction (Fig. 3). No changes in DAP, spermine or spermidine were observed following inoculation of the oat leaves with the inappropriate powdery mildew f.sp. Arginine decarboxylase activity of healthy plants was by mean 51 nmol $^{14}CO_2$ released h^{-1} mg $prot^{-1}$. Increase in agmatine followed the same trend that the increase in arginine decarboxylase activity of the inoculated plants respect to their controls at 12 hours after inoculation. Levels of arginine decarboxylase activity of inoculated leaves were similar to those found in healthy plants at 24 hours after inoculation and slightly lower at 48 hours after inoculation (Fig 2B).

3.4. Effect of exogenous polyamine application on the different resistance responses of oat to powdery mildew

Exogenous spraying of a 1mM solution of each individual polyamine on leaves prior fungal inoculation increased the resistance response of the susceptible cultivar Selma but not of the resistant Charming (Table 1). Interestingly, the effect of all polyamines was observed regarding the penetration resistance. Thus, all assayed polyamines increased the percentage of penetration resistance in Selma up to the levels

observed in Charming (Table 1). This led to a significant decrease in the percentage of established colonies following polyamine spraying. No toxic symptoms in the leaves or in the fungal development were observed following this application. No effect of polyamine application was observed with respect to the percentage of hypersensitive response in any of the cultivars.

Interestingly, when polyamines were applied to stripped-epidermal leaf segments allowing solutions to bath mesophyll cells, a toxic effect on the fungus was observed. In Charming the rate of abnormally germinated conidia (i.e. formation of multi germ tubes, long germ tubes or very thin appresorial tubes) in control plants was 25.3%. However, application of agmatine, putrescine, spermine and spermidine increased significantly ($p<0.001$) the number of abnormally germinated conidia up to 69.4, 49.5, 39.4, and 43.7%, respectively. Similarly, whereas in Selma the rate of abnormally germinated or conidia was 15.8%, application of agmatine, putrescine, spermine and spermidine increased significantly ($p<0.001$) these percentages to 64.6, 35.2, 45.2, and 46.0% respectively.

4. Discussion.

Traditionally it has been assigned a role for polyamines during resistance to abiotic stresses. However, a few reports have recently highlighted the modulation of polyamines profiles during compatible and incompatible interaction of several plants species and their pathogens (Carver et al., 1992; Cowley and Walters, 2002b; Christopher-Kozjan and Heath, 2003; Krippner-Heidenreich et al., 2001). Increased levels of free putrescine and spermine have been associated with the resistance response of barley to powdery mildew (Cowley and Walters, 2002a, b) but also with compatible responses during the later stages of infection (Coghlan and Walters, 1990). Particularly it has been reported an increase of free putrescine and spermine in barley plants of cultivar Delibes carrying the genes *Ml1al* and *Ml(Ab)* conferring hypersensitive response to *Bgh* (Cowley and Walters, 2002b). However, it is difficult to associate this polyamine increase to the hypersensitive response or the penetration resistance since the later response was not microscopically characterised. In this sense, our data showed an early (24 hours after inoculation) increase in putrescine in both resistant and susceptible oat cultivars infected with *Bga*. Since even the susceptible cultivar Selma showed a moderate level of penetration resistance, the increase in putrescine could be associated with this resistance mechanism. Supporting this, exogenous application of putrescine to

Selma leaves increased the level of penetration resistance up to the level observed in the resistant cultivar Charming. No additional effect of putrescine on penetration resistance could be observed in Charming which could indicate that polyamine content in Charming could be near to the maximum threshold at which it exerts its influence. Furthermore an increase in spermidine was also observed in Charming at time of papilla formation and its exogenous application also lead to an increase in penetration resistance. Thus, the combined levels of putrescine and spermidine or a synergistic effect might explain at least in part the higher penetration resistance observed in Charming. The time at which the increase in polyamines was observed fit with the time at which penetration mechanisms are engaged during the oat-*Bga* interaction. Interestingly, an increase in DAP was observed early, at 12 hours after inoculation Since DAP is formed by spermine oxidation mediated by polyamine oxidase, data suggest that the H_2O_2 generated might contribute to the localized oxidative burst which occurs directly beneath the region of attempted penetration (Huckelhoven, 2007; Vanacker et al., 2000). This leads to the rapid accumulation of hydrogen peroxide at the site of papilla formation which is involved in the oxidative cross linking of the papilla component in the callose matrix. Alternatively, the earliness at which DAP was increased in Charming point out to a role for H_2O_2 as messenger for the papilla assembling. Indeed, H_2O_2 together with nitric oxide have been proposed as the earliest signals triggering both penetration resistance and hypersensitive response (Huckelhoven and Kogel, 2003; Prats et al., 2005). Overall our data showed a role for polyamines, particularly for putrescine, spermidine and DAP in the penetration resistance response of oat to its appropriate powdery mildew f.sp, *Bga*. No significant increases in any of the assessed polyamines were observed at later stages of the infection processes correlating with the hypersensitive response. In addition, exogenous application of polyamines did not increase the percentage of cell death, early or late, in any of the cultivars.

A direct fungi toxic role could also be attributed to polyamines since high abundance of these compounds in the epidermal cells following bath of mesophyll cells in polyamines solutions lead to abnormal fungal development at the very early stages. Since one of the functions of the PGT is to gain access to water and other host components directly through epidermis (Carver and Bushnell, 1983), polyamines could entry early into the fungus and exert a toxic effect. Polyamines are important regulators of growth and differentiation in higher eukaryotic organisms including fungi (Pegg,

1988; Walters, 1995). It has been described that whereas polyamine depletion in fungal cells results in growth cessation, excessive intracellular accumulation of polyamines may be cytotoxic (Valdes-Santiago et al., 2012). Thus, an excess of polyamine uptake during this treatment might be responsible for a deregulation of the polyamine metabolism leading to the fungal growth abnormalities observed. However, this direct fungicide effect does not appear to be the cause of the reduction of disease following polyamine spraying where normal fungal development, including appressorium and haustorium formation was observed.

Attempted infection of a cereal in a non-host interaction is commonly arrested by papilla deposition at attempted penetration site and, if cell is penetrated, the challenged epidermal cell dies before haustoria are formed or mature (Bushnell and Bergquist, 1975; Niks and Rubiales, 2002). The frequency of these responses appears to be influenced by fungal f.sp., plant genus and by even genotypic variation in the host (Rubiales and Carver, 2000). However, inappropriate relationships rarely allow fungal development to proceed to sporulation. During the non-host interaction between Selma and *Bgh,* approximately 50% of the penetration attempts were successfully hampered by penetration resistance mechanisms and the penetrated cells developed a programmed cell death so no sporulation of the fungus was observed (data not shown, Carver et al., 1992). Our data shows a very fast increase in the polyamine putrescine from 12 hours after inoculation that might contribute to the penetration resistance observed during the non-host interaction between oat and *Bgh*. The earliness of the response might account for the higher penetration resistance observed during the non-host interaction since the speed at which the resistance machinery is triggered as well as the speed of deposition and compaction of papilla is crucial for successful defense (Huckelhoven, 2007; Prats et al., 2005; von Ropenack et al., 1998). In addition, a significant increase in norspermidine and slight increase in spermidine at later stages, i.e. 48 hours after inoculation might contribute for cell death during the non-host interaction. Norspermidine levels were not changed during the host interaction oat-*Bga*. However, evidence suggests that processes leading to cell death differ between *R*-gene controlled cell death and non-host cell death (Christopher-Kozjan and Heath, 2003). Increased levels of putrescine and spermidine have been reported during the non-host interaction of tobacco plants with the bacteria *Pseudomonas cichorii* which lead to an extensive cell death reaction. These polyamines were shown to serve as the source of hydrogen peroxide during the non-host cell death (Yoda et al., 2009). The increase in agmatine

observed at 12 hours after inoculation together with the increase in arginine decarboxylase activity respect to non-inoculated plants at this time point, suggest the involvement of this pathway in the increase of polyamines observed while we cannot rule out the involvement of the ornithine decarboxylase enzyme in the increase of polyamines observed.

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Tables

Table 1. Effect of polyamine application on resistance responses of oat cultivars Selma, susceptible, and Charming, resistant, to powdery mildew (*Bga*) infection.

Treatment	Pen Res[a]	Early HR	Late HR	Total HR	Established
			Selma		
Control	29,7	0,0	0,0	0,0	70,2
Agmatine	44,0***	0,0	0,0	0,0	56,0***
Putrescine	45,2***	0,0	0,0	0,0	54,7***
Spermine	42,5***	0,0	0,0	0,0	57,5***
Spermidine	41,0***	0,0	0,0	0,0	59,0***
			Charming		
Control	44,0	26,7	20,2	9,0	47,0
Agmatine	$40,7^{ns}$	$30,5^{ns}$	$20,2^{ns}$	$8,5^{ns}$	$50,7^{ns}$
Putrescine	$41,0^{ns}$	$29,5^{ns}$	$18,7^{ns}$	$10,7^{ns}$	$48,2^{ns}$
Spermine	$46,0^{ns}$	$28,2^{ns}$	$17,5^{ns}$	$8,2^{ns}$	$45,7^{ns}$
Spermidine	$44,5^{ns}$	$23,5^{ns}$	$20,2^{ns}$	$11,7^{ns}$	$43,7^{ns}$

[a]Data obtained at 48 hours after inoculation The percentage of germlings that reached different developmental stages (passing from one stage to the next), a) formed an appressorium but not penetrated the cell (Pen Resist), b) penetrated the cell but a rapid hypersensitive response avoid haustorium development (Early HR) c) penetrated the cell and a late hypersensitive response develop but allowing haustorium development (Late HR) d) penetrated the cell and establish a colony (Est) not associated with cell necrosis, were scored from 100 infection units. Analysis of variance was applied to transformed replicate data. *** indicate a significant difference between cultivars at $P<0.001$. Data are mean of 5 replications.

Figure caption

Figure 1. Polyamine content in Selma and Charming cultivars during the host interaction oat-*Bga*. Putrescine, DAP, norspermidine, spermidine, spermine, and agmatine were quantified in susceptible Selma and resistant Charming plants during a time course following inoculation with the host fungus *Bga*. Data are mean of 5 replicates ± standard error. White bar = control, healthy plants; Grey bars = plants inoculated with *Bga*. *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and inoculated plants; absence of stars indicates no significant differences.

Figure 2. Arginine decarboxylase (ADC) activity in oat leaves during the host and non-host interaction. **A.** ADC activity in Selma and Charming cultivars during the host interaction oat-*Bga.* ADC activity of inoculated Selma (white bars) and Charming (black bars) cultivars, respect to healthy plants during a time course following inoculation with the host fungus *Bga*. **B.** ADC activity in Selma plants during the non-host interaction oat-*Bgh.* Bars indicate the ADC activity of inoculated Selma respect to healthy plants during a time course following inoculation with the non-host fungus *Bgh*. Data are mean of 5 replicates ± standard error. *, ** indicate significant differences at $p<0.05$ and 0.01 respectively between cultivars; absence of stars indicates no significant differences.

Figure 3. Polyamine content in Selma cultivar during the non-host interaction oat-*Bgh*. Putrescine, DAP, norspermidine, spermidine, spermine, and agmatine were quantified in Selma plants during a time course following inoculation with the non-host fungus *Bgh*. Data are mean of 5 replicates ± standard error. White bar = control, healthy plants; Grey bars = plants inoculated with *Bgh*. *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and inoculated plants; absence of stars indicates no significant differences.

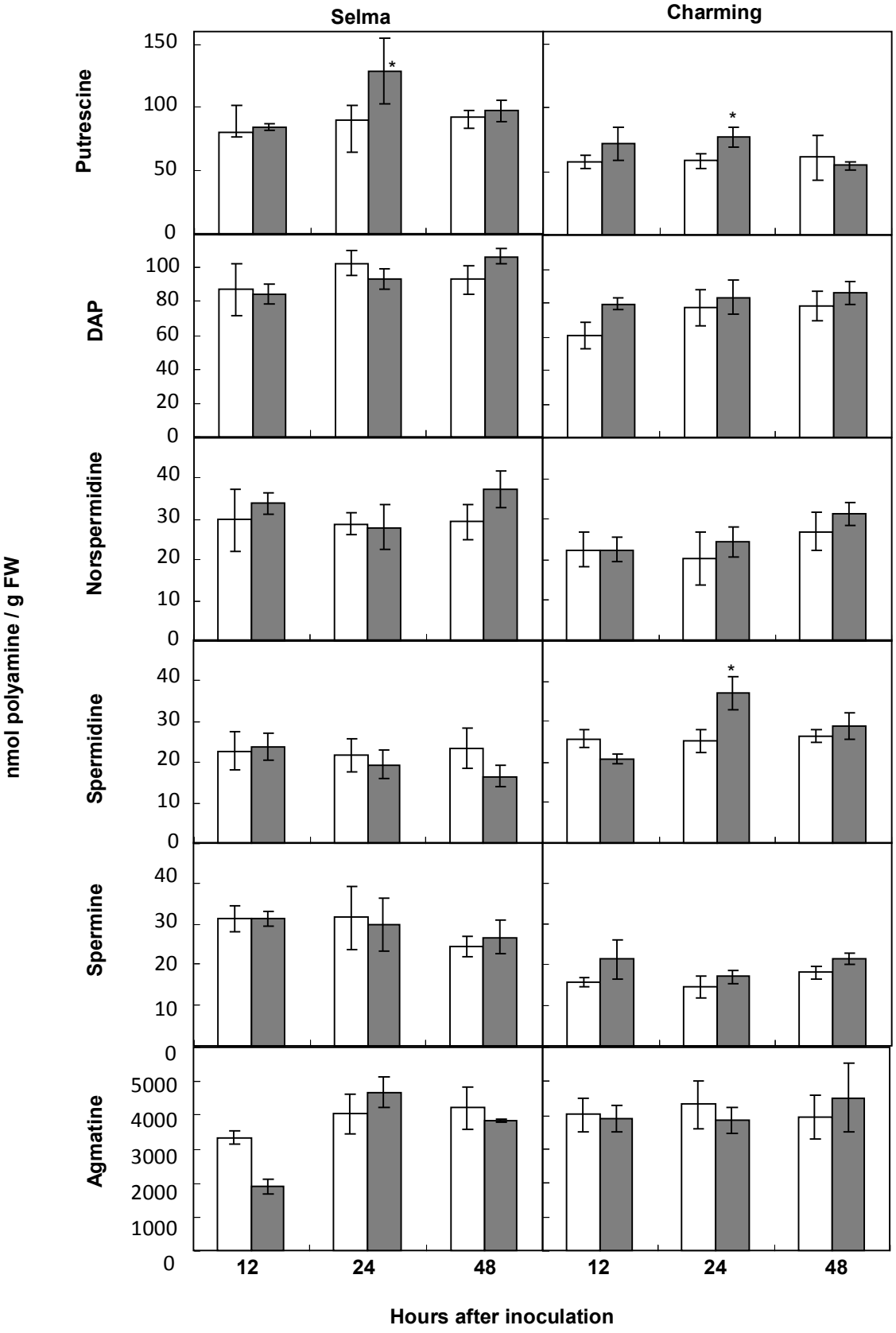
Selma
Charming
Putrescine
DAP
Norspermidine
Spermidine
Spermine
Agmatine
nmol polyamine / g FW
Hours after inoculation
12
24
48

Figure 1.

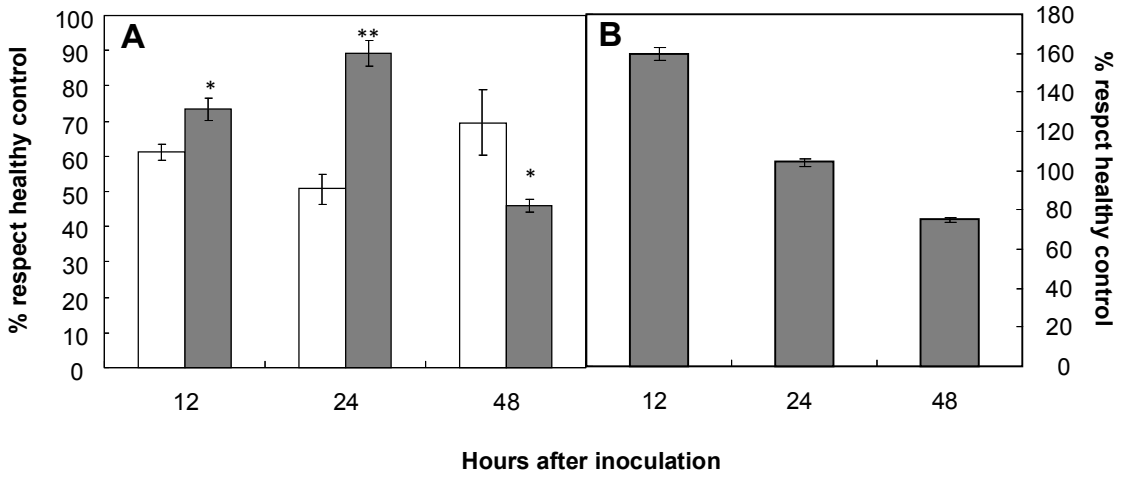
A
B
% respect healthy control
% respct healthy control
100
90
80
70
60
50
40
30
20
10
0
180
160
140
120
100
80
60
40
20
0
*
**
*
12
24
48
12
24
48
Hours after inoculation

Figure 2.

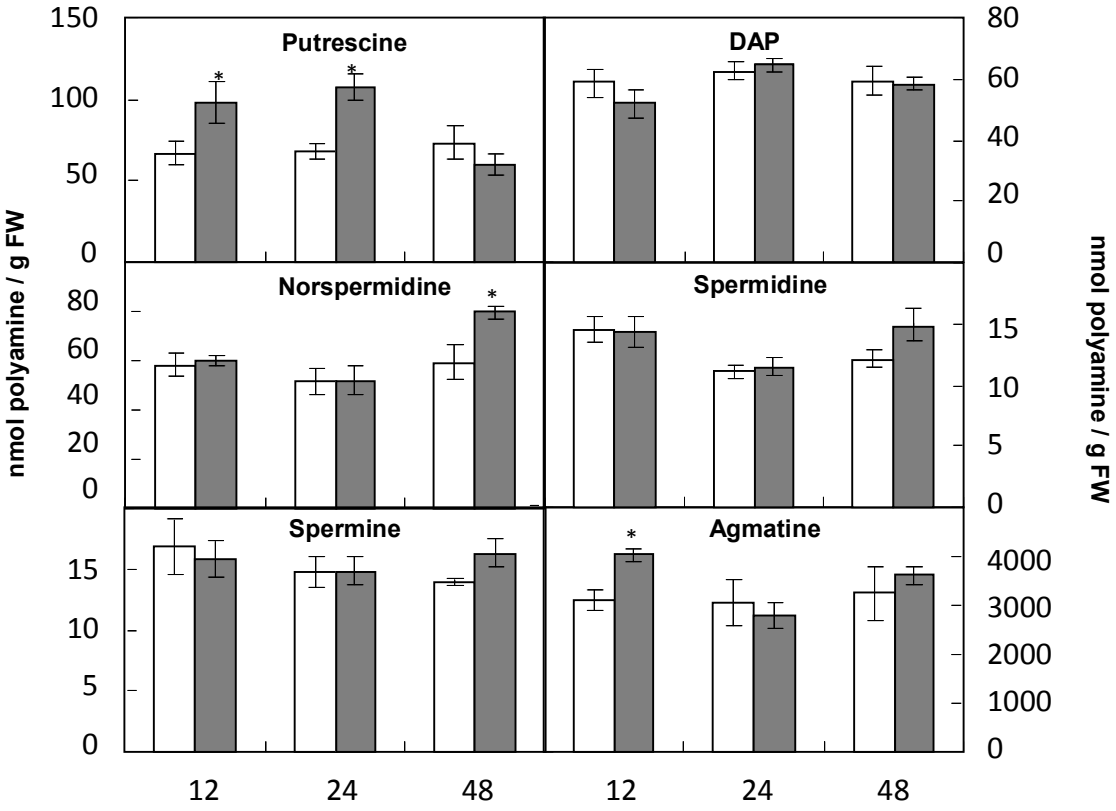
Putrescine
DAP
Norspermidine
Spermidine
Spermine
Agmatine
nmol polyamine / g FW
nmol polyamine / g FW
150
100
50
0
80
60
40
20
0
80
60
40
20
0
15
10
5
0
15
10
5
0
4000
3000
2000
1000
0
12
24
48
12
24
48

Figure 3.