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Dynamics of growth regulators during infection of apple leaves by *Alternaria alternata* apple pathotype

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Abstract Alternaria blotch of apple caused by *Alternaria alternata* apple pathotype has a severe negative effect on apple production. It can cause tissue necrosis on leaves, young shoots and fruit. Recent studies on this pathogen have mostly focused on phytotoxicity and pathogenicity. There are few reports on the roles of signaling and metabolism in the process of infection. In this paper, a filial generation with substantial differences in resistance between individuals with similar genetic background was used as a host. An aggressive strain of *A. alternate*, that can complete the infection process 72 h after inoculation, served as the pathogen. A reproducible and reliable in vitro inoculation system for plant growth regulator determination was established to overcome the difficulties of inoculation of attached leaves. Alterations in growth regulator concentrations were detected, including indole-3-acetic acid (IAA), zeatin riboside (ZR), gibberellin A_3 (GA_3), abscissic acid (ABA) and the polyamines, putrescine (Put), spermidine (Spd) and spermine (Spm). Results indicated the plant growth regulators interacted with each other to modulate signaling and metabolic networks. A biotrophic-like phase was inferred to exist before necrosis developed. Gibberellin A_3 and ABA appeared to be involved in the phase transformation from the biotrophic-like stage to the necrotrophic stage. Cytokinin, Put and Spd appeared to be related to disease resistance. This study advances our knowledge of the pathological mechanisms of Alternaria blotch on apple and provides useful resources for development of disease control and prevention.

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Keywords In vitro inoculation · Plant pathogen interaction

Abbreviations

SA	salicylic acid
JA	jasmonic acid
ET	ethylene
PGRs	plant growth regulators
AOS	activated oxygen species
SEM	scanning electron microscopy
ELISA	enzyme-linked immunosorbent assay
HPLC	high-performance liquid chromatography
PI	post inoculation
IAA	indole-3-acetic acid
ZR	zeatin riboside
GA_3	gibberellin A_3
CK	cytokinin
ABA	abscissic acid
Put	putrescine
Spd	spermidine
Spm	spermine

Introduction

Alternaria blotch of apple is one of most important apple diseases in East Asia (Japan, Korea and China), and in parts of the US, and is spreading worldwide (Filajdić and Sutton 1991). It affects apple production and tree growth quality though infection of leaves, young shoots and fruits and declining tree vigour (Sawamura 1972; Jones and Aldwinckle 1990). This disease is caused by the necrotrophic pathogen, *Alternaria alternata* apple pathotype (*A.alternata* AP) (Roberts 1924; Filajdić and Sutton 1992; Dickens and Cook 1995). It can invade many species of plants, with most strains causing severe pathogenesis (Filajdić and Sutton 1992). The

main pathogenicity factor for apple pathotype is the production of a host specific necrosis-inducing toxin called AM-toxin (Thomma 2003). Previous studies on this toxin have focused on its molecular features, including molecular structure and coding gene (Nishimura and Kohmoto 1983; Johnson et al. 2000). There are also some reports concerning pathogenesis, including the function of the G protein in the process of pathogen invasion (Yamagishi et al. 2006).

For plants, a resistance gene and molecular markers have been found and extensive breeding work has been done to find the underlying resistance mechanism and in order to produce new plants resistant to the pathogen (Banno et al. 1999; Saito et al. 2001; Egusa et al. 2009). Little progress has been made to enhance apple resistance, possibly due to the limited genetic understanding of apple interactions with necrotrophic pathogens and the long breeding cycle of apples. Therefore, application of fungicides is currently the main treatment method to control the disease, which is not only costly and environmentally unfriendly, but also of limited efficacy (Sutton et al. 1994; Xin-ru et al. 2008). Many fungicides are incapable of killing the fungus completely, and the disease can develop again when favorable conditions return, i.e. high temperature and relative humidity. Moreover based on recent reports, differing from the gene-for-gene model for biotrophic pathogens, resistance to *A.alternata* AP is a response to a host specific toxin, whose signaling reaction was found to be involved in the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways (Thomma et al. 1998; Egusa et al. 2008).

Plant growth regulators (PGRs) play important roles in the regulation of metabolism and increasing plant resistance in response to pathogen infection (Davies 1987; Pieterse et al. 2009). Previous studies mostly focused on their function, interaction network and the pathways involved especially those related to the SA, JA and ET pathways (Flors et al. 2008; López et al. 2008). However, there are few related studies on *A. alternata* AP. Indole-3-acetic acid, GA_3, CK and ABA are conventional growth regulators that were shown not only to be involved in plant growth and development, but also to be intimately associated with the SA, JA and ET pathways as signals (Pieterse et al. 2009; Shan et al. 2011). Moreover, polyamines have drawn attention because of their participation in plant stress responses, either in the free or bound state, especially for resistance interactions in the free state (Walters 2003; Takahashi et al. 2004). Polyamines can act in multiple ways in incompatible interactions between plants and pathogens by forming barriers, forming macromolecular cross-linked structures to protect cell stability, binding to DNA, RNA and proteins to change their conformation and modulate metabolism (Angelini and Federico 1989). Evidence also shows that free polyamines affect the plant defense reaction, especially with respect to activated oxygen species (AOS), which are produced by the transformation of Put to Spd and Spm (Angelini and Federico 1989; Bagni and Tassoni 2001). Moreover, it is worth noting that growth regulators are also metabolic products, so their function in plants are restricted by their environment.

It is standard practice to excise tissue after treatment to mimic the plant's natural physical status in metabolism studies. However, this method was not sufficient for this study. Initially, leaves for inoculation were limited because each tree represents isolated trait in the progeny population. Infection rates in the field were too low to meet the requirements of the experiment and the infection model establishment because conditions were difficult to control. Furthermore, the symptoms from inoculation of attached leaves and inoculation of detached leaves were similar (data not shown). Therefore, an in vitro method was developed for this study.

Previously, studies of *A.alternata* AP mostly focused on pathogenicity. However, the roles of PGRs in the regulation of this disease are still poorly understood. We hypothesized that these PGRs participate in the regulation of the occurrence and development of this disease. Therefore, in this study, we performed an analysis on variations of seven PGRs to elucidate their function in the process of the apple disease response. The aim of this study was to further elucidate the mechanisms of the growth regulator network in resistance to *A.alternata* AP, to gain knowledge about the interactions between apple and *A.alternata* AP, and supply the theoretical basis for the establishment of relevant methods to effectively control disease.

Materials and methods

Plant and sample preparation for in vitro inoculation

All plant materials were grown in an experimental orchard at the Institute of Pomology, China Academy of Agricultural Sciences (Xingcheng, Liaoning Province, China, 125100). The resistant and susceptible samples were chosen from the 110 progeny, which were hybridized from a resistant cultivar ('Huacui') and a susceptible cultivar ('Golden Delicious') in 2002, and seed was planted in 2003. Evaluation of a disease rating by field inoculation was applied to this group of progeny in 2008 and 2009 according to guidelines from the Institute of the Control of Agrochemicals (2000) GB/T 17980.124: the Fungicide Control of *Alternaria alternata* apple pathotype in the "Guidelines for Field Trials of Pesticides". Of eight highly susceptible and 12 highly resistant cultivars, four (one highly susceptible and three highly resistant) were chosen to be candidates for the in vitro inoculation test. Ultimately, two cultivars with the most apparent resistance difference, one highly susceptible and one highly resistant, were chosen to be hosts based upon tree vigor, stable infection incidence and severity, and SSR results (data not shown).

One aggressive strain that was isolated from infected leaves of apple trees in the Fruit Plant Protection Research Center, Institute of Pomology, was chosen to be the pathogen strain for later study. This one strain was used to maintain consistency, and chosen because it was more aggressive than other strains and can of inoculation (data not shown). Isolates were kept on potato dextrose agar (Sigma Chemical Co., St. Louis, MO) slants at 4°C and grown on petri plates at 25°C for the harvest of spores. Sporulating mycelia were gently rubbed with a bent glass rod, suspended in sterile water and filtered to remove mycelia by four layers of cheesecloth. The suspension was adjusted to a concentration of 10^6 conidia·mL^{-1}.

The healthy leaves ranged in age from 10 to 20 days depending on growth rate and were randomly selected from current season's shoots. Leaves were picked with the petioles, rinsed gently with distilled water, dried with paper towel and placed upside down on four layers of moist filter paper in petri plates with a moist cotton ball placed at the end of each petiole to maintain moist conditions (70% relative humidity). All samples were observed and selections were made at 24 h after plating in order to ensure that the leaves were free of damage and infection. Two leaves were kept in each plate, susceptible and resistant leaves were inoculated with two drops (20μL) of spore suspension on the abaxial surface. Meanwhile, control plants were treated similarly using water instead of inoculum. All samples were kept in moist petri plates sealed with plastic film and kept at 25°C, 14 h photoperiod and 60% relative humidity. Both infected and control samples were harvested at the same time after inoculation and immediately frozen in liquid nitrogen before storage at −80°C while awaiting analysis.

Scanning electron microscopy (SEM) Analysis

The selected control and inoculated leaves were sampled at 0, 24 and 48 h post inoculation (PI), followed by the harvest of all samples at 72 h PI. Subsequently, these leaves were cut into 1–2 mm^2 pieces and fixed in a 5% glutaraldehyde solution, then treated as described by Mansvelt and Hattingh (Mansvelt and Hattingh 1989). All samples were gold-coated and examined for fungal infection by SEM (Hitachi S-570, Hitachi Co. Ltd., Tokyo, Japan).

Stability and repeatability of infection

In vitro inoculation experiments were carried out on May 11, June 15, July 30, August 10 and September 21, 2009. Each experiment was performed in triplicate, three petri plates for each variety. The infection rate was determined at 72 h PI and was expressed as (number of infected sites/sum of inoculated sites)×100% (Fig.1).

Sample preparation and PGRs determination using enzyme-linked immunosorbent assay (ELISA)

After in vitro inoculation leaves were collected and frozen at 0, 24, 48 and 72 h PI in liquid nitrogen. Afterwards 0.1 mg leaf tissue samples were stored at −20°C for ELISA using the method by You-Ming et al. (2001). ELISA using antibodies against IAA, GAS, ABA and iP+iPA were gained as described by Weiler (1980a, b). The absorbance was recorded at 490 nm.

Sample preparation and growth regulator determination using high-performance liquid chromatography (HPLC)

Samples were harvested and frozen at 0, 24, 48 and 72 h after in vitro inoculation. Free polyamines were extracted and purified using a method modified from that described by Flores (Flores and Galston 1982). Polyamine detection was performed using HPLC (LC-10ATvP, SHIMADZU, Japan) equipped with a C_{18} reversed phase column, UV detector, mobile phase (MeOH: H_2O=70: 30 (V: V)) at a flow-rate (0.5 mL/min), with the detection wavelength set at 254 nm.

Data analysis

Three independent biological replicate experiments (three petri plates of each treatment) with three technological replicates (three observation of each sample) were performed for each PGR. All samples for PGRs determination were from leaf tissue surrounding the lesions. Statistical analysis using Duncan's model was performed using SPSS software 15.0 (SPSS, Chicago, IL), with a 0.05 (p<0.05) significance level.

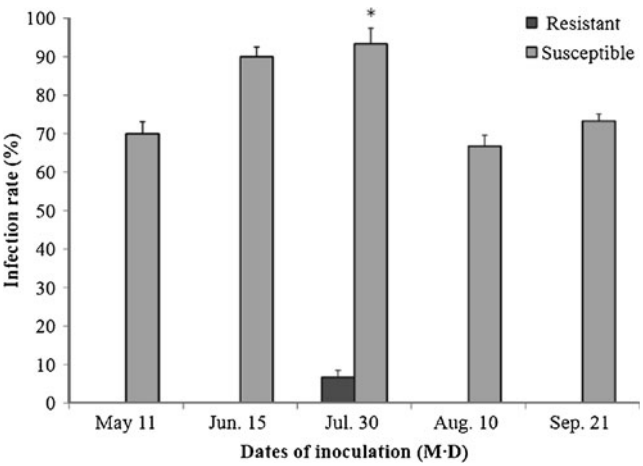

Fig. 1 Infection incidence after inoculation apple leaves by *Alternaria alternata* apple pathotype on May 11, June 15, July 30, August 10 and September 21 in 2009. * significant difference between resistant and susceptible leaves, at $P \leq 0.05$

Results

Determination of sample harvest time

The SEM results indicated changes in leaf microstructure at 24, 48 and 72 h PI. There is not obvious infection on resistant leaves. On susceptible leaves, no penetration was observed on the abaxial surface of leaves at 24 h PI. By 48 h PI, hyphae had pierced through the spongy mesophyll of leaves without reaching palisade tissue, with more development of mycelium on the abaxial surface. By 72 h PI, mycelium was observed to have grown through the leaf lamina and covered the entire surface of the leaf. Based on these evaluations of susceptible line, harvest times of leaves were set at 0, 24, 48 and 72 h PI.

Stability and repeatability of the inoculation system

Infection rates observed from five different inoculation time points showed that the in vitro leaf inoculation system for *A. alternata* AP was stable and repeatable. This system was used to simulate the process of natural non-wounded inoculation for the study of PGRs in apple leaves (Fig. 1).

Dynamics of PGRs

Overall, all seven types of PGRs declined slightly in control plants. Indole-3-acetic acid, ZR, GA_3 and IBA showed more notable variations in the susceptible leaves than resistant ones throughout fungal invasion (Fig. 2).

In susceptible leaves, IAA and ZR showed a dramatic decline after infection followed by a slight increase at 48 h PI. Gibberellin A, which is believed to boost plant growth by promoting cell division, showed a significant increase after an initial decline at 24 h PI. Abscissic acid, which is involved in biotic and abiotic stress, showed a remarkable decline at 24 h PI, followed by a significant increase.

In resistant leaves, the PGRs showed no statistically significant change at all time points, however, there were many

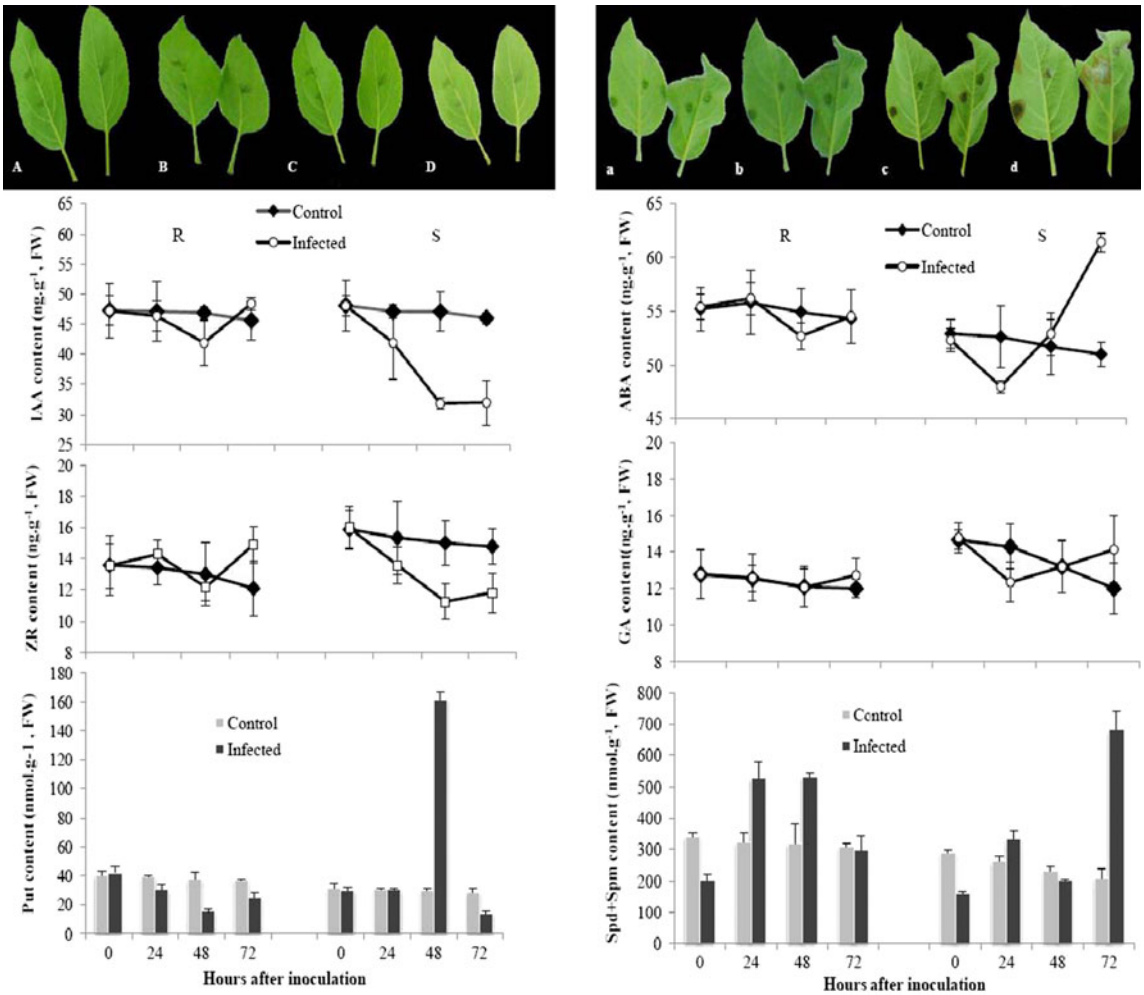

Fig. 2 Symptoms of resistant (A, B, C, D) and susceptible (a, b, c, d) leaves at 0 h, 24 h, 48 h and 72 h PI, in addition to dynamics on plant growth regulators, including indole-3-acetic acid (IAA), zeatin riboside (ZR), gibberellin A_3 (GA_3), abscissic acid (ABA) and three types of polyamines; putrescine (Put), spermidine (Spd) and spermine (Spm). R- resistant leaves, S- susceptible leaves

interesting fluctuations. Indole-3-acetic acid content was increased to a comparably higher level at 72 h PI after a decline at 48 h PI, exhibiting a similar pattern to that observed in the susceptible plants. Gibberellin A_3 shifted in a similar tendency as IAA in susceptible leaf behavior but the trend was less pronounced. Zeatin riboside content increased at 24 h PI followed by a fluctuating pattern, and finally reached a relatively higher level at 72 h PI compared to other time points. Abscissic acid showed a trend similar to that of ZR in resistant plants, this was different from that of ABA in susceptible plants after inoculation.

For polyamines, the sum of Spd and Spm was shown (Fig.2) because they are both the products of a Put catalysed reaction by relevant synthetases accompanied by release of hydrogen peroxide (H_2O_2), this has been reported in many plants (Moschou et al. 2008; Yoda et al. 2009). In inoculated susceptible leaves, Put content increased dramatically at 48 h PI and decreased at 72 h PI. Although Spd+Spm climbed initially at 24 h PI, then decreased during the 24 h PI to 48 h PI period, it went up to a significantly higher level at 72 h PI than compared to 24 h PI. In inoculated resistant leaves, Put content declined before 48 h PI and increased slightly at 72 h PI, this was opposite of the trend observed in inoculated susceptible leaves. In addition, Spm+Spd concentration went up significantly by 24 h PI, and the amount remained in that range until 48 h PI, the concentration then proceeded to decrease to a lower amount by 72 h PI compared to 24 and 48 h PI. Spermine content was generally too low to detect, except for being detectable at 0 h in inoculated susceptible leaves and at 48 h PI in resistant ones, with values at 10 and 93.5 $nmol \cdot g^{-1}$ fresh weight, respectively.

Discussion

In vitro inoculation, primarily using excised leaves, was used for testing plant resistance (Abe et al. 2010). Samples for PGRs determination were from attached tissues after experimental treatment in order to stimulate real life conditions and reactions (de Torres-Zabala et al. 2007; Gaudinová et al. 2008). Due to the previously mentioned difficulties with whole plant inoculations, in vitro inoculation was desirable for *A. alternata* AP. Based on this, a simple and effective infection system was established, evaluated and applied for determining PGRs dynamics. Furthermore, because of evidence that ABA is quite variable in detached leaves under condition of 60% relative humidity, we used control plants in order to keep the interaction model as exact as possible (Forcat et al. 2008).

Variations of PGRs in susceptible leaves were more significant than those in resistant ones following inoculation. Nutrient circulation could account for this phenomenon, for which resistant plants can sustain relatively normal metabolism and physical activity during infection. Moreover contents of IAA, ZR, GA_3 and ABA all decreased before 24 h after active infection began, this could be caused by their involvement as nutrition for the pathogen. They were also reported to stimulate growth of fungi (Kepczyńska and Kepczyński 2005).

Some exceptional PGRs variations were observed. First, ABA concentration increased significantly at 48 and 72 h PI compared to 24 h PI. Gibberellin A_3 content also increased though not as greatly as for ABA. This is unexpected because ABA and GA_3 play different metabolic roles. Gibberellin A can promote cell division and appears to serve as a factor that can reduce plant defenses against necrotrophic pathogens by disabling the JA-mediated resistance to necrotrophic pathogens (Navarro et al. 2008). Based upon this, the increasing levels of GA_3 after 24 h PI would lead to leaves being more susceptible to *A. alternata* AP. In addition, modulation of plant defenses by ABA varies with different diseases. Generally, ABA can lead to plant aging and the loss of defense signaling to necrotrophic pathogens, this could explain the increasing level of ABA at 48 h PI (Flors et al. 2008). In contrast, the content of ABA decreased at 24 h PI compared to 0 h PI, this could be due to another function of the ABA signaling. Abscissic acid plays a role in increasing disease defense by inducing CAT enzyme activation and eliminating the destruction of active oxygen species (AOS) (Guan et al. 2000). The unexpected changes would imply a biotrophic-like stage.

Second, levels of IAA and ZR, that are known to be responsible for promoting plant growth and development, would be expected to decrease in susceptible leaves because of the nutrient depletion by fungi and retarded growth of in vitro leaves, especially after necrosis symptoms appear (Pieterse et al. 2009). However contents of IAA and ZR in the susceptible leaves increased to varying degrees at 72 h PI. A potential

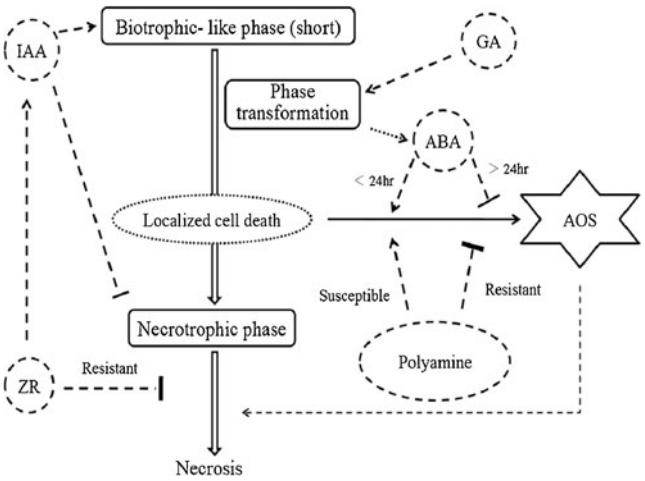

Fig. 3 Schema of plant regulator dynamics and the plant immune response during infection of apple leaves by *A. alternata* AP. After a signal responding to effectors from the pathogen, there is a biotrophic-like phase before necrosis appears. It results in localized cell death, which is used by the pathogen to produce necrosis during the necrotrophic phase.⊥, negative effect; - ➔, positive effect

reason for this is that nutrient sequestration was slowed down based on the observation that the mycelium has already penetrated through leaves and became impaired the tissue structure by 72 h PI. In addition, the increases in auxin and cytokinin levels may be explained by their action as synergistic signals that suppress resistance to necrotrophic pathogens, this is supportive of the existence of a biotrophic-like phase (Siemens et al. 2006). Remarkably, the dynamics of ZR were completely distinct between resistant and susceptible leaves, especially between 24 h PI and 48 h PI. The decline of ZR levels could lead to resistance to *A. alternata* AP according to the finding mentioned above (Siemens et al. 2006). This would imply the vital importance of ZR in resistance responses.

In susceptible leaves, Put accumulated at 48 h PI and declined to pre-infection levels at 72 h PI.. It was assumed that Put changed to Spd and Spm at this stage with emission of H_2O_2 because the content of Spm + Spd increased substantially. Another phase of H_2O_2 production was from 24 to 48 h PI when high Spm + Spd content was generated. In addition, Spm was only detected at 48 h PI in susceptible leaves, this could be another effect of H_2O_2 emission and stimulation of hyphal growth (Kępczyńska 1995; El Ghachtouli et al. 1996). Localized cell death is always the consequence of H_2O_2 accumulation, this is known in the resistance mechanisms of plant to biotrophic pathogens, it is also known to exist in saprophytic fungi (Mehdy 1994; Walton 1996; Sasabe et al. 2000). However, the results for resistant plants, compared to susceptible plants, indicated that the level of Put was low, showing a decrease from 0 to 48 h PI followed by an increase to 72 h PI. In contrast to Put, the Spm + Spd content increased from 0 to 48 h PI and decreased at 72 h PI with no detection of Spm after infection (Fig. 2). This suggests that the sustained Put content and accumulation of the appropriate amount of Spd without the biotrophic-necrotrophic phase change would be a mechanism for enhanced resistance.

To the best of our knowledge, this is the first comprehensive analysis of the dynamics of seven kinds of PGRs in the interaction between apple and the necrotrophic fungus *A. alternata* AP. Our analysis allowed for a schema of plant regulator dynamics during infection of apple leaves by *A.alternata* AP (Fig.3). It is believed that a biotrophic-like stage precedes the necrotrophic infection which can cause programmed cell death (PCD), this corresponds to a previous implication of pathogenesis by the AM-toxin (Otani et al. 1995). It is inferred that the pathogen utilizes localized cell death as the first step in killing leaf tissue. However it is still a hypothesis and more related work needs to be done to prove if there is an independent mechanism accompanying nutrient depletion from dead tissue or a stage in the necrotropic process. We also believe that CK may play a particular role in pathogen resistance and GA_3 can affect the phase change that would act on ABA through some unknown pathway in the biotrophic stage. For free polyamines, the functional agent was assumed to be the AOS they produced, and the results were mostly in accordance with this pathway. We have speculated that Put and Spd would be related to increased resistance and Spm was involved in hyphal growth. We believe that this information will greatly improve our understanding of the interaction between apple and *A. alternata* AP. This information could potentially allow for the development of a selective screen for cultivars and allow for faster breeding for *A. alternata* resistance.

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